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Bor Luen Tang *Editor*

Membrane Trafficking

Second Edition

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Membrane Trafficking

Second Edition

Edited by

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Cover illustration: A431 cells transiently transfected with RFP-Rab5 (red), fixed after a 30 min pulse-chase with EGF-FITC (green) and co-labeled for early endosome antigen 1 (pseudo-colored magenta). Nuclei are visualized with Hoechst 33342 (blue).

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Preface

The term “membrane trafficking” broadly describes selective transfer of proteins and lipids between membranous structures in both prokaryotes and eukaryotes. In principle this entails two different processes. The first is a myriad of membrane traversing events mediating the translocation of proteins *across* membranes. The other refers to vesicle/membranous carrier-mediated transport *between* eukaryotic organelles in the exocytic/secretory and endocytic pathways (the endoplasmic reticulum, Golgi apparatus, plasma membrane, endosome, and lysosome) as well as other membranous structures such as the nucleus and peroxisomes. Membrane trafficking research has been at the frontier of cell and molecular biology for the past 30–40 years, with easily recognized groundbreaking discoveries and top accolades to its researchers. One of the early greats, George Palade (Nobel Prize in Physiology and Medicine, 1974), had used electron microscopy to morphologically map the secretory pathway. Gunther Blobel (Nobel Prize in Physiology and Medicine, 1999) founded the concept of signal sequences in membrane targeting and transport. Membrane trafficking research in the past 20 years has also witnessed a remarkable convergence and synergism from information gained using genetic approaches in yeast cells and biochemical and molecular approaches in mammalian cells. As a result, the principle molecular components mediating membrane trafficking were identified, with the Lasker in 2002 jointly awarded to Randy Schekman and James Rothman and the Nobel in 2013 to Randy Schekman, James Rothman, and Thomas Südhof. Foundations laid down by studies in membrane trafficking have also aided advances of other fields, for example in understanding of disease conditions such as familial hypercholesterolemia and neurodegenerative diseases.

In 2008, an excellent volume in the *Methods in Molecular Biology* series on *Membrane Trafficking* was brought together by volume editor Ales Vancura and series editor John Walker. It included a compendium of articles describing the state-of-art methods and cutting-edge techniques to study macromolecular transport across and between membranes. The past few years have, however, witnessed many new directions, and importantly, new approaches, in membrane trafficking research. These include the discovery of novel, unconventional trafficking pathways as well as the intersections between the classical exocytic and endocytic pathways with other key cellular processes that are membrane-associated (such as autophagy). The advent of Omics-based technologies to perform genome-wide screens or correlative studies, expression profiling, and array-based interrogation of the genome or the functional transcriptome (e.g., sh/siRNA-based screens) has opened up new analytical dimensions that would allow clearer definition of components and deeper understanding of regulatory networks in membrane trafficking. Finally, technological advances in imaging and microscopy have now provided far greater resolution in space and time and facilitated observations in live cells, in ways that were not possible before. All these new advances, in both the science and the technologies, warrant the production of a new volume on *Membrane Trafficking*.

This new volume, with updates of previous chapters and the inclusion of a large number of new chapters, is divided into three parts. Part I includes biochemical and molecular genetics approaches and methods used in analyzing membrane traffic in both yeast and

mammalian cell models. Part II focuses on imaging and microscopy approaches and techniques, while Part III highlights the Omics-type approaches. We hope that this new volume of *Membrane Trafficking* will be a useful desk reference for both experienced researchers and graduate students/junior scientists in the field of membrane trafficking. Furthermore, we hope the wide coverage of the chapters would also be helpful to researchers in other fields who wish to make an excursion into looking at aspects of membrane trafficking as well as those simply wanting to familiarize themselves with the frontiers of this exciting field.

Bor Luen Tang

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Chapter 1

Intracellular Parcel Service: Current Issues in Intracellular Membrane Trafficking

Johannes M. Herrmann and Anne Spang

Abstract

Eukaryotic cells contain a multitude of membrane structures that are connected through a highly dynamic and complex exchange of their constituents. The vibrant instability of these structures challenges the classical view of defined, static compartments that are connected by different types of vesicles. Despite this astonishing complexity, proteins and lipids are accurately transported into the different intracellular membrane systems. Over the past few decades many factors have been identified that either mediate or regulate intracellular membrane trafficking. Like in a modern parcel sorting system of a logistics center, the cargo typically passes through several sequential sorting stations until it finally reaches the location that is specified by its individual address label. While each membrane system employs specific sets of factors, the transport processes typically operate on common principles. With the advent of genome- and proteome-wide screens, the availability of mutant collections, exciting new developments in microscope technology and sophisticated methods to study their dynamics, the future promises a broad and comprehensive picture of the processes by which eukaryotic cells sort their proteins.

Key words Membrane trafficking, Protein sorting, Protein translocation, Vesicular transport

1 Introduction

Cell Biology textbooks typically start with an illustration showing a section through an eukaryotic cell, in which the different cellular compartments are neatly ordered in a well-arranged and static coexistence. In contrast, recent advances in high-resolution microscopy allowed exciting insights into the spatial, three-dimensional orientation of cellular membranes showing that the cell is tightly stuffed with a multitude of membrane structures (for examples *see ref. 1–4*). Solely on the basis of their structural appearance, it is almost impossible to attribute most of these membrane structures to specific cellular compartments. Moreover, these membrane structures are highly dynamic, constantly merging by fusion or dividing by fission, and always moving or are actively transported

by motor proteins throughout the cellular interior. Despite all this seeming chaos, the distribution and dynamics of cellular macromolecules is highly organized and regulated. Like for the moving companies, the logistics of material transport is of uttermost importance for eukaryotic cells. A multitude of factors ensure that each protein is safely delivered to its appropriate position. Over the last two decades, the transport of proteins and intracellular membranes has been one of the major lines of interests for molecular cell biologists and many of these processes were studied in great detail. During this time, most of the basic machinery that governs intracellular transport has been identified. However, the regulation and adaptability of membrane transport processes to the varying cellular demands remain largely elusive.

Thus, despite all current knowledge on membrane trafficking processes, many fundamental questions still remain to be addressed. Examples of such issues are: How are targeting signals recognized by chaperones and translocases? What decides between co-translational or posttranslational targeting of proteins? How is the specific intracellular distribution of lipids mediated? What is the role of organelle contact sites and how are they regulated? How are integral membrane proteins folded or assembled, and which factors control and facilitate these assemblies? How are some proteins specifically recruited into transport vesicles whereas others are excluded? How do cargo proteins regulate the transport of vesicles along cytoskeletal filaments? Do vesicles reach their target membranes randomly or are they actively guided from donor to target compartments? How is the biogenesis and proliferation of peroxisomes and autophagosomes linked to the secretory pathway? How is the functionality of intracellular membrane structures controlled and how do autophagosomes select their substrates? All these questions are currently being addressed by different experimental approaches and first insights have revealed a large variety and complexity of the mechanisms underlying these processes.

2 Protein Sorting

In eukaryotic cells, a huge diversity of signals, sorting components, and energy requirements exists by which proteins are efficiently translocated into the ER, the nucleus, mitochondria, chloroplasts, and peroxisomes, or by which vesicles are precisely sorted between the membranes of the ER, the Golgi, lysosomes, endosomes, autophagosomes, and the plasma membrane (Fig. 1). Nevertheless, all these processes have underlying general principles [5, 6]. Proteins contain specific signals in their primary sequences, which specify them for targeting to individual membranes. These signals (which in some cases resulted from posttranslational modification, or secondary structures) are typically recognized by targeting factors and

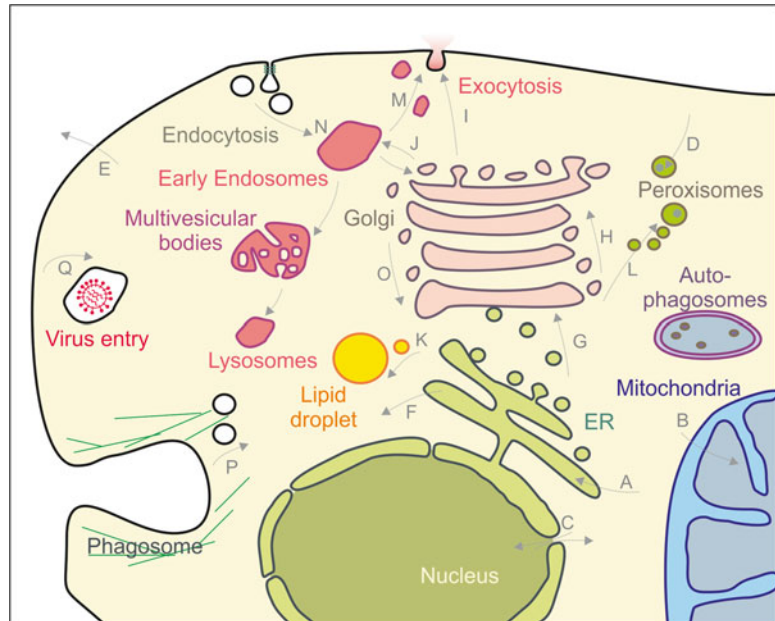


Fig. 1 Schematic representation of the processes of intracellular protein trafficking. The distribution of proteins in eukaryotic cells relies on the translocation of proteins across membranes (A–E) and the dynamics of membrane structures, particularly the coordinated exchange of vesicles (F–P). Whereas endosymbiont-derived organelles (mitochondria and chloroplasts) are presumably rather well-defined structures, all other cellular compartments form an intricately connected endomembrane system that is in constant flux thereby vibrantly exchanging lipid and protein constituents. Given the obvious complexity of these processes one can only wonder how intracellular communication in eukaryotic cells avoids a Babylonian confusion

chaperones which usher their cargo to appropriate translocases at the target membrane. These translocases mediate the transfer of the polypeptides across, or their insertion into the membrane of the target compartment, where proteins are then folded into their native three-dimensional structures with the help of chaperones. Proteins destined for the endomembrane system are initially translocated into the ER, where they accumulate at dedicated budding sites (ER exit sites; ERES) to embark on a journey along the secretory pathway [7–10]. Coat proteins that are recruited by small GTPases onto the surface of the ER mediate the budding of vesicles and are released before the vesicles fuse with the Golgi. The Golgi serves as a sorting station from which vesicles destined for various subcellular locations bud off. An individual protein might pass through several different sorting events before it reaches its final destination.

There are two fundamentally different transport processes that determine the final location of any given protein: (1) Translocation, which is the transport across or into the membranes of the ER or other membrane-bound cellular organelles, like mitochondria, chloroplasts, peroxisomes, and the nucleus and (2) Vesicle-mediated transport processes between cellular compartments. The information that sorts each polypeptide along its specific route is encoded primarily within its sequence. Many of the signals that address proteins to specific compartments of the secretory pathway are complex and still not well understood, as next to the classical linear motifs, multipartite signals also exist. Moreover, cargo receptors and cofactors are often employed that create rather case specific interaction surfaces. Therefore, these areas remain subjects of intensive research (Hong Toh et al., Chapter 17; Tham et al., Chapter 16; Gee et al., Chapter 11).

3 Protein Translocation

Only unfolded polypeptides can be translocated into the ER, mitochondria, and chloroplasts (Fig. 1, A, B). Therefore, translocation and protein synthesis often occur in a mechanistically and kinetically coupled process [11–13]. To allow the initiation of translocation before translation is completed, the targeting signals on these proteins are typically positioned on their very N termini [14]. As soon as these signals emerge from the ribosome, they are recognized by an array of factors, which contribute to the processing, folding and/or targeting of the newly synthesized proteins. Recent studies using ribosome-profiling or other high-throughput methods allowed the proteome-wide identification of the substrate specificity of the different ligands of the ribosomal exit tunnel [15–18]. Cargo is driven across the translocases in a process that often relies on the activity of chaperones, namely, that of members of the Hsp70 family [19–21]. Whereas the translocation machineries of the ER and of mitochondria are studied extensively (Peleh et al., Chapter 3; Becker et al., Chapter 2), those of the chloroplast envelope membranes are still poorly characterized. However, some recent studies have identified a large complex that presumably constitutes the translocase of the inner membrane of chloroplasts (TIC) [22, 23].

Nascent nuclear and peroxisomal proteins fold in the cytosol and, hence, cross the membrane in a folded conformation (Fig. 1, C, D). The same is apparently true for some proteins that are transported through the plasma membrane (Fig. 1, E) in a process called unconventional secretion (Miura and Ueda, Chapter 4). In the case of the nucleus, the nuclear pore complex is large enough to allow translocation of folded proteins and even of fully assembled ribosomes. The translocation across peroxisomal membranes, however, is still one of the big mysteries in cell biology. Interestingly,

some of its mechanistic principles are apparently shared with the ERAD pathway [24], which transports misfolded proteins from the ER to the cytosol to mediate their proteolytic degradation (Fig. 1, F).

4 Vesicular Transport

Along the secretory pathway, proteins are transported from the ER via the Golgi to lysosomes, endosomes, the plasma membrane or the extracellular space (Fig. 1, G–M). How transport to autophagosomes, peroxisomes, and lipid droplets is regulated is still a matter of debate, yet agreement has been reached on the involvement of the ER as a starting point. The retrograde pathway—the endocytic route—mediates the uptake of peptides, proteins, fluids, and nutrients from the extracellular space (Fig. 1, N, O). Moreover, the retrograde transport is critical for the recycling of components of the transport machinery. Whereas the transport processes in the early secretory pathway were studied in quite some detail, the reactions by which autophagosomes, multivesicular bodies, peroxisomes, and lipid droplets exchange their constituents with other compartments are still poorly understood and are intensely pursued in current investigations (Tan et al., Chapter 10; Gireud et al., Chapter 9; Wolinski and Kohlwein Chapter 21; Külzer et al., Chapter 5; Tooze et al., Chapter 12).

Most of these transport steps occur via dedicated vesicles, which serve as specialized transport carriers that shield their content from the cytoplasm. Transport vesicles are formed on a donor membrane by the concerted action of small GTPases of the Arf/Sar family and cytosolic coat proteins [25–28]. The coat proteins can interact selectively with cargo proteins, either directly (in case of membrane proteins) or via specialized membrane receptors [9, 29, 30]. These interactions in turn stabilize the soluble coat components on the membrane, so that accumulations of cargo on the luminal site of the membrane can induce the polymerization of the coat on the cytosolic site. The formation of such coat complexes lead to a deformation of the membrane either by their mechanical properties, or by the sequestration of certain lipids which promote the curvature of the membrane, or a combination of both mechanisms [31–34]. The membrane deforming capacity of coat proteins was first demonstrated in the laboratory of Randy Schekman by the formation of COPII-coated vesicles from synthetic, chemically defined liposomes [35]. Components of the COPII coat form ~70 nm vesicles at the ER membrane, which transport proteins to the Golgi apparatus. Similar activities were reported for COPI and clathrin coats, the two other major classes of coat complexes [36–38]. The vesicles are finally pinched off from donor membranes in a process which in some cases is facilitated by dynamin

family proteins, which specific membrane-binding GTPases [39–42]. In spite of the basic knowledge of the budding machinery, the regulation and adaptability of the process is still ill-understood (Sharpe and Brown Chapter 6; Kuser et al., Chapter 7; En Lin Chua et al., Chapter 18).

After their release from the donor membrane, vesicles can interact with motor proteins, which hook them to the cytoskeleton. Their transport on tracks of the cytoskeleton is critical for long-range transport such as from the ER to the Golgi and subsequently to the plasma membrane in mammalian cells like fibroblasts, and is particularly critical along axons and dendrites of neurons. Similarly, in fungi, vesicles from the Golgi are directed to the plasma membrane on a motor-driven transport route. The interaction of vesicles with motors is often dependent on coat proteins, which adds to the debate on the time when the coat comes off the transport vesicle. Three scenarios for the coordination of uncoating were suggested, which are not mutually exclusive. Firstly, the coat could start to peel off already while the vesicles form, and the small GTPases would only initiate the polymerization of such meta-stable coats [43, 44]. Secondly, the GTPase cycle may act as timer that releases the coat after a certain time period [45–47]. Finally, the coat may be removed just prior to the fusion reaction with the target membrane [48–51]. Vesicles are first recognized by so-called tethering proteins/complex presumably by the recognition of coat components. Tethers come in different flavors and at least one subclass may also act as a GEF for Rab GTPases, while another class may regulate SNARE complex formation. Clearly more work is required to understand the different modes of action (Cheung and Pfeffer, Chapter 14; Willett et al., Chapter 13). One point is generally accepted though: the coat has to be released from the vesicle before fusion can take place. The uptake of large particles by phagocytosis and the entry of many viruses into cells (Fig. 1, P, Q) does not rely on the action of classical coats, such as clathrin, and is mechanistically linked to actin polymerization and movements of the cytoskeleton (Mantegazza and Marks, Chapter 15; Lipovsky, Chapter 30).

Albeit critical for the formation of vesicles, cargo itself does not directly determine the target compartment of a given transport vesicle. The selectivity is at least in part provided by cognate pairs of SNAP receptor proteins on the vesicle (v-SNARE) and on the target membrane (t-SNARE) [52–57]. The achievement of a reconstituted fusion assay using proteoliposomes of defined composition clearly showed that at least *in vitro*, SNARE proteins alone are sufficient to initiate membrane fusion [58]. *In vivo* the system is much more complex and many additional layers of selectivity regulate the fusion processes. It appears unlikely that *in vivo* vesicles try to fuse with all the compartments they encounter until they finally find the right compartment with the matching t-SNAREs.

In some systems, proximity of donor and acceptor organelle alleviates this problem. For example *cis*-Golgi is present in close proximity (about 150 nm) to ER exit sites in yeast, *C. elegans*, and *D. melanogaster* [8, 59–61]. Given that an ER-derived COPII vesicle is about 70 nm in diameter, targeting is easily achieved by diffusion and subsequent engagement with tethering factors. However, how long-range transport, in particular to the plasma membrane is regulated remains poorly understood and predicted regulatory factors are still missing (Beerepoot et al., Chapter 8). Proteomic and protein-protein interaction studies may greatly aid the identification and understanding the function of such regulators (Weber et al., Chapter 20; Gheiratmand et al., Chapter 29). In most in vitro systems used to study intracellular transport, such components may not be essential, and could therefore be missed. It also remains unclear how SNARE proteins are included into vesicles and how t-SNAREs get to their final compartment without causing fusion along the way. It has been shown that some SNARE proteins can interact directly with the COPII component Sec24, and SNARE proteins can interact at least in vitro with COPI components in an ArfGAP-dependent manner [62, 63]. These findings led to the proposal that SNAREs might be the starting point upon which to assemble coat proteins and initiate the formation of a vesicle [44, 64]. However, how SNAREs are distributed into post-Golgi departments is not satisfactorily understood to date. More sophisticated methods will have to be employed to shed light on these important issues.

5 Future Directions

Most of what we know about protein translocation is deduced from the study of a small number of model proteins that were analyzed in a few model organisms. In order to generalize these findings, it will be very important to base our knowledge on a broader ground. The advent of the postgenomic era now allows the analysis of membrane trafficking processes on genome- and proteome-wide scales. For example, the use of yeast knockout collections offers a straightforward and cheap strategy to identify the full complement of components required for a given process. A wonderful example for the influence of such a screen is the study by the laboratory of Bill Wickner, which identified in one study 137 genes that are needed for normal vacuole size and copy number in growing cells [65]. Similarly, siRNA libraries to knockdown individual proteins in tissue culture cells have increased the repertoire of regulatory factors known to date. The use of such yeast knockout arrays and reverse genetic methods is described in several chapters in this book (Dalton et al., Chapter 27; Bard et al., Chapter 28; Gheiratmand, Chapter 29; Lipovsky et al., Chapter 30; Gaziova et al., Chapter 26).

A second novel spike for translocation research came from the identification of entire proteomes of individual cellular compartments. For example, the analysis of the mitochondrial proteome [66] initiated a boom in the search for novel components, which significantly improved our understanding of the composition and function of mitochondrial protein translocases. Comprehensive proteomic studies have been recently published for most compartments of animal, yeast, or plant cells [67–76].

The impact of systems biology on our current understanding of how the cell works is invaluable. Nevertheless, without the detailed analysis of the molecular structure and function of individual components all “OMICS” approaches would only provide inventory lists and interaction networks. Therefore, a thorough characterization of specific factors identified is more important than ever. In addition to classical biochemical and cell biological strategies which are outlined in many chapters of this book, insights into the structure of proteins have shown to be very helpful. An excellent example is the solution of the structure of the Sec translocase by the group of Tom Rapoport [77] which significantly inspired the entire field of protein translocation. In addition to crystallization studies the use of cryo-electron microscopy and modern spectroscopic strategies allowed stimulating insights into the structure of translocation complexes (for examples *see* ref. 78–80). Also the vesicle budding field has greatly benefited from recently published structures like those of the coat components [30, 32, 62, 81, 82]. These studies provided the first evidence for a direct interaction of SNARE proteins with a subunit of the COPII coat.

Two major findings revolutionized the way cell biologist conduct their experiments today: the discovery of green fluorescence protein as a localization tool and the applicability of siRNA knock-downs and CRISP-Cas9 knockouts in different experimental systems [83–85]. These technologies combined with high-throughput automated microscopy have started and will continue to increase the knowledge on the communication between and the dynamics of intracellular organelles tremendously. Moreover, super-resolution microscopy and now the reliable use of correlative microscopy will further enhance understanding of different intracellular transport pathways (MacDonald et al., Chapter 19; Cautain et al., Chapter 22; Baumann et al., Chapter 24; Mironov and Beznoussenko, Chapter 23; Hirschmann et al., Chapter 25). All the puzzle pieces contributed by the different strategies of systems biology, biochemistry, cell biology, genetics, and structural biology promise—when combined into one big picture—an exciting full view on the processes by which eukaryotic cells achieve the intracellular trafficking of their constituents.

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Part I

Biochemical and Molecular Genetics Approaches

Chapter 2

In Vitro Analysis of the Mitochondrial Preprotein Import Machinery Using Recombinant Precursor Polypeptides

Dorothea Becker and Wolfgang Voos

Abstract

The import of proteins into mitochondria represents an essential process for the survival of eukaryotic cells. Most mitochondrial proteins are synthesized as cytosolic precursor proteins. A complex chain of reactions needs to be followed to achieve a successful transport of these precursors from the cytosol through the double membrane system to their final destination inside the mitochondria. In order to elucidate the details of the translocation process, in vitro import assays have been developed that are based on the incubation of isolated active mitochondria with natural or artificial precursor proteins containing the appropriate targeting information. Using this basic system, most of the protein components of the import machinery have been identified and functionally characterized. However, a detailed definition of the molecular mechanisms requires more specialized assay techniques. Here we describe modifications of the standard in vitro import assay technique that are based on the utilization of large amounts of recombinant preprotein constructs. The application of saturating amounts of substrate preproteins is a prerequisite for the determination of translocation kinetics and energy requirements of the import process. Accumulation of preproteins as membrane-spanning translocation intermediates further provides a basis for the functional and structural characterization of the active translocation machinery.

Key words Mitochondria, *Saccharomyces cerevisiae*, Protein import, Precursor proteins, Import kinetics, Translocation intermediates

1 Introduction

According to proteomic studies, yeast mitochondria contain roughly 1,000 different proteins [1]. Only about 1 % of these proteins are encoded by the mitochondrial genome. Consequently, the vast majority of proteins has to be imported into the organelle after their synthesis on cytosolic ribosomes (*see* Fig. 1a). To ensure their proper destination, mitochondrial proteins are generated as precursor polypeptides carrying specific targeting signals. Most matrix-destined precursors, as well as monotopic inner membrane proteins possess N-terminal, typically cleavable presequences. Polytopic outer and inner membrane proteins, by contrast, carry internal signals that are usually distributed throughout the mature protein.

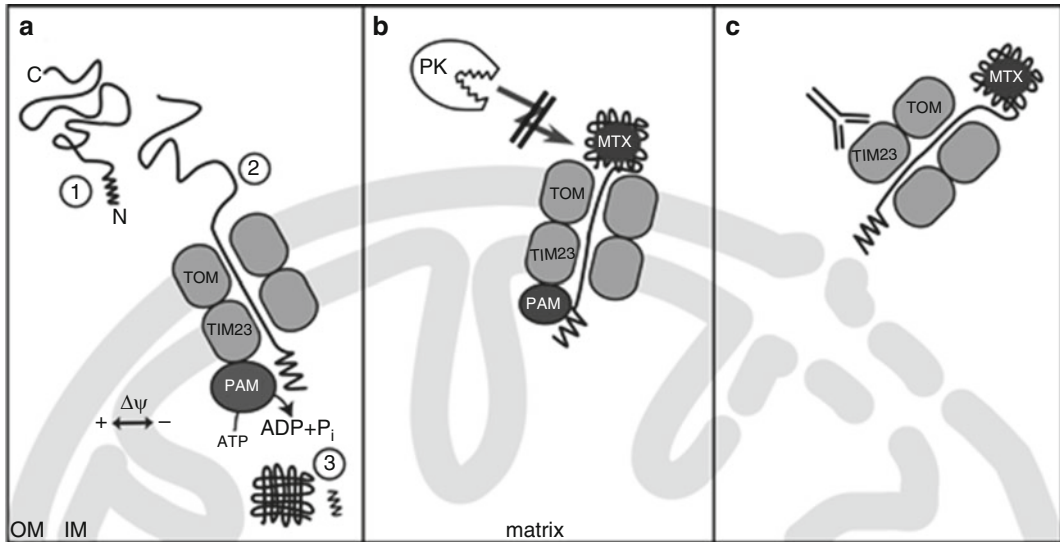


Fig. 1 Principles of the analysis of mitochondrial preprotein import. **(a)** Import of a presequence-containing protein into the mitochondrial matrix. Matrix-targeted preproteins contain N-terminal signal sequences (1) that are recognized by receptors on the outer face of the outer mitochondrial membrane. The preprotein is inserted into the outer membrane by the translocase of the outer membrane (TOM complex), and subsequently handed over to the translocase of the inner membrane (TIM23 complex), where the presequence is inserted in a reaction dependent on the electric potential $\Delta\psi$ (2). Completion of matrix-import requires the mHsp70-containing import motor (PAM) that couples protein import with ATP hydrolysis. Cleavage of the presequence and folding of the protein occurs in the matrix, resulting in the mature size protein in its native state (3). **(b)** Generation of protease-resistant translocation intermediates using preproteins with a C-terminal dihydrofolate reductase (DHFR) domain. Addition of the specific ligand methotrexate (MTX) stabilizes the folding state of the DHFR-domain, thereby preventing its translocation. The N-terminal b_2 -part crosses both membranes and contacts the import motor. Stable folding of the DHFR domain inhibits proteolysis by externally added Proteinase K (PK) when the translocation machinery is active. **(c)** Isolation of the MTX-generated TOM-TIM supercomplex by use of detergent. Addition of digitonin solubilizes the mitochondrial membranes, whereas the protein complex remains stable and can be extracted intact. Detection of the complex can be done by use of anti-Tim23 antibodies. OM: outer membrane; IM: inner membrane

To date, at least five distinct import pathways into the different mitochondrial subcompartments have been identified, all comprising specialized protein components organized as “translocase” complexes [2]. So far, recombinant preproteins have been employed to analyze the targeting pathway into the mitochondrial matrix compartment, also termed “the presequence translocase pathway.” The first step of the preprotein import process into the matrix is the specific recognition of the targeting information by receptor proteins at the mitochondrial surface. This reaction also governs the insertion of preproteins into the Translocase of the Outer mitochondrial Membrane (TOM complex) which represents the general entry pore for essentially all nuclear-encoded proteins. As a second step, translocation across the inner membrane is mediated by the Translocases of the Inner Membrane, the TIM23 complex, typically

also called presequence translocase [2]. Proteins destined for the matrix require a membrane potential across the inner membrane as an initial energy source for insertion of the N-terminal presequence into the inner membrane pore provided by the TIM23 complex. Completion of matrix import furthermore requires chemical energy in the form of ATP-hydrolysis, catalyzed by the matrix heat shock protein of 70 kDa (mtHsp70, Ssc1 in yeast), which provides the driving force for the unfolding of preprotein domains and the translocation of the bulk polypeptide chain across the membrane [3]. MtHsp70 represents the core component of the Presequence-Translocase-Associated Motor (PAM). The motor complex is recruited to the inner face of the inner membrane import complex by an interaction with the peripheral membrane protein Tim44. Further essential PAM-components are the nucleotide exchange factor Mge1 (*Mitochondrial GrpE* homolog), as well as the ATPase-stimulating J-domain protein Pam18, and its partner Pam16. In the matrix, the presequence is cleaved off (often by the *matrix-processing peptidase*, MPP), resulting in the mature-sized protein. Folding of imported proteins is initially promoted by mtHsp70, which stabilizes, and thereby protects unfolded proteins until they acquire their native conformation (assisted by the Hsp60 chaperonin system) or in the case of multimeric proteins, assemble into the respective protein complexes [4].

Due to its convenience, especially with regards to genetic manipulation, the yeast *Saccharomyces cerevisiae* represents an ideal model organism for the study of the mitochondrial biogenesis processes typical for eukaryotic cells. Although research in the recent years has revealed significant additional functions of mitochondria in mammalian cells compared to yeast, the preprotein import machineries are remarkably conserved. An experimental analysis of the mitochondrial protein import first requires the isolation of functionally intact mitochondria, as well as an assessment of their structural integrity. The isolation of yeast mitochondria includes an enzymatic digestion of the cell wall, followed by a mechanical disruption of the resulting spheroblast. Enrichment of the mitochondrial fraction is achieved by subsequent differential centrifugation of the crude cell lysate. The structural integrity of the mitochondrial preparation is assessed by checking the membrane potential across the inner mitochondrial membrane, which is of particular importance for the comparative analysis of mitochondria derived from different yeast strains. The assay described here is based on the reversible interaction of a fluorescent dye with membranes in a membrane potential-sensitive manner [5, 6]. Secondly, an appropriate precursor protein, consisting of a mitochondrial signal sequence and a reporter component has to be generated. The precursor can be either synthesized as a radiolabeled polypeptide by translation in a cell-free system, or isolated from *E. coli* cells as a recombinant protein. The import reaction *in vitro* is finally performed by combining intact mitochondria with precursor polypeptides

under appropriate buffer conditions. For the import reaction, mitochondria are additionally energized by ATP, as well as NADH, which represents the main substrate of the respiratory chain in yeast. A successful translocation reaction is typically monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyzing the shift in relative molecular mass generated by the removal of the targeting sequence by the matrix processing peptidase (MPP) [7]. Information about the completion of polypeptide translocation can be gained by assessing the resistance of the processed preproteins against externally added proteases, which cannot reach the fully imported protein molecules in intact mitochondria.

Precursor synthesized by in vitro translation or generated by heterologous expression are functionally equivalent but their different properties have major implications for the import reaction. Generally speaking, the in vitro synthesis of a radiolabelled precursor in a cell-free system is more convenient and less time-consuming. It is ideally performed in commercially available rabbit reticulocyte lysate, supplemented with [³⁵S]methionine and/or [³⁵S]cysteine, after in vitro transcription from appropriate template DNA using the SP6 or T7 RNA polymerase system (*see* Chapter 27). Template DNA for in vitro transcription can be conveniently generated by PCR reactions directly from yeast genomic DNA since most yeast genes contain no intron sequences. Using this method almost any polypeptide or open reading frame can be tested for its mitochondrial import properties [7]. However, experiments using radioactive precursor proteins, which are usually produced in substoichiometric amounts, only permit a qualitative analysis of the import reaction. In order to apply more stringent conditions, or to obtain reliable kinetic data, saturating amounts of precursor polypeptides have to be used. Recombinant preproteins obtained by expression and purification from *E. coli* cells have been shown to be import competent even in the absence of cytosolic cofactors and are virtually unlimited in their amounts [8]. Using saturating amounts of precursor proteins, conditions can be achieved that represent the maximal rate of the mitochondrial import reaction ($V_{\max}$). These conditions are a prerequisite for the quantitative characterization of the preprotein translocation kinetics [8]. Besides, applying excess preprotein may accentuate potential import defects in mutant forms of individual components of the import machinery.

This chapter describes the expression and isolation, as well as in vitro import of a fusion protein, consisting of a modified mitochondrial presequence derived from the yeast protein cytochrome (b_2), and a heterologous passenger protein (mouse dihydrofolate reductase, DHFR). These so called b_2 -DHFR fusion proteins and their variants have been routinely used in in vitro import analyses with yeast mitochondria [9, 10]. The use of a heterologous protein moiety allows one to take advantage of the highly specific antigen–antibody recognition for analysis of the import reaction,

as interference from endogenous proteins is bypassed. Instead of DHFR other heterologous protein domains or peptide tags can be utilized at the C-terminal end of the respective fusion proteins, provided an appropriated mitochondrial targeting signal is inserted at the N-terminal end. In our lab, we have also successfully employed Strep- and SNAP-tag fusions proteins. An important issue is the availability of a specific and sensitive antiserum for the immunodetection of the respective tag by Western blot. Previous experiments indicated a maximal import rate of about 25 pmol protein per min per mg mitochondrial protein [8], at least under the conditions described here. The absolute amount of imported polypeptides is therefore rather small and has to be reliably detected over the background of the complete mitochondrial proteome.

Based on the import of precursor proteins carrying a DHFR-domain, the mitochondrial protein import can be analyzed in greater detail by the generation of specific translocation intermediates (*see* Fig. 1b). To that end, the DHFR-ligand Methotrexate (MTX) is employed, which strongly stabilizes the native folding state of the DHFR domain. The full translocation of preproteins containing a C-terminal DHFR moiety is thereby prevented while the insertion of N-terminal segments into the translocation channel is not affected [11, 12]. Thus, the precursor accumulates in an intermediate state in which the N-terminal b_2 -part contacts the TIM23 complex (also termed Presequence Translocase), whereas the C-terminal DHFR-domain remains in the cytosol [9, 13]. Since these intermediates represent preproteins engaged in active translocation, they can be used for an assessment of the inward-directed translocation force exerted by the import motor. Provided that the mtHsp70-containing PAM is functional and active, the preprotein is stably held in the intermediate complex, whereas it is lost in cases of a motor defect [14]. Since the preprotein spans both translocation complexes (TOM and TIM23), a physical connection between the machinery of the outer and inner membrane is generated that allows the extraction and analysis of the whole translocation machinery in its active state [13], termed the TOM-TIM supercomplex (*see* Fig. 1c). Formation of this supercomplex is typically analyzed by Blue Native PAGE (BN-PAGE) that allows for the electrophoretic separation of multimeric proteins in their native state [15].

2 Materials

Prepare all buffers in distilled water.

2.1 Preparation of Functional Mitochondria from Yeast Cells

1. DTT softening buffer: 100 mM Tris/ H_2SO_4 pH 9.4; 10 mM DTT.
2. Zymolyase buffer: 20 mM KPi pH 7.4; 1.2 M Sorbitol.

3. Homogenizing buffer (prepare fresh, 4 °C): 10 mM Tris/HCl pH 7.4; 0.6 M Sorbitol; 0.5 % BSA; 1 mM PMSF (0.5 M (500×) Stock: 87 g/ml in EtOH).
4. SEM buffer: 250 mM sucrose; 10 mM MOPS/KOH pH 7.2; 1 mM EDTA; add 1 mM phenylmethanesulfonyl fluoride (PMSF) (from freshly prepared 0.5 M or 500× stock solution) where indicated.

2.2 Measurement of the Mitochondrial Membrane Potential by Fluorescence Spectrometry

1. Isolated mitochondria in SEM buffer (*see* Subheading 2.1): 10 mg/ml protein, freshly prepared or frozen and thawed *on ice*.
2. 1 M KP_i , pH 7.2: Prepare 50 ml each of 1 M KH_2PO_4 and of K_2HPO_4 , mix solutions by adding the appropriate volume of KH_2PO_4 (high pH) to 50 ml of K_2HPO_4 (low pH), until the required pH 7.2 is reached.
3. Potential buffer: 0.6 M sorbitol, 0.1 % (w/V) bovine serum albumin (BSA, for molecular biology, e.g., *Roche*), 10 mM $MgCl_2$, 20 mM KP_i , pH 7.2.
4. 3,3'-dipropylthiadicarbocyanine iodide ($diSC_3(5)$, *Molecular Probes, Life Technologies*), prepare fresh as 1 mM stock in EtOH, wrap tube in aluminum foil as the dye is light sensitive.
5. 100× inhibitor mix: 800 μ M antimycin A (blocks electron transfer within complex III of the respiratory chain), store as 8 mM stock in EtOH p.a. at 20 °C, 50 μ M valinomycin (dissipates membrane potential by acting as K^+ -ionophore), store as 1 mM stock in EtOH p.a. at -20 °C, 2 mM oligomycin (inhibits F_1 subunit of F_1F_0 -ATP synthase), store as 10 mM stock in EtOH p.a. at -20 °C; for preparation of 1 ml 100× working solution, mix 100 μ l of antimycin A stock with 50 μ l of valinomycin stock and 200 μ l of oligomycin stock in 650 μ l EtOH p.a., store at -20 °C, stable for years.
6. 100× valinomycin: dilute 50 μ l of valinomycin stock in 950 μ l EtOH p.a., store at -20 °C, stable for years.
7. Substrates: 1 M succinate in H_2O , pH 8.5 (L-succinic acid, di-sodium salt), 1 M malate in H_2O , pH 8.0 (L-malic acid, di-sodium salt), prepare 1 ml each, store at -20 °C, stable for years.
8. Fluorescence spectrometer (e.g., *AMINCO-Bowman*), Cuvette (1-cm-pathlength) for fluorescence measurements (e.g., *Hellma 101-OS*).

2.3 Preparation of Cytochrome b_2 -DHFR Precursor Proteins from *E. coli*

1. LB-medium: 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl.
2. Isopropyl- β -D-thiogalactopyranoside (IPTG): prepare a 1 M stock and store at -20 °C, stable for years.
3. Pre-lysis buffer: 30 % sucrose, 20 mM KP_i , pH 8.0, 1 mM EDTA, 10 mM DTT (*see* Note 1), 1 mM PMSF (dissolve in

EtOH p.a., keep on ice once dissolved; *see* **Note 2**); precool to 4 °C before use.

4. Buffer A: 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 10 mM DTT (*see* **Note 1**), 1 mM PMSF (*see* **Note 2**), protease-inhibitors (1.25 µg/ml leupeptin, 2 µg/ml antipain, 0.25 µg/ml chymostatin, 0.25 µg/ml elastinal, 5 µg/ml pepstatin, e.g., “Complete EDTA-free,” *Roche*); add 1 mg/ml lysozyme, 0.1 % Triton X-100 (keep as 10 % (V/V) solution at room temperature), and 0.1 mg/ml DNase, as well as 50 % glycerol, as indicated; precool to 4 °C before use.
5. Buffer B: Buffer A plus 1 M NaCl.
6. Sonifier (e.g., *Branson*).
7. Cellulose acetate membranes, 0.22 µm pore size (e.g., *Millipore*).
8. MonoS HR 10/10 cation-exchange column for FPLC, 6 ml (e.g., *GE Healthcare*).
9. Low Molecular Weight-SDS Calibration Kit for electrophoresis (e.g., *GE Healthcare*) consisting of marker proteins for determination of absolute protein amounts.

2.4 In Vitro Import of Recombinant Precursor Proteins into Isolated Mitochondria

1. Isolated mitochondria in SEM buffer: 10 mg/ml protein content, freshly prepared or thawed *on ice*.
2. Preprotein to be imported, freshly prepared, or thawed on ice.
3. Import buffer: 3 % (w/V) fatty-acid free bovine serum albumine (BSA), 250 mM sucrose, 80 mM KCl (*see* **Note 3**), 5 mM MgCl₂, 2 mM KH₂PO₄, 10 mM MOPS-KOH, pH 7.2, store in 10–20-ml aliquots at –20 °C.
4. 0.2 M NADH, prepare fresh, keep solution on ice.
5. 0.2 M ATP, store as stock solution after pH adjustment to 7.0 with 5 M KOH in working aliquots at –20 °C, stable for years, do not refreeze.
6. 1 M KP_i (*see* Subheading 2.2).
7. 100× inhibitor mix (*see* Subheading 2.2).
8. 100× valinomycin (*see* Subheading 2.2).
9. 0.1 M PMSF (*see* **Note 2**).
10. SEM buffer (*see* Subheading 2.2).
11. 2.5 mg/ml Proteinase K (PK, specificity: hydrophobic and aromatic residues), dissolved in SEM buffer, store in working aliquots at –20 °C, stable for years.
12. Import samples need to be incubated on ice for various experimental purposes; we use metal blocks with holes corresponding to the diameter of 1.5 ml reaction tubes, placed on ice.

2.4.1 Import of Urea-Denatured Precursor Proteins

1. Cold-saturated ammonium sulfate, pH 7.2; store at 4 °C, stable for years.
2. Urea buffer: 7 M urea, 30 mM MOPS-KOH, pH 7.2, 1 mM DTT (*see Note 1*), prepare fresh.

2.5 Analysis of the Import Reaction

1. Standard materials required to perform SDS-PAGE and Western blot.
2. Highly specific antiserum against DHFR (or against other utilized protein tags).
3. Quantification of Western blot signals by image analysis software (i. e. ImageJ).

2.6 Dissection of a Functional Import Intermediate on Blue Native PAGE

1. Gel preparation and running settings for Blue Native PAGE.
 - (a) ATP-regenerating system: creatine kinase, 10 mg/ml, prepare fresh; creatine phosphate, store as 1 M stock at -20 °C, stable for years.
 - (b) 5 % digitonin in water, prepare a 5 % stock solution in water (*see Note 4*), keep at 4 °C, stable for months.
 - (c) Solubilization buffer: 1 % digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10 % glycerol, 1 mM PMSF (*see Note 4*). Prepare a 2× solution w/o digitonin, and add detergent prior to use.
 - (d) Acrylamide solution: 49.5 % Acrylamide (e.g., *Roth*), 1.5 % bis-acrylamide, store at room temperature in the dark, stable for months.
 - (e) 3× gel buffer: 200 mM ϵ -amino-*n*-caproic acid, 150 mM bis-tris/HCl, pH 7.0, store at 4 °C, stable for months.
 - (f) 10× Loading Dye: 5 % Coomassie blue G250 (water soluble, whereas Coomassie R is not), 500 mM ϵ -amino-*n*-caproic acid, 100 mM bis-tris/HCl, pH 7.0. Prepare 10 ml and store at 4 °C, stable for months.
 - (g) 10× anode buffer: 500 mM bis-tris/HCl, pH 7.0, store at 4 °C, stable for months; 1× solution can be used for up to three times.
 - (h) 10× cathode buffer: 500 mM tricine, 150 mM bis-tris/HCl, pH 7.0, prepare 1 L, divide into halves, and dissolve 0.2 % Coomassie blue G250 in 500 ml.
 - (i) Marker protein set (e.g., *Sigma-Aldrich*).
 - (j) Gradient mixer, peristaltic pump, cooled vertical gel electrophoresis system (e.g., SE 600 Series, *GE Healthcare*).

2. Generation of the TOM-TIM-Supercomplex.

Materials are the same as listed in Subheading 2.4 with the following variations:

- (a) Import buffer as described in Subheading 2.4 without BSA.
- (b) Methotrexate (MTX): keep as 10 mM solution in 0.1 M MOPS-KOH, pH 7.2, store at -20°C , stable for years; dilute in import buffer w/o BSA as indicated.

2.7 Determination of Matrix-Directed Import Forces Exerted by Hsp70

1. Isolated mitochondria in SEM buffer: 10 mg/ml in protein content, freshly prepared or thawed *on ice*.
2. Import buffer: *see* Subheading 2.4.
3. 0.1 mM Methotrexate in import buffer w/o BSA, store at -20°C , stable for years.
4. 0.2 M NADH, prepare fresh, keep on ice.
5. 0.2 M ATP (*see* Subheading 2.4).
6. 1 U/ μl apyrase, store at -20°C , stable for years.
7. 10 mM oligomycin (*see* Subheading 2.4).
8. ATP-regenerating system (*see* Subheading 2.6).
9. Buffer R1 ($-\text{ATP}$): 20 μM oligomycin, 5 μM MTX, in P80 w/o BSA, prepare fresh.
10. Buffer R2 ($+\text{ATP}$): 2 mM ATP, 20 mM creatine phosphate, 200 $\mu\text{g}/\text{ml}$ creatine kinase, 5 μM MTX, prepare fresh.
11. 100 $\times$ inhibitor mix (*see* Subheading 2.4).
12. 100 $\times$ valinomycin (*see* Subheading 2.4).
13. 0.1 M PMSF (*see* **Note 2**).
14. 2.5 mg/ml Proteinase K (PK, *see* Subheading 2.4).

Miscellaneous: Buffers, solutions, and equipment for SDS-PAGE, Western Blot, and Immunodecoration.

3 Methods

All centrifugation steps are to be carried out at 4°C , unless stated otherwise.

3.1 Preparation of Functional Mitochondria from Yeast Cells

1. Measure OD 660 nm of yeast culture: harvest ideally at 1.5–2.5 OD/ml.
2. Determine and note (!) weight of centrifugation bottles (i.e., 500 ml bottles for Beckman JA-10 rotor). Pellet cells for 5 min at $1,600\times g$ and RT. Discard medium and resuspend cell pellet in monodest. water. Fill up to 3/4 of total volume.
3. Centrifuge cell suspension again for 5 min at $1,600\times g$ and RT. Remove supernatant completely and determine wet weight of pellet.

4. Resuspend pellet in prewarmed DTT buffer, 2 ml/g pellet. Shake gently (about 100 rpm) for 20 min at 30 °C in Erlenmeyer flask.
5. Pellet cells for 5 min at $2,800\times g$. Discard supernatant and resuspend pellet in approx. 100 ml zymolyase buffer (without enzyme).
6. Pellet cells 5 min at $2,800\times g$. Resuspend pellet in prewarmed zymolyase buffer (7 ml/g pellet). Add 3 mg zymolyase per gram wet weight and incubate for 30 min at 30 °C.
7. Mix 50 μ l spheroblast suspension with 2 ml water for zymolyase test (shake vigorously: a clear suspension indicates complete digestion of the cell wall and formation of spheroblasts)—compare lysis efficiency with sample before zymolyase step.
8. Pellet spheroblasts for 5 min at $2,800\times g$. Wash pellet carefully with about 100 ml zymolyase buffer (without enzyme). Centrifuge for 5 min at $1,600\times g$.
9. Perform the following steps on ice.
10. Resuspend in homogenizing buffer 6.5 ml/g wet weight and homogenize 15–20 $\times$ (up and down=1 $\times$). Add additional homogenizing buffer (less than 100 ml total volume) and transfer to centrifuge tubes (i.e., 50 ml tubes for Beckman JA-25.50 rotor).
11. Centrifuge for 2 min at $300\times g$, save supernatant and directly spin again for 4 min at $1,100\times g$. Save supernatant again and centrifuge for 5 min at $2,000\times g$.
12. Save supernatant and centrifuge 15 min at $17,500\times g$, resuspend pellet carefully in 1 ml SEM buffer with blue tips. Rinse centrifugation tube with 1 ml SEM buffer.
13. Fill centrifugation tube (i.e., 50 ml tubes for Beckman JA-25.50 rotor) with resuspended mitochondria to 3/4 of total volume. Centrifuge for 5 min at $2,000\times g$. Save supernatant and centrifuge for 10 min at $17,500\times g$ to obtain a pellet containing the purified mitochondria.
14. Resuspend pellet in a small volume (1–2 ml) of SEM buffer. Determine total protein concentration and adjust mitochondria solution to 10 mg/ml protein (if possible).
15. Prepare aliquots of 200 or 400 μ l and snap-freeze in liquid N₂. Store at –80 °C

3.2 Measurement of the Mitochondrial Membrane Potential by Fluorescence Spectrometry

The assessment of the membrane potential ($\Delta\psi$) by use of a cyanine dye is based on the changes in fluorescence emission in response to a membrane potential [5, 6]. Upon addition of mitochondria to the diSC₃(5)-containing buffer, the dye is potential-dependently partitioned between mitochondria and surrounding medium.

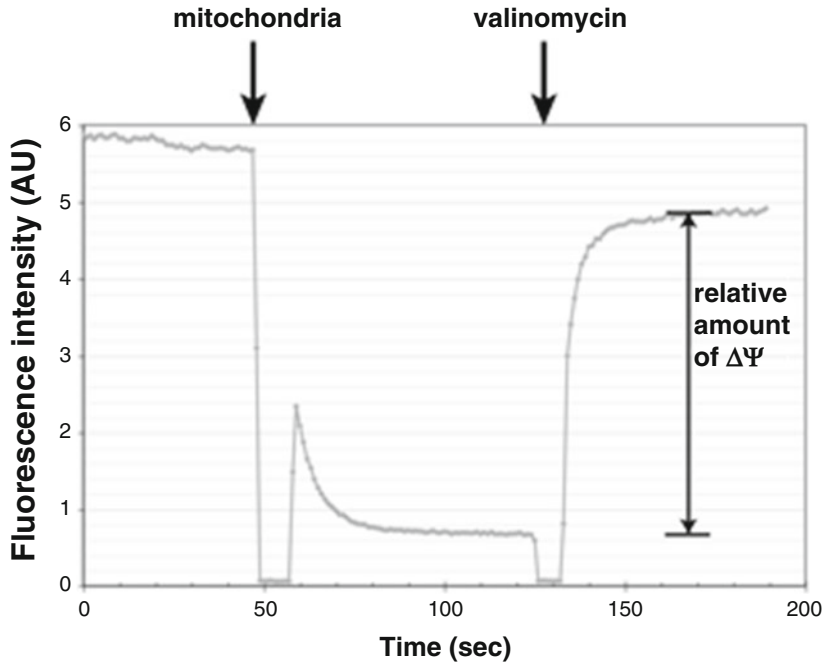


Fig. 2 Assessment of the membrane potential $\Delta\Psi$ by use of the fluorescent dye diSC₃(5). Mitochondria are added to diSC₃(5)-containing potential buffer where indicated. A decrease of the fluorescent signal is observed due to uptake of the dye by the mitochondria and subsequent quenching of the fluorescence. After the signal has stabilized, uncoupling of the respiratory chain and destruction of the membrane potential is achieved by addition of the K⁺-ionophore valinomycin. The relative amount of the membrane potential ($\Delta\Psi$) is reflected by the restoration of diSC₃(5)-fluorescence after addition of valinomycin

The fluorescence emission from mitochondria-associated diSC₃(5) is decreased (quenched) with increasing $\Delta\Psi$. This fluorescence quenching can be reversed by depolarization of the mitochondrial membranes. $\Delta\Psi$ is reflected by the restoration in diSC₃(5) fluorescence emission at 670 nm, in response to the addition of depolarizing (uncoupling) agents (*see Fig. 2*).

1. Add 3 ml of potential buffer to the cuvette.
2. At this step, you may need to add substrates to a final concentration of 5 mM each (*see Note 5*).
3. Add 3 μ l diSC₃(5), mix by covering the cuvette with a piece of laboratory film (e.g., *Parafilm*) and inverting once. Start your measurement using the following settings:
 - Excitation wavelength 622 nm.
 - Emission wavelength 670 nm.
 - Measuring period of 200–250 s.
 - Response interval 1 s.

4. When diSC₃(5) fluorescence has reached a stable value (~40–50 s) add 3–5 µl mitochondria, mix, and continue measurement until the decrease in fluorescence finishes.
5. Add 3 µl of 100× inhibitor mix to uncouple respiratory chain complexes, and trace fluorescent changes until the increase has reached its maximum again (*see Note 6*).

3.3 Preparation of Precursor Proteins from *E. coli*

Authentic cytochrome *b*₂ reaches the intermembrane space (IMS) of mitochondria via a “stop-transfer” mechanism that comprises the insertion of N-terminal segments into the matrix followed by a retranslocation and processing event in the IMS. The fusion proteins described here consist of the N-terminal 167 residues of the cytochrome *b*₂ precursor fused to the complete mouse DHFR [9, 10]. The *b*₂-sequence encompasses the complete mitochondrial targeting signal but lacks residues 47–65 of the intermembrane spacing sorting signal (*see Fig. 3a*). Thus, the resulting preprotein, named *b*₂(167)_Δ-DHFR, is targeted to the mitochondrial matrix instead of being exported to the intermembrane space.

For isolation of the preprotein in high amounts, *E. coli* cells expressing the construct are lysed and the protein is purified using cation-exchange chromatography. The protein is eluted by application of a salt gradient (0–0.5 M NaCl).

1. Grow *E. coli* cells expressing the fusion protein *b*₂(167)_Δ-DHFR at 37 °C in LB medium to an OD₆₀₀ of 0.8.
2. Add IPTG to a final concentration of 1 mM, and continue growth at 30 °C for additional 2–3 h.
3. Harvest cells by centrifugation at 3,500 × *g* for 8 min.
4. Wash cell pellet by resuspension in pre-lysis buffer and centrifugation as in **step 3**.
5. Resuspend cells in buffer A with 0.1 g cell pellet per ml lysis buffer. Destabilize cell walls by freezing the suspension in liquid N₂ and subsequent thawing at room temperature for two times. Add lysozyme to a final concentration of 1 mg/ml, and 0.1 mg/ml DNase, and incubate on ice for 10 min.
6. Add 0.1 % (V/V) Triton X-100 and solubilize cells on ice for 10 min.
7. Disrupt cells by sonication on ice: 3 × 20 pulses (chill cells on ice briefly between pulse intervals), 40 % duty cycle, micro tip setting 7.
8. Pellet cell debris by centrifugation at 15,000 × *g* for 20 min. Take samples of both pellet and supernatant (lysate) to analyze lysis efficiency via SDS-PAGE.
9. Pour crude cell lysate over a cellulose acetate filter membrane. Take sample of filtered lysate for SDS-PAGE analysis.

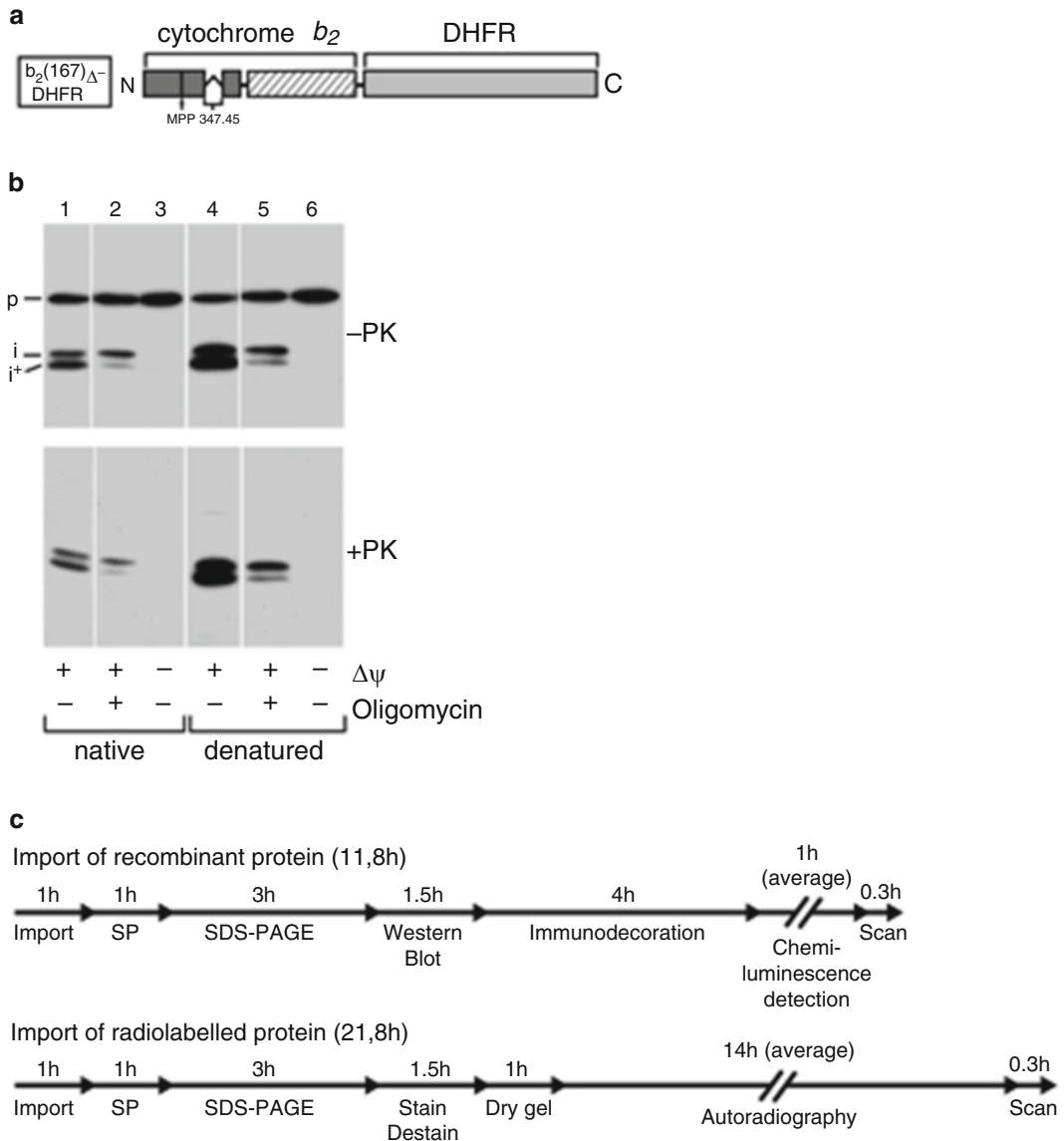


Fig. 3 Import of recombinant presequence-containing DHFR-fusion proteins. **(a)** Schematic drawing of reporter proteins $b_2(167)_\Delta$ -DHFR and $b_2(167)_\Delta$ -DHFR-SNAP representing fusion constructs of the N-terminal 167 residues of the cytochrome b_2 precursor and the complete mouse dihydrofolate reductase (DHFR). The b_2 precursor lacks residues 47–65 of the intermembrane space targeting signal. The cleavage site by the matrix processing peptidase (MPP) is indicated. The lower construct additionally contains the C-terminal SNAP-tag. The site for fluorescence labelling is indicated by an asterisk. **(b)** In vitro import of native (lanes 1–3) and urea-denatured (lanes 4–6) pre- $b_2(167)_\Delta$ -DHFR into the mitochondrial matrix in dependency of $\Delta\psi$. Lanes 1 and 4 represent a standard import reaction showing the appearance of processed, protease protected forms of the precursor protein. Lanes 2 and 5 show decreased protein import after inhibition of the F_1F_0 -ATP-synthase by oligomycin, which reduces the ATP concentration in the matrix compartment. In lanes 3 and 6, the membrane potential was dissipated by addition of the inhibitors antimycin A, valinomycin, and oligomycin, resulting in a complete block of the import reaction. Proteins were separated by SDS-PAGE, transferred to a PVDF-membrane, and immunodecorated with anti-mouse DHFR. The precursor (p) was processed twice (i, i*). In the lower panel, nonspecifically associated preproteins at the outer surface of mitochondria were degraded by treatment with Proteinase K (PK). **(c)** Timelines for import and analysis of recombinant and radiolabelled precursors. Given are the time periods for each individual step, as well as the overall time of the assays presented. SP: sample processing for SDS-PAGE

10. Load filtered lysate on a MonoS HR 10/10 cation exchange chromatography column. Take a sample of the flowthrough for SDS-PAGE.
11. Wash column with three column volumes of buffer A.
12. Elute column by addition of ten column volumes applying a NaCl gradient from 0–50 % (0.5 M NaCl). Collect eluate in 1-ml-fractions, and take a sample each for SDS-PAGE.
13. Check the efficiency of your purification by SDS-PAGE and Western Blot analysis using anti-mouse DHFR.
14. To the fractions containing the $b_2(\Delta)$ -DHFR fusion protein, add glycerol to a final concentration of 50 %, and snap-freeze them in small aliquots in liquid N_2 for storage at $-80^\circ C$.

3.4 In Vitro Import of Recombinant Precursor Proteins into Isolated Mitochondria

A standard import reaction into yeast mitochondria contains 2 mM ATP, 4 mM NADH, as well as 20 mM KP_i , and 25 μg of mitochondria in a total volume of 100 μl import buffer. Substrate-saturating conditions are reached by an addition of at least tenfold excess of recombinant preprotein over available import sites. Based on the assumption of 0.25 pmol import sites per μg of yeast mitochondria [9], this corresponds to 6.25 pmol preprotein for a standard reaction (in the case of the described preprotein $b_2(167)_\Delta$ -DHFR, about 240 ng per reaction). After terminating the import reaction by dissipation of the membrane potential, nonspecifically bound and non-imported precursors are degraded by the addition of Proteinase K. Further analysis requires Western transfer and immunodecoration, using anti-mouse DHFR antiserum. Figure 3b shows the results from in vitro import of $b_2(167)_\Delta$ -DHFR, gained by immunodecoration, under different import conditions. Figure 3c shows a comparison of the typical timelines of an import reaction utilizing radiolabeled versus recombinant preproteins.

1. Thaw 17 μl (170 μg) mitochondria and 7 μl ATP on ice.
2. Prepare an import master mix for 6.6 time points on ice (*see Note 7*; total volume: 654 μl , minus 6.6 $\times$ the amount of precursor added, 6.6 μl ATP, 13.2 μl NADH, 13.2 μl KP_i , pH 7.2, add 660 μl import buffer, minus the amount of precursor added. Add 16.5 μl (165 μg) mitochondria, mix carefully by inverting the tube and spin down at low speed for 2–3 s (*see Note 8*).
3. Divide your master mix into six portions of 100 μl , minus the amount of precursor added.
4. Dissipate the membrane potential in mitochondria of sample #6 by addition of 1 $\times$ inhibitor mix from the 100 $\times$ stock; add 1 μl EtOH p.a. to samples #1–5 (mock). Mix carefully by flicking the tubes with your index finger.
5. Pre-incubate samples at 25 $^\circ C$ for 2 min, add 240 ng precursor (corresponding to 6.25 pmol of $b_2(167)_\Delta$ -DHFR) to sample #6, mix, and leave at 25 $^\circ C$ for the entire import incubation period.

6. Initiate import reaction by addition of precursor to sample #5 (longest incubation period) and subsequent precursor addition to samples #4–1 (with #1 = shortest incubation period). Mix after addition by flicking the tubes.
7. Stop import reactions according to time scheme by addition of $1\times$ valinomycin from $100\times$ stock and moving samples on ice.
8. Prepare six fresh tubes (samples #7–12) with $1\ \mu\text{l}$ PK (f.c.: $50\ \mu\text{g}/\text{ml}$) in $50\ \mu\text{l}$ SEM buffer.
9. Add $50\ \mu\text{l}$ each of samples #1–6 to PK-containing samples #7–12 and incubate for 15 min on ice; add $50\ \mu\text{l}$ SEM buffer to samples #1–6 and likewise keep on ice.
10. Stop PK treatment by addition of 1 mM PMSF from 0.1 M stock (*see Note 5*); for equal treatment of all samples, add PMSF to non-PK-treated samples as well.
11. Keep samples on ice for other 10 min, before pelleting mitochondria by centrifugation at $12,000\times g$ for 10 min.
12. Remove supernatants carefully, wash mitochondrial pellet by addition of $200\ \mu\text{l}$ SEM buffer containing 1 mM PMSF, and centrifugation at $12,000\times g$ for 8 min.
13. After removal of supernatants denature mitochondria in $1\times$ Laemmli buffer.

3.4.1 Import of Urea-Denatured Precursor Proteins

The import of precursor proteins in a denatured state can be useful for the analysis of mitochondrial import defects in more detail. Denaturation of the preprotein to be imported facilitates its import, as the import reaction is no longer dependent on the functionality of the mtHsp70 system for unfolding. ATP-driven precursor unfolding is of particular importance for the import of tightly folded proteins or protein domains. Thus, comparison of the import efficiencies of both natively folded and denatured preproteins in mitochondrial preparations from different strains can have implications both for the tertiary structure of the preprotein imported, and for the mtHsp70 protein (un)folding capacity.

1. Thaw the preprotein to be imported on ice.
2. Add three volumes of cold-saturated $\text{NH}_4(\text{SO}_4)_2$ and mix by vortexing at low speed.
3. Incubate on ice for 30 min with occasional vortexing.
4. Centrifuge precipitates down at $20,000\times g$ for 15 min.
5. Aspirate supernatant carefully, and resuspend protein pellet in the required volume of urea buffer by shaking at room temperature on a thermomixer at maximum speed for 15 min.
6. Pellet non-denatured protein material by centrifugation at $20,000\times g$ for 5 min.

7. Transfer supernatant to a fresh tube and keep on ice until use.
8. Perform import reaction essentially as described in Subheading 3.4.

3.5 Analysis of the Import Reaction

1. Run samples on a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel with a resolving gel acrylamide concentration of 12 %. Always run three control lanes with 1, 2 and 5 % of the preprotein amount used in the assay for quantification.
2. Transfer proteins to a Western Blot membrane (e.g., *Millipore*).
3. Immunodecorate with antibodies directed against mouse DHFR.
4. Detect signals using peroxidase-coupled secondary antibodies and enhanced chemiluminescence.
5. Evaluate raw data with image analysis software (*see* Subheading 3.4.1).

Evaluation of import kinetics can be done by quantification of protein bands using standard image analysis software (i.e., ImageJ) provided that the signals have not saturated. Band intensities in samples not treated with protease provide information on processing efficiencies, while determination of the efficiency of complete import is done from the protease-treated samples. Use the control lanes as references for protein amounts.

3.6 Dissection of a Functional Import Intermediate on Blue Native PAGE

The ligand methotrexate (MTX) stabilizes the tertiary structure of the DHFR domain and thereby prevents translocation of fusion proteins containing a C-terminal DHFR domain across the mitochondrial membranes [11, 12]. When $b_2(\Delta)$ -DHFR is imported in the presence of MTX, it accumulates in a translocation intermediate that spans both mitochondrial membranes. Determination of the constituents of this complex by Blue Native gel electrophoresis (BN-PAGE) revealed the presence of TOM-components, as well as the core components of the TIM23 complex of the inner membrane. The translocase complexes are connected to each other by the preprotein in transit resulting in the formation of a large multiprotein complex comprising both outer and inner membrane components [9, 13]. Formation of the TOM-TIM-supercomplex is dependent on the membrane potential across the inner membrane, and results in an almost quantitative shift of the TIM23 core subunits from a size of ~90 kDa to a size of ~600 kDa, on BN-PAGE (*see* Fig. 4a).

The accumulation of $b_2(167)_\Delta$ -DHFR in import sites is achieved by pre-incubation of the preprotein in the presence of 5 μ M MTX, and addition of MTX in the import reaction, which results in the stable arrest of the preprotein inside the import channels.

Formation of the supercomplex can be monitored by Western blot using anti-Tim23 antiserum. BN-PAGE is specifically suited

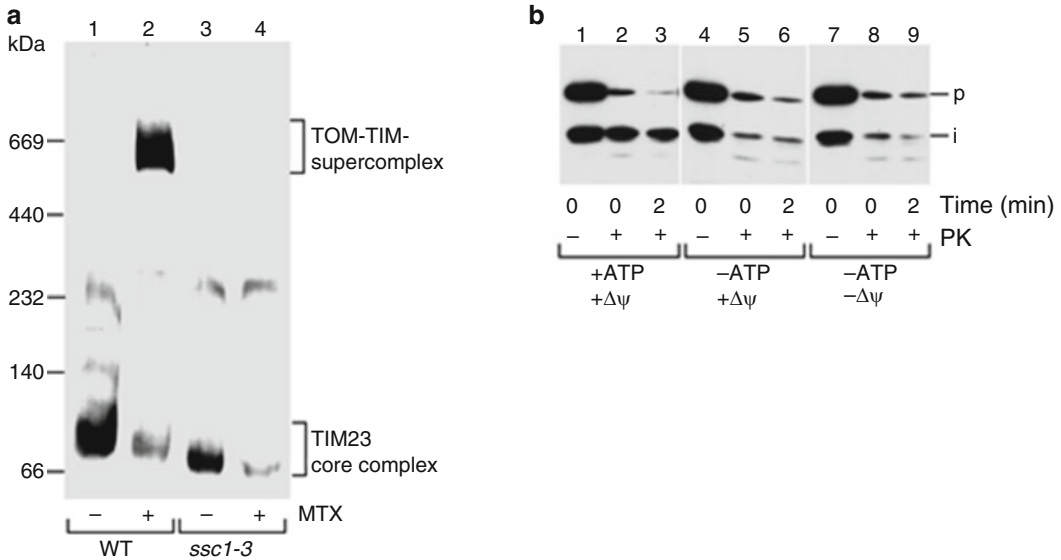


Fig. 4 Generation and analysis of import intermediates. **(a)** Dissection of the TOM-TIM supercomplex on Blue Native gel electrophoresis (BN-PAGE). Wild type (WT; *lanes 1 and 2*) and mtHsp70 mutant (*ssc1-3*; *lanes 3 and 4*) mitochondria were incubated with pre- $b_2(167)_\Delta$ -DHFR in the presence (*lanes 2 and 4*) or absence (*lanes 1 and 3*) of 5 μ M Methotrexate (MTX). Addition of MTX resulted in the accumulation of the preprotein in a location spanning both mitochondrial membranes. Mitochondria were extracted with the mild detergent digitonin and protein complexes resolved by BN-PAGE under native conditions. Analysis by Western blot using anti-Tim23 antibodies indicates the formation of the TOM-TIM23 supercomplex at a size of ~600 kDa only in the presence of MTX (compare *lanes 1 and 2*). The temperature-sensitive mtHsp70-mutant *ssc1-3* is not able to support the completion of the translocation at nonpermissive conditions. Hence, the supercomplex is not observed in *ssc1-3* mutant mitochondria (see *lanes 3 and 4*) **(b)** Assessment of the inward-directed import force exerted by mtHsp70. Mitochondria were incubated in the presence of pre- $b_2(167)_\Delta$ -DHFR and 5 μ M MTX for 10 min. A sample was taken directly after the incubation (*lanes 1, 4, and 7*). Another sample was taken directly after Proteinase K (PK) treatment (*lanes 2, 5, and 8*). A third sample was taken after a 2 min incubation following PK-treatment (*lanes 3, 6, and 9*). In the presence of a membrane potential (+ $\Delta\psi$) and high ATP levels in the matrix (+ATP), an active import motor rendered 20–30 % of the accumulated intermediates resistant to protease treatment (*lanes 2 and 3*). Depletion of the membrane potential (– $\Delta\psi$) or removal of ATP (–ATP) inactivated the translocation machinery resulting in a rapid and complete loss of protease resistance

for the analysis of membrane bound protein complexes [15]. The BN technique includes the solubilization of mitochondria by the non-ionic detergent digitonine (see **Note 9**), and subsequent substitution of the detergent for water-soluble Coomassie blue. Thereby, proteins become negatively charged, and are thus separated mainly by molecular size in the electrophoresis.

1. Dilute MTX stock 200-fold (f.c.: 50 mM) in import buffer w/o BSA.
2. Incubate the appropriate amount of preprotein with 5 μ M MTX for 10 min at 25 °C.
3. Set up an import reaction (see Subheading 3.4) for the accumulation; add creatine kinase, and creatine phosphate to a concentration of 200 μ M, and 20 mM, respectively. Add 5 μ M MTX.

4. Initiate accumulation by addition of a tenfold excess of MTX-coupled precursor (240 ng, corresponding to 6.25 pmol in the case of b₂(167)_Δ-DHFR), and incubate for 15 min at 25 °C.
5. Stop reaction by addition of 1× inhibitor mix, and move samples on ice.
6. Pellet mitochondria by centrifugation at 12,000 × *g* for 10 min.
7. Wash mitochondria by addition of 200 µl SEM buffer and centrifugation at 12,000 × *g* for 8 min.
8. Solubilize mitochondrial pellet in 55 µl ice-cold digitonin buffer by pipetting up and down carefully with a 20–200-µl-pipet-tip for 15–20 times without causing digitonin foam. Keep on ice for 15 min.
9. Spin down unsolubilized material by centrifugation at 20,000 × *g* for 15 min.
10. Prepare fresh tubes with 5 µl 10× Loading Dye, to which 45 µl of solubilized material (supernatants) is added after centrifugation. Take 5 µl of remaining supernatants each, and analyze on SDS-PAGE in parallel.
11. Load samples on a 6–16.5 % acrylamide gradient BN gel (*see* Table 1 and **Note 10**). Cover samples with 1× cathode buffer containing 0.2 % coomassie blue G250. Gel run is performed in a cooled chamber (4 °C). Keep at 100 V for passage through the stack gel, afterwards the voltage can be raised to 600 V. Always keep the current below 15 mA.
12. After the proteins have migrated into the separation gel, the Coomassie blue-containing cathode buffer is replaced by the one without dye (excess dye might interfere with subsequent Western transfer of the proteins).
13. Before Western transfer, the gel is incubated in 1× SDS-PAGE running buffer for 5 min (facilitation of protein transfer).

Table 1
Pipetting scheme for blue native gel preparation

% Acrylamide	6	8	10	13	16.5	Stack gel
3× gel buffer (ml)	3	3	3	3	3	2.5
Acrylamide (ml)	1.07	1.46	1.82	2.35	3.05	0.6
Glycerol (ml)	–	–	1.8	1.8	1.8	–
Water (ml)	4.888	4.507	2.347	1.817	1.117	4.367
10 % APS (µl)	38	38	38	38	38	30
TEMED (µl)	3.8	3.8	3.8	3.8	3.8	3

14. Decorate BN-membrane for Tim23 using TBS(T), supplemented with 5 % skim milk powder (*see* **Note 11**). SDS Western membrane is decorated against anti-mouse DHFR.

3.7 Assessment of the Inward-Directed Translocation Force

The interaction of the precursor protein inserted into the import channels with the functional import motor is required to generate the matrix-directed import force driving polypeptide movement and unfolding [16]. In case of methotrexate-stabilized $b_2(\Delta)$ -DHFR translocation intermediates the protease-resistant DHFR domain is pulled tightly against the outer mitochondrial membrane by the activity of the import motor. The close apposition of stable DHFR and the outer membrane prevents the access of external proteases to the preprotein translocation intermediates. However, resistance against proteases is achieved only in the case of an active ongoing translocation reaction. Therefore the quantification of the protease resistance of translocation intermediates is an indirect measure for the import driving activity of the translocation machinery. In combination with the protease treatment, the membrane potential and the ATP-conditions can be manipulated to assess the role of the mitochondrial energy sources and the activity of the mtHsp70 import motor in the import process (*see* Fig. 4b).

1. Thaw 60 μ l (600 μ g) mitochondria and 12 μ l ATP on ice.
2. Prepare an import master mix on ice (*see* **Note 7**; total volume: 2,080 μ l): 2 mM ATP (23 μ l), 4 mM NADH (46 μ l), 1,978 μ l import buffer, 57.5 μ l mitochondria, and 5 μ M MTX. Mix carefully by inverting the tube and spinning at low speed for 2–3 s.
3. Divide the master mix into two portions of 1,000 μ l.
4. Preparation of precursor: Dilute precursor in import buffer w/o BSA to a concentration of 0.7 pmol/ μ l in 230 μ l. Add 5 μ M MTX and incubate for 5 min on ice.
5. Start accumulation of translocation intermediates: add 110 μ l of precursor to 1,000 μ l import master mix, and incubate for 10 min at 25 °C. Place on ice afterwards.
6. Depletion of ATP: add 11 μ l apyrase (f.c.: 0.01 U/ μ l) and 2 μ l oligomycin (f.c.: 20 μ M) to one of the 1,000 μ l import reactions. The other is treated as mock with 2 μ l ethanol. Incubate for 10 min on ice.
7. Re-isolate mitochondria by centrifugation at 12,000 $\times g$ for 10 min.
8. Resuspend mitochondria carefully in 1,100 μ l buffer R1 (w/o ATP) or 1,100 μ l buffer R2 (w/ ATP).
9. Take 2 $\times$ 500 μ l of each buffer R to achieve the following incubation conditions:

A: + $\Delta\psi$ /–ATP.

B: – $\Delta\psi$ /–ATP.

C: $+\Delta\psi/+ATP$.

D: $-\Delta\psi/+ATP$.

10. Start incubation of conditions A–D (500 μ l) at 25 °C, take sample 1 and 2 (100 μ l each, corresponding to 25 μ g mitochondria) immediately and put on ice.
11. Dissipate the membrane potential in mitochondria for condition B and D by addition of 3 μ l inhibitor mix, and mix carefully by flicking the tube.
12. Add 3 μ l EtOH p.a. to condition A and C (*mock*) and mix carefully flicking the tube.
13. Take sample 3 after 2 min, and sample 4 after 15 min (100 μ l each) and put on ice.
14. PK-treatment: add 2 μ l PK (f.c. 50 μ g/ml) to samples 2, 3 and 4 (condition A–D) and incubate for 15 min on ice.
15. Stop PK treatment by addition of 1 mM PMSF (*see Note 2*); for equal treatment of all samples add PMSF to non-PK-treated samples as well. Keep samples on ice for further 5 min, pellet mitochondria by centrifugation at 12,000 $\times g$ for 10 min.
16. Remove supernatants and wash mitochondrial pellet by addition of 150 μ l SEM buffer, containing 1 mM PMSF, and centrifugation at 12,000 $\times g$ for 8 min.
17. Remove supernatants and denature mitochondria in 1 $\times$ Laemmli buffer.
18. Run samples on a Polyacrylamide gel with a resolving gel concentration of 12 % polyacrylamide.
19. Transfer your samples to a PVDF Western Blot membrane, and immuno-decorate with antibodies directed against mouse-DHFR.

4 Notes

1. Always prepare a fresh 0.5 M stock solution, and add DTT just prior to use.
2. PMSF addition should always be followed by immediate vortexing, otherwise it precipitates. In aqueous solution, PMSF has a half-life of about 30 min. Thus, always add it just prior to use. Alternatively, isopropanol stocks which are stable for up to 6 months can be used.
3. Depending on the preprotein, and the mitochondria used, rising of K^+ concentration up to 200 mM might help to increase import fidelity.
4. It is more convenient to use already recrystallized digitonin that can be directly dissolved in water (e.g., Calbiochem).

Heating up the solution significantly increases the solubility of digitonin.

5. Alternatively, 2 mM NADH plus 2 mM ATP might be added. Wild type mitochondria usually contain sufficient internal substrates, i.e., the addition of substrates will not further increase the fluorescence quenching.
6. Fluorescence intensity will not reach starting (maximal) value again, as you will always have unspecific fluorescence quenching to a certain extent.
7. For preparation of a master mix, it is useful to always use 5–10 % in excess of the volumes needed.
8. To maintain the full import-competence, do not vortex mitochondria at all before performing the import reaction as this might result in damages of the membranes.
9. Other detergents such as Dodecylmaltoside or Triton X-100 may also be suitable.
10. Prepare the BN gel always in advance and keep it at 4 °C until it is loaded.
11. Addition of sodium azide to a final concentration of 0.02 % (from a 100× stock) and storage at –20 °C is useful to preserve the antibody solution for subsequent experiments. Handle sodium azide with care as it is highly toxic.

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Chapter 3

Import of Proteins into Isolated Yeast Mitochondria

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Abstract

Mitochondria are essential organelles of eukaryotic cells. The vast majority of mitochondrial proteins is encoded within the nuclear genome and translocated into various mitochondrial compartments after translation in the cytosol as preproteins. Even in rather primitive eukaryotes like yeasts, there are 700–1,000 different proteins that need to be recognized in the cytosol, directed to the protein translocases in the two mitochondrial membranes and sorted to their appropriate mitochondrial subcompartment. In vitro reconstituted import systems have proved to be important tools to study these processes in detail. Using isolated mitochondria and radioactively labeled precursor proteins, it was possible to identify several import machineries and pathways consisting of a large number of components during the last few decades.

Key words Cell-free protein synthesis, In vitro import, Mitochondria, Protein translocation, *Saccharomyces cerevisiae*

1 Introduction

Mitochondria are complex double-membrane bounded organelles that exhibit a huge variety of functions. Besides their role in cellular respiration, mitochondria participate in biochemical key processes like synthesis of metabolites, lipid metabolism, free radical production, apoptosis and metal homeostasis [1]. Depending on the organism, mitochondria contain about 500–2,000 different proteins [2–4]. In the yeast *Saccharomyces cerevisiae*, only eight of these proteins are encoded by mitochondrial DNA. All the remaining proteins are encoded in the nucleus and synthesized on cytosolic ribosomes. Following synthesis as precursor proteins (preproteins) they have to be imported into the organelle (for review see ref. 5–7). Mitochondria can be divided into four functionally specialized subcompartments: the outer membrane, the intermembrane space (IMS), the inner membrane, and the matrix. Precursor proteins access one of these different compartments as their final location by the use of specific targeting signals. Targeting signals are recognized by receptors on the surface of the organelle.

These receptors are part of the translocase of the outer membrane, the TOM complex [8–10]. This complex appears to interact with all mitochondrial preproteins and either facilitates their insertion into the outer membrane, or their translocation through the outer membrane into the IMS.

Most of the preproteins destined for the inner membrane or the matrix contain cleavable N-terminal targeting sequences, the so-called presequences that are hydrophobic on one face and positively charged on the other [11, 12]. These targeting signals can be predicted on the basis of the primary sequence of proteins by the use of specific algorithms like MITOPRED [13], MitoProt II [14], TargetP [15], or Predotar [16]. Presequences are recognized at the level of the inner membrane by the TIM23 translocase complex. This translocase mediates the cargo's translocation across and the insertion into the inner membrane using ATP and the membrane potential as energy sources. In the matrix, the presequences are proteolytically removed by the mitochondrial processing peptidase (MPP), and the proteins fold into their native structures by the assistance of mitochondrial chaperones.

Some preproteins that are destined for the inner mitochondrial membrane or the IMS contain bipartite signal sequences. In these proteins, the N-terminal matrix-targeting signals are followed by hydrophobic sorting signals that direct the preproteins into the inner membrane. Once inserted, the cargo may remain anchored in the inner membrane or are released into the IMS after a further processing event.

Another group of inner membrane proteins is sorted by the second Tim machinery, the TIM22 complex [17–20]. Substrates of the TIM22 pathway lack presequences and contain patches of targeting information that is scattered throughout the sequence of the mature polypeptide. These proteins, such as members of the metabolite carrier family, cross the TOM channel, are ushered through the IMS by specific chaperones, and eventually inserted in the inner membrane by the TIM22 complex in a membrane potential-dependent manner.

Some soluble IMS proteins are imported independently of ATP hydrolysis or the membrane potential. The import of these proteins seems to be purely driven by the use of cysteine oxidation, which is facilitated by two specialized IMS components, Mia40 and Erv1 [21–24].

To investigate the different protein import machineries and pathways, *in vitro* reconstituted systems are adequate tools. To perform such experiments, preproteins labeled *in vitro* with [³⁵S] can be imported into isolated mitochondria. These assays reveal characteristics and requirements of protein import and were highly effective in the past to characterize the import process of mitochondrial preproteins. In this chapter, a standard protocol for reconstituted

in vitro protein import into isolated yeast mitochondria is provided. Similar approaches can be used to analyze the import of proteins into mammalian mitochondria [25–27].

Radiolabeled preproteins can be easily synthesized in cell-free transcription systems [28]. It should be noted that the protein concentrations obtained by this method is very low and typically below the nanomolar range [29]. However, since the proteins are typically bound by chaperones they are also mostly highly import competent. This high import competence and the simple production of these radiolabeled proteins made cell-free transcription the standard procedure in the field for the generation of preproteins. A protocol for the in vitro synthesis of preproteins and a standard import experiment will be given in this chapter. Alternatively, larger concentrations of preproteins (so-called “chemical amounts”) can be heterologously expressed in bacteria, purified and used for the in vitro import studies (*see* **Note 1**). In order to allow their import, the preproteins need to be unfolded, for example by denaturing them with urea or guanidinium chloride [30]. The imported preproteins can be detected by Western blotting which, however, is not very sensitive and difficult to quantify with satisfying reliability. In any case, large epitope tags on the proteins should be avoided since they may interfere with protein import. Recombinant preproteins can also be purified from metabolically radiolabeled bacteria which allow their sensitive and reliable quantification by autoradiography [31].

In general, preproteins are incubated with purified mitochondria. Following their translocation across the outer membrane, the preproteins become inaccessible to externally added protease. Moreover, the maturation of preproteins by mitochondrial processing peptidases can be easily detected by SDS-PAGE and the import reaction can be thus followed experimentally.

2 Materials

2.1 Yeast Culture

Ingredient amounts are given for 1 L of medium. Sterilize each of the media listed below by autoclaving for 20 min at 121 °C (*see* **Note 2**).

1. Yeast peptone medium (YP): 10 g Bacto-yeast extract, 20 g Bacto-peptone, distilled water added 1,000 mL. Adjust the pH to 5.5.
2. Lactate medium: 3 g Bacto-yeast extract, 1 g KH_2PO_4 , 1 g NH_4Cl , 0.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g NaCl , 0.6 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 3 mg FeCl_3 (or 0.3 mL from a 1 % w/v stock solution), 22 mL 90 % lactic acid (2 % v/v final concentration), distilled water added to 1,000 mL. Adjust the pH to 5.5 with KOH.

3. 5× synthetic minimal medium stock (5× S): 8.5 g yeast nitrogen base (without ammonium sulfate, without amino acids), 25 g ammonium sulfate, distilled water added to 1,000 mL.
4. 5× synthetic lactate medium stock (5× SLac): 8.5 g yeast nitrogen base (without ammonium sulfate, without amino acids), 25 g ammonium sulfate, 110 mL 90 % lactic acid, distilled water added to 1,000 mL. Adjust the pH to 5.5 with KOH.
5. Glucose stock solution: 400 g glucose, distilled water added to 1,000 mL.
6. Galactose stock solution: 300 g galactose, distilled water added to 1,000 mL.
7. Auxotrophic markers stock solutions: Adenine sulfate 200 mg in 100 mL of water; uracil 200 mg in 100 mL of water; L-tryptophan 1 g in 100 mL of water, L-histidine-HCl 1 g in 100 mL of water; L-leucine 1 g in 100 mL of water; L-lysine-HCl 1 g in 100 mL of water (*see Note 3*).
8. Sterile water.

2.2 Isolation of Mitochondria by Differential Centrifugation

1. MP1 buffer: 100 mM Tris base (do not adjust the pH), 10 mM dithiothreitol (DTT, add just before use from a 1 M stock solution).
2. Sorbitol buffer: 1.2 M sorbitol.
3. MP2 buffer: 1.2 M sorbitol, 20 mM phosphate buffer, pH 7.4; chill buffer on ice before use.
4. Zymolyase 20T.
5. MP3 buffer: 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 % (w/v) essentially fatty acid-free bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF, *see Note 4*). Chill buffer on ice before use.
6. SH buffer: 0.6 M sorbitol, 20 mM HEPES, pH 7.4.
7. SEH buffer: 0.6 M sorbitol, 1 mM EDTA, 20 mM HEPES, pH 7.4.
8. Glass Dounce Homogenator (tight-fitting glass pestle).
9. Liquid nitrogen.

2.3 Synthesis of Preproteins

2.3.1 In Vitro Transcription

1. DNA template: use purified plasmid (1 µg/µL) containing the gene for the mitochondrial precursor protein inserted downstream of the SP6 promoter (*see Note 5*).
2. 10x salts buffer: 400 mM HEPES/KOH, pH 7.4, 60 mM Mg-acetate, 20 mM spermidine (*see Note 6*). Sterilize by filtration and store at -20 °C.
3. Premix buffer: 1 mL 10x salts buffer, 20 µL 50 mg/mL essentially fatty acid-free BSA, 200 µL 1 M DTT dissolved in water,

50 μL 0.1 M adenosine triphosphate (ATP), 50 μL cytidine triphosphate (CTP), 50 μL uridine triphosphate (UTP), 10 μL 0.1 M guanosine triphosphate (GTP), 4,620 μL water. Sterilize by filtration and store aliquots at -20°C .

4. Ribonuclease inhibitor (RNasin, 40 U/ μL). Store aliquots at -20°C .
5. Methylguanine cap ($\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$), 2.5 mM: add 483 μL sterile water to 25 A_{250} units of $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ (*see Note 7*). Store aliquots at -20°C .
6. SP6 RNA polymerase (25 U/ μL). Store at -20°C .
7. LiCl: prepare a 10 M solution in water and store aliquots at -20°C .
8. 70 % (v/v) ethanol, store at -20°C .

2.3.2 In Vitro Translation

1. Use commercially available rabbit reticulocyte lysate. Store at -80°C (*see Note 8*).
2. Amino acid mixture minus methionine (*see Note 9*), 1 mM. Store at -20°C .
3. RNasin, 40 U/ μL . Store aliquots at -20°C .
4. Use RNA that was produced in the in vitro transcription reaction.
5. [^{35}S]-labeled methionine, specific activity 1,175 Ci/mmol, 10 mCi/mL. Store in aliquots at 4°C (*see Note 10*).
6. Cold methionine for the chase reaction: 200 mM solution in water. Store at -20°C .

2.4 In Vitro Import into Isolated Mitochondria

1. 2 $\times$ import buffer: 100 mM HEPES/KOH, pH 7.2, 6 % fatty acid-free BSA, 1 M sorbitol, 160 mM KCl, 20 mM Mg-acetate, 4 mM KH_2PO_4 , (*see Note 11*). Store at -20°C .
2. ATP: prepare a 0.2 M solution in water and adjust the pH with KOH to 7.0. Store aliquots at -20°C .
3. NADH: 0.2 M solution in water. Store aliquots at -20°C .
4. VAO buffer: 55.6 μL of 1 mg/mL valinomycin in ethanol, 440 μL of 1 mg/mL antimycin A in ethanol, 425 μL of 2 mg/mL oligomycin A in ethanol, 79.4 μL ethanol. This is a 100 % stock. Store at -20°C (*see Note 12*).
5. Proteinase K (PK): 10 mg/mL solution in water. Store aliquots at -20°C (*see Note 13*).
6. Phenylmethylsulfonyl fluoride (PMSF): always prepare a fresh 0.2 M solution in ethanol.
7. SH buffer: 0.6 M sorbitol, 20 mM HEPES/KOH, pH 7.2. Store aliquots at -20°C .
8. SH/KCl buffer: 0.6 M sorbitol, 80 mM KCl, 20 mM HEPES/KOH, pH 7.2.

9. [³⁵S]-labeled mitochondrial precursor protein prepared in the in vitro translation reaction. Keep in single-use aliquots of 12 μL at -80 °C (*see* **Note 14**).
10. Isolated yeast mitochondria at 10 mg/mL kept in single-use aliquots at -80 °C (*see* **Note 15**).

3 Methods

3.1 Yeast Culture

Yeast cells are grown in Erlenmeyer flasks in liquid cultures under constant agitation (approx. 140 rpm). The optimal growth temperature for *S. cerevisiae* is 30 °C; however, temperature sensitive mutants should be grown at 25 or 15 °C. The growth rate depends notably on the yeast strain. Therefore, it is recommended to measure the generation time for each strain before inoculating the scaled up culture that will be used for isolation of mitochondria (*see* **Note 2**).

1. Inoculate 50 mL of culture with the strain of interest.
2. Follow the growth of the strain over several generations under the same conditions. Take care that it should always be kept at the logarithmic growth phase (OD₆₀₀ should never exceed 2.0).
3. Measure regularly the OD₆₀₀ of the culture, and calculate the generation time of the strain by using the following formula:

$$\text{generation time} = (t * \log 2) / \log (\text{OD}_{600} \text{ final} / \text{OD}_{600} \text{ initial})$$

where t is the time of growth in hours.

4. Inoculate the final culture for the isolation of mitochondria considering the generation time and expecting an OD₆₀₀ of 1.5 at the time of harvesting. The cultures must be in the logarithmic growth phase at the moment of harvest. The amount of culture inoculated depends exclusively on the experimental needs, usually from 10 L of a culture with an OD₆₀₀ of 1.5 approx. 20 g of cells (wet weight) are obtained. The mitochondrial yield from this amount of cells is approx. 20 mg.

3.2 Isolation of Mitochondria by Differential Centrifugation

1. Grow the desired amount of yeasts to an OD₆₀₀ of 0.8–2.0.
2. Collect the cells at 3,000 × g for 5 min and determine the wet weight.
3. Resuspend the pellet in 100 mL distilled water and centrifuge at 3,000 × g for 5 min.
4. Resuspend the cells in MP1 buffer (2 mL/g of wet weight) and incubate the suspension for 10 min at 30 °C under agitation (approx. 140 rpm).

5. Collect the cells by centrifugation at $2,000\times g$ for 5 min. Discard the supernatant and wash the cell pellet with sorbitol buffer (5 mL/g of wet weight).
6. Collect the cells again by centrifugation at $2,000\times g$ for 5 min. Discard the supernatant.
7. Resuspend the cells in MP2 buffer (6.7 mL/g of wet weight).
8. Add the Zymolyase 20T (2 mg/g of wet weight) to the suspension and incubate under agitation (approx. 140 rpm) for 20–40 min at 30 °C until spheroplasts have formed (*see Note 16*).
9. All following steps will be performed on ice using ice-cold buffers, keeping the samples always on ice and centrifugation steps performed at 4 °C.
10. Harvest the spheroplasts by centrifugation at $2,000\times g$ for 5 min.
11. Resuspend the pellet in MP3 buffer (6.7 mL/g of wet weight) by gently shaking or stirring with a pipette.
12. Transfer the suspension to a 50 mL Dounce homogenizer (tight-fitting glass pestle) and homogenize with 15 strokes.
13. Dilute the resulting homogenate with MP3 buffer (again 6.7 mL/g of wet weight).
14. Centrifuge the homogenate at $2,000\times g$ for 5 min to spin down cell debris. Keep the supernatant and transfer it to a fresh centrifugation bucket.
15. Centrifuge at $17,000\times g$ for 12 min. Discard the supernatant.
16. Resuspend the pellet carefully in 10 mL of SH or SEH buffer.
17. Centrifuge at $17,000\times g$ for 12 min. Discard the supernatant.
18. Resuspend the pellet in 0.5 mL of SH or SEH buffer.
19. Take an aliquot to determine protein concentration.
20. Dilute the mitochondrial suspension to a final concentration of 10 mg/mL in SH buffer.
21. Make aliquots of 30–50 μ L, immediately snap-freeze in liquid nitrogen. Store at –80 °C.

3.3 Synthesis of Preproteins

3.3.1 *In Vitro* Transcription

1. To make 50 μ L of the transcription reaction, mix 30 μ L of the premix solution, 2.2 μ L RNasin, 2.5 μ L m⁷G-cap, 0.8 μ L SP6 RNA polymerase, and 15 μ L of the DNA template (*see Note 17*).
2. Incubate for 1 h at 37 °C.
3. Add 5 μ L LiCl and 150 μ L ethanol (–20 °C) in order to pellet the RNA.
4. Incubate for 15 min at –20 °C.
5. Spin down the RNA at $35,000\times g$ and 4 °C for 30 min.

6. Wash the RNA pellet with 70 % ethanol (-20°C).
7. Spin down the RNA at $35,000\times g$ and 4°C for 5 min.
8. Remove ethanol completely at room temperature. Resuspend the RNA pellet in 30 μL water and add 0.8 μL RNasin.
9. Make single-use aliquots and store at -80°C (*see* **Note 18**).

3.3.2 *In Vitro Translation*

1. To make an in vitro translation reaction, mix 140 μL rabbit reticulocyte lysate, 4 μL of the amino acid mixture minus methionine, 4 μL RNasin, 16 μL [^{35}S]-Met, and 30 μL RNA from the transcription reaction.
2. Incubate for 1 h at 30°C .
3. Chase the reaction by adding 8 μL of cold methionine.
4. Incubate for 5 min at 30°C .
5. Make single-use aliquots (12 μL) of the lysate. Freeze in liquid nitrogen and store at -80°C .
6. To assess the translation product, analyze 1 μL of the lysate by SDS-PAGE and autoradiography (*see* **Note 19**).

3.4 *In Vitro Import of Preproteins into Isolated Mitochondria*

The standard import protocol monitors the translocation of pre-proteins across the outer mitochondrial membrane by assessing their resistance against externally added proteases. In addition, the dependency of the import reaction on ATP or the membrane potential can also be tested (*see* **Notes 20–22**). To localize the imported proteins after the import reaction, the mitochondria can be converted to mitoplasts, i.e., mitochondria with the outer membrane ruptured by hypotonic swelling. Upon addition of protease, proteins that have expose domains in the IMS can be degraded by proteases, whereas proteins topologically confined to the matrix will remain intact.

1. Prepare six tubes with 50 μL $2\times$ import buffer.
2. Add in tubes 1–3: 42 μL water, 1 μL ATP, 1 μL NADH, and 5 μL of mitochondria (*see* **Note 23**).
3. Add in tubes 4–6: 43 μL water, 1 μL VAO buffer, and 5 μL of mitochondria.
4. Incubate all tubes for 5 min at 25°C (*see* **Note 24**).
5. Add 1 μL of the radioactive protein lysate to all six tubes (*see* **Note 25**).
6. Incubate for 20 min at 25°C .
7. Add 900 μL SH buffer to tubes 1, 2, 4, and 5. Add 900 μL HEPES/KOH pH 7.4 to tubes 3 and 6. Add 10 μL of PK to tubes 2, 3, 5, and 6 (*see* **Note 26**).
8. Incubate for 30 min on ice.

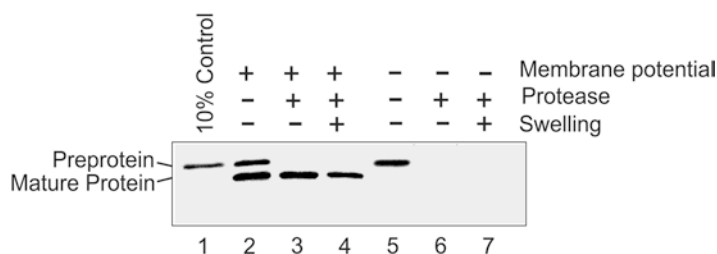


Fig. 1 Import of preSu9-DHFR into yeast mitochondria. A precursor protein consisting of the residues 1–69 of subunit 9 of the ATPase of *Neurospora crassa* fused to mouse dihydrofolate reductase was synthesized in reticulocyte lysate in the presence of [³⁵S]-methionine. The radiolabeled preprotein was incubated with isolated yeast mitochondria in the presence of ATP and NADH (lanes 2–4) or valinomycin (lanes 5–7) for 20 min at 25 °C. The mitochondria were post-incubated on ice in the presence of proteinase K under non-swelling or swelling conditions as indicated. Protease treatment was stopped by the addition of PMSF. The mitochondria were reisolated and washed. Mitochondrial proteins were separated by SDS-PAGE and detected by autoradiography. Lane 1 shows an equivalent of 10 % of the radiolabeled preprotein used per import reaction

9. Add 10 μ L of PMSF to all samples in order to stop the digestion.
10. Spin down the mitochondria by centrifugation at $25,000\times g$ for 10 min at 4 °C.
11. Discard the supernatant and wash the mitochondrial pellets with 500 μ L SH/KCl solution and 5 μ L PMSF.
12. Spin again down the mitochondria by centrifugation at $25,000\times g$ for 10 min at 4 °C.
13. Resuspend the mitochondria in 30 μ L reducing sample buffer. Boil the samples for 5 min at 95 °C (see **Note 27**).
14. The samples are analyzed by SDS-PAGE and fixed on nitrocellulose membranes by blotting. Imported proteins are detected by autoradiography (see **Note 28**). An example of such an import experiment is provided in Fig. 1.

4 Notes

1. The low amounts of in vitro synthesized proteins are generally too small to saturate mitochondrial import sites. When a saturation of mitochondrial import sites is necessary in the experiment, the precursor protein has to be present in excess compared to the available translocases. For this purpose, mitochondrial precursors are heterologously expressed in bacteria and purified as recombinant proteins. It should be noted, however, that this was successfully done for only a few precursors [30].

2. The appropriate carbon source depends on the strains and its ability to grow on non-fermentable conditions. When working with strains that are unable to respire, the use of galactose is preferred to glucose, as glucose-repression of many mitochondrial genes leads to poorly developed mitochondria and consequently a rather poor labeling. When working with strains that are able to respire lactic acid, glucose, galactose, and lactic acid are added to a final concentration of 2 % (w/v). Raffinose can also be used at a final concentration of 1 % (w/v).
3. Auxotrophic markers (amino acids or nucleotides) might be omitted to select for plasmids. L-tryptophan turns into a brown solution during the autoclaving process; alternatively it can be sterilized by filtration through a 0.22 μm filter. L-tryptophan and L-histidine-HCl are photosensitive and therefore the stock solutions must be kept protected from light.
4. PMSF is dissolved at 200 mM in ethanol. Prepare freshly before use. PMSF inhibits serine proteases irreversibly and is toxic. Appropriate safety measurements must be taken when handling it.
5. We usually clone open reading frames (ORFs) into pGEM vectors (Promega) downstream of the SP6 RNA polymerase transcription initiation site. This generally produces transcripts more efficiently than T3 or T7 RNA polymerases. However, the latter enzymes were successfully used as well. It is advisable to inspect the sequence of the mature protein for the presence of methionine residues. To improve the labeling efficiency additional ATG codons might have to be inserted into the DNA sequence or at the 3'-end of the gene during the cloning procedure.
6. Spermidine is made from a 200 mM stock solution. Spermidine may deaminate. Thus, make a small stock solution, keep it at -20°C , and prepare new solutions frequently.
7. $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ is an mRNA cap analog, which is used during the in vitro transcription reactions in order to yield RNA capped at the 5'-end. This cap is required for satisfactory in vitro translations. $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ is shipped in packages with 25 A_{250} units.
8. Avoid multiple freezing and thawing of the lysate. This strongly reduces the translation efficiency. We usually do not freeze the same aliquot more than twice. Lysate is thawed very rapidly by hand warming and subsequently placed on ice.
9. The use of radiolabeled cysteine is possible as well. If cysteine is used, the chase is performed with cold cysteine instead of methionine. We also use radiolabeled methionine-cysteine mixes. This can enhance the labeling efficiency.

10. Refer to the general safety precautions when dealing with radioactivity.
11. We prepare the 2× import buffer with all the ingredients except BSA, dissolve them in water and then adjust the pH with KOH. BSA is added after adjustment of the pH. The purpose of BSA in the buffer is stabilizing some precursor proteins. Although other protocols suggest the usage of 3 % (w/v) BSA, our laboratory found that this is generally not necessary. In fact, the majority of precursors we tested does not need the addition of BSA to be imported, or is even imported more efficiently without BSA.
12. Valinomycin forms ionophores that dissipate the inner membrane potential. Oligomycin is a potent inhibitor of the ATP synthase. Antimycin A blocks transfer of electrons from cytochrome *b* to cytochrome *c*.
13. Keep PK in single-use aliquots, and avoid multiple thawing–freezing procedures as this abolishes the enzymatic activity.
14. When we perform a series of import experiments, we keep the remnants of the preprotein aliquots at -20°C and use them mixed with a new aliquot the next day.
15. We keep mitochondria frozen at -80°C in single-use aliquots of 30 or 50 μL . It is recommended to thaw aliquots that are needed quickly by hand warming and place them immediately on ice. Use them as soon as possible after thawing, since the quality of mitochondria strongly reduces if not kept at -80°C .
16. To test for spheroplast formation, add 50 μL cells to 2 mL water or sorbitol buffer. The suspension in water should clear up as an indication that the cells are being lysed in this hypotonic solution. This effect can be also detected by measuring the OD_{600} and comparing the optical densities from both suspensions.
17. The water used for RNA synthesis should be sterile and of purest quality. In addition, the yield of RNA can be increased by linearization of the DNA template prior to transcription. This is particularly advisable when T3 or T7 polymerases are used instead of the SP6 polymerase.
18. RNA produced in this way can be stable for more than 1 year.
19. Lower molecular weight translation products are often observed in addition to the desired full-length product. This is due to the initiation of translation at internal start codons which can be suppressed by increasing the concentration of Mg^{2+} and K^{+} ions during the translation reaction. However, in most cases an internal initiation is irrelevant since N-terminally truncated proteins lack their mitochondrial targeting signal and will not even bind to receptors of the outer mitochondrial

membrane. Sometimes it is difficult to produce full length proteins. This is frequently observed when the translation products are either very large or hydrophobic. If so, RNA and salt concentrations during the translation reaction have to be optimized. In addition, the plasmid preps should be controlled. Of course, combined transcription-translation reaction systems can be tested, since their yield is usually higher.

20. The import kinetics of a particular preprotein can be assessed using both wild-type mitochondria and mitochondria isolated from mutated yeast strains. This can be used to dissect the import pathway of a preprotein. With this method, one can as well compare established import routes with effects of potential new import components.
21. What is described here is a basic protocol for protein import into isolated mitochondria, which can be modified in many ways. For this, we refer to the methods described in the original literature. For example, the effect of reducing agents like DTT on the import reaction can be tested which strongly interferes with the Erv1-mediated translocation of proteins into the IMS.
22. If an import reaction is strongly ATP-dependent, it may be necessary to add an ATP-regenerating system. Therefore, prepare creatine phosphate (CP; 250 mM solution in water, stored in aliquots at -20°C), creatine kinase (CK; 20 mg/mL solution in water, stored in single-use aliquots at -20°C), malate (200 mM solution in water, stored at -20°C), and succinate (200 mM solution in water, stored at -20°C). Add to the import reaction 1 μL ATP, 1 μL NADH, 4 μL of CP, 0.5 μL of CK, 1 μL of malate, and 1 μL of succinate. This regenerates enough ATP within the mitochondria to keep ATP-driven import running.
23. Mix the components well but avoid vortexing since this breaks the mitochondrial membranes. Be careful when pipetting mitochondria and be as gentle as possible.
24. Import reactions are usually performed at 25°C . Since some precursors are, at this temperature, imported very rapidly, the temperature can be decreased to 12°C in order to observe the import in a linear time range.
25. During imports, we use the term *lysate* for the preprotein within the translation buffer containing the reticulocyte lysate. We normally use 1 % (v/v) of lysate in the import reaction, but this is eventually modified since the quality of the lysate is always different. The amount of lysate depends on its quality.
26. Here, the successful translocation across the outer membrane is assessed. Preproteins that are attached to the surface of the mitochondria are still accessible to the added proteinase

K and degraded. Preproteins that successfully entered the mitochondria are protease-protected. Trypsin may be used instead of proteinase K, but due to its weaker specificity, proteinase K is the preferred protease.

27. Boil all samples immediately at 95 °C. Proteases are never stopped completely by the addition of PMSF. Remaining active proteinase K will degrade all proteins very rapidly upon opening of the membranes in the SDS sample buffer.
28. We always load a 10 % sample of the amount of lysate used in the import experiment in the first lane during SDS-PAGE analysis. This controls the status of the lysate in each reaction. In addition, the efficiency of the import reaction can be estimated. In the end, we blot the gels to nitrocellulose membranes after SDS-PAGE. One can fix the proteins within the gel and dry the gels, followed by autoradiography. Since blotting allows subsequent immunodecoration, additional control experiments on the quality of the mitochondria and the amount of different import proteins may be assessed.

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Evaluation of Unconventional Protein Secretion in *Saccharomyces cerevisiae*

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Abstract

Recent development of large-scale analyses such as the secretome analysis has enabled the discovery of a vast number of intracellular proteins that are secreted outside the cell. Often, these proteins do not contain any known signal sequence required for conventional protein secretion. In order to avoid misidentification of such “leaked” proteins as “secreted” proteins, reconstructing the process of protein secretion is essential. Here, we describe methods for the detection of reconstructed unconventional protein secretion and determination of regulatory proteins of secretion in *Saccharomyces cerevisiae*. We show that conjugating target proteins with a tag-sequence and utilizing various reagents and tools can facilitate quantitative detection of the secretion of target proteins. We expect that these methods will reveal novel unconventional secretion pathways of proteins.

Key words Unconventional secretion, *Saccharomyces cerevisiae*, Western blotting, Recombinant proteins, Phosphoglucose isomerase, Enolase, Glycolytic enzymes, Moonlighting proteins

1 Introduction

An increasing number of unconventionally secreted proteins have been reported to play important roles in cell-cell communication and infection [1–4]. Among these extracellular proteins, the number of proteins with known functions is quite small. Recently, unconventionally secreted proteins have been reported to be the major secreted proteins in cancer cell lines [5], underscoring the urgent need to discover the extracellular functions of such proteins. A number of unconventionally secreted proteins exert “moonlighting” functions [6] which are unrelated to their intracellular roles, making it difficult to predict their extracellular properties; nevertheless, it may be possible to study their functions by inhibiting their secretion. Although a few unconventional secretory pathways [7–9] have been discovered, several other proteins, their secretory pathways, and secretion-regulatory factors remain unknown [10].

To reveal the molecular basis of unconventional protein secretion, the following four steps should be considered: (1) Confirming that the target (endogenous) protein is naturally secreted, (2) Reconstructing the process of protein secretion and ensuring that the extracellular detection of proteins is not due to cell lysis (or “leakage”), (3) Identifying the amino acid sequence required for secretion and visualizing the secretion pathway, and (4) Identifying regulatory proteins of the secretion pathway [11]. This approach has been primarily used in the budding yeast *S. cerevisiae*, since it is one of the most popular and convenient model organisms for the investigation of protein secretion pathways [12].

To determine whether proteins of interest are naturally secreted, performing large-scale secretome analyses is not always feasible and necessary. Numerous reports on secreted proteins in various organisms such as *S. cerevisiae* [13, 14], nematodes [15], and mammals [5, 16] are available, and researchers can access and evaluate the raw data available through proteome databases such as the PRoteomics IDentifications database (PRIDE, <http://www.ebi.ac.uk/pride/archive/>), ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>), PeptideAtlas (<http://www.peptideatlas.org/>), and Global Proteome Machine Database (GPMDB, <http://www.thegpm.org/GPMDB/index.html>). While analyzing the proteome data, it is important to avoid the detection of “leaked” proteins (e.g., by using recombinant proteins as leakage markers [11]) and to primarily focus on quantitative data (e.g., by introduction of quantitative proteome analysis [17]) in order to successfully obtain high-quality data for unconventionally secreted proteins.

Unconventional secretion of proteins can be further investigated by producing recombinant proteins in cells. For most of the proteins that do not have highly sensitive antibodies or methods for detecting the specific extracellular activity, fusing a tag sequence and detecting their secretion by tag-specific antibodies is a realistic approach. It is possible that the tag sequence may affect protein secretion; nevertheless, such tag-specific antibodies have facilitated efficient detection of secreted proteins. In addition, by comparing the unconventionally secreted proteins with non-secreted proteins, it may be possible to determine whether the proteins are actually secreted or leaked from the cells.

Here, we demonstrate methods for detecting unconventionally secreted glycolytic proteins in comparison with enhanced green fluorescent protein (EGFP), which is not secreted from the yeast cells. As a positive control of secreted proteins, a known secretion signal sequence for *S. cerevisiae* was fused with EGFP. We fused both EGFP and a FLAG-tag sequence with the candidate glycolytic enzymes and detected the secretion of proteins by Western blotting using a GFP-specific antibody. Conjugation of target

proteins with EGFP enables visualization of the putative secretion pathway, and may provide clues for the identification of regulatory proteins [11]. For most of the unconventionally secreted glycolytic enzymes, the intracellular localization of full-length proteins is uniform in the cytosol [11]. For any target protein, if the specific region responsible for its localization from the inside to the outside of cells can be identified, then following the changes in localization may facilitate prediction of the secretion pathway. Furthermore, the discovery of such regions may also help to uncover unexpected functions of the target proteins [18].

The regulatory proteins for unconventional secretion can be screened without visualization. Figure 1 depicts all known protein secretion pathways in *S. cerevisiae* [19, 20]. Regulatory factors have been reported for every step of the membrane-driven transport [12, 21–27]. Knockout or temperature-sensitive mutants are available for some genes and these mutants are useful for determining the precise stages when target proteins are secreted during membrane-driven transport. The SNARE proteins, for which the cellular knockout phenotype is not lethal, are shown in Table 1.

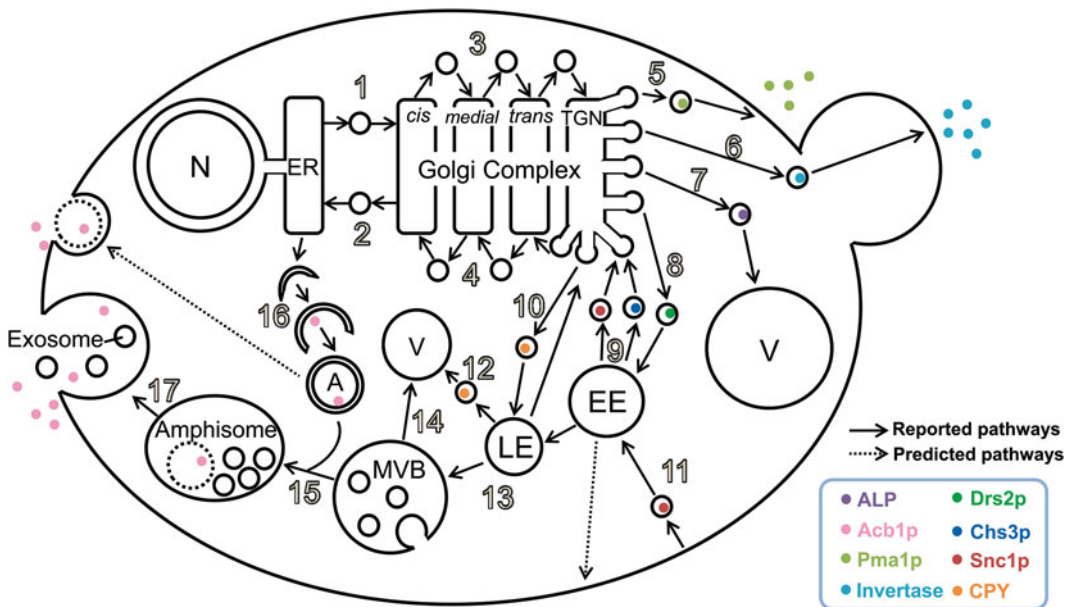


Fig. 1 Overview of the protein secretion pathways in *S. cerevisiae* (in reference to Muthusamy et al. [19] and Bröcker et al. [20]). The numbers denote the names of the intracellular protein transport pathways. 1: ER to Golgi, 2: Golgi to ER, 3: *cis*-Golgi to *trans*-Golgi network (TGN), 4: TGN to *cis*-Golgi, 5: TGN to cell membrane (CM) (for “light” proteins), 6: TGN to CM (for “heavy” proteins), 7: TGN to vacuole (V), 8: TGN to early endosome (EE), 9: EE to TGN, 10: TGN to late endosome (LE), 11: CM to EE, 12: LE to V, 13: LE to multivesicular body (MVB), 14: MVB to V, 15: MVB to Amphisome, 16: ER to Autophagosome (A), and 17: Amphisome to CM. *N* nucleus, *ER* endoplasmic reticulum, *TGN* *trans*-Golgi network, *V* vacuole, *EE* early endosome, *LE* late endosome, *MVB* multivesicular body, *A* autophagosome, *CM* cell membrane

Table 1
SNARE proteins involved in secretion pathway

	Pathway	SNARE protein-coding genes	Essential or nonessential	Description
1	ER to Golgi	SED5	Essential	<i>cis</i> -Golgi t-SNARE syntaxin required for vesicular transport between the ER and the Golgi complex, binds at least nine SNARE proteins
		YKT6	Essential	Vesicle membrane protein (v-SNARE) with acyltransferase activity; involved in trafficking to and within the Golgi, endocytic trafficking to the vacuole, and vacuolar fusion; membrane localization due to prenylation at the carboxy-terminus
		<u>SEC22</u>	<u>Nonessential</u>	R-SNARE protein; assembles into SNARE complex with Bet1p, Bos1p, and Sed5p; cycles between the ER and Golgi complex; involved in anterograde and retrograde transport between the ER and Golgi; synaptobrevin homolog
		BOS1	Essential	v-SNARE (vesicle specific SNAP receptor), localized to the endoplasmic reticulum membrane and necessary for vesicular transport from the ER to the Golgi
		BET1	Essential	Type II membrane protein required for vesicular transport between the endoplasmic reticulum and Golgi complex; v-SNARE with similarity to synaptobrevins
2	Golgi to ER	<u>SEC22</u>	<u>Nonessential</u>	–
		UFE1	Essential	t-SNARE required for retrograde vesicular traffic and homotypic ER membrane fusion; forms a complex with the SNAREs Sec22p, Sec20p, and Use1p to mediate fusion of Golgi-derived vesicles at the ER
		SEC20	Essential	Membrane glycoprotein v-SNARE involved in retrograde transport from the Golgi to the ER; required for <i>N</i> - and <i>O</i> -glycosylation in the Golgi but not in the ER; interacts with the Dsl1p complex through Tip20p
		USE1	Essential	Essential SNARE protein localized to the ER, involved in retrograde traffic from the Golgi to the ER; forms a complex with the SNAREs Sec22p, Sec20p, and Ufe1p
		<u>GOS1</u>	<u>Nonessential</u>	v-SNARE protein involved in Golgi transport, homolog of the mammalian protein GOS-28/GS28
3	Golgi (<i>cis</i> to TGN)	<u>YKT6</u>	Essential	Vesicle membrane protein (v-SNARE) with acyltransferase activity; involved in trafficking to and within the Golgi, endocytic trafficking to the vacuole, and vacuolar fusion; membrane localization due to prenylation at the carboxy-terminus
		<u>GOS1</u> SFT1	<u>Nonessential</u> Essential	– Intra-Golgi v-SNARE, required for transport of proteins between an early and a later Golgi compartment

4	Golgi (TGN to <i>cis</i>)	<u>YKT6</u> <u>SED5</u> <u>VTI1</u> SLT1	Essential Essential Essential Essential	– – Protein involved in <i>cis</i> -Golgi membrane traffic; v-SNARE that interacts with two t-SNAREs, Sed5p and Pep12p; required for multiple vacuolar sorting pathways Essential SNARE protein localized to the ER, involved in retrograde traffic from the Golgi to the ER; forms a complex with the SNAREs Sec22p, Sec20p, and Ufe1p
5	TGN to CM (light)	<u>SSO1/2</u> <u>SNC1/2</u> SEC9	Nonessential <u>Nonessential</u> Essential	Plasma membrane t-SNAREs involved in fusion of secretory vesicles at the plasma membrane Vesicle membrane receptor protein (v-SNARE); involved in the fusion between Golgi-derived secretory vesicles with the plasma membrane; proposed to be involved in endocytosis; member of the synaptobrevin/VAMP family of R-type v-SNARE proteins t-SNARE protein important for fusion of secretory vesicles with the plasma membrane; similar to but not functionally redundant with Spo20p; SNAP-25 homolog
6	TGN to CM (heavy)	<u>YKT6</u>	Essential	–
7	TGN to V	<u>VAM3</u> <u>VTI1</u> <u>VAM7</u> <u>NYV1</u>	<u>Nonessential</u> Essential <u>Nonessential</u> <u>Nonessential</u>	Syntaxin-like vacuolar t-SNARE that functions with Vam7p in vacuolar protein trafficking; mediates docking/fusion of late transport intermediates with the vacuole; has an acidic di-leucine sorting signal and C-terminal transmembrane region – Vacuolar SNARE protein that functions with Vam3p in vacuolar protein trafficking; has an N-terminal PX domain (phosphoinositide-binding module) that binds PtdIns-3-P and mediates membrane binding; SNAP-25 homolog v-SNARE component of the vacuolar SNARE complex involved in vesicle fusion; inhibits ATP-dependent Ca ²⁺ transport activity of Pmc1p in the vacuolar membrane
8	TGN to EE	<u>SNC1/2</u>	<u>Nonessential</u>	–
9	EE to TGN	<u>TLG2</u> <u>VTI1</u> TLG1 <u>YKT6</u>	<u>Nonessential</u> Essential Essential Essential	Syntaxin-like t-SNARE that forms a complex with Tlg1p and Vti1p and mediates fusion of endosome-derived vesicles with the late Golgi; binds Vps45p, which prevents Tlg2p degradation and also facilitates t-SNARE complex formation; homologous to mammalian SNARE protein syntaxin 16 (Sx16) – Essential t-SNARE that forms a complex with Tlg2p and Vti1p and mediates fusion of endosome-derived vesicles with the late Golgi; binds the docking complex VFT (Vps fifty-three) through interaction with Vps51p –

(continued)

Table 1
(continued)

	Pathway	SNARE protein-coding genes	Essential or nonessential	Description
10	TGN to LE	<u>PEP12</u>	<u>Nonessential</u>	Target membrane receptor (t-SNARE) for vesicular intermediates traveling between the Golgi apparatus and the vacuole; controls entry of biosynthetic, endocytic, and retrograde traffic into the prevacuolar compartment; Syntaxin
		VTI1 SYN8	Essential Nonessential	– Endosomal SNARE related to mammalian syntaxin 8
11	CM to EE	<u>SYN8</u> <u>SNCL/2</u>	<u>Nonessential</u> <u>Nonessential</u>	– –
12	LE to V	VTI1 <u>VAM7</u>	Essential <u>Nonessential</u>	– –
13	LE to MVB			
14	MVB to V			
15	MVB to Amphisome			
16	ER to A			
17	Amphisome to CM			

Under line: Unessential. Bold: Used in several pathways. Descriptions are modified from Saccharomyces Genome Database (SGD, <http://www.yeastgenome.org/>)
N nucleus, *ER* endoplasmic reticulum, TGN *trans*-Golgi network, V vacuole, EE early endosome, *LE* late endosome, *MVB* multivesicular body, A autophagosome, *CM* cell membrane

The methods described here are essentially based on previous reports on the analysis of conventional protein secretion [21, 22]. Since the amount of proteins secreted by the unconventional process is considerably smaller than that of the conventionally secreted proteins, our methods have primarily focused on enhancing the protein detection using available reagents and tools. We expect that these methods will contribute to analyzing a broad range of unconventional secretion pathways of proteins.

2 Materials

2.1 Media

1. LB Agar (LBA) plate: Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar in 1 L deionized H₂O and sterilize by autoclaving. Dissolve 100 mg ampicillin in 1 mL deionized H₂O and sterilize by filtration using membrane filter (pore size: 0.45 µm) (store the 1,000× stock solution at -20 °C). Cool the sterilized media (below 55 °C), and add ampicillin from the stock solution to a final concentration of 100 mg/L. Mix the contents and pour into plates.
2. LBA media: Dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 1 L deionized H₂O and sterilize by autoclaving. Add the ampicillin stock solution into cooled sterilized media (below 55 °C) immediately before use, to a final concentration of 100 mg/L.
3. YPD media: Dissolve 10 g yeast extract, 20 g peptone, and 20 g dextrose in 1 L deionized H₂O and sterilize by autoclaving.
4. SD + HM media: Dissolve 6.7 g yeast nitrogen base w/o amino acids (BD Difco, Erembodegem, Belgium), 20 g dextrose, and amino acids according to the selection conditions (20 mg/L L-histidine-HCl and 30 mg/L L-methionine) in 1 L deionized H₂O and sterilize by autoclaving.
5. SDC + HM agar: Dissolve 6.7 g yeast nitrogen base without (w/o) amino acids (BD Difco), 5 g casamino acids, 20 g dextrose, 20 g agar, and amino acids according to the selection conditions (20 mg/L L-histidine-HCl and 30 mg/L L-methionine) in 1 L deionized H₂O, and sterilize by autoclaving. Cool the sterilized media to 55 °C, mix thoroughly, and pour into plates.

2.2 Strains and Plasmids

1. *Saccharomyces cerevisiae* strain BY4741 wild type (Euroscarf, Frankfurt, Germany): The genotype of this strain is *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, and *ura3Δ0*. Typically, secretion of target proteins is analyzed in this strain.
2. *Saccharomyces cerevisiae* strain RSY282 (*MATa*, *leu2Δ*, *ura3Δ*, *sec23-1*) (the temperature-sensitive *sec23-1* strain [19]).

3. BY4741-derived deletion strains are *SSO1* (*sso1Δ*), *SSO2* (*sso2Δ*), *SEC22* (*sec22Δ*), *SNC2* (*snc2Δ*), *TLG2* (*tlg2Δ*), *BTN2* (*btn2Δ*), *PEP12* (*pep12Δ*), *VPS51* (*vps51Δ*), *GOS1* (*gos1Δ*), *ATG1* (*atg1Δ*), *ATG8* (*atg8Δ*), *ATG11* (*atg11Δ*), *ATG17* (*atg17Δ*), *ATG20* (*atg20Δ*), *VAM3* (*vam3Δ*), and *GRH1* (*grh1Δ*) (Euroscarf). The genotypes of these strains are *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *YDR077w::KanMX4*. *SSO1/SSO2/SEC22/SNC2/TLG2/BTN2/PEP12/VPS51/GOS1/ATG1/ATG8/ATG11/ATG17/ATG20/VAM3/ or GRH1* [28].
4. Plasmid pULSG1 [29]: pULSG1 is the plasmid used for the expression of secretory target proteins/peptides and includes (a) the constitutive and strong *GAPDH* promoter expressed in the presence of glucose, (b) a signal sequence of glucoamylase from *Rhizopus oryzae* for secretion, (c) multiple cloning sites (*Bgl*II, *Not*I, *Sph*I, *Nhe*I, and *Xho*I), (d) the EGFP sequence for microscopic observation, (e) the FLAG epitope (DYKDDDDK) for the detection of protein secretion, (f) *URA3* and *leu2-d* auxotrophic markers for the selection of yeast transformants, (g) a 2-μm replication origin for the replication and maintenance of the plasmid in yeast, (h) the ampicillin resistance gene *Amp^r* for selection of *Escherichia coli* transformants, and (i) a *pUC* replication origin for the replication and maintenance of the plasmid in *E. coli*.
5. Plasmid pULGI2 [11]: pULGI2 is constructed from pULSG1 and is used for the intracellular production of unconventionally secreted proteins. pULGI2 does not contain the glucoamylase secretion signal sequence and has modified multiple cloning sites (*Bam*HI, *Xho*I, *Bgl*II, and *Sac*I, newly created by primers [11]).
6. Plasmid pULI1 [11]: pULI1 is the plasmid used for intracellular protein expression and includes (a) the *GAPDH* promoter, (b) multiple cloning sites (*Sac*I, *Kpn*I, *Bam*HI, and *Sal*I), (c) *URA3* and *leu2-d* auxotrophic markers for the selection of yeast transformants, (d) a 2-μm replication origin for the replication and maintenance of the plasmid in yeast transformants, (e) the ampicillin-resistance gene *Amp^r* for the selection of *E. coli* transformants, and (f) a *pUC* replication origin for the replication and maintenance of the plasmid in *E. coli* transformants.
7. Plasmid pUL-ATG-EGFP [11]: pUL-ATG-EGFP is constructed from pULSG1 by substituting the secretory signal sequence with the start codon (ATG) and is used for intracellular production of EGFP-FLAG protein. This plasmid is used as a negative control for unconventional secretion.

2.3 Plasmid Construction

1. KOD-Plus DNA polymerase (Toyobo, Ohtsu, Japan) supplied along with 10× PCR buffer for KOD -Plus-, 2 mM dNTPs, and 25 mM MgSO₄.
2. iProof DNA polymerase supplied along with 10× PCR buffer for iProof and 2 mM dNTPs.
3. MinElute PCR Purification Kit (Qiagen, Valencia, CA) for the purification of PCR products (70 bp to 4 kb).
4. QIAquick PCR Purification Kit (Qiagen) for the purification of PCR products (100 bp to 10 kb).
5. Restriction enzymes (*NotI* and *XhoI*) supplied with the appropriate buffers.
6. Sodium acetate (3 M; pH 5.2): Dissolve 40.8 g sodium acetate trihydrate in 80 mL ultrapure H₂O. Adjust the pH to 5.2 with glacial acetic acid, and make up the volume to 100 mL by adding ultrapure H₂O.
7. 70 % Ethanol: Add ultrapure H₂O to 70 mL of 95.5 % ethanol to a final volume of 100 mL.
8. 50× TAE buffer: Dissolve 242 g Tris base in 57.1 mL glacial acetic acid. Add 18.6 g EDTA·H₂O and make up the volume to 1 L by adding deionized H₂O. Dilute the 50× TAE buffer 50-fold with deionized H₂O to prepare 1× TAE buffer.
9. 1 % (w/v) Agarose gel: Dissolve 2 g Agarose 1600 in 200 mL 1× TAE buffer by heating in a microwave until it boils. Cool the agarose solution until it can be handled safely and pour it into the gel tray.
10. QIAEX II Gel Extraction Kit (Qiagen) (or other kits for the extraction of DNA from agarose gel).
11. Ligation High ligase (Toyobo).
12. *E. coli* DH5α competent cells.
13. Quantum Prep Plasmid Miniprep Kit (Bio-Rad Laboratories, Inc., Hercules, CA).

2.4 Yeast Transformation for Construction of Knockout Strains

1. Frozen-EZ Yeast Transformation II (Zymo Research, Orange, CA) supplied along with EZ 1 (for washing the yeast pellet), EZ 2 (for resuspending the yeast pellet), and EZ 3 (containing polyethylene glycol) solutions for yeast transformation using the lithium acetate method [30].
2. KOD Dash DNA polymerase (Toyobo) supplied along with 10× PCR buffer (KOD Dash-compatible) and 2 mM dNTPs. Other efficient DNA polymerases may also be used.

2.5 Preparation of Extracellular Proteins

1. 0.2-μm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI).
2. Disposable syringe without needles, 30 mL (SS-30ESZ; Terumo, Tokyo, Japan).

3. YM-30 filter-unit size 15 mL (Amicon Ultra centrifugal filter units Ultra-15, MWCO 30 kDa; Millipore, Millford, MA).
4. YM-30 filter-unit size 0.5 mL (Amicon Ultra 0.5 mL centrifugal filters, MWCO 30 kDa; Millipore).
5. YM-10 filter-unit size 15 mL (Amicon Ultra centrifugal filter units Ultra-15, MWCO 10 kDa; Millipore).
6. YM-10 filter-unit size 0.5 mL (Amicon Ultra 0.5 mL centrifugal filters, MWCO 10 kDa; Millipore).
7. 1 M Tris-HCl stock solution (pH 7.8): Dissolve 121.44 g Tris base in 800 mL deionized H₂O and adjust pH to 7.8 with HCl. Make up the volume to 1 L by adding deionized H₂O and remove debris by filtration using membrane filter (pore size: 0.45 μ m).
8. 20 mM Tris-HCl (pH 7.8): Dilute 1 M Tris-HCl stock solution 50-fold with deionized H₂O.
9. Vacuum dryer.

2.6 Preparation of Intracellular Proteins

1. Glass beads (GB-05; diameter 0.5 mm; TOMY, Tokyo, Japan).
2. Bead Smash 12 (Wakenyaku, Kyoto, Japan).
3. 20 mM Tris-HCl (pH 7.8) containing 0.1 % SDS.

2.7 SDS-PAGE

1. 0.5 M Tris-HCl stock solution (pH 6.8): Dissolve 60.72 g Tris base in 800 mL deionized H₂O and adjust pH to 6.8 with HCl. Make up the volume to 1 L by adding deionized H₂O.
2. 2 $\times$ buffer for SDS-PAGE: Mix 2 mL of 0.5 M Tris-HCl (pH 6.8), 4 mL of 10 % SDS, 2 mL glycerol, and 1 mg bromophenol blue. Make up the volume to 10 mL by adding deionized H₂O and store at -20 °C. Add 10 % volume of mercaptoethanol before use.
3. Continuous polyacrylamide gel (5–20 %; 120 $\times$ 100 mm; e-PAGEL; Atto, Tokyo, Japan).
4. 1 $\times$ Running buffer: Dissolve 30.36 g Tris base and 14.4 g glycine in 800 mL deionized H₂O and add 10 mL of 10 % SDS. Make up the volume to 1 L by adding deionized H₂O.
5. FLAG protein (48 kDa cleavage control protein; Novagen, Inc., Madison, WI).
6. Rainbow molecular weight marker proteins (Full-Range Rainbow Molecular Weight Markers, GE Healthcare, Buckinghamshire, UK).

2.8 Western Blotting

1. Nitrocellulose membrane (0.45- μ m pore size).
2. Anti-FLAG M2 antibody conjugated with horseradish peroxidase (HRP; Sigma-Aldrich, St. Louis, MO).
3. Rabbit anti-baker's yeast phosphoglucose isomerase-1 (Pgi1) antibody (Acris Antibodies GmbH, Hiddenhausen, Germany).

4. Anti-rabbit antibody conjugated to HRP (GE Healthcare).
5. Blocking solution (Blocking One; Nacalai Tesque, Inc., Kyoto, Japan).
6. Can Get Signal® Immunoreaction Enhancer Solution (Toyobo).
7. Stripping agent (WB Stripping Solution; Nacalai Tesque, Inc.).
8. ECL Plus Western blotting detection reagents (GE Healthcare) (currently unavailable; we have tested ECL Prime from GE Healthcare, but neither endogenous Pgi1p nor external control were detected by the methods described here. Please consider changing the sample volume or using other detection reagents such as Pierce Western Blotting Substrate Plus from Pierce, Luminata series from Millipore, etc.).
9. ImageQuant LAS 4000 minisystem (GE Healthcare).
10. 1× PBS: Dilute 10× PBS (1,370 mM NaCl, 81 mM Na₂HPO₄, 26.8 mM KCl, 14.7 mM KH₂PO₄, pH 7.4, Sigma-Aldrich,) tenfold with deionized H₂O.
11. PBST: Add 0.1 % Tween 20 to 1× PBS.
12. Hybri-Bag (e.g., Hybri-Bag Hard, Cosmo Bio, Tokyo, Japan).
13. Heat Sealer (e.g., Poly Sealer, Cosmo Bio).
14. Seesaw-motion shaker.
15. Plastic tray (disposable).

3 Methods

To quantitatively evaluate the unconventional secretion of proteins, the following procedures were performed: (1) Preparation of plasmids, (2) Preparation of yeast cells producing recombinant (tagged) proteins, (3) Detection and quantification of extracellular proteins by Western blotting, (4) Evaluation of unconventional secretion by inhibiting conventional secretion pathway, and (5) Determination of secretion-regulatory proteins using knockout strains.

For every experiment, the yeast strain producing EGFP-FLAG protein (i.e., yeast strain transformed with plasmid pUL-ATG-EGFP) was used as a secretion-negative control. Both the EGFP protein and FLAG-tag sequence do not contain signal sequences for secretion; thus, detection of the EGFP-FLAG in culture media of the negative control strain indicates that the proteins were artificially “leaked” during the experimental procedure. This leak often occurs due to careless experimental techniques such as intensive mixing of cells during washes or rapid filtration of culture supernatants before thoroughly eliminating cells by mild centrifugation. If the negative control protein is detected in culture media,

all the other samples taken at the same time should be discarded. For the detection of secreted proteins, positive control (i.e., yeast strain transformed with plasmid pULSG1) was used. Note that sometimes, the amount of unconventionally secreted proteins is less than that of the positive control; weakly secreted proteins may not be detected when compared to the positive control. In such cases, the amount of positive control (but never the negative control) may be reduced. Note that these methods can specifically detect recombinant proteins in which the target proteins are conjugated with EGFP and FLAG-tagged sequences at the C-terminus. Thus, proteins that are detected in the culture media with secretome analyses or other methods may not be detected with this method. In such cases, alternative methods such as preparing a protein-specific antibody or changing the recombination site (e.g. from C-terminus to N-terminus) should be considered.

For the quantification of the secreted proteins, the levels of extracellular target proteins were compared with that of the FLAG protein, which was used as an external control. To standardize the protein production level, the endogenous Pg1p, which was previously found to be unconventionally secreted [11], was first detected. Because the Pg1p signal is weaker than that of recombinant proteins, several reagents (e.g., commercial blocking solution and immunoreaction enhancer solution) were used to enhance the detection. After removing the first antibody, the same membrane was used to detect recombinant proteins. Since the FLAG protein is detected by both the antibodies, it can be used for normalization of the detected proteins. Other marker proteins detected by various antibodies (e.g., MagicMark™ XP Western Protein Standard, Invitrogen) may also be used instead of FLAG protein.

3.1 Construction of an Expression Plasmid for the Evaluation of Protein Secretion

1. Amplify the DNA fragment encoding the target protein to be secreted, together with the recognition sites of restriction enzymes at the 5' and 3' ends, using KOD –Plus or iProof DNA polymerase, according to the manufacturer's instructions.
2. Analyze the PCR product using agarose gel electrophoresis. Add 2 µL of 6× loading dye to 10 µL of the PCR product, and load the mixture onto a 1 % agarose gel. After electrophoresis, visualize the DNA by ethidium bromide staining.
3. Purify the PCR product by using a PCR product purification kit (for DNA fragment size ranging from 70 bp to 4 kb), according to the manufacturer's instructions. Alternatively, similar kits that are efficient for the purification of DNA fragments in the range of 100 bp to 10 kb (e.g., QIAquick PCR Purification Kit) can also be used.
4. Digestion of pULGI2 and the PCR product with restriction enzymes (*Bam*HI and either *Sac*I or *Xho*I): Prepare a mixture

- of 2 μg of DNA sample, 1 μL of restriction enzyme, 10 μL of 10 $\times$ buffer, and ultrapure H_2O to a final volume of 100 μL . Incubate the mixture at 37 $^\circ\text{C}$ for 3 h.
5. Add 10 μL of 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M and mix thoroughly.
 6. Add 275 μL (2.5 volumes) of 99.5 % ice-cold ethanol and mix thoroughly.
 7. Store the solution at -80°C for 10 min and centrifuge at 15,000 $\times g$ for 10 min at 4 $^\circ\text{C}$.
 8. Discard the supernatant and add 500 μL of 70 % ice-cold ethanol.
 9. Centrifuge at 15,000 $\times g$ for 5 min at 4 $^\circ\text{C}$ and discard the supernatant.
 10. Dry the DNA pellet in a lyophilizer.
 11. Dissolve the DNA pellet in 10 μL of autoclaved Milli-Q water and subject it to 1 % agarose gel electrophoresis. Cut out the area of the gel containing the DNA fragments and extract the DNA using QIAEX II Gel Extraction Kit, according to the manufacturer's instructions.
 12. DNA ligation using Ligation High or analogous DNA ligases: Combine 20–400 fmol of DNA fragment, 50–100 fmol of plasmid DNA, an appropriate amount of DNA ligase, and ligase buffer. Incubate the mixture at 16 $^\circ\text{C}$ for 1 h and follow the manufacturer's protocols.
 13. Transform 5 μL of *E. coli* DH5 α competent cells with 1.5 μL of ligation reaction and plate onto an LBA plate using a flame-sterilized spreader. Incubate the plates at 37 $^\circ\text{C}$ overnight.
 14. Inoculate 5 mL of the LBA media with *E. coli* transformants. Incubate at 37 $^\circ\text{C}$ with overnight shaking.
 15. Extract and purify the plasmid DNA using a miniprep kit (e.g., Quantum Prep Plasmid Miniprep Kit), according to the manufacturer's instructions. Determine the concentration and purity of the extracted plasmid DNA from the absorbance measured at 260 and 280 nm, using a spectrophotometer.
 16. Verify the identity of the plasmid DNA constructs by restriction digestion using appropriate enzymes. Digest 2 μg of plasmid DNA as described in **step 4** and perform electrophoresis on a 1 % agarose gel.
 17. Perform sequencing on the ligated DNA fragment by using a forward primer that anneals to the upstream region (GAPDH promoter) of the multiple cloning sites (pGAP_F; 5'-AGA CCGTAGGTATTGATTGTAATTCTG-3') and appropriate primers that can anneal to the internal sequences of the ligated DNA fragment.

3.2 Yeast Transformation with Plasmids for Production of Recombinant (Tagged) Proteins

1. Inoculate a single colony of *S. cerevisiae* strains into 10 mL YPD media in a test tube and incubate at 25 °C with overnight shaking.
2. Prepare yeast competent cells using Frozen-EZ Yeast Transformation II, according to the manufacturer's instructions (*see* **Note 1**).
3. Dispense 50 µL aliquots of the solution into separate tubes and store at -80 °C.
4. Mix 0.2 µg DNA with 5 µL of the competent cells.
5. Add 50 µL EZ 3 solution and mix thoroughly.
6. Incubate at 30 °C for 45 min.
7. Mix vigorously by flicking with a finger or vortexing.
8. Spread 50 µL of the transformant mixture mentioned above on an SDC+HM plate.
9. Incubate the plates at 30 °C for 2–4 days to allow for the growth of transformants (*see* **Note 2**).
10. To confirm the presence of the recombinant expression vector in the transformants, perform direct colony PCR using the efficient KOD Dash DNA polymerase with the appropriate primers, according to the manufacturer's instructions.

3.3 Cell Culture for the Analysis of Intracellular and Extracellular Proteins

1. Inoculate single colonies of transformants into 10 mL of SDC+HM media and incubate at 25 °C for 26 h with continuous shaking until they reach the late log phase (*see* **Note 3**).
2. Transfer the cell culture media into a new 15-mL tube.
3. Centrifuge at 900×*g* for 5 min at room temperature (r.t.).
4. Discard supernatants. Gently resuspend the cells in 5 mL fresh SD+HM media.
5. Repeat the wash in **steps 3** and **4**.
6. Measure the OD₆₀₀ of the cell suspensions.
7. Transfer the cells into 25 mL of SD+HM media to obtain an OD₆₀₀ of 0.3.
8. Incubate at 25 °C for 4 h with continuous shaking until they reach an OD₆₀₀ of 0.5 (*see* **Note 4**).

3.4 Preparation of Extracellular Proteins

1. Centrifuge the cell culture media (**step 8** in Subheading **3.3**) at 900×*g* for 10 min at 4 °C.
2. Filter the culture supernatants through a 0.2-µm syringe filter and keep on ice.
3. Transfer 12 mL of the filtered medium to YM-10 (for media containing proteins less than 35 kDa in size) or YM-10 (for proteins more than 35 kDa in size) filter-unit size 15 mL.

4. Centrifuge at $5,000 \times g$ at 4°C (*see* **Note 5**).
5. Discard the flow through. Transfer the remaining supernatants (**step 2** in Subheading 3.4) to the filter units.
6. Centrifuge at $5,000 \times g$ at 4°C .
7. Transfer the concentrated solution to YM-10 or 30 filter-unit size 0.5 mL.
8. Centrifuge the filter units at $10,000 \times g$ at 4°C .
9. Discard the flow through.
10. Add 400 μL of 20 mM Tris-HCl (pH 7.8) to the filter units.
11. Centrifuge the filter units at $10,000 \times g$ at 4°C .
12. Discard the flow through.
13. Repeat the above steps (**steps 10–12**) two more times.
14. Transfer the concentrated solution into a new 1.5 mL tube.
15. Store the solution at -80°C for 15 min.
16. Lyophilize the solution.
17. Store at -80°C until the samples are used for SDS-PAGE (*see* **Note 6**).
18. Resuspend the samples with 25 μL of $1\times$ buffer for SDS-PAGE and use 10 μL for SDS-PAGE (*see* **Note 7**).

3.5 Preparation of Intracellular Proteins

1. Turn Bead Smash (Wakenyaku) on 20 min before the experiment to cool the interior.
2. Resuspend the cells after centrifugation (**step 1** in Subheading 3.4) with 500 μL of 20 mM Tris-HCl (pH 7.8) containing 0.1 % SDS.
3. Add an equal weight of glass beads with cells to the cell suspension.
4. Homogenize the cell suspension at 4,000 rpm for 20 s using (Bead Smash, Wakenyaku).
5. Centrifuge the samples at $9,700 \times g$ at 4°C for 5 min.
6. Transfer the supernatants into a new 1.5-mL tube (*see* **Note 8**).
7. Add 5 μL of the supernatant to 5 μL of $2\times$ buffer for SDS-PAGE and use 10 μL volume for SDS-PAGE.

3.6 SDS-PAGE

1. Prepare serial dilutions of the FLAG-protein solutions (0.2, 0.4, and 0.8 ng) in 10 μL of $1\times$ buffer for SDS-PAGE (*see* **Note 9**).
2. Heat the samples prepared for SDS-PAGE (from **step 18** in Subheading 3.4, **step 7** in Subheading 3.5, and **step 1** in Subheading 3.6) at 100°C for 3 min.
3. Centrifuge the samples at $21,900 \times g$ (maximum speed) at 4°C for 5 min to remove the debris.

4. Load the 10 μ L of supernatants into a continuous polyacrylamide-gradient gel (*see Note 10*).

3.7 Western Blotting

1. Transfer the proteins to a nitrocellulose membrane.
2. Block the membrane by immersing into Blocking One solution for 30 min with continuous shaking on a seesaw-motion shaker.
3. Pack the membrane in a Hybri-Bag.
4. Add 3 mL of the Can Get Signal® Immunoreaction Enhancer Solution A and 3 μ L of the anti-Pgilp antibody to the membrane. Seal the Hybri-Bag (*see Note 11*) and mix well.
5. Incubate the packed membrane on a seesaw-motion shaker for 1.5 h at r.t.
6. Discard the solution. Transfer the membrane to a plastic tray.
7. Quickly rinse the membrane three times with PBST.
8. Wash the membrane by immersing into PBST for 5 min with continuous shaking on a seesaw-motion shaker (r.t.).
9. Discard the solution.
10. Repeat **steps 8 and 9** two more times.
11. Pack the membrane in a new Hybri-Bag.
12. Add 3 mL of the Can Get Signal® Immunoreaction Enhancer Solution B and 3 μ L of anti-Rabbit antibody conjugated with HRP to the membrane. Seal the Hybri-Bag and mix well.
13. Incubate the packed membrane on a seesaw-motion shaker for 1 h at r.t.
14. Discard the solution. Transfer the membrane to a plastic tray.
15. Quickly rinse the membrane three times with PBST.
16. Wash the membrane by immersing into PBST for 5 min with continuous shaking on a seesaw-motion shaker (r.t.).
17. Discard the solution.
18. Repeat **steps 16 and 17** two more times.
19. Detect the proteins using ECL-Plus (or other suitable) detection reagents and ImageQuant LAS 4000 minisystem by following the manufacturer's instructions (*see Note 12*).
20. After detecting Pgilp proteins, rinse the membrane twice with PBST.
21. Strip the antibodies by immersing the membrane into a stripping solution with continuous shaking on a seesaw-motion shaker (r.t., overnight) (*see Note 13*).
22. Rinse the membrane three times with PBST.
23. Block the membrane (*see step 2*).

24. Pack the membrane in a Hybri-Bag.
25. Add 3 mL of PBST and 3 μ L of anti-FLAG antibody conjugated with HRP to the membrane. Seal the Hybri-Bag and mix well.
26. Incubate the packed membrane on a seesaw-motion shaker for 1 h at r.t.
27. Discard the solution. Transfer the membrane to a plastic tray.
28. Quickly rinse the membrane three times with PBST.
29. Wash the membrane by immersing into PBST for 5 min with continuous shaking on a seesaw-motion shaker (r.t.).
30. Discard the solution.
31. Repeat **steps 29 and 30** two more times.
32. Detect the proteins (*see step 19*).
33. Take biological quartet.
34. Quantify the secreted target proteins. Process the signals gained from the extracellular Pgilp and target proteins conjugated with EGFP-FLAG tag using anti-Pgilp and anti-FLAG antibodies, by setting signals obtained from 0.4 ng/lane of FLAG protein as 1. Calculate the relative amounts of target proteins conjugated with EGFP-FLAG tag as follows: (signal intensities after anti-FLAG treatment)/(signal intensities after anti-Pgilp treatment). Finally, evaluate the protein secretion by Student's *t*-test or other appropriate statistical methods.

3.8 Inhibition of Conventional Secretion by the *sec23-1* Strain

1. Transform the *sec23-1* strain with plasmids (*see* Subheading 3.2) (*see Note 14*).
2. Pre-cultivate the transformants and wash with fresh medium (*see steps 1–6* of Subheading 3.3).
3. Inoculate the cells into fresh 10 mL SD+HM medium to an OD₆₀₀ of 0.3. Prepare two cultures for each sample.
4. Incubate one culture medium at 25 °C and another at 37 °C with continuous shaking for 4 h.
5. Filter the culture medium, concentrate, and lyophilize. Store at –80 °C until further use (*see steps 1–17* of Subheading 3.4).
6. Resuspend the lyophilized sample in 10 μ L of 1 $\times$ buffer for SDS-PAGE.
7. Perform SDS-PAGE and Western blotting (*see* Subheadings 3.6 and 3.7).
8. Compare the amount of target proteins between samples from cultures at 25 and 37 °C. If the target proteins are secreted conventionally, they will not be detected in the culture media at 37 °C (*see Note 15*).

3.9 Inhibition of Unconventional Secretion Using Knockout Strains

1. Transform the knockout strains with the relevant plasmids (*see* Subheading 3.2) (*see* **Note 16**).
2. Pre-cultivate the transformants and wash with fresh medium (*see* **steps 1–6** of Subheading 3.3).
3. Inoculate the cells in fresh 25 mL SD+HM medium to an OD₆₀₀ of 0.3.
4. Incubate the culture medium at 25 °C with continuous shaking for 4 h.
5. Filter the culture medium, concentrate, and lyophilize. Store at –80 °C until further use (*see* **steps 1–17** of Subheading 3.4).
6. Resuspend the lyophilized sample in 25 µL of 1× buffer for SDS-PAGE and use 10 µL for SDS-PAGE.
7. Perform SDS-PAGE and Western blotting (*see* Subheadings 3.6 and 3.7).
8. Perform statistical analysis to evaluate secretion inhibition by the knockout strains (*see* **Note 17**).

4 Notes

1. The competent cells can be stored at –80 °C by freezing slowly.
2. Use transformants within 2 weeks after transformation. It is recommended that freshly transformed cells be used for each experiment.
3. It is recommended that the growth curve of each transformant should be measured before performing the experiment described. In BY4741 wild type strains, it may take about 26 h to reach the late log phase when using freshly transformed cells with the plasmids described here. It is important to treat the cell culture media gently to prevent leaking of the intracellular proteins.
4. It is important to confirm that the cells are not precipitated during cultivation.
5. Centrifugation of culture media will take time (more than 4 h). Place all other samples on ice during the procedure to avoid protein degradation.
6. The lyophilized extracellular protein samples can be stored at –80 °C for at least 1 month.
7. The volume of the 1× buffer used for SDS-PAGE should be altered if the concentration of proteins is too high. Sometimes, the amount of positive control protein may increase to such an extent that secretion of the target proteins may not be detected.
8. The intracellular protein samples can be stored at –80 °C for at least 1 month. Avoid repeated freezing and thawing (no more than twice). It is recommended that after extraction, the protein solution should be immediately used for SDS-PAGE.

9. Preliminary experiments can be performed to compare the signals obtained by Western blotting of serial dilutions of FLAG proteins (or other external control protein markers) with the signals from the internal and secreted proteins. It is recommended that standard curves of control proteins should be prepared for each membrane.
10. The external, negative, and positive controls, and samples for investigation must be loaded onto the same gel.
11. Avoid packing air.
12. The exposure setting (e.g., time and detection modes) among repeated experiments must be identical. For the data acquisition settings, “High” or “Standard” mode is recommended. It is important to confirm that the signals are not saturated.
13. The appropriate incubation time for stripping off the antibodies from a membrane should be determined beforehand.
14. The temperature for cultivating the *sec23-1* strain should always be maintained below 25 °C to avoid problems with cell growth.
15. It is important to confirm that secretion of the positive control protein (EGFP conjugated with secretory signal sequence, produced in yeast cells transformed with plasmid pULSG1) should appear only in the medium cultivated at 25 °C and not at 37 °C.
16. Before beginning the experiments, it is important to ensure that each strain can be cultivated properly at 25 °C. Measuring growth curves is highly recommended. The pre-cultivation times should be altered depending on the growth curves to ensure that all the cultured strains used for the experiments are in the state of late log phase.
17. It is recommended that the results should be verified by compensating for the gene knockout by introducing plasmids for the protein production.

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Fractionation of *Plasmodium*-Infected Human Red Blood Cells to Study Protein Trafficking

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Abstract

Subcellular fractionation is a valuable tool to follow protein traffic between cellular compartments. Here we detail a procedure for fractionating erythrocytes infected with the human malaria parasite *P. falciparum* using the bacterial pore-forming protein Streptolysin O (SLO). Additionally we describe an experimental protocol to determine protein topology by carrying out a protease protection assay on SLO-lysed infected erythrocytes.

Key words Streptolysin O, Malaria, Erythrocyte, Fractionation, Protease protection

1 Introduction

The *Plasmodium*-infected erythrocyte is a multi-compartmentalized cell. Following invasion, the parasite massively alters the biochemical and physical properties of its host cell. These changes are brought about by parasite-encoded proteins that are trafficked to the erythrocyte [1, 2]. In recent years much research attention has been drawn to this process, as a better understanding of protein trafficking mechanisms in this important human pathogen may eventually lead to novel treatment therapies. To follow protein traffic to and through the infected erythrocyte, it is important to be able to prepare subcellular protein fractions for analysis, thus allowing, for example, the study of protein passage from one compartment to another. Most research in this field concentrates on understanding how proteins cross the membrane of the parasitophorous vacuole (PV) and enter the host cell [3–6]. To this end various techniques have been developed to follow this event, one of the most important being the differential permeabilization of cellular membranes using pore-forming toxins such as Streptolysin O (SLO) [7]. Upon addition to infected cells, SLO is capable of incorporating itself into membranes and spontaneously form

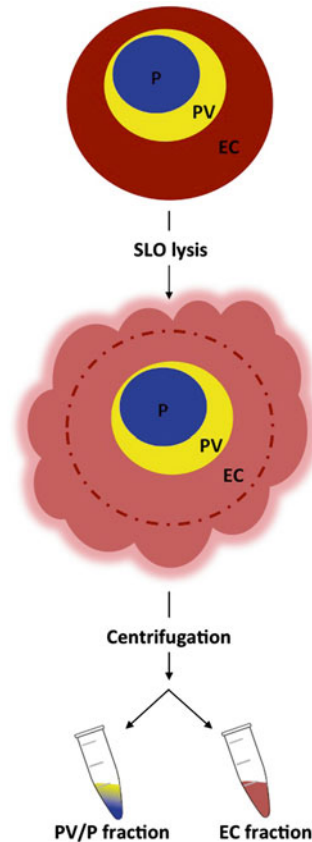


Fig. 1 Principle of subcellular fractionation using SLO. SLO permeabilizes only the erythrocyte plasma membrane, releasing the erythrocyte cytosol fraction (EC). A short centrifugation step allows isolation of the pellet fraction containing parasite cytosol (P) and parasitophorous vacuole fraction (PV)

aqueous pores with a diameter of approximately 30–45 nm [8, 9]. As initial binding of SLO is dependent on the membrane cholesterol content [10], it can be used to differentially permeabilize the infected erythrocyte as the parasitophorous vacuolar membrane (PVM) contains less cholesterol than the erythrocyte plasma membrane [7]. This allows the preparation of subcellular protein fractions for further analysis (Fig. 1). Additionally, permeabilization of infected erythrocytes with SLO permits access to the external face of not only the PVM, but other intra-erythrocytic membrane structures such as Maurer's clefts and the tubulovesicular membrane network (TVN) [11]. A subsequent protease treatment of the SLO-permeabilized cells thus allows determination of the topology of proteins residing within these membranes. Here we describe a detailed protocol for SLO treatment of infected cells and subsequent analysis of the protein fractions obtained.

2 Materials

All solutions are prepared using double distilled water and may be kept at room temperature (RT) unless otherwise indicated. Please read all necessary material safety data sheets (MSDS) and take appropriate safety precautions before beginning this protocol. Follow all guidelines for responsible waste disposal.

2.1 Materials Required for Preparation of SLO Stock Solution

1. Phosphate buffered saline solution (PBS, 140 mM NaCl, 2.7 mM KCL, 1.4 mM KH_2PO_4 , 0.8 mM Na_2HPO_4 , pH 7.4).
2. Streptolysin O (SLO, Sigma S-5265, or *see Note 1*). Dissolve the contents of a vial of SLO in 2.25 ml of PBS. Store 90 μl aliquots at -80°C (*see Note 2*).

2.2 Materials Required for Assaying Hemolytic Units (HU)

1. PBS as per Subheading 2.1.
2. Human erythrocyte concentrate (Buffy coat removed, usually available from local blood bank).
3. Two aliquots of SLO prepared as in Subheading 2.1 above.
4. 1 M Dithiothreitol (DTT). Freeze aliquots and thaw fresh for each assay.
5. Microcentrifuge.
6. Plate reader capable of measuring absorbance at 412 nm.
7. Microtiter plate suitable for plate reader above.

2.3 Materials Required for SLO Lysis and Sample Preparation

1. SLO prepared as in Subheading 2.1 above.
2. 1 M DTT as above.
3. PBS as per Subheading 2.1
4. 2×10^8 (20 μl packed volume) early to mid trophozoite-stage (approx. 24 h post invasion) infected erythrocytes purified to at least 50 % parasitemia (*see Note 3*). Determine parasitemia by counting Giemsa-stained slides.
5. Microcentrifuge.
6. Reducing sample buffer (4 $\times$): 200 mM Tris/HCl pH 6.8, 10 mM EDTA, 40 % glycerol, 8 % SDS, 0.04 % bromophenol blue, 200 mM dithiothreitol. Store aliquots at -20°C for up to a month.

2.4 Materials Required for Protease Protection Assay of SLO Permeabilized Infected Erythrocytes

1. PBS as per Subheading 2.1.
2. Proteinase K (10 mg/ml in PBS, make fresh every time, keep on ice until required).
3. 10 % Triton-X-100 in PBS.
4. 100 mM Phenylmethanesulfonyl fluoride (PMSF)/Isopropanol. Add 0.174 g PMSF to 10 ml of 100 % Isopropanol and allow to dissolve. Keep frozen at -20°C . Allow to come to room temperature before use.

5. Broad spectrum protease inhibitor cocktail (PIC, *see* **Note 4**).
6. 2×10^8 permeabilized cells from protocol in Subheading 3.2.

2.5 Antibodies and Conjugates

1. Mouse anti-human Hsc70/Hsp70 (Santa Cruz, SC-24, 1:500) (*see* **Note 5**).
2. Rabbit anti-PfSERP/SERA5 (available from the author, 1:500 [12]) (*see* **Note 5**).
3. Rabbit anti-PfAldolase (available from the author, 1:5,000 [13]) (*see* **Note 5**).
4. Rabbit anti-PfSBP1 raised against N-terminus (available from Prof. Catherine Braun-Breton, Montpellier, 1:500 [14]) or rabbit anti-Exp1 raised against C-terminus (available from the author, 1:500 [15]).
5. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (DAKO, 1:2,000).

3 Methods

3.1 Determination of Hemolytic Units (HU)

1. Label two sets of eleven 1.5 ml reaction tubes from 1 to 11. Tubes 1–10 are the assay tubes, while tube 11 is a negative control.
2. Referring to Table 1, aliquot the required amount of PBS into each tube.
3. Wash 400 μ l of packed red blood cells (RBC) in 1 ml of PBS. Collect packed cells by centrifugation at $1,000 \times g$ for 4 min at room temperature (RT). Remove and discard supernatant.
4. Referring to Table 1, pipette 20 μ l of packed RBCs into each tube (*see* **Note 6**).
5. Activate SLO. Remove 2×90 μ l aliquots of SLO from storage and thaw. To each add 9 μ l of 1 M DTT. Mix gently and incubate at RT for 15 min.

Table 1
Setup for assaying hemolytic units of SLO

Tube	1	2	3	4	5	6	7	8	9	10	11
PBS	179	178	176	172	168	164	160	155	150	140	180
RBC	20	20	20	20	20	20	20	20	20	20	20
SLO	1	2	4	8	12	16	20	25	30	40	–
Total	200	200	200	200	200	200	200	200	200	200	200

All amounts in microliter

6. Add the amount of activated SLO solution noted in the table below to each tube. Mix gently to resuspend and incubate for 6 min at RT. During this time, invert the tubes ten times every 2 min.
7. Isolate supernatant by centrifugation at $1,000 \times g$ for 4 min. Remove 180 μ l of each supernatant to new tubes.
8. Generate a 100 % positive lysis control. Add 20 μ l of RBC to 180 μ l PBS. Lyse by three freeze/thaw cycles. Centrifuge as above and remove 180 μ l supernatant to a new tube.
9. Make 800 μ l of a 1/200 dilution of each supernatant (4 μ l in 796 μ l PBS). Aliquot 200 μ l of each dilution, in triplicates, into a microtiter plate suitable for the plate reader available.
10. Measure absorbance at 412 nm. This gives a measure of the amount of hemoglobin released.
11. Graph amount of hemoglobin released against volume of SLO used. Set the positive control to 100 %. 1 hemolytic unit (HU) is the amount of SLO required to cause 50 % cell lysis.

3.2 Permeabilization and Subcellular Fractionation of Infected Erythrocytes

1. Wash infected erythrocytes (IE) and resuspend 2×10^8 purified infected erythrocytes in 200 μ l PBS. Centrifuge at $1,000 \times g$ for 2 min at RT.
2. Resuspend IE in a volume of PBS equivalent to (180 μ l – (volume of SLO required to have 4 HU)) (*see Note 7*).
3. Activate SLO. Remove required number of aliquots of SLO from storage and thaw. To each add 9 μ l of 1 M DTT. Mix gently and incubate at RT for 15 min.
4. Add required amount of SLO solution to each tube. Mix gently to resuspend and incubate for 6 min at RT (*see Note 8*). During this time, invert the tubes ten times every 2 min.
5. Centrifuge samples at $1,000 \times g$ for 4 min at RT. From each sample, remove 180 μ l of supernatant (SNT) to a new tube. Retain pellet (P). Re-centrifuge SNT at $1,000 \times g$ for 4 min at room temperature. Remove 150 μ l of SNT to a new tube (*see Note 9*).
6. Wash P three times in 500 μ l PBS ($1,000 \times g$, 4 min, RT).
7. Add 50 μ l of 4 $\times$ protein sample buffer to each supernatant. Resuspend each pellet in 150 μ l of PBS then add 50 μ l of 4 $\times$ protein sample buffer to each. Boil samples for 10 min.
8. Calculate the volume of sample (in μ l) required to load 5×10^6 infected cell equivalents of both SNT and P using the following formulae (*see Note 10*):

Supernatant: Volume to load = $(5 \times 10^6 \div ((2 \times 10^8 \times (\% \text{ parasitemia} \div 100) \times 0.75) \div 200))$.

Pellet: Volume to load = $(5 \times 10^6 \div ((2 \times 10^8 \times (\% \text{ parasitemia} \div 100)) \div 200))$.

9. Load 5×10^6 cell equivalents on a 12 % SDS-PAGE using standard protocols and including commercial protein standards. Load supernatant and pellet fractions from each sample next to each other on the gel to allow direct comparison of band intensity. One entire set of samples is required for detection of control proteins.
10. Transfer to nitrocellulose using standard Western blotting protocols.
11. If using non-stained protein marker, confirm correct transfer by Ponceau S staining the membrane and mark marker bands with pencil. Destain membrane with PBS.
12. Carry out immunodetection. If using antibodies with known specificity (such as those suggested) all control proteins can be detected by carefully cutting the control membrane into three sections as shown in Fig. 2a. A correct result after permeabilization is shown diagrammatically in Fig. 2b.

3.3 *Protease Protection Assay to Determine Protein Topology*

1. Label four tubes (1–4) (*see Note 11*).
2. Use an equivalent of 5×10^7 infected SLO-permeabilized cells (washed three times, from above protocol **step 6**) per tube (*see Note 12*).
3. Referring to Table 2, add the required amounts of PBS, Proteinase K (PK), Triton X-100 (T-X-100), PMSF, and PIC.
4. Mix gently and place samples on ice for 30 min.
5. Add PMSF to a final concentration of 2 mM and PIC to 1× to all samples to inactivate PK.
6. Add 16.5 µl of 4× protein sample buffer to each sample and boil for 10 min.
7. Load 5×10^6 cell equivalents per lane on a 12 % SDS-PAGE using standard protocols and including commercial protein standards.
8. Transfer to nitrocellulose membrane using standard Western blotting protocols.
9. If using a non-stained protein marker, confirm correct transfer by Ponceau S staining the membrane and mark marker bands with pencil. Destain membrane with PBS.
10. Carry out immunodetection. As a control for the effectiveness of PK treatment, detect PfSBP1 or PfExp1 using the antisera listed in Subheading 2. Figure 2c, d shows a diagrammatic representation of a correct result.

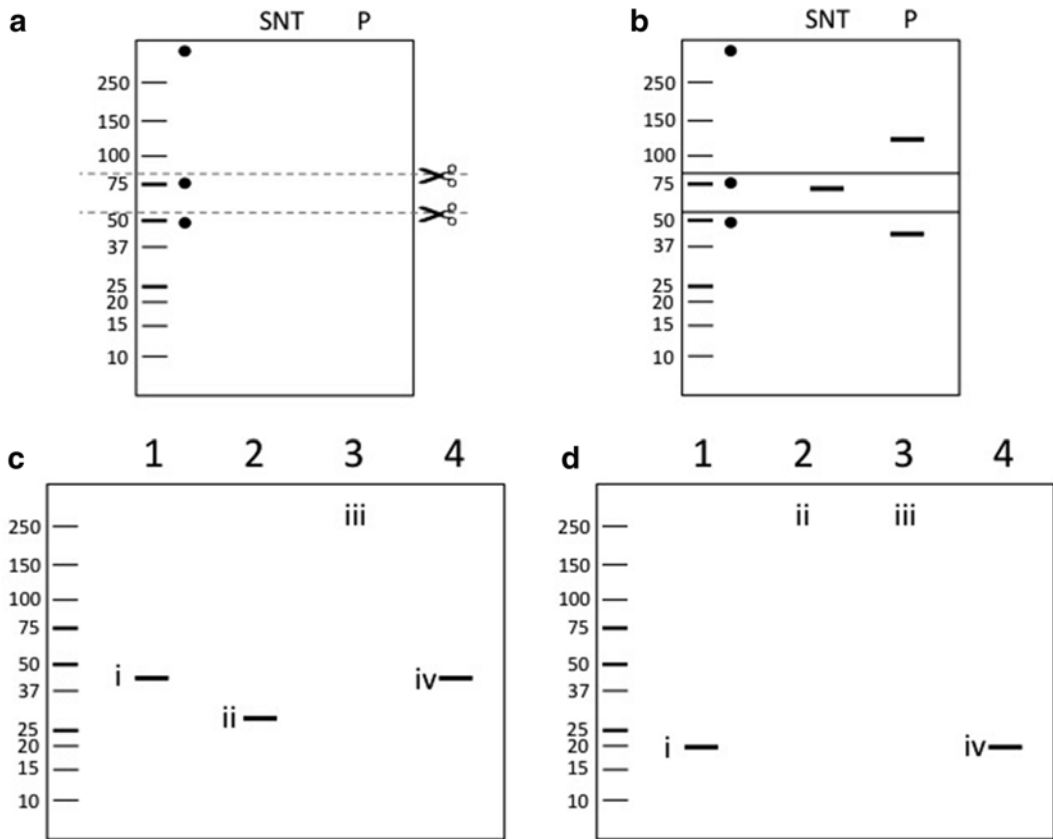


Fig. 2 (a) How to cut membrane for detection of control proteins. Protein standard: Precision Plus Protein™ All Blue Standards (Bio Rad #161-0373). If using other standards: PfSERP $\approx$ 120 kDa; HsHsp70 $\approx$ 70 kDa; PfAldolase $\approx$ 42 kDa. Add a *dot* in the *top left hand corner* of each membrane strip to allow for correct orientation. (b) Correct result for control proteins following SLO fractionation. (c) Correct result following PK protection and detecting using anti-PfSBP1: (i) full-length PfSBP1 ($\approx$ 45 kDa); (ii) protected N-terminal fragment ($\approx$ 30 kDa); (iii) total digestion; (iv) full length PfSBP1. (d) Correct result following PK protection and detecting using anti-PfExp1: (i) full-length PfExp1 ($\approx$ 18 kDa); (ii) total digestion; (iii) total digestion; (iv) full length PfExp1

Table 2
Setup for protease protection assay

Tube	1	2	3	4
10 mg/ml PK	–	0.5	0.5	–
10 % T-X-100	–	–	5	5
100 mM PMSF	–	–	–	1
PIC	–	–	–	1
PBS	50	49.5	44.5	43

All amounts in microliter

4 Notes

1. The original published protocol called for a cysteine–alanine mutant of SLO that is constitutively active (C530A [16]). However this mutant is not commercially available and thus this current protocol describes use of only the commercially available SLO variant which requires activation with DTT prior to use. Those wishing to use the cysteine–alanine mutant are requested to contact Professor Sucharit Bhakdi (University of Mainz, sbhakdi@uni-mainz.de) for further information.
2. SLO does not remain stable at 4 °C for extended periods of time. Be aware that, even when stored correctly SLO loses activity over time, and may have to be re-assayed to ensure the correct number of hemolytic units is used.
3. Parasites may be purified from cultures using various published methods including gelatine flotation, percoll gradient or magnetic activated cell sorting [17–20]. Parasitemia of at least 50 % is required to ensure that it is possible to load enough parasite-derived material on SDS-PAGE gels without problems due to the high hemoglobin content. In our experience, no more than 1×10^7 hemoglobin-containing cell equivalents can be loaded on a typical SDS-PAGE gel without considerable nonspecific reactivity of hemoglobin with the ECL reagent used during immunodetection. The resulting high background typically obscures signals of interest. SLO lysis is most successful on parasites at approx. 24 h post-invasion. At later time points we note that the PVM appears to become more fragile, and release of PV contents upon SLO treatment occurs. For this reason, all cultures should be synchronized to ensure a maximal 4 h spread of parasite development, otherwise the presence of later stage parasites may lead to leakage of control proteins into inappropriate fractions. For reasons that are not clear, this protocol is not scalable. If more than 2×10^8 cells per sample are to be permeabilized, they must be split into aliquots of 2×10^8 for permeabilization to be effective. Resulting subcellular fractions may be pooled following permeabilization.
4. While establishing this protocol we were surprised to note that exogenously added proteinase K, and also endogenous cellular proteases retain a certain level of activity in reducing SDS-sample buffer. It is thus important to inactivate all proteases before adding SDS-sample buffer. Any commercially available PIC is suitable but should be supplemented by 2 mM PMSF.
5. We describe here the antibodies used in our laboratory as they have been verified to be specific to the following cellular compartments: HsHsp70, cytosol of the erythrocyte; PfSERA5/

SERP, parasitophorous vacuolar space; PfAldolase, parasite cytosol. In principle any antibodies that are verified to be specific only to each of these cellular compartments may be used.

6. To do this as accurately as possible, cut the end off the end of a 100 μ l pipette tip to create a larger opening.
7. For example, if you require 14 μ l of SLO, resuspend IE in 166 μ l PBS.
8. We find that best results are achieved if the SLO solution is pipetted onto the side of the reaction tube and then slowly incorporated into the remaining cell suspension.
9. These centrifugation steps are designed to avoid unwanted carryover of permeabilized cells in the supernatant fraction. For added pipetting accuracy, put a 10 μ l pipette tip onto the end of the 100 μ l tip being used.
10. In our experience 5×10^6 cell equivalents is enough material to obtain strong signals for the controls using the antibodies and conditions suggested. Depending on the expression level of the protein of interest, less or more equivalents may be loaded. As noted above, in our experience loading more than 1×10^7 total cell equivalents (including non-infected cells) leads to strong nonspecific signals during immunodetection. A higher initial parasitemia (up to 95 % can be achieved using magnetic cell sorting) allows more parasite cell equivalents to be loaded. The formula takes into account the parasitemia, the initial number of cells (2×10^8), the required number of cells to be loaded (5×10^6), the volume of the sample (200 μ l) and for the supernatant a factor (0.75) to correct for sample loss during the procedure.
11. Tubes 1, 3, and 4 are controls: Tube 1, control that any proteolysis observed is only due to the addition of exogenous Proteinase K and is not due to the activity of endogenous proteases; tube 3, control that the protein of interest is Proteinase K sensitive; tube 4, control that any proteolysis observed took place prior to addition of sample buffer.
12. To divide SLO-permeabilized cells into equal amounts, calculate the desired amount and split cells into four aliquots while performing the third and last washing step. Then, centrifuge, discard washing supernatant, and proceed according to Table 2. Start by resuspending the permeabilized cells in PBS.

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Chapter 6

Investigating Signaling Processes in Membrane Trafficking

Laura J. Sharpe and Andrew J. Brown

Abstract

Signaling and phosphorylation can be very difficult areas to explore, as there can be a lot of cross-talk between signaling pathways, and the stoichiometry of phosphorylation is often very low, and is typically transient. Here we describe an innovative assay using an immunoprecipitation approach, followed by a kinase assay, coupled with a phosphorylated substrate-specific antibody. We also indicate a database and prediction program that can be used in these situations.

We apply these methods to investigate the regulation of ER-to-Golgi trafficking by protein phosphorylation of critical components in the trafficking machinery. Key components of this transport step are well known thanks to the pioneering work of the 2013 Nobel Prize winners James Rothman, Randy Schekman, and Thomas Südhof. However, the regulation aspect of this process is relatively unexplored.

Key words Akt, Immunoprecipitation, Sec24, Kinase, Phosphorylation

1 Introduction

The vesicle transport system within cells has been largely elucidated by the 2013 Nobel Prize winners Randy Schekman, James Rothman, and Thomas Südhof. Firstly, Schekman uncovered the genetic basis for the secretory pathway components [1, 2], followed by Rothman discovering the proteins involved in vesicle fusion with membranes [3]. Südhof then determined how vesicles know when to release their contents [4].

The first part of the secretory pathway involves the transport of proteins from the endoplasmic reticulum (ER), where they are synthesized, to the Golgi, where they are sorted and distributed [5]. This process involves the COPII complex, which consists of a large number of subunits. These were identified from the landmark experiments by Randy Schekman and Peter Novick. These investigations hypothesized that yeast cells with a mutation in any of the genes that encode components of the secretory pathway would be lethal. This led to screening of temperature-sensitive mutants, and ultimately, many of the “Sec” proteins were identified in yeast [1].

There are a large number of these, and the full range will not be discussed here. It has been shown that there is considerable conservation of Sec genes from yeast to mammalian cells, and the mammalian gene products have also been identified [6].

The COPII coat is formed on the surface of the ER where the small GTPase Sar1 and two complexes known as Sec23–Sec24 and Sec13–Sec31 assemble. Sar1 activation takes place via a GDP/GTP exchange which is catalyzed by Sec12, a transmembrane guanine nucleotide exchange factor (GEF) [7]. Once activated, Sar1 recruits the Sec23/Sec24 heterodimer to the membrane, and together these form the Sec23/Sec24/Sar1 complex, an approximately 15 nm long structure in the shape of a bow tie [8]. The side that faces the membrane is concave and positively charged. This is where cargoes bind to Sec24, and are included in the COPII vesicle. The recruitment of Sec23/Sec24 in turn recruits the Sec13/Sec31 heterotetramer, allowing for the export of proteins from the ER in COPII-coated vesicles [9] (Fig. 1). Sec23 acts as a GTPase-activating protein (GAP), hydrolyzing the GTP from Sar1 to release the vesicle from the membrane [10]. Sec24 is the selector of the cargo proteins [11], and Sec13 and Sec31 are responsible for forming the flexible cage around the vesicle in a cuboctahedron shape [12]. It has been suggested that to accommodate larger cargoes, an icosidodecahedron shape may also be possible [13].

We have found that Sec24 is phosphorylated by Akt [14], a kinase involved in cell survival and proliferation, and here we describe the novel method we developed to investigate this. We used an immunoprecipitation of overexpressed, epitope-tagged Sec24, which is then incubated with commercially available Akt in a kinase assay, followed by Western blotting with phosphorylated Akt substrate antibody. To the best of our knowledge, this type of approach is typically used only to assay for activity of immunoprecipitated kinases on an added peptide substrate, rather than vice versa.

We also describe methods for identifying potential phosphorylation sites and kinases using a database and prediction programs. We have recently used these to explore the role signaling processes play in the activity of DHCR24, the final enzyme in cholesterol synthesis [15].

2 Materials

2.1 Immunoprecipitation (IP) of Protein

1. Protein G-Sepharose beads.
2. Dulbecco's Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM disodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate.
3. RIPA buffer: 20 mM Tris–HCl (pH 7.4), 0.1 % (w/v) sodium dodecyl sulfate (SDS), 1 % (v/v) NP-40, 0.5 % (w/v) Na-deoxycholate, 150 mM NaCl, 5 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM Na₃VO₄.

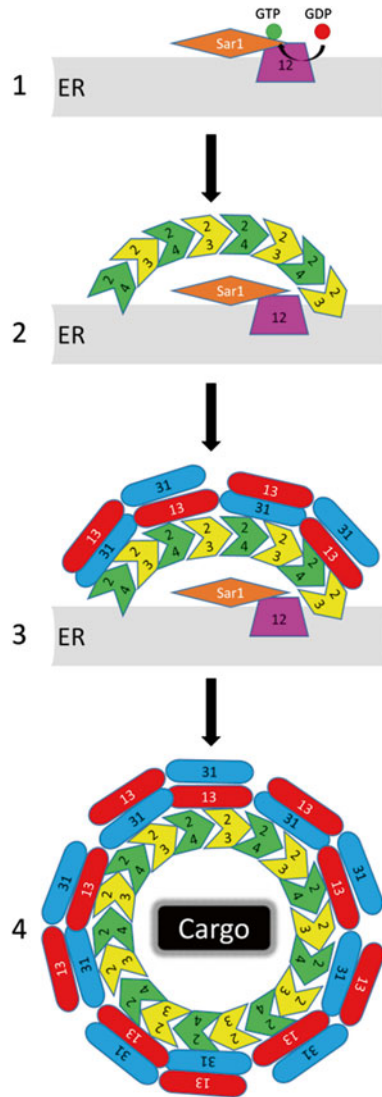


Fig. 1 COPII vesicle budding in ER-to-Golgi trafficking. (1) Sec12 catalyzes the exchange of GDP for GTP on Sar1. (2) Sec23/Sec24 heterodimers are recruited. (3) Sec13/Sec31 heterotetramers form a layer around the Sec23/Sec24 complex. (4) The COPII vesicle is released from the ER membrane, with the cargo packaged inside

4. Protease Inhibitors (PI) and Phosphatase Inhibitors (PPI) (e.g., cocktails of these are available from Sigma).
5. FLAG monoclonal antibody (*see Note 1*).
6. Normal mouse IgG antibody.

2.2 Kinase Assay

1. Kinase buffer: 25 mM Tris-HCl pH (7.4), 5 mM β -glycero-phosphate, 10 mM magnesium chloride, 2 mM DTT, 0.1 mM sodium orthovanadate, 200 μ M ATP, 12.5 μ g/ml bovine serum albumin (*see Note 2*).
2. Recombinant Akt (*see Note 3*).

2.3 Western Blotting

1. Tris-buffered saline with Tween-20 (TBST): 1 l 1 $\times$ TBS, 1 ml Tween-20 (*see Note 4*).
2. Blocking solution: 5 % (w/v) bovine serum albumin in TBST (*see Note 5*).
3. Antibody diluting solution: 5 % (w/v) bovine serum albumin (BSA) in TBST.
4. Primary antibodies: Anti-FLAG and Anti-phospho-Akt substrates (Cell Signaling Technology) (*see Note 6*).
5. Secondary antibody: Anti-rabbit and anti-mouse conjugated to horse radish peroxidase (HRP).
6. ImmobilonTM Western Chemiluminescent HRP Substrate detection system (ECL, Millipore) and LAS 500 Western imaging system (*see Note 7*).

2.4 Identification of Known Phosphorylation Sites and Prediction of Potential Kinases

1. PhosphoSitePlus (phosphosite.org).
2. Scansite (scansite.mit.edu).

3 Methods

To directly determine that Akt phosphorylates Sec24, epitope-tagged Sec24 is immunoprecipitated and a kinase assay with recombinant Akt is performed. The samples are then subjected to Western blotting with an antibody specific to phosphorylated substrates of Akt. *See Fig. 2* for an overview of this process.

To identify known phosphorylation sites in a protein of interest (in our example, DHCR24), a database, PhosphoSite [16], is examined. To determine potential kinases that may phosphorylate DHCR24, a prediction program, Scansite [scansite.mit.edu, [17]], can be employed.

3.1 Immunoprecipitation (IP) of Protein

1. Culture Chinese Hamster Ovary-7 (CHO-7) cells in a 10 cm dish in DMEM/F12 supplemented with 5 % lipoprotein deficient serum prepared from newborn calf serum (*see Note 8*).
2. After 24 h, transfect cells with a plasmid encoding Sec24C fused to a FLAG epitope tag.
3. After another 24 h, rinse cells twice with ice cold PBS.

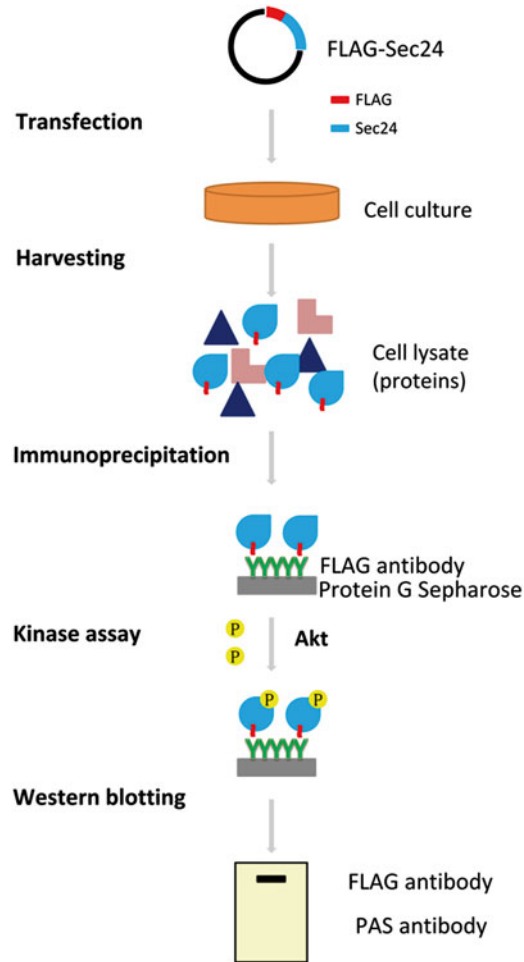


Fig. 2 Immunoprecipitation/kinase assay. *Transfection*: CHO cells are transfected with FLAG-tagged Sec24 plasmid and cell lysates containing a variety of proteins are *harvested*. *Immunoprecipitation*: FLAG-Sec24 is immunoprecipitated using FLAG antibody coupled to protein G sepharose. *Kinase assay*: Recombinant Akt is added and a kinase assay performed, followed by *Western blotting* with FLAG and PAS antibodies

4. Scrape the cells in PBS and transfer to a centrifuge tube.
5. Rinse the dish with PBS and add to the same centrifuge tube.
6. Centrifuge for 5 min at $1,000 \times g$.
7. Discard the supernatant and resuspend cells in 1 ml RIPA buffer containing PI and PPI.
8. Needle 20 $\times$ with a 21 G needle and transfer to a 1.5 ml tube.
9. Centrifuge for 15 min at $20,000 \times g$ (*see Note 9*).
10. Add 50 μ l protein G-sepharose (*see Note 10*) plus 30 μ g normal mouse IgG control antibody.

11. Rotate for 1 h at 4 °C, with end-over-end mixing.
12. Centrifuge for 15 min at 20,000×*g* at 4 °C.
13. Transfer the precleared supernatant to a new tube.
14. Add 30 µg FLAG antibody (*see Note 11*) and 50 µl protein G-sepharose.
15. Rotate overnight at 4 °C with end-over-end mixing.
16. Centrifuge for 5 min at 20,000×*g* at 4 °C.
17. Discard the supernatant (*see Note 12*).
18. Wash the pellet with the following steps:
 - (a) Add 1 ml RIPA buffer (with PI and PPI) to the pellet.
 - (b) Rotate for 30 min at 4 °C.
 - (c) Centrifuge for 5 min at 20,000×*g* at 4 °C.
 - (d) Discard the supernatant.
19. Repeat the washing steps twice for 30 min, and once for 10 min.

3.2 Kinase Assay

1. To perform the kinase assay, the buffer must be changed. This is achieved by washing for 10 min each with the following buffers (*see Note 13*) as in **step 18** of Subheading 3.1:
 - (a) 1:1 ratio of RIPA buffer:kinase buffer.
 - (b) 1:3 ratio of RIPA buffer:kinase buffer.
 - (c) Kinase buffer.
2. Following the wash in kinase buffer, transfer the sample to a 2 ml tube and perform a final wash in kinase buffer for 10 min.
3. Discard all remaining supernatant after the final wash.
4. Resuspend in 40 µl kinase buffer (*see Note 2*).
5. Add 400 ng recombinant active Akt (include a control without kinase).
6. Incubate at 30 °C for 30 min with stirring (*see Note 14*).
7. Add 1 ml kinase buffer and transfer to new 1.5 ml tubes.
8. Wash three times with kinase buffer as in **step 18** of Subheading 3.1.
9. Discard all supernatant.
10. Resuspend in 60 µl SDS-PAGE loading buffer (*see Note 15*) and heat at 95 °C for 10 min with occasional vortexing.
11. Centrifuge for 5 min at 20,000×*g* and load supernatant on an SDS-PAGE gel.

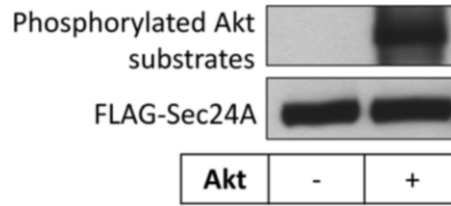


Fig. 3 Immunoprecipitated FLAG-Sec24A is phosphorylated by Akt. CHO-7 cells were transfected with FLAG-Sec24 (isoform A), protein was immunoprecipitated with FLAG antibody, and a kinase assay with or without Akt was performed. Pellets were subjected to SDS-PAGE and Western blotting with phosphorylated Akt substrates and FLAG antibodies

3.3 Western Blotting

1. Once the samples have sufficiently separated (*see Note 16*), transfer to a nitrocellulose membrane using standard Western blotting techniques.
2. Block the membrane in blocking solution with gentle rocking for 1 h.
3. Rinse briefly with TBST.
4. Incubate the membrane in primary antibody (phospho-Akt substrates antibody) with gentle rocking overnight (*see Note 4*).
5. Wash six times for 5 min in TBST with agitation (*see Note 4*).
6. Incubate in secondary antibody with gentle rocking for 1 h.
7. Wash six times for 5 min in TBST with agitation.
8. Visualize labeling using ECL and LAS 500 imaging system.

An example of the results from this method can be found in Fig. 3.

3.4 Identification of Known Phosphorylation Sites

1. Go to PhosphoSitePlus (www.phosphosite.org).
2. Search for your protein of interest.
3. Note the phosphorylation sites that have been found (*see Note 17*).

We found that three known phosphorylation sites on DHCR24 affect its activity [15].

3.5 Prediction of Potential Kinases

1. Go to scansite.mit.edu (*see Note 18*).
2. Search for motifs within your protein of interest (*see Note 19*).

We found that protein kinase C affects the activity of DHCR24 [15], not through a known phosphorylation site, but Scansite predicts eight potential PKC phosphorylation sites, two of which are considered “medium stringency.”

4 Notes

1. We have found that antibodies directed against epitope tags such as FLAG perform far better in immunoprecipitations than those directed against specific proteins.
2. ATP must be added fresh each time. BSA need only be included in the kinase assay with Akt, and should also be added fresh. We have found that PI can interfere with the reaction and should be used very sparingly.
3. Other recombinant active kinases could similarly be used.
4. It is important to use TBST rather than PBST when probing for phosphorylated proteins, as the phosphates can interfere with detection. To improve sensitivity, antibodies should always be incubated overnight. We also use six 5 min washes for phosphorylated antibodies as this improves specificity.
5. BSA should be used for blocking as there are phosphorylated proteins in skim milk which can interfere with detection.
6. Phosphorylated Akt substrates antibody and other phosphorylated substrate antibodies are available from Cell Signaling Technology. Note that the specificities of these antibodies can be exploited to your own advantage—for example, we have used the fact that phosphorylated Akt substrates antibody detects the phosphorylated form of S6 [18] as a control [19], and strengthened our findings by using two versions of the phosphorylated Akt substrates antibody [20].
7. Other detection methods could also be used.
8. Any cell type that provides high transfection efficiency and protein may be used.
9. At this point, a protein assay may be used to ensure equal amounts of protein are used in multiple samples.
10. Thorough washing of the protein G-Sepharose beads in RIPA is necessary beforehand as the commercial stock is preserved in ethanol. To facilitate pipetting of the sepharose, we recommend cutting the ends off tips.
11. The amount of antibody used here can be titrated.
12. The supernatant can be subjected to Western blotting to assess immunoprecipitation efficiency.
13. These ratios are required to ensure that the proteins refold correctly when moving from one buffer to another.
14. Very small magnetic stirrers are required here to ensure mixing in the 2 ml tube.
15. We use a mixture of two parts RIPA buffer, two parts 10 % SDS, and one part 5× loading buffer.

16. Monitor the migration of the molecular weight markers to ensure that the size corresponding to the protein of interest is at least halfway into the gel, to increase resolution in this region. Note that the heavy chain and light chain IgGs from the antibody used for the IP will be detected very strongly if using antibodies from the same species for both the IP and the Western blotting. These will be visible at ~50 and ~25 kDa, so proteins that are of a similar size to this will not be visible due to overshadowing by the detection of the IgG components. One solution to this problem is to add an additional, large epitope tag (e.g., GFP or GST) to alter the migration of the protein on the gel. We have used this approach previously to separate our phosphorylated recombinant protein from the Akt itself in an in vitro kinase assay [20].
17. We urge users to exercise caution with this database, as some references are not publications, but are derived from Cell Signaling Technology's in-house mass spectrometry experiments, which have not been peer-reviewed and therefore may be less reliable.
18. PhosphoSite provides a link to Scansite for each protein, as well as other external links to other databases.
19. Scansite can be searched using low, medium, or high stringency, with high stringency being the most specific and least sensitive. A newer version of Scansite, Scansite 3.0, provides even more sensitive predictions including a "minimum" stringency option. It may also be interesting to particularly compare the known phosphorylation sites from PhosphoSite with the predictions from Scansite.

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Recruitment of Coat Proteins to Liposomes and Peptidoliposomes

Sonja Huser, Gregor Suri, Pascal Crottet, and Martin Spiess

Abstract

Intracellular transport within the cell is generally mediated by membrane vesicles. Their formation is typically initiated by activation of small GTPases that then recruit cytosolic proteins to the membrane surface to form a coat, interact with cargo and accessory proteins, and deform the lipid bilayer to produce a transport vesicle. Liposomes proved to be a useful tool to study the molecular mechanisms of these processes in vitro. Here we describe the use of liposomes and peptidoliposomes presenting lipid-coupled cytosolic tails of cargo proteins for the in vitro analysis of the membrane recruitment of AP-1 adaptors in the process of forming AP-1/clathrin coats. AP-1 recruitment is mediated by the GTPase Arf1 and requires specific lipids and cargo signals. Interaction with cargo induces AP-1 oligomerization already in the absence of clathrin. Without cargo peptides, accessory proteins, such as amphiphysin 2, can be identified that stabilize AP-1 binding to liposomal membranes.

Key words Amphiphysin, Arf1, Clathrin adaptor protein, Coat protein, Liposome, Membrane traffic, Peptidoliposome, Protein sorting

1 Introduction

Liposomes are widely used to study molecular processes at membrane surfaces in vitro. They have been particularly useful in the area of membrane trafficking to reconstitute the assembly of cytosolic coat proteins at the lipid bilayer and the formation of coated vesicles from purified components. Lipid and protein compositions, the order of addition of individual components, and the conditions (temperature, nucleotides, etc.) can be easily manipulated and help to define the minimal machinery of coat assembly and their molecular mechanisms.

The three best characterized coats are coat protein (COP) I mediating intra-Golgi and Golgi to endoplasmic reticulum (ER) transport, COPII for vesicles derived from the ER, and clathrin with various associated adaptor proteins (APs) for pathways between the plasma membrane, endosomes, and the *trans*-Golgi

network [1, 2]. In all systems, coat recruitment is initiated by a small GTPase that is activated at the membrane by nucleotide exchange from GDP to GTP. The generation of COPI vesicles *in vitro* requires the heteroheptameric coatamer complex and ADP-ribosylation factor 1 (Arf1) [3, 4]. COPII consists of two components: Sec23/24 is first targeted by Sar1·GTP to the membrane to recruit the second layer of Sec13/31 [5]. Clathrin coats are similarly composed of two layers, heterotetrameric adaptor complexes and clathrin [6].

In all systems, specific lipid compositions are required for coat recruitment, acidic phospholipids for COPI [4], and phosphoinositides for COPII [5] and clathrin adaptors [7]. In addition, cargo proteins influence coat formation. For example, presentation of cytoplasmic cargo sequences on the membrane surface is important for efficient recruitment of COPI and AP-1 clathrin adaptors [3, 7] and enhances the stability of membrane-bound Sec23/24 of COPII [8]. Different approaches have been used to study the role of cargo proteins. SNAREs, such as Bet1p and Sec22p, were purified and reconstituted into liposomal membranes by detergent dialysis [8]. Alternatively, cytoplasmic sequences of cargo proteins were coupled to liposomes via modified lipids to mimic the presence of cargo. This can be accomplished by preparing fusion proteins with glutathione-*S*-transferase (GST) that bind tightly to a glutathione-phosphatidylethanolamine conjugate incorporated into the liposomal membrane [9] or by covalently coupling synthetic peptides to a maleimide lipid via the sulfhydryl group of a cysteine side chain [3, 7, 10].

In vivo the structural components of the coat, the membrane, and cargo in addition interact with accessory proteins to form an elaborate network, best characterized for AP-2/clathrin in endocytosis at the plasma membrane [11]. The network components include alternative adaptors to expand the cargo spectrum (e.g., β -arrestin), lipid interactors to facilitate membrane curvature (e.g., epsin), inducers of actin polymerization (e.g., intersectins), dynamin recruiters for vesicle fission (e.g., amphiphysins), and finally the lipid phosphatase synaptojanin and auxillin for uncoating. The interactome components stabilize each other at the membrane, but also compete for binding sites and thereby alter the character of the network with time as the process progresses towards vesicle formation [11]. Much less is known about the interactome associated with AP-1/clathrin or other coats. The observation that cytosolic factors mediate Arf1- and lipid-dependent association of AP-1 to liposomes in the absence of cargo peptides pointed to stabilizing accessory components of AP-1/clathrin coats at the *trans*-Golgi network and endosomes. Using liposome recruitment assays, one such factor was identified as amphiphysin 2 [12], a known interactor of AP-2 coats. Amphiphysin 2 contains a C-terminal SH3 domain interacting with dynamin for vesicle fission.

An N-terminal BAR domain mediates dimerization and forms a crescent-shaped positively charged surface to bind and shape curved membranes [13]. In the center, there are sequence motifs shown to interact with clathrin heavy chain and the AP-2 α -appendage domain [14]. The interaction of amphiphysin 2 with AP-1 on liposomes was confirmed in vivo and revealed that the machineries for CCV formation with AP-1 and AP-2 at different locations in the cell thus share more components than previously anticipated [12].

In the present chapter, we describe the methods to study the in vitro recruitment of AP-1 adaptors to liposomes and peptidoliposome by a floatation assay and to analyze the oligomeric state of recruited AP-1 by sedimentation into a density gradient. We provide protocols to produce adaptor complexes from calf brain clathrin-coated vesicles (CCVs), myristoylated Arf1, and amphiphysin from a bacterial expression system, and peptidoliposomes using maleimide lipids, as well as protocols of the floatation and the density gradient sedimentation assays.

2 Materials

Dithiothreitol (DTT) is added just before use from a 1 M frozen stock. All the buffers for fast protein liquid chromatography (FPLC) are filtered through 0.2- μ m nitrocellulose filters (Millipore) and degassed.

2.1 Isolation of Cytosol and CCVs

1. Two calf brains fresh from the slaughterhouse (*see Note 1*).
2. CB-6 Three-speed extra-large capacity blender from Waring Laboratory or equivalent.
3. Loose-fitting dounce homogenizer, small (7 mL) and medium (40 mL) size.
4. Phosphate-buffered saline (PBS): 12.5 mM sodium phosphate buffer, pH 7.6, 125 mM NaCl.
5. 0.5 M Phenylmethylsulfonyl fluoride (PMSF) in dimethyl sulfoxide.
6. Buffer A: 0.1 M MES-NaOH, pH 6.6, 0.5 mM MgCl_2 , 1 mM EGTA, 0.2 mM DTT.
7. Buffer B: 0.1 M MES-NaOH, pH 7.0, 0.5 mM MgCl_2 , 1 mM EGTA, 0.2 mM DTT, 12.5 % (w/v) Ficoll 400, 12.5 % (w/v) sucrose (*see Note 2*).

2.2 Preparation of Mixed Adaptors

1. Fast protein liquid chromatography (FPLC) system (e.g., äkta FPLC system from GE Healthcare).
2. Superose 6 prep grade is packed into an HR 16/60 column (1.6 $\times$ 60 cm).

3. Monoclonal antibodies for immunoblotting (all used at 1:1,000 dilution): 100/3 against γ -adaptin of AP-1 (~90 kDa), 100/2 against α -adaptin of AP-2 (doublet at ~100 kDa; both from Sigma), and anti-clathrin heavy chain (from BD Biosciences).
4. Stripping buffer (3 $\times$): 1.5 M Tris-HCl, pH 7.0, 6 mM ethylenediamine-tetraacetic acid (EDTA), 0.6 mM DTT. This buffer can be stored at -20 °C in 15-mL Falcon tubes. (Note: Falcon tubes do not resist shock-freezing in liquid nitrogen.)
5. Superose running buffer: 0.5 M Tris-HCl, pH 7.0, 2 mM EDTA, 0.2 mM DTT.
6. Protease inhibitor cocktail (500 $\times$): 5 mg/mL benzamidine, 1 mg/mL pepstatin A, 1 mg/mL leupeptin, 1 mg/mL antipain, 1 mg/mL chymostatin (all from Sigma); dissolved in 40 % dimethyl sulfoxide (DMSO)/60 % ethanol and stored at -20 °C.

2.3 Preparation of Myristoylated Arf1

1. Plasmids pET-mArf1* encoding bovine Arf1 with codons 3–7 replaced by those from yeast Arf2p [15] and pBB131 encoding yeast myristoyl-CoA:protein *N*-myristoyltransferase (NMT) [16] were provided by Drs. Stuart Kornfeld and Jeffrey Gordon (Washington University, St. Louis, MO), respectively.
2. Competent *Escherichia coli* BL21(DE3).
3. Luria broth (LB): 10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl.
4. Ampicillin (1,000 $\times$): 100 mg/mL in water; store frozen.
5. Kanamycin (500 $\times$): 50 mg/mL in water; store frozen.
6. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) is dissolved at 1 M in water and stored frozen.
7. Brij 58 is prepared at 10 % (w/v) in water and autoclaved.
8. Myristic acid is dissolved at 0.5 M in ethanol and stored frozen.
9. Diethylaminoethyl (DEAE) Sephacel in an Econo-Column (2.5 $\times$ 10 cm) from Bio-Rad.
10. DEAE buffer: 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 10 μ M GDP, 0.02 % (w/v) NaN₃, 1 mM DTT.
11. Amicon Ultra-15 centrifugal filter devices with a 10 kDa cutoff from Millipore.
12. Superdex 75 column (HighLoad 26/60 prep grade; 2.6 $\times$ 60 cm; from GE Healthcare).
13. Superdex running buffer: 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 10 μ M GDP, 0.02 % (w/v) NaN₃, 10 % (w/v) sucrose, 1 mM DTT.
14. Monoclonal antibody 1D9 for immunoblotting against Arf (used at 1:5,000 dilution; from Alexis).

2.4 Expression and Purification of Amphiphysin

1. *Escherichia coli* BL21(DE3) (Stratagene) containing the plasmid pET24d-Amph1 or pET24d-Amph2. The plasmids correspond to the coding sequence of human amphiphysin 1 or 2, respectively, cloned into the bacterial expression vector pET24d (Novagen), thus C-terminally fused to a His₆ tag. Plasmids containing mutant cDNAs are used in the same manner.
2. Luria broth (LB) with kanamycin (LB/Kana): 10 g/L bacto-tryptone (Applichem), 5 g/L yeast extract (Applichem), 10 g/L NaCl supplemented with 50 µg/mL kanamycin.
3. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) is dissolved at 1 M in water and stored frozen.
4. HisTrapp FF column (1-mL cartridge; from GE Healthcare).
5. Sonication buffer: 50 mM Tris/HCl (pH 8), 300 mM NaCl, 10 % glycerol, and 10 mM imidazole. Wash and elution buffers contain increased imidazole concentrations of 50, 75, 100, 150, 200, or 250 mM.
6. Tip sonicator such as a Branson Cell Disruptor B15.

2.5 Preparation of Liposomes and Peptidoliposomes

1. Synthetic peptides LY (amino acid sequence CRKRS HAGYQTI) and LA (CRKRS HAGAQTI) correspond to the cytoplasmic domain of Lamp1 (lysosome-associated membrane protein-1) and a transport-deficient mutant with the critical tyrosine mutated to alanine, respectively. Peptides were purchased at more than 70 % purity from, e.g., NeoMPS. The N-terminal cysteines were added to the sequences for coupling.
2. Glass tubes resistant to liquid nitrogen, e.g., 12-cm test tubes NS 14.5/23 for vacuum from Glas Keller.
3. Nucleopore polycarbonate membrane with 400-nm pore size and prefilters (drain disc 10 mm PE) from Whatman.
4. Mini-Extruder from Avanti Polar Lipids https://www.avanti-lipids.com/index.php?option=com_content&view=article&id=185&Itemid=193.
5. Egg L-α-phosphatidylcholine (PC) from Avanti Polar Lipids.
6. Soybean L-α-phosphatidylcholine, also called azolectin (Sigma). This is a mixture of phospholipids, containing only 20 % PC, however.
7. MMCC-DOPE (*N*-[4-maleimidylmethyl]cyclohexane-1-carbonyl]-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) from Avanti Polar Lipids is dissolved in chloroform/methanol 2:1 (v/v). Aliquots of 125 nmol are dried in silanized 1.5-mL reaction tubes and stored at -20 °C.
8. Chloroform/methanol 2:1 (v/v).
9. 1,1-Dichloromethane.
10. Liposome buffer: 10 mM HEPES-NaOH, pH 6.5, 100 mM NaCl, 0.5 mM EDTA.

2.6 Floatation Assay

1. Assay buffer: 10 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.2 mM DTT.
2. Sucrose solutions: 20 and 60 % (w/v) in assay buffer.
3. 5'-Guanylylimidodiphosphate (GMP-PNP; Fluka) is prepared at 10 mM and GTP (Sigma) at 100 mM in water and stored at -80 °C.
4. TCA: 100 % (w/v) 2,2,2-trichloroacetic acid in water.
5. Acetone (analysis grade).

2.7 Sedimentation Assay

1. Gradient Master from BioComp Instruments (Fredericton) or alternative gradient maker.
2. Sucrose solutions: 10 and 25 % (w/v) in assay buffer containing 0.2 % (w/v) Triton X-100.
3. Triton X-100 at 20 % (w/v) in water.

2.8 General Materials

1. Standard equipment and materials for sodium dodecylsulfate-polyacrylamide gel electrophoresis (12.5 % acrylamide) and immunoblot analysis.
2. Bradford Protein Assay (Bio-Rad) or equivalent.

3 Methods

For centrifugations, an example of rotors and speeds is provided in parentheses. Unless expressly stated, all procedures are performed at 4 °C.

3.1 Isolation of Cytosol and CCVs

Intracellular membranes released from homogenized calf brains are recovered in a low-speed supernatant and separated from the cytosol by high-speed centrifugation. In a subsequent medium-speed centrifugation with Ficoll and sucrose, CCVs are enriched in the supernatant. After dilution, they are collected by high-speed centrifugation. The procedure is based on ref. 17.

1. Two calf brains fresh from the slaughterhouse are transported on ice to the cold room (*see Note 3*).
2. Fat, brain stem, meninges, and blood clots are removed with paper towels.
3. The cleaned brains are placed in a beaker filled with 1 L PBS to measure their volume (~200 mL per brain).
4. The brains are placed in the Waring blender together with half their volume of buffer A (*see Note 4*). PMSF (0.5 mM) is added and the blender is turned on three times for 8 s on medium speed.
5. The homogenate is centrifuged at 8,000 × *g* for 30 min (Sorvall GS3 rotor, 7,000 rpm). Keep the pellets for **step 7**.

6. The supernatant is carefully collected (*see Note 5*) and the membranes are pelleted by centrifugation at $180,000\times g$ for 80 min (Kontron TFT 45.94 rotor, 40,000 rpm). The high-speed supernatant (i.e., the cytosol) is collected (*see Note 6*), aliquoted, shock-frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$. Protein concentration is typically 15–30 mg/mL. The high-speed pellets are kept for **step 8**.
7. The low-speed pellets of **step 5** are resuspended in an equal volume of buffer A and—after addition of 0.5 mM PMSF—re-homogenized in the blender as before and centrifuged again at $8,000\times g$ for 30 min. The supernatant is collected and all membranes pelleted at $180,000\times g$ for 80 min. This second high-speed supernatant is discarded.
8. The high-speed pellets from **steps 6** and **7** are resuspended in 1 mL buffer A per tube with the help of a spatula, collected, and homogenized with five to six strokes in a dounce homogenizer.
9. The suspension ($\sim 8\text{ mL}$) is mixed with an equal volume of buffer B, dounce homogenized again, and centrifuged at $60,000\times g$ for 40 min (Kontron TFT 45.94 rotor, 24,000 rpm; *see Note 7*).
10. The supernatants are collected, diluted with three volumes of buffer A, and centrifuged at $180,000\times g$ for 80 min (Kontron TFT 45.94 rotor, 40,000 rpm) to pellet the CCVs.
11. The pellets are resuspended in an equal volume of buffer A, homogenized in a small dounce homogenizer, and centrifuged in 1.5-mL tubes in a microfuge for 12 min to remove aggregated material.
12. The supernatant containing the CCVs is collected, frozen as 750- μL aliquots in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$. Typically, four such aliquots are obtained from two brains.

3.2 Preparation of Mixed Adaptors

Clathrin coats are released from CCVs with high concentrations of Tris-HCl, and adaptors enriched by gel filtration. The procedure is based on ref. 18.

1. Two 750- μL aliquots of CCVs are thawed, mixed with an equal volume of stripping buffer containing 0.5 mM PMSF and 1 $\times$ proteinase inhibitor cocktail in a small dounce homogenizer, and kept on ice overnight.
2. The mixture is dounce homogenized again and centrifuged at $100,000\times g$ for 35 min (Beckman TLA 100.3 rotor, 70,000 rpm) to pellet the membranes. If the supernatant is still turbid, it is centrifuged again.
3. Two milliliters of the clear supernatant is fractionated on the Superose 6 column pre-equilibrated in Superose running buffer at a flow rate of 0.5 mL/min and collected in 1-mL fractions.

4. Samples of 25 μL of every second fraction from 41 to 72 are analyzed by SDS-gel electrophoresis and immunoblotting for the presence of AP-1 and clathrin. AP-1 is typically found in fractions 55–63 (*see Note 8*).
5. AP-1-containing fractions are pooled, supplemented with 1 $\times$ protease inhibitor cocktail and 0.5 mM PMSF, and may be stored at 4 $^{\circ}\text{C}$ for several weeks. Typically 10 mL at 100 $\mu\text{g}/\text{mL}$ protein is obtained.

3.3 Preparation of Myristoylated Arf1

Arf1 and NMT are expressed in bacteria and myristic acid is exogenously provided to produce efficiently myristoylated Arf1. The protein is purified from freeze-thawed cells by ion exchange chromatography and gel filtration. The procedure is based on ref. 19.

1. Competent BL21(DE3) bacteria are mixed with approximately 0.1 μg each of pETmArf1* and pBB131 encoding Arf1 and NMT, respectively, incubated on ice for 1 h, and heat-shocked at 42 $^{\circ}\text{C}$ for 2 min.
2. The bacterial suspension is incubated with 1 mL LB at 37 $^{\circ}\text{C}$ for 1 h before addition of 4 mL LB with 100 $\mu\text{g}/\text{mL}$ each of ampicillin and kanamycin, and incubated overnight with shaking to select for transformants carrying both plasmids.
3. The overnight culture is used to inoculate 2.5 L LB with 100 $\mu\text{g}/\text{mL}$ each of ampicillin and kanamycin. The culture is grown until OD_{600} reaches 0.6–0.8.
4. Arf1 and NMT expression is induced by adding 1 mM IPTG. Simultaneously, 0.5 % (w/v) Brij 58 (to increase the solubility of myristic acid) and 500 μM myristic acid are added, and the culture is incubated 4 h at 30 $^{\circ}\text{C}$ (a temperature that favors myristoylation).
5. The cells are harvested by centrifugation at 5,000 $\times g$ for 15 min (Sorvall GS3 rotor, 5,400 rpm).
6. The pellets are resuspended with PBS, transferred into a 50-mL Falcon tube, and pelleted again for freezing at -80°C .
7. To the frozen pellet (~ 5 mL), 25 μL 0.5 M PMSF and 50 μL 500 $\times$ protease inhibitor cocktail are added, and the bacteria are lysed by three cycles of freezing in dry ice/methanol and thawing in lukewarm water.
8. Bacteria are resuspended in 25 mL DEAE buffer and incubated on ice for 1 h before centrifugation at 20,000 $\times g$ for 15 min (Sorvall SS34 rotor, 13,000 rpm).
9. The supernatant is collected and loaded directly onto the 25-mL DEAE Sephacel column equilibrated and run with DEAE buffer at a flow rate of 1 mL/min.
10. The flow-through contains Arf1 and is collected until OD_{280} is below 1.0.

11. These Arf1-containing fractions are concentrated by ultrafiltration (Amicon Ultra filter) to approximately 2 mL.
12. The sample is fractionated at a flow rate of 1 mL/min on the Superdex 75 column equilibrated with Superdex running buffer. Fractions of 3 mL are collected.
13. Fractions 51–80 are tested for the presence of Arf1 and contaminating proteins by analyzing 25- μ L samples on parallel 15 % SDS-gels by immunoblotting and Coomassie Blue staining, respectively.
14. Arf1-containing fractions (typically fractions 60–70) are pooled (*see Note 9*) and concentrated to 1 mg/mL by ultrafiltration.
15. The purified protein is aliquoted, shock-frozen in liquid nitrogen, and stored at -80°C . Approximately 0.5 mg Arf1 could be typically purified from a 2.5 L culture.

3.4 Expression and Purification of Amphiphysin

His₆-tagged wild-type and mutant amphiphysins are expressed in bacteria and isolated in a one-step purification by nickel chelate chromatography adapted from the provider's protocols.

1. Twenty milliliters of an overnight culture of *Escherichia coli* BL21 with plasmid pET24d-Amph1 or pET24d-Amph2 in LB/Kana is used to inoculate 2 L LB/Kana. The culture is grown at 37°C for 3–4 h until OD₆₀₀ reaches 0.7.
2. Expression is induced by addition of 0.5 mM IPTG and further culturing for 6 h at 30°C for amphiphysin 1, and with 1 mM IPTG and further culturing for 4 h at 30°C for amphiphysin 2 (*see Note 10*).
3. The cells are harvested by centrifugation at $5,000\times g$ for 15 min (Sorvall GS3 rotor, 5,400 rpm).
4. The pellets are resuspended in an equal volume of PBS, transferred into a 50-mL Falcon tube, pelleted again, and stored frozen at -80°C .
5. The pellet is resuspended in 20 mL sonication buffer supplemented with $2\times$ protease inhibitor cocktail and $2\times$ PMSF.
6. Sonicate eight times for 15 s at an amplitude of 25 % in 1-min intervals with the tube immersed in ice water.
7. The material is centrifuged for 1 h at $100,000\times g$ (Thermo Scientific T-1270 rotor, 40,000 rpm).
8. The supernatant is loaded twice at a flow rate of 1 mL/min onto the 1-mL HisTrapp FF column equilibrated in sonication buffer.
9. The column is washed and eluted at 1 mL/min with sonication buffer containing increasing concentrations of imidazole: 20 mL with 50 mM, 5 mL with 75 mM, and 2 mL each of

buffer containing 100, 150, 200, and 250 mM imidazole. Collect 1-mL fractions and determine protein concentration.

10. The fraction with the highest protein concentrations are collected, snap-frozen in 30- μ g aliquots, and stored at -80°C .

3.5 Preparation of Liposomes and Peptidoliposomes

Liposomes are prepared by extrusion through a defined pore-size filter. A maleimide-containing lipid is included to allow chemical coupling of cysteine-terminal peptides. The procedure is based on refs. 7 and 20.

1. Five micromoles of the desired lipids, for example 3.8 mg egg PC, soybean lipids, or a 1:1 mixture of the two, is dissolved in 1 mL chloroform/methanol in a test tube that can stand liquid nitrogen.
2. To produce peptidoliposomes, 125 nmol (2.5 mol %) MMCC-DOPE is dissolved in 100 μ L chloroform/methanol and added to the lipids (*see Note 11*).
3. The organic solvent is evaporated under a stream of nitrogen.
4. The lipids are redissolved in 2 mL of 1,1-dichloromethane and dried again under nitrogen.
5. Meanwhile, the extruder is assembled and washed with liposome buffer, and 0.28 μ mol (400 μ g) of the peptides to be coupled (approximately fourfold excess over the reactive lipid, assuming half of it is exposed to the outside of the liposome) is weighed out and dissolved in 100 μ L liposome buffer.
6. The dried lipids are suspended in 1 mL liposome buffer by five cycles of vortexing, shock-freezing in liquid nitrogen, and thawing under warm tap water (*see Note 12*).
7. Lipids are passed 11 times through the extruder unit (*see Note 13*).
8. To couple peptides to the liposomes, they are immediately mixed with 100 μ L 4 mg/mL peptide and incubated for 1 h at room temperature (*see Note 14*).
9. The final peptidoliposomes are stored at 4°C with 0.02 % (w/v) NaN_3 for up to 2 weeks (*see Notes 15 and 16*).

3.6 Floatation Assay

Peptidoliposomes are incubated with coat proteins, Arf1, and nucleotides to allow for Arf1 activation and coat recruitment, and then floated on a sucrose step gradient. Proteins associated with the liposomes or remaining in the loading zone are collected by trichloroacetic acid (TCA) precipitation and detected by immunoblot analysis. The procedure is based on refs. 7 and 10.

1. Cytosol is centrifuged at $170,000\times g$ for 30 min (Beckman TLA100.3 rotor, 85,000 rpm) to remove aggregates.
2. Liposomes or peptidoliposomes (100 μ L containing ~ 0.5 μ mol lipid) are mixed with 5 μ g Arf1, 0.2 mM GMP-PNP or 2 mM

GTP, either 10 μg mixed adaptors or 1 mg cytosol, and reaction buffer to 200 μL . The contribution of amphiphysins is tested by supplementing up to 50 μg purified protein. The mixture is incubated for 30 min at 37 °C to allow nucleotide exchange on Arf1 and protein recruitment.

3. The reaction is mixed in a 4-mL ultracentrifuge tube with 0.4 mL 60 % sucrose solution (to a final concentration of 40 % sucrose). The mixture is carefully overlayed with 1 mL of 20 % sucrose solution. After removal of any foam at the top with a pipette, 20 % sucrose solution is added to a total of 3.82 mL.
4. The tubes are balanced and 180 μL assay buffer is overlayed to facilitate liposome collection afterwards.
5. Samples are centrifuged at $300,000\times g$ for 1 h (Kontron TST60.4 rotor, at 55,000 rpm).
6. Four 1-mL fractions are collected from the top.
7. TCA precipitation: the fractions are mixed with 160 μL TCA in 1.5-mL tubes and centrifuged in a microfuge at maximal speed for 15 min.
8. To the pellets, add 850 μL ice-cold acetone, and the tubes are centrifuged again for 5 min at 4 °C.
9. The pellets are air-dried for 15–20 min at room temperature and dissolved in 60 μL SDS-sample buffer by pipetting up and down and by heating at 95 °C for 10 min (*see Note 17*).
10. Samples are separated by gel electrophoresis on a 12.5 % SDS-gel and analyzed by immunoblotting for AP-1 (γ -adaptin), Arf1, and amphiphysin as appropriate. Example results are shown in Fig. 1 (dependence of AP-1 recruitment on Arf1, lipid composition, and cargo signals), Fig. 2b (left lanes: cargo-independent recruitment of AP-1 using cytosol), and Fig. 3 (stabilization of AP-1 on liposomes by different amphiphysins).

3.7 Sedimentation Assay

To analyze the oligomerization state of liposome-associated protein, membranes in the floated fraction are solubilized and the protein centrifuged into a sucrose density gradient. Protein in the gradient fraction is detected by immunoblot analysis. The procedure is based on ref. 10.

1. The top 350 μL of a floatation gradient (Subheading 3.6, step 5), which contains most of the floated liposomes, is collected and mixed with 350 μL assay buffer to dilute the sucrose.
2. The lipid membranes are solubilized by addition of 0.5 % Triton X-100.
3. The sample is loaded onto a 4.3 mL 10–25 % sucrose gradient with 0.2 % Triton X-100 and centrifuged at $100,000\times g$ for 5 h (Kontron TST 55.5 rotor, at 30,000 rpm).

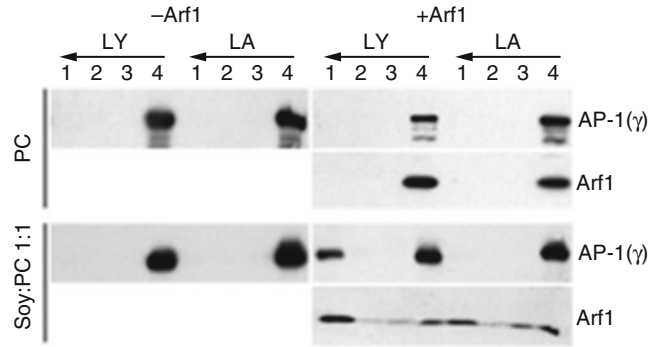


Fig. 1 AP-1 recruitment to peptidoliposomes is Arf1-, lipid-, and signal-dependent. Peptidoliposomes made of 100 % PC or a 1:1 PC/soybean lipid mixture and presenting LY or LA peptides were incubated with mixed clathrin adaptors and with or without Arf1/GMP-PNP. After flotation on a sucrose step gradient, four fractions were collected from the top and analyzed by immunoblotting for γ -adaptin (a subunit of AP-1) or Arf1. Recruitment of AP-1 required Arf1, lipids other than PC, and the presentation of the Lamp1 sequence with an intact tyrosine motif (LY). While Arf1 is activated on PC membranes (not shown), it also requires non-PC lipids for binding to liposomes. *Arrows* indicate the direction of floatation from the bottom to the top fraction of the gradient. (Reprinted in modified form from ref. 7 with permission of The American Society for Cell Biology.)

4. Ten 0.5-mL fractions are collected from the top and 125 μ L of 5 $\times$ SDS-sample buffer is added for analysis by SDS-gel electrophoresis and immunoblotting for AP-1 (γ -adaptin). An example result is shown in Fig. 2.

4 Notes

1. Alternatively, ten pig brains may be used.
2. Ficoll 400 is hard to dissolve. Warm up the solution and add Ficoll 400 slowly with continued stirring.
3. To process two brains, it is convenient in the following procedure to use two TFT45.94 rotors and two ultracentrifuges in parallel. For scaling up to six brains (2 $\times$ 3 brains), one needs two GS3 and three TFT45.94 rotors and appropriate centrifuges.
4. The buffer volume is kept low to retain high cytosol concentration.
5. It is really important to only take the red supernatant. Pour the supernatant over the white pellet into a beaker. It is better to take less supernatant than to have contamination with material from the pellet.

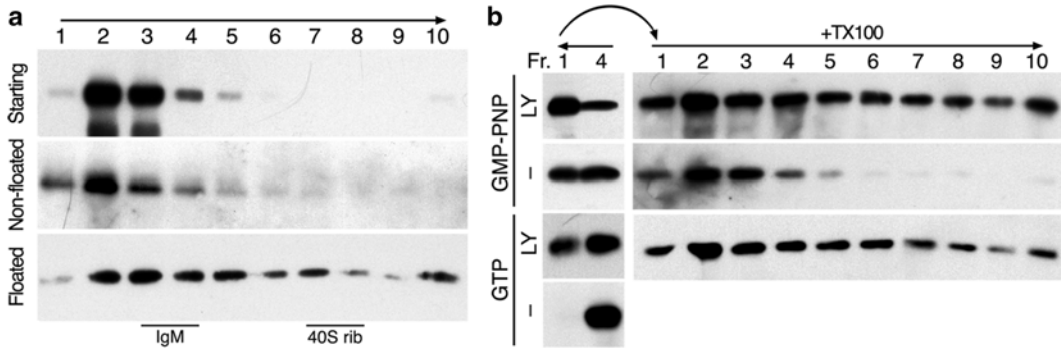


Fig. 2 AP-1 recruited to peptidoliposomes forms high-molecular-weight complexes. **(a)** Peptidoliposomes made of soybean lipids and presenting LY peptides were incubated with clathrin adaptors, Arf1, and GMP-PNP. After floatation on a sucrose step gradient, the floated liposomes were solubilized with Triton X-100 and centrifuged into a 10–25 % sucrose velocity gradient (*horizontal arrow*). Ten fractions were collected and analyzed by immunoblotting for γ -adaptin. For comparison, the nonfloated fraction and the original adaptors were analyzed in parallel. The positions of the sedimentation markers IgM (19S) and 40S ribosomes are indicated. Individual AP-1 adaptors (~ 300 kDa) have a sedimentation coefficient of 7.7S. AP-1 recruited to sorting peptides shows oligomerization to high-molecular-weight complexes even in the absence of clathrin. **(b)** AP-1 recruitment from cytosol. Liposomes with or without LY peptides were incubated with cytosol, Arf1, and GMP-PNP or GTP. After a first step gradient floatation, the floated fraction 1 was solubilized with Triton X-100, sedimented into a sucrose gradient, and analyzed for γ -adaptin as in panel **a**. Unlike AP-1 from purified adaptors, AP-1 from cytosol is recruited to liposomes with Arf1/GMP-PNP even in the absence of sorting peptides. However, LY peptides are required for AP-1 to oligomerize. If GTP is used, stable recruitment is observed only with sorting peptides. These results indicate the presence of cytosolic factor(s) recruiting AP-1 to membranes in a cargo-independent, GTPase-sensitive manner. They further show that membrane recruitment is not sufficient to induce AP-1 oligomerization, but that binding to cargo signals is necessary. (Reprinted in modified form from ref. 10 with permission of The American Society for Cell Biology.)

6. It is important not to contaminate the cytosol with the pellet. Carefully remove the cytosol with a glass pipet, leaving behind the last 1 cm of supernatant above the pellet.
7. To fill tubes to their minimal filling level or to balance them, a 1:1 mixture of buffers A and B may be added.
8. AP-2, AP180, and some clathrin are the major contaminants.
9. The later fractions of the Arf1 peak are usually purer than the earlier ones.
10. The concentration of IPTG to induce maximal expression and the optimal time and temperature of culturing for maximal yield are determined in small-scale cultures beforehand by SDS-gel electrophoresis of lysed bacteria and Coomassie-blue staining. For this, a 40-mL culture is grown to OD 0.7, divided into four 10-mL cultures to be incubated with 0.5 or 1 mM IPTG at 30 °C or 37 °C. After 0, 1, 2, 3, and 4 h 2-mL samples were removed, pelleted, and lysed in 500 μ L loading buffer.

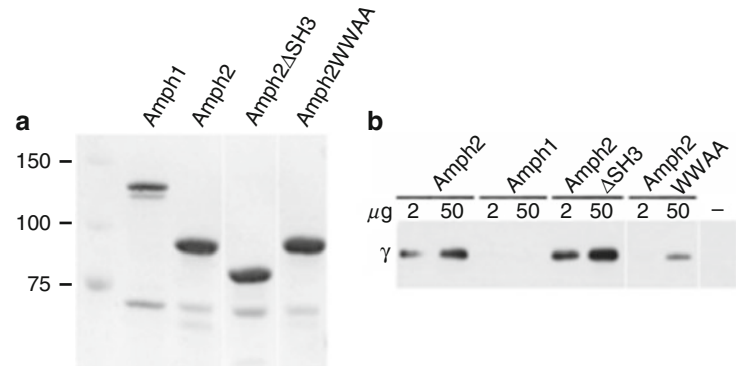


Fig. 3 Amphiphysin 2 stabilizes AP-1 on liposomes in the absence of cargo peptides. (a) Amphiphysin 1 and 2 and mutants lacking the SH3 domain (Δ SH3) or mutated in its PWDLW adaptor-binding motif to PADLA (WWAA) were expressed in bacteria, purified, and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining. Molecular weight size markers are indicated (in kDa). (b) Liposomes made of soybean lipids were incubated with mixed adaptors, Arf1/GMP-PNP in the absence (–) or presence of different amounts of wild-type amphiphysin 1 or 2, or with amphiphysin 2 mutants. The liposomes were floated and the top fraction analyzed for bound γ -adaptin. The results show stabilization of AP-1 on liposomes without cargo signals by amphiphysin 2, but not by amphiphysin 1, in a manner independent of its SH3 domain, but involving the WXXW motif. (Reprinted in modified form from ref. 12 © the Biochemical Society.)

11. In addition, (*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine [NBD-PE]; from molecular probes), a fluorescent phospholipid analog, may be added at 1 mol % to quantify liposome recovery in the subsequent experiments by fluorimetry (λ_{ex} 450 nm, λ_{em} 530 nm).
12. As soon as the liposome buffer is added to the dried lipids it is important to work quickly because of hydrolysis of the maleimide group.
13. Be very careful during the extrusion not to break the membranes. If you are stuck, back up a little before pushing on. After extrusion, disassemble the extruder and make sure the filter is intact. If the filter is damaged, repeat the extrusion.
14. To determine coupling efficiency, *see* ref. 21. The coupling reaction at pH 6.5 is essentially complete within 10 min at room temperature [21].
15. Some peptides induce liposome aggregation with time, which may cause unspecific trapping of protein in the floatation assay. These liposomes have to be used more quickly or even on the same day only.

16. For most uses, the free, excess peptides do not have to be removed (e.g., by gel filtration or dialysis), because the concentration in solution is much lower than that of coupled peptides at the membrane surface.
17. Especially when using cytosol, pellets from the bottom fraction may be hard to dissolve. Also, if samples turn yellow, the pH can be adjusted by pipetting some ammonia vapor above the liquid surface.

Acknowledgments

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A β -Lactamase Based Assay to Measure Surface Expression of Membrane Proteins

Pieter Beerepoot, Vincent M. Lam, and Ali Salahpour

Abstract

Measurement of cell surface expression is an essential part of studying membrane proteins. Traditional techniques for measuring surface expression depend on the availability of appropriate radioligands or antibodies towards extracellular epitopes of a protein of interest. The current protocol outlines the use of an assay to monitor surface expression of membrane proteins tagged with a bacterial β -lactamase in mammalian cell lines. The use of this technique allows for quick, quantitative, sensitive, and inexpensive measurement of surface expression, with the potential for high-throughput screening.

Key words Surface expression, Membrane proteins, Beta-lactamase, GPCR, Transporter

1 Introduction

The functions of membrane proteins at the cell surface, particularly receptors and transporters, are tightly regulated and dynamic due to internalization and recycling processes. Radioligand binding, surface biotinylation, and antibody-dependent assays, including flow cytometry or enzyme-linked immunosorbent assays (ELISA) have been traditionally used to quantify surface expression of membrane proteins [1]. These assays are time-consuming and require reagents that are expensive or often may not be available for a particular protein of interest. We recently developed a new complementary method for measuring surface expression. In our assay, we fused β -lactamase (β -lac) to extracellular motifs of the proteins of interest and quantified surface expression by measuring β -lac activity using a cell impermeable substrate.

β -lac is a bacterial enzyme that hydrolyzes the β -lactam ring present in penicillins. β -lac has previously been used in various reporter assays [2–5]. A number of substrates for this enzyme are available, which either produce a colorimetric change, or a shift in fluorescence properties upon hydrolysis of the β -lactam ring [2, 5]. In our surface expression assay, the colorimetric substrate nitrocefin

is used because it is cell impermeable, allowing for the selective measurement of cell surface/extracellular β -lac [6]. The substrate conversion is monitored over time, where the slope of the reaction (i.e., initial velocity) is directly proportional to surface expression. This is unlike endpoint measurements that are often used in other surface expression assays (ELISA, flow cytometry). We described here the transfection of β -lac fusion protein constructs and the procedure of the β -lac assay in a 48-well format. The assay is performed with live adhered cells and can be scaled down (96 or 384-well plate) for use in higher-throughput applications.

2 Materials

Prepare all solutions using ultrapure water (Milli-Q) and analytical grade reagents. All concentrations are final concentrations unless stated otherwise. Solutions are stored at room temperature unless stated otherwise.

2.1 Reagents

1. Phosphate buffered saline (PBS): NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4 is used to wash the cells and dilute the substrate nitrocefin to the working concentration.
2. Dulbecco's modified Eagles medium (DMEM) (Wisent): Supplement with 10 % (v/v) fetal bovine serum (FBS) (Sigma). 100 U/mL Penicillin, 100 $\mu\text{g}/\text{mL}$ Streptomycin. G418 (500 $\mu\text{g}/\text{mL}$) (Bioshop) or Puromycin (1 $\mu\text{g}/\text{mL}$) (Bioshop) are added as selection agents for the creation of stable cell lines expressing the β -lac constructs of interest.
3. Poly-D-lysine: Dissolve 5 mg poly-D-lysine hydrobromide (mol. Wt. 30,000–70,000) (Sigma) in 50 mL of sterile ddH₂O to obtain a 0.1 mg/mL solution and store at 4 °C.
4. Nitrocefin: Dissolve 5.16 mg of nitrocefin (Calbiochem) in 1 mL dimethyl sulfoxide (DMSO) to obtain a 10 mM stock solution (100 $\times$) and store in 100 μL aliquots at -80 °C.
5. Trypsin/EDTA: 0.25 % w/v trypsin, 0.53 mM EDTA (Wisent) is diluted to 0.05 % trypsin, 0.10 mM EDTA with PBS. Store at -20 °C until use.
6. Polyethylenimine (PEI) (Polysciences). Dissolve PEI at 1 mg/mL in ddH₂O, adjust to pH 7 with 0.5 N HCl, filter-sterilize, and store at -80 °C.
7. Human embryonic kidney (HEK) 293 cells (ATCC CRL-1573).
8. A hemocytometer (Fischer Scientific) is used to count cells.
9. Cellstar clear 48-well plates (Greiner Bio-One) are utilized to conduct the β -lac assay.

2.2 Plasmids

1. pcDNA 3 and 3.1 plasmids were obtained from Life Technologies.
2. The ampicillin resistance gene is cloned from the pcDNA 3.1 plasmid to obtain the β -lactamase cDNA. The β -lac sequence is modified by addition of a His-tag, chicken $\alpha 7$ nicotinic receptor signal sequence [3], and an HA-tag to the N-Terminus. The signal sequence is required for proper surface expression of G protein-coupled receptors (GPCR) that will be N-terminally tagged with β -lac. The His-tag and HA-tag were added for validation purposes using traditional surface expression measurement techniques such as ELISA and FACS [6].
3. For GPCR's, the β -lac constructs were cloned into the pcDNA3 vector (Puromycin resistance) along with DNA for various GPCRs such that the β -lac is located at the N-terminus of the receptors.
4. A dopamine transporter cloned in a peYFP-C1 vector was obtained from Dr Alexander Sorkin [7]. The transporter is modified by addition of an HA-tag followed by the β -lac in frame in the second extracellular loop in place of residues 193–203. This location was chosen because previous studies had shown that insertion of an HA-epitope at this site did not disrupt transporter function [7, 8].

2.3 Apparatus and Software

1. A Biotek EPOCH microplate spectrophotometer using the Gen5 software is used to measure absorbance. Any plate reader able to measure absorbance at 486 nm in 48-well plates can be substituted.
2. Data analysis is performed using Graphpad Prism 5.

3 Methods

3.1 Cell Seeding and Transfection

Wild type HEK-293 cells are cultured in DMEM with penicillin/streptomycin and 10 % FBS at 5 % CO₂ at 37 °C in 100 mm plates. Culture medium should be warmed at least 20 min in a 37 °C water bath before use and 0.05 % trypsin/EDTA should be thawed.

1. Aspirate media and add 5 mL of room temperature (RT) PBS to wash cells.
2. Aspirate PBS and add 1 mL trypsin/EDTA, followed by 1–5 min incubation at RT.
3. Add 5 mL of DMEM to inactivate trypsin and gently pipette up and down with a serological pipet to make a homogenous cell suspension.
4. Add 10 μ L of cell suspension to the chamber of a hemocytometer and count cells.
5. Seed 1–1.5 million cells in 100 mm plate in 10 mL of DMEM.
6. Incubate for 24 h at 37 °C.

3.2 Transfection and Clone Selection (See Note 1)

1. In one tube, pipette 1 μg of β -lac construct plasmid DNA, 2 μg of pCDNA3 plasmid DNA as carrier DNA. To a separate tube, add 9 μL PEI (3 μL of PEI/ μg of DNA). Add 200 μL of DMEM (*no FBS or antibiotics*) to each of the tubes, vortex and incubate for 5 min at RT.
2. Add the PEI/DMEM mixture to the tube with the DNA/DMEM mixture and incubate at RT for 30 min. Add the mixed solution drop-wise to the 100 mm dish containing HEK 293 cells, gently swirl the plate to ensure even distribution of the DNA/PEI mixture.
3. Incubate the cells for 24 h in incubator at 37 °C.
4. Aspirate media and replace with 10 mL of selection media, DMEM containing G418 (500 $\mu\text{g}/\text{mL}$) or Puromycin (1 $\mu\text{g}/\text{mL}$) depending on resistance marker on the plasmid.
5. Incubate several days until extensive cell death takes places, at which point most cells are floating in the media and few adherent cells remain. Aspirate media and replace with fresh selection media.
6. When colonies of cells are clearly visible but still isolated from each other, pick colonies using a pipette tip and grow in wells off a 24-well plate in 1 mL of selection media/well.
7. Expression of β -lac construct can be assessed using western blotting or β -lac assay as outlined in Subheadings 3.3–3.5.
8. Select clones with an expression level such that a color change is seen after 30 min of incubation with nitrocefin but with an absorbance/time relationship that is linear within the assay window (Fig. 1) (*see Note 3*).

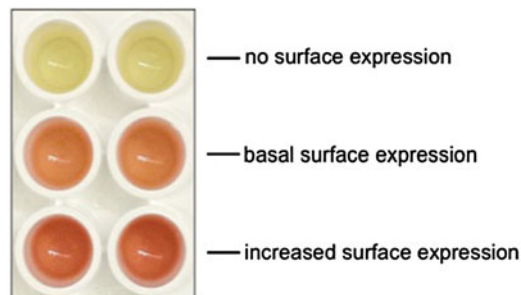


Fig. 1 Change in color after addition of nitrocefin. In mock transfection control cells (*top row*), there is no change in color after half an hour incubation with nitrocefin. However, in β -lactamase fusion construct transfected cells a color change is clearly visible (*middle row*), and is further increased after drug treatment (*bottom row*). Picture taken of assay in white plate for increased contrast

3.3 Coating Plates with Poly-d- Lysine

1. Pipet 0.5 mL of poly-D-lysine solution into each of the wells of the 48-well plates. Incubate for 5 min at room temperature.
2. Remove poly-D-lysine from wells by pipetting into a fresh new 50 mL tube. This recycled poly-D-lysine can be stored for reuse in a subsequent experiment. Wash the wells twice with PBS. Plates can then be used immediately or stored at -20°C for later use.

3.4 Plating Cells

1. Aspirate media from the cells stably expressing the β -lac construct. Wash cells with 5 mL PBS. Then add 1 mL of 0.05 % trypsin/EDTA and incubate for 1–5 min at RT.
2. Stop trypsin reaction by adding 5 mL of DMEM/FBS media. Gently resuspend cells by pipetting up and down with a serological pipette.
3. Count cells using a hemocytometer and dilute to 200,000 cells/mL. Add 0.5 mL/well for 48-well plates (100,000 cells/well) (*see Notes 2 and 3*). Seed cells so that there are three identical wells for each experimental condition (triplicates).
4. Incubate for a minimum of 6 h at 37°C to allow cells to adhere.
5. Apply drug treatment or other desired experimental intervention.

3.5 β -Lac Assay

1. Dissolve 100 μL nitrocefin stock solution (10 mM) aliquot in 9.9 mL of PBS (1:100). This gives a $1\times$ solution (100 μM).
2. Aspirate media from 48-well plate and gently wash cells with 0.5 mL PBS (*see Note 5*). Repeat the washing once.
3. Add 200 μL of nitrocefin solution per well.
4. Immediately read absorbance at 486 nm in each well using a plate reader. Reading should be carried out every minute for a period of 30 min.
5. At the end of the assay, a color change should be clearly evident (Fig. 1) (*see Notes 6 and 7*).
6. Plot the absorbance over time in Graphpad Prism or other statistical analysis software. Perform a linear regression to calculate the slope of the curve (Fig. 2). The slope should be linear with an r^2 value of at least 0.95. Changes in surface expression are calculated relative to untreated control wells, the slope of which is set at 100 %.

4 Notes

1. Although the β -lac assay can be conducted using a transient transfection population, our studies show that for reproducible measurements of internalization studies it is best to use

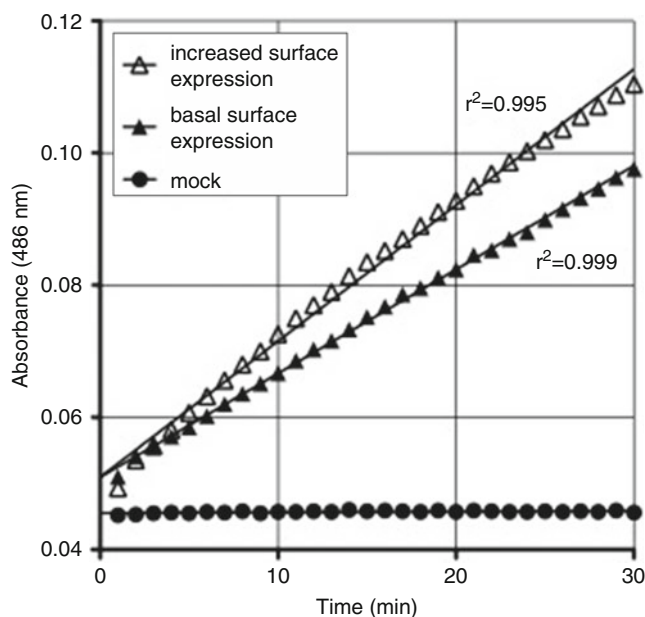


Fig. 2 Change in absorbance at 486 nm over time in individual wells. Linear regression was performed on the absorbance measurements of wells containing mock transfected cells, β -lactamase fusion construct transfected cells treated with PBS, and β -lactamase fusion construct transfected cells treated with a drug that increases surface expression. Note that absorbance in the mock control wells does not change, while the absorbance in wells containing β -lactamase expressing cells increases in a linear fashion

stable cell lines. This is why we detailed the generation of stable cell lines in this chapter. Some optimization of the experimental steps might be required if one chooses to use a transient transfection approach.

2. The assay can be miniaturized to 96-well format or smaller by scaling down reagent volumes accordingly. However, to get robust data it is important to use a plate-reader that can *shake* the plate between reads. Without shaking, local consumption of substrate can affect the reaction rate.
3. The change in absorbance should be linear over time for data analysis (Fig. 2) Deviations from linearity could indicate: (1) that surface expression is too high, causing saturation (*see Note 4*), (2) that local accumulation of product/depletion of substrate at the bottom of the well is occurring, or (3) that surface expression is not stable at room temperature. In the case of local substrate depletion, which would be visible as an uneven color change in a well, a plate reader that can shake between absorbance reads should be used in order for the well contents to remain homogenous. If surface expression is not stable at RT,

absorbance reading can be made over a shorter period of time and less experimental points can be used for the linear regression. This approach is limited by the speed of the plate reader and the number of wells assayed.

4. The amount of surface expression of the molecule of interest is directly proportional to the signal, so when a fusion protein is very highly expressed on the surface, this may lead to signal saturation quickly (within 2–3 min), leaving a very limited window in the linear range of the assay. In such a situation, it is essential that you transfect less DNA (for transient studies) or pick a stable clonal line that has lower expression in order to have linear kinetics throughout the 30 min assay window. Another way to achieve this is to reduce the amount of cells seeded per well; however, at very low cell numbers, increased variability between replicates might become an issue due to loss of cells during the washing steps.
5. Since there are several washing steps involved in this assay and the cells are not fixed, washes can result in removal of cells from the microplates. Gentle washing is therefore essential. For example, dispense PBS using gravity dispensing instead of blow-out.
6. If no color change occurs, it may be useful to test if the fusion protein is retained intracellularly. Cells can be lysed using hypotonic shock by washing with 100 μ L ddH₂O, then adding 100 μ L ddH₂O and incubating for 15 min, followed by vigorous up and down pipetting. Subsequently 100 μ L 2 $\times$ nitrocefin can be added and the color change can be monitored by β -lac assay. In cases where a protein is expressed but retained intracellularly, substrate hydrolysis (color change) should occur upon this hypotonic lysis. Alternatively, protein expression can be assessed using western blot analysis.
7. The assay window/read time can be adjusted for low/high expression. For example, if expression is very low the assay can be run over the course of several hours (2–6 h at RT for example) since baseline nitrocefin hydrolysis is low (*see* mock in Fig. 2). Conversely if the signal is too high and color change is achieved very fast, cell number or expression level can be reduced (*see* Note 4).

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Cell-Free Reconstitution of Multivesicular Body (MVB) Cargo Sorting

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and Andrew J. Bean

Abstract

The signaling activity of cell surface localized membrane proteins occurs primarily while these proteins are located on the plasma membrane but is, in some cases, not terminated until the proteins are degraded. Following internalization and movement through the endocytic pathway en route to lysosomes, membrane proteins transit a late endosomal organelle called the multivesicular body (MVB). MVBs are formed by invagination of the limiting membrane of endosomes, resulting in an organelle possessing a limiting membrane and containing internal vesicles. The fate of an internalized membrane protein depends on whether it buds outwardly from the endosomal membrane, promoting recycling and continued signaling, or is internalized into internal MVB vesicles and is ultimately degraded upon MVB–lysosome fusion. The molecular machinery that regulates the separation of membrane proteins destined for degradation from those resulting in surface expression is not well understood.

To elucidate the molecular mechanisms that underlie membrane protein sorting, we have reconstituted an endosomal sorting event under cell-free conditions. We took advantage of the itinerary of a prototypical membrane protein, the epidermal growth factor receptor (EGFR) and designed a biochemical monitor for cargo movement into internal MVB vesicles that is generally modifiable for other membrane proteins. Since it is not known how internal vesicle formation is related to cargo sorting, morphological examination using transmission electron microscopy (TEM) allows separate monitoring of vesicle formation. We have determined that MVB sorting is dependent on cytosolic components, adenosine triphosphate (ATP), time, temperature, and an intact proton gradient. This assay reconstitutes the maturation of late endosomes and allows the morphological and biochemical examination of vesicle formation and membrane protein sorting.

Key words Multivesicular body, Membrane trafficking, Endocytic, Endosome, Sorting, Cell-free assay

1 Introduction

Endocytosis is required for the uptake of essential nutrients from the extracellular environment and to remove membrane proteins from the plasma membrane [1]. The endocytic pathway is separated into stages based on cargo movement through

morphologically defined compartments (e.g., early endosomes, late endosomes, and lysosomes) [2, 3]. Following internalization, membrane proteins are transported from early to late endosomes [4]. Late endosomes mature into an organelle, the multivesicular body (MVB), formed by invagination of the limiting endosomal membrane resulting in an organelle containing internal vesicles [1–3]. Membrane proteins that remain on the limiting membrane of the endosome can be recycled and sent back to the plasma membrane where they can continue to signal, while proteins that are sorted into internal vesicles are subject to degradation upon MVB–lysosome fusion [5, 6]. The sorting of proteins into the internal vesicles of the MVB is known to require the attachment and removal of ubiquitin tags that allows these ubiquitin-modified proteins to be recognized by a group of multi-protein complexes, the endosomal sorting complex required for transport (ESCRTs) [7], that play a role in the concentration of membrane proteins on the endosomal membrane and formation of the internal vesicles [1, 5, 6, 8]. Understanding the regulation of this critical sorting step at the endosome will allow a better appreciation of molecular mechanisms that affect membrane protein signaling.

To elucidate the mechanisms underlying endosomal sorting, we have established a cell-free assay that measures both MVB formation (using electron microscopy) and the sorting of a cargo protein into the endosomal lumen (Fig. 1). We have taken advantage of the well-known itinerary of the epidermal growth factor receptor (EGFR) and have used classical protease protection analysis to examine its localization (Fig. 1). If the receptor localizes into internal vesicles of the MVB during the reaction, it will no longer be accessible to exogenously added trypsin, and is protected from digestion [1]. We have used antibodies specific to the cytoplasmic tail of EGFR so that only the protected EGFR is detected by western blotting. We have shown that the sorting event is dependent on cytosolic components, adenosine triphosphate (ATP), time, and temperature [1]. Cytosol obtained from mammalian HeLa cells, *Saccharomyces cerevisiae* yeast strains, and *Drosophila melanogaster* fly strains are sufficient to support the MVB sorting of the EGFR with 20–40 % efficiency (Fig. 2a–c) [1, 9]. The ESCRT protein, Hrs, is required for protection of EGFR from trypsin cleavage and formation of internal endosomal vesicles. Cytosol derived from yeast strains deleted of the Hrs yeast counterpart, Vps27, decreased EGFR sorting into endosomes (Fig. 2d). Ultrastructural examination of internal vesicle formation revealed an increase in vesicle formation over time that is attenuated when Hrs is depleted from the cytosol used in the reaction (Fig. 3). This assay reconstitutes the maturation of late endosomes and allows the morphological and biochemical examination of vesicle formation and membrane protein sorting into inwardly budding vesicles.

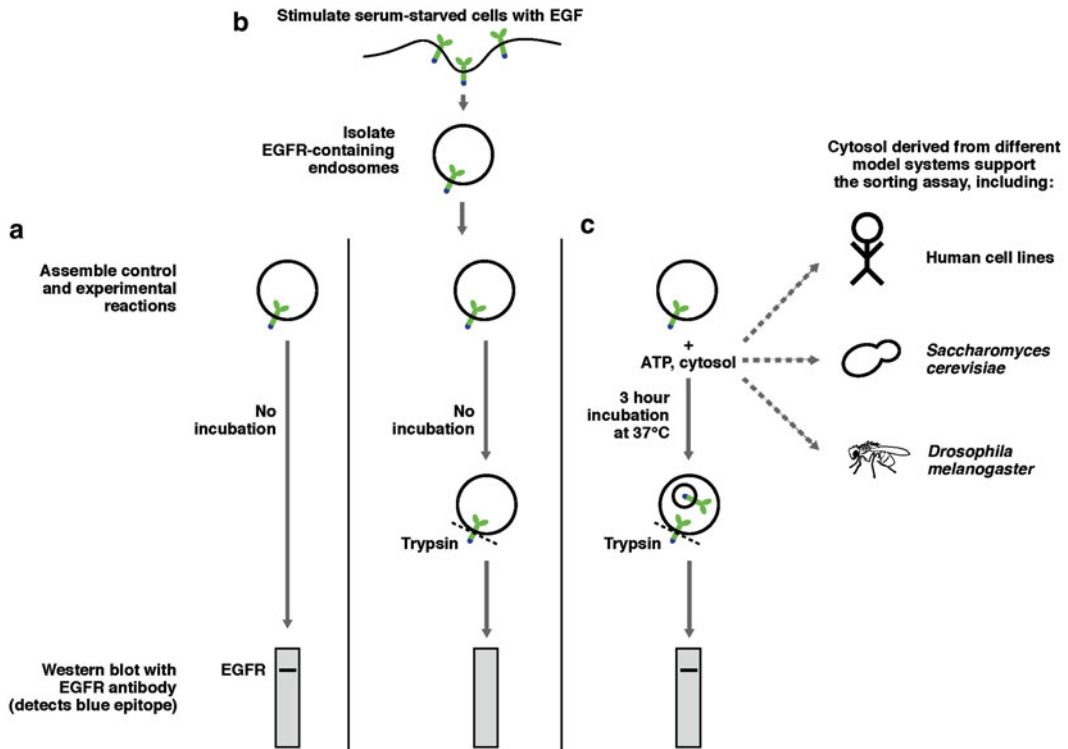


Fig. 1 Schematic for reconstitution of protein sorting into multivesicular bodies. Serum-starved HeLa cells are pulsed (10 min) with EGF (100 ng/mL) to induce internalization of the EGFR from the plasma membrane, resulting in movement of ligand-receptor complex into endosomes [1, 9, 11]. (a) Cells are lysed and partially purified endosomes that contain EGFR are isolated and detected on a western blot using an intracellular epitope specific rabbit polyclonal antibody that recognizes amino acids 1,198–2,110 of human EGFR. (b) Incubation of these endosomes with trypsin removes the C-terminal epitope of the EGFR that protrudes from the plasma membrane resulting in a lack of detection of that C-terminal epitope on a western blot. (c) Incubation with ATP, cytosol (HeLa, *Saccharomyces cerevisiae*, or *Drosophila melanogaster* cytosol), at 37 °C before trypsin treatment results in protection of the C-terminal EGFR epitope from subsequent trypsin cleavage. Hypothetical western blotting results are shown for demonstration purposes

2 Materials

2.1 Cell Culture

1. HeLa cells media: Add 10 % Fetal Bovine Serum (FBS) to Dulbecco's Modified Eagle Medium (DMEM).
2. Starvation media: Add 1 % Bovine Serum Albumin to DMEM.
3. Trypsin/ethylenediaminetetraacetic acid (EDTA).

2.2 Common Reagents

1. 100 ng/mL Epidermal growth factor (EGF) working solution: Resuspend EGF (Sigma, E9644, 200 µg) in 100 µL of filtered 10 mM acetic acid. Then add 100 µL H₂O for a 1 µg/µL stock. Dilute in 2 mL 0.1 % BSA for a 100 ng/µL working solution. Make 30 µL aliquots and store at –80 °C until ready to use.

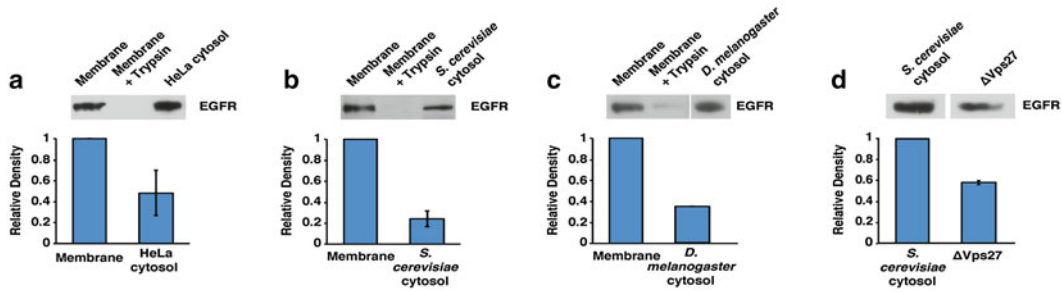


Fig. 2 Protease protection of the EGFR is supported by mammalian, fly, and yeast cytosol. HeLa cells are starved and pulsed (10 min) with EGF (100 ng/mL) to induce internalization of the EGFR from the plasma membrane. These conditions result in the movement of the ligand-receptor complex into endosomes. Cells are lysed and partially purified membranes that contain EGFR are isolated. Incubation of these membranes with trypsin removes the cytoplasmic domain of the EGFR including the epitope that can be detected on a western blot using an epitope specific antibody (that recognizes amino acids 1,198–2,110) of human EGFR. Incubation with cytosol derived from HeLa cells (a), *Saccharomyces cerevisiae* yeast (b), or *Drosophila melanogaster* (c) and ATP, at 37 °C prior to trypsin treatment results in protection of the EGFR epitope from subsequent trypsin cleavage. (d) When endosomes were incubated with cytosol derived from a Vps27 deletion strain the amount of EGFR protected was decreased by 50 % (Lane 2) compared to wild-type *Saccharomyces cerevisiae* yeast cytosol (Lane 1) [1, 9, 11]

To prepare the final EGF concentration, dilute 30 μ L into 30 mL of DMEM for a final concentration of 100 ng/mL.

- Homogenization buffer (HB): 20 mM HEPES pH 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT and a protease inhibitor cocktail (112 μ M PMSF, 3 μ M aprotinin, 112 μ M leupeptin, 17 μ M pepstatin). Store at 4 °C.
- ATP regeneration system: 2 mM MgATP, 50 μ g/mL creatine kinase, 8 mM phosphocreatine, and 1 mM DTT of final concentrations. See Note 1.
- Recombinant proteins: Full length Hrs was subcloned into Hta baculovirus vector (Gibco BRL Grand Island, NY), and Hrs virus produced according to the manufacturer's protocol. The Hrs protein is produced by infecting a 500 mL culture of SF21 cells at an MOI (multiplicity of infection) of 0.1. Seventy-two hours post-infection, harvest the cells by centrifugation and freeze the pellets. Extract proteins by incubation (60 min at 4 °C) with 5 % Betaine in 10 mM Tris base pH 7.5, 1 mM EGTA, 1 mM EDTA, and a cocktail of protease inhibitors including aprotinin, pepstatin, and PMSF. Affinity isolate the proteins using Ni-NTA agarose (Qiagen), and size fractionate on a Superose 6 column using 150 mM KCl, 20 mM HEPES, pH 7.4, and 5 mM EDTA [10]. Add 180 nM of Hrs recombinant protein into experimental reactions.

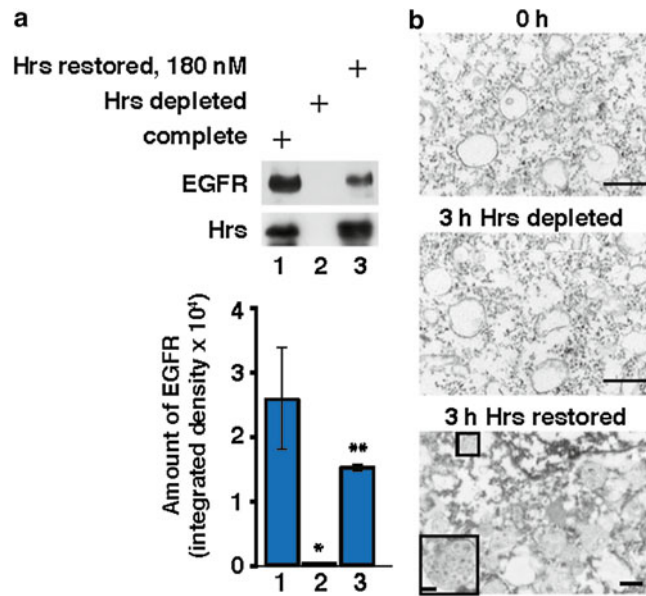


Fig. 3 Hrs is required for EGFR protection from trypsin cleavage and internal endosomal vesicles. HeLa cells are starved and pulsed (10 min) with EGFR (100 ng/mL) to induce internalization of the EGFR from the plasma membrane. Cells are lysed and partially purified membranes that contain EGFR are isolated. (a) Lane 1 (control). Membrane incubation with cytosol derived from rat brain, and ATP, at 37 °C prior to trypsin treatment results in protection of the EGFR epitope from subsequent trypsin cleavage (Lane 1). Lane 2 (Hrs depletion). Hrs was immunodepleted from HeLa cytosol, and endosomal membranes were incubated with ATP, at 37 °C prior to trypsin treatment, resulting in lack of protection of the EGFR epitope from subsequent trypsin cleavage. Lane 3 (Hrs restored). Hrs was immunodepleted from HeLa cytosol followed by addition of recombinant Hrs (180 nM) and incubated with ATP, at 37 °C prior to trypsin treatment, resulting in protection of the EGFR epitope from subsequent trypsin cleavage. (b) Ultrastructural examination of initial endosomal membranes (0 h), membranes from reactions that had been depleted of Hrs, and membranes depleted of Hrs that were subsequently incubated with recombinant Hrs protein. Scale bar indicates 500 nm. * $p \leq 0.05$, ** $p = 0.2$. Scale bar in *inset* is 100 nm. Adapted from Sun et al. [1]

2.3 *Saccharomyces cerevisiae* Reagents

1. YPD media: Add 10 g Bactopeptone and 5 g Yeast Extract to a 500 mL bottle and fill with ddH₂O to 500 mL. Autoclave for 30 min. Add 25 mL of 40 % dextrose after autoclaving.
2. YPD plates: Add 10 g Bactopeptone, 5 g Yeast Extract, and 8 g of Agar to a 500 mL bottle and fill with ddH₂O to 500 mL. Autoclave for 30 min. Add 25 mL of 40 % dextrose after autoclaving, pour plates, let solidify and store at 4 °C.
3. T.P. buffer (TP): 20 mM Tris base, pH 7.9; 0.5 mM EDTA; 10 % glycerol; 50 mM NaCl and a protease inhibitor cocktail (112 μ M leupeptin, 3 μ M aprotinin, 112 μ M PMSE, and 17 μ M pepstatin).

2.4 *Drosophila melanogaster* Reagents

1. Fly Buffer: 30 mM NaCl, 20 mM HEPES, pH 7.5.
2. Stacked sieves (USA Standard Sieves, No. 25 and No. 48).

2.5 *Electron Microscopy Reagents*

1. 0.1 M cacodylate buffer: Add 4.38 g of sodium cacodylate to 100 mL of ddH₂O to make a 0.2 M stock solution. Dilute 0.2 M stock solution 1:2 to get 0.1 M cacodylate buffer.
2. 3 % Glutaraldehyde in 0.1 M cacodylate buffer.

3 Cell-Free Reconstitution of Multivesicular Body Formation Assay

Performed as described in Sun et al. [1] and Gireud et al. [9].

3.1 Cell Culture

1. Grow HeLa cells in 10-cm culture plates in DMEM containing 10 % FBS under 5 % CO₂ at 37 °C. Two days before each experiment, split the cells with trypsin/EDTA and plate into 10-cm tissue culture plates. Allow the cells to grow to 75–80 % confluence. For number of plates per experiment *see* **Note 2**.

3.2 Cytosol Preparation

1. Mammalian cytosol preparation: Grow HeLa cells in DMEM containing 10 % FBS in 10 cm plates to 75–80 % confluence. Place cells on ice and wash with ice-cold PBS (2× with 5 mL). After washing, add 1 mL of PBS and scrape the cells from the plate followed by centrifugation at 2,000×*g* for 15 min at 4 °C. Resuspend the cell pellet in 100 µL of HB. Sonicate the cells (70 % maximum, 5 times with 5 pulses of 1 s each with a microprobe, Branson Sonifier 250, VWR Scientific). Centrifuge the lysate at 2,000×*g* for 10 min at 4 °C to remove cell debris, and then collect the supernatant. Centrifuge the supernatant at 100,000×*g* for 1 h at 4 °C. Collect the supernatant and aliquot into 10 µL, and store at –80 °C. Calculate protein concentration using a Bradford assay.
2. *Saccharomyces cerevisiae* cytosol preparation: Grow *Saccharomyces cerevisiae* strains on YPD plates and incubate at 30 °C for 48 h. Inoculate YPD media (5 mL) with the *Saccharomyces cerevisiae* strains and incubate overnight at 30 °C. Following incubation transfer cells into a secondary culture of YPD media (50 mL) and grow until the OD₆₀₀ reaches 0.8–1.0. Collect the cells (3,000×*g* for 3 min) and wash with 500 µL of water. Then recollect the cells (3,000×*g* for 3 min) and wash with 500 µL T.P. buffer. Collect the cells a final time (3,000×*g* for 3 min) and resuspend in 100 µL of T.P. buffer. Add 50 µL of acid-washed beads to the cells and lyse the cells (1 min vortex/1 min ice 5×). Centrifuge (3,000×*g* for 10 min), collect the supernatant, aliquot into 30 µL, and store at –80 °C. Calculate protein concentration using a

Bradford assay. For cytosol preparation from yeast deletion strains *see* **Note 3**.

3. *Drosophila melanogaster* cytosol preparation: *Drosophila melanogaster* strains should be grown in bottles on standard laboratory fly food. Collect flies at desired ages, weigh them as collected, and freeze at -80°C . When at least 1 g of flies has been collected, consolidate the flies, freeze in liquid nitrogen, and vortex repeatedly (fly heads, bodies, and appendages will be broken apart), making sure that flies remain frozen and do not warm up (or they will stick together). Pour frozen flies through two stacked sieves (No. 25 and No. 48); make sure that sieves are pre-frozen or cooled with liquid nitrogen so that flies do not stick to the sieves. The No. 25 sieve will isolate bodies, the No. 48 sieve will isolate heads, and legs will fall through to the bottom. Without letting them defrost, pour heads into a glass–glass homogenizer. Homogenize heads in ~ 1 mL of fly buffer on ice. Spin homogenate at $1,660 \times g$ in microfuge to pellet chiton; save supernatant. Homogenize chiton in buffer and centrifuge again at $1,660 \times g$ and recover additional supernatant. Supernatants should be combined, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until ready to use. Calculate protein concentration using a Bradford assay.

3.3 Isolating Endosomal Membranes

1. Grow HeLa cells on 10 cm plates to 75–80 % confluence.
2. Before harvesting, serum starve cells for 2 h by washing cells with 6 mL of DMEM twice, and then adding 4 mL of starvation media to each plate and placing at 37°C in 5 % CO_2 for 2 h.
3. After 2 h, stimulate cells for 10 min at 37°C with warm 100 ng/mL of EGF.
4. Place cells on ice, aspirate supernatant and wash with 5 mL of ice-cold PBS. Repeat two times.
5. Aspirate supernatant, and add 1 mL of ice-cold PBS to one plate. Scrape the cells from the plate and transfer to the next plate. Repeat until all plates have been scraped and all cells have been collected.
6. Centrifuge the cells at $1,500 \times g$ for 10 min at 4°C .
7. Resuspend the cell pellet in 100 μL of HB.
8. Lyse the cells by drawing them through a 30-gauge needle into a 1 mL syringe 30 times within approximately 6 min.
9. Centrifuge the lysate at $800 \times g$ for 5 min to remove debris.
10. Collect the supernatant and centrifuge for 15 min at $1,500 \times g$.
11. Aspirate the supernatant and recover endosomal membranes to be used for reconstitution reactions.

3.4 Reaction Conditions

1. Resuspend endosomal membranes in HB buffer. Volume is dependent on the number of reactions (10 μ L for control reactions, and 15 μ L per experimental reaction). Example: For seven experimental reactions add 115 μ L of HB.
2. Starting material controls.
 - (a) Add 5 μ L of endosomal membranes (starting material) to a microcentrifuge tube and place on ice (no incubation) for 3 h (Fig. 1a). Skip ahead to step 7.
 - (b) Add 5 μ L of endosomal membranes (starting material) to a microcentrifuge tube and place on ice for 3 h (no incubation) (Fig. 1b). Then add 6 μ L of Trypsin (0.27 μ g/ μ L) and place on ice for 30 minutes. Move to step 7.
 - (c) For additional controls *see* **Note 4**.
3. For experimental reactions (Fig. 1c):
 - (a) For the mammalian cytosol reactions add 15 μ L membranes, 6 μ L ATP regeneration system, 25 μ g of HeLa cytosol, and HB up to 50 μ L (Fig. 2a).
 - (b) For the *Saccharomyces cerevisiae* cytosol reactions, add 15 μ L membranes, 6 μ L ATP regeneration system, 70 μ g of *Saccharomyces cerevisiae* cytosol, and HB up to 50 μ L (Fig. 2b, d).
 - (c) For the *Drosophila melanogaster* (fly) cytosol reactions, add 15 μ L membranes, 6 μ L ATP regeneration system, 25 μ g of *Drosophila melanogaster* cytosol, and HB up to 50 μ L (Fig. 2c).
 - (d) For the experimental reactions immunodepleted of Hrs, incubate the membranes with a monoclonal antibody against Hrs (2 μ g per reaction) for 30 min on ice followed by centrifugation. Additionally, immunodeplete Hrs from cytosol by incubation with a monoclonal anti-Hrs antibody for 1 h at 4 °C. Add 15 μ L of membranes, 6 μ L ATP regeneration system, 25 μ g of Hrs-immunodepleted HeLa cytosol, and HB up to 50 μ L (Fig. 3).
 - (e) For the experimental reactions immunodepleted of Hrs plus recombinant Hrs protein, incubate the membranes with a monoclonal antibody against Hrs (2 μ g per reaction) for 30 min on ice followed by centrifugation. Additionally, immunodeplete Hrs from cytosol by incubation with a monoclonal anti-Hrs antibody for 1 h at 4 °C. Add 15 μ L of membranes, 6 μ L ATP regeneration system, 25 μ g of Hrs-immunodepleted HeLa cytosol, 180 nM of recombinant Hrs protein, and HB in a final reaction volume of 50 μ L (Fig. 3).
4. Incubate the experimental reactions for 3 h at 37 °C. *See* **Note 5**.
5. After incubation, place the reactions on ice and add 6 μ L trypsin (0.27 μ g/ μ L). Incubate for 30 min. *See* **Note 6**.

6. Centrifuge the experimental reactions at $15,000 \times g$ for 30 min at 4 °C.
7. For control reactions, resuspend in sample buffer for biochemical examination. For experimental reactions, aspirate supernatant and resuspend the pellet in sample buffer for biochemical examination. *See Note 7* for ultrastructural analysis instructions.
8. Boil the samples and run on a gel immediately or store samples at 4 °C overnight.

3.5 Western Blotting

1. Separate proteins by SDS-PAGE, followed by transfer to nitrocellulose using standard conditions. After blocking with 5 % nonfat dry milk in PBS, probe the blot with an antibody that recognizes the intracellular domain of EGFR (ABR Inc, 1:1,000 dilution, overnight at 4 °C) followed by goat anti-rabbit polyclonal antibody conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich Inc, for 1 h at room temperature). Proteins are detected using enhanced chemiluminescence (ECL; Pierce) and visualized by exposure to X-ray film.

4 Notes

1. For best results make the ATP regeneration system fresh every experiment.
2. Use one 10-cm plate per reaction.
3. Grow the *Saccharomyces cerevisiae* deletion strains on YPD plates containing G418 (500 µg/mL) and incubate at 30 °C for 48 h. Inoculate YPD media (5 mL) containing G418 (500 µg/mL) with the *Saccharomyces cerevisiae* strains and incubate overnight at 30 °C. After incubation, transfer cultures into a secondary culture of YPD media (50 mL) containing G418 (500 µg/mL) and grow until the OD₆₀₀ reaches 0.8–1.0. Collect the cells ($3,000 \times g$ for 3 min) and wash with 500 µL of water. Then recollect the cells ($3,000 \times g$ for 3 min) and wash with 500 µL T.P. buffer. Collect the cells a final time ($3,000 \times g$ for 3 min) and resuspend in 100 µL of T.P. buffer. Add 50 µL of acid-washed beads to the cells and lyse the cells (1 min vortex/1 min ice 5×). Centrifuge ($3,000 \times g$ for 10 min), collect the supernatant, aliquot into 30 µL, and store at –80 °C. Calculate protein concentration using a Bradford assay.
4. Experimental reactions containing no cytosol can be used as negative controls. Additionally, incubating the experimental reactions for 3 h at 0 °C can also serve as a negative control.
5. During the incubation period, gently mix the reactions by tapping the tube every 30 min.
6. During the incubation period, gently mix the reactions by tapping the tube every 5 min.

7. Electron Microscopy Analysis: After harvesting the membranes at $15,000\times g$ for 30 min at 4 °C, fix the pellets with 3 % glutaraldehyde in cacodylate buffer for 2–16 h at 4 °C. Subsequently, embed the samples in epon or another embedding material, cut sections, and view on an electron microscope.

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Chapter 10

Analysis of Biogenesis of Lipid Droplets by Examining Rab40c Associating with Lipid Droplets

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Abstract

The biogenesis of lipid droplets (LDs) is regulated by multiple proteins. Rab40c is a recently characterized small GTPase associating with LDs. Here we describe our approaches to analyze the involvement of Rab40c in the biogenesis of LDs. Co-localization of Rab40c and LDs was revealed through fluorescence confocal microscopy by expressing GFP-Rab40c and labeling LDs with dyes Oil Red O, Nile Red, or BODIPY 493/503. Association of Rab40c with LDs was further confirmed by fractionation with sucrose density centrifugation. The relationship of Rab40c with adipocyte differentiation was examined by detecting the protein level of Rab40c during adipocyte differentiation of 3T3-L1 cells. The roles of Rab40c in the biogenesis of LDs were analyzed by inducing LD formation in HepG2 cells.

Key words Lipid droplets, Fluorescence microscopy, Cell fractionation, Adipocyte differentiation, Lipid droplets formation, Rab40c

1 Introduction

Intracellular membrane compartmentalization generates varied organelle with divergent biological functions. Lipid droplets (LDs), also named adiposomes, have been long ignored but have attracted extensive attention in the past decade. LD is a common organelle produced by animal cells, yeasts, plants, and even bacteria. LDs are surrounded by phospholipid monolayer and membrane associated proteins. However the lumen of the LDs always contains neutral lipids, mainly triacylglycerol (TAG), and cholesterol esters (reviewed in ref. 1). Investigations revealed that LDs are not only structures for the storage of triglyceride and cholesterol ester but also dynamic organelles that maintain lipids homeostasis, generate energy, and regulate cell signaling, and involved in many metabolic disorders, such as obesity, diabetes, and arteriosclerosis (reviewed in refs. 2, 3).

Investigation on the biogenesis of LDs is not only important for exploring the regulatory mechanisms and the biological functions

of this organelle but also significant for designing drugs to treat LDs-related diseases. Although LDs are most likely derived from endoplasmic reticulum (ER), there are specialized proteins associated with LDs. Since these are lipid storage organelles, there are enzymes for lipid metabolism associating with LDs. In particular, there are special proteins associated with the membrane of LDs to regulate the biogenesis of LDs. The PAT family proteins [perilipin, ADRP (adipocyte differentiation related protein or adipophilin) and TIP47 (tail-interacting protein 47 kDa)] are the best characterized proteins crucial for the biogenesis of LDs [4–9]. Proteomic investigations have also discovered that the protein components of LDs vary depending on cell types or physiological conditions [10–13]. SNARE and Rab proteins are key regulator for membrane trafficking in eukaryotes. Some Rab proteins have been reported to be involved in the biogenesis of LDs [14, 15]. Although specific SNARE complex mediating LDs trafficking have not yet been identified, SNARE proteins may be involved in regulating LDs membrane fusion [16].

The roles of some LD-associated proteins in regulating the biogenesis of LDs have been examined by immuno-electron microscopy, immunofluorescence microscopy, biochemical methods, as well as in animal models [17, 18]. Our previous work has demonstrated that Rab40c is a novel LDs associated Rab protein [19]. Here we describe the approaches applied to identify the LD association of Rab40c, including fluorescence confocal microscopy, LD fractionation, stimulation of adipocyte differentiation with 3T3-L1 cells, and induction of biogenesis of LD in HepG2 cells with oleic acid. The methods described here would be useful in general for the characterization of proteins associating with LDs, particularly other Rab proteins.

2 Materials

2.1 Cell Culture and Transfection

1. NRK (Normal rat kidney), 3T3-L1 and HepG2 cell lines were from the American Type of Culture Collection (ATCC) (*see* **Note 1**).
2. DMEM medium (4,500 mg/L glucose, Gibco) containing 10 % (v/v) fetal bovine serum (FBS, Hyclone) or 10 % fetal calf serum (FCS, Hyclone).
3. Transfection reagent: Lipofectamine 2000 (Invitrogen).
4. Trypsin solution: 0.25 % trypsin in PBS containing 1 mM EDTA.

2.2 Expression Plasmids

GFP-Rab40c was constructed by inserting the coding sequence of human Rab40c cDNA into the EcoRI/SalI sites of pEGFP-C1 vector (BD Clontech) [19].

2.3 Fluorescence Microscopy

1. Laser scanning confocal microscope: Carl Zeiss LSM5 EXITER.
2. Phosphate-buffered saline buffer (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄ in double-distilled water.
3. PBSCM: PBS containing 1 mM CaCl₂ and 1 mM MgCl₂.
4. Oil Red O stock containing 0.5 g Oil Red O (Sigma) in 100 ml isopropanol. Dilute 6 ml of Oil Red O stock in 4 ml double-distilled water to make working solution.
5. Nile Red stock containing 1.0 mM Nile Red dissolved in DMSO. Nile Red working solution is prepared by diluting the Nile Red stock to 1.0 μ M with PBS.
6. BODIPY 493/503 stock containing 1.0 mg BODIPY 493/503 dissolved in ethanol. BODIPY 493/503 working solution prepared by diluting BODIPY 493/503 stock to 1.0 μ g with PBS.
7. Saponin: 0.1 % (m/v) in PBSCM.
8. Cell fixation buffer: 3 % paraformaldehyde in PBSCM.
9. Mounting buffer: VECTASHIELD mounting medium (Vector Laboratories).

2.4 Lipid Droplets Fractionation

1. Cell disruption buffer: 25 mM Tris/HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, and 5 mM EGTA, EDTA-free protease inhibitor cocktail (Roche).
2. Sucrose solution: prepare 1.0 M sucrose solution stock using disruption buffer, prepare 0.33 M, 0.25 M, or 0.125 M sucrose solution by diluting 1.0 M sucrose solution stock using disruption buffer.
3. Cell scraper and 27# gauge needle.
4. Beckman ultracentrifuge and SW41Ti swinging-bucket rotor.
5. 13.2-ml polycarbonate ultracentrifuge tubes.

2.5 Western Blot

1. 10 % Resolving gel: 10 ml of resolving gel containing 6.6 ml of resolving gel buffer, 3.3 ml of 30 % (m/v) Acrylamide/Bis (29:1), 60 μ l of 10 % (m/v) APS, and 2.4 μ l of TEMED.
2. Stacking gel: 5 ml of stacking gel containing 3.75 ml of stacking gel buffer, 1.25 ml of 30 % (m/v) Acrylamide/Bis (29:1), 30 μ l of 10 % (m/v) APS, and 3 μ l of TEMED.
3. Running buffer: containing 25 mM Tris-base, 0.2 M glycine, and 0.3 % (w/v) SDS in double-distilled water.
4. Gel transferring buffer: containing 25 mM Tris-base, 0.2 M glycine, and 15 % (v/v) methanol in double-distilled water.
5. Antibodies: PolyAb against Rab40c, polyAb against TIP47, polyAb against ADRP, mAb against β -tubulin, mAb against KDEL receptor, Rabbit mAb against Perilipin (*see Note 2*).

6. Secondary antibody: HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti mouse IgG (Pierce).
7. Nitrocellulose membrane (Millipore) and western blot ECL substrate (Pierce).

2.6 Adipocyte Differentiation

1. IBMX solution (fresh-making): Dissolve 11.5 mg isobutyl methylxanthine in 1 M NaOH to a final concentration of 50 mM, and sterilize through a 0.22 μ m syringe filter.
2. Insulin stock solution: Dissolve 1.0 mg insulin in 1 ml of 0.02 M HCl, and sterilize by passing through a 0.22 μ m filter. Store at -20°C for long term, or at 4°C for short term.
3. Dexamethasone stock solution: Dissolve 0.39 mg dexamethasone in ethanol to a final concentration of 1 mM (store at -20°C). Filter-sterilize through a 0.22 μ m filter.
4. Medium 1: DMEM (4,500 mg/L glucose) containing 10 % (v/v) newborn calf serum.
5. Medium 2: DMEM (4,500 mg/L glucose) containing 10 % (v/v) FBS, 0.5 mM isobutyl methylxanthine, 1.0 μ M dexamethasone, and 1.0 μ g/ml insulin.
6. Medium 3: DMEM (4,500 mg/L glucose) containing 10 % FBS (v/v) and 1.0 μ g/ml insulin.

2.7 Induction of LD Biogenesis with Oleic Acid

1. 1,000 mg/ml oleic acid stock: 1.0 g oleic acid pre-dissolved in 1.0 ml ethanol.
2. 1 % (m/v) BSA stock: 1.0 g fatty acid-free BSA (Sigma) dissolved in 10 ml 0.1 M Tris-HCl (pH 8.0), and sterilized by using 0.45 μ m filter unit and stored at 4°C .
3. 10 mg/ml oleic acid-BSA solution: Mix 10 μ l of oleic acid stock with 10 ml BSA stock, and sterilize through 0.45 μ m filter unit (*see Note 3*).
4. Induction medium: add 100 μ l of 10 mg/ml oleic acid solution to 900 μ l DMEM (4,500 mg/L) containing 10 % (v/v) FBS, with 100 μ g/ml of oleic acid in final concentration.

3 Methods

3.1 Analysis of Rab Protein Co-localizing with Lipid Droplets by Fluorescence Confocal Microscopy

1. NRK cells are seeded on coverslip in the 6-well plate and cultured in DMEM medium supplemented with 10 % fetal bovine serum in a 5 % CO_2 incubator at 37°C .
2. Grow the cells on coverslip for 16–18 h to 50 % of confluence, then transfect the cells with GFP-Rab40c plasmids using Lipofectamine 2000 reagents according to the manufacturer's protocol (*see Note 4*).

3. After 24 h of transfection, remove the culture medium and wash the cells twice with cold PBSCM.
4. For cell fixation: add 2.0 ml of cold 3 % paraformaldehyde to each well of the plate, and keep the cells at 4 °C for 30 min.
5. For cell permeabilization, wash the cells thrice with PBSCM containing 50 mM NH₄Cl followed by two washes with PBSCM (1 min per wash), then add 2.0 ml of 0.1 % saponin to each well of the plate at room temperature for 15 min to permeabilize the cells (*see* **Note 5**).
6. For labeling the lipid droplets: Remove the coverslip to a new plate. Incubate the coverslip with 100 µl of Oil Red O, Nile Red, or BODIPY 493/503 dyes for each staining for 20 min at room temperature, respectively, with the face seeded with cells down immersed in the staining dyes.
7. After staining, wash the cells thrice with PBSCM (3 min per wash).
8. Mount the coverslip on a glass slide with VECTASHIELD mounting buffer with the face seeded cells down. Seal the coverslip with nail polish.
9. The labeled cells are analyzed with Carl Zeiss LSM5 EXITER laser scanning confocal microscope. GFP-Rab40c is viewed directly under GFP channel in microscope, while lipid droplets are revealed by the dyes Oil Red or Nile Red (Fig. 1).

3.2 Lipid Droplets Fractionation by Sucrose Density- Gradient Centrifugation

1. Grow 7–10 cm dishes of HepG2 cells in DMEM as mentioned above.
2. Replace the medium with induction medium (refer to Subheading 2.7) until the culture reach 80 % of confluence, and grow the cells for another 24 h.
3. Wash the cells twice with 10 ml ice-cold PBS per dish
4. Scrape the cells into a 15 ml falcon tube with 2 ml disruption buffer. Keep the cells on ice 30 min, and then disrupt the cells by passing the cell suspension through a 27# gauge needle (*see* **Note 6**).
5. Remove the cell debris by spinning at 1,000×*g* for 5 min at 4 °C.
6. Adjust the sucrose concentration of the supernatant to 0.33 M and the final volume to 3.0 ml (*see* **Note 7**).
7. To make the sucrose density-gradient, 3.0 ml of cell lysate with 0.33 M of sucrose layered in the bottom of the ultracentrifuge tube for SW41Ti rotor, followed by 3 ml of 0.25 M, 3 ml of 0.125 M sucrose solution, and 3 ml of disruption buffer.
8. Run the centrifuge at 150,000×*g* at 4 °C for 2 h.

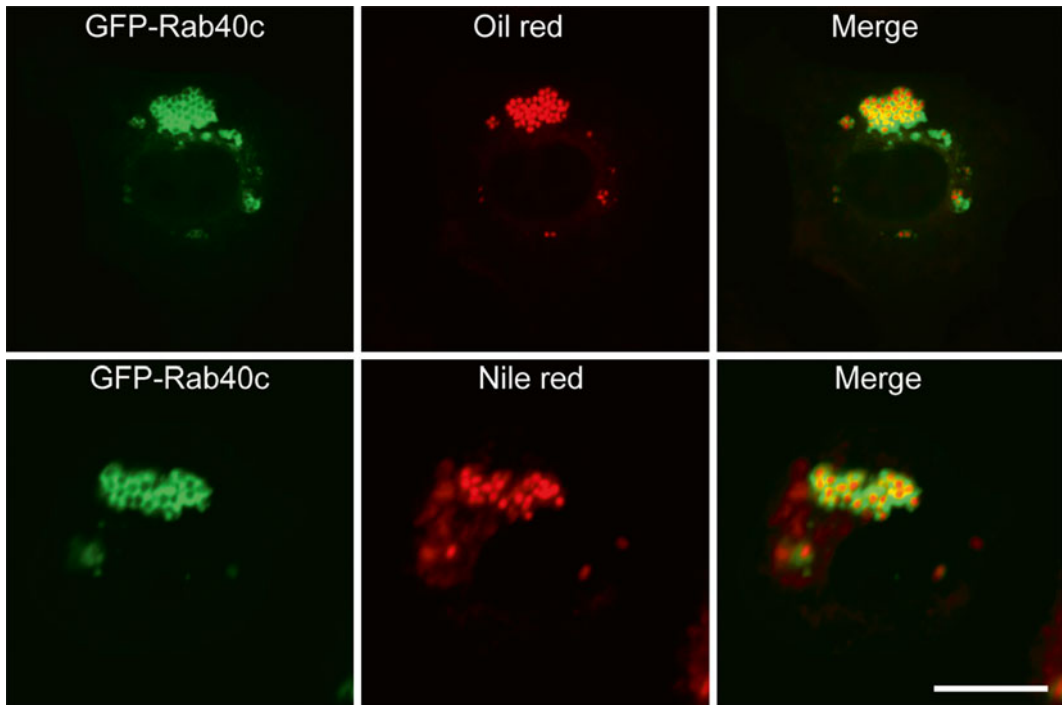


Fig. 1 Analysis of Rab40c associating with lipid droplets. NRK cells were transfected with GFP-Rab40c and labeled with Oil Red O or Nile Red, then processed for confocal microscopy. Rab40c was revealed by GFP and lipid droplets were revealed by Oil Red O or Nile Red. The results showed that GFP-Rab40c associates with lipid droplets. Bar = 20 μ m. This figure is reproduced from the open access journal PLoS One [19]

9. After centrifugation, fractions are collected sequentially from the top to bottom of the tube, with 1.5 ml of sample per fraction as demonstrated in Fig. 2a.
10. For the characterization of lipid droplets fraction, 50 μ l sample from each fraction is assessed by western blot to detect marker of LDs.
11. For western blot, 50 μ l samples mixed with 5 $\times$ SDS sample buffer are resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane is blocked with 5 % milk in PBS and then incubated with primary antibody for 1 h at room temperature, followed by the incubation with HRP-conjugated secondary antibody. The blots are detected using ECL system.
12. As indicated in Fig. 2b, Rab40c is co-fractionated with the marker of lipid droplets TIP47.

3.3 Adipocyte Differentiation Stimulation

1. 3T3-L1 fibroblasts are grown in medium 1 (Subheading 2.6) (*see* Note 8).
2. Plate the cells in the 6-well plate and culture in medium 1 for 24 h. When cells are confluent, the medium is replaced with

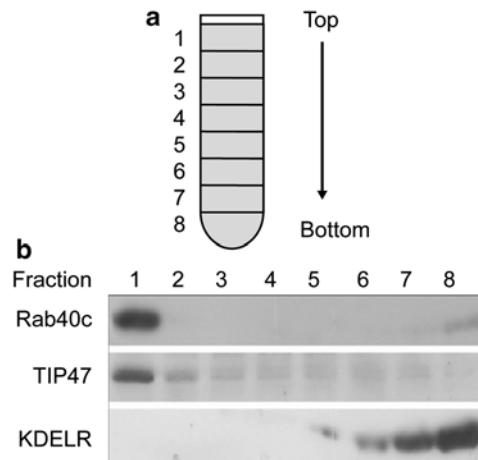


Fig. 2 Analysis of lipid droplets fractionation. **(a)** Diagram demonstrated the fractions sampled after sucrose density-gradient centrifugation. **(b)** Western blotting analysis of fraction samples. The results demonstrated that both TIP47 and Rab40c were enriched in the top floating LD-containing fraction (lane 1). The ER-Golgi recycling protein KDEL receptor was detected in the bottom fractions. Figure **(b)** is reproduced from the open access journal PLoS One [19]

fresh medium 1, and maintained for another 24–48 h, then go to the next step (*see* **Note 9**).

3. Replace medium 1 with medium 2, and maintain the culture in stimulation medium for 2 days, then go to the next step.
4. Replace medium 2 with medium 3, and maintain the culture for 2 days.
5. Feed the cells with fresh medium every 2 days (*see* **Note 10**).
6. To examine the differentiation process, the cells are stained by BODIPY 493/503 and analyzed by confocal microscopy or western blot as mentioned above (Fig. 3).

3.4 Induction of LD Biogenesis in HepG2 Cells

1. HepG2 cells are cultured as mentioned above.
2. Seed and grow the cells on coverslips for 24 h to 60 % confluence.
3. Transfect the cells with GFP-Rab40c plasmids as mentioned in Subheading 3.1, and grow the cells for 18–24 h.
4. Replace the medium with induction medium (refer to Subheading 2.7), and grow the cells for another 24 h.
5. To examine the formation of lipid droplets, the cells are stained by Oil Red O and analyzed by confocal microscopy as mentioned in Subheading 3.1. As demonstrated in Fig. 4, the number and the size of LDs increased with the stimulation of oleic acids in a time course dependent manner, and Rab40c staining was gradually associated with LDs.

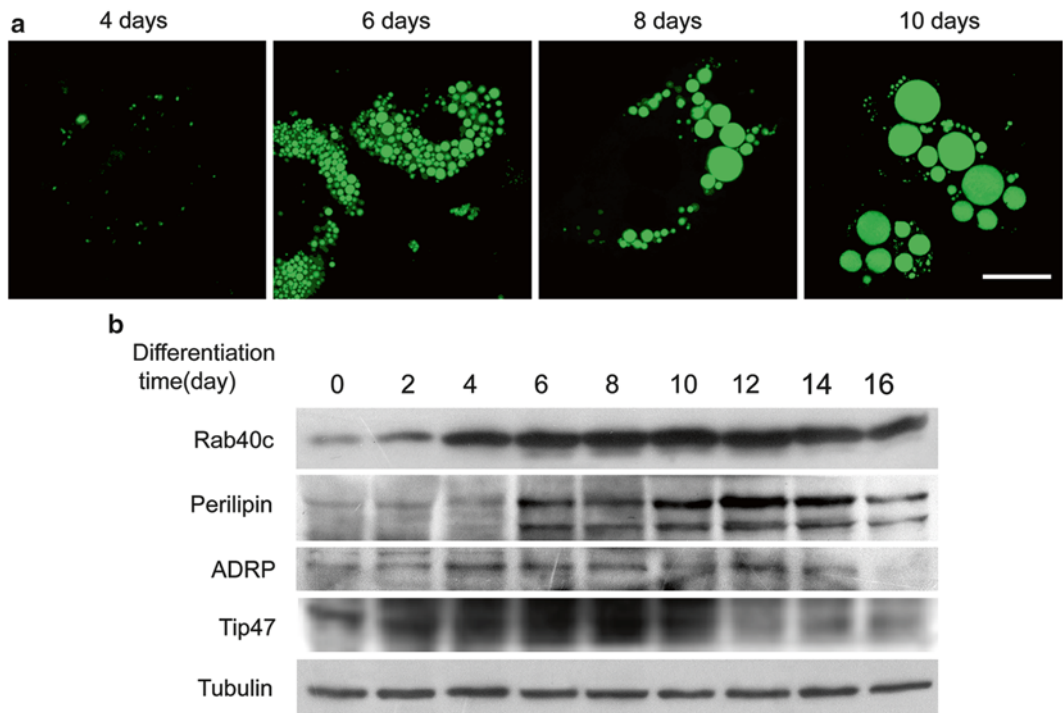


Fig. 3 Analysis of adipocyte differentiation. **(a)** 3T3-L1 cells were induced to differentiate into adipocytes for the indicated times, and processed for confocal microscopy. Lipid droplets were revealed by the dye BODIPY 493/503. **(b)** 3T3-L1 cells were induced to differentiate into adipocytes. The cells were lysed and subjected for western blot to detect Rab40c and the markers Perilipin, ADRP, or TIP47 for lipid droplets. β -tubulin serves as loading control. The results indicated that the level of Rab40c increased following with the adipocyte differentiation. Bar = 20 μ m. Figure **(b)** is reproduced from the open access journal PLoS One [19]

4 Notes

1. NRK cells give a better vesicular structure of Rab40c in our experiments. 3T3-L1, the pre-adipose cell line, was usually used to induce adipocyte differentiation [12], and HepG2 cells were used to mimic LD formation in adipocytes by stimulation with oleic acids [20].
2. PolyAb against Rab40c has been described previously [19]. PolyAb against TIP47 (AnaSpec), Rabbit mAb against Perilipin (Cell Signaling Technology), and polyAb against ADRP (Santa Cruz) serve as markers for lipid droplets. mAb against β -tubulin (Sigma) serves as loading control. mAb against KDEL receptor (BD Biosciences) serves as marker for endoplasmic reticulum.
3. Oleic acid must be pre-complexed with fat free BSA to prevent detergent effects of the unbound fatty acids as described [21]. When pipetting oleic acid, the tips of pipette should be cut off

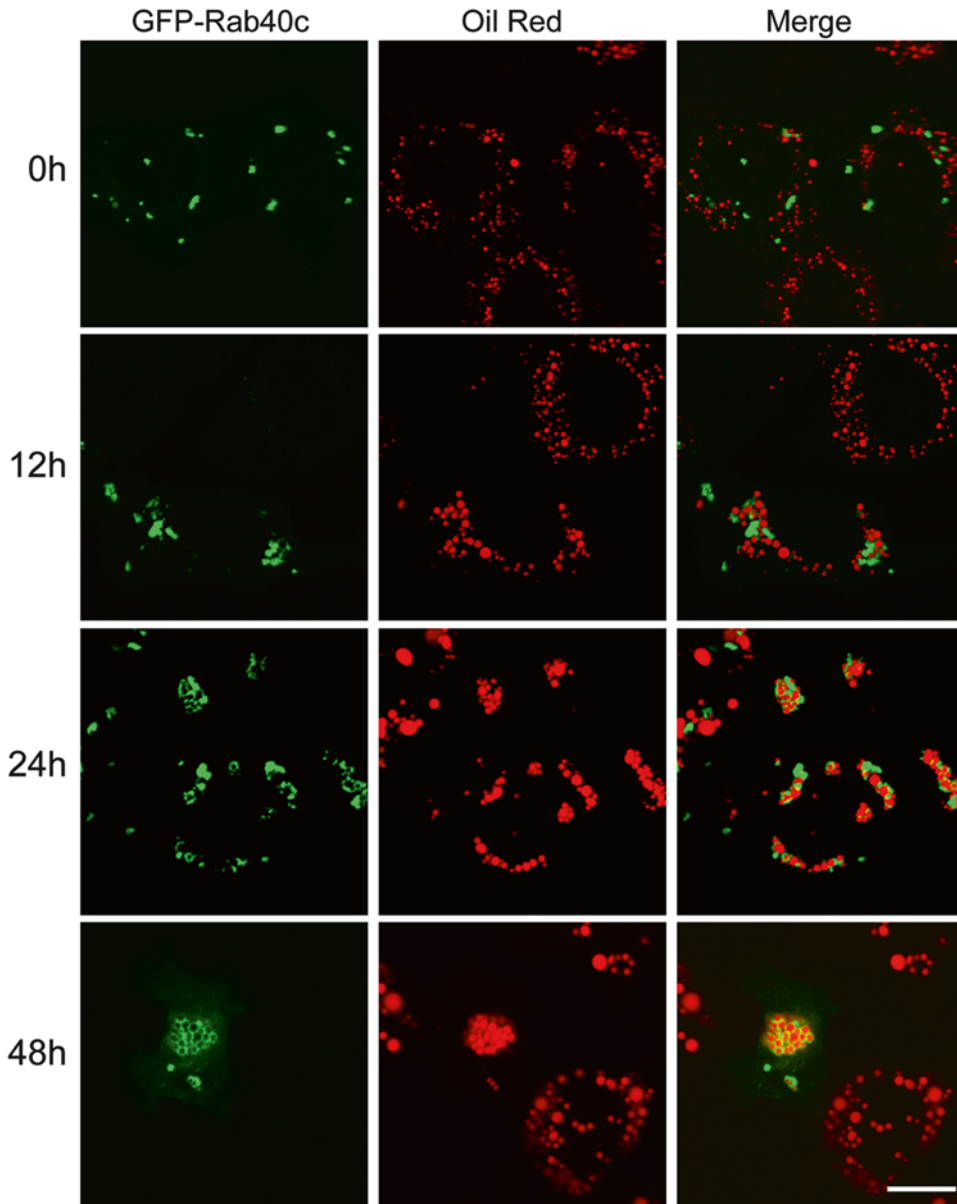


Fig. 4 Analysis of the biogenesis of LDs in HepG2 cells. HepG2 cells were transfected with GFP-Rab40c and induced by oleic acid 0, 12, 24, and 48 h, respectively. Rab40c was revealed by GFP and LDs were revealed by Oil Red under confocal microscopy. The results showed that GFP-Rab40c is recruited to LDs upon stimulation in a time course dependent manner. Bar = 20 μ m. This figure is reproduced from the open access journal PLoS One [19]

because oleic acid is viscous. For complexing oleic acid and BSA, oleic acid and BSA were mixed by gentle inversion and swirling to avoid excessive foaming in a 50 ml tube until the solution becomes clear.

4. To achieve high transfection efficiency, lower cell confluency is better. In our experiments, 50 % confluence cells give about 30 % of transfection efficiency with NRK cells. Other transfection reagents may also work for NRK cells, but Lipofectamine 2000 works better in our hands.
5. Fixation is achieved by paraformaldehyde (3–5 %) in PBS followed by permeabilization using either 0.1 % saponin or 0.01 % digitonin which permits optimal labeling of LDs while preserving good morphology of LDs. 0.1 % Triton X-100 is not suitable in our experiments.
6. To avoid damaging the tube, the tip of the 27# gauge needle must be blunted. Cells were homogenized by 6 passes through the needle. To avoid excessive disruption, check the extent of cell lysis under a microscope.
7. Usually, the volume of cell lysates exceeds 3.0 ml. To avoid generating large volume of diluted lysates, 2.0 ml of disruption buffer in total should be used to scrape the cells. If the volume of the lysates exceeds 3.0 ml after adjusting the concentration of sucrose, use only 3.0 ml of lysates for further procedures.
8. The murine embryonic 3T3-L1 cell line is a well-characterized cell model to study regulated lipolysis. 3T3-L1 cells can differentiate into adipocytes that respond in a similar manner to primary adipocytes [17]. 3T3-L1 cells were always maintained in the medium containing newborn calf serum and not FBS before stimulation, because FBS contains factors which may induce differentiation.
9. To achieve high adipocyte differentiation efficiency, it is crucial to use 3T3-L1 cells that have not been subjected to more than 20 passages. It would be better if the culture in 6-well plate is seeded at a density that will become confluent within 24 h.
10. 3T3-L1 cells induced to differentiate are very fragile and detach easily from the plate. It is better to use pipette with the tip cut off to remove or add the medium carefully. Usually, the differentiation can continue for 12–16 days, depending on the cell status.

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Chapter 11

Analysis of Conventional and Unconventional Trafficking of CFTR and Other Membrane Proteins

Heon Yung Gee, Joo Young Kim, and Min Goo Lee

Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is a polytopic transmembrane protein that functions as a cAMP-activated anion channel at the apical membrane of epithelial cells. Mutations in *CFTR* cause cystic fibrosis and are also associated with monosymptomatic diseases in the lung, pancreas, intestines, and vas deferens. Many disease-causing CFTR mutations, including the deletion of a single phenylalanine residue at position 508 ($\Delta F508$ -CFTR), result in protein misfolding and trafficking defects. Therefore, intracellular trafficking of wild-type and mutant CFTR has been studied extensively, and results from these studies significantly contribute to our general understanding of mechanisms involved in the cell-surface trafficking of membrane proteins. CFTR is a glycoprotein that undergoes complex N-glycosylation as it passes through Golgi-mediated conventional exocytosis. Interestingly, results from recent studies revealed that CFTR and other membrane proteins can reach the plasma membrane via an unconventional alternative route that bypasses Golgi in specific cellular conditions. Here, we describe methods that have been used to investigate the conventional and unconventional surface trafficking of CFTR. With appropriate modifications, the protocols described in this chapter can also be applied to studies investigating the intracellular trafficking of other plasma membrane proteins.

Key words Membrane protein, Unconventional trafficking, Cystic fibrosis transmembrane conductance regulator (CFTR), Surface biotinylation, Immunofluorescence staining

1 Introduction

Defects in the folding of secretory proteins are often accompanied by intracellular trafficking defects, which underlie a number of human diseases. A well-known example is the cystic fibrosis transmembrane conductance regulator (CFTR) (MIM 602421). CFTR is a key transepithelial transport membrane protein in the respiratory, gastrointestinal, and genitourinary systems. CFTR functions as a cAMP-activated anion channel that permeates Cl^- and HCO_3^- and acts as a regulator of other membrane transport proteins at the apical membrane of epithelial cells [1, 2]. Loss of CFTR function resulting from gene mutations causes a range of epithelial disorders, including the classical form of cystic fibrosis (CF),

chronic pancreatitis, and infertility in males. The most common disease-causing mutation of CFTR is the deletion of phenylalanine at position 508 ($\Delta F508$ -CFTR), which results in protein misfolding, retention in the endoplasmic reticulum (ER), and eventual degradation through the ER-associated degradation (ERAD) pathway [3]. Consequently, very little or no $\Delta F508$ -CFTR reaches the plasma membrane. However, although $\Delta F508$ -CFTR has protein folding and trafficking defects, it retains some Cl^- channel activity once embedded in the plasma membrane. Therefore, molecular mechanisms involved in the intracellular trafficking of wild-type and $\Delta F508$ CFTRs have been extensively studied with the hope of developing methods for facilitating the cell-surface expression of $\Delta F508$ -CFTR [4].

The WT-CFTR glycoprotein undergoes complex glycosylation as it passes through Golgi-mediated conventional exocytosis. Therefore, the Golgi-mediated complex-glycosylated mature WT-CFTR, also known as band C, is generally expressed at the apical membrane of epithelial tissues. On the other hand, $\Delta F508$ -CFTR appears in Western blot as an ER core-glycosylated immature form, also known as band B, because $\Delta F508$ -CFTR is primarily retained in the ER [5]. It is commonly assumed that the complex-glycosylated CFTR (band C) is the only form expressed in the plasma membrane. However, we recently reported that the core-glycosylated CFTR (band B) can also reach the plasma membrane under certain conditions through an unconventional alternative route that bypasses the Golgi [6]. Here, we describe the methods used to investigate both the conventional and unconventional surface trafficking of CFTR [3, 7], with a focus on methods based on cell-surface biotinylation and immunofluorescence techniques. These protocols can also be modified for use in studying the intracellular trafficking of other plasma membrane proteins.

2 Materials

2.1 Cell Culture and DNA Transfection

1. Use culture media listed in Table 1 for culturing HEK 293 cells and the epithelial origin of CAPAN-1, PANC-1, CFPAC-1, and T84 cells.
2. Permeable supports fabricated from Transwell-Clear Polyester membrane (0.4 μm pore diameter; Costar) for polarized culture of epithelial cells.
3. Prepare the Lipofectamine PLUS reagent for DNA plasmid transfection into HEK 293 and PANC-1 cells and the AMAXA Nucleofector transfection kits (Lonza) for CAPAN-1, CFPAC-1, and T-84 cells (Table 2).

Table 1
Cell culture media

Cell line	Media	Serum	Anti-biotic
HEK 293 CAPAN-1, PANC-1 CFPAC-1 T84	DMEM-high glucose RPMI 1640 medium Iscove's modified Dulbecco's medium 1:1 mixture of Ham's F-12 and DMEM medium	10 % FBS	Penicillin (50 units/mL)/ streptomycin (50 µg/mL)

Table 2
DNA transfection reagents

Cell line	Transfection reagent	Transfection efficiency	Survival rate
HEK 293, PANC-1	Lipofectamine PLUS Lipofectamine 2000	~85 %	~95 %
CAPAN-1, CFPAC-1	AMAXA Nucleofector V kit	~25 %	~78 %
T-84	AMAXA Nucleofector T kit	~55 %	~82 %

2.2 Antibodies and Markers

1. Anti-CFTR antibodies.
 - (a) Anti-CFTR R4 (rabbit polyclonal) raised against peptides corresponding to amino acids 1,458–1,471 of human CFTR (CKSKPQIAALKEET) and affinity-purified [7].
 - (b) Anti-CFTR M3A7 (monoclonal, Millipore).
 - (c) Anti-CFTR 24-1 (monoclonal, R&D Systems).
2. Organelle markers.
 - (a) Anti-calnexin (Abcam), ER marker.
 - (b) Anti-BiP (Cell Signaling), ER marker.
 - (c) Anti-EEA1 (monoclonal, 610456, BD Biosciences), endosome marker.
 - (d) Anti-ERGIC-53 (monoclonal, SC-365158, Santa Cruz), ERGIC marker.
 - (e) Anti-SEC16 (A300-648A, Bethyl Lab), ER-exit site marker.
 - (f) Anti-Giantin (ab24586, Abcam), Golgi marker.
 - (g) Anti-LAMP1 (monoclonal, ab25630, Abcam), lysosome and autophagosome marker.
 - (h) Anti-LC3 (L7543, Sigma), autophagosome marker.

- (i) Anti- γ -tubulin (monoclonal, T-6557, Sigma), centrosome and aggresome marker.
 - (j) LysoTracker Red (Invitrogen), lysosome marker.
 - (k) MitoTracker Green and Red (Invitrogen), mitochondria marker.
 - (l) RRX-conjugated actin-phalloidin (Invitrogen), F-actin and terminal web marker.
 - (m) Anti- β actin (Santa Cruz), marker for housekeeping protein.
 - (n) Anti-aldolase (Santa Cruz), marker for cytosolic protein in biotinylation assay.
3. Nucleus staining.
 - (a) DAPI (4, 6-diamidino-2-phenylindole).
 - (b) DRAQ5™ far-red fluorescent DNA dye (Cat #:DR50200, ImmunoChemistry Technologies).
 4. Antibody for epitope-tagged proteins.
 - (a) Anti-HA (monoclonal F-7 and polyclonal Y-11 from Santa Cruz; monoclonal #2367 from Cell Signaling).
 - (b) Anti-Flag (monoclonal 9E10 and polyclonal F3165 from Sigma).
 - (c) Anti-Myc (monoclonal sc-40 from Santa Cruz; monoclonal #9B11 and polyclonal #2272 from Cell Signaling).
 5. Secondary antibodies with fluorophore.
Anti-mouse, anti-rabbit, or anti-goat IgG conjugated with Alexa Fluor 488, 568, or 633 (Invitrogen).
 6. Secondary antibodies with HRP.
Anti-mouse, anti-rabbit, or anti-goat IgG conjugated with HRP (Thermo Scientific Inc.).

2.3 Materials for Immunofluorescence Experiments

1. 10× phosphate buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, pH 7.2. Use 1× PBS working solution for preparing following solutions.
2. Fixative solution: PBS supplemented with 3.7 % formaldehyde or 4 % paraformaldehyde.
3. Solution for cell permeabilization: 0.1 % Triton X-100 in PBS (for 10 min at room temperature).
4. Blocking solution: PBS containing 5 % goat serum, 1 % bovine serum albumin, and 0.1 % gelatin. For experiments with primary antibodies originated from goat, 5 % goat serum must be replaced with 5 % horse serum.
5. Optimal Cutting Temperature (OCT) compound (Miles).

6. Liquid N₂.
7. Pap Pen (Cat. SPR0905, Super PAP Pen, Cancer Diagnostics).
8. Humid chamber: a square container with wet paper towel in the bottom. The container was wrapped entirely in foil for light protection.
9. Mounting medium: DakoCytomation fluorescence mounting medium (Dako).
10. Slide glass, Corning Frosted End, One Side, 75×25 mm (Marienfeld).
11. Cover glass (Marienfeld).

2.4 Materials for Cell-Surface Biotinylation

1. PBS-CM: PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂.
2. DL-dithiothreitol (DTT) (Sigma).
3. Bovine serum albumin (BSA) (Sigma).
4. Sulfo-NHS-LC-biotin (EZ-Link Sulfo-NHS-LC-Biotin, Pierce).
5. Sulfo-NHS-SS-biotin (EZ-Link Sulfo-NHS-SS-Biotin, Pierce).
6. Streptavidin agarose or NeutrAvidin agarose beads (Pierce).
7. Lysis buffer: 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, pH 7.4, and Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics).
8. 2× sample buffer: 20 % glycerol, 4 % sodium dodecyl sulfate, 80 mM DTT, 0.02 % bromophenol blue, and 125 mM Tris-Cl, pH 6.8.
9. Sodium 2-mercaptoethanesulfonate (MESNA) (Sigma).
10. Iodoacetamide (Sigma).
11. TNB buffer: 100 mM NaCl, 0.2 % BSA, and 50 mM Tris-Cl, pH 8.6.
12. MESNA stripping buffer: 100 mM MESNA in TNB buffer.

2.5 Materials for Glycosidase Treatments

1. Peptide-N-Glycosidase F (PNGase F; New England Biolabs).
2. Endoglycosidase H (Endo H; New England Biolabs).
3. 10× glycoprotein denaturing buffer: 5 % SDS, 0.4 M DTT (10 % β-mercaptoethanol can be used as substitute for 0.4 M DTT).
4. 10× PNGase F reaction buffer: 0.5 M sodium phosphate, pH 7.5 at 25 °C.
5. 10× Endo H reaction buffer: 0.5 M sodium citrate, pH 5.5 at 25 °C.
6. 10 % NP-40.

3 Methods

3.1 Introduction to Immunofluorescence-Based Assays

Immunofluorescence-based imaging is one of the most widely used techniques for evaluating the intracellular trafficking of membrane proteins. Although immunofluorescence imaging is a subjective method, the data obtained from immunofluorescence methods can be analyzed and presented in a quantifiable form when combined with various morphometric assays. For example, the surface-to-total ratio of CFTR can be used to determine the proportion of CFTR in the plasma membrane and to evaluate the amount of $\Delta F508$ -CFTR translocated to the plasma membrane under basal and certain rescue conditions [3, 7]. The cell-surface expression of membrane protein can be more directly determined using the extracellular epitope-tagged protein with staining in non-permeabilized membrane conditions (Fig. 1). When epithelial cells are cultured in permeable supports, the membrane-specific localization of cell-surface protein can be evaluated [8]. For example, the amount of

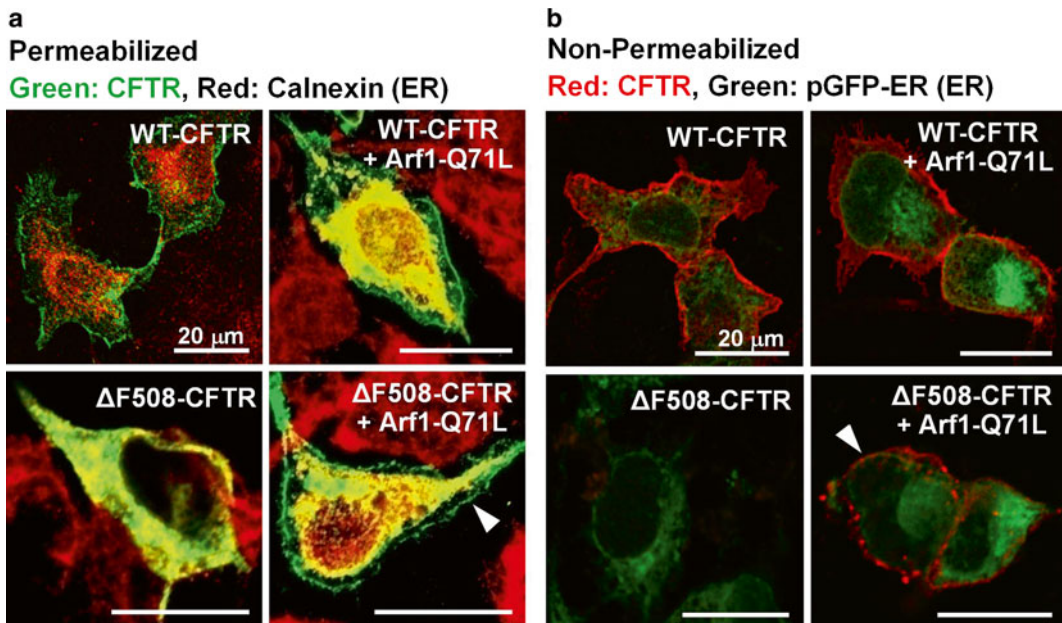


Fig. 1 Examination of CFTR cell-surface expression by immunocytochemistry. CFTR was co-stained with the ER marker protein calnexin or pGFP-ER after blockade of the conventional ER-to-Golgi traffic by co-expression of Arf mutant (Arf1-Q71L). **(a)** In membrane-permeabilized control cells, most wild-type (WT) CFTR is expressed at the cell surface. When conventional ER-to-Golgi trafficking was blocked by Arf1-Q71L, a large amount of WT-CFTR is confined to the ER. However, a significant fraction of WT-CFTR was still found on the plasma membrane. Interestingly, a significant amount of $\Delta F508$ -CFTR that was located only in the ER in control cells reached the cell surface when ER-to-Golgi trafficking was blocked by Arf1-Q71L. **(b)** Surface expression of $\Delta F508$ -CFTR induced by Arf1-Q71L was more evident after immunostaining non-permeabilized cells with extracellular HA-tagged CFTR constructs. Arrows indicate cell-surface expression of $\Delta F508$ -CFTR. Modified from Gee et al. [3] with permission

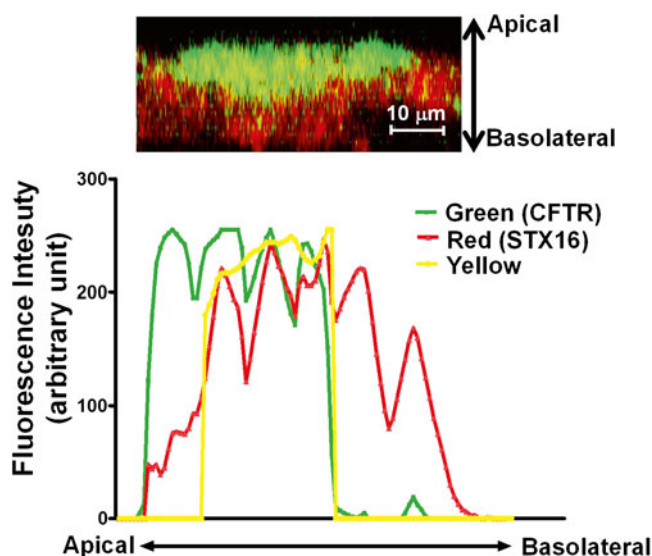


Fig. 2 Localization of CFTR and STX16 in T84 monolayers. Colonic T84 epithelial cells were cultured in permeable supports to form polarized monolayers, and exogenous CFTR was expressed by transient transfection with pCMV-CFTR. Fluorescent images of the vertical z axis section were obtained after staining with anti-STX16 (*red*) and anti-CFTR (*green*) using a Zeiss LSM 510 confocal microscope. Fluorescence intensities were analyzed with the MetaMorph software. Note that *red* and *green* colors are highly co-localized at subapical regions (*yellow*). Reprinted by permission from the American Society for Biochemistry and Molecular Biology, reprinted from Gee et al. [3]

endogenously or exogenously expressed CFTR in the apical plasma membrane of T84 and CFPAC-1 cells can be quantified using a Z-axis scan of a polarized cell sheet after proper membrane culture and immunostaining (Fig. 2). In addition, immunohistochemistry using tissue samples from gene-manipulated animals can provide information related to the *in vivo* effects of a gene of interest on membrane trafficking. As shown in Fig. 3, mice expressing transgenic GRASP55 showed improved expression of $\Delta F508$ -CFTR at the apical membrane in colonic crypt cells [3].

3.2 Immuno-cytochemistry

1. HEK293 cells cultured on coverslips or epithelial cells cultured on permeable supports can be used for immunostaining 36–48 h after transfection with plasmids or recombinant viruses for expressing CFTR or other proteins of interest.
2. Fix cells grown on coverslips (or permeable supports) with 3.7 % formaldehyde for 10 min and permeabilize the cell membrane with 0.1 % Triton X-100 for 10 min. Alternatively, 100 % methanol (–20 °C freezer chilled) can be used for both fixation and permeabilization simultaneously (*see Note 1*).

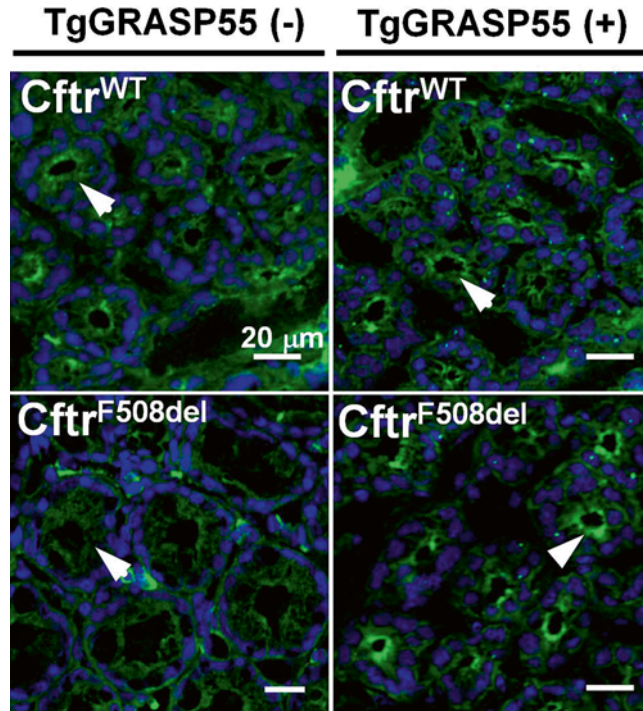


Fig. 3 Apical expression of Δ F508-CFTR when Δ F508-CFTR-bearing mice are crossed with TgGRASP55 mice. Cross-sections of mouse colonic crypts were immunostained with an anti-CFTR R4 rabbit polyclonal antibody. TgGRASP55 expression results in the rescue of CFTR expression at the apical membrane of crypt cells in the Δ F508-CFTR mouse colon. *Arrowheads* indicate the apical region of colonic crypts. Modified from Gee et al. [3] with permission

3. Block nonspecific binding sites by incubation in blocking solution for 1 h at room temperature.
4. After blocking, cells are stained by incubation with appropriately diluted primary antibodies (e.g., anti-CFTR R4, 1:100) in blocking solution for 1 h at room temperature (or overnight at 4 °C). Wash cells three times with 1× PBS for 5 min. For each wash, lift up the one corner of coverslip gently and then suction the fluids at the other corner of coverslip.
5. Treat cells with diluted fluorophore-tagged secondary antibodies (usually 1:100) in blocking solution for 1 h at room temperature. For nucleus staining, DAPI can be co-treated at this step. Wash cells with 1× PBS for 5 min (three times).
6. For double or triple labeling, repeat **steps 4 and 5** with appropriate antibodies being sure that the primary antibody was made from different species of origin and secondary antibody was conjugated with different fluorophore from those used for the first staining step.

7. Remove the residual fluids from the coverslip by standing the edge of the coverslip on the paper towel.
8. Mount the samples by inverting each coverslip into 20 μ L of mounting medium on a microscope slide glass.
9. Dry the slide glass in a flat, dark place for 4–5 h. After they are completely dry, clean the surface area of the cover glass with 70 % ethanol and Kimwipes.
10. Acquire images by confocal microscopy with appropriate excitation and emission settings for each fluorescent dye. For fluorescence quantification, images can be exported as TIFF files and analyzed with image analyzing software (e.g., MetaMorph, Molecular Devices Corp.).

3.3 Immuno-histochemistry

1. Embed tissue samples in the OCT compound and quickly freeze in liquid N₂. Cut frozen tissues into 4 μ m sections using a microtome and transfer the sections into a slide glass. The tissue sections can be stored in a freezer (–20 °C) for later use at this step.
2. Mark the surrounding area of tissue sections that are adherent on the slide glass with a Pap Pen to create a hydrophobic barrier.
3. For staining, dissolve the OCT compound and hydrate the tissues in a jar containing 1 $\times$ PBS for 5 min at room temperature. Next, fix the tissues with 3.7 % formaldehyde for 10 min and permeabilize the cell membrane with 0.1 % Triton X-100 for 10 min. Alternatively, 100 % methanol (–20 °C freezer chilled) can be used for both fixation and permeabilization simultaneously (*see Note 1*). A slide glass rack for staining and a matching staining jar are recommended for **steps 3, 5, and 6**.
4. Place the slide glass in a humid chamber and cover the tissue area with 100–200 μ L of blocking solution for 1 h at room temperature.
5. Remove the blocking buffer and treat the tissue area with the appropriately diluted primary antibodies (e.g., anti-CFTR R4, 1:100) in blocking solution for 1 h at room temperature (or overnight at 4 °C). Wash tissue samples three times in a jar containing 1 $\times$ PBS for 5 min at room temperature.
6. Treat the tissue with 100–200 μ L of diluted fluorescently tagged secondary antibodies (usually 1:100) in blocking solution for 1 h at room temperature. For nucleus staining, DAPI can be co-treated at this step. Wash tissue samples with 1 $\times$ PBS three times.
7. For double or triple labeling, repeat **steps 5 and 6** with appropriate antibodies ensuring that the primary antibody was made from different species of origin and secondary antibody was conjugated with different fluorescence from those used for the first staining step.

8. Remove the residual fluids by standing the edge of the slide glass on a paper towel.
9. Drop 30 μ L of mounting media onto the slide, and put the cover slip over the tissue area without making an air bubble.
10. Dry the slide glass in a flat and dark place for 4–5 h. After completely dry, clean the surface area of cover glass carefully with 70 % ethanol and Kimwipes.
11. Acquire images by confocal microscopy using the appropriate excitation and emission settings for each fluorescent dye. For fluorescence quantification, images can be exported as TIFF files and analyzed using image analyzing software (e.g., MetaMorph, Molecular Devices Corp.).

3.4 Introduction to Cell-Surface Biotinylation-Based Assays

Cell-surface expression of the protein of interest can be assessed by chemical biotin labeling reagents that target specific functional groups or residues, including primary amines, sulfhydryls, carboxyls, and carbohydrates. Biotinylation reagents consist of biotin, a reactive moiety, and a spacer that cross-links the biotinylation reagent to the targets. For primary amines, sulfonated N-hydroxysuccinimide (NHS) ester is used as a reactive moiety. Commonly used spacers include a cleavable disulfide (sulfo-NHS-SS-biotin), aminocaproic acid (sulfo-NHS-LC-biotin), and polymers of 4 or 12 polyethylene glycol (PEG) moieties (sulfo-NHS-PEO4-biotin or sulfo-NHS-PEO12-biotin, respectively; <https://www.piercenet.com/method/biotinylation>). The reactive NHS ester forms an amide bond with exposed lysine side chains (~7 % of protein is lysine) or the unblocked N-terminus of polypeptides on the cell surface (*see Note 2*). The biotinylated proteins are frequently precipitated on avidin beads for electrophoresis and subsequently analyzed by immunoblot analysis (*see Note 3*). The cell-surface biotinylation technique can be also applied to estimate the internalization or recycling of cell-surface proteins by using biotin-stripping agents, such as MESNA [9] (*see Note 4*). In addition, information about carbohydrate groups attached to the surface proteins is helpful for identifying the intracellular trafficking route of the protein. Therefore, the cell-surface biotinylation technique is frequently combined with glycosidase treatments to analyze the conventional and unconventional trafficking of CFTR and other membrane proteins. Finally, various treatments that affect intracellular protein trafficking are used to investigate the basic characteristics of intracellular trafficking pathways in combination with the cell-surface biotinylation technique (*see Note 5*). Some exemplary methods are introduced in Subheading 3.8.

3.5 Cell-Surface Biotinylation

1. Wash cells transfected with plasmids for expressing CFTR (or other proteins of interest) or epithelial cells grown on a permeable support twice with PBS-CM. In the case of some epithelial

monolayers, such as T84 cells, incubate cells with PBS-CM containing 5 mM DTT for 10 min with gentle rocking to remove the accumulated surface mucus [10]. To remove all excess DTT, wash epithelial monolayers three times with PBS-CM for 10 min.

2. Add 0.1 (or up to 0.3) mg/mL sulfo-NHS-LC-biotin (or sulfo-NHS-SS-biotin) in PBS and incubate for 30 min with gentle agitation. Sulfo-NHS-based reagents are inherently prone to hydrolysis. Dissolve sulfo-NHS-SS-biotin in PBS immediately before adding to cells. For CFTR biotinylation in epithelial monolayers, the biotinylation reagent should be treated on the apical side, and the basolateral side should be blocked with PBS-CM containing 1 % BSA.
3. Wash cells twice with PBS-CM and then incubate with PBS-CM containing 1 % BSA (to quench the unbound biotin) for 10 min.
4. Wash cells twice with PBS. Collect cells into a 1.5 mL tube with lysis buffer, and then sonicate cells briefly to break cell membranes and centrifuge for 15 min at $15,000 \times g$. Transfer supernatant to the new 1.5 mL tube, and determine total cellular protein content using the Bradford assay at 595 nm.
5. Add 40 μ L of a 50 % NeutrAvidin slurry or Streptavidin beads to a 1.5 mL tube, microcentrifuge for 30 s at $4,000 \times g$, and remove residual fluid with a gel loading tip.
6. Wash beads once by adding lysis buffer to the bead pellet. Add the supernatant prepared in **step 4** to the washed avidin beads, and incubate them for 4 h—overnight at 4 °C on an end-over-end rotator.
7. Microcentrifuge briefly, remove the supernatant, and wash beads five times with lysis buffer.
8. Elute biotinylated proteins in 2 $\times$ sample buffer. Resolve the elute by SDS-PAGE and carry out immunoblotting.

3.6 Endocytic Assay

1. The internalization of cell-surface protein can be assayed by stripping the biotinylated protein after cell-surface biotinylation (Fig. 4). Cells are allowed the designated time for endocytosis of surface proteins, and then proteins remaining at the cell surface were stripped of biotin using MESNA stripping buffer. The following is a representative protocol for endocytic assay.
2. Wash cells with PBS-CM and add 0.1 mg/mL sulfo-NHS-SS-biotin in PBS for 30 min with gentle agitation.
3. Incubate cells at 30 °C or 37 °C for the indicated times. Typically, endocytosis is tested at 0, 5, 10, 20, and 40 min for CFTR. Endocytosis is slower at 30 °C, which may allow for easier detection of differences between conditions.

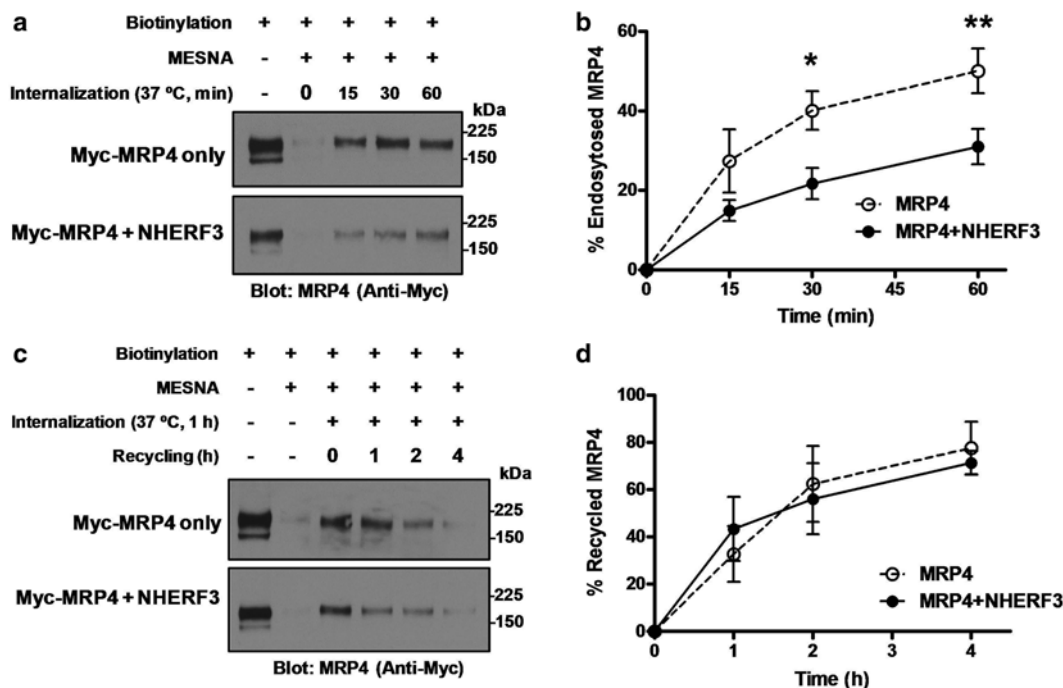


Fig. 4 Surface MRP4 internalization and recycling assays. **(a and b)** The internalization of cell-surface MRP4 protein was assayed in HEK 293 cells with or without NHERF3 co-expression. Cell-surface proteins were biotinylated and then internalized for the indicated times at 37 °C. Proteins remaining at the cell surface were stripped of biotin with the sodium 2-mercaptoethanesulfonate (MESNA) stripping buffer. Internalized, biotinylated proteins were immunoblotted. **(a)** A representative MRP4 immunoblot of the internalization assay is shown. Lane 1, total cell-surface MRP4; lane 2, MESNA stripping control; lanes 3, 4, and 5, internalized MRP4 that remained in the cells after the indicated times. **(b)** Shown are the quantitative analysis of internalized MRP4 in cells with control or NHERF3 co-expression ($n=4$). MRP4 internalization was significantly reduced by NHERF3 co-expression. **(c and d)** The recycling of cell-surface MRP4 protein was assayed in HEK 293 cells with or without NHERF3 co-expression. Cell-surface proteins were biotinylated and then internalized for 1 h at 37 °C. Proteins remaining at the cell surface were stripped of biotin with the MESNA stripping buffer. Non-recycled, biotinylated proteins were collected at the indicated times after additional MESNA treatment. **(c)** A representative MRP4 immunoblot for a recycling assay in HEK 293 cells is shown. Lane 1, total cell-surface MRP4; lane 2, MESNA stripping control; lane 3, internalized MRP4 after 1 h at 37 °C; lanes 4, 5, and 6, internalized MRP4 that remained in the cells after 1, 2, and 4 h. The difference between lane 3 and lanes 4, 5, and 6 represents recycled MRP4. **(d)** The quantitative analysis of recycled MRP4 in control cells or those with NHERF3 co-expression are shown ($n=4$). MRP4 recycling was not significantly altered by NHERF3 co-expression. * $p<0.05$, ** $p<0.01$. Modified from Park et al. [9] with permission

4. Wash cells for 15 min four times with MESNA stripping buffer. MESNA cleaves disulfide bonds between the biotin group of sulfo-NHS-SS-biotin and the target protein. It acts on cell-surface-exposed proteins but not on internalized proteins. At this point in the protocol, biotinylated proteins reside within the endosomal compartment.
5. Follow **steps 4–8** outlined in Subheading 3.5.

3.7 Recycling Assay

1. Cell-surface recycling of the membrane protein can be assayed by a modification of the internalization assay described in Subheading 3.6. Because it is difficult to positively visualize the recycled protein, the amount of recycled surface protein is estimated by subtracting non-recycled protein from the total internalized protein (Fig. 4). The following is a representative protocol for the recycling assay.
2. Wash cells with PBS-CM and add 0.1 mg/mL sulfo-NHS-SS-biotin in PBS for 30 min with gentle agitation.
3. Incubate cells for 30 min at 37 °C to induce the internalization of biotinylated proteins. Keep at least one well or filter without incubating at 37 °C for a cell-surface labeling control.
4. Wash cells four times with MESNA stripping buffer for 15 min to remove biotin from the biotinylated proteins remaining at the cell surface.
5. Wash cells twice with PBS. Inactivate MESNA by adding 300 mM iodoacetamide in the TNB buffer, and incubate cells for 30 min on ice. Keep at least one well or filter for a stripping control.
6. Wash cells with PBS. Incubate cells at 37 °C for the indicated times. The internalized, biotinylated membrane proteins can recycle to the plasma membrane during this time. For the CFTR recycling assay, add 5 μ M forskolin to stimulate recycling of CFTR at this step [11].
7. Quickly cool cells to 4 °C after the indicated times. Wash cells for 15 min four times with MESNA stripping buffer. This step removes biotin from the recycled CFTR.
8. Follow steps 4–8 in Subheading 3.5.
9. The recycling of endocytosed membrane protein is calculated as the difference between the amount of biotinylated protein after the first and second MESNA treatments.

3.8 Methods for Evaluating Unconventional Protein Secretion

1. PNGase F treatment: Glycosidases are used to obtain information about carbohydrate groups attached to glycoproteins and glycopeptides. PNGase F is an amidase that cleaves the innermost GlcNAc and removes all types of N-linked glycosylation. Therefore, PNGase F is used to detect all forms of N-glycosylated proteins that have traveled through the ER and Golgi (Fig. 5).
Protocols: (a) Place 10–20 μ g of the protein samples in 10 μ L of 1 $\times$ glycoprotein denaturing buffer. (b) Incubate the sample at 100 °C for 10 min. Alternatively, for membrane proteins like CFTR, protein samples can be incubated at 37 °C for 10 min to avoid aggregation. (c) Add 2 μ L of 10 % NP-40, 2 μ L of 10 $\times$ PNGase F reaction buffer, 1–2 μ L of PNGase F, and enough distilled water for a total reaction volume of 20 μ L. Incubate the samples at 37 °C for 1–2 h. (d) Perform SDS-PAGE and immunoblotting.

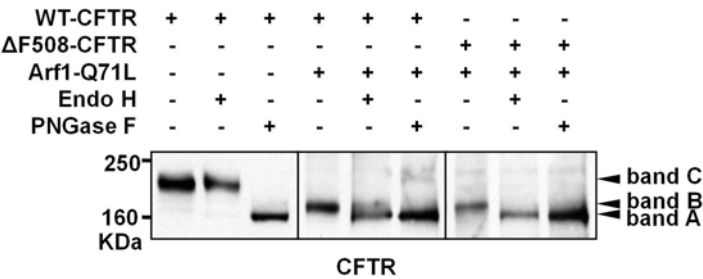


Fig. 5 Glycosidase treatment of surface biotinylated CFTR. Surface biotinylated WT and ΔF508 CFTRs were treated with endoglycosidase H (Endo H), which deglycosylates ER core glycosylation (band B) but not complex glycosylation (band C). CFTRs were also treated with N-Glycosidase F (PNGase F), which deglycosylates all N-glycan chains to remove carbohydrate residues from proteins (band A). Data indicate that surface expressed WT-CFTR in control cells (*left 3 panels*) is resistant to Endo H. However, surface expressed WT (*middle 3 panels*) or ΔF508 (*right 3 panels*) CFTRs in cells treated with Arf1 mutant (Arf1-Q71L) are sensitive to Endo H, indicating that they underwent only ER core glycosylation. Modified from Gee et al. [3] with permission

2. Endo H treatment: Endo H is a recombinant glycosidase that cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. This conversion takes place in the medial Golgi region. When proteins are correctly processed in the ER and travel through the Golgi, they become resistant to Endo H. Therefore, sensitivity to Endo H indicates the presence of proteins that have not been processed beyond the ER.

Protocols: (a) Place 10–20 μg of the protein samples in 10 μL of 1× glycoprotein denaturing buffer. (b) Incubate the samples at 100 °C for 10 min. Alternatively, for membrane proteins like CFTR, protein samples can be incubated at 37 °C for 10 min to avoid aggregation. (c) Add 2 μL of 10 % NP-40, 2 μL of 10× Endo H reaction buffer, 1–5 μL of Endo H, and enough distilled water for a total reaction volume of 20 μL. Next, further incubate the samples at 37 °C for 1–2 h. (d) Perform SDS-PAGE and immunoblotting.

3. N-ethylmaleimide (NEM) treatment: NEM can be used to detect NEM-sensitive factor attachment protein receptor (SNARE)-mediated vesicular trafficking, because NEM inhibits membrane fusion by disrupting SNARE functions [12] (Fig. 6).
Protocols: (a) Incubate cells at 20 °C for 2 h. (b) Treat cells with NEM (1 mM) for 15 min at 20 °C. (c) Incubate cells in serum-free media without NEM for 2 h at 37 °C. This step is required to identify the effects of NEM on intracellular trafficking in comparison with control cells that had not been

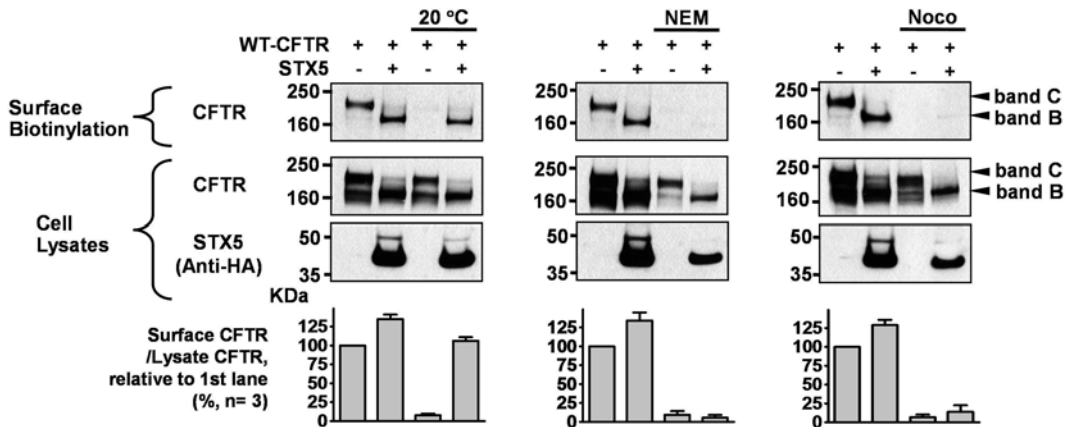


Fig. 6 Surface biotinylation in cells treated with low temperature, NEM, and nocodazole. HEK 293 cells were transfected with WT-CFTR and surface biotinylated. STX5 overexpression was used to block the conventional ER-to-Golgi trafficking. 24 h after transfection, batches of cells were (1) incubated at 20 °C for 24 h, (2) incubated at 20 °C for 2 h then treated with N-ethylmaleimide (NEM; 1 mM) for 15 min followed by serum-free media without NEM for 2 h at 37 °C, or (3) treated with nocodazole (10 μ M, 24 h). Data are shown as mean $\pm$ SEM. Note that conventional surface trafficking of CFTR is blocked by all three treatments (lane 1 and 3 of each panel), but unconventional surface trafficking of CFTR is blocked by only NEM and nocodazole. These results indicate that unconventional surface trafficking of CFTR in STX5 transfected cells is SNARE mediated and microtubule dependent, but does not appear to involve the TGN-to-plasma membrane transport (see **Note 5**). Modified from Gee et al. [3] with permission

treated with NEM in **step (b)**. (d) Lyse cells with lysis buffer and perform SDS-PAGE and immunoblotting.

4. Nocodazole treatment: Nocodazole disrupts microtubules and, thus, can be used to identify the involvement of microtubules in newly identified intracellular trafficking pathways (Fig. 6). Protocol: (a) Treat cells with nocodazole (10 μ M) for 24 h at 37 °C. (b) Lyse cells with lysis buffer and perform SDS-PAGE and immunoblotting.

4 Notes

1. For non-permeabilized staining of extracellular epitope-tagged proteins, treat cells only with 3.7 % formaldehyde for 30 min. For staining with RRX-conjugated actin-phalloidin, treatment with methanol is not recommended.
2. NHS-modified biotin has a low water solubility but is readily soluble in dimethyl sulfoxide (DMSO) or dimethyl fluoride. Sulfo-NHS-biotin is polar because of the negative charge contributed by the sulfonate group and therefore is very soluble even at low temperatures. In addition, the negative charge reduces biotin reagent uptake by cells, which limits the contamination of biotinylation due to intracellular protein labeling [13].

3. Biotinylation of cell-surface proteins varies by cell type and protein. However, cell-surface biotinylation of the same plasma membrane protein in the same cells is a useful technique for comparing treatments that affect cellular trafficking, provided that identical conditions are used to treat the samples.
4. In the endocytic and recycling assays, maintaining cells at 0–4 °C during biotin labeling, antibody incubations, and washing steps is important for minimizing both the endocytosis and exocytosis of cell-surface molecules. It is also essential to keep the cells viable and intact during the different incubation and washing steps. Addition of 0.1 % BSA or 5–10 % FBS to the washing buffer may increase cell viability and recovery.
5. The protocols described here can be combined with plasmids or small molecules available to interfere with intracellular trafficking. This helps to decipher the route of a particular cell-surface protein follows. Some common drugs and plasmids to inhibit membrane trafficking are described below.
 - (a) ER-to-Golgi transport: brefeldin A (BFA) and overexpression of Sar1-H79G, Sar1-T39N, Arf1-Q71L, Rab1-N124I, and Syntaxin5 [14–16]. BFA blocks nucleotide exchange on Arf1 by stabilizing a transient complex between Arf1-GDP and Sec7 domain-containing GEFs. Treating cells with 5 µg/mL BFA causes the Golgi to disassemble and redistribute into the ER.
 - (b) Golgi to plasma membrane transport inhibitor: sulfonamides [17].
 - (c) Microtubule disruptor: nocodazole (5 µg/mL) and colchicine (1 µM) [18].
 - (d) Actin depolymerizing drugs: cytochalasin B (1–30 µM) and latrunculin A (0.2–10 µg/mL) [18].
 - (e) Membrane fusion inhibitor: N-ethylmaleimide (NEM, 0.5 mM). NEM inhibits membrane fusion by disrupting the function of the soluble NEM-sensitive factor attachment protein receptor (SNARE) [12].
 - (f) ATP depletion: Intracellular trafficking is sensitive to ATP depletion. ATP depletion can be achieved by incubating cells in glucose-free media containing the metabolic inhibitors cyanide (5 mM) and 2-deoxy-D-glucose (5 mM) [19].
 - (g) Protein synthesis inhibitor: cycloheximide (10 mg/mL) and puromycin (0.2–1 mM).
 - (h) ER-associated degradation (ERAD) inhibitor: eeyarestatin 1 (10 µM) [20]. Eeyarestatin 1 specifically inhibits Sec61-mediated protein translocation at the ER.

- (i) Proteasome inhibitor: lactacystin (100 μ M), N-carbobenzoxyl-L-leuciny-L-leuciny-L-leucinal (MG132, 50 μ M) and N-acetyl-L-leuciny-L-leuciny-L-norleucinal (ALLN, 50 μ M) [21].
- (j) Lysosomal inhibitor: chloroquine (200 μ M) [22].
- (k) Temperature: Incubation cells at 15 °C causes accumulation of cell-surface proteins to a pre-Golgi compartment, whereas incubation at 20 °C leads to accumulation of these proteins in the trans-Golgi network [23]. Low temperatures (e.g., 28 °C) are known to rescue the trafficking defects associated with Δ F508-CFTR [24].
- (l) Endocytosis inhibitor: overexpression of dominant-negative dynamin (e.g., Dynamin-1 K44A), dynamin inhibitors (e.g., Dynasore) [25], and wortmannin [26].

Acknowledgments

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Chapter 12

Assessing Mammalian Autophagy

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Minoo Razi, and Martina Wirth**

Abstract

Autophagy (self-eating) is a highly conserved, vesicular pathway that cells use to eat pieces of themselves, including damaged organelles, protein aggregates or invading pathogens, for self-preservation and survival (Choi et al., *N Engl J Med* 368:651–662, 2013; Lamb et al., *Nat Rev Mol Cell Biol* 14:759–774, 2013). Autophagy can be delineated into three major vesicular compartments (the phagophore, autophagosome, autolysosome, *see* Fig. 1). The initial stages of the pathway involve the formation of phagophores (also called isolation membranes), which are open, cup-shaped membranes that expand and sequester the cytosolic components, including organelles and aggregated proteins or intracellular pathogens. Closure of the phagophore creates an autophagosome, which is a double-membrane vesicle. Fusion of the autophagosome with the lysosome, to form an autolysosome, delivers the content of the autophagosome into the lysosomal lumen and allows degradation to occur.

Autophagy is a dynamic process that is initiated within 15 min of amino acid starvation in cell culture systems (Köchl et al., *Traffic* 7:129–145, 2006) and is likely to occur as rapidly in vivo (Mizushima et al., *J Cell Biol* 152:657–668, 2001). To initiate studies on the formation of the autophagosomes, and trafficking to and from the autophagic pathway, an ideal starting approach is to do a morphological analysis in fixed cells. Additional validation of the morphological data can be obtained using simple Western blot analysis. Here we describe the most commonly used morphological technique to study autophagy, in particular, using the most reliable marker, microtubule-associated protein 1A/1B-light chain 3 (LC3). In addition, we describe a second immunofluorescence assay to determine if autophagy is being induced, using an antibody to WD repeat domain, phosphoinositide interacting 2 (WIPI2), an effector of the phosphatidylinositol (3)-phosphate (PI3P) produced during autophagosome formation.

Key words Autophagy, Autophagosome, LC3, Atg8, Phagophore, Autolysosome

1 Introduction

When cells are subjected to stress, either from internal and external origin or pathologies that cause stress (e.g., ROS produced by damaged mitochondria), they respond in many ways, including the induction of autophagy [1]. If the reader is considering the assays in this chapter, they have most likely had some indications

that the autophagic pathway is altered by the appearance of membrane-bound lysosome-like vesicles in their cells, a dysregulation of protein turnover or increased cell death not easily explained by apoptosis or necrosis. Here we describe the most widely used assays to assess if autophagy has been altered in the experimental scenario being studied and how the results from these assays will determine the follow-up experiments [3, 4]. The primary questions that will be addressed are as follows: (1) is the response an alteration in the magnitude or extent of the autophagy initiation and autophagosome formation, or (2) is the maturation of the autophagosomes and autophagic flux (degradation of content) altered? The answers to these two questions will determine what the next step in the research should be. Finally, it is likely that the results associated with question 1 can be either an increase in the initiation and formation or an inhibition. However, for question 2 it is most probable that if flux is altered, the results will commonly indicate an inhibition of flux. It would be rather unusual to have observed an acceleration of flux without a corresponding increase in initiation or formation.

If the results are supportive of an increase in autophagy initiation/autophagosome formation, both LC3 and WIPI2 spot (or puncta) number would be elevated compared to controls (*see* Fig. 2). In addition, LC3-II levels should be increased compared to controls, and this increase can be further augmented by lysosomal inhibitors (*see* Fig. 3). The key point of including WIPI2 in the immunofluorescence assay is that it provides evidence that the autophagy-specific pool of PI3P has been generated by the class III PI3 kinase complex containing Beclin-1 and Atg14 [5]. In addition, by examining if the lysosomal-associated membrane protein 1 (LAMP1) co-localizes with LC3, it is possible to determine if the nascent

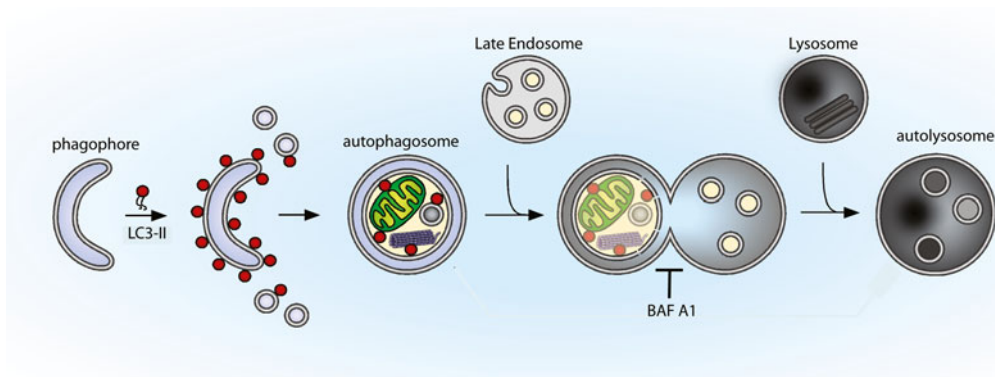


Fig. 1 Autophagic pathway in mammalian cells [2]. Lipidated LC3 is on early autophagosome structures called phagophores. During maturation and closure of the autophagosome, LC3-II is captured and retained inside the autophagosome, but eventually degraded when the autophagosome fuses with late endosome and lysosomes. The step which is inhibited by BafA1 is indicated

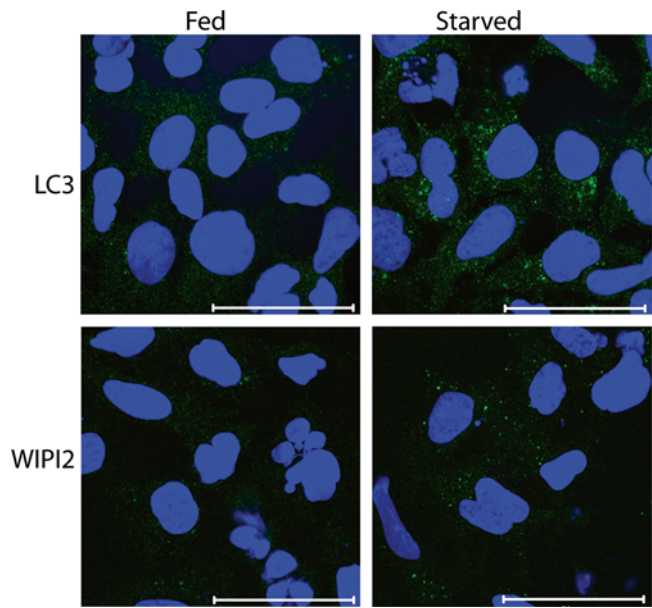


Fig. 2 Autophagy induction using LC3 and WIPI2 spot/puncta formation. HEK293 cells were either incubated in growth medium (fed) or EBSS (starved) for 2 h. After fixation (in this example with PFA followed by methanol), the cells were stained with an anti-LC3 antibody (rabbit) and anti-WIPI2 antibody (mouse). Scale bars equal 5 μ m

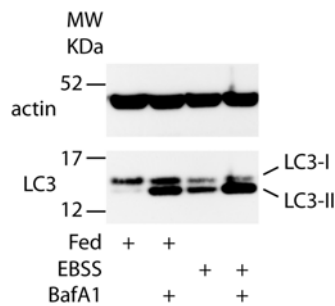


Fig. 3 Autophagy flux revealed by Western blot analyses using antibodies LC3 and actin. HEK293 cells were either incubated in complete cell culture medium (fed) or in EBSS (starved) either with or without BafA1

autophagosome has undergone fusion with the late endosome or lysosome, in confirmation of the lysosomal inhibitor data. Subsequent analysis would be to determine if the observed processes occur through the canonical autophagy pathway, which requires all of the core Atg proteins. For further information, *see* Codogno et al. [6].

Alternatively, if maturation and/or flux is altered, then the next step should be to investigate lysosome function, in particular the degradative capacity of the lysosome, including the biogenesis of the

lysosome, and the upstream endocytic pathway (late endosomes, early endosomes). Other areas of investigation would be to monitor the efficiency of fusion of autophagosomes with the lysosome as a possible source for the inhibition of flux. Additional assays can also be employed to validate the conclusions reached from these preliminary assays. Interested readers are directed to the recommended “Guidelines” for such investigations [7].

2 Materials

2.1 For Immuno-fluorescence Microscopy

Glass coverslips (pre-cleaned) 13 mm round.
 Frosted-edge microscope slides (pre-cleaned).
 Poly-D-lysine: 1 mg/ml in dH₂O.
 PFA: 3 % paraformaldehyde (diluted from 16 % liquid stock from Agar Scientific) in phosphate buffered saline (PBS), 0.9 mM CaCl₂, 0.5 mM MgCl₂.
 Methanol.
 CMF-PBS: calcium- and magnesium-free PBS.
 PBS-gelatin blocking buffer: 2 % fish skin gelatin in CMF-PBS.
 50 mM NH₄Cl in CMF-PBS.
 0.2 % Triton X-100 (w/v) in CMF-PBS.
 Mowiol 4-88 (Calbiochem) in CMF-PBS (*see Note 1*).
 LC3 antibody (there are numerous suppliers, but we recommend mouse monoclonal 5F10 from Novus and rabbit polyclonal cat. No. ab48394 from Abcam).
 WIPI2 antibody mouse 2A2 cat. No. ab105459 from Abcam.
 LAMP1 antibody mouse cat. No. CD107A from BD.

2.2 For Western Blot Analysis of LC3-II

SDS-PAGE gels, 12 % or ideally Bis-Tris 4–12 % gradient gels (from Invitrogen) run in MES buffer (Invitrogen).
 Lysis buffer: 20 mM Tris-HCl pH 7.2–7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100. Complete protease inhibitor (Roche).
 SDS-PAGE reagents and equipment.
 5× Laemmli sample buffer: 15 % SDS (w/v), 312.5 mM Tris-HCl, pH6.8, 50 % glycerol (w/v), 16 % β-mercaptoethanol, a sprinkle of bromophenol blue.
 Western blot reagents and transfer equipment.
 PVDF or nitrocellulose membrane.
 Methanol.
 CMF-PBS.

LC3-lipidation blocking buffer for LI-COR quantitative infrared fluorescence Western blots: 5 % BSA in PBS.

LC3-lipidation blocking buffer for ECL: 5 % skimmed milk, 0.1 % Tween-20, PBS.

Washing buffer: PBST (PBS, 0.1 % Tween 20).

2.3 Autophagy Inducers and Inhibitors

Earle's balanced salt solution (EBSS): 5.56 mM D-glucose, 123.08 mM NaCl, 5.37 mM KCl, 1.82 mM CaCl₂, 0.81 mM MgSO₄, 0.99 mM Na₂HPO₄, 13.10 mM NaHCO₃.

Rapamycin: 11 μM in complete cell culture medium.

Torin 1: 100 nM final in complete cell culture medium.

Bafilomycin A1: 100 nM final in complete cell culture medium or EBSS (Calbiochem).

Wortmannin: 100 nM final in complete cell culture medium or EBSS (Calbiochem).

3 Methods

3.1 Immuno-fluorescence Microscopy

All steps were performed at room temperature, and solutions are kept at room temperature unless otherwise indicated. We routinely use HEK293A cells as they respond robustly to amino acid starvation. Note: all PBS is CMF-PBS, i.e., calcium- and magnesium-free:

1. Prepare a 12-well plate by adding cleaned, microscopy quality 13 mm round glass coverslips. Coat with poly-D-lysine if cells are not very adherent (*see Note 2*). If rabbit anti-LC3 is being used with the mouse anti-WIP12 antibody, then only one set of coverslips is needed. If both mouse anti-LC3 and mouse anti-WIP12 are used, then set up two separate sets of coverslips. For additional information about autophagosome maturation, use LAMP1 antibodies in combination with LC3 (*see Note 3*).
2. Plate cells in a 12-well dish at a density which will yield a 60–70 % confluency at the point of fixation, e.g., plate $5-7 \times 10^4$ HEK293A cells per well for fixation the following day.
Return to incubator and incubate overnight.
3. Perform experimental manipulations or starvation experiment as appropriate. All medium should be pre-warmed to 37 °C. To prepare samples for LC3 staining under amino acid starvation, wash ½ of the coverslips twice with 1 ml complete cell culture medium (fed), and wash the other ½ twice with 1 ml EBSS.
4. To dishes washed with growth medium, add:
Fed: add 1 ml growth medium.
Fed plus BafA: add 1 ml growth medium plus 100 nM bafilomycin A1.

To dishes washed with EBSS, add:

Starved: add 1 ml EBSS.

Starved plus BafA: EBSS plus 100 nM bafilomycin A1.

Return to incubator and incubate for 90–120 min.

See Note 4 for alternative treatments to induce or inhibit autophagy.

5. Wash cells on glass coverslips once with 2 ml PBS.
Fix in 3 % PFA in CMF-PBS for 20 min (*see Note 5*).
6. Wash twice with PBS, and quench aldehydes with 50 mM NH_4Cl in PBS for 10 min.
7. Wash twice with PBS, and permeabilize with 0.2 % Triton X-100 in PBS for 3 min maximum (*see Note 5* for alternative permeabilization protocols).
8. Wash 3 times with PBS, replace final wash with PBS, 0.2 % gelatin, and incubate for 20 min.
9. Set up a moist chamber for the antibody incubation. We suggest lining the lid of a plastic box with wet filter paper and covering it with parafilm. The bottom of the box would be placed down on the lid to create the chamber (resulting in the plastic box sitting upside down on its lid). Number or divide the area of the parafilm to correspond to the number of coverslips using a fine-tipped marker.
10. Prepare the primary antibodies. Make a cocktail of all 3 primary antibodies in PBS-gelatin (*see Note 6*) if primary antibodies are compatible. Prepare 80 μL per coverslip.
11. Drop 80 μL of primary antibody on parafilm. Remove the coverslip from well with fine-point forceps. Drain excess liquid from coverslip by touching on a piece of absorbent tissue. Place coverslip cell-side down on drop of antibody. Incubate 1 h at room temperature or 4 °C overnight. Ensure coverslips do not dry out while floating on the drop of antibody.
12. Remove the coverslip from drop using fine-tipped tweezers, and place back into 12-well plate with cell-side facing up.
13. Wash 3 times with PBS-gelatin.
14. Dilute fluorescent secondary antibodies in PBS-gelatin. *See Note 6*. Use fluorescent antibodies validated in laboratory. Use 80 μL per coverslip.
15. Replace parafilm in chamber and remark. Repeat **step 11**.
16. Repeat **step 12**. Wash twice with PBS-gelatin and then once with PBS.
17. Prepare glass slides. Mark frosted region with pencil. Drop about 20 μL Mowiol mounting medium onto slide. It is

possible to fit 3–4 coverslips on each slide which makes imaging and comparison easier.

18. Remove coverslip, and keep track of which side the cells are on, dip the coverslip into a beaker of dH₂O to remove PBS, then drain carefully on absorbent paper, and invert cell-side down onto a drop of Mowiol. Ensure no air bubbles are trapped under the coverslip. Repeat.
19. Store at room temperature preferably in slide box or in dark area until Mowiol has hardened. After Mowiol has set, store slides at 4 °C.
20. Visualize with fluorescent microscope. We typically acquire confocal images with an optical slice thickness of 0.7 µm using a Zeiss Axioplan 2 LSM510 or LSM 710 laser scanning microscope equipped with a 63×, 1.4 NA, oil lens.
21. Quantify LC3 and WIPI2 spots using blinded samples, and capture about 10 representative or random fields from approximately 100 different cells. Use available software such as ImageJ or Imaris to quantify LC3 or WIPI2 spots. For example, using Imaris image analysis software, distinct spots were identified using the spot-create function and the total divided by the number of cells per field (spot/cell). All images were batch analyzed using the same thresholds [8].

3.2 Western Blot Analysis of LC3-I and LC3-II

All steps were performed at room temperature, and working solutions are kept at room temperature unless otherwise indicated. Note: all PBS is CMF-PBS, i.e., calcium- and magnesium-free:

1. Plate cells in a 12-well dish pretreated with poly-D-lysine if cells are not very adherent (*see Note 2*), e.g., plate 1×10^5 HEK cells per well. Cells should be plated for an analysis in triplicate.
Incubate overnight in incubator at 37 °C.
2. Following day cells should be 80 % confluent for amino acid starvation or at appropriate density for experimental analysis. *See Note 7*.
3. Prepare samples to assess LC3 flux under amino acid starvation (*see Note 8*). All medium should be pre-warmed to 37 °C. Wash $\frac{1}{2}$ of the wells twice with 1 ml complete cell culture medium (fed), and wash the other $\frac{1}{2}$ twice with 1 ml EBSS.
4. Perform experimental manipulations or starvation experiment as appropriate.
To dishes washed with complete cell culture medium, add:
Fed: add 1 ml growth medium.
Fed plus BafA: add 1 ml growth medium plus 100 nM bafilomycin A1.

To dishes washed with EBSS, add:

Starved: add 1 ml EBSS.

Starved plus BafA: EBSS plus 100 nM bafilomycin A1.

Return to incubator and incubate for 90–120 min.

See Note 4 for alternative treatments to induce or inhibit autophagy.

4. Wash cells 3 times with PBS at 4°C. Remove all of the final PBS wash, and lyse each well in 100 µL ice-cold lysis buffer with dishes containing cells placed on ice.
5. Clear lysate by spinning for 5 min at 5,000×*g*. Remove and keep 80 µL of the supernatant, toss pellet.
6. Add 5× sample buffer and incubate at 65 °C for 10 min (*see Note 9*).
7. Load the maximum sample volume (*see Note 10*) (Invitrogen Bis-Tris 4–12 % 20 well gels have a maximum volume of 25 µL, thus the need to keep lysis volume to a minimum). Running buffer for Bis-Tris gels is MES buffer.
8. Semi-dry transfer overnight, especially if you use a 1.5 mm gel (*see Note 11*).
9. Stain membrane with Ponceau S or similar protein stain to check loading.
10. Block membrane with blocking buffer for 1 h at room temperature. The membrane can be cut horizontally at different parts to allow Western blotting of proteins with different molecular weights with different antibodies. If only looking at LC3 and actin/tubulin in the experiment, use the 30 kDa molecular weight marker to separate membrane into two halves. Top half is for actin/tubulin and bottom half for LC3.
11. For the optimum LC3 staining and quantification using LI-COR infrared fluorescence dye, use anti-LC3 5F10 at 0.5 µg/ml in PBS, overnight at 4 °C. For ECL use the 5F10 at 0.5 µg/ml or the polyclonal LC3 at 0.4 µg/ml. Also, probe for actin or tubulin as a loading control.
12. For LI-COR infrared fluorescence dye analyses, wash membrane 3 times, 10 min each with PBST at room temperature. For enhanced chemiluminescence (ECL) analysis, wash 3 times, 10 min each with PBST.
13. To detect LC3, incubate with LI-COR secondary antibodies or HRP-conjugated secondary antibodies previously validated in the laboratory. For actin or tubulin, use an antibody previously validated in the lab. Incubate for 1 h at room temperature or overnight at 4 °C.
14. Wash membrane 3 times, 10 min each with PBST, and then 2 times, 5 min each with PBST.

15. Reveal using LI-COR or with ECL reagent and X-ray film. Ensure that exposures are in the linear range.
16. Quantify your bands using imageJ or MetaMorph, acquire, and subtract background (BG) (*see Note 12*). Measure LC3-I (minus BG) (your top band running at about 16 kDa), actin (minus BG), and LC3-II (minus BG) (running just underneath LC3-I).
Quantify LC3-II over actin (*see Note 13*).

4 Notes

1. Mowiol is prepared from a powder stock. Add 6 g of 100 % glycerol to a 50 ml conical tube. Add 2.4 g Mowiol 4-88 and mix well.
Add 6 ml of dH₂O and mix. Allow to sit for 2 h at RT. Add 12 ml 0.2 M Tris-HCl, pH 8.5. Heat to 53 °C until Mowiol has dissolved mixing occasionally. Aliquot and store at -20 °C. Keep aliquots at 4 °C for immediate use.
2. Poly-D-lysine is essential for cells that are loosely attached (especially during a 2 h amino acid starvation when they tend to detach). Add 0.5 ml per well, covering the bottom of the well or coverslip. Allow to sit for 5 min. Wash 3 times with 1 ml dH₂O and once with 1 ml PBS.
3. Additional information about the maturation of the autophagosomes can be obtained by using antibodies specific for LAMP1, which is a lysosomal membrane protein found in lysosomes and late endosomes. If the LC3-positive spots colocalize with LAMP1, this indicates they have fused with late endosomes and lysosomes and have become mature autophagosomes or autolysosomes. We use a mouse anti-LAMP1 at 0.3 µg/ml that can be combined with rabbit anti-LC3 at 0.4 µg/ml. The WIPI2 antibody should be used at 0.1 µg/ml. Note: optimization of concentration of primary antibody may be required due to efficiency of secondary antibodies.
4. Both rapamycin and Torin 1 can be added to normal growth medium to induce autophagy, as they inhibit mTOR activity. They can be combined with BafA to assess autophagy flux. Wortmannin will inhibit production of PI3P and so also inhibit formation of autophagosomes. All treatments should have a similar effect of LC3 and WIPI2 spot formation. However, LC3 remains associated with the autophagosomes until they are consumed by lysosomes and thus marks all stages. WIPI2 dissociates from the autophagosomes before they become LAMP1 positive.

5. Fixation with PFA is the most widely used approach. However, there are some antibodies which epitopes are destroyed by PFA. Alternatively, 100 % methanol fixation (either -20°C or RT) for 5 min can be used after PFA fixation or as a fixative alone. In this case, use BSA blocking buffer (5 % BSA fraction V in CMF-PBS) and BSA dilution buffer (1 % BSA fraction V in CMF-PBS). Digitonin (50 $\mu\text{g}/\text{ml}$ digitonin in PBS) or saponin (0.2 % in PBS) can be used instead of 0.2 % Tx-100. 100 % methanol at room temperature for 5 min also works well to permeabilize after PFA fixation for LC3 antibodies.

For antigens which have a cytosolic and membrane-associated pool, it is worth considering a pre-permeabilization step using very low concentrations of saponin (between 0.01 % and 0.001 % in 80 mM pipes, 1 mM MgCl_2 , 5 mM EGTA, pH 7.4) before PFA fixation.

6. Primary antibodies from different species can be mixed together in PBS-gelatin to save time. For instance, a common mixture might be a rabbit polyclonal, a mouse monoclonal, and a sheep polyclonal. The only caveat is the use of secondary antibodies made in goat (e.g., goat anti-mouse) that will cross-react with sheep primary antibodies. To avoid this, we use secondary antibodies raised in donkey. Both primary and secondary antibodies should be used at the lowest possible dilution. There should always be a control of secondary antibody/antibodies alone.
7. Quantification is more robust if equal numbers of cells are plated in each well as the samples are not normalized to protein content.
8. The investigators own treatment can be used here either in parallel to amino acid starvation or as a stand-alone treatment. Caution should be used when treating cells for prolonged times (greater than 8 h) with BafA because of cytotoxicity.
9. It is best to process and run the samples ASAP; however, samples can be left in lysis buffer in the dish at -80°C for a week (put dish directly onto metal shelf in freezer).
10. Decrease 5 $\times$ sample buffer if using gels with small well volumes. It's important to load as much as possible (without compromising sample solubilization) to maintain a good signal to noise ratio.
11. Must use PVDF or PVDF-FL membranes with LI-COR. Nitrocellulose membranes give a high background with LI-COR but can be used for ECL.
12. To obtain good data with MetaMorph, keep the boxes as small as possible.

13. The difference between fed and fed + BafA is your basal rate of autophagy (basal flux).

The difference between fed + BafA and starved + BafA is how much you have induced autophagy.

The difference between Starved and Starved + BafA is induced flux.

If there is no difference between plus or minus BafA, then you have a maturation phenotype (i.e., no induction but inhibition of turnover).

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Chapter 13

Expression of Functional Myc-Tagged Conserved Oligomeric Golgi (COG) Subcomplexes in Mammalian Cells

Rose A. Willett, Tetyana A. Kudlyk, and Vladimir V. Lupashin

Abstract

Docking and fusion of transport carriers in eukaryotic cells are regulated by a family of multi-subunit tethering complexes (MTC) that sequentially and/or simultaneously interact with other components of vesicle fusion machinery, such as SNAREs, Rabs, coiled-coil tethers, and vesicle coat components. Probing for interactions of multi-protein complexes has relied heavily on the method of exogenously expressing individual proteins and then determining their interaction stringency. An obvious pitfall of this method is that the protein interactions are not occurring in their native multi-subunit state. Here, we describe an assay where we express all eight subunits of the conserved oligomeric Golgi (COG) complex that contain the same triple-Myc epitope tag and then an assay for the (sub) complex's interaction with known protein partners. The expression of all eight proteins allows for the assembled complex to interact with partner proteins, and by having the same tag on all eight COG subunits, we are able to very accurately quantify the interaction with each subunit. The use of this assay has highlighted a very important level of specificity of interactions between COG subcomplexes and their intracellular partners.

Key words COG, Conserved oligomeric Golgi complex, Golgi, SNARE, Vesicle tethering, Multi-expression, Co-immunoprecipitation, Subcomplexes, Protein-protein interaction

1 Introduction

The conserved oligomeric Golgi (COG) complex is a peripheral membrane protein complex in the subfamily of multi-subunit tethering complexes (MTC) [1]. The COG complex functions to tether retrograde intra-Golgi vesicles to the Golgi cisternae, a critical step in vesicle docking that occurs prior to SNARE-mediated membrane fusion [2]. The COG complex is required for the proper recycling of Golgi-localized glycosylation enzymes [3–5], with defects in COG subunits resulting in a class of disorders known as congenital disorders of glycosylation [6]. According to the current “maturation” model of the Golgi [7], vesicle-mediated recycling of Golgi enzymes is essential for proper glycosylation of glycoconjugates that traffic through the Golgi apparatus. Vesicle tethering is

hypothesized to occur in two steps, the initial capturing of a vesicle, achieved by the subfamily of long coiled-coil tethers, and then the organization of the vesicle on the acceptor membrane, achieved by the MTCs [8]. Currently, there is little understanding of how the steps of vesicle tethering and fusion are coordinated.

The COG complex is comprised of eight different protein subunits (named COG1–8) [9, 10] that are organized into two functionally distinct lobes: COG1–4 in lobe A and COG5–8 in lobe B [11, 12]. The two lobes, or subcomplexes, are bridged together via an interaction between lobe A subunit COG1 and lobe B subunit COG8 [11, 13]. Previous studies on the interactome of the COG complex have revealed interactions with many different families of trafficking regulatory proteins including SNAREs, SNARE-interacting proteins, Rab GTPases, coiled-coil tethers, and COPI subunits [14–22], and COG membrane attachment relies heavily on these interactions [22]. COG interactions with its partners are likely to be transient and tightly regulated and as a result are difficult to detect and measure in biochemical assays with the endogenous proteins. Therefore, insight into the COG complex function/mechanism has relied heavily on the use of exogenous overexpression of individual subunits [19, 23–25]. Furthermore, the COG subunits are not thought to exist in the cell in a monomeric state [11]; thus, interactions with COG subunits are believed to be occurring with the assembled complex/subcomplexes.

In this chapter, we describe an assay based on simultaneous expression of all eight COG subunits in mammalian cells. Importantly, we express all eight proteins with the same C-terminus triple-Myc epitope tag (Fig. 1a), which allows for the accurate quantification of each COG subunit in relation to the entire COG complex (a task that is not possible with detection by individual antibodies). Using this multi-expression assay, we can probe for COG interactions in the COG's native state. Indeed, when we express all eight COG subunits in HEK293T cells, we see that the complex is both soluble and bound to the Golgi (P15) and vesicle (P100) membranes, indicating that our expressed proteins are physiologically similar to the endogenous complex [26, 27]. In a previous study, we and others [23, 24] have demonstrated that the *trans*-Golgi SNARE Syntaxin 6 (STX6) very specifically interacted with COG6. Now, using our multi-expression assay, we show that there is a clear preference for GFP-STX6 to bind to lobe B and not to lobe A or the assembled complex (Fig. 2a, b). Similarly, we see that there are certain proteins which prefer to bind to either lobe A or the fully assembled complex [22]. This demonstrates specificity in the subcomplexes' interactions and is further indicative that the subcomplexes have different functional activities in the cell. Finally, we propose that this assay is a more physiologically relevant method to study interactions of the COG complex and perhaps all other multi-subunit protein complexes.

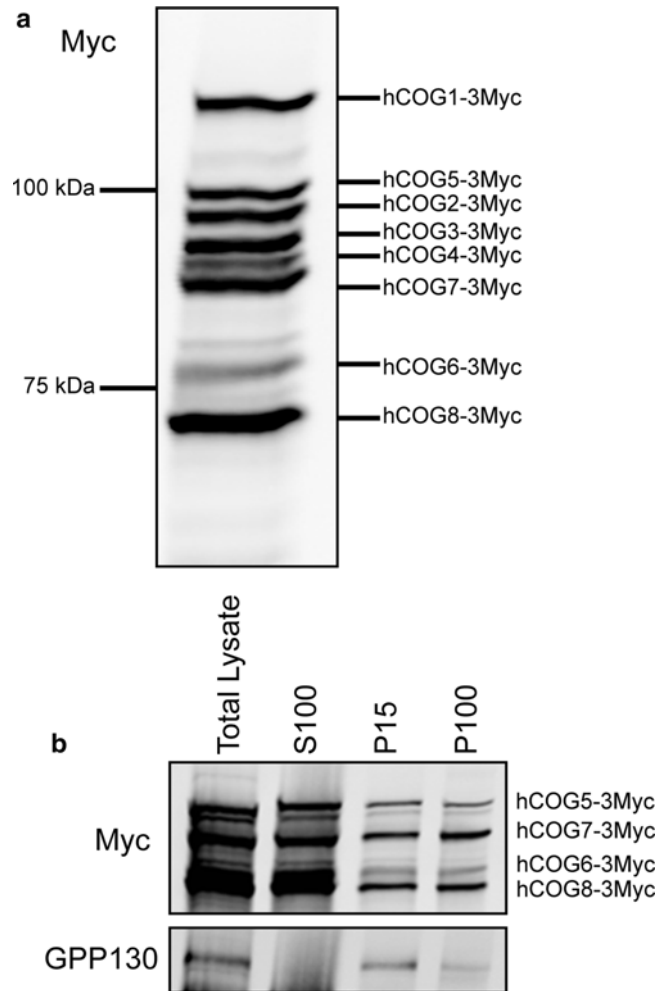


Fig. 1 Expression of all 8 COG subunits in mammalian cells. **(a)** HEK293T cells were transfected with 4 plasmids containing all 8 COG subunits with C-terminal 3 Myc epitope tag. Cells were lysed, loaded on SDS-PAGE gel, and then blotted with anti-Myc antibodies. **(b)** HEK293T cells transfected with lobe B plasmids were homogenized by passage through a needle and then fractionated into cytosol and membrane by differential centrifugation. Lobe B subunits can be detected both on Golgi membranes (P15) and vesicular membranes (P100), indicating that expression of the full subcomplex is physiologically similar to the endogenous subcomplex

2 Materials

2.1 Constructing Multi-expression Plasmid of COG Components

Mammalian expression constructs were generated using standard molecular biology techniques.

1. Individual COG ORFs were cloned into a vector plasmid containing three sequential Myc epitope tags (*see Note 1*).

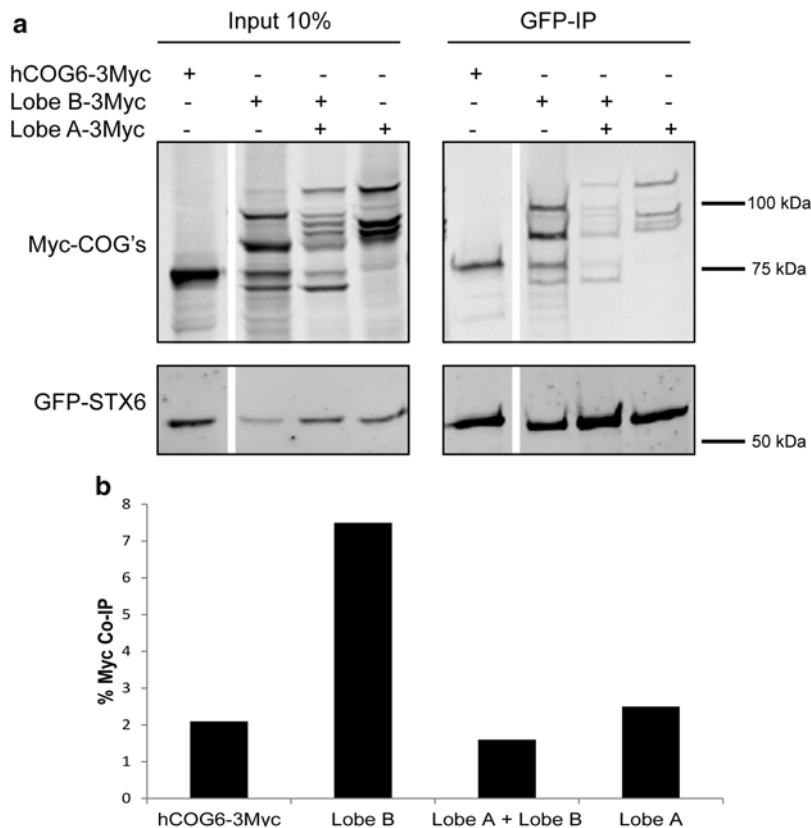


Fig. 2 COG subcomplexes selectively interact with COG partner protein Syntaxin 6. **(a)** Single COG subunit hCOG6-3Myc, 2 plasmids containing 4 Lobe B COG-Myc subunits, 2 plasmids containing 4 Lobe A COG-Myc subunits, or 4 plasmids containing all 8 COG-Myc subunits were transfected in HEK293T cells along with GFP-tagged STX6. Cells were lysed in IP lysis buffer and then incubated with GBP beads. Bead eluates were run on an SDS-PAGE gel and blotted with anti-Myc and anti-GFP antibodies. There are significantly more of the lobe B COG subunits that are co-immunoprecipitated by GFP-STX6 compared to individual COG6, lobe A, or even the fully assembled COG complex **(b)**. These results indicate a preference and level of selectivity of COG interactions with assembled (sub)complexes

2. Primers designed to add AscI and PacI restriction digestion sites on the COG-3Myc genes:
 - (a) Forward primer: GATAGGCGCGCCTGACCGCCCAA CGACCC.
 - (b) Reverse primer: GAGCTTAATTAAGGGACCCCGTCC CTAACCCACGG.
3. Multi-label kit from ATG Biosynthetics (Germany): contains donor and acceptor fusion vectors ML-DGZ2x and ML-DSZ2cx, donors and ML-AAZ6 and ML-AKZ1, acceptors, as well as competent piRHC + bacteria for cloning.
4. Cre recombinase (New England Biolabs, Ipswich, MA).
5. AscI and PacI restriction enzymes (New England Biolabs, Ipswich, MA).

2.2 Lipofectamine 2000 Transfection Components

1. Culture dishes: 12-well tissue culture plates (TPP).
2. HEK293T (ATCC CRL-3216) cells grown on 12-well plate to 90 % confluency.
3. Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA).
4. Plasmids: Rat Syntaxin 6 (rSTX6) cloned into pEGFP-C1. *See* Subheading 3.1 for COGs with Myc tag.
5. Dulbecco's Phosphate Buffered Saline (DPBS 1×) without calcium and magnesium (Thermo Fisher Scientific Inc., Waltham, MA).
6. Transfection media: Opti-MEM® I Reduced Serum Media buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors (Invitrogen, Carlsbad, CA).
7. Growth media: dilute 50 mL of heat-inactivated Fetal Bovine Serum (FBS) (Atlas Biological, Fort Collins, CO) in 450 mL of in DMEM/F-12 50/50 medium supplemented with 15 mM HEPES, 2.5 mM L-glutamine (Invitrogen, Carlsbad, CA). Filter solution in 0.45 µm PES Corning (Lowell, MA) filtration system.
8. Gibco® 0.25 % Trypsin-EDTA (1×) phenol red (Invitrogen, Carlsbad, CA).

2.3 Immuno- precipitation of Components

1. HEK293T cells transfected with COG complex multi-expression plasmids for 24 h (*see* Subheading 3.1).
2. 1.7 mL microcentrifuge tubes.
3. Centrifuge equipped with microcentrifuge rotor.
4. Dulbecco's Phosphate Buffered Saline (DPBS 1×) without calcium and magnesium (Thermo Fisher Scientific Inc., Waltham, MA).
5. Immunoprecipitation (IP) lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 5 µL/mL protease inhibitor cocktail, 2 µL/mL 20 % PMSF in DMSO.
6. GFP-binding protein (GBP)-conjugated glyoxal agarose beads (ABT, Tampa, FL) (*see* **Notes 2 and 3**).
7. Clip bar model microcentrifuge rotator.
8. Washing buffer: 0.05 % Triton X-100 in PBS.
9. 6× Laemmli sample buffer: 30 % glycerol, 12 % SDS, 303 mM Tris-HCl pH 6.8, 0.006 % bromophenol blue, 5 % 2-mercaptoethanol.
10. Vacuum apparatus for collecting waste: Büchner flask, with extended intake tubing, connected to a vacuum source.
11. Elution buffer: 2× Laemmli sample buffer (BioRad) with 10 % 2-mercaptoethanol.

2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Components

1. 7.5 % SDS-PAGE mini gels.
2. Vertical mini gel electrophoresis unit (BioRad).
3. SDS-PAGE running buffer: 25 mM Tris, 0.192 M glycine, 0.1 % SDS.
4. 0.2 μ m Whatman Protran Nitrocellulose Blotting Membranes (GE Life Sciences, Pittsburgh, PA).
5. Blotting paper.
6. Sponges.
7. Transfer chamber: Wet-Tank blotting unit (BioRad).
8. Transfer buffer: 25 mM Tris, 0.192 M glycine, 20 % methanol, 0.02 % SDS.
9. PBS.
10. Flat platform model rotator.
11. Odyssey™ blocking buffer (LI-COR Biosciences, Pittsburgh, NE) containing 0.01 % Tween-20.
12. Secondary antibody incubation solution: PBS containing 5 % milk.
13. Antibodies:
 - (a) Primary antibodies: c-Myc (A190-205A: Bethyl Laboratories, Montgomery, TX), GFP (MMS-118P: Covance, Princeton, NJ).
 - (b) Secondary antibodies: LI-COR Donkey anti-Mouse 680 (926-32222), LI-COR Donkey anti-Rabbit 800 (926-32213) (LI-COR Biosciences, Lincoln, NE).
14. LI-COR Odyssey® imaging system.

3 Methods

3.1 Construction of Multi-COG Expression Plasmid

1. COG ORFs with the triple-Myc epitope tag were amplified by PCR to add AscI and PacI restriction digestion sites at the 5' and 3' ends of the gene, respectively, and then inserted into either donor or acceptor vectors (*see* **Notes 4** and **5**).
2. Using the manufacturer's protocol, donor and acceptor vectors were fused using cre recombinase into combinations of one, two, or three COGs: hCOG5-3Myc/hCOG7-3Myc, hCOG6-3Myc/hCOG8-3Myc, hCOG1-3Myc/hCOG2-Strep-3Myc/-hCOG4-3Myc, and hCOG3-3Myc.
3. Isolate DNA from bacteria using the QIAprep Spin Miniprep Kit (Qiagen).

3.2 Transfection

1. Plate HEK293T cells on 12-well culture dishes 1 day prior to transfection in 10 % FBS DMEM/F-12 media that does not contain any antibiotics so that the cells are 90 % confluent and

evenly spread on the day of the transfection (*see Note 6*). Grow cells at 37 °C and 5 % CO₂ in a 90 % humidified incubator.

2. Prepare transfection solutions with the modified manufacturer's protocol. For a 12-well plate, in a 1.5 mL microcentrifuge tube, dilute 3 µL of Lipofectamine™ 2000 in 50 µL of Opti-MEM®, set aside, and let incubate for 5–10 min (*see Note 7*).
3. In a separate tube, combine 1.6 µg total DNA (multi-expression COG DNA, 0.2 µg of each plasmid, and 0.8 µg of GFP-tagged partner protein) with 50 µL of Opti-MEM® gently mixing the solution (*see Note 8*).
4. After 5–10 min, combine the diluted DNA with the diluted Lipofectamine™ 2000 and incubate for 20 min.
5. After 20 min of incubation, add in dropwise manner the DNA-Lipofectamine™ 2000 complexes to their corresponding wells. Mix gently by rocking the plate back and forth (*see Note 9*).
6. Incubate the cells for 8–12 h at 37 °C, 5 % CO₂, and 90 % humidity, then remove transfection solution and replace with 10 % FBS DMEM/F-12 growth media and allow cells to recover.
7. 24 h after the transfection, proceed to harvesting for co-immunoprecipitation.

3.3 Co-immunoprecipitation

All steps of the immunoprecipitation are done at room temperature, with room temperature reagents.

1. HEK293T cells that have been transfected for 24 h with COG multi-expression plasmids were collected by gentle resuspension in 1 mL of growth media (*see Note 10*) and placed in a 1.7 mL microcentrifuge tube.
2. Centrifuge cells for 2 min at 1,000 × *g*.
3. Remove growth media from tube using a vacuum and resuspend the cell pellet in 1 mL of 1× PBS (*see Note 11*).
4. Centrifuge cells for 2 min at 1,000 × *g*.
5. Remove PBS from the tube and resuspend the cell pellet in 0.5 mL of immunoprecipitation lysis buffer.
6. Incubate the cells in lysis buffer and allow lysis to proceed for 30 min.
7. Centrifuge the cell lysate for 10 min at 20,000 × *g*.
8. Aliquot 50 µL of lysate supernatant (S20) to a new 1.7 mL microcentrifuge tube for analysis. Add 5 µL of 6× sample buffer and boil for 5 min.
9. Transfer the remaining S20 to a new microcentrifuge tube.
10. Add 25 µL of 50 % washed GBP bead suspension (*see Note 12*).
11. Incubate the beads and the lysate sample for 2 h on the rotator.

12. After 2 h, centrifuge the bead and sample mixture for 1 min at $3,000 \times g$ to pellet beads.
13. Remove the sample supernatant with a pipette and set aside.
14. Wash the beads by resuspension in 0.5 mL of washing buffer (*see Note 13*).
15. Centrifuge the beads for 1 min at $3,000 \times g$ to pellet beads.
16. Remove wash buffer by vacuum aspiration (*see Note 11*).
17. Repeat the wash step 2 more times (**steps 12–14**).
18. After the 3rd wash, remove wash buffer using vacuum aspiration. Then, using a fine pipette tip, carefully remove the residual wash buffer from the beads.
19. Resuspend the beads in 45 μ L of elution buffer. Boil the sample for 5 min at 95 °C.

3.4 SDS-PAGE Electrophoresis and Western Blot

1. Load boiled samples onto the SDS-PAGE gel and run at 150 V until the dye front reaches the bottom of the gel.
2. When the gel is almost finished running, prepare the transfer apparatus: soak sponges (2) and filter paper (2) in transfer buffer. Soak the nitrocellulose membrane in 20 % methanol solution.
3. After electrophoresis, pry open the glass plates and gently place the gel onto the filter paper/sponge soaked in transfer buffer.
4. Gently place the nitrocellulose membrane on top of the gel (*see Note 14*).
5. On top of the membrane, add the second layer of the filter paper and sponge that was soaked in transfer buffer.
6. Close the cassette and place into the transfer chamber.
7. Fill the chamber with transfer buffer and insert stir bar and ice block.
8. Run the transfer at 100 V for 1 h on top of a stir plate running at maximum speed.
9. After the transfer, trim the membrane and place inside a clean blotting box with PBS (*see Note 15*).
10. Block the membrane for 30 min using Odyssey blocking buffer containing 0.01 % Tween. Incubate the membrane at room temperature on a table rocker (*see Note 16*).
11. After blocking, add primary antibodies (Myc: 1:10,000, GFP: 1:2,000) and incubate for 1 h while rocking at RT.
12. After the primary antibody incubation, wash the membrane 4 times by adding 5–10 mL of PBS and rocking for 5 min.
13. After washing, add the secondary antibody solution diluted in PBS containing 5 % milk (both secondary antibodies diluted

- 1:40,000). Let incubate for 1 h while rocking at room temperature.
14. Repeat the wash step (**step 12**).
 15. Scan in blots using LI-COR Odyssey® system.

4 Notes

1. COG ORFs were cloned as previously described [19, 22, 24].
2. GFP-binding protein (GBP) [28] with a six-histidine tag (pET24b vector) was transformed into the BL21 strain of competent *E. coli*. The bacteria were induced with 1 mM IPTG for 4 h at OD 0.4. The bacteria were lysed, and the GBP protein was purified on a column containing Talon resin (Clontech, Mountain View, CA) and eluted with 0.1 M imidazole. The purified protein was then dialyzed in a 0.1 M bicarbonate buffer with 10 % glycerol (pH 8.5) overnight. The next day, the purified protein was dialyzed in 0.1 M bicarbonate buffer with 10 % glycerol pH 10.5, and then the protein was conjugated to glyoxal agarose beads (Agarose Bead Technologies, Tampa, FL) using the manufacturer's protocol.
3. GBP beads are not required for this protocol. Additionally, we have used the standard method of GFP antibodies and protein G beads to capture the protein interactions [19, 22, 24].
4. Because we were only successful in combining 2 or 3 COGs into one plasmid, we chose to combine them by their specific interactions with the other COG proteins. Lobe B COG5 and COG7 were combined into one plasmid because they are known to form a stable dimer [11]. COG6 and COG8 were combined because they are strong protein partners [19], and COG1, COG2, and COG4 were combined into one plasmid.
5. Multi-expression plasmids were generated as per the manufacturer's protocol. Positive clones were selected on dual resistance media and were confirmed to contain the COGs both by restriction enzyme digestion and western blot.
6. HEK293T cells are loosely adhered to the plate. It is important not to add media directly to the cells as they will detach.
7. We found that by decreasing the volume of the Opti-MEM used to dilute the transfection reagent and DNA, we achieved higher transfection efficiency.
8. The amount of GFP-rSTX6 was kept even with the total concentration of Myc-tagged COGs (0.8 µg each). For the COGs, the 0.8 µg was divided evenly between the 4 plasmids that contain all 8 COG subunits.
9. Add the Lipofectamine/DNA solution very gently to the cells in a dropwise fashion so as not to disrupt them.

10. Because the HEK293T cells are only loosely adherent, the cells can be collected directly in the growth media by gentle resuspension with a pipette.
11. When using a vacuum, be careful not to get too close to the cell pellet/bead pellet. Leave approximately 25–50 μL of volume to protect the pellet.
12. Before adding the GBP beads to the lysate, it is important to wash the beads 3 times in PBS to remove any unbound GBP from the beads. Unbound GBP protein will decrease the overall efficiency of the IP because it will readily bind the GFP-tagged proteins. To wash the beads, simply add PBS to the beads, spin for 1 min at $3,000\times g$, remove PBS by vacuum aspiration, and repeat 2 more times.
13. When washing the beads, add washing buffer gently to the side of the tube. Gently tap the tube to completely resuspend the bead pellet.
14. Remove all air bubbles between the gel and the membrane by gently swiping your fingers across the top of the membrane or with a plastic roller.
15. Before placing the membrane in the blotting box, you may choose to do a Ponceau S stain to determine the efficiency of the transfer.
16. The amount of buffer to be added is dependent on the size of the container used for blotting. Use enough solution to completely cover the membrane.

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Molecular and Cellular Characterization of GCC185: A Tethering Protein of the *Trans*-Golgi Network

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Abstract

Transport vesicle tethers are proteins that link partner membranes together to permit subsequent SNARE protein pairing and fusion. Despite the identification of a relatively large number of tethering proteins, little is known about the precise mechanisms by which they act. Biochemical isolation of tethers permits direct analysis of their physical characteristics and molecular interactions. Here, we describe the expression and purification of GCC185, a *trans*-Golgi-localized, 190-kDa coiled-coil tethering protein. In addition, we present a gene rescue approach to analyze the function of this tether after its depletion from cells using siRNA.

Key words Golgi complex, Membrane traffic, Tethering factor, Secretion, Protein purification

1 Introduction

Transport vesicle tethering is the process that physically links incoming vesicles to target membranes [1–3]. To date, two classes of tethers have been described: (1) multi-subunit complexes such as the exocyst, COG, Dsl1, and TRAPP complexes [4] and (2) larger, coiled-coil-containing proteins such as golgins and EEA1 [5, 6]. Despite significant progress, how vesicle tethering occurs remains poorly understood. One of the major limitations is the availability of purified tethering proteins. There are expression and purification protocols available for smaller tethering complexes that function at the *cis*-Golgi or for truncated versions of longer proteins [7, 8]. However, the very large size of some tethering proteins (>100 kDa) [9] makes bacterial expression difficult, if not impossible to achieve.

Here we demonstrate the expression and purification of the *trans*-Golgi-localized, coiled-coil tether, GCC185, using a mammalian expression system. The constructs presented here include both the full length and the N-terminal half of the protein; both contain an N-terminal GFP tag, followed by a FLAG-tag, prior to

the start codon. Purification of GCC185 highly relies on affinity chromatography using anti-FLAG resin and a size exclusion step to remove eluting FLAG peptides and residual contaminants. The N-terminal GFP can be used for multiple purposes: (1) as a fluorescent marker to follow protein expression in cells or to detect the protein molecules in fluorescence-based assays, (2) as a globular marker to identify protein's orientation in high-resolution microscopic techniques, and (3) as an affinity tag for immobilization on resins to capture binding partner(s).

Cellular characterization of the tethering function of GCC185 is based on its role in the retrograde transport of the mannose 6-phosphate receptors (MPRs) from late endosomes to the *trans*-Golgi network (TGN) [10, 11]. MPRs bind newly synthesized lysosomal hydrolases in the Golgi complex and deliver them to late endosomes. After releasing these enzymes, MPRs are recycled back to the TGN [12]. The cellular distribution of MPRs can be assessed by immunofluorescence microscopic staining using anti-cation-independent MPR antibody. Normally, at steady state, MPRs are in perinuclear late endosome and to a lesser extent in the TGN [13]. When the tethering is impaired, MPR staining is much more dispersed and distributed in cell periphery. This lab has shown that GCC185 is required for the transport of MPRs from late endosomes to the TGN: in the absence of GCC185, MPRs accumulate in peripheral Rab9⁺ and AP-1⁺ vesicles [14–16]. In this manner, the functionality of a tethering protein and its mutants can be examined by plasmid rescue after siRNA–protein depletion, in this case using immunofluorescence microscopic staining of MPRs to determine their intracellular localization.

2 Materials

2.1 Protein Expression in Mammalian Cells in Suspension

1. Orbital shaker housed in a 37 °C incubator with a humidified atmosphere supplied with 5 % CO₂.
2. Erlenmeyer cell culture flasks or glass flasks (*see Note 1*).
3. FreeStyle™ 293-F cells and FreeStyle™ 293 Expression Medium (GIBCO, Grand Island, NY).
4. Expression constructs: Ligate the cDNA encoding the tethering protein of interest (here we use human GCC185 full length and its N-terminal half (residues 1–889)) in the EGFP-C1 plasmid; insert a FLAG-tag (DYKDDDDK) downstream of the GFP flanked by the GGATCC linker (AS) for purification purposes.
5. Opti-MEM® I Reduced Serum Media (GIBCO) or other medium without serum.
6. Polyethylenimine (PEI) (Polysciences Inc., Warrington, PA): 1 mg/ml PEI pH 7.4. Dissolve 50 mg of PEI in 40 ml sterile water. Mix the solution on a magnetic stirrer at 50–60 °C until

the solution turns colorless. Adjust the pH to 7.4. Add water to a final volume of 50 ml such that the final concentration is 1 mg/ml and sterile-filter the solution through a 0.22- μ m membrane (*see Note 2*). Prepare 0.5 ml aliquots and store them at -20°C (*see Note 3*).

2.2 Protein Purification

Except for the stock solutions, all buffers in this section should be prepared fresh before use and kept on ice (unless otherwise indicated).

1. 10 $\times$ phosphate buffered saline (PBS) stock: 1.35 M NaCl, 17.5 mM KH_2PO_4 , 100 mM Na_2HPO_4 , 27 mM KCl, pH 7.4.
2. 1 $\times$ PBS: Dilute the 10 $\times$ PBS stock with distilled water.
3. Protease inhibitor stocks: Phenylmethanesulfonyl fluoride (PMSF, 100 $\times$ stock solution), 0.1 M in isopropanol; aprotinin (100 $\times$ stock), 0.1 mg/ml in ddH₂O; leupeptin (100 $\times$ stock), 0.1 mg/ml in ddH₂O; pepstatin (1,000 $\times$ stock), 1 mg/ml in methanol. All the protease inhibitors are used together as a 1 $\times$ cocktail in lysis buffer. The stock solutions are stored at -20°C (stable for months).
4. Lysis buffer: 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.5 % Triton X-100, 1 mM EDTA, 10 % glycerol (v/v), and protease inhibitors.
5. Anti-FLAG M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO) (*see Note 4*).
6. High-salt wash buffer: 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.5 % Triton X-100, 10 % glycerol.
7. Storage buffer: 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 10 % glycerol.
8. 3 $\times$ FLAG peptide (Sigma-Aldrich): Stock solution can be prepared by dissolving the peptide in 50 mM Tris-HCl pH 7.4, 150 mM NaCl at a concentration of 5 mg/ml (*see Note 5*).
9. Sepharose CL-4B (Sigma-Aldrich).
10. Basic buffers and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Reagents for Transfections in Functional Rescue Experiment

1. HeLa cells (ATCC) and Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 7.5 % fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.
2. Coverslips (22 $\times$ 22 mm (or smaller) that fit into the wells of a six-well plate).
3. Opti-MEM[®] I Reduced Serum Media (GIBCO) or other medium without serum.
4. Oligofectamine (Invitrogen, Grand Island, NY) or other siRNA transfection reagent.
5. FuGENE6 (Roche, Indianapolis, IN) transfection reagent.

6. siRNA targeting the sequence 5'-GGAGTTGGAACAATCA CAT-3' of GCC185 [14].
7. Constructs for rescue experiments (here, GCC185 wild type): cDNA encoding rescue plasmid ligated into pcDNA3.1(+) modified with an N-terminal Myc tag. It contains eight silent mutations in the siRNA-targeted region to prevent destruction [14, 15].

2.4 Cell Fixation and Immuno-fluorescence Staining

1. 1× PBS (as in Subheading 2.2, item 2).
2. Fixative: 3.7 % paraformaldehyde (PFA) in 200 mM HEPES pH 7.4 (*see* Note 6).
3. Permeabilization buffer: 0.2 % Triton X-100 in PBS (prepared fresh from 20 % stock).
4. Blocking/washing buffer: 1 % bovine serum albumin in PBS. It is better to prepare the blocking buffer fresh every time and filter the buffer before use.
5. Primary antibodies: monoclonal mouse anti-cation-independent MPR (2G11; [15]) and chicken anti-Myc (Bethyl Laboratories, Inc., Montgomery, TX) antibodies.
6. Secondary antibodies: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-chicken (Invitrogen).
7. Mowiol mounting medium (*see* Note 7).

2.5 Imaging Equipment

1. Fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan): fitted with a 60×/NA 1.4 plan apochromat objective lens, a Sedat Quad filter set (Chroma Technology Corp., Bellows Falls, VT), and a charge-coupled device camera (CoolSnapHQ, Photometrics, Tucson, AZ) at room temperature.
2. MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) or other software to control the instrument.
3. softWoRx (v.4.1.0; Applied Precision, Inc., Issaquah, WA) or other image deconvolution software.

3 Methods

3.1 Protein Expression and Purification

Expression scale: 100 ml.

1. FreeStyle™ 293-F cells are maintained in a 37 °C humidified incubator on an orbital shaker rotating at 125 rpm (*see* Note 8). Use a 500-ml Erlenmeyer cell culture flask for 100 ml culture (*see* Note 9). Depending on the type of flask used, loosen the cap of the flask if needed, to allow CO₂ exchange.
2. One day before transfection, determine cell viability and density. Seed cells at a density of 0.6×10^6 cells/ml (target cell density on the day of transfection should be $\sim 1 \times 10^6$ viable cells/ml).

3. On the day of transfection, take a small aliquot of cells to determine the viability and density before transfection.
4. Spin down the rest of the cells at $\sim 200 \times g$ for 5 min and remove the medium. Resuspend the cell pellet and dilute cells to 1×10^6 viable cells/ml in fresh, pre-warmed medium. Return cells to the incubator.
5. To prepare the transfection mixture, dilute 100 μ g of DNA in 9.5 ml of Opti-MEM® I Reduced Serum Medium; mix well and add 500 μ l PEI into the mixture without touching the wall of the tube. Incubate at room temperature for 15 min (*see Note 10*).
6. Add the transfection mixture dropwise to the cells and incubate the cells on a shaker at 37 °C for 22–24 h (*see Note 11*).
7. Before purifying the protein, put a small aliquot (50–100 μ l) of the culture into a well plate and check the transfection efficiency or the expression of the GFP fusion protein using a fluorescence microscope. The green fluorescence signal should be readily apparent.
8. To harvest, spin down the cells at $\sim 200 \times g$ for 5 min at 4 °C and discard the medium. Wash cells gently by resuspending the pellet with 20 ml of PBS and spin down the cells again at $\sim 200 \times g$ for 5 min at 4 °C; discard supernatant.
9. Prepare lysis buffer and keep it cold on ice. Add protease inhibitors to lysis buffer right before use.
10. Lyse cells by resuspending the pellet in 30 ml lysis buffer. Incubate the lysate on ice for 15 min.
11. Spin the lysate at $20,000 \times g$ for 30 min at 4 °C in a Fiberlite F15 (8 $\times$ 50c) (Thermo Scientific, Waltham, MA) or comparable rotor.
12. Equilibrate 100 μ l of anti-FLAG M2 affinity gel with 30 column volumes of lysis buffer (1 ml $\times$ three times).
13. Incubate supernatant from **step 11** with equilibrated anti-FLAG M2 affinity gel on a rocking platform for 4 h at 4 °C.
14. Wash affinity gel three times with ten column volumes of high-salt wash buffer (*see Note 12*).
15. Wash affinity gel two times with ten column volumes of storage buffer.
16. Elute bound FLAG fusion protein by incubating the affinity gel with 300 μ l of 0.2 mg/ml 3 $\times$ FLAG peptide overnight at 4 °C with rotation.
17. Perform a final elution with 200 μ l of 0.1 mg/ml 3 $\times$ FLAG peptide in storage buffer at 4 °C for 30 min (*see Note 13*).

The FLAG peptide eluate of GFP-FLAG-GCC185 full length and N-terminal half has a purity of about 90 % when

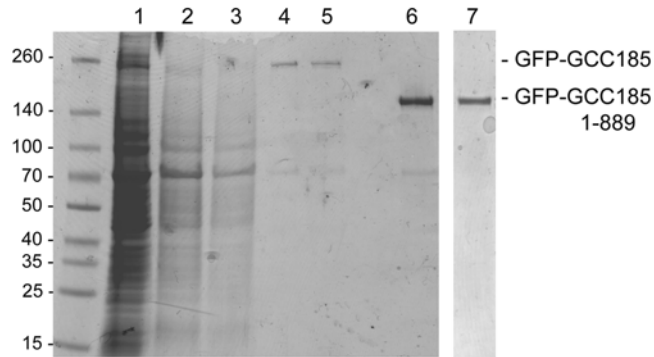


Fig. 1 Purification of the *trans*-Golgi-localized tethering protein GCC185. Coomassie-stained SDS-polyacrylamide gel of lysate input (*lane 1*), high-salt washes (*lanes 2 and 3*), purified GFP-FLAG-GCC185 full length (*lanes 4 and 5*), and N-terminal half (1-889) before (*lane 6*) and after gel filtration on Sepharose CL-4B (*lane 7*)

assessed by Coomassie Blue staining of SDS-PAGE gels. The smaller, N-terminal half of GCC185 has higher yield than the full-length protein (Fig. 1). The next step is designed to remove 3× FLAG peptide and residual contaminants by size exclusion.

18. Prepare a 12.5 ml Sepharose CL-4B column (*see Note 14*) and equilibrate the column with two column volumes (25 ml) of storage buffer (*see Note 15*).
19. Carefully load 300 µl of the FLAG peptide eluent without disturbing the resin and allow the sample to enter the packed bed completely (*see Note 16*). Discard the flow-through.
20. Place tubes for sample collection under the column.
21. Elute with a total of one column volume (12.5 ml) of storage buffer and collect 0.5 ml fractions.
22. Perform SDS-PAGE to detect fractions containing the target protein and to evaluate purity (*see Note 17*). The N-terminal half of GCC185 elutes at 6.0–6.5 ml from a 12.5 ml Sepharose CL-4B column with high purity (Fig. 1).

3.2 Monitoring Tethering Function by Immunofluorescence Microscopic Staining

In this type of functional rescue experiment, cells are depleted of the tether with siRNA for a total of 72 h. Rescue constructs are transfected into cells 24 h after initial siRNA treatment (*see Note 18*) so that the time permitted for rescue is 48 h (*see Note 19*) overall.

1. HeLa cells are cultured in DMEM supplemented with 7.5 % fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37 °C tissue culture incubator supplied with 5 % CO₂.

2. One day before transfection, plate HeLa cells in a 10-cm dish at ~30 % confluency in medium without antibiotics.
3. On the day of transfection, check to make sure the cells are healthy and viable at ~50–60 % confluency.
4. Prepare the transfection mixture by diluting 800 pmol siRNA (20 μ l from 40 μ M stock) in 625 μ l of Opti-MEM® I Reduced Serum Medium. In a separate tube, dilute 16 μ l of Oligofectamine in 44 μ l of Opti-MEM® I Reduced Serum Medium. After incubating the two tubes at room temperature for 5 min, mix the two tubes and incubate the mixture at room temperature for another 20 min to allow formation of siRNA–Oligofectamine complex.
5. Slowly add the siRNA–Oligofectamine complex dropwise to cells using a 200- μ l micropipette tip, and incubate the transfected cells in a 37 °C incubator for 22 h.
6. Place sterile coverslips into each well of a six-well dish. 22 h after siRNA transfection, trypsinize the transfected cells in the 10-cm dish and seed them into each well of the six-well plate containing sterile coverslips, to achieve a confluency of ~40 %.
7. Return the plate to the incubator and allow cells to adhere to the coverslips for at least 3 h before transfection of rescue constructs.
8. Set up transfection reactions in sterile polystyrene tubes:
 - (a) Calculate the volume of DNA to be added in each reaction. The amount of DNA needed for each well of a six-well plate is 1 μ g.
 - (b) To each reaction tube, add pre-warmed Opti-MEM® I Reduced Serum Medium (volume of medium = 100 μ l, volume of DNA to be added).
 - (c) Add 3 μ l of FuGENE®6 transfection reagent to the medium (transfection reagent: DNA = 3:1), mix immediately, and incubate at room temperature for 5 min (*see Note 20*).
 - (d) Add 1 μ g of Myc-tagged siRNA-resistant rescue constructs to the mixture from step (c); mix well and incubate at room temperature for 15 min (*see Note 21*).
9. Slowly add the 100 μ l transfection reactions dropwise to each well of the six-well using a 200- μ l micropipette tip. After 48 h, remove the medium and wash each well three times with 2 ml PBS.

Procedures below are performed at room temperature unless specified.

10. Incubate the cells in 3.7 % formaldehyde in 200 mM HEPES pH 7.4 for 20 min for fixation. Remove the fixative and wash cells twice with PBS.

11. Permeabilize the cells with 0.2 % Triton X-100 in PBS for 5 min. Remove the permeabilization buffer and wash cells twice with PBS.
12. Incubate the cells in blocking buffer for 15 min.
13. Prepare primary antibodies by diluting the chicken anti-Myc antibody at 1:1,000 in monoclonal mouse anti-cation-independent MPR antibody culture supernatant.
14. Add the diluted primary antibodies on top of each coverslip and incubate at room temperature for 1 h (*see Note 22*). Make sure the antibody solution covers the entire surface of the coverslips.
15. Remove primary antibodies and wash coverslips three times for 5 min each with wash buffer on a Belly Dancer or other platform shaker.
16. Prepare secondary antibodies by diluting the Alexa Fluor 488 goat anti-mouse antibody 1:2,000 and the Alexa Fluor 555 goat anti-chicken antibody 1:2,000 in wash buffer.
17. Add secondary antibodies on top of each coverslip and incubate at room temperature for 1 h (*see Note 22*).
18. Remove the secondary antibodies and wash the coverslips two times for 5 min each with wash buffer and once with PBS for 5 min on a Belly Dancer.
19. Clean glass slides and put a drop of Mowiol on top.
20. Transfer 500 ml of distilled water into a beaker. Dip the coverslip into distilled water for 10 s; absorb any residual water droplets by placing the edge of the coverslip onto a piece of Kimwipe or other tissue.
21. Carefully put the coverslip (cells facing down) on top of the Mowiol droplet; avoid bubbles and clean away excessive Mowiol using a Kimwipe. Let the glass slides dry in the dark overnight at room temperature before imaging. Glass slides can be transferred to 4 °C in the dark for long-term storage.
22. Acquire images using a fluorescence microscope at 60× [13]:
 - (a) Acquire 3–5 Z-sections with a Z-axis drive (MFC-2000, Applied Scientific Instrumentation, Eugene, OR) in 0.2-mm steps.
 - (b) Use software to deconvolve images using a theoretical point spread function.
23. Score rescue experiment images. Rescued cells display a more compact perinuclear staining for MPRs, whereas cells depleted of the tethering protein or non-rescued cells show a more dispersed punctate staining that extends to the cell periphery (Fig. 2) (*see Note 23*).

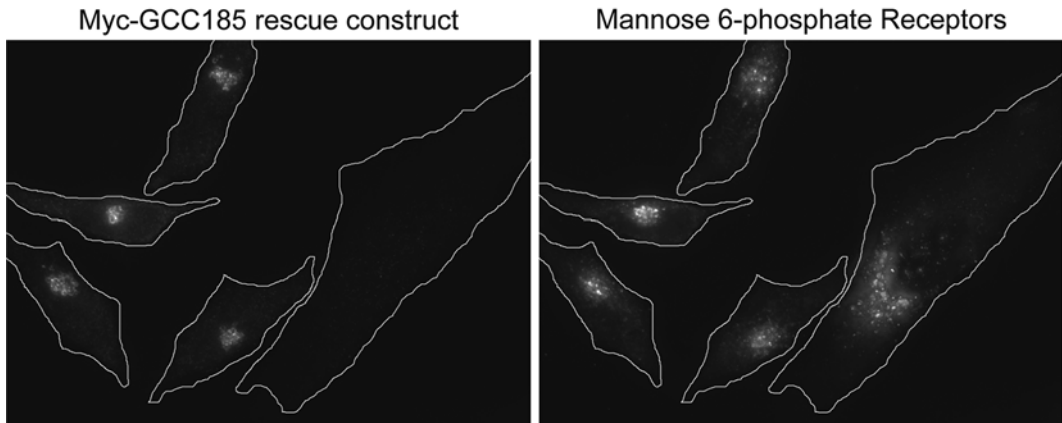


Fig. 2 Immunofluorescence micrographs of HeLa cells depleted of GCC185 by siRNA for 72 h followed by transfection of GCC185 rescue plasmid. *Left column*, expression of the indicated rescue construct detected using chicken anti-Myc and Alexa Fluor 555 goat anti-chicken antibodies. *Right column*, MPR localization detected by 2G11 mouse anti-cation-independent MPR and Alexa Fluor 488 goat anti-mouse antibodies. Approximate cell outlines are indicated

4 Notes

1. Glass flasks may be used after thorough cleaning and autoclaving to avoid potential contamination. Glass flasks can be first soaked in dilute bleach (5–10 %) overnight. Rinse the flasks with water to remove bleach and any residual detergent. Fill each flask with water up to 50 % of the total volume before the first autoclaving. Pour out the water and autoclave the flasks for a second time.
2. Filtering the PEI solution is both for sterility and to enhance the efficiency of transfection, as the presence of undissolved PEI can interfere with transfection by precipitating DNA plasmids.
3. Thawed PEI can be kept at 4 °C and should be used within 2 weeks. Test the transfection efficiency before using thawed PEI that has been kept at 4 °C for more than 2 weeks.
4. Anti-FLAG M2 is a mouse monoclonal antibody that binds FLAG-tag (or tandem FLAG) at the N-terminal, Met-N-terminal, C-terminal, and internal locations of fusion proteins. The calcium-dependent anti-FLAG M1 is an alternative, if working with N-terminal (not Met-N-terminal) FLAG fusion proteins. In addition to the use of FLAG peptide, proteins can also be eluted with a buffer containing EDTA when using the M1 resin.
5. Construct containing 3× FLAG fusion must be eluted with 3× FLAG peptide. If the construct contains only 1× FLAG-tag fusion, FLAG peptide can be used for elution.

6. Caution: PFA is toxic. To prepare 3.7 % PFA, dissolve 1.48 g of PFA in 30 ml sterile water by adding four drops of 10 N KOH and heat the mixture at 50 °C in a hood for 15 min; vortex gently a few times. Add 8 ml 1 M HEPES pH 7.4, mix well, and filter the solution through a 0.22- μ m membrane. 3.7 % PFA can be prepared fresh before immunofluorescence staining experiments; it can also be stored at -20 °C.
7. To prepare Mowiol mounting medium, add 2.4 g Mowiol 4-88 to 6 g of glycerol, and mix by stirring thoroughly, followed by the addition of 6 ml distilled water. Incubate the mixture at room temperature for 2 h. Add 12 ml of 0.2 M Tris pH 8.5 and incubate at ~53 °C until the Mowiol has dissolved. Clarify by centrifugation at 5,000 $\times g$ for 20 min. Aliquot the supernatant into vials and store at -20 °C.
8. The FreeStyle™ 293-F cells are mammalian cells derived from the HEK293 cell line that is adapted to growth in suspension, with a doubling time of about 24 h depending on the age and handling of the cells. Cells should be split to 0.2–0.5 $\times 10^6$ cells/ml when the density reaches 1–3 $\times 10^6$ cells/ml. Cell viability and density can be assessed by trypan blue staining and hemocytometer counting.
9. The culture volume should always be about 20 % of the flask volume. The performance of the cells may be affected if the culture volume is 30 % or more of the flask volume.
10. The amount of DNA required for transfection depends on the volume of culture, the expression construct, and the size of the protein. A strong promoter or a smaller protein usually requires less DNA and shorter transfection time to reach a similar level of protein production. A recommended starting condition is 1 μ g DNA/ml of culture; further optimization may be required. In addition to PEI, 293fectin™ reagent (Invitrogen) can be used for transfecting FreeStyle™ 293-F cells. In our hands, 293fectin™ reagent gives higher protein production for secreted proteins when compared with the use of PEI, but the two reagents appear to perform almost equally well for cytosolic proteins. Please refer to the manufacturer's protocol for details regarding the 293fectin™ reagent.
11. Time required for optimal expression varies between proteins. If the protein is expressed in FreeStyle™ 293-F cells for the first time, harvest cells at multiple time points to determine the shortest time required to get the highest level of protein and whether the protein is intact or degraded.
12. High-salt wash can help reduce nonspecific, ionic binding.
13. The elution conditions vary between proteins. Please refer to **Note 5** for the choices of FLAG peptides. A total of five column

volumes of peptide elution are sufficient to elute most FLAG fusion proteins. Proteins with high thermal stability can be eluted at room temperature for shorter times. Allow longer incubation times when working with larger proteins.

14. Sepharose CL-4B has a fractionation range of 6×10^4 – 2×10^7 for globular proteins.
15. The storage/elution buffer in the size-exclusion step depends on the buffer requirement of the assay(s) that the purified protein will be used for. Check the pI of the protein, since basic proteins at low ionic strengths may adsorb to the resin due to the presence of a small number of ionic sulfate and carboxyl groups on Sepharose; in this case, high ionic strength buffer may be necessary.
16. A sample volume of 1–3 % of the column volume usually gives the maximum resolution in size exclusion chromatography; do not exceed 5 %.
17. The protein is ready to use. Depending on the concentration and the amount of protein needed, concentration of the final product or larger-scale expression may be required.
18. Cells depleted of GCC185 appear more elongated and larger in size; this can be seen 22 h after siRNA transfection.
19. The time required for incubating the cells with rescue constructs depends on the phenotype being assessed, the size of the protein being expressed, and the half-life of the endogenous protein being depleted. MPR retrieval phenotype is usually assessed 48 h post-transfection.
20. The ratio between transfection reagent and DNA should be optimized as it varies with the cell line and construct used. Always add transfection reagents directly into medium without touching the sides of the tube.
21. Long incubation times (e.g., >45 min) may affect the performance of transfection.
22. Fixed cells on coverslips can be incubated in primary and secondary antibodies at 4 °C for longer times (at least 4 h).
23. When scoring for the MPR dispersal phenotype, do not include dividing cells or multinucleated cells.

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Chapter 15

Visualizing Toll-Like Receptor-Dependent Phagosomal Dynamics in Murine Dendritic Cells Using Live Cell Microscopy

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Abstract

Dendritic cells are professional phagocytes that are highly specialized to process and present antigens from internalized particles to prime naïve T cells. To achieve their functions, the phagocytic machinery and membrane dynamics of these cells have been adapted to optimize presentation of antigens from phagocytosed particles that bear ligands of pattern recognition receptors, such as toll-like receptors (TLRs), and that are thus perceived of as “dangerous.” We have recently shown that phagosomes that are engaged in TLR signaling in dendritic cells emit numerous long tubules that facilitate content exchange with other signaling phagosomes and favor presentation of particle-derived antigens. This chapter describes the methods used to study the formation of these tubules, which we refer to as “phagotubules,” by live cell imaging of mouse dendritic cells after the phagocytosis of fluorescent latex beads. We also describe methods to assess the effect of TLR signaling on this process.

Key words Dendritic cells, Phagocytosis, Phagosome, Phagotubules, Toll-like receptors, Live cell imaging, Antigen presentation

1 Introduction

Dendritic cells (DCs) link innate immune signaling to adaptive immunity. Classical DCs (cDCs) are a subset of hematopoietic cells that reside both in lymphoid and non-lymphoid tissues, and are distinguished from plasmacytoid DCs that function largely as sources of type I interferon during infection [1–3]. In peripheral tissues, cDCs continually sample the extracellular milieu for potential pathogens. Pathogens are sensed by a series of pattern recognition receptors (PRRs) that are present on the DC surface and/or within intracellular organelles [4–6]. Triggering of these PRRs stimulates a maturation response that includes the release of proinflammatory and/or antiviral cytokines, migration to lymph nodes, and processing of internalized material for presentation to

naïve T lymphocytes. This process provides the initial stimulus for adaptive immunity and tunes the immune response to the type of encountered pathogen [7, 8].

cDCs internalize pathogens and other extracellular material by different mechanisms. Soluble materials, including viruses and contents released from bacteria or parasites, are internalized by either receptor-mediated endocytosis or micro- or macropinocytosis, with the latter being highly upregulated in immature cDCs [9, 10]. cDCs are also among a number of professional phagocytic cell-types that are specialized to capture large particles such as bacteria by an actin-dependent mechanism known as phagocytosis [11]. Upon completion of phagocytosis, the internalized particle is enclosed within a phagosome, an organelle that undergoes progressive remodeling through a series of interactions with the endosomal system [12]. This remodeling process, referred to as phagosome maturation, provides a mechanism to kill invading particulate organisms and to break them down through hydrolysis. It also provides a means to allow signaling via phagosome-associated PRRs, including Toll-like receptors (TLRs), following recruitment of PRRs and their signaling platforms [8].

Phagosome maturation manifests distinct features in different phagocytic cell types. In cDCs, the maturation of both phagosomes and classical endosomes is skewed toward preservation of antigenic peptides for assembly with major histocompatibility complex (MHC) class I (MHC-I) and class II (MHC-II) molecules for presentation to T cells. For example, maturing phagosomes and endolysosomes in cDCs are less acidic than in other cell types, at least in part due to impaired assembly of the vacuolar ATPase and to the consumption of protons by superoxide generated by a highly active NADPH oxidase [13, 14]. This restrains proteolysis by lysosomal hydrolases and thus prolongs survival of antigenic peptides. In addition, cDCs are specialized to form tubules from both endolysosomes and phagosomes following TLR stimulation. Long tubules that emerge from endolysosomes following stimulation with soluble TLR ligands favor the delivery of newly assembled MHC-II-peptide complexes to the DC plasma membrane and towards the immunological synapse with T cells [15, 16]. Similarly, we have shown that tubules form from maturing phagosomes in cDCs several hours after uptake of bacteria or TLR ligand-coated latex beads [17]. In contrast to the endolysosomal tubules, these “phagotubules” are not essential for the transport of MHC-II-peptide complexes to the cell surface and do not appear to fuse with the plasma membrane. Rather, they favor content exchange among distinct TLR signaling phagosomes within the same DC. Content exchange favors optimal antigen presentation, likely by exposing MHC-II molecules on multiple phagosomes to more antigen sources [17].

TLRs such as TLR2 and TLR4 can signal from phagosomes independently of plasma membrane TLRs [18–20]. They trigger distinct signaling pathways through two sets of adaptors: TIRAP-MyD88 and TRAM-TRIF [21]. Upon TLR stimulation at the plasma membrane or phagosomes, MyD88 recruits IL-1 receptor-associated kinases (IRAK) 1 and 4. Subsequent activation of IRAK4 and IRAK1 initiates a signaling cascade that ultimately activates MAP kinases and the transcription factors AP-1 and NF- κ B. TLR activation of the TRAM-TRIF pathway on endosomes or phagosomes specifically induces the phosphorylation of the transcription factor interferon regulatory factor-3 (IRF-3) by activating the protein kinases TANK-binding kinase-1 (TBK-1) and IKK-I [22–24]. The importance of each of these pathways from phagosomes or other cellular sites for a variety of downstream responses can be dissected by exploiting mice that lack functional genes encoding the adaptors MyD88 [25], TIRAP/MAL [26], TRIF [27], and TRAM [28] or by using pharmacological inhibitors of IRAK1/4 or TBK-1. Because of the inducible formation of phagosomes following particle uptake, addition of pharmacological inhibitors at different times following exposure to TLR ligand-coated particles can be used to distinguish between signaling from the plasma membrane and from the phagosomes themselves.

In the following sections we describe the procedure that we have used to visualize the formation of tubules from phagosomes by live cell imaging of dendritic cells and to define the role of downstream effectors of TLR4 signaling on this process. We focus on bone marrow-derived DCs (BMDCs) as a model system. These cells represent a population of CD11b⁺/CD11c⁺/B220[−] cells that resemble *in vivo* inflammatory or migratory DCs [1, 29]. This procedure can also be applied to resident DCs purified from different tissues such as mouse spleen.

2 Materials

2.1 Bead Coating and Phagocytosis

1. BMDC complete medium: RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, Logan, UT, Defined FBS, cat. #SH30070.03), 2 mM glutamine, 100 U/ml penicillin/100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol. For BMDC culture conditions *see* **Note 1**.
2. BMDC conditioned-complete medium: Same as BMDC complete medium with 30 % granulocyte-macrophage colony stimulating factor (GM-CSF)-transfected NIH-3T3 fibroblast conditioned medium containing 10–20 ng/ml GM-CSF ([31]; *see* **Note 2**). Alternatively, recombinant granulocyte-macrophage colony stimulating factor can be provided directly at 1,000 U/ml (R&D Systems, Minneapolis, MN).

3. Phosphate buffered saline without CaCl_2 or MgCl_2 (PBS).
4. 0.25 % Trypsin-EDTA (Life Technologies, cat # 25200-052).
5. Poly-L-Lys coated glass bottom 35 mm culture dishes (MatTek, Ashland, MA, cat. # P35G-1.5-14-C).
6. Polybead polystyrene 3 μm microspheres (Polysciences Inc., Warrington, PA, cat. # 17134).
7. Polybead amino 3 μm microspheres (Polysciences Inc., Warrington, PA, cat. # 17145).
8. Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 strain (Sigma, St. Louis, MO, cat. # L4391 or InvivoGen, San Diego, CA, cat. # tlr1-3pelps). Prepare as 1 mg/ml stock solution in endotoxin-free phosphate-buffered saline (PBS, Life Technologies, cat. # 14190-136) or water (Life Technologies, cat. # 15230-147). The stock can be stored at 4 °C and diluted to 100 $\mu\text{g}/\text{ml}$ for the working solution.
9. Polyinosine-polycytidylic acid (poly(I:C)) (InvivoGen, cat. # tlr1-pic). Prepare as 1 mg/ml stock solution in endotoxin-free water and dilute to 100 $\mu\text{g}/\text{ml}$ for the working solution. Aliquot and store stock solution at -20 °C.
10. Ovalbumin (OVA) conjugates (Life Technologies-Molecular Probes; Texas Red ovalbumin, cat. # O-23021; Alexa Fluor 488 ovalbumin, cat. # O-34781). Prepare as 5 mg/ml stock solution in endotoxin-free PBS; use as 1 mg/ml working solution. Aliquot and store stock solutions at -20 °C.
11. 8 % Glutaraldehyde (Polysciences).
12. 0.5 M Glycine in PBS.

2.2 Pharmacological Treatments

1. Interleukin-1 Receptor-Associated Kinase-1/4 (IRAK-1/4) inhibitor (EMD Biosciences, Billerica, MA, cat. #407601). Prepare 12.6 mM stock in DMSO; use as 5–50 μM working solution in BMDC complete medium. Aliquot and store stock solution at -20 °C.
2. TBK1/IKK ϵ inhibitor BX795 (InvivoGen, cat. #tlr1-bx7). Prepare 10 mM stock solution in DMSO; use as 0.1 μM working solution in BMDC complete medium. Aliquot and store stock solution at -20 °C.
3. 3-Methyladenine (3-MA) (Sigma, cat. # M9281). Prepare 100 mM stock solution in PBS with heating the day of the experiment; use as 5 mM working solution in BMDC complete medium.
4. Bafilomycin A1 from *Streptomyces griseus* (Sigma, cat. # B1793). Prepare 100 μM stock solution in DMSO; use as 0.5 μM working solution in BMDC complete medium. Aliquot and store stock solution at -20 °C.

5. Methyl-(5-[2-thienylcarbonyl]-1 H-benzimidazol-2-YL) carbamate (nocodazole, Sigma, cat. # M1404). Prepare 16.6 mM stock solution in DMSO; use as 10–20 μ M working solution in BMDC complete medium. Aliquot and store stock solution at -20°C .
6. Latrunculin B from *Latruncula magnifica*. (Sigma, cat. # L5288). Prepare 1 mM stock solution in ethanol; use as 1 μ M working solution in BMDC complete medium. Aliquot and store stock solution at -20°C .
7. Cytochalasin D (Sigma, cat. # C8273). Prepare 10 mM stock solution in DMSO; use as 1 μ M working solution in BMDC complete medium. Aliquot and store stock solution at -20°C .

2.3 Detection of Phosphorylated Proteins

1. Antibody to interferon regulatory factor (IRF)-3 (Cell Signaling, Beverly, MA, cat. # D83B9).
2. Antibody to phospho-interferon regulatory factor (IRF)-3 (Ser396) (Cell Signaling, 4D4G rabbit monoclonal antibody).
3. Antibody to p38 MAP kinase (Cell Signaling, cat. #9212).
4. Antibody to phospho-p38 MAP kinase (Cell Signaling, cat. #9211).
5. Either phosphatase inhibitor cocktail tablets (PhosStop, Roche Diagnostics, Indianapolis, IN) or a solution containing 2 mM Na_3VO_4 , 25 mM NaF, 5 mM β -glycerophosphate.
6. Ice-cold PBS without CaCl_2 or MgCl_2 .
7. 96-well round-bottom plates.

3 Methods

3.1 Coating of OVA Fluorophore-Conjugated to $3\mu\text{m}$ Amino Beads

This procedure has been adapted from ref. [30].

1. Calculate the amount of beads required for the experiment. Plan to use 6 μ l of bead suspension per condition to test, per time point. Consider performing each condition in triplicates or more to allow for imaging at different time points. You may need to perform preliminary experiments to test the amount of beads needed such that the average cell will take up the desired number of beads. Phagotubules are best observed when cells take up 4–6 beads per cell.
2. Resuspend bead suspension in 1 ml PBS per 100 μ l of suspension.
3. Centrifuge for 5 min in a microcentrifuge at 4°C at maximum speed (14,000 rpm/20,000 $\times g$ in an Eppendorf 5417R microcentrifuge). Remove supernatant carefully and discard it.
4. Add 1 ml PBS and centrifuge as in **step 3**.

5. Repeat **step 4** once more.
6. Activate the beads for conjugation by resuspending them in 0.5 ml of 8 % glutaraldehyde.
7. Mix for 4–6 h at room temperature on an end-to-end mixer (preferably a rotating wheel, but a shaker will suffice).
8. Centrifuge for 5 min at $20,000\times g$ and discard supernatant.
9. Repeat **step 4** once.
10. Add 1 mg/ml of fluorescent OVA conjugate and 100 $\mu\text{g}/\text{ml}$ LPS in 0.5 ml PBS final volume.
11. Mix overnight at 4 °C with end-to-end mixing (a rotating wheel is preferred).
12. Centrifuge for 5 min at $20,000\times g$ and discard supernatant.
13. Resuspend pellet in 1 ml of 0.5 M glycine and mix for 30 min at room temperature. This step is performed to block unreacted sites on the beads.
14. Centrifuge for 5 min at $20,000\times g$ and discard supernatant.
15. Repeat **step 4** twice. Fluorescent OVA is now covalently conjugated to the beads.
16. Resuspend the pellet in the original volume of beads. Fluorescent OVA-coated beads can be stored for 1 week at 4 °C.

3.2 Phagocytosis of OVA Fluorophore Conjugated–Coated Latex Beads

1. Differentiate and culture BMDCs in BMDC conditioned-complete medium for 7–10 days on non-tissue-culture-treated plastic dishes, as previously described (*see Note 1*). For isolation of mouse tissue-resident DCs *see Note 3*.
2. On day 6 of BMDC culture, harvest BMDCs by washing the dishes once with PBS and then treating them with trypsin-EDTA for no longer than 5 min (*see Note 4*). Collect detached cells and recover by centrifugation for 5 min at $150\times g$ (1,000 rpm in a Sorvall ST40R tabletop centrifuge with a TX-750 rotor). Resuspend the cells at 200,000 BMDCs/2 ml of BMDC conditioned-complete medium for each sample to be analyzed. Seed 2 ml/dish in poly-L-Lys-coated 35 mm culture dishes. Seed one dish per condition per time point, to allow for imaging at different time points.
3. On day 7, remove medium and add 1.5 ml of BMDC complete medium without or with inhibitors. Add inhibitors either 2.5 h before the pulse, at the time of the pulse or at the start of the chase at the concentrations indicated above (*see Note 5* regarding testing the efficacy and specificity of the inhibitors). Use DMSO at a comparable dilution as vehicle control.
4. Pulse the cells with beads. Carefully remove media from the dishes and add 1.5 ml of BMDC complete medium (plus/minus inhibitors) plus OVA-Texas Red and/or OVA-Alexa Fluor 488-coated beads at a 1:200 dilution.

5. Leave dishes on ice for 15–30 min to allow the beads to adhere to the cells.
6. Incubate at 37 °C for 30 min to allow for phagocytosis.
7. Carefully remove media and wash dishes three times with RPMI 1640 medium (without FBS) to remove non-phagocytosed beads. Add BMDC complete medium for the 2 h chase.
8. Analyze by live cell imaging using an inverted fluorescence microscope (optimally a spinning disk confocal microscope) equipped with an environmental chamber kept at 37 °C and 5 % CO₂. If addition of CO₂ is not possible, add 20 mM HEPES buffer (pH 7.4) to the cells immediately before imaging. *See Note 6* regarding imaging requirements. Figure 1 shows examples of single frames from movies taken at 1 frame/s using an Olympus IX71 inverted spinning disk confocal microscope equipped with a Hamamatsu ImageM EMCCD camera and LCI Chambridge stage-top incubation system for live cell imaging. Movies were acquired over a 5-min time period using MetaMorph software (Molecular Devices, LLC, Sunnyvale, CA, USA). The image sequences were saved as TIF files, and then further analyzed using Image J (NIH) as described below.

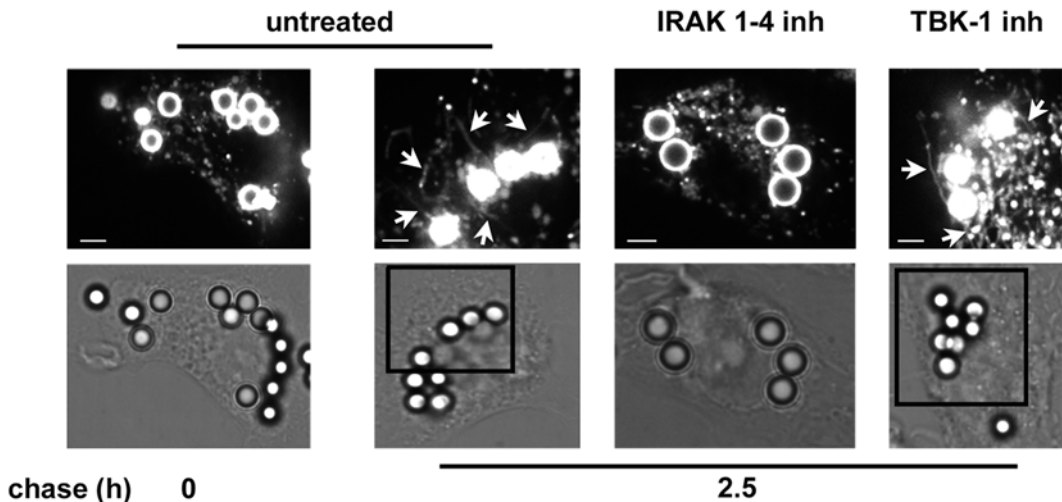


Fig. 1 Visualizing BMDC phagotubules and the effects of inhibitors of TLR signaling on their formation. BMDCs from C57BL/6 mice were left untreated or were pretreated for 2 h with 5 μ M IRAK 1-4 inhibitor (inh) or 0.1 μ M BX795 (TBK-1 inh). Cells were then pulsed for 15 min with OVA-Texas Red coated beads and chased for 2.5 h, all in the absence or presence of inhibitor as indicated. Cells were then imaged by spinning disk confocal microscopy. Shown are still images of Texas Red fluorescence (*top*) and corresponding DIC images (*bottom*). Bars, 2.6 μ m

3.3 Quantification of Tubule Number and Length Using Image J Software

See **Note 7** for alternative analysis options.

1. Open the image sequence file in .tiff extension. Quick time movie format can be opened in the 32-bit version of Image J.
2. Go to “Image,” “Type” and set to 8-bit.
3. Go to “Process,” “Binary” and click on “Make binary.”
4. Go to “Process,” “Binary” and click on “Skeletonize.” This mode allows for a better visualization of tubular structures by stylizing the images. If the tubules are predominantly arranged independently of each other, they can be analyzed automatically as described below in **step 5**. If the tubules are interconnected in a network structure, they must be analyzed manually. This is described in **step 6** below.
5. Go to “Analyze” and click on “Analyze particles” to count tubules of the desired length on each movie frame (for example, you might want to count tubules that are greater or shorter than 1 μm in length). A dialog box will appear. Type the tubule length and the program will count structures of the indicated length on each movie frame. When the program completes the command, it will show a “Summary” of the number of tubules of that length per frame. If any tubules are interconnected within a network, the network will be counted as a single tubule.
6. Because a network of interconnected tubules are counted as a single tubule using “Analyze particles,” movie frames with interconnected tubules are best counted manually in each frame. To do this, advance the movie frame by frame. Go to “Plugins,” “Cell counter.” A window will let you select among different counter symbols; hence you can select one counter symbol per tubule length that you wish to count (for example, tubules that are greater or shorter than 1 μm in length). Click on every tubule on each frame. Then go to “Results” to visualize a chart with a list of tubule counts of each length category per frame.

4 Notes

1. BMDCs were cultured following the procedure described by Winzler et al. [31] for D1 and splenic DCs. Briefly, cells were flushed from mice tibias and femurs and seeded on 10 cm non-tissue-culture-treated petri dishes at a concentration of 1×10^6 cells/ml and passaged twice a week using trypsinization for 7–10 days. Alternatively, BMDCs can be cultured according to Inaba et al. [32]. For a detailed protocol on bone marrow cell isolation and BMDC culture see Gross [33]. The characteristic phenotype of BMDCs should be analyzed by flow cytometry as described by Winzler et al. [31].

The cell population should be between 80 and 90 % CD11b⁺/CD11c⁺ (BMDCs) at the time of the experiment. In an immature state, BMDCs display low staining for major histocompatibility complex class II molecules (MHC-II) and the costimulatory molecules CD86 and CD80. Maturation is initiated by the addition of LPS (100 ng/ml) or LPS-coated beads; 16–24 h later, MHC-II and costimulatory molecule expression strongly increases.

2. Aliquot and store GM-CSF-containing supernatant at –80 °C for multiple uses. Once thawed, the supernatant can be stored at 4 °C for approximately 1 month.
3. Tissue resident DCs can be isolated by tissue homogenization followed by purification with Miltenyi antibody-coated beads following the manufacturer instructions (Miltenyi Biotec, San Diego, CA). Cells are then plated and treated as described for BMDCs.
4. BMDCs can be harvested using trypsin-EDTA or with PBS without CaCl₂ or MgCl₂ alone. Trypsin-EDTA is preferred when passaging cells in culture and when cells will not be used for experiments within 24 h after trypsinization. After a 5 min treatment with trypsin-EDTA, cells that remain attached are considered macrophages and discarded. *See Note 5.2* for BMDC harvesting with PBS for experiments performed on the same day of the cell harvest.
5. To test the functionality and specificity of IRAK 1-4 and TBK-1 inhibitors, it is recommended to perform a parallel experiment to ensure that they have the expected effects on another well-characterized consequence of TLR engagement. IRAK 1-4 inhibitor blocks kinase activation downstream of MyD88 [23, 24], while the TBK-1 inhibitor impedes kinase activation downstream of TRAM-TRIF [28, 34]. The most straightforward way to assay their effects is to test for inhibition of the TLR-induced secretion of the proinflammatory cytokines IL-6, IL-12, or TNF α 3–6 h after stimulation with TLR ligands such as LPS or LPS-coated beads that trigger both the TIRAP-MyD88 pathway and the TRAM-TRIF pathway. To test for TBK-1 inhibitor efficacy specifically, use a TLR3 ligand (like poly(I:C)-coated beads) that stimulates only the TRAM-TRIF pathway. An alternative to measuring cytokine secretion is to monitor phosphorylation of downstream effectors, such as IRF3 after stimulation of the TRAM-TRIF axis or p38 MAP Kinase after stimulation of the TIRAP-MyD88 axis. Below we describe these procedures:

5.1. Coating of LPS or poly(I:C) to polystyrene beads.

1. Calculate the amount of coated beads required for the experiment. Plan to use 3 μ l of bead suspension per

condition, in triplicates, per time point. For assaying by ELISA, consider testing at least two time points. For assaying by immunoblotting, consider testing at least three time points. Leave some beads uncoated, washed three times with PBS (**steps 2–5** below), to be used as a negative control.

2. For coating: resuspend bead suspension in 1 ml PBS per 100 μ l of suspension.
3. Centrifuge for 5 min in a microcentrifuge at $20,000\times g$, 4 °C. Remove supernatant carefully and discard it.
4. Add 1 ml PBS and centrifuge as in **step 3**.
5. Repeat **step 4** once more.
6. Add LPS or poly(I:C) to a final concentration of 100 μ g/ml in 1 ml of PBS.
7. Mix overnight at 4 °C with end-to-end mixing (a rotating wheel is preferred).
8. Centrifuge at $20,000\times g$ for 5 min at 4 °C in the microcentrifuge and discard supernatant.
9. Repeat **step 4** three times.
10. Resuspend the pellet in the original volume of beads. The coated beads are now ready to use.

5.2. Detection of IL-6 and IL-12 secretion by ELISA.

1. On days 7–10 of BMDC culture, collect BMDCs using PBS only and without trypsinization (*see Note 4*) as follows. Transfer the culture medium containing BMDCs in suspension to a 50 ml conical tube. Collect mildly adherent BMDCs by adding 5 ml of ice cold PBS to each 10 cm dish and rigorous pipetting, and transfer the resuspended cells in PBS into the same 50 ml tube. Finally, add 8 ml of ice cold PBS to each dish and incubate for 30 min at 4 °C. Rigorously pipette again and transfer the final suspension into the same conical tube.
2. Centrifuge for 5 min, $150\times g$, at 4 °C.
3. Discard supernatant and resuspend BMDCs in BMDC complete medium. Count BMDCs.
4. Seed BMDCs into 96-well round-bottom plates (1 well per time point) at 60,000 BMDCs/well in 50 μ l of BMDC complete medium. Plate enough wells to test the following conditions in triplicate: (1) unstimulated (no beads added); (2) stimulated with uncoated beads; (3) stimulated with LPS coated beads; and (4) stimulated with poly(I:C) coated beads. Inhibitors or vehicle control should be tested under all stimulating conditions.

5. Add 50 μ l of BMDC complete medium to the untreated or “after pulse” conditions, and add inhibitors as 2 $\times$ concentrates in 50 μ l of BMDC complete medium for all treated samples in which agents are to be added 2 h before or at the pulse.
 6. Dilute bead suspension 1:16.6 in BMDC complete medium (3 $\times$ bead suspension). Separate the bead suspension volumes required for the conditions with inhibitors, and add the corresponding inhibitors (1 $\times$ concentrated).
 7. Add 50 μ l of 3 $\times$ bead suspension to each well (final dilution 1:50).
 8. After 30 min add 50 μ l of 4 $\times$ concentrated inhibitors in BMDC complete medium to the “after pulse” condition. Add 50 μ l of 1 $\times$ concentrated inhibitors in BMDC complete medium to the “before pulse” and “at pulse conditions” and 50 μ l of BMDC complete medium to the untreated conditions. Final volume: 200 μ l.
 9. Incubate plates at 37 °C for 3 and 6 h.
 10. Collect supernatants and transfer them to 96-well round-bottom plates. Store them at –20 °C until use.
 11. Perform ELISA according to manufacturer’s instructions. Dilute supernatants 1/2, 1/4, and 1/8.
- 5.3. Detection of phosphorylated proteins by Western blot.
1. Collect BMDCs with PBS, as described above.
 2. Seed cells on 96-well round-bottom plates (1 per time point) at 100,000 BMDCs/well in 50 μ l of BMDC complete medium. Conditions to test (in triplicates): unstimulated, uncoated bead-stimulated, LPS coated bead-stimulated, poly(I:C) coated bead-stimulated; all conditions treated with the inhibitors to test, or left untreated.
 3. Perform **steps 5–8**.
 4. Incubate plates at 37 °C for 1, 1.5, and 3 h.
 5. Remove supernatants. They can be stored at –20 °C to test for cytokine secretion.
 6. Add cold PBS to plates and centrifuge for 5 min at 150 $\times g$.
 7. Discard supernatants and add 30 μ l of Laemmli sample buffer 1 $\times$ containing phosphatase inhibitors to the wells.
 8. Store plates at –20 °C until use.
 9. Analyze samples by 10 % SDS-PAGE and Western blot. Run 2 separate gels, one to detect IRF3 and the other one for P-IRF3.

6. Due to the fast-changing dynamics of tubule formation, extension and contact with neighboring phagosomes, the use of spinning disk confocal microscopy is advised to allow for rapid acquisition of images in a single plane (at least 1 frame/s). This is particularly important under circumstances in which multiple fluorescence channels are analyzed and/or in which bright-field, phase-contrast, or differential interference contrast (DIC) detection is used to better visualize cell morphology in addition to the fluorescence associated with the beads. Environmental conditions that ensure cell health are also required, as cellular stresses impact poorly on membrane dynamics. Therefore, a microscope equipped with an environmental chamber providing stable temperature and 5 % CO₂ is strongly suggested. See details above for specifications on the microscope that we have used in our studies.
7. Multiple software packages are available for image acquisition and analysis, and most would be suitable for acquiring and analyzing the formation of tubules from phagosomes or endolysosomes. Image J is a versatile program that is supported by the National Institutes of Health and can be downloaded for free from the NIH website (<http://imagej.nih.gov/ij/>); FIJI (*FIJI Is Just ImageJ*) is a complete Image J package with multiple useful Plugins and a convenient interface, and is available for free download at <http://fiji.sc/Fiji>. Another useful tool for analysis is Matlab software (MathWorks, Natick, MA), which allows for the development and application of algorithms to track particles and detect particle merging and splitting events in live-cell time-lapse sequences. A useful tracking software for Matlab platform is u-track 2.0, which is available online from Harvard Medical School (<http://lccb.hms.harvard.edu/software.html>).

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Understanding of Complex Protein Interactions with Respect to Anchorage Independence

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Abstract

Anchorage-independent growth of cells in soft agar is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth. It may be considered as one of the most stringent assays for detecting malignant transformation of cells. Here, we describe a retroviral infection of a library of small secretory proteins and the use of the soft agar assay to obtain and study novel interacting protein combinations that cause cell transformation.

Key words Viral particle stock, Multiple viral infections, Soft agar assay, PCR genomic screening, Secreted proteins in chemically defined medium, TCA precipitation, Immunoblotting

1 Introduction

In recent years, the study of proteomics has led to great interest in the study of how proteins interact with each other to affect neighboring cell development. Many systems use small molecular arrays as a tool for discovering either protein–small molecule or protein–protein interactions [1, 2] as well as computational approaches [3, 4]. Tandem affinity purification coupled with mass spectrometry (TAP/MS or QTAX) has also been used for the identification of novel endogenous protein–protein interactions [5, 6]. One of the best in vitro indicators of cell transformation is the ability to grow in an anchorage-independent environment [7]. In Van Huffel et al. [8], it was shown that high multiplicity of infection in human breast epithelial cells of a library of human ECM-associated genes led to the discovery of combinations of proteins with the potential for cellular transformation by way of anchorage-independent growth in MCF10A cells. The random introduction of multiple genes allowed various combinations of protein–protein interactions (PPI) within living cells, forming monoclonal anchorage-independent

cytospheres that can then be further studied. The interaction of such combinational PPIs may lead to increasing the understanding of cellular transformation with respect to various aspects of cancer. In Part I (Subheading 3.1), we describe a method of introducing secretory proteins carried by a retroviral library into MCF10A cells with the goal to achieve multiple infections within individual cells and to identify novel protein–protein combinations that bring about cellular differentiation with the aim of further understanding the development of cancer. In Part II (Subheading 3.2) and Part III (Subheading 3.3), we describe several means of evaluating the protein combinations by intracellular as well as extracellular ways using the anchorage-independent assay (soft agar assay) [9].

2 Materials

2.1 Part I

2.1.1 Creation of Viral Library and Infection of MCF10A

1. Amphotropic Phoenix packaging cell line (AMPHO cells; Nolan Laboratory, Stanford University) [10, 11], MCF10A (ATCC), and pBABE human cDNA library [8].
2. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10 % Fetal Bovine Serum (FBS; HyClone).
3. Phosphate buffered saline (PBS; Sigma): 137 mM NaCl, 2.7 mM KCl, 4.2 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4.
4. 10A-medium: Dulbecco's modified Eagle's medium supplemented with 5 % horse serum (Invitrogen), 20 ng/mL epidermal growth factor (PeproTech), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 µg/mL insulin (Sigma).
5. 0.25 % Trypsin-Versene (HyClone).
6. Effectene Transfection Reagent (Qiagen).
7. Hemocytometer.
8. 5 mg/mL Polybrene (Hexadimethrine bromide, Sigma).
9. Puromycin (Sigma) (*see Note 1*).
10. 37 °C, 5 % CO₂ cell culture incubator.
11. 32 °C, 5 % CO₂ cell culture incubator.
12. 0.45 µM cellulose acetate (CA) vacuum filter units (Corning).
13. Sterile 15 mL polystyrene tubes with screw closure; 50 mL sterile polypropylene centrifuge tube with PP screw closure (Nalgene).
14. High-speed centrifuge, capable of speeds >32,000 rcf, and 50 mL tube rotors.

**2.1.2 Soft Agar Screen
and Soft Agar (Anchorage-
Independent) Assay**

1. 3 % Noble Agar (Difco) in water, sterilized by autoclaving (*see Note 2*).
2. 41 °C dry heating block with 15 and 50 mL blocks or small water bath with an appropriate rack to hold 15 and 50 mL tubes.
3. 10 and 15 cm diameter cell culture dishes and 6-well plates (Corning).
4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) prepared at a concentration of 5 mg/mL in PBS. Stock is kept at -20 °C in the dark.
5. 1 mg/mL MTT-10A-medium: Add 2 mL of stock MTT to 8 mL of 10A-medium (made fresh).
6. 10 mM KH₂PO₄ (pH is approximately 4.1; Sigma).
7. Sterile 15 and 50 mL conical tubes (Corning).
8. Epson scanner system (Epson).

**2.1.3 PCR Screen
for Library Inserts**

1. FlexiGene DNA kit (Qiagen).
2. Primers for confirmation of pBABE library inserts: For: ctaagcctccgctctcttcttcc and Rev: gcctccctaccggtagaattgtc.
3. Primer for sequencing: gcctctcttcttccatccg.
4. QIAEX II Gel Extraction System (Qiagen).
5. GoTaq Flexi Kit (Promega).
6. Tetrad 2 Peltier Thermal Cycler (Bio-Rad).
7. UltraPure Agarose (Invitrogen).
8. 50× TAE buffer: Dissolve completely 242 g Tris base and 18.61 g Na₂EDTA·2H₂O in 700 mL RO water first (*see Note 11*). Add 57.1 mL glacial acetic acid to the solution and adjust with water to a final volume of 1 L.
9. 1× TAE buffer: To make 1 L, add 20 mL 50× TAE buffer to 980 mL H₂O.
10. 5× DNA loading dye: 5 mL glycerol, 1 mL 10× TAE, 1 mL 10 % bromophenol blue, 1 mL 10 % xylene cyanol, and 2 mL H₂O.
11. 5 mg/mL ethidium bromide.
12. 1 Kb Plus DNA Ladder (Invitrogen).
13. Microwave oven.
14. Wide Mini-Sub Cell GT Cell system (Bio-Rad).
15. Qiaquick Gel Extraction Kit (Qiagen).
16. InGenius™ gene documentation system and Fluorescence UV transilluminator (Syngene).

2.2 Part II

2.2.1 Establishing of Stable Cell Lines

1. Dulbecco's modified Eagle's medium (DME medium; Invitrogen) supplemented with 10 % Fetal Bovine Serum (FBS; HyClone).
2. 10A-medium: Dulbecco's modified Eagle's medium supplemented with 5 % horse serum (Invitrogen), 20 ng/mL epidermal growth factor (PeproTech), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), and 10 µg/mL insulin (Sigma).
3. Phosphate buffered saline (PBS; Sigma) 137 mM NaCl, 2.7 mM KCl, 4.2 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4.
4. 0.25 % Trypsin-Versene (HyClone).
5. Effectene Transfection Reagent (Qiagen).
6. Puromycin (Sigma) (*see* **Note 1**).
7. Cellulose acetate (CA) 0.45 µM syringe disk filter (Corning).
8. Sterile 5 mL polystyrene capped tubes.

2.2.2 Preparation of Cellular Lysates for SDS-PAGE and Immunoblotting

1. Cell scrapers (TPP Techno Plastic Products AG, Switzerland).
2. cComplete™ Lysis-M reagent, EDTA-free (Roche Diagnostics) for cell lysis: Add one cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablet (PI) (Roche) and one PhosSTOP™ (Roche) tablet to 5 mL of the Lysis-M reagent (lysis buffer).
3. Coomassie Plus™ Protein Assay reagent and Albumin Standard (Thermo Scientific) for determination of total protein concentration from cell lysates.
4. Vortex mixer.
5. 20 % SDS solution.
6. 10× PAGE running buffer: 303 g Tris base and 1,440 g glycine in 10 L of H₂O.
7. For 1 L of 1× SDS-PAGE running buffer, add 100 mL 10× running buffer and 5 mL 20 % SDS to 895 mL H₂O.
8. For 1 L of transfer buffer, add 200 mL methanol and 100 mL 10× running buffer to 700 mL H₂O.

2.2.3 Production of Secreted Proteins in Serum-Free (SF) Medium

1. Pro293a™ Chemically Defined, Serum-free Media (Lonza Inc.).
2. Cellulose acetate (CA) 0.45 µM 150 mL filter units (Corning).
3. Amicon Ultra Ultracel-3K & Ultracel-50K, Membrane (Millipore).
4. Trichloroacetic acid (100 % saturated) (Sigma).
5. Ice-cold methanol–acetone 1:9 (v/v) solution.
6. –20 °C freezer.
7. 10 mM Tris pH 8.0.
8. 1 M Tris pH 8.8.
9. FOCUS FASTsilver™ (G-Biosciences).

2.2.4 SDS-

Polyacrylamide Gel Electrophoresis (SDS- PAGE) and Immunoblotting

1. 40 % Acrylamide/Bis Solution 29:1 (3.3 % C) (Bio-Rad).
2. 6× Laemmli sample buffer: 197 mM Tris-HCl pH 6.8, 6.3 % SDS, 80 % glycerol, and 5 and 0.03 % bromophenol blue. To every 850 μ L of 6× sample buffer, add 150 μ L of β -mercaptoethanol. For 2× Laemmli loading buffer, dilute the 6× buffer with β -ME in a ratio of 1:3 with H₂O.
3. 4× lower gel buffer: 1.5 M Tris-HCl pH 8.8, 0.4 % SDS, filtered.
4. 4× upper gel buffer (stacking buffer): 0.5 M Tris-HCl pH 6.8, 0.4 % SDS, filtered.
5. 1.5 mg/mL Ammonium Persulfate (APS) (kept as 500 μ L frozen aliquots) (Promega).
6. *N,N,N,N*-Tetramethylethylenediamine (TEMED) (Bio-Rad).
7. H₂O saturated butanol. Add 5 mL of RO H₂O to 50 mL butanol. Shake and allow the two phases to settle. The lower layer is the aqueous phase, and the upper layer is the butanol phase saturated with water.
8. Precision Plus Protein Dual Color Standards (Bio-Rad).
9. SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, and 1 % SDS (*see Note 17*).
10. Bio-Rad Mini-Protean 3 electrophoresis and Mini Trans-Blot module (Bio-Rad).
11. FOCUS FASTsilver™ (G-Biosciences).
12. Nitrocellulose membrane 0.45 μ M (Bio-Rad).
13. Chromatography 3mm CHR paper (Whatman, GE Healthcare).
14. Immunoblot transfer buffer: 25 mM Tris, 192 mM glycine pH 8.3, 20 % methanol (*see Note 17*).
15. 20× Tris-buffered saline (TBS), pH 7.6: Dissolve 48.4 g Tris base and 160 g NaCl in 900 mL H₂O and pH the solution to pH 7.6. Adjust the volume to 1 L.
16. 20 % Tween 20 (Sigma).
17. TBS containing 0.1 % Tween 20 (TBST): Add 5 mL 20 % Tween 20 to 995 mL 1× TBS solution.
18. Blocking buffer: 5 % nonfat milk in TBST. Store at 4 °C.
19. 50 and 15 mL conical tubes.
20. Plastic container and thick PE plastic bags.
21. Plastic sealer.
22. Flat-tipped forceps.
23. Orbital shaker or 360° rotator.
24. SuperSignal West Pico (Thermo Scientific).
25. Fuji Medical X-ray Film Super RX (Fujifilm) and X-ray developing equipment.

3 Methods

3.1 Part I

3.1.1 *Creation of Viral Library and Infection of MCF10A*

1. Creation of viral library: Create a library of cDNAs in a choice viral vector (*see Note 3*). CDNAs (*see Note 4*) can be created by reverse transcription followed by PCR from mRNA or by cloning from commercial cDNA libraries.
2. Purify and quantitate each plasmid. Mix equimolar amounts of the plasmids in 13 random equal subgroups. This will make transfection easier to handle. Ensure the concentration of the DNA is sufficiently high.
3. Seed AMPHO cells into five 175 cm² flasks so that they are 80 % confluent in DME medium on the day of the experiment (*see Note 5*). Similarly, seed MCF10A into three 175 cm² flasks so they are 80 % confluent when needed in Subheading 3.1.1, step 11.
4. Trypsinize the AMPHO cells and determine cell numbers using a hemocytometer. Dilute the cells and plate 39 dishes with a diameter of 15 cm, each containing 7.5×10^6 cells, in a volume of 20 mL. Incubate in normal tissue culture conditions (37 °C, 5 % CO₂).
5. On the day of transfection, ascertain that the cells are about 60 % confluent before proceeding.
6. Prepare 13 transfection mixes, one from each library subgroup, using the Effectene transfection reagent; gently mix 37.5 µg library plasmid DNA with 60 µL enhancer and 1,125 µL buffer EC mixture in a 15 mL polystyrene tube (*see Note 6*). Tap the sides gently to mix and incubate at room temperature for 5 min.
7. Add 225 µL of Effectene directly into the DNA mixture, tap to mix, and allow the Effectene–DNA complex to form by incubating it at room temperature for 10–20 min.
8. In the meantime, replace the medium in the dishes with 15 mL of fresh DME medium and put the dishes back into the 37 °C incubator while waiting for the DNA–Effectene complex to form.
9. Add 12 mL pre-warmed fresh medium to each DNA–Effectene complex and gently mix by drawing the mixture up and down twice with a 10 mL pipette. Gently layer one third of the complex-medium dropwise onto the adherent cells in a dish in a concentric spiral. This will ensure that the complex covers the entire surface area of the dish without having to swirl the dish too much to distribute the complex uniformly. Continue spreading the rest of the transfection mix over two more plates, working until each transfection mix is spread over the three dishes each. Incubate at 37 °C 5 % CO₂ for 16–24 h (*see Note 7*).

10. Remove the Effectene–DNA complex by replacing the growth medium with 20 mL 10A-medium, and incubate for a further 24 h in a 32 °C CO₂ incubator.
11. Plate the MCF10A to be infected the next day: Trypsinize the MCF10A cells, and determine the cell number using a hemocytometer. Plate six 15 cm diameter dishes with 3×10^6 cells per dish, and allow to grow overnight at 37 °C, 5 % CO₂.
12. Collect the supernatant from the AMPHO cells, which contains the viral particles. Pool all supernatant together, and filter through 0.45 µM cellulose acetate (CA) filter units.
13. Aliquot the filtered viral supernatant in sterile centrifugation tubes, and centrifuge at a minimum of 32,000 rcf for 2 h at 4 °C (*see Note 8*).
14. Carefully remove the supernatant. There will be a very small transparent pellet on the base of the centrifuge tubes.
15. Resuspend the viral pellets with 90 mL fresh pre-warmed 10A-medium (*see Note 9*). Add Polybrene to a final concentration of 5 µg/mL.
16. Remove the medium from the MCF10A cells, and replace it with the virus-containing medium prepared in Subheading 3.1.1, **step 14**, evenly distributing the medium over all plates. A total volume of 15 mL medium suffices for each dish.
17. Incubate the MCF10A cells at 32 °C for 16–24 h (*see Note 10*).
18. Trypsinize the infected MCF10A cells, and proceed with soft agar assay as described in Subheading 3.1.2.

3.1.2 Soft Agar Screen

1. Melt the 3 % agar in a microwave oven at 30 % power and allow the agar to cool to 41 °C. Warm 41 mL aliquots of 10A-medium supplemented with 1 µg/mL puromycin to 41 °C. Working one aliquot at a time, add 9 mL 3 % agar, mix gently by inversion, and apply 25 mL each to two 15 cm dishes. Gently swirl the dishes to cover the entire surface, without creating air bubbles. Set the dishes on a level surface at room temperature, to solidify (*see Notes 11 and 12*).
2. To add the top agar layer, heat 3 % agar and cool to 41 °C (*see Note 13*), and warm 10A-medium supplemented with 1 µg/mL puromycin to 37 °C. Dilute the infected MCF10A cells in tubes of 1.5×10^6 cells in 22 mL medium. Working one tube at a time, add 3 mL 3 % agar, mix gently, and spread 12.5 mL each to two base agar dishes. Tilt the dishes to evenly spread the agar–cell layer over the base layer (*see Note 14*).
3. Allow the top agar layer to solidify completely at room temperature (at least 20 min) before moving the dishes to a 37 °C, 5 % CO₂ incubator (*see Note 15*).

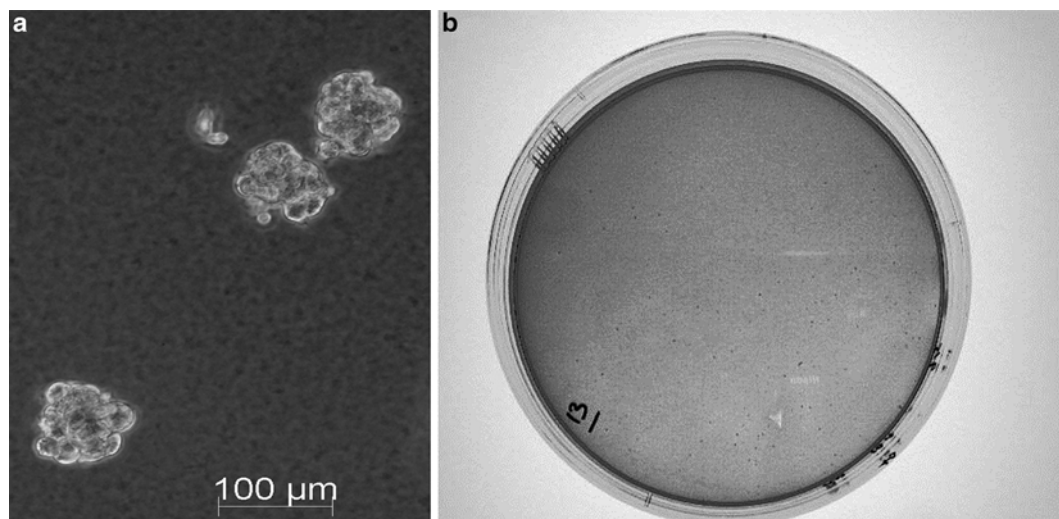


Fig. 1 Phase-contrast photomicrograph of MCF10A spheroid colonies that were selected for the ability of anchorage-independent growth (**a**) from MCF10A retroviral-infected cells in a soft agar screen (**b**)

4. After 24 h, gently overlay the agar with 15 mL 10A-medium, supplemented with 1 µg/mL puromycin. Twice a week, add 5 mL fresh medium (containing puromycin) to each dish (*see* **Note 16**).
5. Regularly inspect the dishes; after approximately 10 days, small specks, indicating developing colonies, may be observed. After 3–4 weeks, colonies would have grown sufficiently to be clearly visible to the naked eye (*Fig. 1*).
6. When colony size is satisfactory, move individual colonies to individual wells in a 24-well plate.
7. Set up 24-well plates that contain 1 mL 10A-medium containing puromycin in each well. Label clearly, identifying the originating dish.
8. Using a sterile 1 mL pipette tip, while depressing the plunger of the pipette, position the tip very close to the colony. While slowly releasing the plunger, move the colony into the tip. With some practice, this can be achieved with aspiration of minimal amounts of liquid and agar.
9. Visually examine the contents of the tip to ascertain that a single colony has been picked up. If multiple colonies are observed, discard the colonies. Only proceed when single colonies are observed.
10. Expel the contents of the tip into one of the wells of the prepared 24-well plate. Rinse the inside of the tip by pipetting up and down a few times. This will also help to break up the spheroid of cells. Incubate in TC incubator.

11. Inspect the picked colonies daily under an inverted microscope. Those colonies that have not started to adhere can be dispersed by pipetting to aid in breaking up the colony.
12. Five days after picking the colonies, change the medium. Daily inspect the 24-well plates, and, upon reaching confluency, transfer the cells to a 25 cm² flask.
13. When cells in the 25 cm² flask reach confluency, trypsinize the cells, freeze half of the cells, and store in liquid nitrogen. From the remaining cells, extract genomic DNA using FlexiGene DNA kit (Qiagen).

3.1.3 Identification and Confirmation of Multiple Gene Inserts

1. Verify the quality of the genomic DNA by PCR using primers for a housekeeping gene, e.g., G3PDH (*see* **Note 17**).
2. The identification of the multiple inserts is done by using the vector-specific primers (For: ctaagcctccgcctctcttcttcc; Rev: gcctcccctaccggtagaattgtc) with the PCR program in **Table 1** using the GoTaq Flexi Kit from Promega with the addition of 3 % DMSO in the reaction mix (**Table 2**).
3. Separate the PCR bands by 1 % agarose gel electrophoresis (*see* **Note 18**).

Table 1
PCR program for identification of viral inserts in total genome

Step 1	95 °C for 3 min
Step 2	95 °C for 15 s
Step 3	72 °C for 1 min
Decrease the temp. by 0.5 °C every cycle	
Cycle to step 2, 7 times more	
Step 4	94 °C for 15 s
Step 5	66 °C for 30 s
Step 6	72 °C for 1 min
Decrease the temp. by 1 °C every cycle	
Cycle to step 4, 14 times more	
Step 7	94 °C for 15 s
Step 8	55 °C for 30 s
Step 9	72 °C for 1 min
Cycle to step 7, 25 times more	
Step 10	72 °C for 10 min
Step 11	End

Table 2
PCR reaction mix per sample using the GoTaq Flexi Kit (Promega)

Primers: For (10 μ M)	1 μ L
Rev (10 μ M)	1 μ L
dNTP (10 mM)	1 μ L
5 $\times$ buffer	4 μ L
MgCl ₂ 25 mM	2 μ L
Taq polymerase	0.5 μ L
DMSO	0.6 μ L
DNA template (1 ng/mL)	0.5 μ L
H ₂ O	9.4 μ L
Total volume	20 μ L

- Visualize and document the PCR products by UV light. Excise each band individually, and purify the PCR fragment using QIAEX II Gel Extraction System (Qiagen; *see* **Notes 19** and **20**).
- Sequence the individual PCR fragments with the vector-specific primer gcctctctcttccatcg. The sequencing results (*see* **Note 21**) are subjected to nucleotide BLAST analysis on the NCBI web site: <http://blast.ncbi.nlm.nih.gov/Blast.cgi> [12] for insert identification.

3.2 Part II: Reconstructive Analysis by Direct Viral Infection

3.2.1 Direct Infection and Stable Recombinant MCF10A Cells

Viral supernatant carrying selected recombinant genes is produced and used in combinations to infect MCF10A cells for verification. Integration of the genes into the genome is confirmed by direct sequencing or their expression by immunoblotting.

- From 75 cm² flasks of 80 % confluent AMPHO cells in DME medium, trypsinize the cells, resuspend in fresh medium, and count with a hemocytometer and seed at 2.5×10^6 cells per 10 cm diameter dish. Allow to grow overnight at 37 °C, 5 % CO₂. The confluency of cells in each dish should be about 60 % as viewed by microscopy on the day of transfection.
- Add 5 μ g of DNA of the plasmid construct to a 16 μ L enhancer and 300 μ L buffer EC mixture in a 5 mL polystyrene (PS) tube (*see* **Note 6**). Tap the sides gently to mix, and incubate for 5 min at room temperature.
- Add 60 μ L of Effectene directly into the DNA mixture, tap to mix, and allow the Effectene–DNA complex to form by incubating it for 10–20 min.

4. After 24 h, change the medium of the transfected cells with 8 mL of fresh 10A-medium and incubate overnight at 32 °C, 5 % CO₂.
5. Seed the MCF10A cells at a cell concentration of 10⁵ in 6-well dishes and grow overnight at 37 °C, 5 % CO₂.
6. The following day, harvest the viral particle-containing medium and filter using a 0.45 µM cellulose acetate (CA) syringe filter. The virus is ready for use. It can be stored up to 2 weeks at 4 °C.
7. On the day of infection, remove the medium from the MCF10A wells, and replace it with 2 mL of viral supernatant supplemented with 5 µg/mL Polybrene. Incubate at 32 °C overnight with two changes of viral supernatant within 8 h intervals. Leave the dishes to incubate overnight after the last medium change.
8. Start the antibiotic selection the next day by changing the medium to 10A-medium containing 2 µg/mL puromycin and incubating the cells at 37 °C, 5 % CO₂.
9. Refresh the medium after 3 days (*see Note 16*) and continue to selection medium for at least 4 days or till cell count is sufficient for the assay.

3.2.2 Soft Agar Assay (Anchorage Independence)

The soft agar assay consists of a 0.5 % agar base layer and a 0.36 % top agar layer in which the cells grow. Each sample should be done in triplicate along with the selected negative control sample (empty vector or GFP).

1. To prepare base agar, heat the 3 % agar stock and cool to 41 °C (*see Note 2*). Pre-warm the 10A-medium at 41 °C. Calculate the number of wells required for the assay. Each well will contain 1.5 mL of 0.5 % medium agar. For three wells, 4.5 mL medium agar is needed (3.75 mL 10A-medium + 0.75 mL 3 % agar). Gently mix the medium and agar and layer 1.5 mL in the dish, covering the surface completely. Do not create bubbles as it will make the surface of the base agar uneven. Allow the base agar to cool on a level surface.
2. Trypsinize the infected MCF10A cells, and determine cell concentration using a hemocytometer. For MCF10A cells, 2.5×10^4 cells are used per well. For triplicate wells, 7.5×10^4 cells are required.
3. Top agar: dispense the warm 10A-medium in 4.0 mL aliquots in 15 mL tubes and incubate them at 41 °C (*see Note 22*). Add 7.5×10^4 cells to pre-warmed 10A-medium and then add 0.5 mL 3 % agar (41 °C) (*see Notes 23 and 24*). Mix gently and immediately layer 1.5 mL aliquots carefully into each well. Swirl the plate when the three wells are filled, and allow to solidify on a level surface at room temperature.

4. Incubate the plates at 37 °C, 5 % CO₂ overnight to allow the surface of the soft agar to dry slightly. The next day, gently add 1.5 mL of 10A-medium containing 2 µg/mL puromycin on the surface of the top agar, without disturbing the agar. Change the medium twice a week (every 2 or 3 days; *see* **Note 25**).
5. After 10–21 days, observe the colony shape, size, and number under a microscope. Then prepare to stain the viable spheroids with a MTT-10A-medium solution (*see* **Note 26**).
6. Remove most of the medium from the wells. Add 1 mL MTT-10A-medium to each well. Cover the plates with foil and replace the plates back into the incubator for 1–2 h. The viable spheroids will begin to develop a purple stain.
7. When the stain has developed to a satisfactory color, remove the plates and chill them for 30 min at 4 °C. This will stop the reaction as well as firm up the top layer. Carefully remove as much as possible the solution from the surface of the soft agar. Slowly fill the well with 10 mM KH₂PO₄ (*see* **Note 27**) and return the plates to the cold. Change the phosphate solution two or three times over a period of 24 h.
8. Prepare the plates for documentation by removing as much of the liquid as possible. Place the plates on the scan bed right side up, with documentation for the plate on the side, and scan as 800-pixel color images. Save the images as TIFF or JPEG files.

3.3 Part III: Extracellular Effect of Secreted Proteins on Anchorage- Independent Growth

Here, we describe a method to study the effect of secreted recombinant proteins on anchorage-independent growth. This is a way to test the properties of the various combinations of proteins that were observed to play a role in cell transformation, in an extracellular environment. These combinations of cell-secreted proteins are examined for potential transforming as well as proliferation effects.

3.3.1 Establishment of 293 Stable Cell Lines

1. Transfection of 293 cell lines. The day before, from a 75 cm² flask of 80 % confluence, trypsinize and seed 5×10^6 cells per 10 cm diameter dish and allow to grow overnight at 37 °C, 5 % CO₂. The confluence of cells in each dish should be about 60 % as viewed by microscopy on the day of transfection.
2. Add 5 µg of DNA of the plasmid construct to a 16 µL enhancer and 300 µL buffer EC mixture in a 5 mL polystyrene (PS) tube (*see* **Note 6**). Tap the sides gently to mix and incubate for 5 min at room temperature.
3. Add 60 µL of Effectene directly into the DNA mixture, tap to mix, and allow the Effectene–DNA complex to form by incubating it for 10–20 min.

4. Replace the medium on the cells with 6 mL of fresh DME medium and put the dishes back into the 37 °C incubator while waiting for the complex formation.
5. Add 2 mL of pre-warmed fresh medium to the Effectene–DNA complex and gently mix by drawing the mixture up and down twice with a 2 mL pipette. Gently layer the complex medium dropwise onto the adherent cells in the dish in a concentric spiral. This will ensure that the complex covers the entire surface area of the dish without having to swirl the dish too much to distribute the complex uniformly. Incubate at 37 °C 5 % CO₂ for 16–24 h. Remove the Effectene–DNA complex by changing the growth medium and incubate for a further 24–48 h. This will allow the recombination of the transfected gene to stabilize.
6. The growth medium is changed to the antibiotic selection medium of 2 µg/mL puromycin DME medium and incubated as before. Refresh the selective medium every 3 days. Many of the cells will die off leaving clustered colonies. Allow these colonies to mature for 7–10 days. Trypsinize the cells and transfer them to two 25 cm² flasks. Continue to allow the cells to grow to a 70–80 % confluent state.

3.3.2 Confirmation of Stable Cell Lines by Immunoblotting

1. For each cell line created, split the cells of one of the flasks into half and seed one well of a 6-well dish. Remember to create a control well of untransfected cells. Seed the other half of the cells into a new 25 cm² flask to freeze down. Sufficient protein lysate will be generated from 6-well dishes.
2. Allow the cells to grow until they become confluent in the wells.
3. Remove the medium from the well, and rinse once with PBS. Add 200 µL lysis buffer with protease and phosphatase inhibitors to each well, incubate for 5 min at room temperature, and scrape the cells from the dish. Transfer the cellular lysate to a microfuge tube and vortex for 3 min at maximum speed.
4. Spin the lysates for 10 min at maximum speed, 4 °C, to remove cellular debris and unbroken cells. Transfer the cleared lysates to fresh microfuge tubes.
5. To determine the total protein concentration, prepare lysate dilutions of 1 in 10 and 1 in 20 (3 µL lysate in 27 µL H₂O and 3 µL in 57 µL, respectively) and assay with the Coomassie Plus™ Protein Assay reagent using the Albumin Standard as instructed in the protocol supplied. Determine the volume of each lysate and add 6× Laemmli dye to make 1× dye + lysate mixture (*see* **Note 28**) and heat the sample on a dry-block for 10 min at 95 °C. Vortex the sample after heating and briefly spin down the sample. The samples are stored on ice, ready to be loaded on an SDS-PAGE gel.

6. Set up the glass plates as instructed by the mini gel electrophoresis system using the 1.5 mm spacer and short plates and the casting module.
7. Mix 5 mL Lower Gel Buffer, 5 mL of the 40 % acrylamide/bis-acrylamide reagent, and 10 mL ddH₂O in a 50 mL plastic tube. Add 100 μ L APS and 10 μ L TEMED to initiate the cross-linking process. Working quickly, invert and rotate the tube two or three times to mix gently without introducing air or frothing (*see Note 29*). Pour approximately 7.5 mL between the glass plates until the level is about 15 mm from the top of the short plate, immediately overlay the surface with water-saturated butanol, and allow the gel to set. The butanol phase will seal the gel from air, allowing the cross-linking to proceed. It should take about 45 min.
8. When the gel is set, remove the butanol and rinse the gel well with ddH₂O. Tilt the gel upside down and place it on a piece of absorbent paper to soak up all the remaining liquid from between the plates. Prepare 10 mL of 4 % stacking gel by adding 2.5 mL upper gel buffer and 1 mL of acrylamide reagent to 6.5 mL of ddH₂O. Mix gently. Add 60 μ L APS and 6 μ L TEMED and mix and pour to the top of the plates. Immediately and carefully slide the combs in place until they are flush with the glass plate and allow the gel to set.
9. Remove the combs and set the gel plates into the electrophoresis module and tank as instructed. Fill the inner chamber to the top and the outer chamber till it is 5–6 cm from the base with 1 $\times$ SDS Running Buffer. Ensure that there are no leaks between the seal of the glass plates and the rubber gaskets of the module. Load the gel with 30–50 μ g of the protein samples and 5 μ L of standard markers.
10. Run the gel at 15 mA for 15 min to run the protein into the stacking gel; then increase the current to 25 mA and run until the loading dye reaches the base of the glass plates.
11. Freshly prepare 1 L of 1 $\times$ transfer buffer and cut a piece of nitrocellulose membrane 8 cm by 10 cm. Also cut six pieces of 3mm CHR paper to the size of 8 cm by 10 cm. Soak the membrane and CHR paper in 1 $\times$ transfer buffer.
12. Using a clean tray, add 500 mL of 1 $\times$ transfer buffer. Submerge an open transfer cassette (black side down) in the tray. On the black surface of the transfer cassette, layer a transfer pad, followed by two soaked 3mm CHR paper.
13. Insert a plastic separator between the glass plates to release one glass plate from the gel. Run flat-tipped forceps between the spacers and gel to release the gel. Place a soaked 3mm CHR paper on the gel, invert the plate, gel and paper, and remove the glass plate from the gel and paper. Place the gel and paper onto the setup of paper, transfer pad and cassette in the tray.

14. Layer the nitrocellulose membrane carefully, ensuring that there are no bubbles. Pour some transfer buffer over the gel-paper stack and layer a piece of soaked 3mm CHR paper. Using a clean 15 mL plastic tube as a roller, roll the tube like a rolling pin, outwards to the edge of the paper. This will push out any bubbles that may be between the gel and the membrane. Layer the last two soaked 3mm CHR sheets and roll out any bubbles that may be trapped between them. Add another transfer pad to the top of the stack and carefully clamp the cassette in place. Slot it into the transfer module in the correct orientation, gel side (black side) to the cathode end (black panel). Place an ice block in the tank beside the module, top up with 1× transfer buffer, and fix the lid on. Run the transfer system at 200 mA for 2 h.
15. Remove and discard the gel. Soak the membrane in PBST buffer for 5 min. Block the membrane by incubating it in blocking buffer for 1 h at room temperature. Dilute the primary antibody according to the company specifications into the appropriate volume of blocking/diluent buffer (*see Note 30*).
16. Place the membrane between two sheets of PE plastic cut from a new plastic bag and seal three sides. Add the diluted primary antibody. Push out the air from the bag by rolling a pipette on the outside surface of the bag, so that the air escapes through the opening. Seal the opening close to the membrane edge to minimize the capacity of the bag, thus maximizing the exposure of the membrane to the antibody mixture. Allow the membrane to rotate 360° or place it on an orbital shaker for 1 h at room temperature.
17. Remove the membrane from the bag and place it in a clean plastic container. Wash the membrane for 10 min with PBST buffer three times.
18. Dilute the secondary antibody-HRP and process the membrane as with the primary antibody. Incubate the membrane for another 1 h at room temperature.
19. After the secondary antibody-HRP incubation, wash the membrane in the plastic container three times with TBST as before.
20. Prepare the detection solution as instructed by the manufacturer (*see Note 31*). Recommended exposure times for the X-ray film are 15 s, 30 s, and 2 min. A further exposure can be determined by assessing the intensity of the exposed X-ray film if required.

3.3.3 Production of Recombinant Proteins in Chemically Defined Serum-Free Medium

1. Seed a 75 cm² cell culture flask to get 60 % confluency of recombinant stable cells. Prepare a control flask containing empty vector (EV) stable cell line. Incubate overnight at 37 °C in the CO₂ incubator.
2. The cells should be about 70–80 % confluent. Remove the media from the flasks and replace with 20 mL Pro293a™

Chemically Defined Serum-free Media (Pro293a-CDM) without the selection antibiotic. Grow the cells for 2 days. The Pro293a media (spent media) is harvested and prepared for immunoblotting or sensitivity assays. The medium is passed through a 0.45 μM filter for sterilization.

3.3.4 Confirming Secretion by Immunoblotting

1. To detect the presence of secreted protein in the media, aliquots of 1 mL of spent media (*see* Subheading 3.3.3) are transferred to 1.5 mL microfuge tubes. Add 200 μL 100 % (v/v) trichloroacetic acid (TCA) to each aliquot (*see* **Note 32**). Mix well and incubate the samples on ice for 30 min. Centrifuge the tubes for 10 min at max speed, 4 °C.
2. Remove the supernatant without disturbing the fluffy pellet. Wash the pellet by adding 1 mL cold methanol–acetone 1:9 (v/v) and inverting the tubes several times. Spin the tubes for 5 min at max speed, 4 °C, and remove the supernatant.
3. Wash the pellet two more times with 300 μL of methanol–acetone 1:9. Remove the supernatant and dry the pellet by placing the tubes in a 95 °C heating block for 5 min.
4. For SDS-PAGE, add 50 μL 2 $\times$ sample buffer to the pellet and mix well. Heat the samples on a dry-block for 5 min at 95 °C (*see* **Note 33**). Vortex the sample after heating and briefly spin down the samples. The samples are stored on ice, ready to be loaded on an SDS-PAGE gel.
5. Twenty-five microliter aliquots of the samples are run on an SDS-PAGE as described in Subheading 3.3.2, **steps 4–6**, and transferred onto nitrocellulose membrane and analyzed by immunoblotting as in Subheading 3.3.2, **steps 11–17**.
6. The rest of the samples can be run on a second SDS-PAGE gel and process with the FOCUS FASTsilver™ staining kit to visualize the amount of protein secreted (Fig. 2).

3.3.5 Preparation of Medium Carrying Secreted Proteins for Assay

1. About 150 mL of spent media (SM) is collected from the stable cell lines (*see* Subheading 3.3.3). An experimental control of spent media is collected from cells carrying the empty vector (EV). The media is sterilized by unit filtration of 0.45 μM pore size.
2. The sterile spent media is mixed with fresh medium in a ratio of 1:4 spent medium to fresh medium (refreshed media, RM; *see* **Note 34**).

3.3.6 Soft Agar Assay (Anchorage Independence) Using Refreshed Media

1. The 0.5 % base agar is prepared as in Subheading 3.2.2, **step 1**.
2. Trypsinize the infected MCF10A cells, and determine cell concentration using a hemocytometer. For MCF10A cells, 2.5×10^4 cells are used per well. For triplicate wells, 7.5×10^4 cells are required.

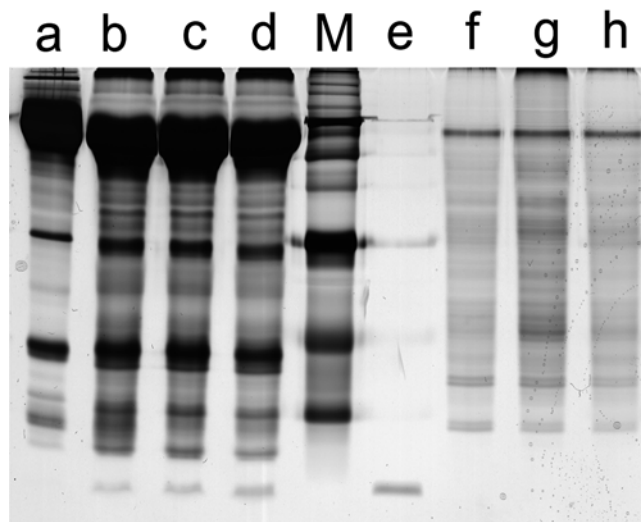


Fig. 2 An example of protein secretion in Chemically Defined Serum-free Medium (Pro293a) versus DME medium. Silver staining of TCA-precipitated media obtained from 293 stable cells grown in DME medium (*b, c, d*) and those grown in Pro293a Chemically Defined Serum-free Medium (*f, g, h*). Fresh DME medium and fresh Pro293a medium are shown in lanes (*a*) and (*e*), respectively. Empty vectors (EV) carried in 293 stable cell lines are represented by lanes (*b*) and (*f*). Lanes (*c*) and (*g*) represent spent media from secretogranin III (SCG3) 293 stable cells, and lanes (*d*) and (*h*) are spent media from IGFBP7 293 stable cells. Lane (*M*) is the protein marker (250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa)

3. Prepare the top agar as in Subheading 3.2.2, **step 3**, using refreshed media. Aliquot RM in 4.0 mL aliquots into each 15 mL tube and incubate at 41 °C (*see Note 22*). Add 7.5×10^4 cells to pre-warmed refreshed media and then add 0.5 mL 3 % agar (41 °C) (*see Note 23*). Mix gently and layer 1.5 mL aliquots carefully into each well. Swirl the plate when the three wells are filled and allow to solidify on a level surface.
4. Incubate the plates overnight to allow the surface of the soft agar to dry slightly. The next day, gently add 1.5 mL refreshed media on the surface of the top agar. Change half of the surface medium every 2–3 days.
5. After 10–21 days, observe the colony shape, size, and number under a microscope. Then prepare to stain the viable spheroids with an MTT-10A-medium solution as in Subheading 3.2.2, **step 5**, and process the soft agar as in Subheading 3.2.2, **steps 6–8** (*see Note 26*).

4 Notes

1. Puromycin is made as a 5 mg/mL stock with sterile water and is filtered with a 0.22 μ M filter and syringe.
2. The 3 % agar can be made and sterilized in advance and slowly heated in a microwave oven (at 30 % power) at the time of use. It is not advised to reheat the 3 % agar stock multiple times, as the solid agar becomes softer with each heating.
3. A choice of retroviral or lentiviral vector must be made; retroviral vectors can only infect dividing cells, while lentiviral vectors can also infect nondividing cells.
4. Preferably, all cDNAs should be of a similar size. Extremes in cDNA size can result in varied production of viral particles or of protein expression by the infected cells, which can skew results because some library proteins are under- or overexpressed.
5. Setting up the screen relies on a timed interplay, where large quantities of two different cell lines need to be ready at the right time. In the days before the start of the screen, grow up the AMPHO and MCF10A cell lines. The day before transfection, 3×10^8 AMPHO cells are needed. This corresponds with approximately five 175 cm² tissue culture flasks. The day before infection, 1.8×10^7 MCF10A cells are needed. This corresponds with approximately three 175 cm² tissue culture flasks or seven 75 cm² flasks.
6. For transfections, it is best to use polystyrene (PS) tubes rather than polypropylene (PP) tubes because the DNA–transfection complex sticks to the PP surface and decreases the efficiency of the transfection into the cells. Low-retention microfuge tubes can also be used for small volume reactions.
7. As soon as the AMPHO cells are transfected, there is the possibility of viral particle formation. All manipulation with viral stock should be done in a biosafety level 2 tissue culture room. This safety level should stay in place at least until 4 days after infection.
8. This corresponds with 18,000 rpm ($38,700 \times g$) in a Sorvall SS-34 rotor or 15,000 rpm ($32,500 \times g$) in a Sorvall SA-600 rotor.
9. Collecting 790 mL from the AMPHO cells and resuspending the pellets in 90 mL medium correspond to a concentration factor of 8.8.
10. The infection time is kept short, and no selection is applied before the cells are seeded in soft agar. This is to avoid much cell proliferation, which would duplicate individual clones, and also to avoid cross-influence of secreted proteins from one infected cell to another

11. Place the dishes away from the air vents at the back of some TC hoods, as they could unevenly dry out the agar layer. Dishes that do not have the entire bottom surface evenly covered or that have air bubbles in the base agar layer cannot be used.
12. The base agar layer can be prepared the day before and stored at 4 °C overnight. Wrap the dishes in plastic bags to prevent drying out of the agar layer. Ensure that the base layer has reached room temperature before the top agar layer is added.
13. The agar temperature should not exceed 41 °C, as it might induce a heat shock in the cells, undermining cell viability.
14. Any air bubbles that form can carefully be removed with a sterile tip. Allow the top agar layer to solidify before moving the dishes to the 37 °C incubator. If the agar layer is liquid at the time of placing at 37 °C, the agar layer will not solidify.
15. Move the dishes carefully, as even modest shaking can separate the top agar layer from the base layer, allowing the medium to come between them and the top layer becoming extremely fragile. The incubator that holds the soft agar dishes should sit on a sturdy surface and preferably be infrequently used, as repeated opening and closing of the door can result in shaking of the dishes.
16. The medium atop the agar may be removed and replaced with fresh medium, but there is a danger that the fragile agar layer is disturbed. If not necessary, it is better not to remove the medium.
17. Primers for G3PDH are: For: tgaaggtcggagtcaacggatttggt and Rev: atgtggccatgaggtccaccac.
18. Prepare a 1.0 % TAE agarose gel by weighing out 3.0 g agarose and adding it to 300 mL of 1× TAE buffer. Melt the agarose with a microwave and allow the gel mixture to cool to approximately 56 °C before casting the gel. Set up the casting tray and comb of the horizontal gel system on a level surface. Add 15 µL of 5 mg/mL ethidium bromide to the agarose solution. Mix gently and pour the gel into the casting tray and allow it to set. After it has set, remove the comb and transfer the tray to the electrophoresis chamber. The comb end should be at the cathode end. Add the 1× TAE buffer so that the gel is submerged. Mix the PCR sample with loading dye, and load the samples into the wells, loading a 100 bp DNA marker alongside for reference. Run the gel until the dye front has reached just past midway.
19. Wear safety glasses or a face shield when cutting out the bands as UV light can be harmful for the eyes.
20. Purification of DNA fragments with the QIAEX II system is based on solubilization of agarose and selective adsorption of

nucleic acids onto QIAEX II silica gel particles in the presence of chaotropic salt. It is designed for batch purification of a wide range of DNA fragment sizes from 40 bps to 50 kb.

21. If the sequencing trace gives an indication that two PCR fragments are mixed, these fragments can be separated by cloning in a T/A cloning vector and sequencing several colonies to identify the fragments.
22. It is important that the medium and agar are not warmer than 41 °C, as the cells might be subjected to heat shock.
23. It is important to keep the final concentration of the top agar close to 0.36 %. If the percentage of agar is low, the top agar layer will be very soft, and the spheroids could be removed when the medium is being refreshed. So the volume of 10A-medium can be adjusted accordingly.
24. Do one tube at a time; otherwise, the agar may solidify before it is overlaid in the 6-well dish. Do not keep the cell-medium-agar mixture at 41 °C. The cells will lose their viability and will give a lower spheroid count.
25. It may be difficult to remove the medium from the top agar completely. In this case, remove half of the old medium and replace it with the same volume of fresh medium.
26. MTT is a colorimetric biochemical reaction used for assessing the viability of the cells. It utilizes the electrons produced by the mitochondria to reduce the yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan compound, which has a purple color, thus staining the cells or spheroids purple.
27. The pH of 10mM KH₂PO₄ is 4.68 which changes the phenol red dye in the medium from red to yellow. This will contrast better with the colonies for scanning or photography documentation.
28. For a lysate volume of 200 µL, 40 µL of 6× dye is added to make a 1× dye mixture. An alternative, rough cell lysis method is to use the dye, diluted to 2× concentration and added to the cells directly. This is a rough protein lysate preparation and does not allow for normalization of the protein expression against the starting cell line. The Laemmli buffer will lyse the cells entirely, and the DNA will be released. Hence, the lysate at this stage will be very viscous. The viscosity will disappear after the 95 °C heat treatment for 10 min. Some researchers like to heat the samples twice for a period of 5 min each with brief vortexing of the sample in between the two treatments.
29. The presence of air in acrylamide prevents the cross-linking process. In some protocols, degassing of the acrylamide mixture is performed before APS and TEMED are added.

30. For an 8 cm by 10 cm membrane, a minimum volume of 3 mL of antibody diluent buffer solution is needed. The 5 % nonfat milk in PBST buffer is used for both blocking and diluting the primary and secondary antibodies for immunoblotting.
31. Depending on the strength of the signal, you may need to dilute the detection solution if the illumination of the membrane is too strong. A good starting dilution is 1:1:8 of luminol/catalyst/H₂O, but this requires initial testing.
32. Adding 1 volume of TCA stock to 4 volumes of protein sample works more efficiently than a precipitation using a final concentration of 10 % TCA. However, the pellet requires more methanol–acetone washes.
33. The samples should be blue. If the sample turns yellow, there is residual TCA present, turning the sample acidic. This may interfere with the electrophoretic separation. Adjust the samples by adding a few microliters of 1 M Tris pH 8.8 until the color returns to blue.
34. Various different cell lines can undergo treatment with refreshed media by mixing the spent medium (Pro293a) with medium required by the selected cell line.

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Chapter 17

Application of Flow Cytometry to Analyze Intracellular Location and Trafficking of Cargo in Cell Populations

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Abstract

Pulse shape analysis (PulSA) is a flow cytometry-based method that involves the measurement of the pulse width and height of a fluorescently labeled molecule simultaneously, enabling a multidimensional analysis of protein localization in a cell at high speed and throughput. We have used the method to detect morphological changes in organelles such as Golgi fragmentation, track protein trafficking from the cell surface, and also discriminate cells with different target protein localizations such as the Golgi, lyso-endosomal network, and the plasma membrane. Here, we describe the basic experimental setup and analytical methods for performing PulSA to examine membrane trafficking processes. We illustrate in particular the application of PulSA for monitoring the trafficking of the membrane-bound enzyme furin and morphological changes to the Golgi caused by Brefeldin A.

Key words Flow cytometry, Fluorescence pulse shape, Intracellular location, Membrane trafficking

1 Introduction

Methods to investigate and quantitate the cellular localization of cargo proteins rely heavily on microscopy. While the strength of microscopy lies in the ability to provide information on intracellular location, it has limited quantitative throughput capacity due to the complex data collection process and analysis after image acquisition. Although recent methods have been developed to address the limitations in throughput [1], specialized equipment and complex methods of quantitation and analysis are still needed. In contrast to microscopy, flow cytometry is capable of analyzing >1,000 cells per second. The hydrodynamic focusing ability of flow cytometry enables cells to be ordered into a stream of single particles which allows for the analysis of cells individually, hence eliminating the need for segmentation when analyzing clustered cells.

Classically, flow cytometry has been widely used to analyze expression of cell surface and intracellular molecules, to define

different cell types in a heterogeneous cell population, and to analyze cell size and volume. This use of flow cytometry typically involves the output parameter of “pulse area,” which is a measure of the total fluorescence emitted by the particle. Also commonly used is the pulse height, which is the maximum fluorescence intensity of the particle at the center of the focused laser beam. Most flow cytometers can also acquire information on the pulse width which provides information about the length of time a fluorescence signal is detected as the particle passes through the laser beam. Pulse width can be used to discriminate cells of different sizes, single cells from clumped cells [2], and the nuclear sizes of cells [3]. However, pulse-width measurement can have much broader applications, as recently demonstrated [4, 5].

The collective use of all these flow cytometry-acquired parameters enables additional features to be extracted from cells, which can be defined as pulse shape analysis (PulSA) (Fig. 1). In particular, PulSA enables multidimensional analysis of protein localization in a cell [4]. We have applied PulSA to detect morphological changes in organelles such as Golgi fragmentation, track retrograde protein trafficking from the cell surface to the Golgi, and discriminate cells with different target protein localizations such as the Golgi, lyso-endosomal network, and the plasma membrane (Fig. 1) [4, 5]. In addition, we have also shown that PulSA can be used to sort cells based on protein localization [5] and to monitor protein aggregation and nucleocytoplasmic translocation of proteins [4].

We describe the experimental setup of the PulSA technique, including data acquisition, analysis, and interpretation and also some helpful tips to note for applications in protein localization in cells relating to trafficking pathways and organelle structure.

2 Materials

1. Windows XP-based (or newer operating system) computer.
2. Flow cytometry analysis software—BD FACSDiva (BD Biosciences) or FlowJo (Tree Star, Inc).
3. Microsoft Office Excel program.
4. Flow Cytometry Standard (FCS) extract software (FCS extract 1.02 and above) (*see Note 1*).
5. Prism software (GraphPad).
6. Flow cytometer that can collect pulse area, height, and width information (*see Note 2*).
7. FACS tubes (BD round-bottom polypropylene tubes).
8. Media for cell culture.
9. 0.05 % trypsin-EDTA or 5 mM EDTA/PBS to detach cells.

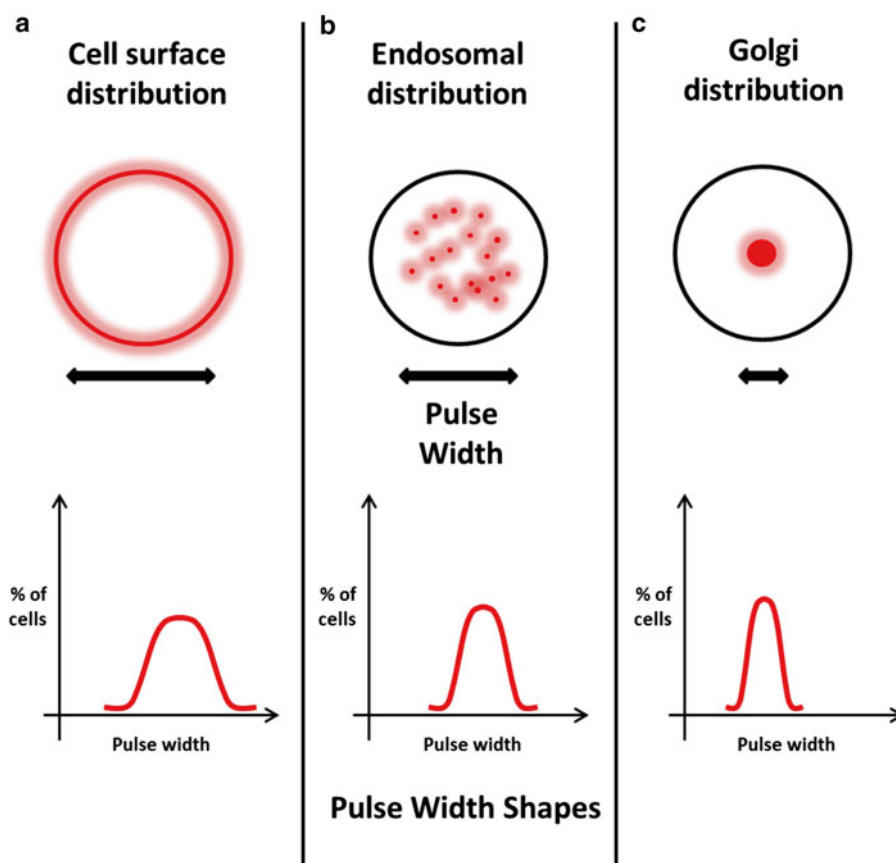


Fig. 1 Principle of pulse shape analysis (PulSA). Proteins located at the cell surface (a) would generate a larger pulse width than proteins with intracellular localization. Proteins that localized to the Golgi (c) would give a much smaller pulse width as compared to those with an endosomal distribution (b)

10. Control tubes for the setting of gates in flow cytometry (*see* Subheading 3.3 for gating).
11. Control tubes for setting up spectral compensation if staining with two or more different fluorophores (*see* **Note 3**).
12. Antibodies to stain organelle markers, e.g., Golgi Marker, GM130.
13. Cells that are genetically engineered for flow cytometry (protein of interest must be fluorescently labeled or tagged) (*see* Subheading 3.1 for sample preparation methods).

3 Methods

To visualize protein of interest in the cells, the fluorescence-labeled protein can be endogenous or ectopically expressed. In HeLa cells, if required, we transfect cells for 24 h before carrying out any

trafficking studies. Fluorescence microscopy can be used to complement PulSA as it would be important to correlate results from PulSA with the localization of the protein of interest in the cell using microscopy. To ensure a successful setup and interpretation of PulSA results, controls for setting up the gating and spectral compensation on flow cytometer and internal controls are needed. The requirements for each control are outlined in the subsequent sections below.

3.1 Sample Preparation

1. The protein of interest has to be fluorescently labeled either by fusion with a fluorescent tag protein (e.g., GFP or mCherry) or labeled by indirect immunofluorescence (*see Note 4*). Fluorescent dyes like the MitoTracker and Tubulin Tracker or the DNA dye Hoechst 33342 can be added to the cells to mark different compartments in the cell. For a multicolor analysis, the flow cytometer must be capable of detecting multiple colors without spectral spillover between channels (*see Note 3*). We recommend using at least 1×10^6 cells per sample for PulSA.
2. Cells can be analyzed either live or fixed (*see Note 5*).

3.1.1 Retrograde Trafficking Assays of FLAG-Furin Using PulSA

PulSA can be used to monitor kinetics of cargo transport from the cell surface to the Golgi. Furin is a membrane protein that cycles between the Golgi and the plasma membrane (PM) via the late endosome [6, 7]. We monitored the retrograde transport of FLAG-tagged furin from the PM to the Golgi in transfected HeLa cells in suspension using a FLAG antibody internalization assay [7]. The general protocol is illustrated in the flow chart in Fig. 2:

1. Lift cultured cell monolayers using 5 mM EDTA/PBS rather than trypsinization for 4 min, 37 °C (*see Note 6*).
2. Suspend cells in C-DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/μL penicillin, and 0.1 % (w/v) streptomycin) before washing cells in phosphate buffered saline (PBS) twice.
3. Incubate cells with rabbit anti-FLAG antibodies diluted in serum-free media (SFM) (5 μg/mL) for at least 30 min on ice.
4. Wash cells in cold PBS.
5. Incubate cells for 15, 60, and 90 min time points in SFM at 37 °C for the appropriate durations (*see Note 7*). Fix cells for the 0 min time point in 4 % paraformaldehyde/PBS for 15 min/room temperature (RT). Cells for other time points are fixed after each respective time points.
6. After the internalization assay, permeabilize cells in 0.1 % Triton X-100/PBS for 4 min/RT and then wash in PBS. Cells

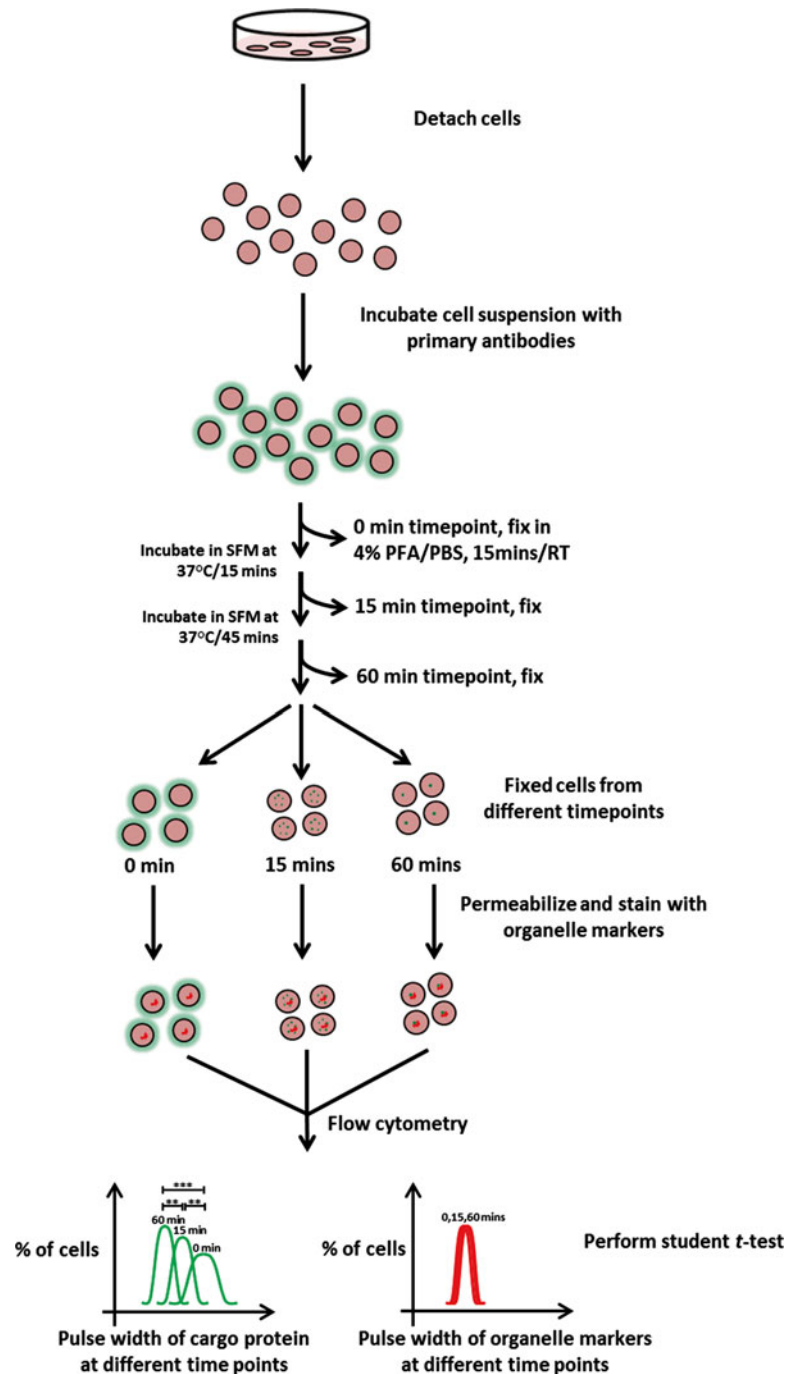


Fig. 2 Flow chart to monitor cargo trafficking from the plasma membrane to intracellular compartments by pulse shape analysis (PulSA). Shown here is the protocol for monitoring cargo trafficking from the cell membrane to intracellular compartments by PulSA. Briefly, cells are detached from the plates or dishes before incubation with epitope-specific antibodies for 30 min on ice. After 30 min, cells are washed in cold PBS before incubation with serum-free media for different durations at 37 °C or fixed (0 min). Cells were fixed in 4 % PFA/PBS for 15 min after each time points. Cells were then permeabilized in 0.1 % Triton X-100 for 4 min and then blocked in 5 % FCS/PBS. After which, the cells can be stained with additional organelle markers before flow cytometry

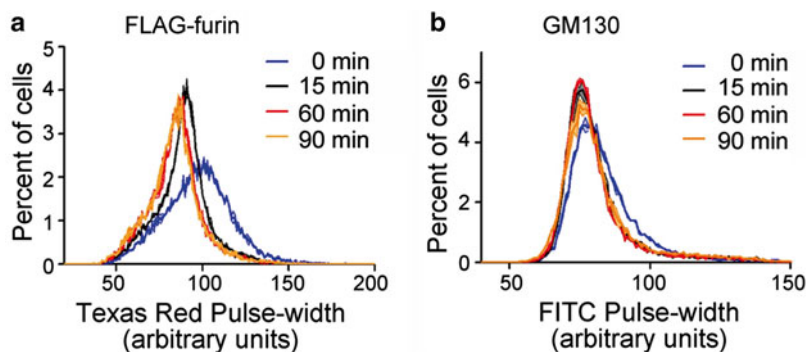


Fig. 3 Use of pulse shape analysis (PulSA) to monitor the trafficking of FLAG-furin from the plasma membrane to the Golgi apparatus in cell populations. Histograms of PulSA values for FLAG-furin and GM130 for each time point ($n=2$ mean $\pm$ SEM) are shown here. The thickness of line represents SEM of the dataset at each mean value. Figure adapted from Chia et al. [5]

are blocked in 5 % FCS/PBS for at least 30 min/RT to reduce unspecific binding of antibodies.

7. Stain cells with Alexa568-conjugated anti-rabbit IgG (Red) for 30 min/4 °C before washing with PBS.
8. The Golgi marker GM130 is stained with mouse monoclonal anti-GM130 antibodies for 30 min/4 °C, followed by Alexa488-conjugated anti-mouse IgG (green) for 30 min/4 °C. Wash with PBS once between the application of the primary and secondary antibodies (*see Note 8*). The Golgi marker, GM130, is used as an internal control to test for the pulse width of the Golgi in the cell population and also to test for changes in the Golgi during the trafficking assay. Other organelle markers for the early endosomes or late endosomes can be used as well.
9. Wash cells in PBS before resuspending in 1 % (w/v) BSA/PBS for flow cytometry.
10. Results are analyzed using the method described below. Briefly, analysis shows similar kinetics of transport for furin from the PM to the Golgi, as previously reported [7] (Fig. 3). The pulse width for the internal control—GM130—remained similar throughout all the time points indicating that the change in the pulse width of furin was due to the changes in its localization during trafficking.

3.1.2 Analysis of Golgi Disruption After Brefeldin A Treatment Using PulSA

The ability to quantitate the impact of drugs on intracellular cargo transport and organelle structure in a cell population is highly advantageous, and this ability is limited in microscopic approaches. Many agents or small molecules that inhibit Golgi trafficking often affect Golgi morphology and cause fragmentation of the Golgi. For example, Brefeldin A (BFA) is an inhibitor of Arf guanine

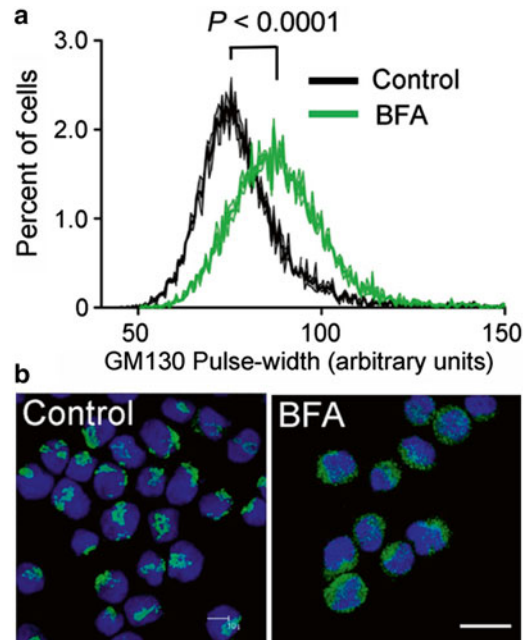


Fig. 4 Use of pulse shape analysis (PulSA) to monitor effects of Brefeldin A in cell populations. **(a)** HeLa cells were treated with 2 $\mu\text{g}/\text{mL}$ BFA or carrier alone (control) in suspension for 60 min. The cells were then fixed, permeabilized, blocked, and stained for Golgi marker, GM130 (*green*). Histograms of PulSA values for GM130 for each condition ($n=3$ mean $\pm$ SEM) are shown here. Statistical analysis is done using the median pulse-width values (median $\pm$ SEM; assessed by Student's *t*-test). **(b)** Fluorescence images are 3D reconstructions, presented as a maximum intensity projection of cells collected by cytospin. GM130 (*green*) and DAPI (*blue*). Bars represent 10 μm . Figure adapted from Chia et al. [5]

nucleotide exchange factors which play a role in membrane trafficking and organelle maintenance [8]. We describe below the use of PulSA for a high-throughput quantitative analysis of Golgi fragmentation in cell populations (Fig. 4):

1. Trypsinize HeLa cells for 4 min at 37 $^{\circ}\text{C}$.
2. Suspend cells in C-DMEM before washing cells in PBS twice.
3. Treat cells with 2 $\mu\text{g}/\text{mL}$ BFA/C-DMEM (stock solution of BFA is dissolved in DMSO) or carrier alone (DMSO)/C-DMEM for 60 min/37 $^{\circ}\text{C}$.
4. Wash cells in PBS.
5. Fix cells in 4 % PFA/PBS for 15 min/RT and then wash in PBS.
6. Permeabilize cells in 0.1 % Triton X-100 for 4 min/RT and then wash in PBS. Cells are blocked in 5 % FCS/PBS for at least 30 min/RT to reduce unspecific binding of antibodies.

7. The Golgi marker, GM130, is stained with mouse monoclonal anti-GM130 antibodies for 30 min/4 °C, followed by Alexa488-conjugated anti-mouse IgG (green) for 30 min/4 °C. Wash with PBS once between the application of the primary and secondary antibodies (*see* **Note 8**).
8. Wash cells in PBS before resuspending in 1 % (w/v) BSA/PBS for flow cytometry.

3.2 Parameters for PulSA

1. Flow cytometer should be equipped with the appropriate laser lines and filter sets needed to detect and analyze the fluorescent markers of choice. This information determines the default settings of the flow cytometer.
2. Flow cytometer must be able to detect pulse area, height, and width all at the same time for each channel that is set up (*see* **Note 2**).
3. Before the acquisition of PulSA information from experimental samples, it is important to ensure that there is no spectral spillover between the fluorescence channels set up. Compensation can be done to correct any spillover (*see* **Note 3**).
4. The flow rate used for sample acquisition may vary between cells or experiments. We have obtained similar results for any flow rate chosen on the BD LSRFortessa flow cytometer.
5. It is recommended to collect results for 50,000–100,000 cells of interest.

3.3 Gating Considerations

An outstanding feature of flow cytometry is the ability to selectively visualize the cells of interest while eliminating unwanted particles such as dead cells or debris. This procedure is called gating:

1. *Single and uniform cells*: To choose a representative cell population and to remove subcellular debris, clumps, and doublet cells, gate (Fig. 5a—P1 gate) on the main population based on the forward scatter and side scatter. The forward scatter and side scatter are usually viewed on a density plot or contour diagram. Forward scatter is proportional to the cell size, while side scatter is proportional to the granularity or inner complexity of the particle. An example of the difference in the forward and side scatter of single cells versus doublets or clumps of cells is shown (Fig. 5a). It is paramount to select single cells as doublets or clumps of cells would affect PulSA readout, since PulSA provides information with regard to particle brightness over an area (*see* **Note 9**).
2. *Cells containing protein of interest*: After gating for single cells, we use a second gate to select for the population of cells that is fluorescently labeled. Cells that are fluorescently labeled are known as

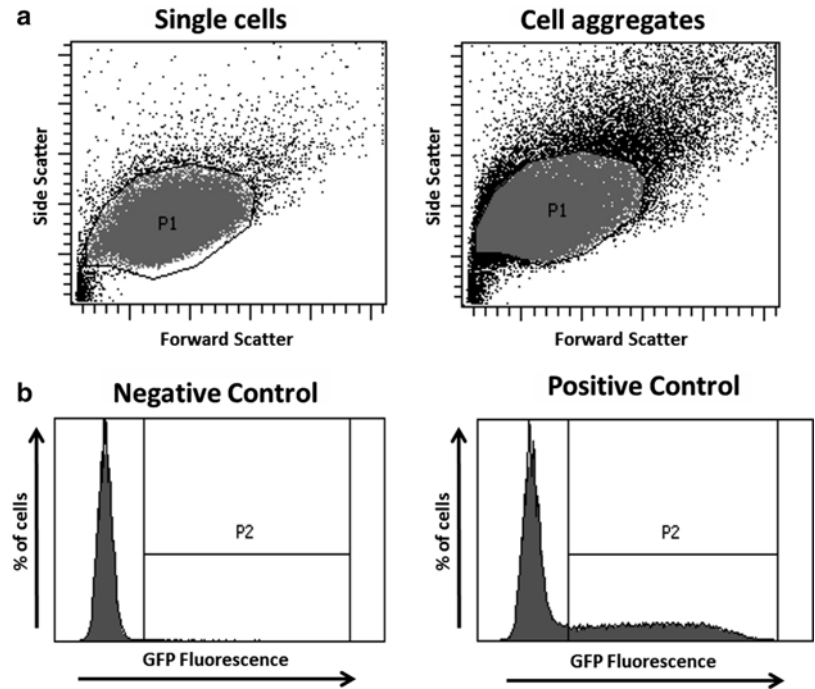


Fig. 5 Selection of cells to be included for pulse shape analysis (PuISA). **(a)** Density plots showing forward and side scatter information for a suspension of single HeLa cells and a suspension also containing cell aggregates, as indicated. The forward scatter and side scatter for cell aggregates are higher than those of single cells. They can be manually excluded for the PuISA analysis. **(b)** Typical histograms for a transfected protein of interest. By comparing with the negative control, cells that are fluorescently labeled can be gated (P2) for further analysis

the positive dataset (Fig. 5b—P2 gate). For a transient transfection, there will be a population of untransfected cells with some background fluorescence as compared to transfected cells, which form a brighter fluorescence population. This data can be viewed as a histogram of pulse area versus the count or percentage of cells. Using a negative control (untransfected cells) and a positive control (transfected cells expressing the fluorescent protein) would enable the area to be set as a gate (Fig. 5b) (*see Note 10*).

3.4 Analysis of Pulse-Width Histogram

3.4.1 Export of Pulse-Width Data into Excel

In this section, we briefly show the steps needed to export flow cytometry data for statistical analysis. For a more detailed explanation for each step, refer to Ramdzan et al. [9].

1. Open flow cytometry dataset in BD FACSDiva program and set up gates based on density plots, etc.
2. Select all sample data of interest, e.g., test samples, not the negative controls.

3. Use median rather than mean data.
4. Export FCS files as FCS 3.0:
 - (a) Gated events, e.g., P2 FITC.
 - (b) File version, e.g., FCS 3.0, not FCS 2.0.
 - (c) Parameter type: tick “none” for all parameters and “linear” for only the width readouts required, e.g., FITC-W.
 - (d) Save to the experiment folder (will create another folder within that folder).
5. Use FCS extract 1.2 to convert exported FCS 3.0 data into a format that can be recognized by Excel:
 - (a) Select “batch extraction.”
 - (b) Select directory where data folder was stored.
 - (c) Select parameters required, e.g., you may have exported DAPI, FITC, and 568, but only need to process the FITC data.
 - (d) Select process batch—data will disappear as it is processed.
 - (e) Excel (comma-separated data) will be saved into the same folder.
6. Open data in Excel.

3.4.2 Generation of Frequency Table

1. Create an Excel spreadsheet template of bin ranges (e.g., 0, 500, in 500 increments to 300,000). Use a formula to add +500 down a column.
2. Copy in columns of data from the process batch, e.g., 0 min, 30 min of a time point, triplicates of conditions, and untreated vs. treated data.
3. Calculate the frequency of bins using the Histogram analysis tool in Excel:
 - (a) Click on “Data” tab at the top of Excel page.
 - (b) Click on “Data Analysis” tab.
 - (c) Choose “Histogram” from the list.
 - (d) Select one of your data columns as the “Input Range”—note do not choose empty cells.
 - (e) Select your bin column as your “Bin Range”—note do not choose empty cells.
 - (f) Select an empty cell at the top of an empty column for the “Output Range”—this is where the generated data will be placed.
 - (g) Click “OK.”
4. The bin and frequency will be generated in the empty column.
5. Label columns so that you do not lose track of which data is being processed.

6. Copy “Frequency” data into “Scaled to Percent Population” Excel spreadsheet, containing:
 - (a) Bin range copied into the first column.
 - (b) Calculated frequency data in subsequent columns.
 - (c) Percent of population is calculated by Excel formula: $\text{cell}/(\text{sum of column}) \times 100$.
7. Copy “Percent of population” data into Prism to draw histogram graphs.

3.4.3 Presenting Histogram Values as a Scatter Plot

1. Plot bin values against percentage of population.
2. Add error bars corresponding to the standard error of the mean (SEM).

3.4.4 Statistical Analysis of Pulse-Width Differences

Differences between the median pulse widths are analyzed with the two-tailed Student’s *t*-test using the Prism software.

4 Notes

1. FCS extract 1.02 software is from Earl F Glynn, Stowers Institute for Medical Research. The program can be downloaded from <http://research.stowers-institute.org/mcm/efg/ScientificSoftware/Utility/FCSExtract/index.htm>. However, if you are using the FlowJo software, you do not need the FCS extract software.
2. An example of a flow cytometer that can collect pulse area, height, and width information simultaneously is the BD LSRFortessa. For the BD LSRFortessa, these options can be added under the Inspector—cytometer settings menu item. The BD FACSCalibur is unable to collect pulse-width information.
3. Methods to compensate for spectral spillover can be found in the BD FACSDiva Software 6.0 Reference Manual. The manual can be found in the program’s **Help** menu (**Help** → **Literature** → **Reference Manual**).
4. We normally stain cells with either primary or secondary antibodies in suspension for 30 min at 4 °C. Wash cells with 1× PBS before the application of the next antibody.
5. We recommend fixing cells with 4 % w/v PFA/PBS instead of organic solvents like methanol. Organic solvents may cause shrinkage of cells which would affect the PulSA readout.
6. It is useful to compare different methods of cell detachment as certain reagents like trypsin can cleave the protein of interest at the PM.
7. Always pre-warm SFM before adding to cells for incubation.

8. It is good to note or test whether the antibodies used are suitable for both flow cytometry and immunofluorescence. In this way, cells used in flow cytometry can be then placed on a slide for correlative immunofluorescence analysis.
9. PulSA seems to work best for large cells like HeLa. Primary cells or small cells would post some problems when interpreting the PulSA readout.
10. Additional application notes can be obtained from Ramdzan et al. [9].

Acknowledgments

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Chapter 18

Approaches to Analyze the Role of Rab GTPases in Endocytic Trafficking of Epidermal Growth Factor Receptor (EGFR)

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Abstract

The epidermal growth factor receptor (EGFR), a member of the erythroblastic leukemia viral oncogene homologue (ErbB) receptor tyrosine kinase family, plays key mitogenic signaling roles in development, cellular, and tissue physiology, as well as a myriad of malignancies. EGFR signaling occurs concurrently with ligand–receptor binding and subsequent endocytosis, and its signaling strength and engagement of different downstream signaling components are modulated by its endocytic trafficking itinerary. Understanding the factors and mechanisms that modulate ligand-bound EGFR's endocytic trafficking is therefore important for deciphering its role in pathophysiological processes. Endocytic trafficking of EGFR is regulated by a bunch of Rab small GTPases associated with the endocytic pathway. In this chapter, we describe a suite of relatively standard protocols in dissecting the role of a particular Rab protein in EGFR endocytic trafficking steps/stages. The approach constitutes a combination of genetic/molecular manipulations, followed by confocal imaging and a range of biochemical analyses. We shall mainly focus on Rab31 in our illustrations, but the approaches would be equally applicable to any Rab and its associated regulators/effectors.

Key words Confocal microscopy, Density gradient sedimentation, Epidermal growth factor receptor (EGFR), Rab, Small interfering (si)RNA

1 Introduction

Upon ligand binding, EGFR dimerization stimulates its intrinsic tyrosine kinase activity, resulting in autophosphorylation of several tyrosine residues in its intracellular C-terminal domain. These phosphorylated tyrosine residues attract signaling molecules with phosphotyrosine-binding SH2 domains, resulting in the activation of mitogen-activated protein kinase (MAPK) and other mitogenic pathways [1, 2]. Receptor cross talk also allows EGFR to be transactivated by other signaling receptors [3], and activated

EGFR could also phosphorylate tyrosine residues of other molecules in close proximity. Ligand binding triggers receptor-mediated endocytosis of the complex, ultimately culminating in the degradation of both receptor and ligand in the lysosome [2, 4]. For EGFR and several other growth factor receptors such as tropomyosin receptor kinases (Trks), signaling is not limited to the plasma membrane and does not stop upon internalization. In fact, signaling continues in the endosomes, and the strength and duration of EGFR signaling are therefore dependent on, and thus modulated by, its endocytic trafficking itinerary [4, 5].

Like the secretory pathway, endocytic trafficking is regulated by a suite of small GTPases of the Rab subfamily [6, 7], which is part of the Ras superfamily of GTPases. The human genome contains over 60 different Rab and Rab-like genes [6]. While their general working principles are known [8], the exact role and mechanisms of function of many of these are not yet well understood. In general, Rabs are activated by guanosine triphosphate (GTP)–guanosine diphosphate (GDP) exchange mediated by guanine nucleotide exchange factors (GEFs) and are inactivated by GTPase-activating proteins (GAPs). In their GTP-bound active form, Rabs engage downstream effector proteins, which include tethering factors, membrane fusion factors, and motor proteins [9]. Rab proteins that are known to be important for EGFR's endocytic traffic include the early endosomal Rab5 [10], the late endosomal Rab7 [11], and Rab11 of the recycling endosome [12]. We have recently shown that Rab31, a Rab that is phylogenetically related to Rab5 and Rab22, has a specific role in mediating EGFR trafficking from the early to the late endosome [13]. Furthermore, Rab31 appears to associate with EGFR and its effector EEA1 in a putative trafficking complex and is dependent on GAPex-5 for its function [13].

In the ensuing paragraphs, we shall describe some relatively standard protocols to functionally dissect the role of a particular Rab in EGFR endocytic trafficking steps/stages, using Rab31 for our illustrations. Rab activities and levels are amenable to some fairly standard genetic and pharmacological manipulations. Constitutively active (possible but not always) and dominant negative mutants could be generated based on analogous mutations in key conserved residues in other Rabs and other members of the Ras superfamily. Findings made with these mutants could be complemented by silencing and rescue experiments. Identification of molecular partners and functional interactions could be achieved by affinity pulldowns or co-immunoprecipitation. The consequence of these manipulations could be monitored, often with at least semiquantitative readouts by confocal fluorescence microscopy imaging in conjunction with a range of common biochemical analytical methods.

2 Key Materials and Equipment

A brief listing and description of materials, reagents, and equipment that are key or unique for the approaches are given below.

2.1 Cell Lines

The human epidermoid carcinoma cell line A431 (ATCC® CRL1555™), which expresses high levels of EGFR, has been used extensively in the study of mitogenic and oncogenic signaling [1]. The human cervical cancer line HeLa (ATCC® CRMCL2™) expresses much less EGFR and is often useful for comparative analyses. Both cell lines are sturdy and readily cultured in either DMEM or RPMI medium with 5–10 % fetal bovine serum (FBS) under standard conditions. Their well-spread, epitheloid shapes are well suited for membrane compartment staining and analysis at the light microscopy level. The above cell types are the most commonly used for studying EGFR trafficking, but other cell types may serve specific investigations (*see Note 1*).

2.2 Molecular, Immunological, and Biochemical Reagents

For silencing experiments, Dicer substrate RNAi 27-mer RNA duplexes (Integrated DNA Technologies, Inc., Coralville, IA, USA) could effectively achieve population-wide gene silencing by transient transfection. Alternatively, small hairpin RNA (shRNA) expressing constructs or viral vectors may be used (*see Note 2*).

2.2.1 Molecular Reagents

2.2.2 Immunochemicals

1. Anti-EGFR antibodies.
2. Antibody against Rab protein of interest. Many Rabs have commercially available antibodies, although the quality of any of these has to be empirically determined. For generating “homemade” antibodies, the reader is referred to relevant chapter in the issue “Antibody methods and protocols” [14].
3. Antibodies against early endosome markers (e.g., Rab5, EEA1) and late endosome markers (e.g., CD63/Lamp-3).

2.2.3 Biochemicals

1. EGF conjugated to fluorophores (EGF-FITC, EGF-Texas Red, or EGF-Alexa Fluor 488/568).
2. GTPγS (a non-hydrolyzable analogue of GTP).

2.3 Confocal Microscope System and Image Analysis Software

For very basic or qualitative analysis of immunostained fixed cells, any fluorescence microscope with a digitized readout will suffice. Confocal imaging, however, offers high resolution that would be particularly useful in colocalization analyses. As we have used the Carl Zeiss LSM710 confocal system, the description of imaging analysis in Subheading 3.2 below shall be largely based on this

system (*see* **Note 3**). Quantitative and semiquantitative image analyses are often necessary and could be performed with software provided by the vendor or those in the public domain (such as ImageJ (NIH, Bethesda, MD, USA) plug-ins).

3 Methods

A description of the key approaches and their associated protocols are given below.

3.1 Manipulation of Rab Levels and Activity by Mutant Constructs and RNA Interference/ Rescue

Based on analogous activating/inactivating mutations first discovered in Ras, standard mutations of key residues that would predictably render a Rab constitutively active or inactive could be generated. Disrupting the GTPase activity of a Rab (typically by changing a key catalytic residue equivalent to H-Ras, Q61 → L) would generate a constitutively active mutant. This, however, need not always be the case [15], and it is best that increased Rab activity is verified by GTPase assay or some other functional assays. On the other hand, GTP-binding mutations (typically an equivalent of H-Ras S17 → N) would render the Rab inactive. When transiently expressed in cells, these mutants can dominantly affect the Rab function positively or negatively as their high levels of expression overwhelm the endogenous protein.

While potentially useful, overexpression of Rab and its mutants would also likely lead to nonspecific, gain-of-function phenotype. One reason why this is so is because Rabs do share regulators and effectors, and overexpression could simply be massively disruptive in terms of stoichiometric associations required for normal function. A complementary approach is to perform RNAi-mediated silencing of Rabs and to show that the resulting phenotype or deficiency could be reversed or “rescued” by an exogenous transcript that is not susceptible to degradation by the RNAi method used.

3.1.1 Rab Mutants

Generation of Rab mutants in mammalian expression constructs can be routinely achieved by PCR and nested PCR-based mutagenesis or the use of site-directed mutagenesis kits available from multiple vendors. The various cloning techniques involved are the subject of other issues in these series, and the reader is referred to the chapters in these [16]. If there is an intention to add an epitope tag to the Rab or its mutant to facilitate detection, important points to note are:

1. Tagging, if at all, should be done at the N-terminus, as the C-termini of Rabs contain the signal for geranylgeranylation.
2. Tagging may disrupt functional properties of certain Rabs, at least partially. Although it would be difficult in mammalian cells to perform functional complementation analysis as is routinely performed in yeast, some salient functional features

could still be checked to ensure that tagging was not grossly disruptive. One of these is cellular localization, which is quite specific for some, but not all Rabs, and could be readily verified by confocal imaging (*see* Subheading 3.2 below). If the Rab has known interacting partners and effectors, biochemical approaches (*see* Subheading 3.3 below) could also be used to verify integrity of function upon tagging.

3.1.2 Manipulation of Rab Levels with RNAi Silencing/Rescue Experiments

1. Seed cells on glass coverslips such that it is ~30–40 % confluent the next day.
2. Transfect cells with siRNA using a suitable transfection reagent (such as Lipofectamine® RNAiMAX Reagent (Life Technologies) or infection with viral particles generated with retro- or lentiviral constructs). The degree of silencing can be estimated by Western immunoblot as described in Subheading 3.3 below or by quantitative reverse transcription polymerase chain reaction (RT-PCR) (*see* **Note 4**).
3. Perform imaging analysis as outlined in Subheading 3.2 below.
4. *Rescue experiments*: the nature of the rescue construct would vary depending on the nature of the silencing method (*see* **Note 5**). Typically, rescues are done by transfecting or infecting siRNA-/shRNA-treated cells for 24 h with expression constructs of a non-RNAi degradable form of the Rab (or infected with viruses expressing such) and the reversal of phenotype assessed as in Subheading 3.2 below.

3.2 Confocal Imaging Analysis of Ligand-Bound EGFR

3.2.1 Pulse–Chase Experiments and Compartment Localization of Ligand-Bound EGFR

1. Seed cells on glass coverslips and grow to ~60–80 % confluency. Serum-starve cells overnight in basal DMEM.
2. Incubate cells with 0.25–0.5 µg/ml of Texas Red (TxR) or FITC-tagged epidermal growth factor (EGF), or nonconjugated EGF, on ice for 20 min to allow ligand–receptor binding (*see* **Note 6**).
3. Transfer cells to 37 °C for internalization for 5 min.
4. Acid wash (150 mM NaCl, 50 mM glycine, pH 3.0, ice-chilled) cells to remove un-endocytosed EGF before incubating in complete DMEM pre-warmed to 37 °C for the chase.
5. Fix cells at various time points with 4 % paraformaldehyde. Wash cells with 50 mM NH₄Cl to quench autofluorescence caused by unreacted aldehydes. After rinsing with phosphate buffered saline (PBS), permeabilize cells with 0.1 % saponin to permit antibody access to antigen.
6. Label fixed cells with primary antibodies for colocalization analyses, followed by secondary antibodies with the desired fluorescence tag, in blocking buffer consisting of 5 % fetal bovine serum (FBS) and 2 % BSA in PBS. To track the location of EGF/EGFR complexes within the cell, primary antibodies

against early endosome markers (e.g., EEA1) or late endosome markers (e.g., CD63) can be used for colocalization studies with the tagged EGF/EGFR complex.

7. View and image cells under the microscope. At different chase times, ligand (EGF)-bound EGFRs (marked by fluorescence-labeled EGF) accumulate in membranous endosomal structures with different morphologies, which can be verified based on size (*see* Fig. 1) and specific marker co-labeling (*see* Fig. 2).

3.2.2 Membrane Feature and Colocalization Analyses of Ligand-Bound EGFR with Compartment Markers

With the confocal microscope system, collect images in separate *z* sections. Final analysis of images could be from collapsed *z* stacks or orthographic projections. For the LSM710 system and other systems from the same vendor, image processing was done using Zen 2010 software (Carl Zeiss):

1. Size of membrane puncta can be measured using ImageJ (US NIH, Bethesda, Maryland, USA), which enables the user to perform particle size distribution analyses.
2. Specific marker co-labeling can be performed to identify the membranous structures in which EGF-bound EGFR is found. For example, the early endosomal marker EEA1 (early endosomal antigen 1) or late endosomal marker CD63/Lamp-3 can be used. Alternatively, other endosomal localized Rabs, such as Rab11 in the recycling endosome, can also be used. Visual inspection or calculation of overlap coefficient can be performed using the Zen 2010 software to determine the percentage of EGF/EGFR membranous structures bearing the various markers.

3.3 Biochemical Analysis of Rab-EGFR Complexes

To identify, confirm, or study protein–protein interaction between Rab proteins and their regulators and effectors, the simplest and cheapest methods involve affinity pulldown and co-immunoprecipitation. Both methods are of low to moderate sensitivity and rely on the interactions investigated being sufficiently stable in cell lysates (*see* Note 7). Co-immunoprecipitation, in addition, relies on the availability of suitable antibodies. Both methods could at best infer biochemical associations between

Fig. 1 (continued) of the different localizations of EGF/EGFR complex as it is trafficked from cell surface (0 min) to early (10 min) to late endosome (30 min). Colocalization between the ligand, EGF, and the receptor, EGFR, is observed at all time points, indicating that EGFR remains ligand associated. Scale bar = 20 μ m. **(b)** Size of puncta can be quantified using ImageJ to determine the effect of various treatments on EGFR trafficking. A431 cells transfected with scrambled (Scr) or Rab31 siRNA were pulsed with 0.5 μ g/ml EGF–TxR and fixed 30 min after pulse. Size of EGF puncta was quantified and presented here as a distribution of the number of puncta at various sizes. Depletion of Rab31 shifted the distribution of puncta to smaller sizes, suggestive of inhibited trafficking to the late endosome

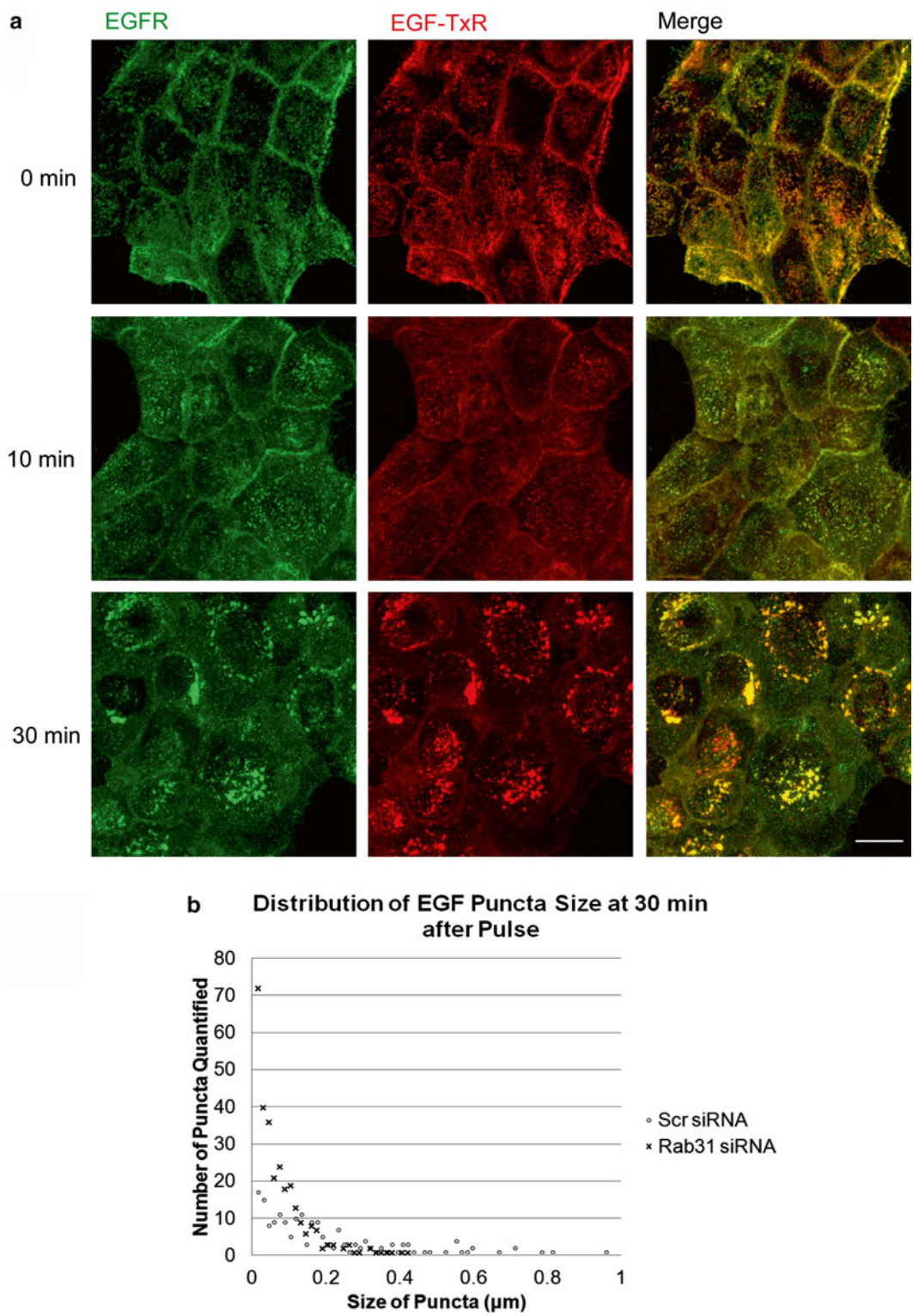


Fig. 1 Pulse-chase and puncta size analysis of EGFR traffic with EGF-TxR. **(a)** A431 cells were pulsed with 0.5 $\mu\text{g}/\text{ml}$ EGF-TxR, (red) and fixed at various time points (0, 10, and 30 min after pulse) for immunofluorescence labeling of EGFR (green). Change in distribution and size of the EGF and EGFR-positive puncta is indicative

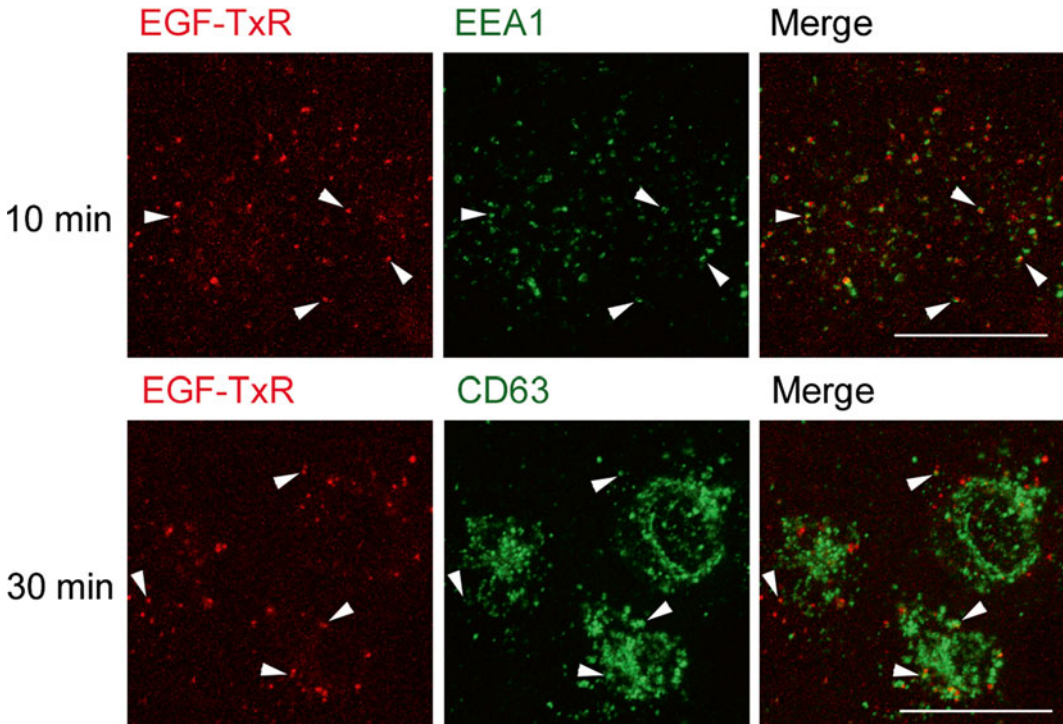


Fig. 2 Analysis of EGFR entry into early and late endosomes. (a) A431 cells were pulsed with 0.5 $\mu\text{g/ml}$ EGF–TxR (red) and fixed after a 10 min chase for immunofluorescence analysis with the co-labeling of early endosome marker EEA1 (green). Arrowheads indicate examples of colocalization between EGF (and by extension, EGFR) and EEA1, indicative of entry into the early endosome. Scale bar = 20 μm . (b) Cells were pulsed with 0.5 $\mu\text{g/ml}$ EGF–TxR (red) followed by a 30 min chase before fixation and immunofluorescence analysis with the co-labeling of the late endosome marker CD63 (green). Arrowheads indicate examples of colocalization between EGF (and by extension, EGFR) and CD63, indicative of entry into the late endosome. Scale bar = 20 μm

two proteins, without any confirmation of direct physical interaction, and cannot be extrapolated to surmise the formation of a multiprotein complex containing more than two proteins. The formation of a multiprotein complex could be further gleaned from a shift from a monomeric, low molecular size fraction to a higher molecular size fraction by density gradient sedimentation analysis.

For Rabs, interactions with effectors, in particular, could be enhanced or stabilized with the addition of the non-hydrolyzable GTP analogue, GTP γ S. GTP γ S effectively locks the Rab in an activated form. Resultant increases in Rab interaction in the presence of GTP γ S would be deemed “GTP dependent,” attesting to the specificity of the observed interaction. A concomitant shift of Rabs and other factors shown to be associated with these by affinity pull-down or co-immunoprecipitation to a higher molecular size fraction upon exposure to GTP γ S could further support the notion of a multipartite complex formation. The procedures for the abovementioned approaches are outlined below.

3.3.1 Affinity Pulldowns

1. Prepare affinity bait constructs by conjugating Rab protein (and its mutants) of interest (or other factors of interest) to affinity tags such as glutathione S-transferase (GST) in suitable vectors (such as the pGEX4T series, GE Healthcare, Singapore). Refer to [16] for general cloning techniques.
2. Express and affinity purify the GST fusion proteins in a suitable *E. coli* host strain (such as BL21-DE3) using glutathione immobilized on agarose or sepharose beads. The GST-Rab and its mutants could be used directly for affinity pulldown with cell lysates.

If the tagging is known to affect the function of Rab, the GST tag may be cleaved with the appropriate protease (such as thrombin or factor X). Subsequently, the cleaved Rab can be coupled to cyanogen bromide (CNBr)-activated sepharose beads.

3. Lyse cells with lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl, and 1 % Triton X-100 with a protease inhibitor cocktail (Roche Diagnostics, Alameda, CA, USA)) for 60 min.
4. Treat an aliquot of the lysate with 1 mM GTP γ S (Merck Millipore, Singapore), and dilute to a final solution containing 0.5 % Triton X-100. Have a control without GTP γ S.
5. Incubate lysate with GST-Rab on glutathione sepharose or Rab bound to CNBr sepharose at 4 °C for 24 h in a microcentrifuge tube.
6. Pellet the beads down with a benchtop microcentrifuge. Wash with lysis buffer with 0.5 % Triton X-100, followed by lysis buffer without Triton X-100 (or for convenience, PBS can be used).
7. Elute bound proteins at 72 °C with 1 $\times$ SDS sample buffer (50 mM Tris pH 6.8, 2 % SDS, 10 % glycerol, 0.02 % bromophenol blue), and resolve by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins can be identified by Western immunoblot (*see* below) with suitable antibodies. Always check for loading of GST fusion proteins by Coomassie blue/Ponceau S staining of the eluate if GST fusion protein is used for affinity pulldown (*see* Fig. 3a).

3.3.2 Co-immunoprecipitation

1. Lyse cells with lysis buffer and treat an aliquot of the lysate with GTP γ S as above. Have a control without GTP γ S.
2. Incubate lysate with Rab (or other) antibodies at 4 °C for 24 h. Add protein A (for rabbit antibodies) or protein G (for mouse antibodies) sepharose slurries to the lysate, and continue the incubation for another 2 h in a microcentrifuge tube.
3. Wash with lysis buffer with 0.5 % Triton X-100, followed by lysis buffer without Triton X-100 (or for convenience, PBS can be used).

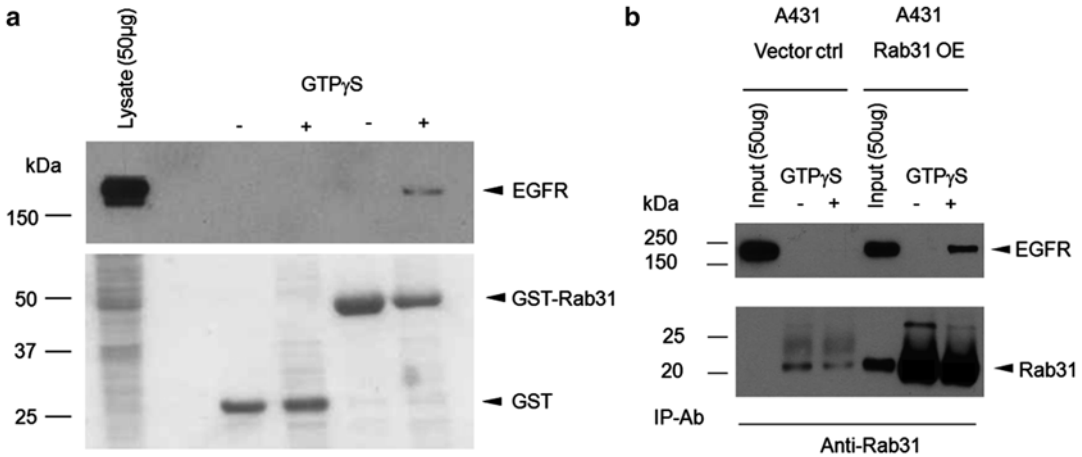


Fig. 3 GTP-dependent engagement of effectors. GTP-dependent engagement of effectors by Rab proteins can be determined by affinity pulldowns or co-immunoprecipitation. Here, the ability of Rab31 to engage EGFR in a GTP-dependent manner is shown. **(a)** 1 mg of A431 cell lysate with and without GTP γ S was incubated with 20 µg GST or GST-Rab31 and glutathione beads, and the ability of the GST fusion proteins to pull down EGFR was analyzed by Western blot. The GST fusion proteins were visualized with Ponceau S stain. **(b)** EGFR was co-immunoprecipitated with Rab31 antibody, using 1 mg lysates from cells transfected with vector alone (Vector ctrl) and Rab31 (Rab31 overexpression (OE)), respectively, with or without GTP γ S loading (*Note: The figure originally appeared in the authors' earlier paper [13]*)

4. Elute bound proteins, and resolve by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as above. Proteins can be identified by Western immunoblot (*see below*) with suitable antibodies. For Western immunoblot, always probe also for the primary target of the antibody use (*see Note 8*) (*see Fig. 3b*).

3.3.3 Western Immunoblot

1. Load 25–100 µg of total protein from lysate in an SDS–PAGE of an appropriate percentage, and electrophorese to resolve proteins.
2. Electroblot proteins onto nitrocellulose membranes, e.g., Hybond-C extra (Amersham Biosciences, UK), followed by blocking the membrane with 5 % skimmed milk powder in PBS with 0.05 % Tween 20 (PBST). Probe the blocked membrane with primary antibodies diluted in 3 % BSA in PBST, followed by labeling the primary antibodies with a horseradish peroxidase (HRP)-conjugated secondary antibody (Thermo Scientific) diluted in 5 % skimmed milk powder in PBST.
3. Western blot signals could then be detected by the enhanced chemiluminescence detection system and imaged with instruments such as ChemiDoc MP (Bio-Rad, Hercules, CA, USA) or by exposure to an X-ray film.

3.3.4 Density Gradient Sedimentation Analysis

1. Lyse cells with lysis buffer (10 mM HEPES, 2 mM MgCl_2 , 10 mM KCl, 0.5 mM EDTA, 150 mM NaCl, and 1 % Triton X-100).
2. Treat an aliquot of the samples with 1 mM GTP γ S (Merck Millipore), and dilute to a final solution containing 0.5 % Triton X-100. Have a control without GTP γ S.
3. Prepare gradient in 14×89 mm ultracentrifuge tubes for a swing-out rotor (e.g., Beckman SW41Ti rotor) by gradual layering of 1 ml of 45, 41, 37, 33, 29, 25, 21, 17, 13, 9, and 5 % glycerol solutions prepared in lysis buffer.
4. Load 2 mg of lysate (in a volume of 200 μ l) onto glycerol gradient column. Perform ultracentrifugation for 18 h at 178,000×*g* maintained at 4 °C. Run molecular size standards applied to another glycerol gradient in parallel.
5. Collect 1 ml fractions, and precipitate proteins in a final concentration of 20 % trichloroacetic acid (TCA) on ice (*see Note 9*).
6. Pellet the precipitate by centrifuging at 16,000×*g* for 15 min. Wash three times with cold acetone. Redissolve in PBS with 1× SDS sample buffer.
7. Resolve proteins by SDS–PAGE and probe by Western immunoblot analysis as above (*see Fig. 4*).

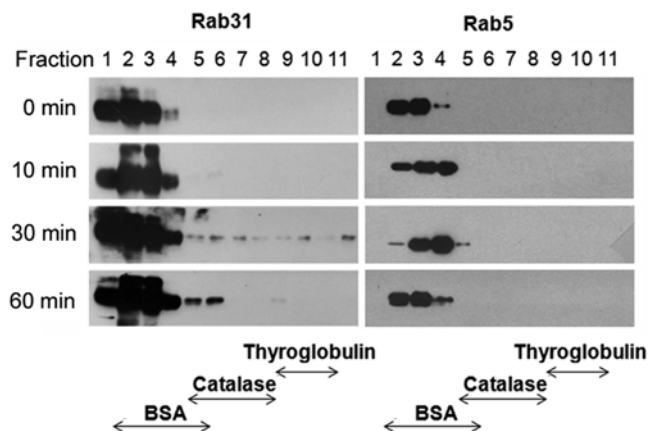


Fig. 4 Density gradient sedimentation analysis. A431 cells overexpressing Rab31 cells were pulsed with 0.5 μ g/ml EGF and harvested at various time points, with and without GTP γ S. 2 mg of lysates were separated by glycerol gradient sedimentation. Fractions were collected, TCA precipitated, and analyzed by Western blot for Rab31 and Rab5. The position in the gradient that contains the molecular size markers bovine serum albumin (BSA) (67 kDa), catalase (240 kDa), and thyroglobulin (660 kDa) is indicated. The results suggest that Rab31 can be found in high-molecular-weight complexes in the presence of GTP γ S, but not Rab5, and that this complex forms 30 min after pulse with EGF

4 Notes

1. For example, for the studies on polarized EGFR transport in apico-basolateral trafficking in epithelial cells, the Madin-Darby canine kidney (MDCK) cells have been used [17].
2. Both retroviral and lentiviral vectors could be used for these proliferating cell lines.
3. Several other vendors have comparable setups (e.g., Leica Microsystems, Olympus, Nikon Instruments, and Molecular Devices).
4. Silencing conditions (amount of reagents used and time of incubation) need to be optimized. Typically the decrease in protein level, rather than mRNA, is monitored, as protein turnover may be much slower, particularly for abundant house-keeping proteins.
5. If the silencing method targets the coding region, the rescue construct would need to generate a transcript that will not be recognized by the si-/shRNA. One could of course make mutations, but one simple way is to use the cDNA of the orthologue of another species (e.g., mouse orthologue to rescue silencing in human cells). However, if the RNAi method targets an untranslated region, the coding region itself without the untranslated parts could be used.
6. Concentration of EGF used may need to be optimized. Typically, the concentration of EGF used ranges from 0.05 to 0.5 $\mu\text{g}/\text{ml}$. It has been shown that the levels of EGF affect whether clathrin-mediated or clathrin-independent internalization of the ligand-bound receptor predominates, and subsequent recycling versus degradation of the ligand-bound receptor also varies depending on ligand concentration.
7. Biophysical methods, such as those based on surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR), require sophisticated equipment and smaller fragments of the interacting proteins, but could investigate direct physical associations.
8. This is largely for the purpose of normalization. Not all antibodies can perform immunoprecipitation (and this therefore needs to be verified), and there is a chance that antibody binding fortuitously disrupts the interaction being investigated.
9. This is a simple method that generates relatively less volume of solutions to work with. However, if the acid precipitation destroys the antibody epitope, other precipitation methods such as acetone or chloroform/methanol precipitation needs to be considered.

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Part II

Microscopy Methods and Imaging Approaches

Does Super-Resolution Fluorescence Microscopy Obsolete Previous Microscopic Approaches to Protein Co-localization?

Laura MacDonald, Giulia Baldini, and Brian Storrie

Abstract

Conventional microscopy techniques, namely, the confocal microscope or deconvolution processes, are resolution limited to approximately 200–250 nm by the diffraction properties of light as developed by Ernst Abbe in 1873. This diffraction limit is appreciably above the size of most multi-protein complexes, which are typically 20–50 nm in diameter. In the mid-2000s, biophysicists moved beyond the diffraction barrier by structuring the illumination pattern and then applying mathematical principles and algorithms to allow a resolution of approximately 100 nm, sufficient to address protein subcellular co-localization questions. This “breaking” of the diffraction barrier, affording resolution beyond 200 nm, is termed super-resolution microscopy. More recent approaches include single-molecule localization (such as photoactivated localization microscopy (PALM)/stochastic optical reconstruction microscopy (STORM)) and point spread function engineering (such as stimulated emission depletion (STED) microscopy). In this review, we explain basic principles behind currently commercialized super-resolution setups and address advantages and considerations in applying these techniques to protein co-localization in biological systems.

Key words Super-resolution microscopy, Fluorescence microscopy, STORM, SIM, STED, Confocal microscopy

1 Introduction

In practice, protein co-localization within cells is most commonly done by fluorescence microscopy, a technique in which the planar distribution of light emitted by protein-specific fluorophores is compared [1]. Until recently, this approach had a resolution of no better than 200 nm due to the diffraction properties of light. As stated by Ernst Abbe in 1873, diffraction prevents resolution of two objects if their distance apart is less than half of the full width half maximum (FWHM) generated by their point spread function [2]. Fluorescence microscopy cannot alter physics. However, when combined with the cleverness of the human mind, the diffraction limit can be overcome. Here, we consider any technique that

provides a resolving power beyond that of the diffraction limit to be super-resolution microscopy.

In reality, the development of fluorescence microscopy can be compared to the process of peeling an onion in which one layer after another is revealed. In practice, the first limit was not diffraction but brightness. In the early 1970s, the introduction of epifluorescence microscopy revolutionized the field by overcoming much of the brightness limitations at high magnifications (40–100 $\times$), making possible localization studies at the full diffraction-limited resolution of the light microscope. In this configuration, the objective serves both as the condenser and objective, and the light source was placed 90° to the objective. Light was reflected onto the specimen using a dichroic mirror. This arrangement minimized background contributions from the excitation light, and more importantly, the intensity of the collected fluorescence light now increased with the objective's numerical aperture. In contrast, for previous optical configurations in which the condenser and objective were separate optical elements, fluorescence intensity failed to increase with numerical aperture of the objective, and brightness decreased with magnification [3]. With these advances, routine studies of protein co-localization became possible with a resolution approaching 200 nm in nearly flat, two-dimensional space, conditions found most commonly at the periphery of well-spread tissue culture cells.

Cells, though, are not two-dimensional objects. Even tissue culture fibroblasts are thicker toward the cell center where the nucleus is located. Cell types such as epithelial cells lining an organ (e.g., the intestine) are columnar in three-dimensional space. The overall planar resolution of the epifluorescence microscope, often simply referred to as the wide-field microscope, is limited in practice by sample thickness. Light from both above and below the plane of focus is collected. As shown in Fig. 1a, this affects image acuity even for small cells such as human platelets that are discoid in shape with a thickness of about 1.5 μm and a diameter of about 3 μm . Hence, an image co-localizing two different α -granule proteins, von Willebrand factor and fibrinogen, in an isolated platelet appears hazy making the extent of co-localization difficult to assess. Deconvolution algorithms in which the light distribution is corrected on the basis of the point spread function allow for contrast improvement as shown in Fig. 1b, but not a gain in resolution [4].

In this review, we compare developing super-resolution techniques including structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), and photoactivated localization microscopy/stochastic optical reconstruction microscopy (PALM/STORM) with conventional wide-field and confocal microscopy. We briefly explain the basic principles of each technique and consider advantages and challenges in confronting these new possibilities in microscopy. Microscope setup and probe use

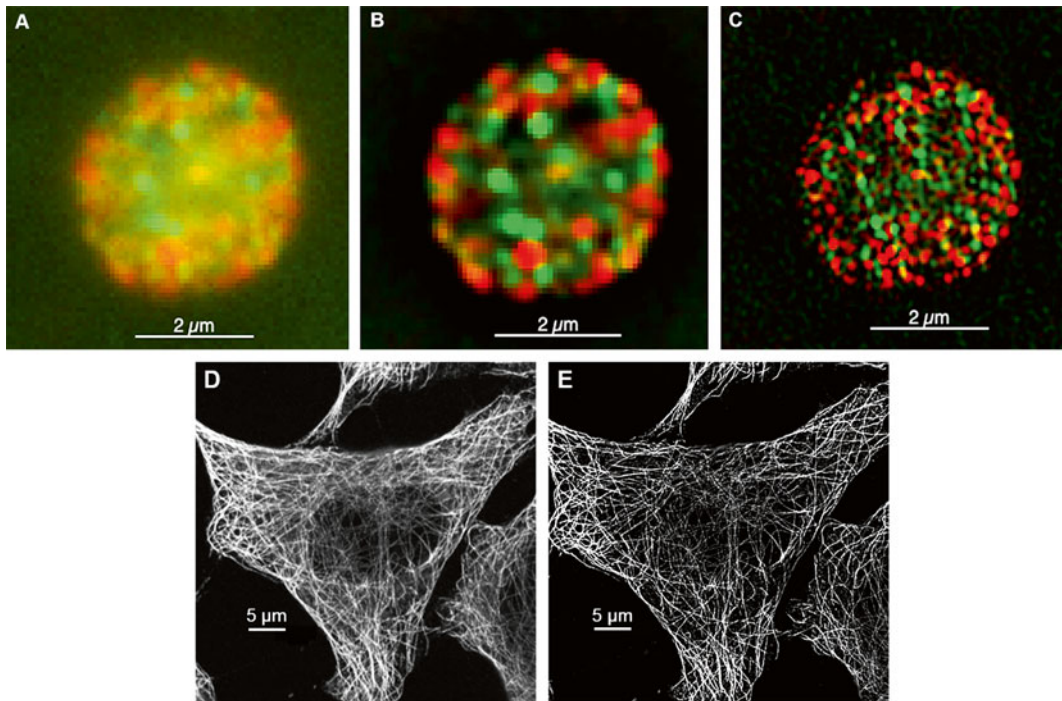


Fig. 1 SIM technology increases resolution in the XY dimension and increases contrast over conventional techniques. A single image plane from a human platelet viewed by wide-field microscopy (**a**), after deconvolution (**b**), and by 3D SIM (**c**). As a resolution example, we scanned microtubules in cells by either confocal microscopy (**d**) or 3D SIM (**e**). These 24 nm objects imaged by Cy3 emission (*red*) had an apparent XY width (diameter) of 250 nm by confocal microscopy and 100 nm resolution by 3D SIM demonstrating approximately twofold better XY resolution than that achieved by confocal microscopy

are illustrated by examples from our own work in cellular membrane trafficking and platelet biology. Biological applications to the problem of protein co-localization in cells are highlighted. We close our review with a brief commentary on additional tools needed to make super-resolution techniques truly accessible to the general life science community.

2 Conventional Light Microscopy: Wide-Field + Deconvolution and Confocal Microscopy

Current microscopy techniques take advantage of high numerical aperture objectives, refractive index-matched mounting media, and immersion oils to approach the diffraction barrier of approximately 200 nm [2]. In practice, obtaining resolution is easier at shorter wavelengths of light as predicted quantitatively by Abbe's equation for diffraction-limited resolution. Unfortunately, dyes that fluoresce at shorter wavelengths have lower quantum yield and often have poor photostability that negates increases in exposure time.

As proteins and nucleic acids absorb in the UV range, the use of ever shorter wavelengths of light is not possible. Collectively, this limits resolution in the XY dimension. Resolution in the Z dimension is distinctly worse, approaching only 500–700 nm [5]. The optical system of the laser scanning confocal microscope in which pinholes are used to eliminate out-of-focus light when combined with the algorithms of deconvolution software is presently the workhorse of conventional techniques. Pinpoint apertures are used to exclude background light emitted from fields below and above the plane of interest [3, 6]. A three-dimensional image set is obtained by moving the focal plane up and down through the specimen in the Z dimension, giving a stack of images. In brief, these techniques provide gains in signal to noise ratio and a reduction of background signal. Image stacks of 10–50 μm thickness can be obtained and three-dimensional images generated by rendering techniques. Deconvolution is sufficiently powerful to mathematically “haze-bust” wide-field images as shown in Fig. 1b [4]. These techniques lend themselves to answering general questions about localization of proteins to membranes or organelles as well as three-dimensional structure of a whole sample. As discussed previously, algorithms can then be used to quantitatively compare protein distributions at the individual pixel or voxel level of the images [1].

However, conventional fluorescence microscopy cannot address questions regarding protein complex dynamics as molecular machines. The average molecule is approximately 2 nm in the XY dimension. If we consider an assembly of proteins resulting in a 50 nm complex, the diffraction limit of 200 nm results in a point spread function nearly four times larger in each individual dimension than the average molecule. One can easily see the impossibility of resolving individual complexes in the three-dimensional space of a cell under these limitations. The objective, then, of super-resolution microscopy is to bring these resolution parameters to a level more closely approaching the underlying protein complexes being studied. With small fluorescent probes, recent work suggests that the resolution of fluorescence microscopy, nearing approximately 10 or 20 nm, can approach that of electron microscopy. In localizing near the molecular level, fluorescence microscopy has the obvious benefit of being a nondestructive technique that can support live cell imaging.

3 Super-Resolution Microscopy Approaches

There are three emerging approaches to super-resolution microscopy: structured illumination microscopy (SIM), localization based using either photoactivated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM), and point spread function engineering (stimulated emission depletion

microscopy or STED). These three approaches are currently being commercialized for use in biological sciences. Most of these use mathematical approaches to extend resolution beyond the diffraction limit. SIM improves resolution to 100 nm in the XY direction and 250 nm in the Z direction [7], whereas PALM/STORM techniques improve resolution to 20–50 nm in the XY direction and 50 nm in the Z dimension [8, 9]. As a comparison, STED improves resolution to less than 50 nm in the XY direction under optimal conditions [10]. We highlight basic concepts underlying super-resolution techniques in this section.

3.1 Structured Illumination Microscopy (SIM)

SIM techniques take advantage of the physical observation of Moiré fringes [11], and biophysicists have applied these observations to calculate light distribution. The general principle is that a grid pattern can be superimposed upon another grid pattern at different angles to yield a third pattern containing Moiré fringes. These three patterns are mathematically related such that if the spatial frequencies of two of the three patterns are known, the frequency of the third can be back calculated. Thus, in SIM, light is structured upon a sample (pattern 1) in a grid pattern (pattern 2), and the resulting Moiré fringe (pattern 3) is collected via the objective in different planes [7]. An associated advanced workstation and software package calculate the three-dimensional structure of the sample based on the presented Moiré fringes and imposed light grid. The sample is examined from multiple angles to improve resolution in the XY and Z direction. SIM is limited primarily by the illumination pattern, which is deliberately chosen to be close to the diffraction limit, resulting in a finer pattern and a higher gain in resolution. Illumination patterns are introduced in the focal plane by one of several methods. Gustafsson's method is to project a diffraction grating with multiple rotations on the rear aperture of the objective [12], while other variations take advantage of spatial light modulators to move the pattern [13]. Using spatial modulators is advantageous so that samples and microscope components need not be rotated but may in practice be limited by pixel density and the flatness of the spatial light modulator. The last method that has been employed is to introduce the illumination pattern into the focal plane using total internal reflection fluorescence (TIRF) microscopy [14]. The spatial frequency of the imposed pattern can be increased by a factor of 2, and therefore a corresponding increase in resolution results in the XY and Z dimensions would give a total of eightfold increase in overall resolution [15]. Thus, SIM increases resolution in the XY dimension to 100 nm and the Z dimension to approximately 250 nm.

3.2 Super-Resolution Techniques Based on Localization Mapping

First we must address the concept of reversible saturable optical fluorescence transitions (RESOLFT). Briefly, the principle is that fluorescent molecules can be turned reversibly “on” in an excited, fluorescent state and “off” in a ground, nonfluorescent state.

Importantly, at high light excitation intensity, the probability of a molecule returning to the ground state is reduced [16]. Probes used for super-resolution microscopy are chosen because these have the ability to switch repetitively between the “on” and “off” without being destroyed. These concepts are of utmost importance for techniques based in single-molecule localization microscopy.

3.2.1 PALM/STORM

PALM/STORM techniques have been compared to the pointillism techniques developed in the 1880s by the painters Georges Seurat and Paul Signac. If we consider Abbe’s law of diffraction, resolution is limited by how close together two molecules are. For example, consider a protein cluster of five fluorescent proteins as shown in Fig. 2. The proteins in most organelles will be too close together to resolve effectively if the entire protein population is excited. Using the PALM technique, only a subset of proteins will be randomly excited at a time and recorded at that point in time and space. This subset is then turned off by a short-wavelength, high-intensity laser and a second subset randomly activated. In this manner, it is possible to visualize disperse proteins one at a time, isolate the individual molecule’s point spread function, and then mathematically predict the XY center of that emission event [8, 9, 17]. The success of PALM/STORM depends on photo-switchable (PS) and photoactivatable (PA) proteins or dyes. A photoactivatable probe exists natively in the inactive and nonfluorescent state. Upon brief laser excitation, the fluorophore becomes active and, if irradiated sufficiently long, will be photobleached [17]. In contrast, a photoswitchable fluorophore starts in an active, fluorescent state (state A) and, upon laser excitation, switches to a second active fluorescent state (state B) [18, 19]. Lasers are used to stochastically, i.e., randomly, excite “blinking” fluorescent molecules in the sample. Only a random subset of the molecules will be active and fluoresce. The image is recorded, and the molecules



Fig. 2 Diagram of RESOLFT and single-molecule localization principles. Five GFP proteins clustered together are shown in the first panel. The idea behind PALM/STORM technologies is that only a subset of proteins is turned “on” by a laser. This subset is imaged and recorded and is then turned “off.” The laser then activates a second random subset, and an image is recorded. An off-line microscope workstation compiles the data to regenerate a full image

return to a ground or “dark” state. The laser is again used to excite a new subset of molecules. The same field is imaged over thousands of frames, and an image is constructed based on the region of space molecules occupied relative to one another. In this way, coordinates of a few molecules are recorded at a time until all of the molecules are imaged to give a complete molecular map. Overall, PALM/STORM techniques could ideally bring resolution in the XY dimension to 20 nm [8, 9].

3.2.2 *Specific Considerations for Successful PALM/STORM*

The success of PALM/STORM techniques depends on high molecular labeling density and low nonspecific sample background [20]. Additionally, the switching rate, switching contrast, and image buffer are important considerations when choosing probes for PALM/STORM. Photobleaching is prevented using an oxygen-scavenging buffer system. However, it should be noted that different probes demand different buffering conditions [21]. For example, rhodamine and oxazine dyes (most Alexa Fluor and ATTO dyes) blink very well under a cysteamine, glucose oxidase, catalase buffering system, whereas the carbocyanine dyes (Cy5, Cy5.5, Alexa Fluor 647) blink optimally when an alternate enzymatic oxygen-scavenging system is employed [22, 23]. For two-color imaging, matching buffers is crucial for success. An explanation of photo-switching mechanisms and brief commentary of which buffer conditions are optimal for conveniently available dyes are provided in reference [20].

3.2.3 *PALM vs. STORM*

PALM differs from STORM primarily in probe choice. The original PALM method makes use of GFP variants including Dronpa fluorescent protein, photoactivatable GFP, photoswitchable cyan fluorescence, and photoactivatable mCherry1 [24–28]. Early applications of STORM made use of dye pairs, whereby the protein of interest is labeled with two dyes, typically Cy3 and Cy5. Cy5 emits fluorescence upon excitation with a red laser length and is then switched off. Exposure to green laser light switches Cy5 back “on”; however, this depends on the proximity of the second dye, Cy3 [19]. They can be switched on and off hundreds of times before photobleaching occurs. Additional combinations of dyes include Alexa Fluor 405 and Alexa Fluor 647. Dye pairs have been largely replaced by direct STORM (dSTORM), a third mechanism of single-molecule localization whereby single dyes (versus a dye pair) can be turned on and off, including Cy5, Alexa Fluor 647, and ATTO 488 [18, 29, 30]. Importantly, all of these systems benefit from the inclusion of a reducing agent and oxygen scavenger in the visualization media [21, 22].

3.2.4 *STED*

Stimulated emission depletion (STED) microscopy was developed by Stefan Hell in 1994 as a point spread function engineering strategy [10]. The principle takes advantage of photoswitchable probes

and the concept that a second laser is used to reduce the size of the recorded point spread function of a fluorescent molecule. The first laser provides an excitation beam and excites RESOLFT-compatible fluorescent dye molecules or proteins. The first laser pulse is short, approximately 0.1 fs. Immediately after excitation, the second laser, or emission beam, irradiates the molecules before they reemit photons at their emission spectrum. The shape of the second beam is in the shape of a ring or donut (also called the depletion or STED beam). This forces the molecules to quench, depleting their fluorescence. This allows shaping of a point spread function because while those molecules within the ring are not quenched, those that lie in the path of the STED beam are [10]. The benefit of using photoactivatable probes is that STED is a scanning technique not unlike confocal laser scanning microscopy, so that the laser path scans the specimen. Illumination of the next molecule subset depends on the ability of the first set to return to a ground state. Resolution depends on the intensity of the STED beam laser, and resolution directly increases with increase in laser intensity by allowing molecules at the outer ring of the STED beam to be switched off, decreasing the diameter of the PSF [10].

4 Advantages of Super Resolution

Improved ability to distinguish between two point objects is implied by the term “super resolution.” We highlight the advantages of super resolution using our studies of platelet’s α -granule structure, a platelet organelle that is approximately 200–500 nm in diameter. As shown in Fig. 1a versus b, wide-field microscopy coupled with deconvolution cuts background haze significantly, but SIM methods result in clearer images with a high signal to noise ratio and substantial contrast improvement, as shown in Fig. 1c. We also scanned microtubules by both laser scanning confocal microscopy and SIM, and as shown in Fig. 1d, e, it is clear that SIM technologies greatly enhance resolution and contrast. As a clear example of SIM resolution of a membrane structure, we show the resolution of the demarcating membrane of alpha 1-antitrypsin mutant inclusion bodies by 3D SIM in Fig. 3a, b, a situation not resolvable by confocal microscopy. It is important to note that while a combination of wide-field microscopy and deconvolution software improves contrast, they do not increase resolution. PALM/STORM increases the lateral resolution to approximately 20 nm under ideal conditions. We labeled platelet α -granules with P-selectin, a membrane marker. Distinguishing demarcating membranes of organelles has been impossible with conventional techniques, and as shown in Fig. 3c, it is apparent that dSTORM techniques allow substantial improvement in resolving small granular and vesicular structures. Organelles the size of an α -granule

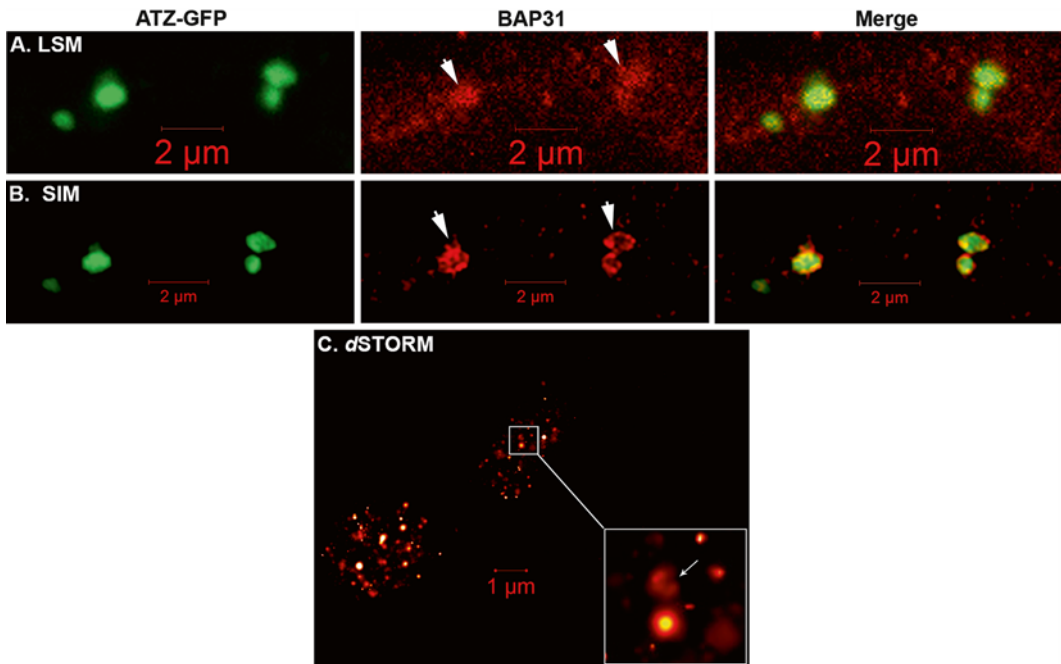


Fig. 3 Super-resolution techniques resolve membrane versus organelle contents. Alpha-1 antitrypsin-tagged GFP protein (ATZ-GFP) was transfected into HEK293 cells. An ER protein, BAP31, was labeled using antibodies, and cells were imaged using either (a) laser scanning confocal microscopy (LSM) or (b) SIM. Arrowheads point to the inclusion bodies of interest. Note that a clear membrane structure surrounding the inclusion body can be resolved by SIM, but not LSM. P-selectin, a platelet α -granule marker, was labeled using a P-selectin antibody and an anti-goat Alexa Fluor 647 secondary antibody and imaged using dSTORM (c). The white arrow in the inset (5 $\times$) points to a “cupped” outline of the α -granule, previously unresolved by wide-field or confocal technologies. Note that the ability to develop a full circular outline of the granule membrane may be limited by density of the P-selectin molecules

typically resolve merely as a diffraction-limited bright fluorescent spot. Here P-selectin partially surrounds what may be the nonfluorescent lumen of the granule; a half-moon pattern is observed. The result is encouraging, and we speculate that antibody labeling density and steric hindrance likely prevent the generation of a full-membrane structure.

Because SIM and PALM/STORM techniques both report an intensity value at each pixel/voxel position, they immediately lend themselves to being useful for quantitative distribution and hence co-localization comparisons. As shown in Fig. 4, Golgi structures from SIM images are easily converted to an XY intensity array in multiple colors using currently available dyes and proteins that work well in multichannel conventional imaging. Overall, it is evident that these approaches provide advantages that improve the precision of co-localization over the traditional wide-field and confocal practice, particularly by improving contrast and resolution.

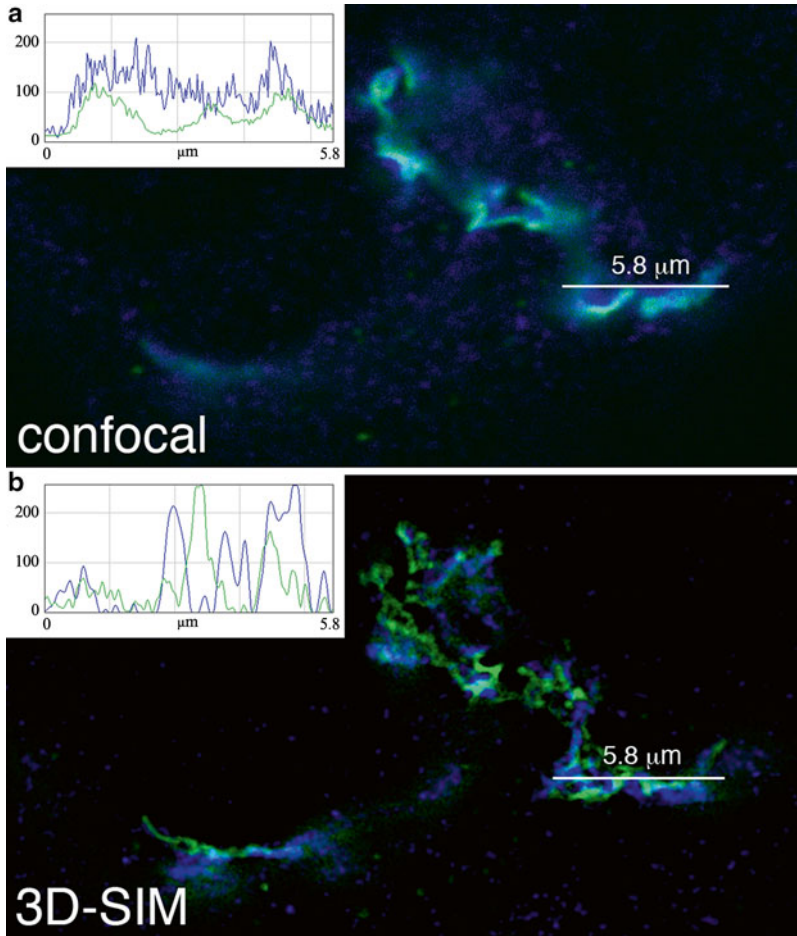


Fig. 4 Super-resolution techniques are inherently quantitative. The resolution of 3D SIM (**b**), but not confocal (**a**), is sufficient to separate cisternal Golgi apparatus (*green*) and the trans-Golgi network (*blue*): a quantitation of intensity and resolution in *XY* plane for laser scanning confocal versus 3D SIM. HeLa cell Golgi apparatus is labeled by GalNAcT2-GFP (cisternal Golgi, *green*) and antibody against Rab6 (trans-Golgi network, *blue*). *Insets* give quantitation of intensity versus distance

5 Technical Considerations in Super-Resolution Techniques

The spatial benefits of super-resolution microscopy are evident. However, acquisition speed, camera selection, post-processing, and probe use must be considered when developing an application of these technologies to your research problem. To give just one very simple example, a dSTORM approach in which 40,000 frames are collected to give the final image is hardly appropriate for a research goal of resolving events occurring over time. Each aspect of the imaging system setup should be carefully planned and optimized to obtain the best possible data for the individual laboratory, and it is evident that one type of super-resolution microscopy may

be better suited for one's experiment than another. We highlight important technical choices in this section and consider different applications in the next.

5.1 Probes and Dyes

For confocal systems, the issues of selective detection and photobleaching of dyes have been resolved in practice by using multiple lasers, optical filters, and photobleach-resistant dyes including cyanine dyes or the Alexa Fluor dye line combined with anti-fade reagents such as *n*-propyl gallate [21]. Currently available antibodies, dyes, and fluorescent proteins provide useful data for up to four channels with most wide-field and confocal systems, provided labeling conditions are optimized. By comparison, super-resolution techniques that rely on RESOLFT dyes are complex, and good choices are not always available even when the goal is as basic as a two-channel localization. Therefore, in considering a super-resolution microscopy system, care must be taken to choose probes, proteins, and dyes that work optimally.

A brief compilation of available dyes for SIM and other super-resolution techniques can be found in Table 1. On the whole, SIM systems optimally utilize currently available dyes, proteins, and antibodies. Of all the super-resolution techniques, SIM is least limited by dye and probe choice, and in our experience, multichannel imaging of samples can be done easily. In contrast, such limitations make PALM/STORM and STED techniques a less robust choice for multichannel co-localization work. Although Alexa Fluor 647 dye is well documented as a strong blinking photoswitchable fluorophore that has been very successful in localization mapping techniques [31], its use as a dye to give co-localization may be more difficult. Most biologists are interested in two-color fluorescence as a minimal requirement for investigating molecular co-localization. Other dyes, such as ATTO 488 and Cy3B, have been recommended as partners for Alexa Fluor 647 [19, 30, 32]. We show in Fig. 5 one brilliantly successful example of two-color dSTORM localization. In this case, clathrin (ATTO 488-conjugated second antibody, green channel) is localized by antibody staining versus TGN46 (Alexa Fluor 647-conjugated second antibody, red channel). This is our first success in showing coated vesicles or buds in selective association with the trans-Golgi network by fluorescence microscopy. However, we must consider that clathrin is concentrated as a multicopy protein in Golgi-associated budding structures and vesicles, which lends itself to success.

In our more general experience, the photophysics of these dyes together can be less than optimal. For best super resolution, literature and resources from microscope companies suggest that probes be used for direct immunofluorescence in which the probe is conjugated to the primary antibody, rather than for indirect immunofluorescence where the conjugation is to the secondary antibody. For many laboratories, this would be an additional challenge as

Table 1
Probes for super-resolution use

	SIM	PALM	STORM	STED
<i>Synthetic dyes</i>				
ATTO 425	X			X
Alexa Fluor 488	X		X	X
ATTO 488	X		X	
ATTO 532	X			X
Cy3B	X		X	
ATTO 565	X			X
Alexa Fluor 568	X		X	
Alexa Fluor 594	X			X
Alexa Fluor 647	X		X	
Cy5	X		X	
ATTO 590	X			X
ATTO 633	X			X
Cy7	X		X	
DyLight 750	X		X	
C-Rhodamine	X		X	
C-Fluorescein	X		X	
<i>Fluorescent proteins</i>				
EGFP	X			X
EYFP	X			X
Citrine	X			X
<i>Photoactivatable fluorescent proteins</i>				
PA-GFP	X	X		
PS-CFP2	X	X		
PA-mCherry1	X	X		
<i>Photoactivatable fluorescent proteins</i>				
tdEos	X	X		
mEos	X	X		
PSmOrange	X			
<i>Photoswitchable fluorescent proteins</i>				
Dronpa	X	X		
rsFastLime	X			

(continued)

Table 1
(continued)

	SIM	PALM	STORM	STED
bsDronpa	X			
rsCherry	X			
rsCherryRev	X			

Note that not every dye and probe listed has been tested for every technique. Thus, while ATTO 488 has been tested as effective for SIM and STORM, this does not mean it will not work for STED, merely that it may not have been tested for this application

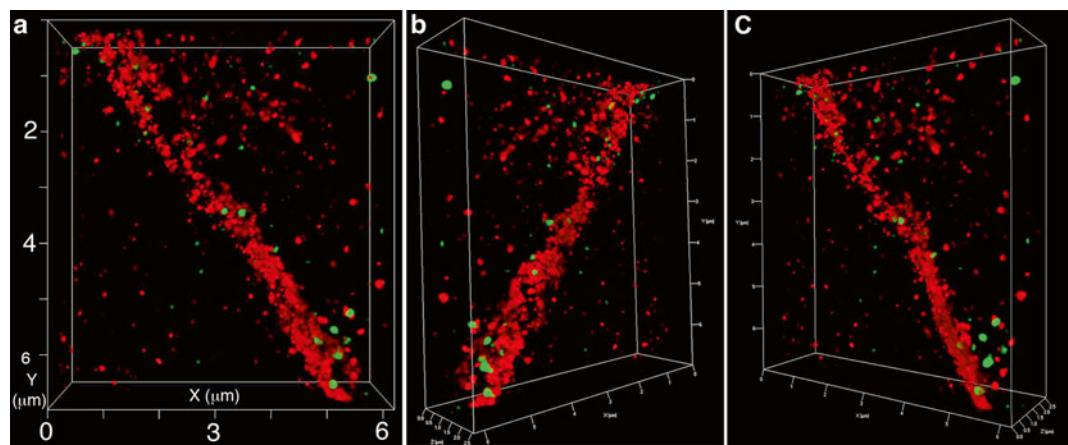


Fig. 5 Three-dimensional views are helpful in interpreting STORM images. Clathrin (*green*) and the trans-Golgi network (*red*) were labeled with antibodies and imaged by dSTORM. A region of interest was cropped, and different angles were manipulated to observe co-localization of molecules in the *Z* dimension. Viewing structures in three dimensions provides added information helpful for drawing co-localization conclusions from super-resolution data

few commercialized primary antibodies are directly conjugated to these dyes. Moreover, optimization of antibody labeling density is required to ensure high quantum yield without background [31]. Expression of photoactivatable or photoswitchable proteins (PA-GFP, PA-mCh, etc.) is an enticing option; however, not all biological systems can be optimized for expressing these proteins. For example, platelets lack a transcriptional system and hence cannot be productively transfected with DNA. Another specific example is overexpression of certain proteins within cells. Overexpression of certain proteins can lead to alteration of signaling pathways or induction of apoptosis. Indirect immunofluorescence is still the cornerstone of co-localization and imaging techniques. The microscopy field must focus on ways to tag molecules that prevent potential steric hindrance and hence effective

co-localization studies below 50 nm. Likely better chemistry will prevail and at least partially solve the problem of multichannel co-localization. However, depending on how the labeling is done, steric issues may still remain.

Scientists interested in temporal evaluation of cellular processes require live cell techniques. The necessity of taking thousands of frames for PALM/STORM to produce one full image prevents the ability to conduct live cell imaging experiments, a distinct disadvantage. For such temporal studies today, SIM provides the best time resolution, ~1–2 images per second. An extensive review of fluorescent probes and their application in answering biological questions is provided in reference [27].

5.2 Sample Preparation

Preparing samples for super-resolution microscopy demands precision and cleanliness. Using very clean, very flat, high-precision #1.5 coverslips is central to sample preparation. All reagents used for preparing SIM or PALM/STORM samples should be filtered to reduce possible background noise. Preparation of fixed samples for PALM/STORM should include an extra fixative step post-secondary antibody incubation. Mounting media is a growing area of development as soft mount media are recommended for super-resolution techniques to avoid sample compression and image distortion. Refractive mismatch should be considered when choosing mounting media [21]. Some commercial mounting media may be useful for both SIM and PALM/STORM [21].

5.3 Imaging System Considerations

All super-resolution microscopes require a highly sensitive EMCCD or CMOS detector, laser modules with optimized filter and dichroic mirror sets, and especially stage stability and vibration reduction modifications. Vibration system tables and very stable microscope stands are recommended for all systems, and as always, high numerical aperture objectives are recommended for best resolution. Most systems can also be optimized for wide-field microscopy for general focusing. The most important microscope requirement for SIM is an illumination module with different grating frequencies to match the light illumination pattern to the laser wavelength and objective lens [20]. Pattern rotation is also required. PALM/STORM systems usually include a TIRF system for minimization of background fluorescence, allowing detection of faint emission from single molecules. Hence, a TIRF numerical aperture objective is used. A laser system with 50–200 mW is required to give appropriate energy for single-molecule switching and imaging [8, 9].

STED microscopes require two laser wavelengths. Resolution depends on the intensity of the STED beam laser, and resolution directly increases with increase in STED laser intensity [10]. As such, these systems often have lasers that operate at approximately 700 mW of power [10]. Additional components required for multicolor and 3D imaging are considered below.

5.4 Reducing Background, Drift, and Alignment Issues

A distinct concern with regard to all super-resolution techniques is that alignment of fields must be precise. Given the nature of super-resolution microscopy, movement of field by just a few nanometers can deliver compromised results. There are a variety of fiducial and non-fiducial markers on the market to compensate for drift, and these ultimately need to be optimized for each scientist's system. SIM specifically comes with additional problems because grid background remains unless software applications are designed to remove it. This is illustrated in comparison with a wide-field and SIM image of the Golgi, as shown in Fig. 6. Note the distinct grid appearance in Fig. 6d. There is some concern that increased resolution reveals decreased organelle continuity. As shown in Fig. 7, some loss in Golgi continuity is apparent in the image collected from dSTORM techniques. Whether this loss is due to the failure to collect enough image frames or the inherent clustering of the labeled protein requires further experimentation. It is therefore very important to use appropriate controls and optimize dye conditions for the imaging system.

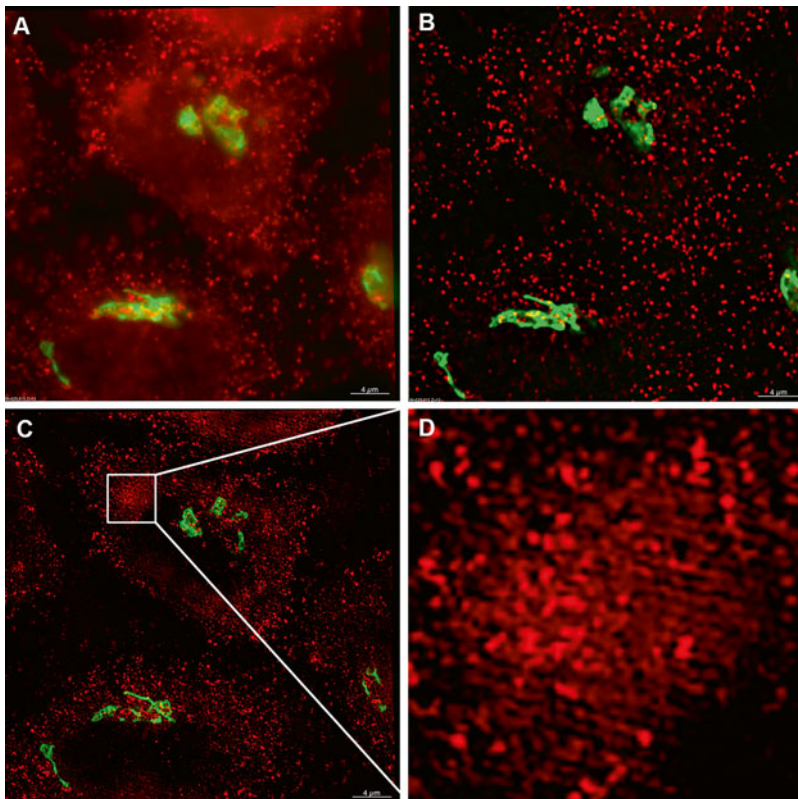


Fig. 6 3D SIM can produce artifacts. Clathrin (*red*) and the trans-Golgi network (*green*) were labeled with antibodies and imaged by wide-field microscopy (**a**), confocal microscopy (**b**), and 3D SIM (**c**). The blowup of a region of the image reconstruction (**d**) reveals a grid pattern which is an artifact

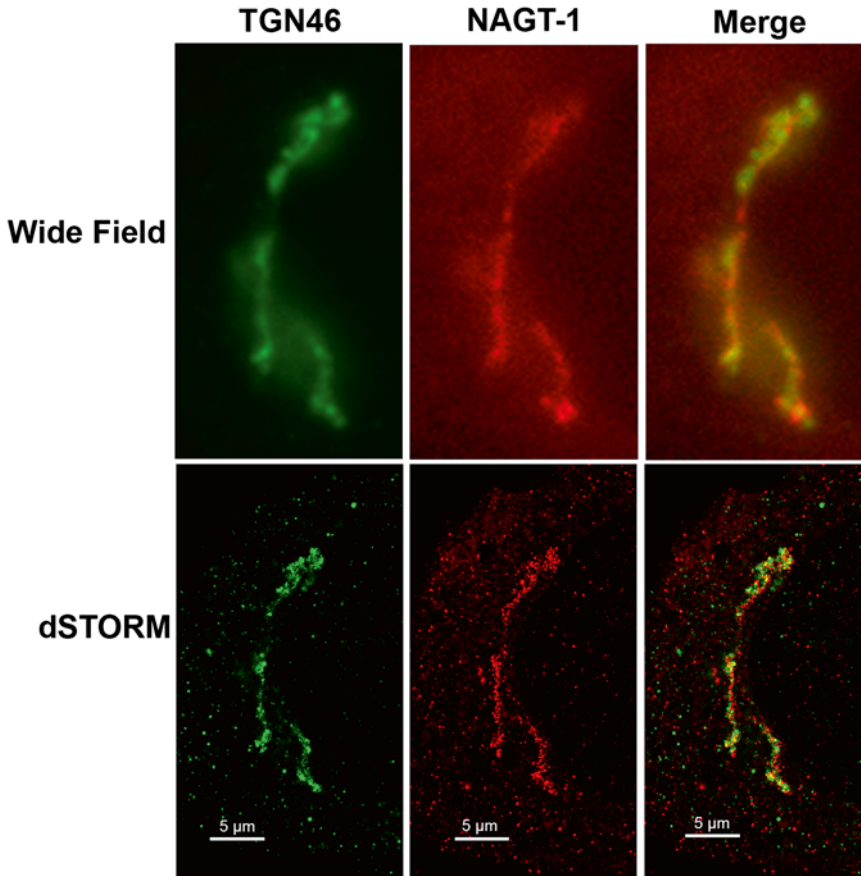


Fig. 7 With super resolution, the structural continuity of an organelle can be less apparent. The trans-Golgi network (TGN46) (*green*) and medial-Golgi network (NAGT-1) (*red*) were labeled with the respective antibodies. Alexa Fluor 647 (*green*) was used for the trans-Golgi network secondary antibody, and Cy3B was used for the medial-Golgi network secondary antibody, and samples were imaged via either wide-field microscopy (*top panel*) or dSTORM (*bottom panel*). It is clear that some structural continuity loss in the Golgi apparatus resulting from dSTORM is likely due to protein clustering or limitations in the efficiency of dye photophysics. Investigators will often superimpose a SIM or confocal image on the dSTORM outcome to reveal a better sense of organelle structure

5.5 Post-processing

Because most super-resolution techniques rely on mathematical algorithms to extract frequency data of fluorescence intensities, post-processing is extensive. As previously described, SIM records a series of images by rotating different light grid patterns on the sample. A Fourier transform-based algorithm transforms grid patterns into frequency space to generate a full, reconstructed image. Reconstructions from 2D SIM are acquired by rotating the light pattern grid in the XY plane by 3–5 rotations and up to five phase shifts for up to 25 images, and gaining resolution in the Z dimension requires image stacking. In our experience, the raw data files for a three-color, 10 μm image stack result in approximately

5–10 Gb per stack. Image reconstruction based on these stacks requires complementary alignment and grid background removal software. Collectively, this means that SIM requires a high-end off-microscope workstation with high computing power. Acquisition of thousands of image frames is required for generation of one full image in PALM/STORM techniques, and each of those frames must be aligned and corrected for drift. By necessity, processing of these images is time-consuming and again requires a high-end workstation computer. In concept, STED is not a computationally dependent technique and hence holds an advantage over other super-resolution techniques. Nevertheless, deconvolution software is available specifically to enhance final STED resolution (e.g., Scientific Volume Imaging, Hilversum, the Netherlands).

6 Application of Super-Resolution Techniques: Specificity Is Key

The resolution and contrast-enhancing ability of super-resolution microscopy approaches make them attractive solutions for life scientists. However, one super-resolution technique may be more appropriate than another depending on the application and experimental questions. Specificity and knowledge of how to best answer one's scientific question are key in selecting a super-resolution system. We highlight three of the major applications in biology and address context-specific application of super-resolution techniques below. A brief summary is provided in Table 2.

6.1 Multicolor Imaging

In our experience, SIM lends itself most easily to multicolor imaging, as shown in Fig. 4. Implementation of SIM is immediately accessible for most life scientists due to the conventional nature of dyes and fluorescent proteins. By comparison, PALM/STORM

Table 2
Applications of super resolution

Application	Technique		
	SIM	PALM/STORM	STED
Typical resolution (XY)	100 nm	20–50 nm	25–80 nm
Typical resolution (Z)	250 nm	50 nm	150–600 nm
Conventional probes available	All	Some	Some
Multicolor imaging	4-Color	2-Color ideally	2-Color ideally
Live cell imaging	X	Limited by sampling approach	Inherently difficult
3D imaging	X	X	X

techniques are less easily optimized for more than two colors and often work best under conditions of one color localization study. It is possible to do two-color imaging, as shown by Figs. 5 and 7. Although STED systems can be used for two-color imaging, they have been primarily used for studies utilizing just one photoactivatable or photoswitchable probe. Dye choice primarily limits multi-color imaging for PALM/STORM and STED techniques.

6.2 3D Imaging

Three-dimensional data are a distinct advantage at the super-resolution level. We illustrate this with a dSTORM example. As shown in Fig. 5, the interpretation of two-color STORM images is clearer in 3D as illustrated by two observational angles. We note here that both SIM and STED were designed from the beginning to be 3D techniques in which vertical stacks of images are taken. PALM/STORM can be a 3D approach as illustrated by implementations from various manufacturers such as Nikon, Vutara, and Zeiss (listed in alphabetical order). The acquisition of 3D data is particularly essential to demonstrating *Z* resolution and generally comes at the cost of increased image collection time and processing time. One striking example of successful 3D STORM is provided by the mapping of the mitochondrial network in whole cells using dye pairs by Zhuang laboratory in [33].

6.3 Live Cell Imaging

Live cell imaging primarily requires the ability to maintain focus and collect images at short exposures as quickly as possible without shifts in temperature or movement. By necessity, it requires as little photobleaching as possible and reduced background noise from outside light sources. As has been mentioned, super-resolution techniques falter with live cell imaging primarily due to speed and the absolute requirement for stability. To date, variations on SIM have been most successful. Recent developments have shown vast improvements in utilizing super-resolution for three-dimensional microscopy of living *Drosophila* cells in TIRF mode in which samples were flattened against a pad of agarose gel [15]. Of particular significance in this application was the use of spatial light modulators to generate phase shifts without mechanical movement. Camera efficiency is important in any imaging application but is particularly imperative under live cell imaging considerations. Increased sensitivity and speed and reduced specimen damage could be gained by using either a high-efficiency EMCCD camera or a scientific CMOS camera. For live cell, multichannel 3D SIM approaches, the use of individual cameras for each channel increases collection speed with some cost in system complexity. STED as a laser scanning technique has most of the advantages and disadvantages of laser scanning confocal microscopy. STED has indeed been applied to live cell imaging but is limited presently to the *XY* dimension and to small fields, for which this strategy is most efficient [34]. Perhaps parallel STED image collection can someday be implemented

as is the case with confocal microscopy; much live cell work is done with spinning disk systems in which light from many pinholes are collected in parallel.

PALM/STORM systems present an inherent challenge in terms of acquisition speed. By their nature, these systems require thousands of frames to produce a stochastic image. For now, it is our opinion that these techniques are unlikely to have general applicability in live cell imaging. That said, PALM has been used to study adhesion-complex dynamics [35]. In this case, the movement being studied was slower than most biological movements.

7 Conclusions and Comments

New techniques are developed in the hope of obtaining concrete answers to biological questions and to be able to ask better questions. Ideally, super-resolution microscopy approaches provide both. However, at present, there is no single ideal approach to super-resolution co-localization studies. The techniques differ significantly in resolution and in practicality in their application to individual research problems. In addition, convenience, time, and cost must also be considered in making any decision. For many uses, SIM provides advantages over PALM/STORM or STED. SIM is practical using conventionally available dyes, while PALM/STORM relies on a subset of blinking dyes that work well individually and less so for two-color co-localization studies. Robust dye choices will need to be added to the repertoire for PALM/STORM techniques to be a truly valuable technique for a general cell biology community. One can imagine a repertoire of dyes that all switch under the same buffer conditions, allowing for easily obtained multicolor readouts. Additionally, SIM systems are being optimized and further developed for the important biological applications of live cell and multicolor imaging. SIM also lends itself easily to multicolor 3D imaging, whereas PALM/STORM techniques are less adept at multicolor 3D. The primary benefit of PALM/STORM and STED strategies is the further improvement in resolution. This gain is typically available only for fixed samples. A trend within the industry appears to be the implementation of a SIM and a 3D PALM/STORM system in the same microscope stand. For many, that choice may give the best of both worlds.

For our own study of platelets, SIM has been very helpful in addressing questions of platelet α -granule structure and protein packaging [36], and we look forward to implementing this method for live cell imaging. In part, this is because platelet granules are relatively large cylinders, about 250 nm in diameter and 500 nm in length. We consider 3D SIM to be an easily applicable and quickly implementable technique to augment our current studies using electron microscopy. Likely, the use of a combined

SIM and PALM/STORM system will further increase the range of questions that we can answer with light microscopy techniques. We look forward in the future to applying a combined system to more general problems in membrane trafficking and problems in diabetes and hunger signaling.

Super-resolution techniques are still on the “rugged edge” of development and are sure to improve greatly over the next decade. It is likely that the repertoire of photoactivatable and photoswitchable proteins, dyes, and probes will continue to grow. Recent molecular tagging strategies (reviewed in reference [27]) are in progress, which will likely lead to new gains in resolution. Overall, super-resolution techniques will obsolete previous fluorescence approaches, particularly as fluor choices improve, instrument implementations improve, and algorithms advance.

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Bimolecular Fluorescence Complementation (BiFC) Technique in Yeast *Saccharomyces cerevisiae* and Mammalian Cells

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Abstract

Visualization of protein–protein interactions in vivo offers a powerful tool to resolve spatial and temporal aspects of cellular functions. The bimolecular fluorescence complementation (BiFC) makes use of nonfluorescent fragments of green fluorescent protein or its variants that are added as “tags” to target proteins under study. Only upon target protein interaction is a fluorescent protein complex assembled, and the site of interaction can be monitored by microscopy. In this chapter, we describe the method and tools for the use of BiFC in the yeast *Saccharomyces cerevisiae* and in mammalian cells.

Key words Bimolecular fluorescence complementation, EYFP, Fluorescence microscopy, GFP, Venus, Protein interactions, *Saccharomyces cerevisiae*, Yeast, Mammalian

1 Introduction

Proteins carry out a vast number of cellular functions through interactions with one or multiple binding partners. A given protein may display differential interactions depending on its cellular localization. Examples of such differential interactions are proteins that shuttle between nucleus and cytosol having different interaction partners at these two cellular compartments. A central issue in modern cell biology is to reveal such differential interactions and how they are regulated in space and time (e.g., during cell cycle progression or in response to extracellular stimuli).

The identification and analysis of protein interactions by biochemical approaches based on pull-down or immunoprecipitation experiments, or analysis of protein interactions with different

variants of the yeast two-hybrid assay, are extremely useful techniques. However, typically none of these techniques reveal the cellular localization where the protein interaction normally takes place. In the case of immunoprecipitation and pull-down experiments, cells need to be broken, and the assays are performed under *in vitro* conditions. Such conditions do not favor preservation of weak protein–protein interactions that nevertheless are typical for regulatory molecules. Different two-hybrid interaction assays usually require that the interaction occurs at a predetermined site within the cell such as the nucleus. A complicating feature in these assays is that all cofactors needed for an efficient protein interaction to take place may not be present in this unnatural site of interaction.

Development of green fluorescent protein (GFP) technology has enabled visualization of protein localization and movement dynamics under *in vivo* conditions [1]. However, the visualization of the total localization pattern of GFP-tagged target protein does not reveal where within its overall distribution the proteins interact with different binding partners. GFP technology has recently been utilized in two *in vivo* protein interaction methods that can be used to reveal the cellular site of interaction: the fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) [2, 3]). In FRET, the difference in fluorescence signal or its lifetime is detected when the fluorophores are close enough in the same complex [4]. In contrast, in BiFC, a fluorescence signal is generated only when nonfluorescent fragments of GFP are brought together by interaction of the target molecules fused to GFP fragments. Thus, BiFC is potentially very sensitive with a low background. The drawback of BiFC is that it does not allow interaction dynamics studies as an assembly of the GFP results in significant stabilization of the target protein–GFP complexes [2, 5]. Recently, BiFC has been shown to function in various cells from bacteria to mammals [5, 6]. At the moment, there exist several versions of BiFC that make use of the different spectral properties of GFP variants. Protein interactions have been visualized with the help of fragments of enhanced yellow fluorescent protein (EYFP) or its variants Venus and Citrine, cyan fluorescent protein (CYFP) or its variant Cerulean, and blue fluorescent protein (BFP) and monomeric red fluorescent protein (mRFP) fragments [7–9]. Combinations of these variants enable simultaneous visualization of several protein interactions [10].

In this chapter, we report the method and tools for the use of BiFC system with EYFP fragments in the yeast *S. cerevisiae* and Venus fragments in mammalian cells.

2 Materials

2.1 Transformation/ Transfection

2.1.1 Yeast Transformation

1. 10× TE: 100 mM Tris-HCl, 10 mM ethylenediamine-tetraacetic acid (EDTA), pH 7.5. Store at room temperature. Working concentration is 1×.
2. 10× LiAc solution: 1 M LiOAc, pH 7.5 (adjusted with acetic acid). Store at room temperature. Working concentration is 1×.
3. 50 % polyethylene glycol (PEG): 50 g PEG-4000 in 100 mL. Store at room temperature. Working concentration is 40 %.
4. Salmon sperm DNA: 10 µg/µL, store in small aliquots at -20 °C.
5. Vectors: *see* Fig. 1.

2.1.2 Mammalian Cell Transfection

1. Lipofectamine™ 2000 (Invitrogen).
2. Opti-MEM® I Reduced Serum Medium (Gibco).

2.2 Verification of Expression

2.2.1 Lysate Preparation

Yeast Lysate Preparation

1. 2 % SDS-containing protease inhibitors: add one tablet protease inhibitors (cOmplete, EDTA-free [Roche]) to 25 mL of 2 % sodium dodecyl sulfate (SDS) in water, and store in aliquots at -20 °C.
2. Acid-washed glass beads 0.45 mm diameter (Sigma).
3. Laemmli sample buffer: 0.3 M Tris-HCl (pH 6.8), 30 % glycerol, 10 % SDS, 1.54 M dithiothreitol (DTT), 0.4 mg/mL bromophenol blue. Store in 1 mL aliquots at -20 °C.

Mammalian Lysate Preparation

1. Wash: PSB (phosphate buffered saline).
2. Lysis buffer: 10 mM HEPES, pH 7.6, 150 mM NaCl, 0.5 mM MgCl₂, 10 % glycerol, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, protease inhibitor cocktail [cOmplete, EDTA-free (Roche)].
3. Laemmli sample buffer (*see* Subheading “Yeast Lysate Preparation,” **item 3**, of Subheading 2.2.1).

2.2.2 SDS-PAGE

1. 12 % separating gel: 4.65 mL distilled water, 1.25 mL 3 M Tris-HCl (pH 8.8), 0.05 mL 20 % SDS, 4 mL acrylamide/bis-acrylamide (30 %/0.8 %), 0.05 mL 10 % ammonium persulfate (APS), 0.005 mL tetramethyl-ethylenediamine (TEMED). Mix directly before use. This amount is sufficient for preparing two gels with a spacer thickness of 0.75 mm when using a GE Healthcare gel running system.
2. 4 % stacking gel: 4.12 mL distilled water, 0.21 mL 3 M Tris-HCl (pH 6.8), 0.025 mL 20 % SDS, 0.67 mL acrylamide/bis-acrylamide (30 %/0.8 %), 0.025 mL 10 % APS, 0.005 mL

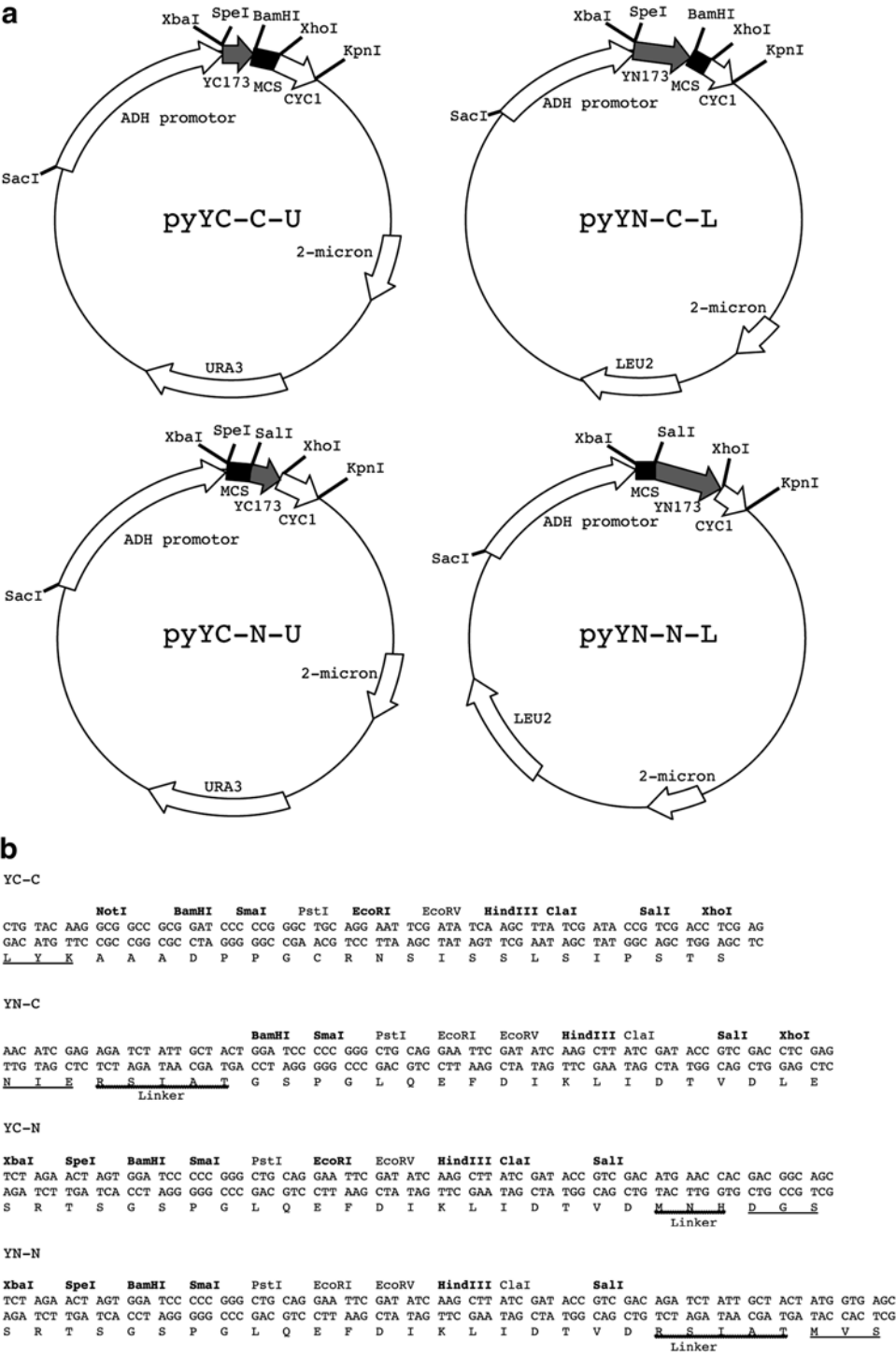


Fig. 1 Yeast plasmid maps and sequences of the multiple cloning sites. Yeast plasmids enable tagging of your target protein either at the amino (YC-C and YN-C)- or carboxy (YC-N and YN-N)-terminus. *Underlined* single-letter amino acid sequence is EYFP sequence. Unique restriction enzyme sites are shown in *bold*

TEMED. Mix directly before use. This amount is sufficient for preparing two gels with a spacer thickness of 0.75 mm when using GE Healthcare gel running system.

3. 10× running buffer: 30 g Tris–HCl, 144 g glycine, 10 g SDS, add distilled water to 1 L. Store at room temperature. Working concentration is 1×.
4. BCA™ Protein Assay Kit (Pierce).

2.2.3 Western Blotting

1. Western blotting buffer: 3 g Tris–HCl, 14.4 g glycine, 200 mL methanol, add distilled water to 1 L. Store at +4 °C.
2. Ponceau S solution: 100 mg Ponceau, 1 mL acetic acid, add distilled water to 100 mL. Store at room temperature.
3. 10× TBS: 30 g Tris–HCl, 80 g NaCl, 2 g KCl, add distilled water to 1 L. Adjust pH to 7.4 with HCl. Working concentration is 1× and add 500 µL Tween-20 to obtain Tris-buffered saline Tween-20 (TBST). Tween-20 dissolves faster if you add it into 10× TBST before dilution with water to 1 L. Store at room temperature.
4. Enhanced chemiluminescence (ECL) detection reagents: Pierce ECL Western Blotting Substrate kit (or equivalent).
5. Incubation chambers for filters: plant tissue culture container (PLANTCON) or equivalent.
6. Secondary antibodies: horseradish peroxidase-conjugated immunoglobulin G specific for your primary antibody.

2.3 Microscopy

1. Wash: PSB (phosphate buffered saline).
2. Fixation: 4 % paraformaldehyde solution (Electron Microscopy Sciences no. 15713-3) in PBS.
3. Sample mounting for fixed cells: dissolve 5 g Mowiol in 20 mL Tris–HCl (pH 8.0) overnight. Add 10 mL 100 % glycerol and let it mix overnight. Store at –20 °C.

3 Methods

3.1 Generation of Target Gene–YFP Fusions

The plasmids presented here enable the introduction of the target gene in the multiple cloning sites indicated in Figs. 1 and 2. Yeast plasmids reported here enable high-copy expression of the target protein tagged either at the amino- or carboxy-terminus with EYFP fragments (*see Note 1*). The amino-terminal (YN) fragment encodes amino acids 1–172, whereas the carboxy-terminal (YC) fragment encodes amino acids 173–238 of EYFP. All plasmids have *ADHI* promoter and *CYCI* terminator and are based on the Mumberg et al. plasmid series [11]. Mammalian plasmids enable

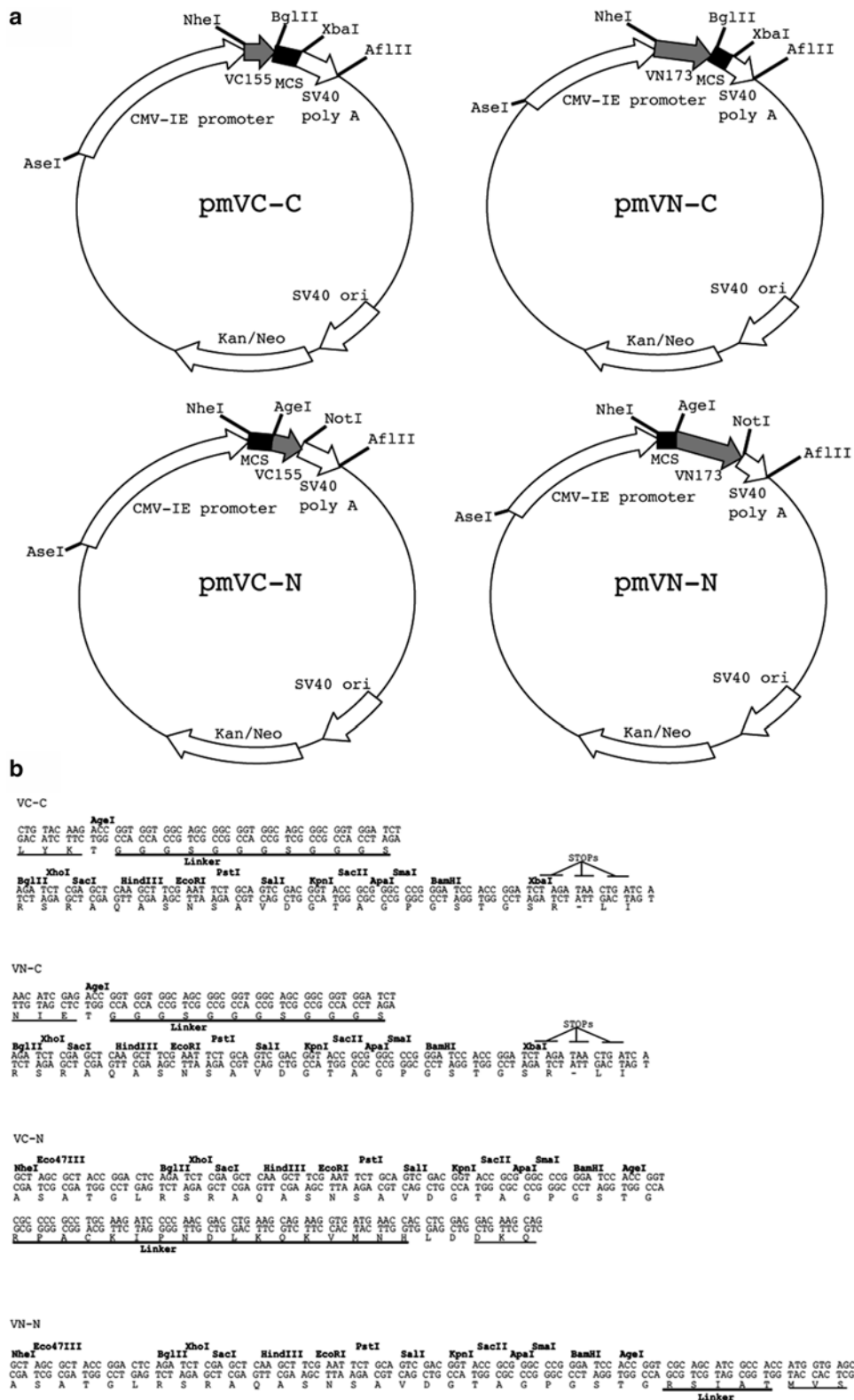


Fig. 2 Mammalian plasmid maps and sequences of the multiple cloning sites. Mammalian plasmids enable high-level transient expression of the target protein tagged either at the amino (VC-C and VN-C)- or carboxy (VC-N and VN-N)-terminus with Venus fragments. Underlined single-letter amino acid sequence is Venus sequence. Unique restriction enzyme sites are shown in *bold*

high-level transient expression of the target protein tagged either at the amino- or carboxy-terminus with Venus fragments (*see Note 1*). The amino-terminal (VN) fragment encodes amino acids 1–172, whereas the carboxy-terminal (VC) fragment encodes amino acids 155–238 of Venus. All plasmids have CMV-IE promoter and SV40 poly A terminator and are based on pEYFP-N1 and pEYFP-C1 from Clontech. Plasmids can be requested from the corresponding author.

3.2 Transformation/ Transfection

3.2.1 Yeast Transformation

1. Inoculate yeast cells into 5 mL liquid YPD [12] medium and grow overnight at 30 °C with shaking to approx 2×10^7 cells/mL ($A_{600} = 1$).
2. Dilute to OD₆₀₀ 0.1–0.2 in 20 mL of fresh, warm YPD, and regrow to $1\text{--}2 \times 10^7$ cells/mL (OD₆₀₀ <1). Typically, this takes 4–5 h.
3. Harvest the cells by centrifugation at $3,200 \times g$ for 2 min, and wash in 1 volume of sterile water.
4. Pellet the cells by centrifugation at $3,200 \times g$ for 2 min, pour off the water and resuspend the cells in 1.0 mL 1× TE/LiAc solution made fresh from sterile 10× stocks, and transfer the suspension to a 1.5 mL Eppendorf tube.
5. Pellet the cells in microfuge at top speed for 5 s and remove the supernatant with a micropipet.
6. Resuspend the cells to 2×10^9 cells/mL with 1× TE/LiAc.
7. Incubate carrier DNA for 10–15 min at 100 °C and place it immediately on ice.
8. Mix 50 µL of yeast cell suspension with 0.5–1 µg transforming plasmid DNA and 50 µg single-stranded carrier DNA (salmon sperm DNA) in a 1.5 mL Eppendorf tube. The protocol provided here transforms both YN and YC plasmids at the same time. Remember to prepare one negative control that is lacking the plasmid DNA.
9. Add 300 µL of 40 % sterile polyethylene glycol (PEG)-4000 in 1× TE/LiAc made fresh from a 50 % PEG stock and 10× TE and LiAc stocks. Mix by gentle pipetting until the cell pellet has been completely resuspended.
10. Incubate with agitation for 30 min at 30 °C.
11. Add 40 µL tissue culture grade dimethyl sulfoxide (stored at –70 °C in small aliquots) and heat shock at 42 °C for 15 min.
12. Spin down in microfuge for 5 s and wash cells gently with 1 mL of sterile water.
13. Spin down again and resuspend the cell pellet in 200 µL of 1× TE.
14. Plate 50 µL and the rest on SC-leu-ura plates. Grow in an incubator at 30 °C. Colonies appear in 2 days. There should be no colonies in the negative control lacking the plasmid DNA.

3.2.2 Mammalian Cell Transfection

Mammalian cell transfection was performed as described in the Lipofectamine™ 2000 (Invitrogen) plasmid DNA transfection protocol (*see* **Note 2**).

3.3 Verification of Expression

It is important to verify that your constructs produce a fusion protein of expected size (*see* **Notes 1** and **3**).

3.3.1 Lysate Preparation Yeast Lysate Preparation

1. Inoculate one yeast colony into 20 mL of liquid Sc-ura-leu medium and grow overnight with shaking at 30 °C.
2. Dilute cell culture in fresh, warm 20 mL Sc-ura-leu medium to OD₆₀₀ 0.2, and regrow to OD₆₀₀ 0.8–1.0 (*see* **Note 4**).
3. Harvest cells by centrifugation at 3,200×*g* for 2 min; discard the supernatant.
4. Resuspend the cells in 1 mL of water, transfer into a 1.5 mL microfuge tube, and collect by centrifugation.
5. Resuspend the cells in 400 µL 2 % SDS containing the protease inhibitors (Subheading 2.2.1), and vortex.
6. Add 200 µL of 0.45 mm acid-washed glass beads and heat for 3 min in a 95 °C block heater or equivalent.
7. Vortex twice for 90 s with maximum speed.
8. Centrifuge for 5 min at full speed in a microfuge and collect the clear lysate in a new tube. Avoid the pellet.
9. Measure the protein concentration by BCA method (BCATM Protein Assay Kit, Pierce) or equivalent (*see* **Note 5**). Typically a lysate with a protein concentration 8–12 µg/µL is obtained.
10. Dilute the lysate to a concentration of 4 µg/µL with 6× Laemmli sample buffer and water to yield 1× sample buffer concentration.

Mammalian Lysate Preparation

1. Use 1 day transfected cells grown on a 2 cm dish to about 90 % confluence.
2. Wash cell monolayer gently with PBS.
3. Add 1 mL lysis buffer per dish.
4. Break the cells by shearing by pipetting 20× up and down and rotating the lysate for 15 min at 4 °C.
5. Remove unbroken cells by spinning the lysate for 5 min at top speed in a microcentrifuge. Collect the supernatant in a new tube.
6. Measure the protein concentration, and dilute the sample to 1× Laemmli sample buffer as described in Subheading “Yeast Lysate Preparation” of Subheading 3.3.1.

3.3.2 SDS-PAGE

Apply 20–40 µg total protein of every sample to 12 % mini SDS-PAGE gel. This percentage of gel gives optimal resolution for 20–60 kDa proteins:

1. Heat the samples for 5 min in a 95 °C block heater with the lid closers on.
2. Centrifuge the sample in microfuge for 5 min at top speed.
3. Load 5–20 µL of sample per well.
4. Run at 20 mA for each gel until the dye front is at the bottom of the gel.
5. Remove the gel from the running apparatus. Discard the upper gel.

3.3.3 Western Blotting

For the electrophoretic transfer of proteins from the gel to nitrocellulose membrane, we use Bio-Rad Mini Trans-Blot Cell system:

1. Assemble a sandwich for transfer by prewetting the fiber pads, two Whatman no. 3 filter papers, and the nitrocellulose membrane (slightly bigger than the gel) in Western blotting buffer. The order of constituents in the “sandwich” is as follows:
 - (a) Pad
 - (b) Filter papers
 - (c) Gel
 - (d) Nitrocellulose membrane
 - (e) Filter papers
 - (f) Pad
2. Remove air bubbles by stroking the assembled “sandwich” (e.g., with the flat side of a test tube). Put the “sandwich” into the gel holder cassette, and mount the assembled cassette into the electrophoresis blotting module so that the membrane is facing the positive charge.
3. Transfer at 400 mA for 1 h with the cold pack and pre-chilled buffer or overnight at 50 mM at room temperature toward the plus.
4. When finished, remove the nitrocellulose membrane with forceps (avoid touching the filter without gloves), and put membrane into an incubation chamber (*see Note 6*) and cover with Ponceau S solution. Shake for 1 min. The Ponceau S solution can be reused many times.
5. Rinse with distilled water until protein bands appear. If the filter looks fine, continue to the next step.
6. Immerse membrane in 20 mL of blocking buffer (5–8 % non-fat milk powder in TBST) and incubate for 1 h at room temperature with shaking.
7. Pour the blocking buffer and rinse the membrane once with TBST.

8. Incubate with primary antibody specific for your target protein (*see* **Note 3**, dilution in TBST or blocking buffer) in 10 mL of the antibody dilution for 60 min at room temperature or overnight at 4 °C.
9. Remove the primary antibody and wash at least three times for 10 min each with 20–30 mL of TBST at room temperature.
10. Incubate with horseradish peroxidase-conjugated secondary antibody (dilution in TBST or blocking buffer) for 60 min at room temperature.
11. Remove the secondary antibody and wash at least three times for 10 min each with TBST at room temperature.
12. Detect with Pierce ECL Western Blotting Substrate kit (or equivalent) and autoradiography film or by other means as specified by the manufacturer of the kit.

3.4 Microscopy

3.4.1 Cell Growth

Yeast Cell Growth

1. Inoculate a 10 mL overnight culture of the yeast strain in Sc-ura-leu medium at 30 °C.
2. Dilute cultivation to A_{600} 0.2 and regrow to A_{600} 0.8–1.0. This will take 4–5 h at 30 °C.

Mammalian Cell Growth and Fixation

It is important to grow cells in incubation chambers containing a cover slip:

1. Grow freshly transfected cells overnight at 37 °C.
2. Wash cells once with PBS (phosphate buffered saline) warmed up to 37 °C.
3. Fix cells for 30 min with 4 % paraformaldehyde in PBS.
4. Wash once with PBS.

3.4.2 Cell Mounting

Mounting of Living Yeast Cells

Preferably, use synthetic yeast medium lacking tryptophan (presence of tryptophan can result in background fluorescence):

1. To obtain optimal density of cells, it is useful to collect cells by spinning down 1 mL of the culture, and resuspend it in about 50 μ L medium.
2. Pipet 5 μ L of the collected cells on a microscope slide and push the cover slip gently on top.
3. Seal the edges of the cover slip with tape.

Mounting of Fixed Mammalian Cells

1. Pipet a 6 μ L drop of the mounting solution on a microscope slide.
2. Dip cover slip in water to rinse.
3. Touch cover slip edges with tissue to remove excess water.
4. Lay the cover slip with the fixed mammalian cells facing the mounting solution drop on the microscope slide. Avoid air bubbles.

3.4.3 Microscopy

The microscope used needs to have a light source and filters optimal for YFP/Venus excitation and signal detection. For yeast microscopy, 100× oil immersion objectives should be used because of the small size of the cells. However, 60× can also be used. For bright-field observation of the yeast cells, the microscope should optimally have DIC (Nomarski) optics. For mammalian microscopy, 60× objectives are sufficient. For more detailed observation, higher magnification and/or confocal microscopy is recommended. As for any method, appropriate negative controls should be used. When available, one should prepare a negative control of cells expressing a mutant form of one of the binding partners that does not interact with the other target protein. In case no such mutant is known, one should use a functionally unrelated protein (of similar size) as the other binding partner. One should also test that no signal is obtained when only the YFP/Venus fragments are expressed in the cells (*see Note 7*).

4 Notes

1. Care should be taken to maintain the correct reading frame when fusing your target gene with the EYFP fragments. When feasible, it is advisable to test the tagging of your target proteins both at the amino- and at the carboxy-terminus. In many cases tags in a specific combination of orientations give the best signal.
2. In our experience, transfection of cells 2 days after splitting them in new chambers has given the best results.
3. In case there are no good antibodies available for your target protein, it is useful to generate a peptide tag (e.g., HA or Myc) to your protein by introducing a DNA sequence encoding the tag in one of the oligonucleotide primers used for cloning your target gene as a PCR fragment into the plasmids. This will enable verification of the expression level of your target protein and detection of the total distribution of the target proteins (e.g., with indirect immunofluorescence).
4. Do not grow the culture to a stationary phase because the *ADHI* promoter activity decreases gradually when glucose is consumed. We usually collect the cells around A_{600} 1.0.
5. In contrast to many other protein assays, the BCATM Protein Assay Kit, Pierce (or equivalent), is compatible for SDS concentrations up to 5 %.
6. We have found plant cell cultivation chambers (Subheading 2.2.3) handy for membrane staining with Ponceau S and incubation with antibodies. 10 mL of antibody dilution is sufficient when the lid is on. The antibody dilutions can in most cases be used several times. For storage at +4 °C,

it is advisable to add 0.02 % (w/v) NaN_3 . However, do not use NaN_3 for antibodies coupled with horseradish peroxidase as it inhibits peroxidase activity.

7. In order to be confident with the results obtained with BiFC, it is advisable to test the *in vivo* functionality of the tagged target genes. For this purpose the ability of the tagged target gene to rescue the phenotype of the conditional or knockout mutants of your gene of interest should be used. Knockout strains can be ordered with a very small cost (e.g., from EUROSCARF collection that has deletions for almost all genes in the *S. cerevisiae* genome: <http://web.uni-frankfurt.de/fb15/mikro/euroscarf/>).

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Chapter 21

Microscopic and Spectroscopic Techniques to Investigate Lipid Droplet Formation and Turnover in Yeast

Heimo Wolinski and Sepp D. Kohlwein

Abstract

In spite of some progress in understanding the molecular basis of lipid-associated disorders, major questions about the regulation of synthesis and degradation of lipids and the interaction of these processes with other aspects of cellular physiology are still unresolved. Studies in reference organisms such as various yeast species, the fruit fly *Drosophila melanogaster*, or the nematode *Caenorhabditis elegans* complement efforts in mouse models as well as clinical studies in humans to address these questions. Imaging techniques play a pivotal role in understanding lipid droplet biology, and the implementation of imaging-based high-content screens of mutant collections has led to the identification of novel molecular players. This study focuses on novel fluorescent probes as well as spectroscopic imaging techniques to investigate lipid droplet formation and turnover in yeast. The application and limitations of such techniques in understanding lipid storage and turnover are discussed.

Key words Yeast, *Saccharomyces cerevisiae*, Neutral lipid storage, Lipolysis, Fluorescence microscopy, Green fluorescent protein, BODIPY 493/504, LD540, Nile Red, CARS microscopy

1 Introduction

Storage of neutral lipids such as triglycerides (TAG) in cytosolic lipid droplets (LDs) is an important cellular process and essential under conditions of fatty acid overload. Research on LD physiology has attracted great interest in cell biology and biomedicine in recent years [1–4]. The molecular function of a key regulator of TAG synthesis, the phosphatidate phosphatase Pahl1, and its mammalian ortholog lipin was first described in yeast, underscoring the important role of yeast as a reference organism [5–7]. The discovery of novel enzymes and their regulators executing lipid degradation in mammalian cells [8–13] has led to a paradigm shift in our understanding of how cells execute fat degradation. These studies have unveiled molecular links between lipid degradation, signaling, and cancer development and progression. Similarly, yeast expresses the Tgl4 lipase, an ortholog of the mammalian adipose triglyceride

lipase, ATGL [14, 15], that is regulated in a cell cycle-dependent manner, thus linking lipolysis to cell proliferation. In the fission yeast *S. pombe*, LD formation is also linked to the cell cycle [16]. In addition, numerous similarities in terms of lipid metabolism and physiology exist between yeast and cancer cells [17]. Taken together, despite millions of years of evolutionary distance between yeast(s) and mammalian cells, basic biochemical and physiological reactions in LD biology display significant levels of conservation, which facilitates discovery of critical components in LD homeostasis across species.

Since LDs represent unique subcellular structures that are morphologically very distinct from other organelles, imaging-based screens have been very powerful in identifying proteins associated with LDs, as well as in identifying factors that are important for LD morphology and inheritance [18]. In previous microscopic studies, the fluorescent dye Nile Red has played a prominent role in characterizing LDs [19], due to its high specificity for hydrophobic environments. On the negative side, Nile Red tends to bleach very rapidly and shows redshifted emission when inserted into the hydrophobic core of phospholipid bilayers. Although it may be used advantageously, this property may be misleading with membrane accumulations in mutant scenarios [20] that may obscure LDs. The novel LD dye LD540 [21] provides an excellent alternative to Nile Red staining. In addition, a label-free spectroscopic imaging technique, coherent anti-Stokes Raman scattering (CARS) that has now move from physics laboratories to the commercial market, provides an attractive alternative technology to investigate LDs. CARS utilizes a multiphoton process to excite molecular vibrations (e.g., C-H vibrations which are particularly frequent in the hydrocarbon chains of lipids), which can then be imaged by a photon scattering process [22–25]. Since infrared photons are used, this technology is especially benign for live cell imaging and allows for monitoring LD dynamics over extended periods of time, especially in mammalian cells [24]. Caution is needed, however, when CARS is applied to yeast cells due to the high photon density required for high resolution in small cells.

2 Materials

2.1 Yeast Strains

Wild-type strain BY4742 and strains expressing chromosomally integrated GFP fusion constructs are obtained from EUROSCARF (Institute of Microbiology, Johann Wolfgang Goethe-University Frankfurt, Germany) as well as from Invitrogen, Inc. [26].

2.2 Cell Culture

Bacto™ yeast extract (BD, Inc., USA).
D(+)-glucose monohydrate (Roth, Inc.).
Bacto™ peptone (BD, Inc., USA).

Bacto™ agar (BD, Inc., USA).
12-well microtiter plates (Greiner, Inc.).

For preparation of yeast media, yeast extract (1 % w/v) and peptone (2 % w/v) are weighed in an appropriate flask and filled up with distilled water to 9/10 of the final volume. Autoclave at 121 °C for 20 min; add 1/10 volume of filter-sterilized 10× glucose solution (2 % w/v final concentration) to the media, mix well, and dispense into sterile flasks or glass tubes. For solid media plates, 2 % Bacto™ agar is added to the solution prior to autoclaving.

2.3 Dyes and Reagents

BODIPY 493/503 (Invitrogen, Inc.). 1 mg/ml stock solution in DMSO.
LD540 ([21] see below). 1 mg/ml stock solution in DMSO.
Nile Red (Sigma-Aldrich, Inc.). 1 mg/ml stock solution in DMSO.
Oleic acid-D₃₄ (Sigma-Aldrich, Inc.). 1 % (w/v) stock solution in 10 % Tergitol.
Palmitic acid (Sigma-Aldrich, Inc.). 1 % (w/v) stock solution in 10 % Tergitol.
Tergitol NP-40S 70 % stock solution (Sigma, Inc.).
Tergitol working solution 10 % (aqua dest. diluted stock solution).
Tris base (Merck, Inc.).
D-Sorbitol (Merck, Inc.).
NaCl (Merck, Inc.).
Agarose electrophoresis grade (Invitrogen, Inc.).
RediGrad™ (Amersham Biosciences, Inc.).

2.4 Cell Preparation

COREX™ high-speed glass centrifugation tubes (38 ml).
Thermomixer (Eppendorf Comfort, Eppendorf, Inc.).
50 ml plastic tubes (Greiner, Inc.).

2.5 Fluorescence Microscopy Hardware and Imaging Software

Microscope slides 76×26 mm (Roth, Inc.).
Coverslips 50×24 mm #1, 0.17 mm (Menzel, Inc.).

Leica Microsystems SP5 confocal microscope with differential interference contrast DIC (transmission imaging), spectral detection, and sequential scanning feature. HCX PL APO 63×/1.4 OIL CS oil-immersion objective. HCX PL APO 63×/1.2 W CORR water-immersion objective. Leica LAS AF ver. 2.6.3 (Leica Microsystems, Inc.) microscope control software.

2.6 CARS Microscopy Hardware and Imaging Software

The CARS (coherent anti-Stokes Raman scattering) system is based on a Leica SP5 confocal microscope (Leica Microsystems, Inc.). The CARS signal is generated using a picosecond laser source with integrated optical parametric oscillator (*pico*Emerald; APE, Germany; HighQ Laser, Austria). For the detection of neutral lipids and lipid droplets, the laser is tuned to 2,845 cm⁻¹, which enables the imaging of CH₂ symmetric stretching vibrations. To detect deuterium-labeled fatty acids, the laser is tuned to

2,105 cm^{-1} (CD_2 stretching vibrations [27, 28]). Both, CH_2 and CD_2 stretching vibrations are detected in epi(E)-CARS mode using suitable emission filters (650/210, 770SP) and a non-descanned detector (NDD) [29, 30]. The appropriate wave number is set via the user interfaced of the SP5 microscope control software.

2.7 Image Processing Software

Fiji is an ImageJ-based public domain image processing software (<http://fiji.sc>; Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). Leica LAS AF ver. 2.6.3 (Leica Microsystems, Inc.) is used for microscope control and basic image processing.

3 Methods

3.1 Cell Cultivation

Fluorescence labeling efficiency (BP 493/503, LD540, and specifically Nile Red) and GFP expression levels are strongly dependent on cell growth phase and cell viability. Thus, great care has to be taken to maintain reproducible growth conditions. A more homogeneous labeling in a cell population can be achieved by two to three cycles of pre-cultivation, 8–12 h each (*see* **Note 1**).

3.1.1 Stationary Phase Cells

1. Cultivate yeast cells in culture flasks containing 5 ml YPD for 12 h at 30 °C on a rotary shaker (180 rpm).
2. Inoculate 100 ml fresh YPD in a 500 ml flask with 100 μl of the preculture and cultivate cells for 72 h, to stationary phase.

3.1.2 Logarithmically Growing Cells

1. Cultivate yeast cells in culture flasks containing 5 ml YPD medium for 12 h at 30 °C on a rotary shaker (180 rpm).
2. Inoculate 100 ml fresh YPD medium in a 500 ml flask with 100 μl of the preculture and cultivate cells for 72 h, to stationary phase.
3. Inoculate 2–10 ml fresh YPD medium with 1/100 volume of stationary phase culture and cultivate for 4–8 h at 30 °C on a rotary shaker (180 rpm).

3.2 Separation of Quiescent Yeast Cells by Density Gradient Centrifugation

This protocol is based on the method described by Allen et al. for the isolation of quiescent cells from stationary phase cultures [31]. The procedure proved to be extremely valuable for microscopic investigation of cells due to the quantitative elimination of necrotic or apoptotic cells and stationary phase cells with large vacuoles. Thus, vital staining and GFP expression patterns turn out to be very homogeneous in the cell population (*see* **Note 2**).

1. Add 9 ml RediGrad™ and 1 ml sterile 1.5 M NaCl to 38 ml high-speed centrifuge tubes (e.g., COREX™ glass tubes).

2. Establish density gradient by centrifuging tubes at $19,000\times g$ for 15 min at room temperature in a fixed angle rotor.
3. Centrifuge 2 ml of stationary phase yeast culture (72 h) for 5 min at $1,000\times g$ in a tabletop centrifuge. Aspirate off supernatant.
4. Resuspend cell pellet in 1 ml sterile 50 mM Tris-HCl pH 7.5.
5. Overlay the cell suspension onto the previously formed RediGrad™/NaCl gradient in the high-speed centrifuge tubes.
6. Centrifuge for 1 h at $430\times g$ in a tabletop centrifuge with a swing-out rotor; do not use brakes to decelerate the rotor.
7. Carefully remove the tubes from the centrifuge; avoid shaking.
8. Collect the lower fraction (~1 ml) of quiescent cells with a syringe and transfer it into a 15 ml polypropylene tube. Add 500 μ l of sterile 50 mM Tris-HCl pH 7.5; vortex briefly. Harvest cells at $1,000\times g$ for 5 min. Wash cells once with sterile 50 mM Tris-HCl pH 7.5.

3.3 Immobilization of Yeast Cells for Long-Term Microscopic Observation

3.3.1 Immobilization of Yeast Cells Using Agar Sheets [32]

1. Weigh in Bacto™ agar in an appropriate flask and fill up with distilled water to the final volume to obtain a 2 % (w/v) solution.
2. Autoclave at 121 °C for 20 min.
3. Transfer 5 ml of melted agar into a 15 ml plastic tube. Pour the agar on a standard microscope slide (76×26 mm) on a flat surface. Take care that the edges of the slide are fully covered with agar.
4. Allow polymerization of the agar for 15 min at RT.
5. Add 1 μ l of (labeled) yeast cell suspension to the center of the agarose layer. Mount the cell preparation with a large (50×24 mm) coverslip (Fig. 1: Illustration of the agarose immobilization method; see Notes 1 and 3).

3.3.2 Immobilization of Yeast Cells Using Fluorescently Labeled Agar Sheets

This immobilization method is particularly useful for four-dimensional (4D) live cell imaging of fluorescently labeled LD during cellular growth [19, 33] (see Notes 1 and 3).

1. Prepare (medium) agar (see Subheading 3.1).
2. Transfer 5 ml of melted (medium) agar into a 15 ml plastic tube and cool to approx. 60 °C in a water bath (the agar must remain liquid).
3. Add the corresponding fluorescent dye in the same concentration as used for liquid media to the warm agar solution (see Subheading 3.5). Vortex thoroughly but gently to avoid air bubbles.
4. Pour the fluorescently labeled agar on a microscope slide. Take care that the edges of the slide are fully covered with agar.
5. Allow polymerization of the agar for 15 min at RT.

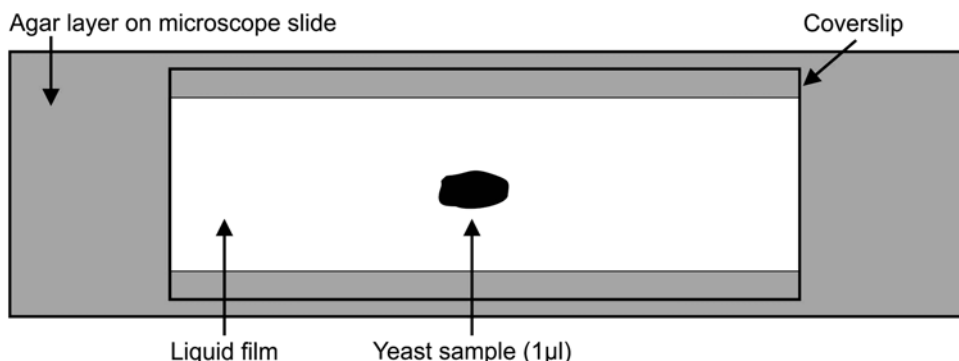


Fig. 1 Immobilization of yeast cells using the “agarose sheet” method for long-term microscopic observation. Slides are covered with melted agarose (optionally containing appropriate growth components and dyes). After polymerization, cells are placed in the center of the slide and covered with a coverslip. Cells are spread out in a liquid film as a monolayer right underneath the coverslip, providing least distortion of the laser beam and thus optimum resolution for transmission and fluorescence microscopy. Note that the condenser position of the microscope needs to be adjusted to the appropriate level, to obtain optimal illumination of the specimen. Conditions are appropriate for maintaining cell viability and monitoring cellular growth and division over extended periods of time (>10 h)

3.4 Preparation of Yeast Cells for CARS Microscopy (See Note 4)

1. Cultivate yeast cells to the desired growth stage (see Subheading 3.1).
2. Transfer 1 ml of yeast culture to a 1.5 ml Eppendorf reaction tube.
3. Centrifuge cells at $1,000 \times g$ for 1 min in a tabletop centrifuge; aspirate off supernatant.
4. Resuspend cells in 1 ml aqua dest.
5. Repeat steps 3–4 two times.
6. Mount 1 µl of dense cell suspension on a standard microscope slide under a large 50×24 mm coverslip.

3.5 Fluorescence Labeling of Neutral Lipids

Media for yeast cultivation such as complete media (YPD) contain large quantities of fluorescent metabolites and may thus cause significant fluorescence signals at the interface between the coverslip and the liquid phase. This phenomenon may interfere with high-resolution imaging of fluorescently labeled yeast structures. In this case the cells may have to be washed prior to the staining procedure. However, to avoid significant changes of the cellular physiology and morphology during the staining procedure and washing steps (e.g., the formation of large vacuoles), we prefer to stain the cells directly in the corresponding cultivation media (see Notes 1, 5, and 6).

3.5.1 Labeling of Lipid Droplets Using BODIPY 493/503

BODIPY 493/503 labeling of neutral lipids is highly efficient. Thus, the dye is an excellent choice for advanced imaging techniques such as 4D live cell imaging of LD [34] as well as for quantification of LD in yeast cell populations [35, 36] (*see* **Notes 6** and **7**).

1. Cultivate yeast cells to the desired growth stage (*see* Subheading 3.1).
2. Transfer 1 ml of the cell suspension to a 1.5 ml Eppendorf tube.
3. Add 1 μ l of BODIPY 493/503 stock solution (final concentration 1 μ g/ml) to the cell suspension. Stain for 15–20 min.
4. Centrifuge cells at $1,000\times g$ for 1 min in a tabletop centrifuge.
5. Mount 1 μ l of dense cell suspension on a standard microscope slide for short-term analysis or on a microscope slide covered with agar (*see* Subheading 3.3), for long-term observation.

Figure 3 shows BODIPY 493/503 labeling of cells cultivated to exponential and stationary growth phase.

3.5.2 Labeling of Lipid Droplets Using LD540

LD540 enables spectral discrimination of labeled LDs against green fluorophores (e.g., GFP) and therefore facilitates imaging of multi-labeled specimens (*see* **Notes 6–8**).

1. Cultivate yeast cells to the desired growth stage (*see* Subheading 3.1).
2. Transfer 1 ml of the cell suspension to a 1.5 ml Eppendorf tube.
3. Add 1 μ l of LD540 stock solution (final concentration 1 μ g/ml) to the cell suspension; stain for 15–20 min.
4. Centrifuge cells at $1,000\times g$ for 1 min in a tabletop centrifuge.
5. Mount 1 μ l of dense cell suspension on a standard microscope slide for short-term analysis or on a microscope slide covered with agar (*see* Subheading 3.3), for long-term observation.

3.5.3 Nile Red Staining of Unfixed Stationary Phase Yeast Cells

Nile Red staining efficiency is high in stationary phase cells; thus, fixation with formaldehyde can be omitted under these conditions to obtain reliable lipid droplet staining (*see* **Notes 6, 7, and 9**). In contrast to other neutral lipid-specific dyes, the spectral properties of the dye enable discrimination of neutral lipids and phospholipids in Nile Red-labeled samples both in stationary phase cells and exponentially growing cells (*see* Subheading 3.6, **step 2**).

1. Transfer 1 ml of a stationary phase yeast cell suspension to a 1.5 ml reaction tube. Wash the cells two times with 1 ml sterile 50 mM Tris-HCl, pH 7.5.

2. Centrifuge cells at $1,000 \times g$ for 1 min in a tabletop centrifuge; aspirate off supernatant.
3. Resuspend cells in 1 ml sterile 50 mM Tris-HCl, pH 7.5
4. Add 1 μ l of the Nile Red stock solution to the cell suspension (final concentration, 1 μ g/ml). Vortex briefly.
5. Incubate cells for 20 min at RT. Do not close the lid of the reaction tube during staining.
6. Centrifuge cells at $1,000 \times g$ for 2 min using a tabletop centrifuge. Aspirate off supernatant and resuspend cells in the remaining liquid.
7. Mount 1 μ l of dense cell suspension on a standard microscope slide for short-term analysis or on a microscope slide covered with agar (*see* Subheading 3.3), for long-term observation.

3.5.4 Nile Red Staining of Logarithmically Growing Cells

For labeling logarithmically growing or stationary phase cells, and in combination with GFP detection, cells are preferentially fixed with formaldehyde prior to Nile Red staining (*see* **Notes 9** and **10**).

1. Transfer 1 ml of yeast culture to a 1.5 ml reaction tube.
2. Centrifuge cells at $1,000 \times g$ for 1 min in a tabletop centrifuge. Aspirate off supernatant.
3. Resuspend cells in 500 μ l sterile 1 M sorbitol. Incubate for 60 s at RT.
4. Add 27 μ l of a 37 % formaldehyde solution (v/v) to the sample (final concentration 2 %, v/v). Incubate cells for 1–5 min at RT (*see* **Note 10**). Vortex briefly every 30 s.
5. Centrifuge cells for 2 min at $1,000 \times g$ in a tabletop centrifuge.
6. Wash cells three times with 500 μ l sterile 50 mM Tris-HCl pH 7.5.
7. Add 0.2 μ l of the Nile Red stock solution to the cell suspension (final concentration, 0.4 μ g/ml); vortex briefly.
8. Stain cells for 10 min at room temperature.
9. Centrifuge cells at $1,000 \times g$ for 2 min using a tabletop centrifuge. Aspirate off supernatant and resuspend cells in the remaining liquid.
10. Mount 1 μ l of dense cell suspension on a standard microscope slide for short-term analysis or on a microscope slide covered with agarose (*see* Subheading 3.3), for long-term observation.

3.6 Fatty Acid Treatment of Yeast Cells for CARS Microscopy

1. Cultivate yeast cells to the desired growth stage (*see* Subheading 3.1).
2. Add 100 μ l of the deuterium-labeled oleic acid stock solution (final concentration, 0.05 %) and 100 μ l of the unlabelled palmitic acid stock solution (final concentration, 0.05 %) to 2 ml

fresh YPD in a 12-well microtiter plate. Inoculate 3×10^7 cells (OD 1; *see Note 11*) of the preculture into the pre-warmed YPD/fatty acid medium.

3. Cultivate the cells for 16 h at 30 °C on a thermomixer (350 rpm).

3.7 Microscopy

3.7.1 Transmission Light Microscopy: Differential Interference Contrast (DIC, Nomarski)

1. Mount cells on standard microscope slides or on agarose-coated slides.
2. Visualize cells using DIC optics. The condenser position needs to be adjusted for cells mounted on agarose-covered slides, due to the extended distance from the condenser lens, for optimal results (Fig. 2: differential interference contrast imaging of quiescent cells).

3.7.2 Confocal Laser Scanning Microscopy of Fluorescently Labeled LD

BODIPY 493/503 fluorescence is excited at 488 nm (Argon laser) and emission detected between 500 and 550 nm. Thus, the dye is not compatible with simultaneous GFP detection (GFP $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 488/500–550 nm); however, it is compatible with red fluorescent proteins, e.g., mCherry or dsRed [37, 38] (Fig. 3).

Nile Red fluorescence can be excited both at 488 nm (Argon laser) and 543 nm (HeNe laser). For specific lipid droplet staining, Nile Red is preferentially excited at 488 nm and fluorescence emission detected between 550 and 575 nm. For detection of membranes in addition to lipid droplets, Nile Red is preferentially excited at 543 nm, and emission is detected between 550 and 575 nm for lipid droplets and between 600 and 700 nm for lipid droplets plus intracellular membranes. Due to the rapid saturation

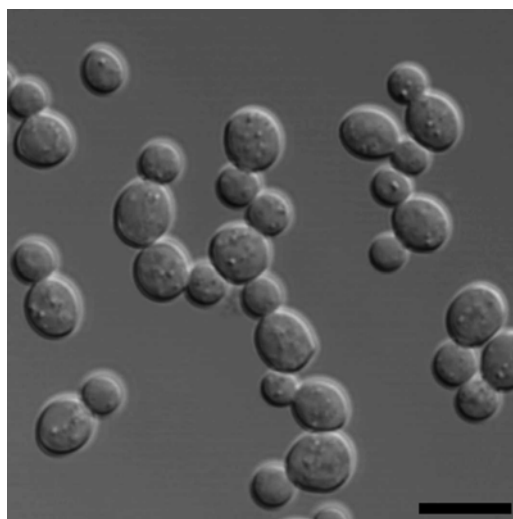


Fig. 2 Differential interference contrast image of yeast cells prepared by density gradient centrifugation and subsequent cultivation in complete media for 90 min. Note the lack of any damaged cells and the high level of synchrony of cell division. Bar = 10 μm

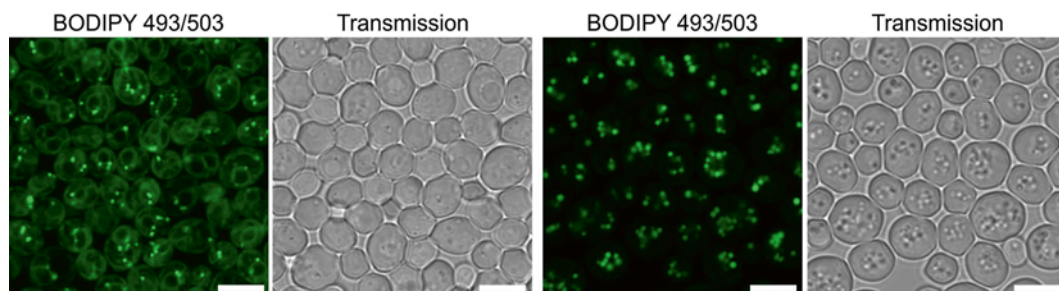


Fig. 3 BODIPY 494/504 fluorescence of labeled subcellular yeast structures in stationary phase cells and in exponentially growing cells. Strong signal of phospholipid membranes and of small LD in actively growing yeast cells (images at *left*). Note high-contrast detection of LD in stationary phase cells. The “membranous” background can hardly be detected due to the high affinity of the dye for neutral lipids (images at *right*). Bar = 5 μ m

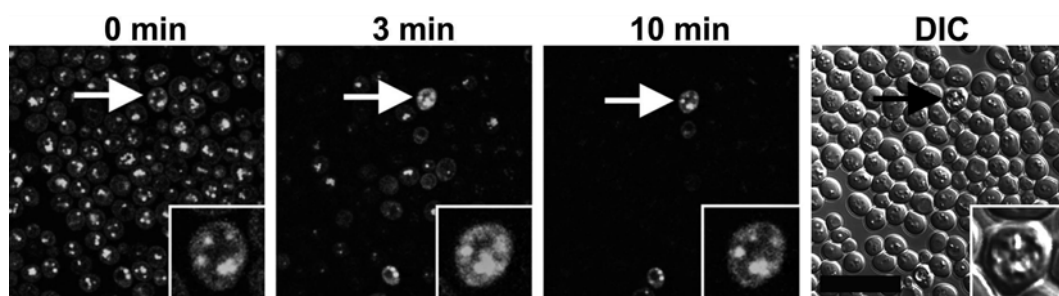


Fig. 4 Time-lapse record showing fast disappearance of Nile Red from stationary phase yeast cells. Yeast cells were labeled with Nile Red (Subheading 3.3) and mounted on agarose-covered slides without Nile Red. Three minutes after mounting, a significant loss of the Nile Red signal is detectable in most cells. After 10 min only visibly damaged cells retain fluorescence (*white arrow*; the corresponding cell is displayed in the *lower right corner* of each time-lapse image at higher magnification). Nile Red excitation and emission were at 543 nm and 600–700 nm, respectively. These data show that Nile Red labeling is reversible and that Nile Red preferentially stains damaged or dead cells, which limits its use for live cell imaging, in particular of mutant cell cultures. Bar = 20 μ m

and bleaching characteristics, Nile Red excitation should be performed with reduced laser intensity (1–5 % of maximum laser power at 488 nm excitation and 50 % laser power at 543 nm excitation) (Fig. 4) (*see Note 12*).

LD540 fluorescence is excited at 543 nm or at 561 nm (DPSS laser) and emission detected between 550 and 700 nm or 570 and 700 nm. Thus, LD540 is compatible with simultaneous imaging of GFP (Fig. 5) (*see Note 13*).

3.7.3 CARS Microscopy of (Deuterium Labeled) Neutral Lipids (Fig. 6) (*See Note 14*)

1. Tune the CARS system to 2,840 cm^{-1} to probe for CH_2 vibrational frequencies of unlabeled (neutral) lipids.
2. Set the pump beam and the Stokes beam to low output power. Acquire an image using 8–16 $\times$ line averaging. Use a lookup table that indicates scanned regions above the

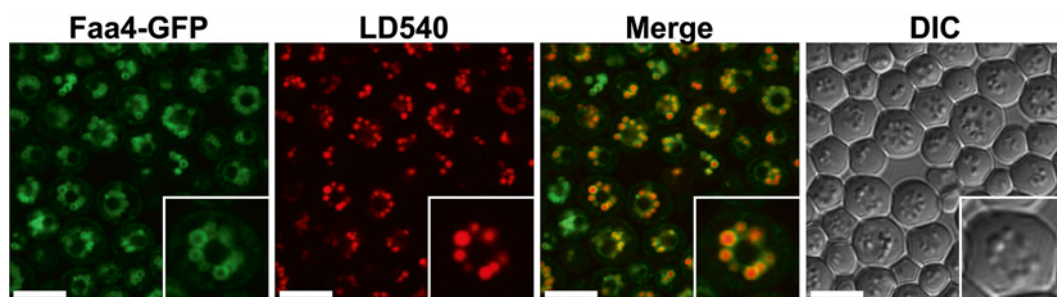


Fig. 5 High-resolution imaging of Faa4p-GFP and LD540 double-labeled live yeast cells. The localization of Faa4p-GFP at the periphery of the lipid droplets as well as the LD540-labeled lipid droplet core is clearly resolved. GFP excitation was at 488 nm and emission was detected between 500 and 535 nm. LD540 excitation was at 561 nm and emission was detected between 570 and 700 nm. Fluorescence images were acquired simultaneously. DIC image was recorded sequentially to avoid fluorescence bleaching and distortion of the fluorescence light by the DIC optics. Bar = 5 μ m

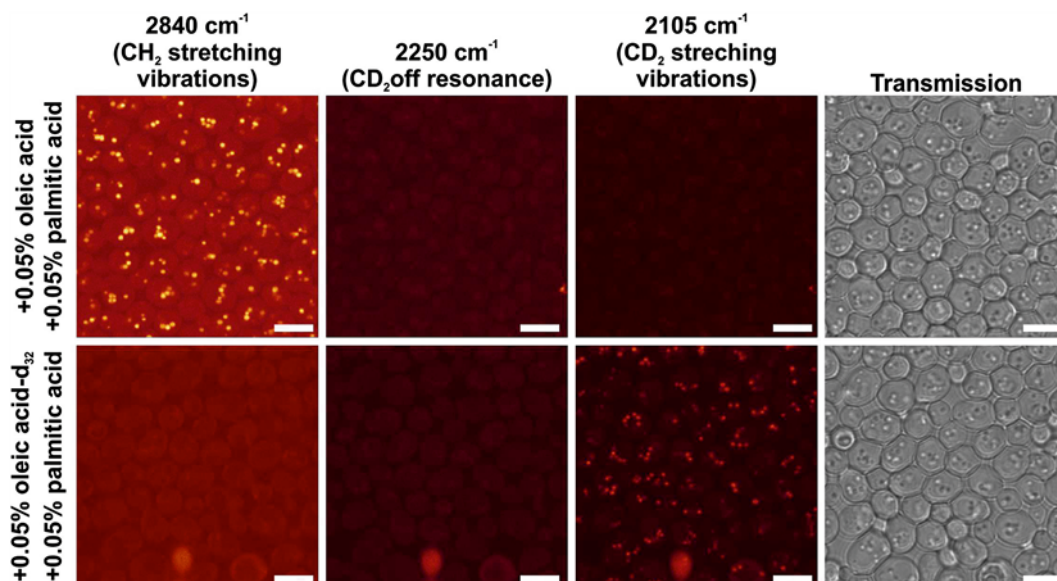


Fig. 6 Detection of deuterium-labeled fatty acids that are incorporated into neutral lipids of living yeast cells. Detection of CH_2 stretching vibrations in cells grown for 16 h in the presence of deuterium-labeled oleic acid and unlabeled palmitic acid. Lipid droplets yield an intense CARS signal when CH_2 stretching vibrations of neutral lipids were excited at 2,840 cm^{-1} (*top panel, left*). The CARS signal of neutral lipids is very weak at 2,250 cm^{-1} , i.e., “off resonance” (*top panel, middle*). Almost no CARS signal is detectable in unlabeled cells at 2,105 cm^{-1} (CD_2 stretching vibrations; *top panel, right*). A strong signal appears at 2,105 cm^{-1} in cells supplemented with fully deuterated oleic acid- d_{32} (*bottom panel, right*). No CARS signal of deuterium-labeled or unlabeled cells at 2,250 cm^{-1} “off resonance.” Very weak signal of CH_2 stretching vibrations at 2,840 cm^{-1} in cells treated with both oleic acid- d_{32} and unlabeled palmitic acid, indicating that oleic acid was preferentially incorporated into neutral lipids rather than palmitic acid (*bottom panel*). Bar = 5 μ m

dynamic range of the detector. Evaluate the sample and image for potential light-induced movement of LD, for cell destruction, and for pixels exceeding the dynamic range of the detector. Increase laser power and optimize image acquisition (*see* **Notes 15–17**).

3. Acquire an image at $2,840\text{ cm}^{-1}$ (CH_2 stretching vibrations).
4. Tune the CARS system to $2,105\text{ cm}^{-1}$ to probe CD_2 stretching vibrations of deuterium-labeled neutral lipids.
5. Acquire an image at $2,105\text{ cm}^{-1}$.
6. (Figure 6: CARS images of deuterium-labeled and unlabeled fatty acids incorporated into neutral lipids of yeast cells).

4 Notes

1. Media for yeast cultivation, such as complete media (YPD), contain large quantities of fluorescent metabolites, which may cause significant spurious fluorescence signals especially at the interface between the coverslip and the liquid phase. This phenomenon may interfere with high-resolution imaging of fluorescently labeled yeast structures, and cells may have to be washed prior to the staining procedure. However, to avoid significant changes of the cellular physiology and morphology during the staining procedure and washing steps (e.g., the formation of large vacuoles), we prefer to stain the cells directly in the respective cultivation media. Appropriate emission filter settings (spectral detector) may help to eliminate background fluorescence emitted from the medium.
2. RediGrad™ density gradient centrifugation was previously shown to be largely inert on yeast cell physiology [31]. The resulting cell population is very homogeneous in terms of cell size, labeling characteristics, and growth. If cells are grown for up to 7 days in stationary phase prior to RediGrad™ enrichment, they are arrested in G0 phase of the cell cycle; after transferring to fresh media, cells rapidly and synchronously enter the cell cycle [15].
3. After mounting the coverslip, the cells are spread out as a monolayer in a liquid film, which should not extend to the horizontal borders of the coverslip, indicative of too much liquid that results in floating of the cells. By cutting several vertical and horizontal slits into the agar, a large number (up to 96) of different small yeast colonies can be mounted on a single agar sheet without mixing. This setup enables image-based high-content screens [33, 39].
4. For CARS imaging of yeast LD, we apply high-numerical-aperture objectives such as a $63\times$ NA1.4 oil-immersion objective

or a 63× NA1.2 water-immersion objective. For 2D imaging of yeast cells with optimized sampling rate, a line frequency of 400 Hz (512×512 pixels) and bidirectional scanning mode are applied. Optionally, 8× or 16× line averaging is performed. Slower scan speed may result in light-driven artificial movement of LD or may lead to cell destruction. For 3D imaging of yeast cells, an increased line frequency of 700 Hz (512×512 pixels) and bidirectional scanning mode are applied. With this setup no visible cell damage was observed. Using this setup small LD (<200 nm) can also be imaged multidimensionally [36]. However, in 4D experiments over extended periods of time, significantly impaired cell growth, most likely caused by cell stress, has been observed, using CARS. Yeast LD can also be imaged at video-rate using a resonant scanner [24]. However, by using this fast scanning mode, imaging of smaller LD (<200 nm) is limited.

5. To supply cells with nutrients during long-term microscopic observation, the agarose (agar) can be dissolved in growth media. Using an objective heater system, this preparation technique also enables cultivation and microscopic analysis of yeast cells directly on the microscope table, over extended periods of time under close-to-physiological conditions.
6. Nile Red, BODIPY 493/503, and LD540 are practically non-fluorescent in the aqueous environment. Thus, we typically do not wash the cells after staining, and confocal imaging of cells in the presence of excess dye in the media has no significant effect on the background signal. Brightly fluorescing entities are typically dead cells. Of note, all three dyes have a high affinity for neutral lipids (i.e., LDs), but they also label the more polar phospholipids. Thus, especially in growing cells that contain reduced levels of neutral lipids and LDs, a significant staining of subcellular membranes is observed. Also, the staining of membrane accumulations in mutant cells lacking neutral lipids [20] may lead to misinterpretations.
7. BODIPY 493/503, LD540, and particularly Nile Red are actively pumped out from actively growing yeast cells by the activity of pleiotropic drug resistance pumps [33, 40]. This effect can be partially compensated for by maintaining cells on fluorescently labeled agar sheets (*see* Subheading 3.4) or by chemical fixation of cells (*see* also **Note 9**).
8. LD540 is not yet commercially available. It is synthesized according to Patent No. EP 2284541 A1: Fluorescence-based imaging and analysis of cells and cellular components using lipophilic dyes with improved specificity, spectral property, and photostability. Patent owner: Christoph Tiehle, Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V., Germany.

9. To overcome limitations of the imaging of both GFP- and Nile Red-labeled stationary phase yeast cells, i.e., photobleaching of Nile Red or PDR-mediated active export of Nile Red from yeast cells, we apply this short fixation protocol in combination with sequential imaging. In the sequential scan mode, parameters such as laser intensity and emission wavelength are optimized for both GFP and Nile Red individually and are then applied to the double-labeled fixed yeast samples automatically and in a sequential order. Fixed cells can be mounted in the presence of anti-fading reagents to reduce photobleaching.
10. The fixation time depends on the type of GFP fusion and on the growth stage of the cells and has to be determined experimentally. In our lab, stationary phase wild-type cells are fixed for 2–5 min and exponentially growing yeast cells are fixed for a maximum time of 60 s. In general, fixation times should be as short as possible since longer fixation times may influence morphology of lipid droplets and GFP fluorescence. Stabilization during fixation by sorbitol efficiently supports GFP integrity and maintains its fluorescence, but may interfere with vacuole morphology.
11. The cell density is critical for efficient fatty acid uptake. Cell densities should be kept low to increase the specific cellular fatty acid uptake.
12. We observed rapid photobleaching of Nile Red when applying optimized zoom settings required for high-resolution confocal imaging. However, the rate of Nile Red photobleaching is strongly dependent on the intensity of the illuminating laser source. Setting the laser intensity of the 488 nm argon laser line or the 543 nm HeNe laser line of the confocal microscope system to ~1–5 % or 50 %, respectively, of the maximum output power, significantly reduces Nile Red photobleaching. However, these values may need adjustment as the laser power decreases over time. Due to the excellent fluorescence properties of Nile Red, high-contrast images with optimum resolution are obtained even at very low excitation intensities that also enable reliable multidimensional imaging of Nile Red-labeled structures.
13. LD540 is compatible with simultaneous detection of GFP. However, the dye shows cross emission (“fluorescence crosstalk”) with the GFP channel when applied in higher concentrations. The emission wavelength for GFP detection should therefore be further restricted and set between 500 and 530 nm and/or the concentration of LD540 should be reduced to the lowest possible amount. Notably, at higher concentrations and upon intense light illumination, LD540 may induce fusion of LD (Wolinski, unpublished observations, [22]).

14. CARS microscopy enables the detection of a resonant CARS signal that is generated by a specifically excited chemical bond. For the detection of neutral lipids, the symmetric CH_2 molecular vibration of fatty acids is excited at $\sim 2,845 \text{ cm}^{-1}$. However, the signal from the molecules of interest may be impaired by the nonresonant signal of a solvent or—in the case the live yeast cell imaging—by the aqueous growth media [25, 41]. Fortunately, the nonresonant background produced by yeast media (e.g., rich medium or synthetic medium) does not significantly interfere with the detection of LD in F-CARS mode. Thus, yeast cells can be imaged directly in the particular growth media. For critical experiments and for imaging of very small LD, cells are typically washed $3\times$ with distilled water, which further reduces the nonresonant background. For detection of CARS signals both in E-CARS or F-CARS modes, the cells are mounted on standard microscope slides or on agar slides (*see* Subheading 3.4). No background signal of the agar that is used for cell immobilization was observed in E-CARS or in F-CARS mode detection of CH_2 molecular vibrations excited at $2,845 \text{ cm}^{-1}$.
15. For CARS imaging of yeast LD, we apply high-numerical-aperture objectives such as a $63\times$ NA1.4 oil-immersion objective or a $63\times$ NA1.2 water-immersion objective. For 2D imaging of yeast cells with optimized sampling rate, a line frequency of 400 Hz (512×512 pixels) and bidirectional scanning mode are applied. Optionally, $8\times$ or $16\times$ line averaging is performed. Slower scan speed may result in light-driven artificial movement of LD or may lead to cell destruction. For 3D imaging of yeast cells, an increased line frequency of 700 Hz (512×512 pixels) and bidirectional scanning mode are applied. With this setup no visible cell damage was observed. Using this setup also small LD ($<200 \text{ nm}$) can be imaged multidimensionally [36]. However, in 4D experiments with CARS over extended periods of time, cell growth may become significantly impaired, most likely caused by cell stress. Yeast LD can also be imaged at video-rate using a resonant scanner [23]; however, imaging of smaller LD ($<200 \text{ nm}$) is limited.
16. GFP fluorescence is rapidly bleached upon illumination with a laser intensity that is used for CARS microscopy. Thus, CARS signals and GFP fluorescence are acquired sequentially.
17. Fluorescent dyes for labeling LD also stain phospholipids and membrane proliferations, which may occur in specific mutants. In such cases, the differentiation between the “true” neutral lipid signal and the “artificial” membrane signal is difficult. In such cases the resonant CARS signal at $2,845 \text{ cm}^{-1}$ can be used as a control for neutral lipids in fluorescently labeled specimens. It should be noted, however, that the fluorescent

dye may also be excited by a two-photon process as it is used for CARS microscopy, which thus obscuring the CARS signal. To differentiate between the fluorescence and a CARS signal, the pump and Stokes lasers are alternately switched on and off, which ablates the CARS signal but enables two-photon excitation of the fluorophore. A limitation of quantitative LD imaging by CARS is the occurrence of “shadow” effects. The incident light beam may be scattered strongly at objects located above another one, resulting in a dark shadow on a deeper localized object along the optical axis. Consideration of this phenomenon and the analysis of multiple optical sections are critical for the evaluation of CARS signal homogeneity [41].

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Image-Based Identification of Nuclear Export Inhibitors from Natural Products

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Abstract

High-content imaging with robotic microscopy has been widely used for phenotype-based cellular screening research. This technology is ideally suited to monitor intracellular translocation of macromolecules. Here, we describe in detail the procedures for screening microbial natural extracts for their capability to inhibit the general nuclear export machinery. The method is based on human cells that stably express a fluorescently-tagged reporter protein that contain a nuclear export signal capable of mediating its translocation from the nucleus to the cytoplasm through the nuclear pore. In the presence of a small molecule nuclear export inhibitor, the fluorescent signal is trapped to varying degrees within the nucleus. In order to analyze complex libraries of compounds, the assay has been scaled to 96- or 384-well formats and optimized for high-throughput screening (HTS). Active microbial extracts undergo confirmation screening, bioassay-guided fractionation, chemical dereplication, and compound purification. The active purified compound is characterized in secondary assays that monitor the nuclear export of disease-relevant proteins. Nuclear export inhibitors hold promise as potential cancer and antiviral drugs.

Key words High-content screening, Nuclear export inhibitors, Image analysis, Natural products, Fractionation, CRM1

1 Introduction

1.1 Nuclear Export

The compartmentalization of the eukaryotic cell imposes the requirement of transporting macromolecules between the nucleus and the cytoplasm [1, 2]. Abnormal regulation of the nuclear import and export machinery is a key driver of various disease states. Cancer cells utilize the aberrant localization of tumor suppressor proteins as a means for their inactivation and to effectively evade antineoplastic therapies. The aberrant localization of oncoproteins can also lead to their aberrant activation. Examples of unbalanced nucleocytoplasmic shuttling factors that are correlated with tumor formation, progression, or resistance to treatment include FOXO factors, p27, β -catenin, and NF- κ B [3–6]. On the other hand, many viruses including

human HIV-1, influenza A, dengue, respiratory syncytial virus, rabies, Rift Valley fever virus, and Venezuelan equine encephalitis virus rely on the transport of specific viral proteins into the host cell nucleus to avoid the antiviral response [7]. Therefore, inhibiting the nuclear trafficking of viral proteins has been proposed as a therapeutic strategy.

1.2 *Natural Products*

Natural products have been and still are the single most productive source of leads for the development of drugs [8]. Natural products are an extremely valuable source of chemical structure diversity for the discovery of new drugs against diverse pathologies. In particular, microorganisms use a relatively limited number of basic biosynthetic pathways to generate an outstanding diversity of chemical structures including polyketides, nonribosomal peptides, and terpenoids. It is important to note that we have only explored a fraction of the diversity of bioactive compounds, and opportunities for discovering new natural products leading to new drugs are enormous. In view of such notable structural diversity, it should not be surprising that we keep finding microbial metabolites with biological activity of interest for a wide range of therapeutic uses, most importantly for anticancer and anti-infective drugs. Interestingly, fifteen years after the advent of high-throughput screening and the post-genomic era and the resulting focus on screening huge libraries of synthetic compounds, we are witnessing a comeback of natural products in drug discovery. Although the process of drug discovery from natural products is still complex and difficult, significant technological progress made in the last years has improved our understanding of the key factors required for success. At present, it is widely accepted that natural products are the future of drug discovery. Their unparalleled structural diversity, their optimized interaction with molecular targets derived from a long evolutionary process, the still unexplored vast microbial diversity, and the existing potential of novel approaches to trigger the expression of silent pathways have been prompting current research that is fostering in the field of future natural product drug discovery [9].

Over a hundred natural product-derived compounds are currently undergoing clinical trials and at least a hundred similar projects are in preclinical development [8, 10]. In order for natural product drug discovery to continue to deliver novel leads, new and innovative approaches that were successful in basic research, with very elegant examples reported in literature, must be set up in a high-throughput “industrial format.” It might be possible to increase the current efficiency in identifying and developing new drugs from natural products by applying new approaches in a systematic manner to natural product drug discovery. Any new efforts in this area will benefit from the continued implementation of new technologies, such as imaging-based high-content screening, that will contribute to improving the efficiency of the process [9].

The structural diversity of natural products might also prove to be useful to target challenging processes such as functional analysis of the components of the nuclear export machinery which are mostly regulated by protein–protein interactions.

1.3 High-Content Screening

High-content screening (HCS) is a technology that combines automated fluorescence microscopy and quantitative image analysis to collect quantitative data from complex biological systems [11]. HCS is ideally suited to systematically observe translocation events in response to chemical perturbation on a large scale in time and space [12]. The subcellular localization of target proteins that lie downstream of the perturbation provides a visual readout for measuring the activity of signaling pathways and supports the identification of small molecules that might be exploited pharmaceutically to restore or interfere with the protein localization and function. HCS-based protein translocation assays have been widely employed to probe cellular signaling pathways [12]. These assays monitor the subcellular localization of a reporter protein either tagged with fluorescent protein biosensors such as GFP or visualized using fluorescent immunodetection in fixed cells. We describe here a high-content translocation assay which is based on the human osteosarcoma cell line U2OS stably expressing a green fluorescent protein (GFP)-tagged Rev protein. This reporter protein contains a strong heterologous nuclear export signal (NES) [13]. The fluorescent signal of untreated U2nesRELOC cells predominantly localizes to the cytoplasm. Upon treatment with nuclear export inhibitors, the GFP-labeled reporter protein accumulates rapidly in the cell nucleus (Fig. 1).

2 Materials

2.1 Equipment

Multidrop dispenser (Multidrop™ Combi Reagent Dispenser, Thermo Scientific).

Robotic Workstation (cell culturing robot, SelecT, TAP Biosystems).

BD Pathway 855 High-Content Bioimager (BD Biosciences, San Jose, CA).

Leica SPE confocal imaging system.

Gilson preparative high-performance liquid chromatography (HPLC) system.

LC-MS: Agilent 1200 RRLLC coupled to an ESI-TOF Bruker maXis mass detector.

NMR: Bruker Avance III HD NMR spectrometer (500 MHz) equipped with a 1.7 mm MicroCryoProbe.

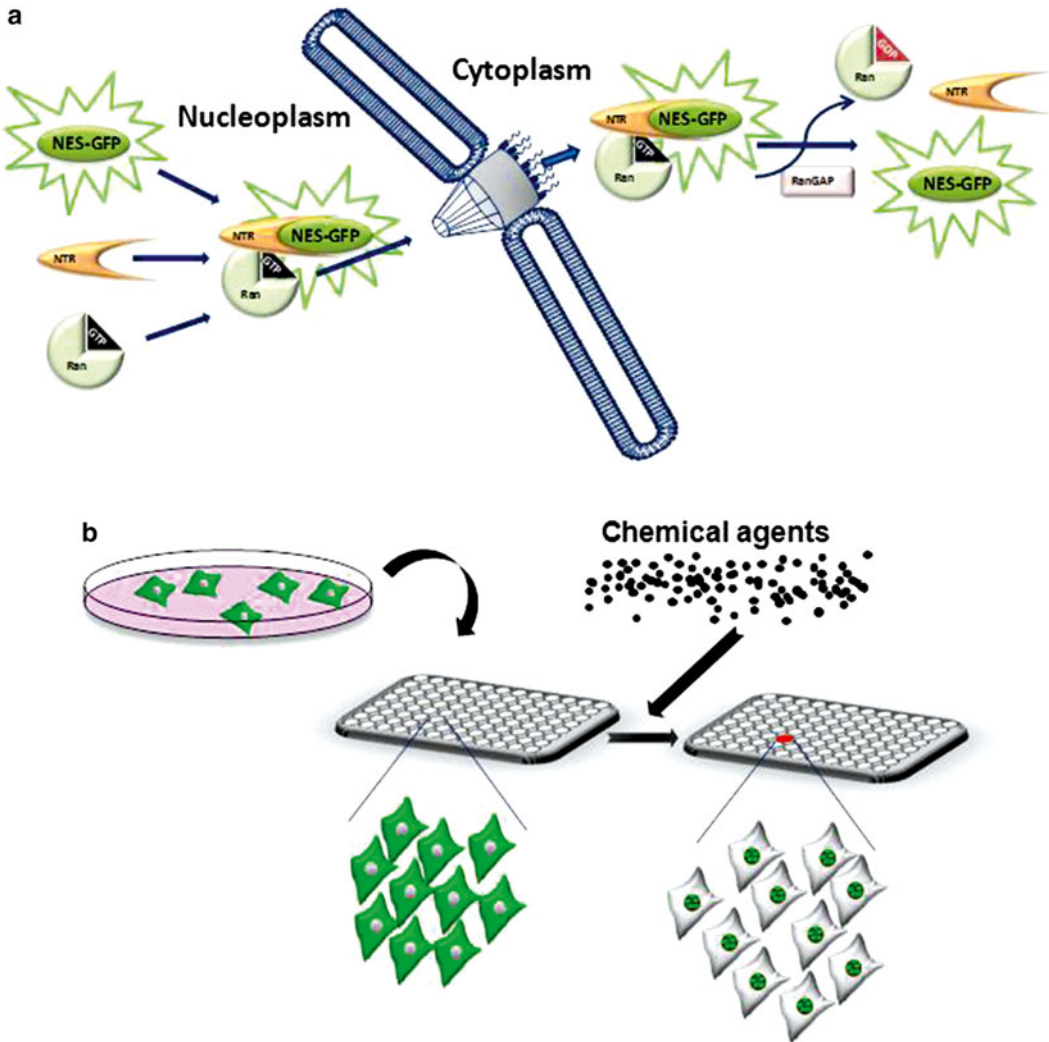


Fig. 1 Nuclear export and the identification of nuclear export inhibitors. **(a)** The assay is based on a reporter protein that contains a fluorescent tag (GFP) and a nuclear export sequence (NES). The export receptor CRM1 recognizes and binds to the NES of the reporter protein and escorts it through the nuclear pore complex. The export complex is formed by binding RanGTP. Once in the cytoplasm, the export complex dissociates when the GTP of Ran is hydrolyzed. **(b)** The assay has been adapted to a multiwell format. Treatment of the stable reporter cell line with inhibitors of the nuclear export that block the interaction between CRM1 and NES-containing cargo proteins leads to an accumulation of fluorescent signal in the cell nucleus

2.2 Software

AttoVision software (BD Biosciences, San Jose, CA).
 BD Image Data Explorer (Genedata AG, Switzerland).
 Genedata Screener software (Genedata AG, Switzerland).

2.3 Reagents, Buffers, Compounds, Dyes, and Antibodies

Methanol, bovine serum albumin (BSA, Sigma-Aldrich, #05470), and Tween (PBST) were purchased from Sigma-Aldrich (#p1379); nuclear factor (NF)- κ B2 p100/p52 antibody was from Cell Signaling (4882; Cell Signaling Technology, Danvers, MA); the secondary antibody ab96921 DyLight 594 was from Abcam, Cambridge, UK (donkey anti-rabbit IgG (H+L)); and DAPI was purchased from Sigma-Aldrich (#32670).

Leptomycin B (LC Laboratories, Woburn, MA, USA), fixatives: Our fixative of choice is 6 % paraformaldehyde prepared in PBS at a pH of 7.2–7.4. To prepare 100 mL of paraformaldehyde, add 4 g of paraformaldehyde powder to 100 mL of PBS and gradually heat to 60 °C under a fume hood. To clear the solution, slowly add drops of 1 N NaOH while stirring. Do not allow the temperature of the solution to exceed 65 °C. The final pH can be adjusted with NaOH or HCl if necessary. Blocking buffer: 1 % BSA (IgG- and protease-free) in phosphate-buffered saline (PBS) to block nonspecific staining. If there are problems associated with high background, 5 % normal serum from the host of the secondary antibody may be added to the blocking buffer.

2.4 Cells and Cell Culture

The human osteosarcoma cell line U2OS was obtained from the American Type Culture Collection (Manassas, VA). The U2nesRELOC cells stably express the reporter construct pRevMAPKKnesGFP, which has been described earlier [14]. pRevMAPKKnesGFP carries the NES from MAPK kinase (MAPKK) cloned between the BamHI and AgeI sites of pRev(1.4)-GFP and sandwiched between the Rev and the green fluorescent protein (GFP) coding sequences.

U2nesRELOC cells are maintained in T-175 cm² flasks at 37 °C with 5 % CO₂ in Dulbecco's modified Eagle's medium (Gibco Life Technologies, #11960-044) and supplemented with 10 % fetal bovine serum (Sigma) and Antibiotics-Antimycotics (Gibco Life Technologies, #10378-016). Upon reaching about 80 % confluency (about every 4–5 days), the cells were subject to passage in the following steps:

1. Wash cells twice with DPBS buffer. This step removes general debris as well as facilitates cell detachment.
2. Trypsinize the cells using TrypLE™ Express (Gibco Life Technologies, #12604-021) at room temperature (RT) for 5 min.
3. Gently tap the flask and neutralize the TrypLE™ Express with a cell culture medium.
4. Split a desired volume of cells to a new flask or imaging 96-well plates (Greiner Bio-One #655087).

3 Methods

3.1 High-Content Nuclear–Cytoplasmic Translocation Imaging Assay

1. Figure 2 illustrates the workflow of the assay procedure. The protocol described in this review uses the U2nesRELOC system [13, 15] which is an image-based methodology compatible with a high-throughput evaluation of small molecule inhibitors of the nuclear export. The U2nesRELOC is based on a human osteosarcoma cell line U2OS stably expressing green fluorescent protein (GFP)-labeled Rev protein that contains a strong heterologous nuclear export signal (NES). The fluorescent signal of untreated U2nesRELOC cells localizes exclusively to the cytoplasm. Upon treatment with nuclear export inhibitors, the reporter protein accumulates rapidly in the cell nucleus.
2. Grow U2nesRELOC cells until they reach 80–90 % confluency.

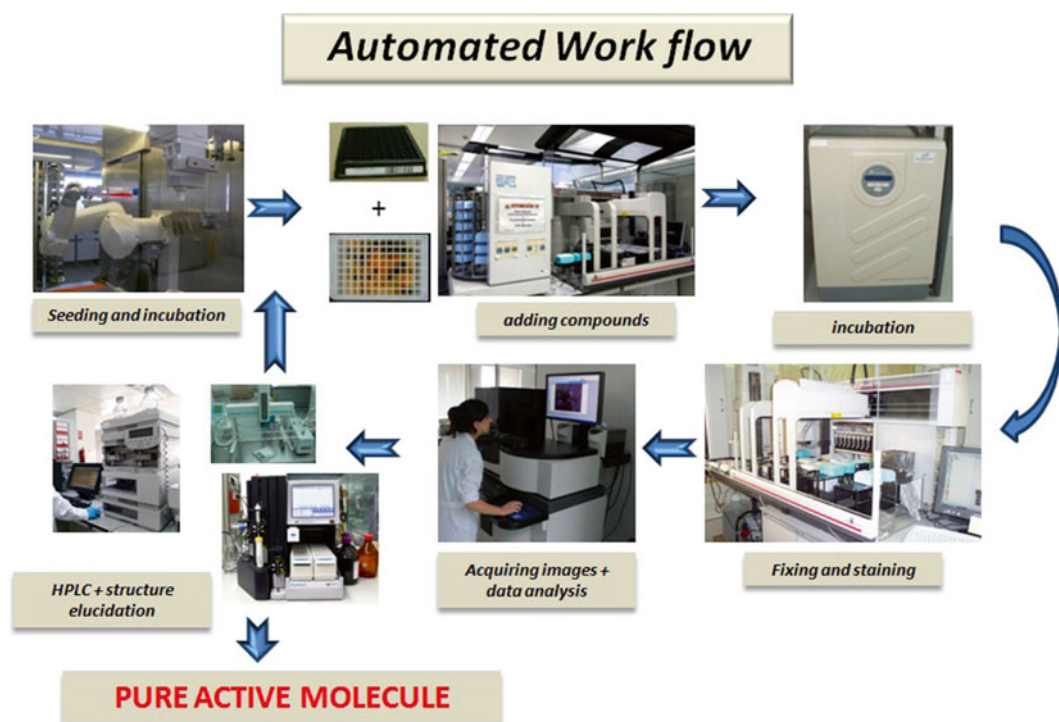


Fig. 2 Schematic workflow that illustrates the process of identification and purification of nuclear export inhibitors from natural products

3. Trypsinize the cells using TrypLE™ Express (Gibco Life Technologies, #12604-021) at room temperature (RT) for 5 min.
4. Gently tap the flask and neutralize the TrypLE™ Express with a cell culture medium.
5. Split a desired volume of cells to a new flask or imaging 96-well plates (Greiner Bio-One #655087).
6. Cells were seeded at a density of 10,000 cells/well into black-wall clear-bottom 96-well microplates (Greiner Bio-One #655087, *see* **Note 1**) and incubated at 37 °C 5 % CO₂. The final volume of the cell suspension was 200 µL in each well. 24 h later, the medium was replaced with a volume of 195, and 5 µL of extracts and controls (Leptomycin B (LMB) and DMSO) were added to the plates. Cells were incubated in the presence of the extracts for 1 h at 37 °C. After this time, the culture medium was removed, and the plates were washed twice with 1× PBS and fixed in 100 µL of 6 % paraformaldehyde for 30 min at room temperature. After aspiration of paraformaldehyde, fixed cells were washed twice with 1× PBS and, in order visualize the cell nucleus, stained with 4',6-diamidino-2-phenylindole (DAPI) 10.9 mM for 20 min at room temperature in the dark. DAPI concentration in cells was 300 nM. The DAPI solution was removed by aspiration. Finally, the plates were washed with 1× PBS twice and stored at 4 °C before analysis.
7. Samples were read in a high-content analyzer in the wide-field mode. Acquired images were processed using AttoVision software (BD Biosciences). The Bioimager was equipped with a 488/10 nm enhanced GFP (EGFP) excitation filter, a 380/10 nm DAPI excitation filter, a 515LP nm EGFP emission filter, and a 435LP nm DAPI emission filter. Images were acquired in the DAPI and GFP channels of each well using a 10× dry objective. The plates were exposed 0.066 ms (Gain 0) to acquire DAPI images and 0.85 ms (Gain 30) for GFP images (Fig. 3). An image algorithm was applied to allow the cell nucleus segmentation based on a local thresholding (AttoVision software). The segmentation strategy assumes that the cell's cytoplasm surrounds the nucleus. Consequently, cytoplasmic fluorescence intensity is calculated from all the pixels within a circumferential ring surrounding the nuclear ring mask. Based on the definition of cell compartments, the nuclear and cytoplasmic levels of GFP fluorescence were quantified with BD Image Data Explorer (Genedata AG, Switzerland) and Genedata Screener software (Genedata AG, Switzerland).
8. The BD Pathway Bioimager outputs its data in standard text files. Data were imported into the data analysis software BD

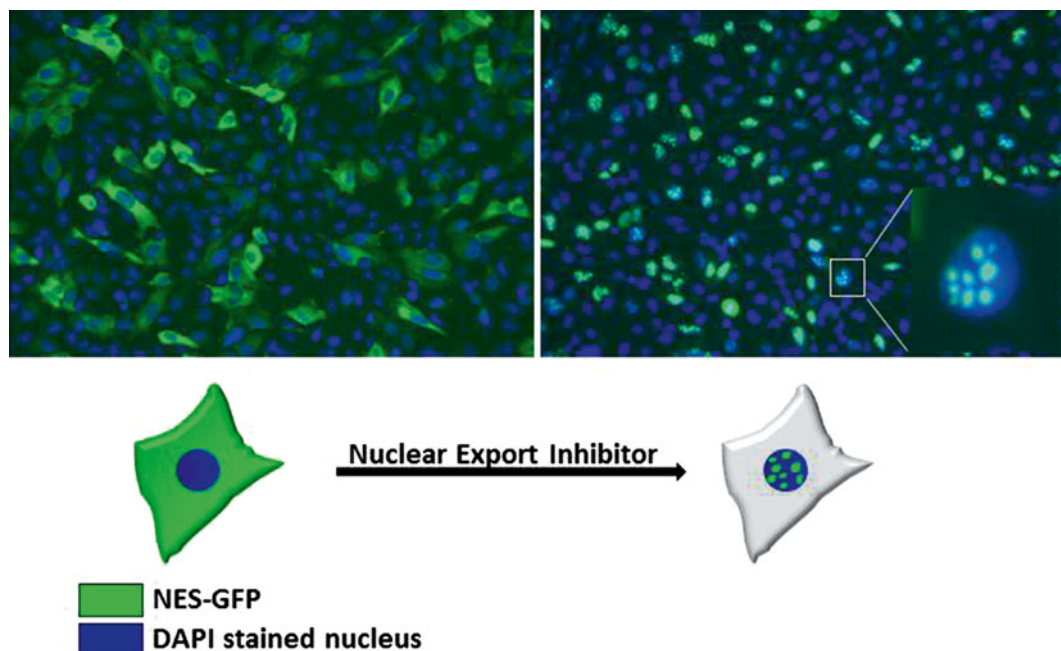


Fig. 3 Nuclear export inhibitors induce the accumulation of fluorescent signal (NES-GFP) in the nucleus of the reporter cell line. Cells were treated with DMSO (*right image*) or 4 nM Leptomycin B (*left image*) for 1 h. Then, cells were fixed and the cell nucleus stained with DAPI

Image Data Explorer. The nuclear–cytoplasmic ratios of fluorescence intensity were determined by dividing the fluorescence intensity of the nucleus by the cytoplasmic fluorescence intensity. A threshold ratio greater than 1.8 was employed to define nuclear accumulation of fluorescent signal for each cell. Based on this procedure, we calculated the percentage of cells per well displaying nuclear translocation or inhibition of nuclear export. Compounds that induced a nuclear accumulation of the fluorescent signal greater than 60 % of that obtained from wells treated with 4 nM LMB (LC Laboratories, Woburn, MA, USA) were considered to be hits.

9. The active extracts were fractionated on a Gilson Preparative HPLC system (ver. 3.2), and each fractionated plate is treated as previously described (Fig. 2).

3.2 Scale-Up Fermentation and Bioassay-Guided Fractionation of Selected Extracts

Microorganisms were isolated and cultured in EPAs tube (10 mL) or flasks (50 mL) at 28 °C on a rotary shaker (220–225 rpm) for 7–14 days in optimized medium for each microorganism [10]. These cultures were extracted with acetone and DMSO, and the extracts were arranged in 96-well plates.

The active wells were identified, and a new culture was made using two vials (10 mL) and a flask (100 mL). Following the same

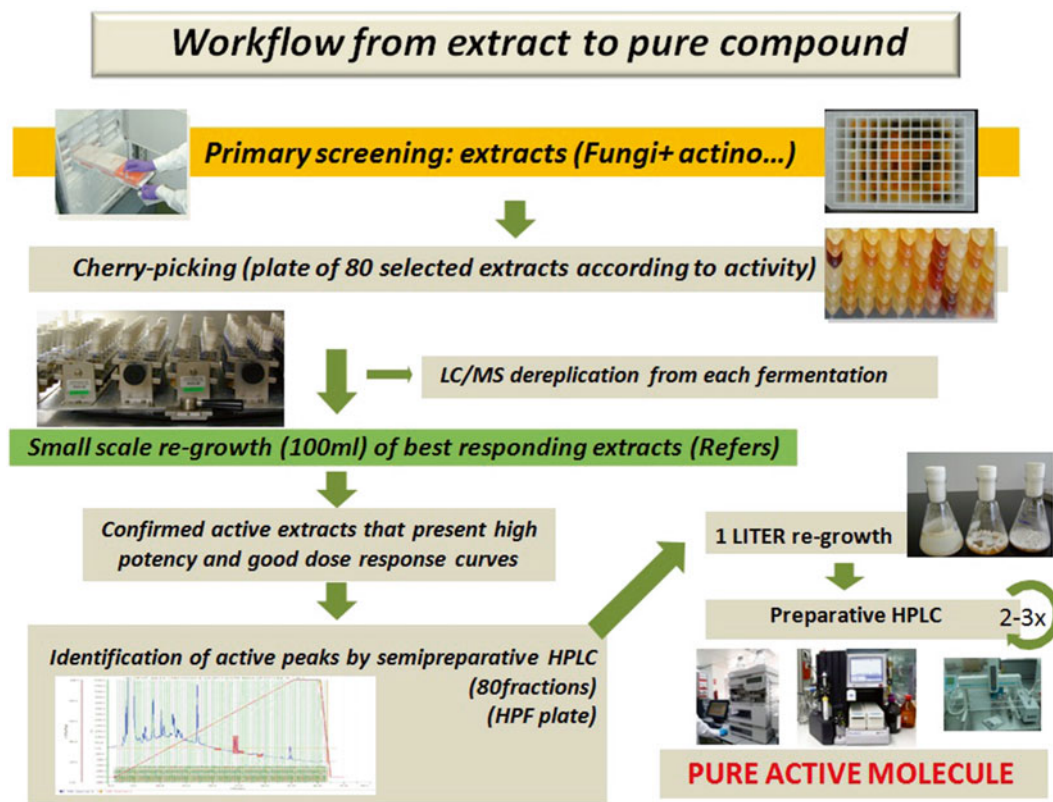


Fig. 4 Schematic workflow for scale-up fermentation and bioassay-guided fractionation of selected extracts

process described earlier, these new extracts were arranged in 96-well plates called “refers” for re-fermentation. These plates were tested again to confirm the activity and to know if the micro-organism produces the same active metabolite at 100 mL growth condition. Active extracts identified were cross-checked with those listed in Natural Products Dictionary (distributed by CRC Press Taylor and Francis Group: <http://www.crcpress.com/>), and the ones already known were excluded from further analysis, a process known as dereplication. Semi-preparative HPLC was made with the active extracts into 80 fractions. This new plate (called HPF) was tested again in the assay to detect the active fraction/s.

Active extracts with clear chemical profiles were cultured in a larger scale using a 1 L flask. Different fractionation processes were made by following the peak activity and the process end with the purification and the elucidation of the molecular structure of the active compound using liquid chromatography–mass spectrometry (LC-MS) and NMR (Fig. 4).

3.3 Extraction of Microbial Culture Broths and Fractionation of Extracts

1. Extract liquid culture broths (1 L) by addition of an equal volume of acetone (1 L) under continuous shaking at 220 rpm for 1 h.
2. Separate biomass by centrifugation, and concentrate the supernatant to 1 L under a stream of nitrogen.

3. Load the remaining solution (with continuous 1:1 water dilution, discarding the flow through) on a column packed with SP-207ss resin (brominated styrenic polymer, 65 g, 32 × 100 mm) previously equilibrated with water.
4. Wash the column with water (1 L).
5. Elute the column at a flow rate of 8 mL/min using a linear gradient from 10 to 100 % acetone in water (in 12.5 min) with a final 100 % acetone step (for 15 min), collecting fractions of 20 mL.
6. Concentrate fractions to dryness on a centrifugal evaporator and test for biological activity.
7. Further fractionate these fractions using one or several steps of semi-preparative or preparative reversed-phase HPLC with columns packaged with C8 or C18 silica gel. Fractionation method will depend on the chemical nature of the bioactive components.
8. Test the biological activity of the HPLC fractions.
9. Repeat HPLC fractionation changing elution method and/or column until a bioactive compound of the desired purity is obtained as determined by LC-MS and NMR analyses (typically >90 % purity).

3.4 Characterization of the Pure Compound

3.4.1 Dose–Response Analysis

Determination of IC₅₀ values were carried out in automated U2nesRELOC assays. Cells were cultured as described above and exposed for 1 h to equal volumes of test compounds (2 µL). IC₅₀ values were calculated as being the inhibitor concentration that increases nuclear accumulation of the reporter protein by 50 % using Genedata Screener software (Genedata AG, Switzerland). Cells were treated with eight twofold serial dilutions of each compound in 1 % DMSO final:

1. Prepare serial dilutions of the active compound.

Cells were seeded at a density of 10,000 cells/well into black-wall clear-bottom 96-well microplates (Greiner Bio-One #655087) and incubated at 37 °C 5 % CO₂. The final volume of the cell suspension was 200 µL in each well. Eight twofold serial dilution of pure compound were prepared in 100 % DMSO; 2 µL of each point is collected and mixed with 198 µL of complete medium. 24 h after cell seeding, medium was replaced with the medium/compound or controls mix (LMB at 4 nM final and 1 % DMSO). After 1 h treatment, plates are treated as previously described. Compound activity was normalized to the in-plate negative and positive controls using DMSO and LMB, respectively. We chose 4 nM LMB as maximal inhibition and 1 % DMSO as minimal inhibition of nuclear export in U2nesRELOC (Fig. 5).

Representative images of the compound-treated U2nesRELOC cell line

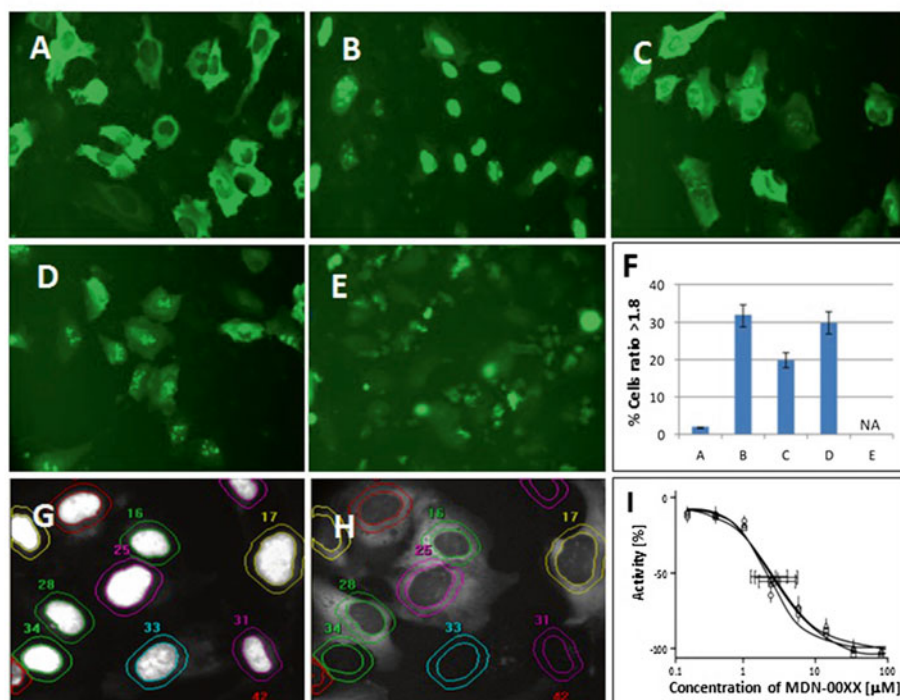


Fig. 5 Representative images of the compound-treated assay cell line using the high-throughput format (ABCDE) of the U2nesRELOC system: (a) vehicle control (1 % DMSO), (b) positive control (4 nM Leptomycin B [LMB]), (c) crude extract, (d) active fraction from high-performance liquid chromatography (HPLC), (e) sample that was eliminated by the cytox measure, (f) *bar graphs* that represent the quantification of the effect on the nuclear export of the reporter protein, and (g, h) *panels* demonstrating the segmentation. (i) *Graphs* represent an IC₅₀ determination curve for a putative compound. Activity is relative to cells treated with 4 nM LMB (100 %) and carrier (DMSO, 0 %)

3.4.2 Confirmation of Compound Activity by Confocal Microscopy

1. U2OS cells were cultured as described above and exposed for 1 h to equal volumes of pure compound (2 μL).
2. Obtain cell cultures and rinse the cell culture media away with several quick washes of PBS at 37 °C.
3. The cells were 100 % methanol fixed (100 μL/ well, 5 min) and then incubated in 100 μL/ well 1 % BSA/10 % normal goat serum/0.3 M glycine in 0.1 % PBS Tween for 1 h to permeabilize the cells and block nonspecific protein–protein interactions.
4. Rinse cells in two changes of PBS with 1 % BSA for 10 min per change.

5. The cells were then incubated with 25 μ L/ well NF- κ B2 p100/p52 antibody (Cell Signaling Technology® #4882) and diluted at appropriate concentration (*see* **Note 2**) in the above blocking buffer overnight at +4 °C.
6. Rinse three times at 10 min each in blocking buffer.
7. Incubate in fluorochrome-conjugated secondary antibody ab96921 DyLight®594 (Abcam) donkey anti-rabbit IgG (H+L) at 37 °C for 1 h or 4 °C overnight. Keep dishes covered with foil to prevent fading of fluorochrome.
8. Rinse and repeat for staining with DAPI to stain the cell nuclei (blue) at a concentration of 1.43 μ M.
9. Rinse three times at 10 min each in PBS.

Samples were imaged at 22 °C using 63 $\times$ water immersion lenses on a Leica SPE confocal imaging system. Images were obtained using Leica software.

4 Notes

1. The authors achieve a homogenous distribution of the cells by using the Multidrop™ Combi Reagent Dispenser which provides precise fast-dispensing and high-throughput operation with minimal dead volume and back flushing.
2. Optimal dilution for immunohistochemistry must be determined by investigator (from 1/100 to 1/500) depending of the type and the lot of antibody.

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Chapter 23

Correlative Video-Light–Electron Microscopy of Mobile Organelles

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Abstract

Correlative microscopy is a method when for the analysis of the very same cell or tissue area, several different methods of light microscopy (LM) and then electron microscopy (EM) are used consecutively. The combination of LM and EM allows researchers to study phenomena at a global scale and then to look for unique or rare events for their subsequent EM examination. Unfortunately, the observation of living cells under EM is still impossible. LM provides the possibility to examine quickly many live cells, whereas EM provides the high level of resolution. On the other side, the final goal of any morphological analysis of a biological sample, whether it is an organism, organ, tissue, cell, organelle, or molecule, is to get an averaged three-dimensional model of the structure examined and to determine the chemical composition of it. This chapter describes the methodology of imaging with the help of CVLEM. The guidelines presented herein enable researchers to analyze structure of organelles and to obtain the three-dimensional model of the structure examined, and in particular rare events captured by low-resolution imaging of a population or transient events captured by live imaging can now also be studied at high resolution by EM.

Key words Electron microscopy, Correlative light–electron microscopy, Time-lapse analysis, Three-dimensional imaging, Electron tomography, Focused ion beam–scanning electron microscopy, Serial ultrathin sections, Intracellular localization, Intracellular transport, Culture cells

1 Introduction

The maximal goal of a morphological study is to make the average three-dimensional (3D) model of the structures examined and to determine its chemical composition with maximally possible resolution. At the EM level, it means that it is necessary to make the average three-dimensional (3D) model of the structure examined and to determine its average chemical composition.

However, often we have rare structure and fast rare events, which is important to examine with high resolution but which cannot be examined on the basis of random EM imaging. Now, EM has the single limitation, namely, it cannot be used to study

living cells, whereas light microscopy is not able to reach the same high level of resolution as EM. In this case, one needs correlative light–electron microscopy.

Correlative light–electron microscopy (CLEM) was proposed in 1958 [1]; more than several improvements were developed [2, 3]. The correlative video-light–electron microscopy was published in 2000 [4]. However, only after 2007, its development greatly accelerated (reviewed in ref. 5). The typical CVLEM procedure includes several stages: (1) observation of the structures labeled with fluorescent protein (FP, i.e., green FP) or other fluorescent markers in living cells, (2) immobilization (fixation or freezing), (3) immune labeling or other types of labeling with gold particles or other markers suitable for EM, (4) embedding (or freezing), (5) identification of the just examined cell in the resin block or within the frozen sample, (6) sectioning of thin or thick serial sections (or analysis of the sample under FIBSEM) and identification of the cell on the resin block and cutting of thin or thick serial sections, and (7) EM analysis and structure identification. Each of these steps could be performed by different ways, and all techniques have their own advantages and disadvantages.

Recently, CLEM became the leading edge of EM where dozens of protocols could be combined for different purposes. This field became extremely wide and includes now almost all existing methods of light and electron microscopy [6]. Now, there exist more than 10,000 different methods of CLEM because at each step of CLEM, there are several methods which could be used (as such, the full description of the CLEM within one chapter is equal to the description of almost all the methods of EM). Therefore, it is necessary to select the best combination of methods, which could be used for the specific goal of one's study. It is also necessary to use stereology for measurement [7].

The first choice is to select what state of cells or tissue will be examined under LM: in living or fixed state. If it is necessary to study living cells, one should select what kind of organelle will be examined: quickly moving or static. This determines the type of LM, which is necessary to select. The final selection is what type of 3DEM is necessary to use. If you need the highest level of resolution along *Z*-axis, the selection is EM tomography. If you need to have rather high level of resolution, but the most important is to have high thickness of the volume examined, the best choice is FIBSEM. The resolution of 3DEM based on serial images depends on what kind of image is formed. If this image represent the projection of a structure localized within a defined layer of a sample, the resolution will be a double thickness of serial layers (sections, virtual layer, or layer, which is eliminated during milling [scraping of a layer of sample with the help of focused ions]). If serial images represent the image of the very superficial zone of the layer examined, the *Z*-resolution is equal to the thickness of the single layer (section). In EM tomography, this resolution is equal to the

double thickness of virtual section. Here, we describe the simplest procedure for the examination of living cells containing quickly moving organelles.

Initially, CVLEM was demanding, and to master its various steps requires a whole array of skills. However, microscopy is developing fast both in the field of live-cell imaging and in the field of EM, and new powerful technologies are rapidly becoming available in user-friendly versions.

This should make the use of CLEM appealing to a number of cell biologists. Through the use of fluorescent proteins of different colors, two or more marker proteins can be observed simultaneously. This will allow the analysis of the interactions between different organelles and organelle sub-domains. Combining these and other methods with electron microscope tomography increases the subtlety and range of the questions that can be answered by the CLEM approach.

Taken together, all the steps of CLEM represent quite a long procedure (*see below*) and require significant effort of a researcher or technician. Hence, it would be especially disappointing to lose such tour-de-force experiments due to small troubles in specimen handling. The entire CVLEM procedure requires quite a long time to be completed (4–5 days). During the first day, the cells have to be transfected with cDNA. The second day, it is possible to make observations in living cells, fix them, and start the immune labeling. The third day is required to complete the immune labeling and resin embedding of the cells. During the fourth day, the cell of interest has to be identified in the resin block and be cut in serial sections. However, it is possible to store already embedded specimen for a long time and to cut them later.

Our experience suggests that people often ask us whether treatment of sample with 70 % ethanol could be longer than 5–10 min or whether it is possible to leave samples in glutaraldehyde for more than one day before Epon embedding. Here, we give the minimal times, and in the brackets, one could find the time during which it is possible to leave samples without significant alteration of the structure.

Before deciding what method of CLEM could be used, it is necessary to consult with the established specialist in EM. The scientist planning to perform correlative microscopy should decide which combination he will apply: CLLM, CLEM, or CLLM and CLEM. It is necessary to decide whether one needs to examine the rare event/structure using simple 3D reconstruction describing the shape and connections or it is necessary to combine this with immune labeling. For example, the strategy of CLEM will be different if the identification of the organelle will be in the flat cell or in the tall cell. It is necessary to select what kind of sections he will be applying for TEM or STEM tomography and so on: tangential or vertical sections, simple serial section, or thick serial sections.

1.1 Equipment

1. MatTek Petri dish with CELLocate (MatTek, Ashland, MA).
2. Anti-capillary self-closing tweezers (Ted Pella Inc., Redding, CA).
3. Ultra 35° and 45° diamond knives (Diatome, Hatfield, PA 19440, USA, or Electron Microscopy Sciences, Hatfield, PA).
4. Eyelash with handle (Ted Pella Inc., Redding, CA).
5. Pick-up loop (Agar, Cambridge, UK).
6. Slot grids covered with formvar–carbon supporting film (Electron Microscopy Sciences, Fort Washington, PA, or Agar, Cambridge, England).
7. A stereomicroscope equipped with a fiber optic illuminator (i.e., Leica Microsystems Spa, Milan, Italy, www.leica-microsystems.com).
8. A digitalized fluorescent-inverted microscope or laser scanning confocal microscope (i.e., from Leica Microsystems Spa, www.leica-microsystems.com or Zeiss).
9. Ultramicrotome Leica EM FC6 or EM US7 (Leica Microsystems Spa, www.leica-microsystems.com). Only ultramicrotomes from Leica are equipped with everything necessary for CLEM.
10. A transmission electron microscope with a motorized goniometer.
11. A scanning electron microscope with ion beam device.

2 Materials

Cells of interest, i.e., HeLa cells (No. CCL 185; American Type Culture Collection, Rockville, MD) or other cells.

2.1 Reagents

1. cDNA and transfection reagents (from corresponding companies).
2. The highest purity HEPES, NaOH, HCl, BSA, paraformaldehyde, potassium ferrocyanide, thiocarbonylhydrazide, 100 % ethanol, hydrofluoric acid, and chloroform (all from, i.e., Sigma-Aldrich S.r.l., Milano, Italy, www.sigmaaldrich.com, or other companies).
3. NANOGOLD-conjugated monovalent Fab fragments; a gold enhancement kit, HQ Silver (Nanoprobes, NY, USA; <http://www.nanoprobes.com/products/Silver.html#hq>); or LM/EM Silver Enhancement Kit 1 (SPI Supplies/Structure Probe, Inc. West Chester, PA, USA).
4. 50 % glutaraldehyde (EM grade); sodium cacodylate, 4 % water solution of OsO₄ in ampoules, tannic acid, TAAB 812,

1 × 2-mm slot grids, 1 × 2-mm slot grids covered with formvar and carbon (Electron Microscopy Sciences, Hatfield, PA, or Agar, UK).

5. Whatman number 1 filter paper (Fisher Scientific, Pittsburgh, PA).

2.2 Solutions

1. 150 mM NaCl.
2. 10× PBS.
3. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 2 mM l-glutamine (Gibco BRL, Life Technologies).
4. HEPES buffer (0.2 M). Dissolve 4.77 g HEPES in 100 ml distilled water, and add of 1 N HCl droplet by droplet to provide a pH of about 7.2–7.3.
5. PBS–BSA buffer: 20 mM phosphate, 150 mM NaCl, pH 7.4; 1 % bovine serum albumin (BSA), optional, may reduce background, 0.5 M NaCl, 0.05 % Tween 20.
6. Fixative 1. 0.05 % glutaraldehyde plus 4 % formaldehyde in 0.15 M HEPES (pH 7.2–7.3). Dissolve 8 g paraformaldehyde powder in 50 ml 0.2 M HEPES buffer, stirring and heating the solution to 60 °C. Add drops of 1 N NaOH to clarify the solution. Add 1.25 ml 8 % glutaraldehyde and 50 ml 0.2 M HEPES buffer. Dilute twice before use.
7. Fixative 2. 4 % formaldehyde in 0.15 M HEPES (pH 7.2–7.3). Dissolve 4 g paraformaldehyde powder in 100 ml HEPES buffer, stirring and heating the solution to 60 °C. Add drops of 1 N NaOH to clarify the solution.
8. Blocking solution. Dissolve 0.50 g BSA, 0.10 g saponin, 0.27 g NH₄Cl in 100 ml of 0.2 M HEPES (pH 7.2–7.3).
9. Gold enhancement mixture. Using equal amounts of the four components (Solutions A, B, C, and D from a gold enhancement kit), prepare about 200 µl of reagent per Petri dish (a convenient method is to use an equal number of drops from each bottle). First, mix Solution A (enhancer; green cap) and Solution B (activator; yellow cap). Wait for 5 min, and then add Solution C (initiator; purple cap) and finally Solution D (buffer; white cap). Mix well. Development starts with the addition of Solution C (initiator), so apply to sample as soon as possible after adding C and D to minimize auto-nucleation background. The development is not highly light sensitive, so it may be conducted under normal room lighting. Secondary GEEM solution: for slower development, substitute the following buffer for Solution D (buffer), 0.05 M sodium phosphate, 0.1 M NaCl, and pH 6.1.

10. Silver-enhance mixture. Prepare HQ SILVER™ using equal amounts of the three components included into the silver enhancement kit. Dispense initiator (A) into a clean tube or dish, add moderator (B), and mix thoroughly, then add activator (C), and mix thoroughly again to prepare the reagent. Use immediately after preparation.
11. 1 % glutaraldehyde in 0.2 M HEPES buffer (pH 7.2–7.3). Mix 1 ml of 50 % glutaraldehyde with 49 ml of 0.2 M HEPES (pH 7.2–7.3). Store at 0–4 °C.
12. 0.2 M cacodylate buffer. Dissolve 2.12 g sodium cacodylate in 100 ml of distilled water. Add 1 N HCl to provide a pH of about 6.9.
13. Reduced OsO₄. Mix 2 % OsO₄ in water 3 % potassium ferrocyanide in 0.2 M cacodylate buffer (pH 7.2) 1:1.
14. 0.3 % thiocarbohydrazide. Dissolve 0.1 g of thiocarbohydrazide in 33.3 ml 0.2 M cacodylate buffer (pH 6.9). Use immediately after preparation.
15. 1 % OsO₄. Mix 2 ml of 4 % OsO₄ with 6 ml of 0.2 M cacodylate buffer (pH 6.9). Use immediately after preparation.
16. 1 % tannic acid. Dissolve 0.1 g tannic acid (TAAB Laboratories Equipment Ltd) in 0.05 M sodium cacodylate buffer (pH 6.9) prepared by the dilution of one volume of 0.2 M cacodylate buffer (pH 6.9) in three volumes of distilled or deionized water.
17. Ethanol. To prepare N% ethanol, mix N ml of 100 % ethanol (Electron Microscopy Sciences, Hatfield, PA) with 100-N ml of deionized water.
18. Epon (or other resins prepared according to the manufacturer's instructions) mixture (TAAB 812, DDSA, MNA, DMP30 in a ratio of 24:9.5:16.5:1, respectively). Put 20.0 g Epon, 13.0 g dodecenyl succinic anhydride (DDSA) and 11.5 g methyl nadic anhydride (MNA) into the same test tube. Heat the tube in the oven for 2–3 min at 60 °C and then vortex it well. Add 0.9 g tri-dimethylaminomethyl phenol (DMP-30, all from Electron Microscopy Sciences, Fort Washington, PA), and immediately vortex the tube again. It is possible to freeze the Epon in aliquots and to store it for a long time at 20 °C before use.
3.3.9. 1:1 Epon/100 % ethanol mixture. Mix 10 ml of 100 % ethanol with 10 ml of Epon mixture.
19. DAB solution. Just before incubation, dissolve 0.05 g diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) in 50 ml 0.1 M sodium cacodylate buffer, pH 7.4, to get 1 mg/ml concentration, filtered through a 0.22 micron syringe filter (Millipore), place on ice, and add to the cells. In general,

solutions freshly prepared containing DAB at concentrations from 0.5 to 2 mg/ml in Tris-HCl pH 7.4–8.2 are in use. Diaminobenzidine solutions should be freshly prepared, filtered through folded filters (Macherey-Nagel, Germany), and stored at 4 °C no more than 2 h. For incubation, the samples are kept cold and in the dark. Preincubation should ensure equilibrium of DAB within the samples; times from 10 to 60 min are tuned to the sample thickness, from a few microns of cell monolayers to several hundred microns of tissue sections. The DAB solutions are recommended to be renewed, in order to provide fresh and clear (and maybe also oxygen-enriched) solutions.

20. 10-nm colloidal gold conjugated with protein A. This always-fresh reagent could be ordered from Utrecht University, The Netherlands (Dr. G. Posthuma).

3 Methods

3.1 Transfection

1. Grow, i.e., HeLa cells in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum and 2 mM l-glutamine at 37 °C and 5 % CO₂.
2. Suspend HeLa cells using standard procedures, and plate cells for CLEM on a MatTek Petri dish with the CELLocate cover slip attached to its bottom. The CELLocate cover slip contains an etched grid with coordinates that allow the localization of the cell of interest at any step in the preparation.
3. Transfect HeLa cells with cDNA of the protein fused with a fluorescent tag (i.e., green fluorescent protein; GFP) using any standard method of transfection or microinjection of cDNA into the nucleus.
4. By 6–48 h (depending of the fusion protein and method of transfection used) after transfection, place the dish under an inverted fluorescence microscope or laser scanning confocal microscope.
5. Select the transfected cell of interest, and identify its position related to the coordinates of the CELLocate grid.
6. Draw (or photograph) the position of the cell on the map of the CELLocate grid. For instance, the cell could be near the cross of horizontal line A and vertical line 3. Then the figure will be visible after the polymerization of Epon as the replica of the roof on the cover slip.
7. Another way to map the cell of interest is to scrap cells around the cell of interest using a wood stick, and cells remaining in the center of a ring could be visible; scrapping of cells in a parallel and perpendicular way could help to mark the position of

the cell of interest. Finally, the pattern of cell culture could be used for the labeling of the cell position.

8. Observe the dynamics of the GFP-labeled structures in the selected living cell using a multiphoton—a laser scanning confocal—or a digitalized fluorescent-inverted microscope, which allows the grabbing of a time-lapse series of images by a computer.
9. At the moment of interest, add fixative A to the cell culture medium while still grabbing images (fixative A: medium volume ratio is 1:1). Fixation usually induces the fast fading of GFP fluorescence and blocks the motion of labeled structures in the cell.
10. Stop grabbing time-lapse images and keep the cells in fixative for 5–10 min (during this time, it is useful to grab a Z-series of images of the cell).
11. Replace the mixture with the fixative 1 and keep the cells in the fixative 1 for 5 min.
12. Replace fixative 1 with fixative 2 and keep cells there for 30 min.
13. Wash with 0.2 M HEPES (pH 7.2–7.3) for 10 min (*see Note 1*).

3.2 Correlation of Video Live Imaging and Additional Immune Labeling of the Same Cell

1. Fix cells (*see above*).
2. Wash.
3. Incubate cells in the block solution.
4. Incubate with the first primary antibody.
5. Wash.
6. Incubate with the secondary antibody against the first primary one.
7. Wash.
8. Incubate with the second primary antibody.
9. Wash.
10. Incubate with the second secondary antibody against the second primary antibody and so on.
11. Photo cells after labeling with four different antibodies and prepare them for EM.

3.3 Immune Labeling for EM with NANOGOLD

1. Wash the fixed cells for 3×5 min with 0.2 M HEPES (pH 7.2–7.3).
2. Incubate the cells with the blocking solution for 30 min and then with the primary antibodies diluted in blocking solution, overnight.
3. Wash the cells for 6×2 min with 0.2 M HEPES (PH 7.2–7.3).

4. Dilute the NANOGOLD-conjugated Fab fragments of the secondary antibodies $\times 50$ times in the blocking solution, and add this to the cells; incubate for 2 h.
5. Wash the cells again for 6×2 min with 0.2 M HEPES (pH 7.2–7.3).
6. Fix the cells with 1 % glutaraldehyde in 0.2 M HEPES buffer (pH 7.2) for 5 min.
7. Wash the cells for 3×5 min with 0.2 M HEPES (pH 7.2–7.3) and then for 3×5 min in distilled water.
8. Incubate the cells with the gold enhancement mixture for 6–10 min according to instruction of the manufacturer. The cells will become violet–gray in color if the gold enhancement is successful.
9. Wash cells for 3×5 min with distilled water (*see Note 2*).

3.4 Silver Enhancement for Double Labeling

1. Incubate cells with 1 % bovine serum albumin in PBS at pH 7.4 (PBS–BSA) for 5 min; this manipulation blocks any nonspecific protein-binding sites and minimizes nonspecific antibody binding.
2. Incubate with mixture of primary antibodies obtained from different species and diluted at usual working concentration in PBS–BSA (30 min–1 h or usual time)
3. Rinse with PBS–BSA (3×1 min).
4. Incubate cells with monovalent conjugated with NANOGOLD Fab fragments of antibody against IgG of the species where the first primary antibody was developed (NANOGOLD® reagent should be diluted 1/40–1/200 in PBS–BSA with 1 % normal serum from the same species as the NANOGOLD® reagent) for 30 min at room temperature.
5. Rinse with PBS–BSA (3×1 min) and then PBS (3×1 min).
6. Rinse with deionized water (2×1 min).
7. Optional (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3×5 mins).
8. Prepare HQ SILVER™ using equal amounts of the three components. Dispense initiator (A) into a clean tube or dish, add moderator (B), and mix thoroughly, then add activator (C), and mix thoroughly again to prepare the reagent. Develop specimen for 30 s–4 min. More or less time can be used to control particle size and intensity of signal. A series of different development times (i.e., 30, 60, 120 s) should be tried to find the optimum time for your experiment. Select the time, which gives the size of gold–silver particles of 20 nm. Incubate cells with HQ SILVER for time, which gives 20 nm gold–silver particles.

9. Rinse in deionized water for 5 min, twice.
10. Incubate cells with monovalent conjugated with NANOGOLD Fab fragments of antibody against IgG of the species where the second primary antibody was developed (NANOGOLD[®] reagent should be diluted 1/40–1/200 in PBS–BSA with 1 % normal serum from the same species as the NANOGOLD[®] reagent) for 30 min at room temperature.
11. Rinse with PBS–BSA (3 × 1 min) and then PBS (3 × 1 min).
12. Rinse with deionized water (2 × 1 min).
13. Optional (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 × 5 min).
14. Prepare HQ SILVER[™]. Incubate cells with HQ SILVER for time, which gives 20 nm gold–silver particles.
15. Rinse with deionized water (2 × 1 min).
16. Fix with 1 % glutaraldehyde in 0.2 M HEPES buffer (pH, 7.2) for 10 min.
17. Wash in the same 0.2 M HEPES buffer.
18. To avoid dissolution of silver particles in OsO₄, fix cells with 1 % OsO₄ intermitting with thiocarbohydrazide (*see* below) using the shortened scheme and embed in Epon (*see* **Note 3**).

3.5 Immunolabeling for EM with Antibodies Conjugated with HRP

1. Wash the fixed cells for 3 × 2 min with PBS.
2. Incubate the cells with the blocking solution for 30 min and then with the primary antibodies diluted in blocking solution, overnight.
3. Wash the cells for 6 × 2 min with PBS.
4. Incubate the cells with the HRP-conjugated Fab fragments of the secondary antibody for 2 h.
5. Wash the cells again for 6 × 2 min with PBS.
6. Fix the cells with 1 % glutaraldehyde in 0.2 M HEPES buffer for 5 min.
7. Wash the cells 3 × 5 min with PBS, and then incubate them with the DAB solution. A successful peroxidase reaction results in a slightly brown staining of the cells.
8. Finally, wash the cells for 3 × 2 min with PBS and start embedding procedure (*see* below).

Protocol 6: Enhancement of Sample Contrast, Sample Locating, and Embedding

1. Wash cells three times with 0.2 M cacodylate buffer (pH 6.9).
2. Treat cells with the reduced osmium for 1 h on ice in the darkness (if one uses silver enhancement during the next steps, the incubation time should be 5 min).

3. Wash cells with 0.2 M cacodylate buffer (pH 6.9) 3×5 min.
4. Treat samples with 0.3 % thiocarbohydrazide in 0.2 M cacodylate buffer (pH 6.9) for 20 min.
5. Wash cells with 0.2 M cacodylate buffer (pH 6.9) 3×5 min.
6. Treat cells with the reduced osmium for 1 h on ice in the darkness (if one uses silver enhancement, the incubation time should be 5 min).
7. Wash cells with 0.2 M cacodylate buffer (pH 6.9) 3×5 min.
8. Treat samples with 0.3 % thiocarbohydrazide in 0.2 M cacodylate buffer (pH 6.9) for 20 min.
9. Wash cells with 0.2 M cacodylate buffer (pH 6.9) 3×5 min.
10. Treat cells with the reduced osmium for 1 h on ice in the darkness (if one uses silver enhancement, the incubation time should be 5 min).

If you do not plan to use TEM or STEM tomography, **steps 6–10** could be omitted.

11. Rinse sample in 50 % ethanol 2×5 min.
12. Dehydrate cells stepwise with 70, 90, and 100 % ethanol (3×5 min for each) for the dehydration of the specimens.
13. Prepare Epon solution as described in materials.
14. Prepare a 1:1 Epon/100 % ethanol mixture (acetone and propylene oxide could dissolve plastic Petri dishes). Vortex until thoroughly mixed and store all solutions in the fume hood.
15. Treat sample with a 1:1 Epon/100 % ethanol mixture for 1 h.
16. Replace the 1:1 Epon/100 % ethanol mixture with Epon alone for 4 h with one exchange of Epon after 2 h.
17. Keep the cells in Epon for 1–2 h at room temperature, and then leave them in an oven at 60 °C for 12 h.
18. After 12 h of polymerization of the Epon, place a small droplet of a fresh resin on the site where the examined cell is located, and insert a resin cylinder (prepared before by polymerization of the resin in a cylindrical mold) with a flat lower surface; leave the samples for an additional 18 h in the oven at 60 °C. The resin cylinder should be placed on the semi-polymerized Epon just over the cell of interest. The central position of the cylinder is important to ensure the possibility of using the transmission light for the examination of Epon samples. If the wall of the cylinder is situated near the cell of interest, the light will be scattered.
19. Polymerize for 24 h at 60 °C.

20. Carefully pick up the resin from the Petri dish and glass; this is easy to do by gentle bending of the resin cylinder to and fro. The resin block and the empty MatTek Petri dish after block detachment could be separated easily. If the cover glass with a coordinated grid cannot be detached from the cells included into the resin, the latter should be placed into commercially available hydrofluoric acid (do not use glassware for this) for 30–60 min.
21. Remove the glass bottom of tissue culture dishes by hydrofluoric acid for 30–60 min.
22. Control the completeness of the glass dissolution under a stereomicroscope.
23. Wash the samples in water after the complete removal of the glass.
24. Leave the samples in 0.2 M HEPES buffer (pH 7.3) for 60 min to neutralize the hydrofluoric acid.
25. Wash the samples in water and allow them to dry. The round basement of the sample with diameter of about 1 cm or more should not be cut.

3.6 Identification of the Cell of Interest on Epon Blocks

1. Place the embedded sample under a stereomicroscope using a fiber optic illuminator.
2. If the gridded cover slip was used, the furrows on the cover slips are filled with Epon, and after polymerization and dissolution of the glass or its detachment, the furrows appeared as combs because Epon forms a replica from the gridded glass. The relief of grid is visible under a stereomicroscope. Using numbers and letters on the grid, it is easy to find the position on the surface of the sample where the cells are situated.
3. Use the composite of bright-field DIC images or manual drawings of the region of interest to locate the region of interest on the Epon block. Another possibility for the identification of our cell could be the overall general pattern of the surrounding cells and the localization of the cell within the cover slip. The cell of interest could be surrounded by scrapping of the ring. Finally, the position of the cell could be determined on the basis of rectangular coordinates made by scrapping of the confluent monolayer of cell. After scrapping, the cell of interest is easily identified. Instead of a ring, one could make a perpendicular coordinated grid (*see Note 4*).

3.7 Sectioning

1. Place the sample under the transmission light microscope.
2. Find the cell of interest among the cells within the sample according to the coordinated grid (Figs. 3a and 4a) or pattern of the cell layer (Fig. 3b, c) or using scrapping (Fig. 3c–f).

3. Put the resin block into the holder of an ultramicrotome and examine it under a stereomicroscope.
4. Place the holder with the sample under the stereomicroscope, and using a steel needle and rotating the sample in the holder, make two small cavities in such a way that they form a horizontal line with our cell appearing in the center of the sample.
5. Introduce the holder into the ultramicrotome in such a way that the segment arc of the ultramicrotome is in the vertical position and the two cavities form a horizontal line.
6. By rotating the glass knife stage, align the bottom edge of the pyramid parallel to the knife-edge. Using the segment arc, orient the plane of the sample vertically.
7. Bring the sample as close as possible toward the glass knife.
8. Adjust the gap (which is visible as a bright band if all three of the lamps of an ultramicrotome are switched on) between the knife-edge and the surface of the sample. The gap has to be identical in width between the most upper and lower edges of the sample during the up and down movement of the resin block. This ascertains that every point of the sample surface containing the cell of interest is at the same distance from the knife-edge.
9. Slowly moving the sample up and down, continue its approach until the knife begins to cut one of the edges of the sample. The sectioning begins from either the upper or the lower part of the sample; the middle part of the sample where the cell of interest is situated will be unaffected because the length of the radius passing through the cell is shorter than the radii passing through the upper and the lower edges of the sample.
10. If the sectioning is to begin from the upper part of the sample, tilt the segment arc to approach the lower edge toward the knife. If the sectioning is to begin from the lower edge of the sample, tilt the segment arc and approach the upper edge of the sample toward the knife. A vertically oriented sample should produce equal sections from both the upper and lower edges of the samples (Fig. 4b). Note down precisely all of parameters relating to the position of the sample in the ultramicrotome, i.e., the degree of rotation of the sample in the holder, the degree of tilting of the segment arc, and the degree of rotation of the knife in its stage. Do not take the sample from the holder and do not rotate the sample inside the holder.
11. Take the sample and trim it to provide a narrow horizontal pyramid. The pyramid should be as narrow as possible, and the cell of interest should be at the center of the pyramid. The length of the pyramid should be smaller than 0.9 mm and its height smaller than 0.1 mm. In this case, you could place on the slot 18 serial sections. An experienced person can trim a pyramid directly with a razor blade.

12. Introduce the sample back into the ultramicrotome, and lock it in exactly the same position as before (preserving all of the parameters of sample positioning; this is very important).
13. Replace the glass knife with the diamond one, and position the latter toward the pyramid. If the sample is not parallel to the knife, adjust the angle of the diamond knife by rotating the knife stage to make its edge parallel to the plane of the pyramid. Do not change any other parameters of the sample position.
14. Approach the sample toward the edge of the knife until the gap is extremely narrow. Using a 200-nm approaching step, begin the sectioning. Take serial sections according to the instructions with the ultramicrotome. It is enough to take only ten sections to pass 2 μm from the bottom of the cell.
15. Identify the position of the organelle of interest within the EM sample according to the Z-stacking, and select those thick sections that should correspond to this position. For instance, if the organelle of interest is situated at 500 nm from the surface of the sample (bottom of the cell), it is enough to collect only the first three 200-nm serial sections (or ten 50-nm sections). If the position is at 1 μm in height, it will be necessary to collect from the fourth to the eighth serial 200-nm sections (or twenty 50-nm sections; *see Note 5*).

3.8 Picking Up the Serial Sections with the Empty Slot Grid (See Fig. 1)

1. Clean the empty (not covered) slot grid (the donor transfer grid) and one slot grid covered with formvar-carbon film (the acceptor grid).
2. When the normal (not bent) slot grid is taken by the tweezers, it is not convenient to take sections from the water, because the axes of grid cannot be oriented paralleling to the plane of water surface. One needs a holder on the grid. The holder on the slot grid could be done by bending just the small part of the grid from horizontal position. Using a tweezers, bend just the small part of the edge of the empty slot grid from horizontal position. This bent rim will serve as a holder for the tweezers.
3. Take the slot grid with supporting film from a container by the self-closing tweezers, and place it film-down on parallel holders covered with scotch tape.
4. Prepare short sequences of serial sections could be numbered and floated to the specific boxes within the bath of the diamond knife.
5. Take the empty slot grid using the just made holder, and place it over the band of serial sections not touching the water and in such a way that the slot is projected on the band of serial section.

6. Touch water with the donor grid in such a way that sections should be inside the slot, and move the slot grid away. The droplet of water and sections will be inside the slot.
7. To avoid dirt on the sections, it is better to place a small droplet of glass-handled distilled water on the acceptor slot grid. Using stereomicroscope, put a small droplet of water on the supporting film of the acceptor grid.
8. Put the donor grid with serial sections on acceptor grid as it is shown in Fig. 1.
9. Using sharp and very narrow (to avoid too fast elimination of water between slot grids which could give incorrect positioning of grids) filter paper, orient slot in parallel to each other, and eliminate the excess of water from the space between slot grids.
10. Dry grids during at least 20 min.
11. Carefully eliminate the donor grid.
12. Take the dried acceptor grid and check whether sections are in correct position.
13. Use serial semithin sections if the cell of interest is too tall (Fig. 1y, z; see **Note 6**).

EM examination, EM tomography, or analysis of samples under FIBSEM should be done according to the instruction of the manufacturer (see **Notes 7 and 8**).

4 Notes

1. It is necessary to make photos at all steps of the sample preparation and be very careful during all steps of preparation. If cells were transfected with two fusion proteins tagged with fluorescent proteins of different colors, i.e., green and red, it is possible to photo cells immediately after fixation and made Z-stacking and then bleach both fluorescent proteins with the laser beam of maximal intensity in order to use the light waves for the next fluorochrome which will be used for labeling after fixation. Double labeling of live cells together with quadruple labeling with four distinct antibodies (using light with length of wave of 454, 488, 536, and 633) and double labeling (using silver enhancement) within the immune EM result in eight different markers within the same organelle. This approach is doable with culture cells and rather difficult with tissue sections.
2. Usually, the immunogold protocol is suitable for the labeling of the large majority of antigens, while the immunoperoxidase protocol allows good labeling of only antigens residing within

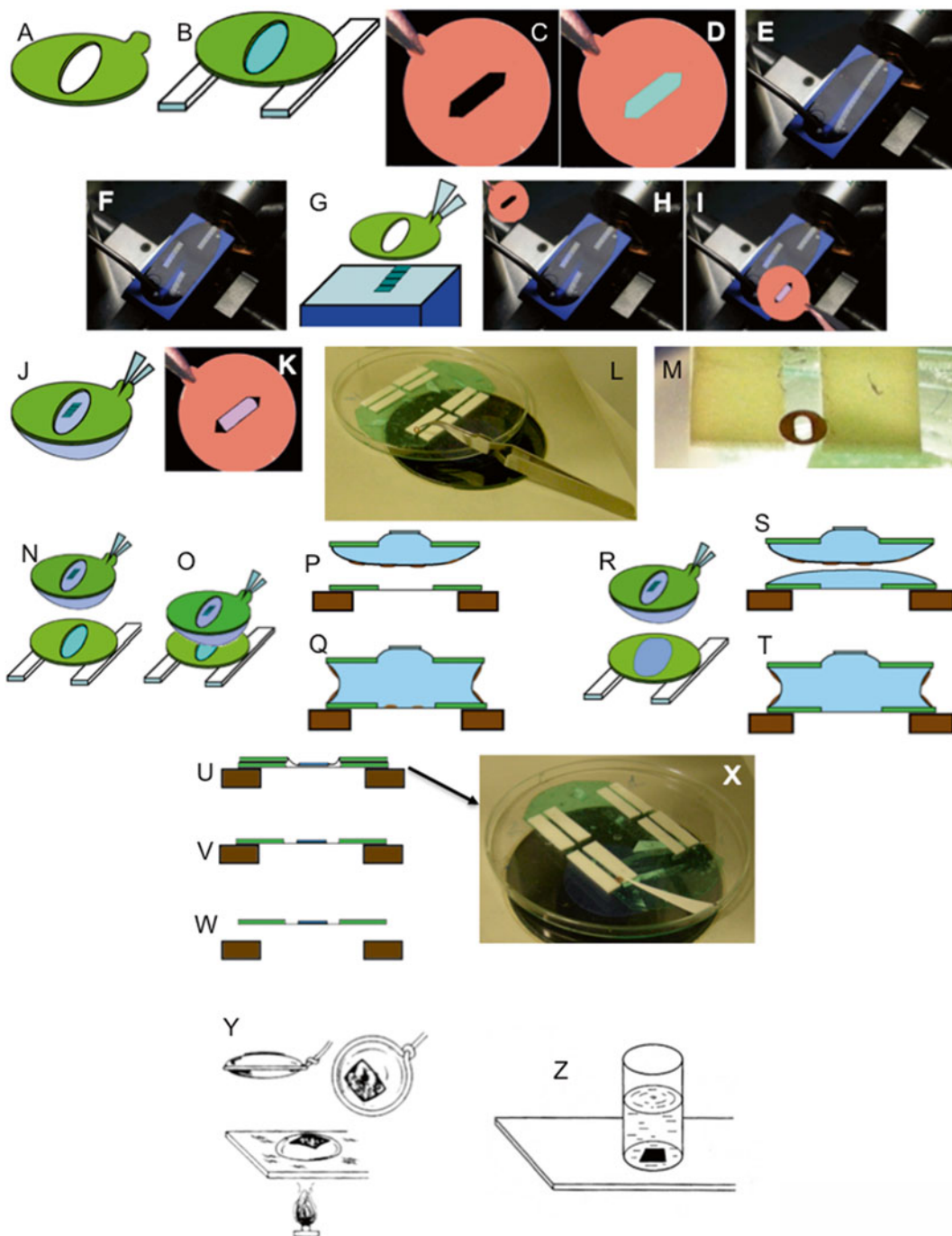


Fig. 1 The collection of the serial sections on the slot grid with the formvar–carbon film. (a) The empty (not covered) slot grid (the donor transfer grid). (b) The acceptor slot grid covered with formvar–carbon film. (c, d) Photos of donor (c) and acceptor (d) grids. (e) Serial sections in the bath of diamond knife. (f) The division of the single tape of serial sections into three parts. (g) Donor empty slot grid over the first band of serial sections. (h) The empty grid over diamond knife. (i) The empty grid over the tape of serial sections. (j) The empty grid contains the first part of the serial section tape over the droplet of water. (k) The photo of the empty grid after the collection of serial sections from the bath. The droplet of water and sections is inside the slot. (l) Placement of the acceptor grid on parallel holders covered with scotch tape made from the paper and scotch tape.

small membrane-enclosed compartments, because the electron opaque products of the peroxidase reaction tend to diffuse from the place of antibody binding [1].

An advantage of HRP-DAB immunostaining is that when the antibody is directed against an intraluminal epitope, the stain delineates the profile of the labeled structure. This facilitates the recognition of the organelle of interest in consecutive serial sections and hence its 3D reconstruction. A limitation of this method, however, is that the pre-embedding technique requires the balance between the need to preserve the intracellular structures (which ideally requires strong fixation) and the necessity of preserving epitopes and allowing the penetration of the antibody (both properties are usually negatively affected by strong fixatives). This can be overcome by appropriate fixation protocols, but avoiding immune labeling and using instead chimeras containing both GFP and HRP can also overcome this problem perhaps more elegantly. Since HRP maintains its activity even in strongly fixed cells, fixation will no longer be a problem. Another limitation is linked to the use of HRP itself; if the epitope is extra luminal, the HRP-induced oxygen radicals leading to the precipitation of DAB might diffuse from the site of production, thus reducing the precision of labeling. This can be avoided by pre-embedding immune labeling with a second antibody tagged not with HRP, but with nanogold (*see above*), or with a fluorochrome suitable for inducing DAB photo oxidation, as this reaction is much less prone to the problem of diffusion.

An alternative is to conjugate the monovalent Fab fragments of a secondary antibody with both a fluorochrome and nanogold, since after a suitable (silver or gold) “enhancement” procedure (Nanoprobes, NY, USA), the latter becomes visible by EM. A fluorescent ultrasmall immunogold probe, FluoroNanogold, has been used for immunocytochemistry on ultrathin cryosections. FluoroNanogold has the properties of both a fluorescent-dye-conjugated antibody for FM and



Fig. 1 (continued) **(m)** Position of the acceptor slot grid on the holders. **(n, o)** The donor grid with serial sections is moving toward the on acceptor grid. **(p, q)** If the dry acceptor grid is used, the dirt (*brown lines* on the acceptor grid) on the water surface could be placed on the serial sections. **(r–t)** To avoid dirt on the sections, it is better to place a small droplet of glass-handled distilled water on the acceptor slot grid. Using stereomicroscope, put a small droplet of water on the supporting film of the acceptor grid. **(u)** Elimination of excessive water and orientation of the donor grid in parallel to the acceptor grid with the help of the very narrow filter paper (to avoid too fast elimination of water between slot grids which could give incorrect positioning of grids; the photo is shown in panel **(x)**). **(v)** Position of serial sections on the acceptor grid after drying and elimination of the donor grid. **(w)** Detachment of the acceptor grid with serial sections from the sticky holders. **(y)** Application of semithin serial sections instead of ultrathin sections for CLEM. Collection of semithin sections is shown. **(z)** Re-embedding of serial semithin sections

a gold-particle-conjugated antibody for EM. Therefore, this bifunctional immunoprobe allows CM of the very same cell structure labeled in a single labeling procedure by these two imaging methods. It is useful to do additional Z-stacking after labeling with FluoroNanogold and then to use silver/gold enhancement.

The maximal resolution of immune EM is about 20 nm in the case of 5 nm gold and 25 nm in the case of 10 nm gold. Moreover, in reality, nanogold–gold enhancement gives rather low resolution for labeling—40–60 nm due to shadows from large gold particles shielding organelle structures. Therefore nanogold–gold enhancement allows rough calculations, but these calculations are only semiquantitative (our unpublished observations).

For visualization of ultrasmall (<2 nm) immune-gold particles in TEM, these labels need to be enlarged by silver or gold enhancement. The increase in particle size reduces the resolution of the antigen detection and the large particles obscure ultrastructural details in the tomogram. Nanogold–gold-enhanced particles are not dissolved by OsO₄. However, gold enhancement represents the procedure where the size of the final gold particles is regulated very difficultly. Silver enhancement is much more reproducible but is very sensitive to the treatment of samples with OsO₄ and usually is dissolved. In order to reduce the level of dissolution of the silver it is better to decrease the time of the treatment with OsO₄ up to 5 min, subsequent treatment of thiocarbohydrazide up to 20 min, the second OsO₄ treatment – up to 5 min, the second treatment of thiocarbohydrazide up to 20 min; the third OsO₄ treatment up to 5 min. Then, samples should be washed out and dehydrated in ethanol (*see below*). Under these conditions, the dissolution of silver is less strong, whereas the high contrast of membranes remains almost the same.

3. If the use of EM tomography is planned, it is very important to have uniform contrast through the whole sample. To get this, we recommend using OsO₄–thiocarbohydrazide repetitive procedure.
4. It is extremely important to be able to find the cell of interest at any step in the CVLEM procedure. So only cells located on the grid of the MatTek Petri dish can be selected for time-lapse observations. The position of the cell of interest on the grid should be noted; otherwise, it will be difficult to find it again. The low-magnification images showing the field surrounding the cell of interest can greatly help then in the trimming of the resin block around the right cell as well as finding it later under the electron microscope. In this case, neighboring cells can be used as landmarks to identify the cell of interest. It is also more

convenient to work with cells that are plated at a lower confluence (50–60 %). Furthermore, during the analysis of serial sections under the electron microscope, it is useful to have the fluorescent- and phase-contrast images of the target cell because particular structures (filopodia, pseudopodia, inclusions, etc.) greatly help to find both the cell and the structure of interest.

MitoTracker could help in the identification of this position because we could determine the position of mitochondria. Z-stacking of fixed cells is an almost obligatory procedure making the identification of the organelles much easier.

If available, infrared laser beam could be used for the marking of cell of interest. In fixed tissue or cell culture, well-defined fiducial marks in three dimensions could be made with the help of near-infrared laser with the 910 nm length of laser wave.

5. All procedures should be performed under stereomicroscope. It is better to use self-closing tweezers and slot grids that are previously tested under the stereomicroscope. Once stained, the cells must be prepared for EM by traditional epoxy (or other) embedding, and the cell and the structure of interest must be identified in EM sections. Finding of an individual subcellular structure in a single thin section can be complex and sometimes impossible, simply because most of the cellular organelles are bigger than the thickness of a routine EM section and may lie in a plane different from the plane of a random section. So analysis of serial EM sections of the whole cell is required to identify the structure observed previously *in vivo*. Finally, if TEM or STEM tomography is used, the thickness of EM sections should be 200–250 and 350–450 nm correspondingly. In the case of routine serial sections, the thickness could be from 40 to 80 nm. Thinner sections demand more grids necessary for the picking up of serial section. Thus, one should find a compromise between the difficulty, cost, and demands. Most of the methods of CLEM are based on tangential sections. If tangential (horizontal) sectioning is selected, it is necessary to get the very first serial section. In order to avoid the necessity for vertical orientation and gluing of samples in the case of the use of SIB SEM, it is better to use the universal copper holder (*see* Figs. 2 and 3)
6. One of the most important questions is how to cut and how to collect sections. Our (the above described) method of collection of serial section is better because the possibility to dirt sections is eliminated. The standard method to pick up the serial sections for routine EM is not sufficient for CVLEM due to significant rate of possible mistakes during manipulations with sections. The demands of CVLEM for the reproducibility of this step are much higher. The method should give almost 100 % of success.

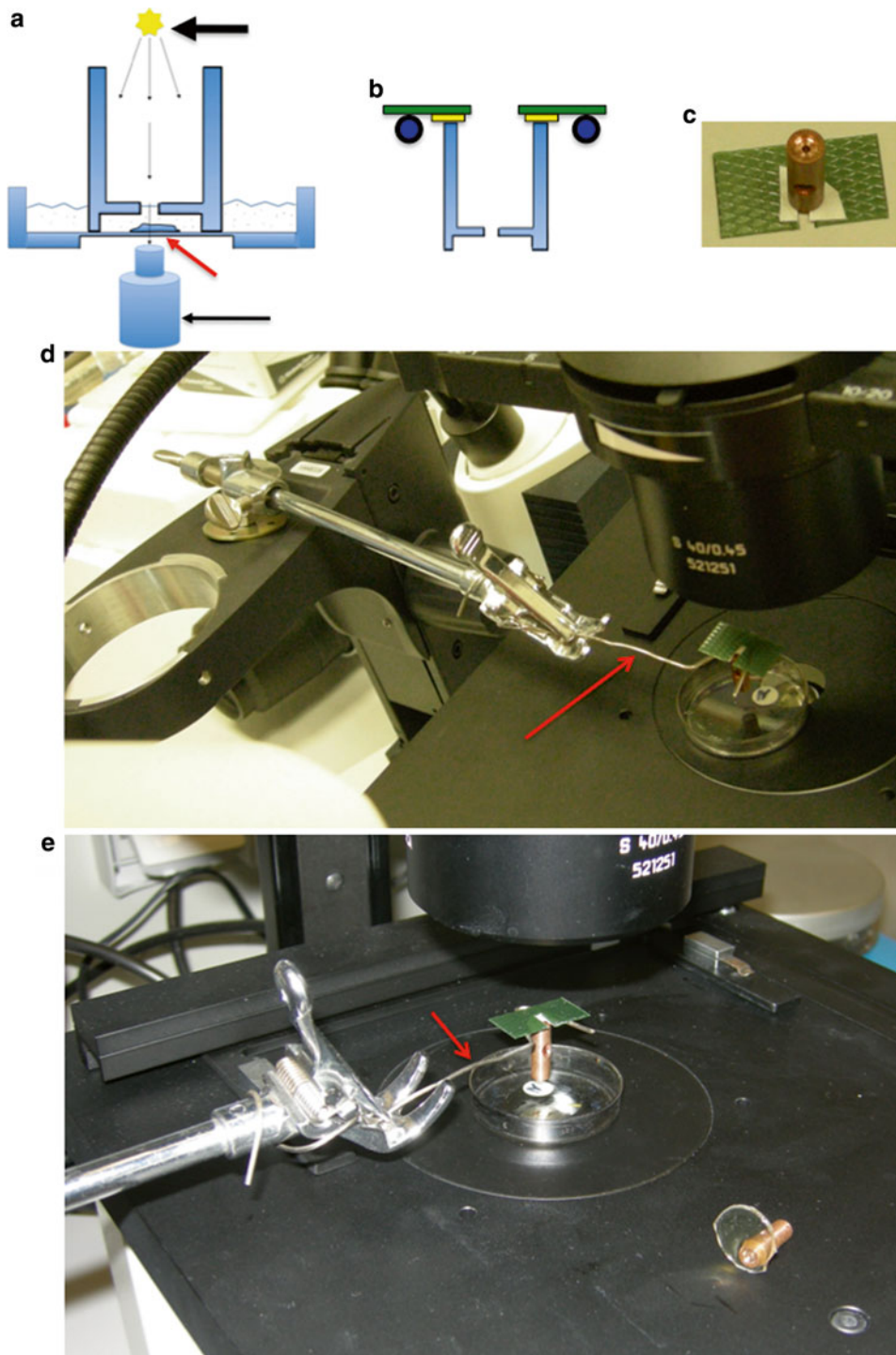


Fig. 2 Embedding for CVLEM using a copper holder. (a) The scheme of the universal holding for the embedding of culture cells. It should be done from copper, which can be easily cut by diamond knife. The external diameter of the holder is 5 mm and its height is 10 mm; the thickness of the cylinder is 1 mm; it is possible to order it personally in Leica Microsystems Spa (www.leica-microsystems.com). *Thick black arrow* shows the source of light; the *thin black arrow* shows the objective of the inverted light microscope; the *red arrow* shows the cell

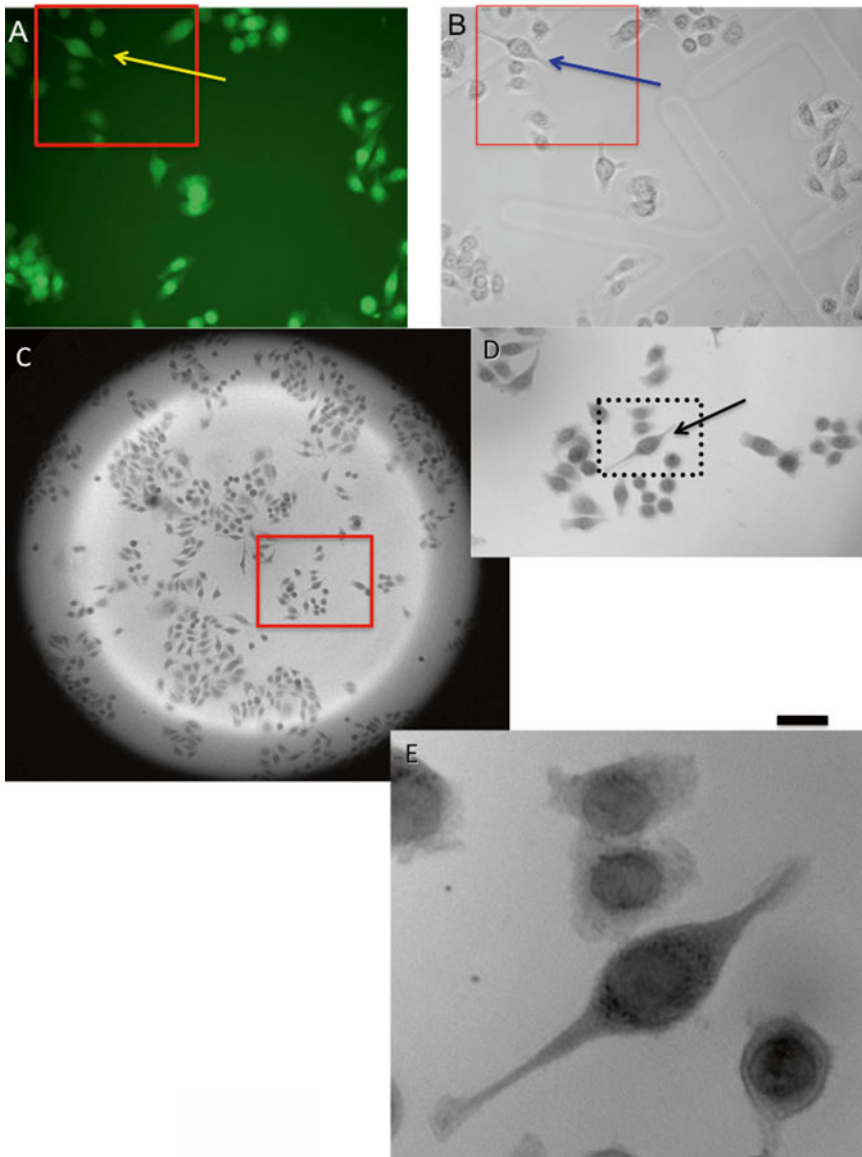


Fig. 3 Identification of the cell of interest under light microscopes. **(a, b)**. The cells of interest (the *yellow arrow* in **(a)** and the *blue arrow* in **(b)**) are visible under the light fluorescence **(a)** and phase-contrast light **(b)** microscope. **(c)** The area with the cell of interest (*red box*) is situated almost in the center of the round window visible through the holder. **(d)** The cell of interest is shown with the *black arrow*. **(e)** The higher magnification of the area shown in *black box* in plate **(d)**. Bar: 15 μm in **(e)**

Fig. 2 (continued) of interest growing on the cover slip of the MatTek Petri dish. **(b)** The scheme of the attachment of the holder to the hard plate (colored in *green*) with the hole in the center using two-sided scotch tape (colored in *yellow*). The supporting holder is shown by *blue ring*. **(c)** The view on the holder from the *bottom* after attachment of the plate to our holder. **(d, e)**. The holder could be placed on the sample manually or using the lifting system of the stereomicroscope under the stereomicroscope. The supporting holder is shown by *red arrows*

7. Once considerations for specific localization of the cell and the organelle have been optimized, the precise 3D structure of the organelle and its connections with other organelles at different moments of its life circle can be identified. EM analysis of serial sections can be supported by digital 3D serial reconstruction or high-voltage EM tomography. This method is applicable for CLEM even if cells were looked at as video CLEM. EM tomography represents an alternative to the serial section TEM approach for imaging tissue. The method therefore relies on the mechanical microtome approach, with a diamond knife cutting thin sections from the block face. Unlike TEM, in which the contrast in the images is generated by the elastic scattering of electrons in regions of higher density, the block-face scanning approach captures electrons backscattered from below the surface of the block itself. More backscattering occurs in regions of higher electron-dense stain, making it possible to image conventionally prepared biological tissue. This technique can be highly automated, removing many of the problems associated with manual serial section manipulation and imaging in the TEM, automatically generating aligned serial images. In EM from FEI, available 3D imaging software (Xplore3D) automatically collects tomography tilt series and reconstructs detailed 3D models of intricate biological structures.

Finally, the EM analysis of serial sections can be supported by high-voltage EM tomography and/or digital 3D reconstruction. In electron tomography, a biological specimen of thickness from 0.1 to 0.5 μm is imaged by EM over a range of tilt angles to provide a series of projections onto planes perpendicular to the beam direction. By back-projecting the images and summing over all the orientations, it is possible to obtain a 3D reconstruction of the specimen. In addition to correlative imaging at multiple scales by EM, it is imperative that methods for correlating 3D light microscopy data with high-resolution tomography data of the same region at the ultrastructural level are properly developed, together with techniques for mapping antigens of interest in 3D reconstructions.

Recent success in electron tomography has depended on advances in automated data acquisition, which have greatly reduced the collection times for tilt series and lowered the required electron doses [1]. This powerful technique allows for 3D reconstructions of intracellular objects with extremely high spatial resolution, and its use in CLEM is currently being investigated, with satisfactory results obtained so far. At the end of the procedure just described, the result is equivalent to a “snapshot” of the 3D ultrastructure at high resolution of a single moving intermediate taken at any time chosen by the experimenter while monitoring the carrier in vivo under light microscope.

The main question is whether the reader needs super-resolution or it is enough to make rough 3D reconstruction of a significant volume of the cell. Knowing this, it is possible to decide what is necessary for your experiment to examine serial sections or to make EM tomography. If you need 3D reconstruction of a large volume of the cell, it is better to deal with serial thick sections and their consecutive EM tomography reconstructions.

To achieve success, the researcher should make all procedures with the reproducibility of about 99 %. Therefore, we recommend performing all manipulations with grids under stereomicroscope. During manipulations with sections and samples, it is better to use self-closing tweezers and slot grids that are previously tested under the stereomicroscope. The addition of TOMO EM into CLEM only slightly increases the time of preparation.

There are several methods for 3DEM (Table 1). Their advantage and disadvantages are shown in Table 2. We recommend using EM tomography and FIBSEM.

FIBSEM possess the following advantages in comparison to EM tomography: (1) high deepness of 3D reconstruction, (2) good alignments of images (although there could be a small shift of images), and (3) applicability of nanogold-enhanced samples. There are also disadvantages of FIBSEM: (1) resolution of FIBSEM along Z-axis is lower than that of EM tomography and (2) during examination of the sample in SEM, the Epon undergoes damaging.

Table 1
Methods of three-dimensional electron microscopy

Resolution along the Z-axis	Name of the method	EM methods used for analysis				
		TEM	SE SEM	BSE SEM	STEMTEM	STEM SEM
Pseudo 3D	Routine SEM		+			
	Metal replicas	+	+		+	+
Low resolution (>100 nm)	Stereo pairs	+	+		+	+
	Video films of sample tilting	+	+		+	+
Middle resolution (15 nm > <100 nm)	Serial sections obtained out of the EM	+				
	Serial images after sectioning inside SEM (SBF SEM or 3-VIEW)		+	+		
High resolution (<15 nm)	FIB SEM (resolution along Z-axis is 5–10 nm)		+	+		
	EM tomography (resolution along Z-axis)	Tomo TEM (6 nm) Tomo STEM (4 nm)	+			+

Table 2
Methods of 3D analysis in EM

Method of 3D		Resolution (nm)			Surface area of an image		Deepness	Dependency on resin	Difficulty of preparation
Name	Mode of EM	X/Y	Z						
Routine SEM	SEM	2	50		Small	Small	Moderate	Low	
Metal replicas	TEM	2			Small	Very small	No	Low	
Stereo pairs	SEM TEM/STEM	2	?		Small	Small	Low	Low	
Video of tilting	TEM/STEM	2	?		Small	Small	Low	Low	
Serial sections	Inside EM column (3-VIEW)	3	100		Large (1 × 1 cm)	>50 μm	High	High	
	Outside column	2	50		Small	Limited by difficulty of work	High	Very high	
	BSE SEM	3	50		Small	Limited by difficulty of work	High	Moderate	
FIB SEM		3	5		Intermediate.	30 μm	No	Low	
EM tomography	TEM	2	5		Small	Limited by difficulty of work	Moderate	High	
	STEM	1	3		Small	Limited by difficulty of work	High	High	

To avoid mistakes, it is necessary using stereomicroscope to place the slot grid into the holder of EM and introduce it into EM and then to find the very first serial EM sections using the traces (usually letters) of the coordinated grid filled with the resin. These are visible mostly on the first two sections. If the central position of the cell of interest within the pyramid is used as a marker, identify the central cell within the sections. It is important to take consecutive photographs (or grab the images with a computer using a video camera) of the serial sections beginning from the very first section since the organelle of interest (just observed under the LSCM) appears and until it is no longer seen. Having images from FL is useful to identify the cell of interest. If EM and FM images are reflective, it is necessary to change the reflection position of FM images.

8. The example of CVLEM is shown in Fig. 4.

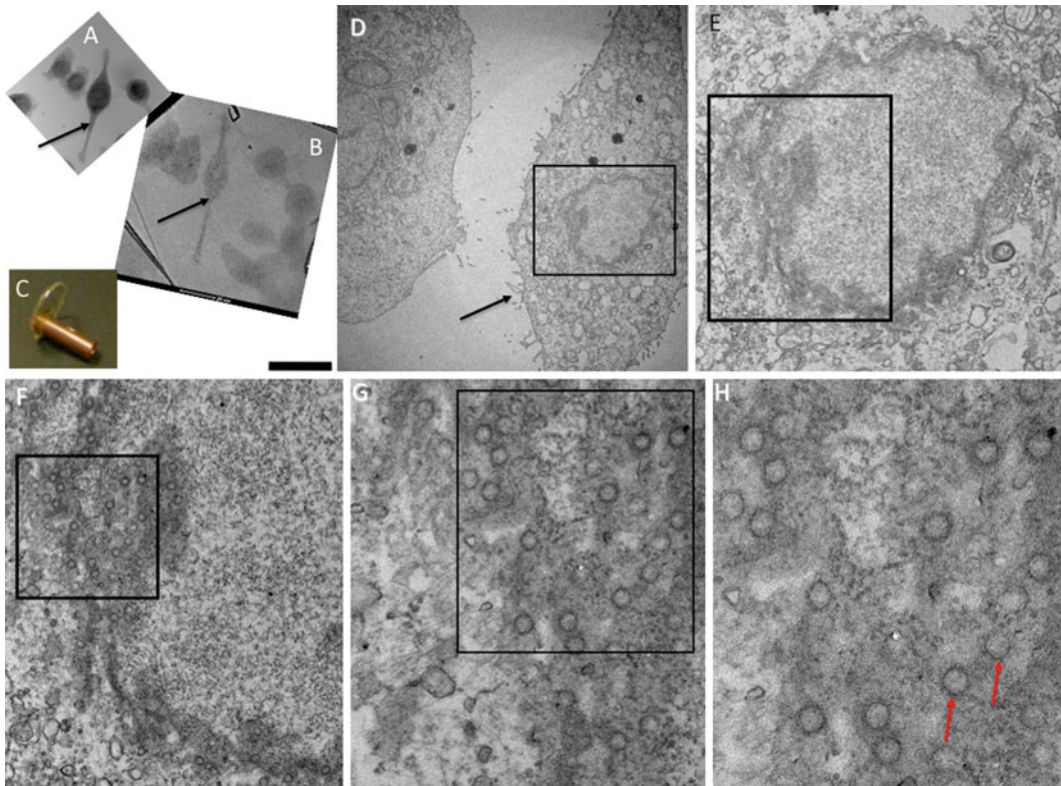


Fig. 4 Identification of the cell in prophase with the help of universal holder. Consecutive images of the same cell (*black arrows*) under different magnifications. (**a**) Photo of the cell of interest after its embedding into Epon under the light microscope. (**b**) The same cells at low magnification in the EM section. (**c**) The holder embedded into the Epon. (**d**) Ultrathin section of the cell of interest (*black arrow*) at higher magnification. (**e**) The higher magnification of the area shown in *black box* in plate (**c**). (**f**) The higher magnification of the area shown in *black box* in plate (**d**). (**g**) The higher magnification of the area shown in *black box* in plate (**f**). (**h**) The higher magnification of the area shown in *black box* in plate (**g**). Nuclear pores (*red arrows* in (**h**)) are shown in the prophase cells. Bar: 260 nm

Acknowledgment

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Live Cell Imaging of Endosomal Trafficking in Fungi

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Abstract

Endosomes are multipurpose membranous carriers important for endocytosis and secretion. During membrane trafficking, endosomes transport lipids, proteins, and even RNAs. In highly polarized cells such as fungal hyphae, they shuttle bidirectionally along microtubules mediated by molecular motors like kinesins and dynein. For in vivo studies of these highly dynamic protein/membrane complexes, advanced fluorescence microscopy is instrumental. In this chapter, we describe live cell imaging of endosomes in two distantly related fungal model systems, the basidiomycete *Ustilago maydis* and the ascomycete *Aspergillus nidulans*. We provide insights into live cell imaging of dynamic endosomal proteins and RNA, dual-color detection for colocalization studies, as well as fluorescence recovery after photobleaching (FRAP) for quantification and photo-activated localization microscopy (PALM) for super-resolution. These methods described in two well-studied fungal model systems are applicable to a broad range of other organisms.

Key words Endosomes, Rab5, Microtubules, mRNA transport, RNA live imaging, Dual-color microscopy, FRAP, PALM, Ascomycete, Basidiomycete

1 Introduction

The vast majority of fungi grow in the form of hyphae, which are highly polarized cells like neurons and pollen tubes. Cellular processes at their growth apex are optimized for efficiency in order to deliver new building blocks for the cell membrane and enzymes for cell wall biosynthesis as well as hydrolytic enzymes enabling nutrition [1, 2]. Essential for polar growth is a highly sophisticated machinery for membrane trafficking. Secretory vesicles, for example, accumulate at the hyphal tip in high quantity for fusion with the plasma membrane. In addition to exocytosis, endocytosis plays an important role in polar growth [3, 4]. Endocytosis is important for recycling of membranes and membrane-bound proteins and could be involved in the organization of cell-end marker proteins at the tip [4–6]. After internalization, such endosomes shuttle bidirectionally by the action of molecular motors with opposite

directionality: minus end-directed dynein and plus end-directed kinesins such as Kin3/UncA [7–10]. Hyphae serve as excellent model systems to study membrane dynamics, because they provide sufficient resolution to perform the studies in a single cell. Compartments like nuclei, ER, and mitochondria are clearly resolvable, and endosomes are transported over long distances. In this chapter, we focus on two distantly related fungi, the basidiomycete *Ustilago maydis* and the ascomycete *Aspergillus nidulans*, which share a comparable machinery for long-distance membrane trafficking [8, 9, 11].

U. maydis is a pathogen that infects corn [12]. Prerequisite for infection is the mating of two yeastlike cells that form a filamentously growing dikaryon. The transcription factor bE/bW is the master regulator for the switch to polar growth. Its activity is regulated at the level of heterodimerization with subunits derived from different mating partners [12]. This knowledge allowed the generation of laboratory strains, in which the expression of an active bE/bW heterodimer is under control of a nitrate-regulated promoter. Thus, switching the nitrogen source results in efficient and synchronous elicitation of hyphal growth (Fig. 1a, b; see below).

Active microtubule-dependent transport of endosomes, which are positive for the small G protein Rab5a and the SNARE Yup1, is essential for efficient hyphal growth [7, 8]. A key factor for the function of these endosomes is the RNA-binding protein Rrm4

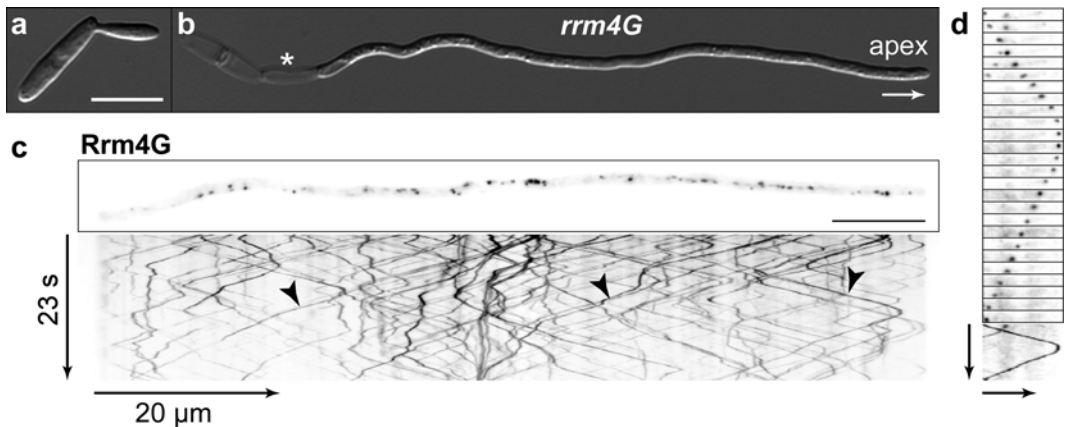


Fig. 1 Moving endosomes decorated with Rrm4G in hyphae of *U. maydis*. (a) Budding cell of *U. maydis* strain AB33rrm4G. (b) Hypha of AB33rrm4G, expressing Rrm4 fused to eGfp, 7 h after induction of hyphal growth; arrow indicates direction of growth, asterisk marks latest basal empty section. (c) Principle of a kymograph: (top) individual frames of a movie containing a selected region with a single Rrm4-decorated endosome moving forth and back. (Bottom) In a kymograph time on Y-axis plotted against the distance on X-axis, the Rrm4G signal is compressed to a single pixel on Y-axis (vertical arrow=7.6 s, horizontal arrow=5 μm). (d) Fluorescence micrograph of Rrm4G. Depicted is the first frame of a movie (top) with corresponding kymograph (bottom). Kymographs were generated from movies with 150 ms exposure time (inverted contrast, bars=10 μm). Arrowheads indicate Rrm4-positive endosomes shuttling in both directions

that mediates endosomal transport of mRNAs and ribosomes most likely to guarantee translation throughout the entire length of the hypha [13–15]. Importantly, Rrm4 also mediates endosome-coupled translation of specific mRNAs such as *cdc3* mRNA for loading of endosomes with septin. The translation product is delivered by these endosomes to the hyphal tip for correct assembly into higher-order septin filaments [14]. Thus, these endosomes are versatile multipurpose carriers transporting not only obvious cargo such as lipids but also cytoskeletal components, ribosomes, and mRNAs.

The ascomycete *A. nidulans* is an excellent model system for filamentous fungi. It completes the asexual life cycle, starting from a single conidiospore, within 24 h and is well suitable for genetic screens [16]. These resulted in the discovery of important eukaryotic factors such as the first eukaryotic tubulin genes—including γ -tubulin [17, 18]. Furthermore, the kinesin motor *bimC* was the founding member for a whole class of mitotic kinesins, and, last but not least, the isolation of nuclear distribution mutants (*nud*) revealed important insights into the human disease lissencephaly [19, 20]. In recent years, *A. nidulans* became an attractive model to study polar growth. The multinucleated hyphal tip compartment grows remarkably fast (about 0.5 $\mu\text{m}/\text{min}$) in an actin- and microtubule-dependent manner [21]. This growth speed depends on the predominant secretion of vesicles at the apex and also on endocytosis, which is required for membrane recycling. Its essential role was shown with different mutants of the endocytotic machinery, e.g., fimbrin (an F-actin cross-linking protein that plays a role in endocytotic internalization) and ArfB (a small GTPase) that leads to polarity defects [22, 23]. Shuttling Rab5-positive endosomes can be labeled with two paralogues RabA and RabB. These endosomes show characteristic long-distance bidirectional movement on microtubules, driven by the corresponding motor proteins dynein and most likely a kinesin-3 type motor UncA [10, 24, 25].

Here, we describe important techniques for live cell imaging of endosomal trafficking such as (1) millisecond alternating laser excitation (msALEX) for dual-color acquisition applied during in vivo colocalization studies on moving endosomes. msALEX allows for the alternating excitation of different fluorophores with a minor time shift, but with the advantage of a longer observation time because of reduced bleaching of the respective fluorophores. (2) RNA live imaging to demonstrate endosomal mRNA transport [13, 14, 26] relies on the insertion of binding sites for a specific peptide derived from a heterologous RNA-binding protein like λN in the 3' untranslated region (UTR) of the mRNA of interest. Thereby, the localization of these mRNAs can be visualized by expressing a fusion protein consisting of λN -Gfp, because the fluorescent protein is specifically recruited to these mRNAs. (3) FRAP (fluorescence recovery after photobleaching) is used to

quantify the interaction of endosomal cargo with proteins at the final destination [14]. (4) PALM (photo-activated localization microscopy) uses photoswitchable fluorophores to achieve temporal control of the emission. The fluorophore is converted between a fluorescent (“on”) state and a dark (“off”) state or states that fluoresce at different wavelengths, such as photoconversion from green to red fluorescence. Therefore, when activation light of a sufficiently low intensity is applied to the sample, only a random, sparse subset of fluorophores is activated to the on or red fluorescent state at any time, allowing these molecules to be imaged individually. Signals can be precisely localized and then deactivated by switching to a reversible dark state or permanent bleaching. Repetitive cycles of activation, imaging, and deactivation allow the construction of super-resolution images [27]. One fluorescent protein, mEosFP, can be photoconverted from green to red fluorescence by 400 nm light [28]. The red signals of mEosFP activated at random are used during PALM imaging. Here, we show a PALM analysis of TeaR, a membrane-associated cell-end marker that controls the growth direction of hyphae by focusing polarity markers at the growing tip [6, 29].

2 Materials

2.1 Preparing Hyphae for Live Cell Imaging

Ustilago maydis

1. Complete medium (CM): 0.25 % (w/v) casamino acids (Difco), 0.1 % (w/v) yeast extract (Difco), 1.0 % (v/v) vitamin solution, 6.25 % (v/v) salt solution, 0.05 % (w/v) herring sperm DNA (Sigma), and 0.15 % (w/v) NH_4NO_3 ; add $\text{H}_2\text{O}_{\text{bid.}}$, adjust pH with 5 M NaOH to 7.0, and add glucose (glc) or arabinose (ara) solution after autoclaving (1 % f.c.).
2. Nitrate minimal medium (NM): 0.3 % (w/v) KNO_3 , 6.25 % (v/v) salt solution.
3. Vitamin solution: 0.1 % (w/v) thiamine hydrochloride, 0.05 % (w/v) riboflavin, 0.05 % (w/v) pyridoxine, 0.2 % (w/v) calcium pantothenate, 0.05 % (w/v) p-aminobenzoic acid, 0.2 % (w/v) nicotinic acid, 0.2 % (w/v) choline chloride, 1 % (w/v) *myo-inositol*; add $\text{H}_2\text{O}_{\text{bid.}}$ and sterile filtration.
4. Salt solution: 16 % (w/v) KH_2PO_4 , 4 % (w/v) Na_2SO_4 , 8 % (w/v) KCl, 1.32 % (w/v) $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$, 8 % (v/v) trace elements, 1 % (w/v) MgSO_4 (water free); add $\text{H}_2\text{O}_{\text{bid.}}$ and sterile filtration.
5. Trace elements: 0.06 % (w/v) H_3BO_3 , 0.14 % (w/v) $\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$, 0.4 % (w/v) ZnCl_2 , 0.4 % (w/v) $\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O}$, 0.1 % (w/v) $\text{FeCl}_3 \times 6 \text{ H}_2\text{O}$, 0.04 % (w/v) $\text{CuSO}_4 \times 5 \text{ H}_2\text{O}$; add $\text{H}_2\text{O}_{\text{bid.}}$ and sterile filtration.
6. Glass reaction tubes, baffled flasks 125 ml.

Aspergillus nidulans

1. Minimal medium: 5 % (v/v) salt solution, 0.1 % (v/v) trace elements, 2 % (w/v) glucose or 2 % (v/v) glycerol; add $\text{H}_2\text{O}_{\text{bid.}}$, and adjust pH with 10 M NaOH to 6.5.
2. Salt solution: 12 % NaNO_3 ; 1 % KCl, 1 % $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 3 % KH_2PO_4 ; add $\text{H}_2\text{O}_{\text{bid.}}$.
3. Trace elements: 2.2 % $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$; 1.1 % H_3BO_3 ; 0.5 % $\text{MnCl}_2 \times 4\text{H}_2\text{O}$; 0.5 % $\text{FeSO}_4 \times 7\text{H}_2\text{O}$; 0.16 % $\text{CoCl}_2 \times 5\text{H}_2\text{O}$; 0.16 % $\text{CuSO}_4 \times 5\text{H}_2\text{O}$; 0.11 % $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$; 5 % $\text{Na}_4\text{-EDTA}$; adjust pH with 10 M KOH to 6.5.
4. Glass-bottom FluoroDish (World Precision Instruments, Berlin, Germany).

2.2 General Microscopy Equipment for Live Cell Imaging

1. Wide-field-fluorescence microscope (Axio Observer.Z1, Zeiss, Jena, Germany).
2. Metal-halide lamp or laser for excitation of eGfp (488 nm/50 or 100 mW) or Rfp/mCherry (561 nm/50 or 150 mW).
3. Filter sets (eGfp ET 470/40 $\times$, ET525/50 m, T495_PXR and mCherry ET560/40 $\times$, ET 630/75 m, T585lp, AHF Analysentechnik, Tübingen, Germany).
4. Zeiss objectives: Plan-Apochromat (63 $\times$, NA 1.4) and EC Plan-Neofluar (100 $\times$, NA 1.3).
5. CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ, USA).
6. Software package MetaMorph (version 7, Molecular Devices, Sunnyvale, CA, USA).
7. VisiFRAP 2D control software for Meta Series 7.6 (Visitron Systems, Munich, Germany) to adjust laser excitation power.
8. Microscope slides 76 $\times$ 26 mm (Marienfeld, Lauda-Königshofen, Germany).
9. High precision cover slips 18 $\times$ 18 mm, $D=170 \pm 5 \mu\text{m}$ (Zeiss, Jena, Germany).
10. Agarose (Bio-Rad, Munich, Germany).

2.3 Microscopy Equipment for Local Photobleaching and Dual-Color Acquisition

1. A VS-LMS4 Laser-Merge-System (Visitron Systems, Munich, Germany) with solid-state lasers for excitation of eGfp (488 nm/50 or 100 mW) and Rfp/mCherry (561 nm/50 or 150 mW).
2. Multiband filter set for eGfp/mCherry (59022, Chroma, Bellows Falls, VT, USA).
3. VisiFRAP 2D-System (Visitron Systems, Munich, Germany) including a 405 nm/80 mW diode laser, regulated by a UGA-40 controller (Rapp OptoElectronic GmbH, Hamburg, Germany).
4. VisiFRAP 2D control software for Meta Series 7.6 (Visitron Systems, Munich, Germany).

2.4 FRAP Setup

1. 40× EC Plan-Neofluar objective (NA 1.3, Zeiss, Jena, Germany).
2. 472 nm LED (CoolLED, precisExcite, Andover, UK) (*see Note 1*).
3. *See items 3 and 4* in Subheading 2.3.
4. PIFOC P-737 high-speed Piezo Z-stage (Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany).
5. Software-driven autofocus.

2.5 PALM Setup

1. Modified inverted microscope (Axiovert 200, Zeiss, Jena, Germany) equipped with a 63× C-Apochromat water immersion objective (NA 1.2, Zeiss, Jena, Germany).
2. Three diode-pumped solid-state lasers, with wavelengths 561 nm/up to 150 mW (Cobolt Jive, Cobolt, Solna, Sweden), 473 nm/up to 600 mW (LSR473-200-T00, Laserlight, Berlin, Germany), and 405 nm/up to 150 mW (CLASII 405-50, Blue Sky Research, Milpitas, CA, USA) for excitation and photoactivation of the fluorophores.
3. Excitation dichroic (z 405/473/561/635, AHF, Tübingen, Germany), band-pass filter for emission (607/50, AHF, Tübingen, Germany).
4. Back-illuminated EMCCD camera (iXon Ultra 897, Andor, Belfast, Northern Ireland).
5. Appropriate dichroic mirrors (AHF, Tübingen, Germany) and AOTF (AOTF_{nC}-400.650, A-A, Opto-Electronic, Orsay Cedex, France).
6. Single-mode fiber (OZ Optics, Ottawa, Ontario, Canada).
7. Custom-written analysis software, a-livePALM, ran in MATLAB R2010b (The MathWorks, USA) environment [30].
8. Personal computer using an Intel(R) Core™ i7-2600 processor clocked at 3.4 GHz with 8 GB memory. A NVIDIA GeForce GTX 560 Ti graphics card with 1 GB memory was used for GPU-based computation.

3 Methods

An important prerequisite for live cell imaging in fungal model systems is the generation of transgenic strains expressing the proteins of interest fused N- or C-terminally to fluorescence proteins such as the green and red fluorescent protein (eGfp or mRfp). Preferably, this is achieved by integration at the homologous locus in order to avoid overexpression artifacts. To this end, sophisticated strategies have been described [31, 32]. Alternatively, it is

possible to express the protein ectopically in addition to the wild-type version at defined loci such as the *ip^s* locus in *U. maydis* [33] (see **Note 2**). Importantly, it needs to be verified that the expression of fusion proteins are functional and do not cause altered hyphal growth (Fig. 1a, b). The test can be easily done by complementation of a deletion mutant with the construct of interest, if it is a nonessential gene and if deletion causes a phenotype. Full complementation of the mutant phenotype is desired.

3.1 Live Cell Imaging of Dynamic Endosomes in *U. maydis*

1. Inoculate 3 ml of CM-glc (1 % f.c.) in a glass tube and grow on wheel incubator for 20–24 h at 28 °C.
2. Dilute cells 1:5,000 in 30 ml of CM-glc (see **Note 3**), and incubate for 17–18 h in a 125 ml baffled flask shaking at 200 rpm and 28 °C.
3. Adjust OD₆₀₀ to 0.5 (final volume 20 ml), and shift the cells to NM-glc by centrifugation at 3,500×*g* for 3 min (see **Note 4**). Wash once in NM-glc, centrifuge again, and resuspend in 20 ml NM-glc. Transfer cells into a new 125 ml baffled flask.
4. Pipette about 300 µl of 3 % liquid agarose on a microscope slide. Immediately place a second slide on the agarose drop, perpendicular to the lower slide. Remove one microscope slide by sliding it slowly sideways. This results in a thin agarose cushion on the microscope slide (see **Note 5**).
5. Pipette 0.3 µl of cell suspension onto the cushion, let it dry for 1 min, and position cover slip on top.
6. To visualize endosomes in whole hyphae, use a 63× objective and full camera frames (see **Note 6**).
7. Choose appropriate illumination for excitation of fluorophores (metal-halide lamp or laser) and filter sets (excitation/emission/dichroic beam splitter).
8. Drive camera in stream mode with 150 ms exposure time and in overlapping exposure and readout mode (“normal” mode) to obtain the fastest acquisition (see **Note 7**).
9. Convert resulting movie into a kymograph (Fig. 1c, d; see **Note 8**).
10. Analyze kymographs for endosome behavior such as direction of movement, pausing events, velocity, and turning frequency.

3.2 Live Cell Imaging of Dynamic Endosomes in *A. nidulans*

1. Inoculate spores from colony in 0.5 ml minimal medium (about 10³ spores per ml) with 2 % glycerol (derepression of the *alcA* promoter) mounted on a sterile cover slip. Incubate the cells for 16–20 h at 28 °C for germination and hyphal growth (Fig. 2a; see **Note 9**).
2. Mount the cover slips upside down on a microscope slide.

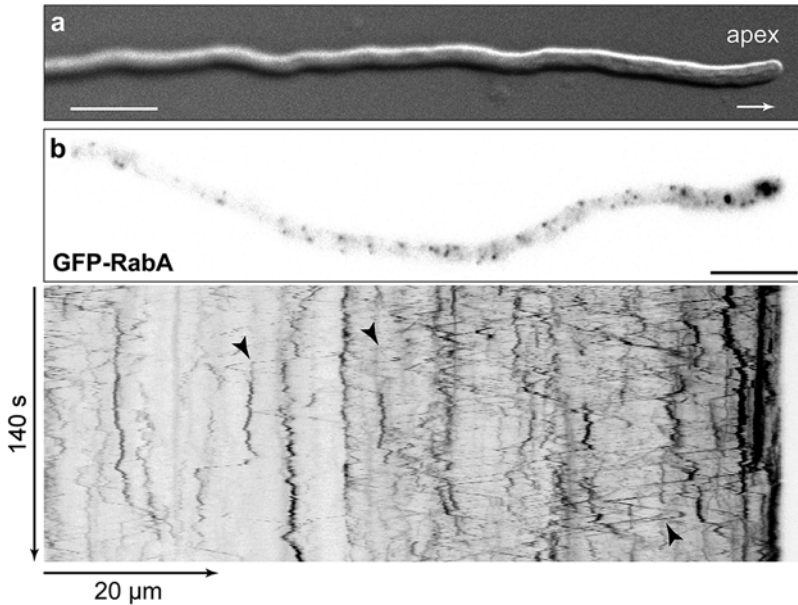


Fig. 2 Moving endosomes decorated with Gfp-RabA in hyphae of *A. nidulans*. (a) DIC image of *A. nidulans* hyphae. (b) Fluorescence micrograph of RabA tagged with Gfp. Depicted is the first frame of a movie (top) with corresponding kymograph (bottom). Kymographs were generated from movies with 450 ms exposure time (*inverted contrast*). Arrowheads indicate RabA-positive endosomes shuttling in both directions. Scale bars = 10 μ m

3. To visualize endosomes, use the 63 $\times$ objective and an appropriate camera frame to visualize the whole hypha.
4. Choose appropriate illumination for excitation of fluorophores, and filter sets (excitation/emission/dichroic beam splitter) and exposure time.
5. Drive camera in stream mode to obtain the fastest acquisition.
6. Convert resulting movie into a kymograph (Fig. 2b).
7. Analyze kymograph for endosome behavior like direction of movement, pausing events, speed, and turning frequency (*see Note 10*).

3.3 Colocalization of Proteins at Dynamic Endosomes Using Dual-Color Acquisition

1. Follow steps 1–6 in Subheading 3.2 (*see Notes 11 and 12*).
2. Use a 63 $\times$ or 100 $\times$ objective, without or with camera chip binning 2, respectively (*see Notes 6 and 7*).
3. Alternating laser excitation is operated by the microscope control software (*see Note 13*).
4. Drive camera in stream mode with 80 ms exposure time for each fluorophore and in overlapping exposure and readout mode to obtain the fastest acquisition, and choose region of interest (*see Notes 7 and 14*).

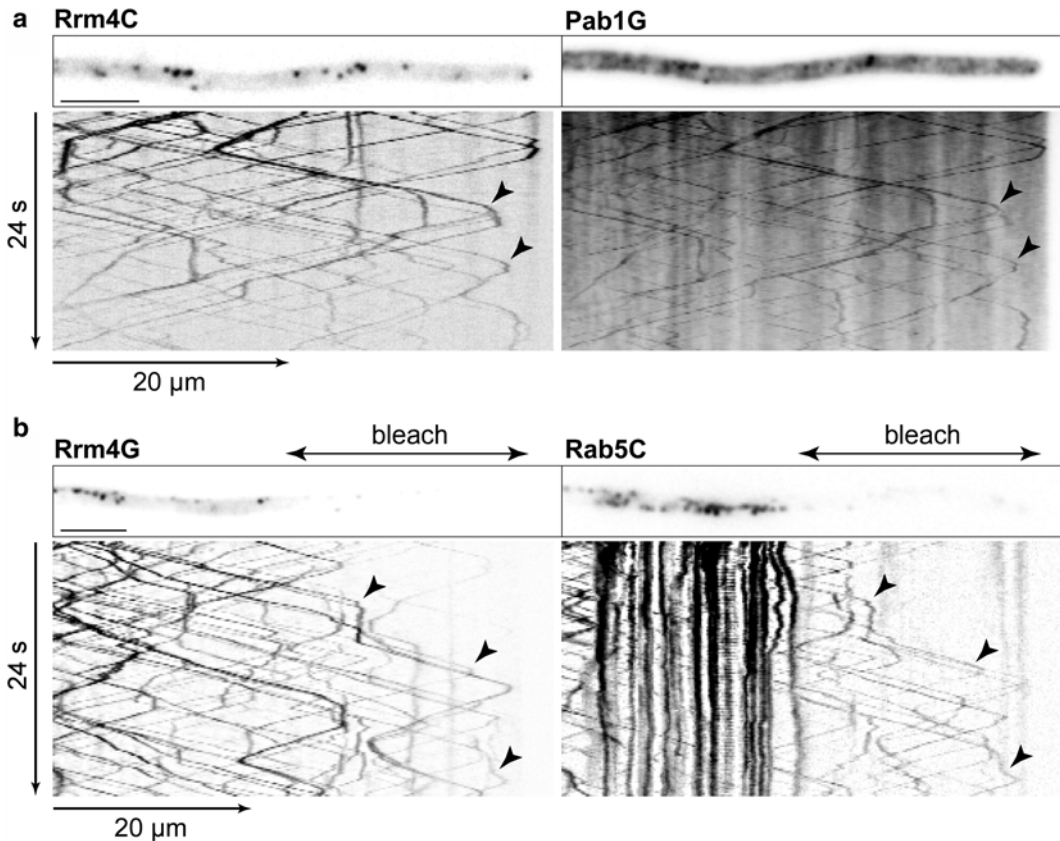


Fig. 3 Colocalization of proteins on dynamically moving endosomes. **(a)** Dual-color acquisition with msALEX of hypha expressing RrmC and Pab1G. Kymographs were generated from a movie that was acquired with a 63× objective and 80 ms exposure time per fluorophore. **(b)** Dual-color acquisition with local photobleaching followed by msALEX of hypha expressing Rrm4G and Rab5aC. Kymographs were generated from a movie that was acquired with a 100× objective, 80 ms exposure time per fluorophore, and camera chip binning 2; prior to acquisition, the indicated area was photobleached

5. Adjust laser intensity (*see Note 15*).
6. Convert resulting movies into kymographs (Fig. 3; *see Note 8*).
7. Analyze kymographs for protein colocalization (*see Note 16*).

3.4 RNA Live Imaging

1. Generate the λN^* -Gfp² fusion construct (Fig. 4a, b; *see Note 17*).
2. Insert *boxB* hairpins into the 3' UTR of your gene of interest (Fig. 4a, b; *see Note 18*).
3. Transform both constructs successively into *U. maydis*. Verify correct strain generation by Southern blot analysis.
4. Inoculate 3 ml of CM-glc (1 % f.c.) in a glass reaction tube for 20–24 h at 28 °C on an incubation wheel.
5. Dilute cell culture 1:2,500 in 30 ml of CM-ara (1 % f.c.; *see Notes 3 and 19*), and regrow in a baffled flask for 18 h at 200 rpm and 28 °C.

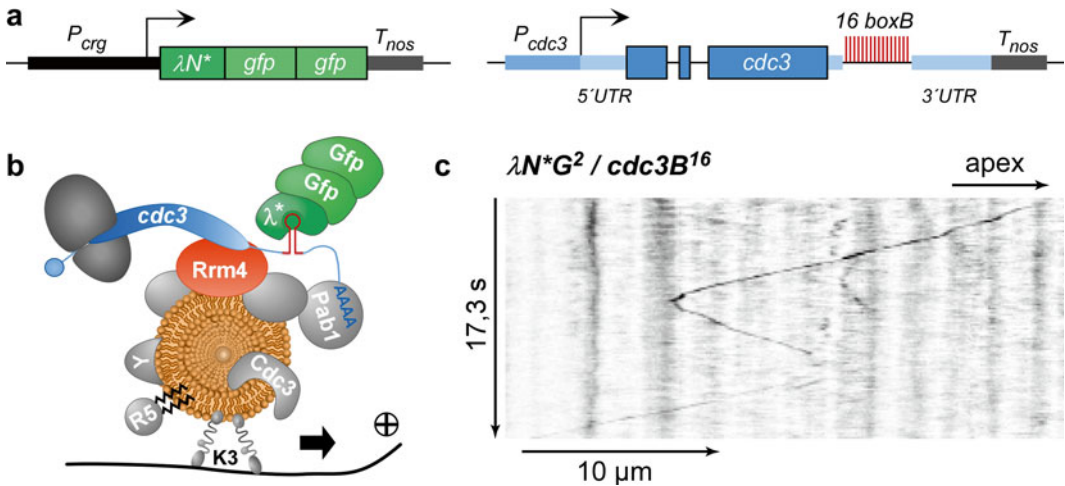


Fig. 4 λN -based RNA live imaging in *U. maydis*. **(a)** Schematic representation of constructs: the gene encoding the λN^* -peptide fused to tandem eGfp is under control of the arabinose-inducible *crg1* promoter (left; T_{nos} = heterologous terminator); the *cdc3* gene is tagged with 16 copies of the λN^* -binding sites *boxB* in its 3' UTR (right). **(b)** Schematic representation illustrating the concept of RNA live imaging: the λN^*G^2 fusion protein is recruited to the *cdc3* mRNA via *boxB* binding sites (for the sake of simplicity, only a single interaction is shown), the Rrm4 links this complex to endosomes which are transported via molecular motors along microtubules, and different endosomal cargos are indicated, R5 Rab5a, YYup1, and K3 Kin3. **(c)** Kymograph showing shuttling *cdc3B*¹⁶ mRNA labeled with λN^*G^2

6. Adjust OD₆₀₀ to 0.5 (final volume 20 ml) and shift the cells to NM-ara by centrifugation at 3,500×*g* for 3 min (see **Note 4**). Wash once in NM-ara, centrifuge again, and resuspend in 20 ml NM-ara. Transfer cells into a new 125 ml baffled flask. Filamentous growth should be induced for at least 8 h prior to microscopic analysis.
7. Follow **steps 4–6** in Subheading 3.2.
8. Use a 63× objective or 100× with camera chip binning 2 (see **Notes 6** and 7).
9. Follow **steps 8–11** in Subheading 3.2 (Fig. 4c; see **Note 20**).

3.5 FRAP Experiments

1. Prepare hyphae of *U. maydis* expressing septin-Gfp-fusion protein Cdc3G according to **steps 1–6** in Subheading 3.2.
2. Determine the minimum laser power necessary for bleaching the region of interest.
3. Operate camera in the most sensitive settings: set readout speed to 10 MHz to reduce readout noise (see **Note 21**), operate camera in “alternate normal” mode to provide best quantum efficiency, and set camera gain to 3.
4. Use 472 nm LED for illumination.

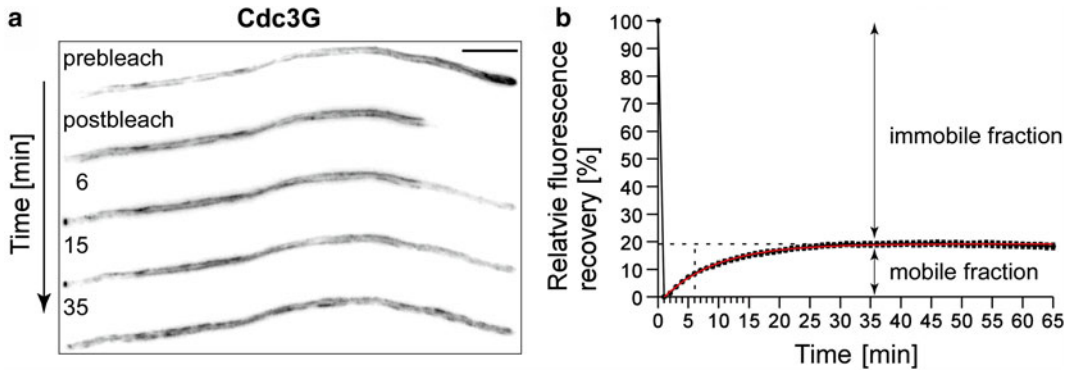


Fig. 5 FRAP of Cdc3G at hyphal tips of *U. maydis* (a) Fluorescence micrographs illustrating the chronology of a FRAP experiment: fluorescence recovery underlies endosomal transport of Cdc3G [14]; different steps and selected time points are indicated (bar = 10 μ m). (b) Fluorescence recovery is plotted against time, half-time recovery ($t_{1/2}$, vertical dashed line), and mobile as well as immobile fractions (double arrows) are deduced from the fitted curve (red), and horizontal dashed line indicates plateau

5. Before bleaching, take a z-stack of 10 planes with an exposure time of 500 ms and a distance between single planes of 0.5 μ m (see Note 22).
6. Bleach 15 μ m of the hyphal tip in ten z-planes through fungal hyphae with a z-distance of 0.5 μ m.
7. Immediately acquire fluorescence recovery in ten z-planes with an exposure time of 500 ms each with a z-distance of 0.5 μ m (open camera shutter).
8. Take a z-stack every minute for a period of 65 min (Fig. 5a; see Notes 22 and 23).
9. For data analysis, convert all z-stacks to maximum projections and correct for drift of cells during the experiment (see Note 24).
10. Subtract average background in each image (see Note 25).
11. Determine the amount of acquisition bleaching and correct all images (see Note 26).
12. Normalize postbleach intensities to prebleach intensities, plot fluorescence recovery against time, apply curve fitting, and determine half-time recovery as well as mobile and immobile fraction of protein with Prism 5 software (GraphPad, Fig. 5b).

3.6 PALM Experiments

1. Inoculate spores of *A. nidulans* strain expressing mEosFP-TeaR in a glass-bottom chamber with minimal media. Incubate at 25 $^{\circ}$ C for 12 h.
2. With low 473 nm illumination, identify a cell that expresses a high level of fluorophores.
3. Adjust laser intensity to image mEosFP. Initially, illuminate the sample with the 561 nm laser to bleach the small population of mEosFP that is already converted, e.g., emits red fluorescence (see Note 27).

4. Fluorescent proteins are converted one by one from their green to their red emitting forms using low intensity ($0\text{--}50\text{ W/cm}^2$) 405 nm light and excited at 561 nm simultaneous illumination ($200\text{--}400\text{ W/cm}^2$).
5. Use a 607/50 band-pass filter after passing through the excitation dichroic (λ 405/473/561/635) to detect the red fluorescence.
6. Detect and record the signals from individual red fluorescent mEosFP molecules with a back-illuminated EMCCD camera typically at 50 ms time resolution (*see* **Note 28**).
7. Analyze the PALM data with custom-written analysis software, a-livePALM, ran in MATLAB R2010b environment [30]. Use the molecule identification threshold value, P value, of 0.04 to identify and fit all data (*see* **Note 29**).

4 Notes

1. We observed the lowest acquisition bleaching during recovery time using the 472 nm LED.
2. Different marker proteins are available in *U. maydis* to visualize endosomes: Rab5a, the SNARE Yup1, and the RNA-binding protein Rrm4. We recommend using Rrm4 as endosomal marker, as Rrm4 localizes exclusively to moving endosomes (Figs. 1d and 3b). In contrast, Rab5a and Yup1 stain additional structures.
3. Culture volume can be adjusted. The ratio for inoculation should be 1:5,000 at best. Do not exceed 1:1,000 since this might affect hyphal growth.
4. Some mutant strains might require an extended period of spinning at higher velocities, e.g., 5 min at $8,000\times g$.
5. Agarose cushions ensure a plane orientation of hyphae in a single focal plane. We recommend drying the agarose for 10–20 min before usage.
6. Use high numerical aperture objectives, e.g., NA 1.3 or higher.
7. With these settings, frame rate equals exposure time. Endosomes in *U. maydis* move 2–3 μm per second. Therefore, camera exposure time should not exceed 200 ms. If fluorescent signals are weak, choose a $100\times$ objective and apply binning to the camera chip.
8. Microscope equipment is controlled by MetaMorph (version 7), which is used to generate kymographs. Free software, e.g. ImageJ, is also available [34]. In a kymograph, the pixel with the highest intensity of each moving structure is plotted against time in a coordinate system. This results in a 2D projection of 3D data (Fig. 1c).

9. Use high precision cover slips ($170 \pm 5 \mu\text{m}$) to avoid intensity changes due to local thickness differences in your resulting image. For longtime imaging, use glass-bottom dishes with correct cover slip thickness (#1.5), and hyphae will stay healthy for an extended period of time.
10. Kymographs are generated using Zeiss software (Zen) or Fiji (ImageJ, [34]).
11. For slow processes, a metal-halide lamp combined with an excitation filter wheel or with the microscope filter turret is sufficient for colocalization studies.
12. If dual-color experiments are performed, objectives should be tested for chromatic aberrations. Usually objectives of the apochromat type are well corrected. We use a 100 $\times$ EC-Neofluar objective (NA 1.3, Zeiss) that has no detectable shift in z-planes if Gfp and mCherry are visualized. Use commercially available fluorescent beads to test for chromatic aberrations.
13. In Subheading 2.4 (items 6 and 7), the applied software is listed.
14. We recommend exposure times no longer than 80 ms. Check the frame rate limit of your camera and choose a region of interest if required. Alternatively use a 100 $\times$ objective with binning 2, which enhances signal intensity and allows shorter exposure times at the cost of resolution.
15. When performing msALEX, one should be aware that the 488 nm laser excites mCherry to a low degree. Depending on the expression level of the tagged protein, this might result in a moderate false-positive signal which can be detected with the dual emission filter. Therefore, in control experiments, the protein tagged with mCherry should be excited with 488 nm light without expressing a Gfp-fusion protein simultaneously. The degree of cross talk should be checked and laser intensity be adjusted accordingly.
16. If the protein of interest exhibits a cytoplasmic localization in addition to the endosomal localization, local photobleaching might be needed before dual-color acquisition. For photobleaching, a 405 nm laser is required. Identify a region of interest and bleach prior to acquisition (Fig. 3b).
17. The gene encoding the λN^* -peptide carries three point mutations derived from a related arginine-rich RNA-binding peptide of phage P22. The gene encoding λN^* -Gfp² is inserted at the *ip^s* locus. Expression is controlled by the arabinose-inducible promoter P_{crg1}.
18. With this setup, we are able to detect mRNAs under endogenous expression levels. RNA sequencing data on the MUMDB

[35] help to determine the length of 3' UTRs; for tagging *cdc3* (um10503) mRNA, *boxB* hairpins were inserted 100 nt downstream of the stop codon.

19. At first, P_{crg1} is induced because switching on P_{nar1} and P_{crg1} promoters simultaneously results in a delay of filament induction.
20. Due to the relatively high cytoplasmic signal intensity of λN^*G^2 , we recommend to draw the line for the kymograph point by point to ensure the selection of the pixel with the highest intensity for the kymograph line. The thickness of the kymograph line should not exceed three pixels.
21. This setup is optimized for comparably slow fluorescence recovery processes in the order of minutes. For recovery in the order of seconds, settings have to be adapted for faster acquisition.
22. Before acquiring Gfp fluorescence with the LED, the software-driven autofocus was used in the DIC channel to assure that acquisition started in the correct focal plane. The whole procedure can be automated with the “multidimensional acquisition” tool of MetaMorph 7.
23. In our experiment, we determined fluorescence recovery of a cortical protein. Acquiring *z*-stacks might not be required if studying cytoplasmic proteins and are not applicable if recovery processes in the range of seconds are analyzed.
24. Shrinking of the agarose cushion requires drift correction.
25. Identify a background region of a certain size that can be used in all experiments.
26. With every *z*-stack taken, the Gfp fluorescence gets photo-bleached to a certain amount. One has to correct for this acquisition bleaching. Also correct for collateral bleaching. The high intensity of the 405 nm laser causes bleaching in areas outside the determined area.
27. A small population of mEosFP emits red fluorescence already before irradiation with 405 nm light. That is an original character of mEosFP.
28. As the pool of non-converted mEosFP is depleted during data acquisition, it is advisable to increase the power of the activation laser to keep the number of activated fluorophores per frame constant.
29. For a typical image size of 256×256 pixels per image in 16-bit tif format, the processing time is 15–30 ms per image, depending on the complexity of constructed image. The PALM micrograph in Fig. 6a is constructed by 1,000 frames with a time resolution of 50 ms.

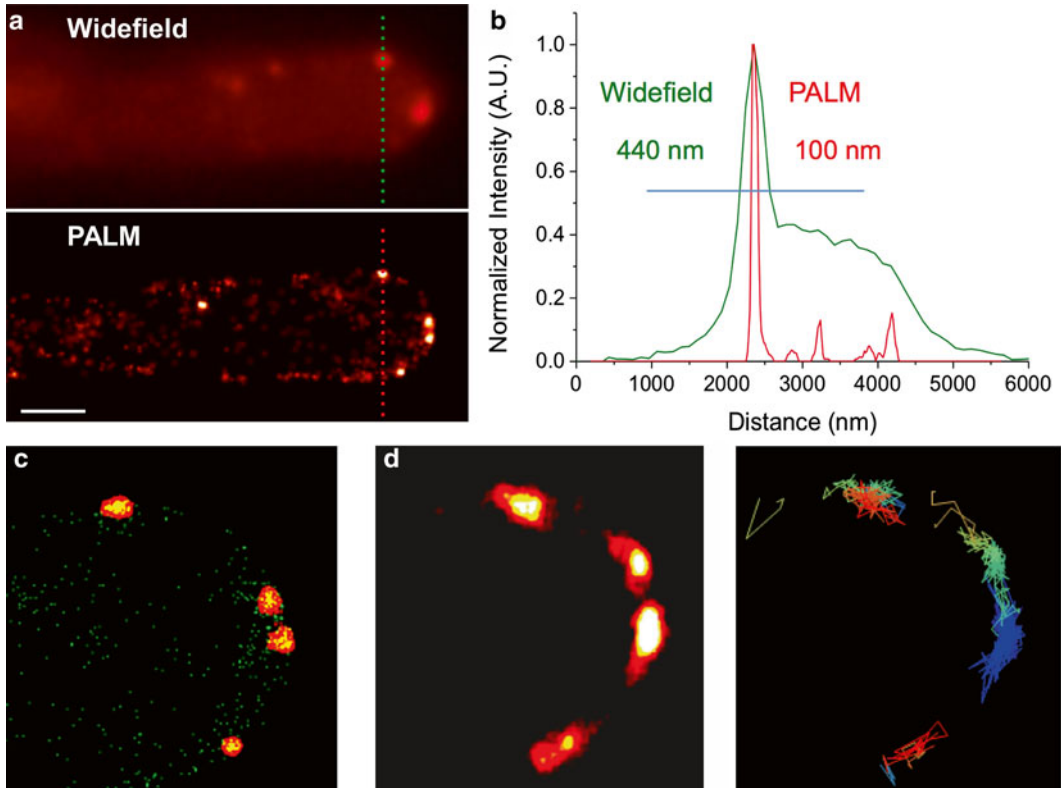


Fig. 6 PALM microscopy in *A. nidulans*. (a) Localization of mEosFP-TeaR at hyphal tip. Conventional wide-field epifluorescence microscopy (*top*) and PALM microscopy (*bottom*) are compared. The PALM micrograph is constructed by 1,000 frames with a time resolution of 50 ms (bar = 1 μ m). (b) Intensity profile of a TeaR membrane domain illustrating the increased resolution achieved with PALM. Intensity was measured along the *green* and *red line* in (a). (c) Four membrane domains identified by cluster analysis (to be published elsewhere), setting the threshold on the number of neighboring molecules (ten molecules) within a certain distance r (50 nm). (d) Tracking the dynamic behavior of single molecules for a few seconds until they are photobleached. Each line indicates the movement of a single TeaR molecule. Separate *colors* indicate different molecules

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Quantitative Analysis of Transferrin Cycling by Automated Fluorescence Microscopy

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Abstract

Surface receptors are transported between the plasma membrane and intracellular compartments by various endocytic mechanisms and by recycling via different pathways from sorting or recycling endosomes. The analysis of cellular components involved in mediating or regulating these transport steps is of high current interest and requires quantitative methods to determine rates of endocytosis and/or recycling. Various biochemical procedures to measure uptake of labeled ligand molecules or internalization and reappearance of surface-labeled receptors have been developed. Here, we describe a quantitative method based on fluorescence microscopy of adherent cells taking advantage of the transferrin (Tf) receptor as the prototype of cycling transport receptors. Tf is endocytosed with bound Fe^{3+} and, upon release of the iron ion in endosomes, recycled as apo-Tf together with the receptor. To follow the ligand–receptor complex, fluorescently labeled Tf is used and detected microscopically with or without releasing Tf from cell surface receptors by acid stripping. To go beyond the observation of a few individual cells, automated fluorescence microscopy is employed to image thousands of cells at different time points and in parallel with different treatments (such as chemical inhibitors, siRNA silencing, or transfection of candidate genes) in a 96-well format. Computer-assisted image analysis allows unbiased quantitation of Tf content of each cell and to distinguish between different cell populations.

Key words Endocytosis, Fluorescence microscopy, Image analysis, Receptor, Recycling, Transferrin

1 Introduction

In eukaryotic cells, membrane transport is essential to distribute proteins between intracellular organelles and the plasma membrane, to export proteins via the secretory pathways, and to internalize macromolecules by endocytosis. A multitude of proteins are involved in membrane traffic as building blocks for vesicular coats, as regulators, and as tethering and fusion factors and in further functions. This is exemplified by the known interactome involved in clathrin-mediated endocytosis [1]. Much less is known about other uptake pathways [2–4] and about intracellular transport and sorting steps at endosomes [5, 6]. To investigate the role

of candidate proteins in transport by gene silencing or expression of mutant proteins, quantitative assays are required. Chemically modified and/or radioactive ligands or surface-labeled receptors may be followed biochemically by monitoring the disappearance or reappearance of surface accessibility. Fluorescent molecules may be followed microscopically with the added benefit of morphological information.

A particularly useful system to analyze receptor endocytosis and recycling is the transferrin (Tf) receptor, the prototype of a transport receptor cycling between the plasma membrane and endosomes. Tf acts as the transporter of Fe^{3+} ions in the bloodstream. Loaded holo-Tf binds to the Tf receptor at neutral pH and is rapidly endocytosed at rates of $\sim 15\%$ /min. In the more acidic environment of early endosomes, the iron is released, while apo-Tf remains bound to the receptor, which is recycled to the plasma membrane either directly from early sorting endosomes or via recycling endosomes. At neutral extracellular pH, apo-Tf is released from the receptor [5, 7, 8]. At steady state, approximately 80 % of the Tf receptor is intracellular, while 20 % is at the plasma membrane [9–11].

To follow trafficking of Tf, it needs to be labeled. Iodination with radioactive [^{125}I] iodine provides high sensitivity. Biotinylated Tf (which is also commercially available) allows easy detection by streptavidin–horseradish peroxidase blotting after SDS-gel electrophoresis or directly on the culture dish with a microplate reader. A further possibility is the use of fluorescent Tf to measure the fluorescence intensity of either entire cell populations or single cells using flow cytometry or fluorescence microscopy. To study endocytosis, cells are incubated with fluorescent Tf, and endocytosis is measured as the increase of intracellular fluorescence over time. To investigate recycling from endosomes back to the plasma membrane, cells are first loaded with fluorescent Tf and subsequently the surface-bound Tf is removed. The cells are then incubated at 37 °C to allow recycling of Tf in the presence of an iron chelator to prevent rebinding of the recycled Tf. The time course of release of fluorescent Tf is monitored.

Here, we describe the use of fluorescently labeled Tf and fluorescence microscopy for quantitative endocytosis and recycling assays using automated fluorescence imaging and image analysis (Fig. 1). Using this automated analysis process, it is possible to measure the fluorescence intensity of Tf in thousands of individual cells at different time points. It is further possible to measure Tf in transiently transfected cell populations distinguishing transfected from untransfected cells, high expressers from low expressers, or large cells from small ones. The simultaneous measurement of two conditions in the same experiment provides additional confidence in the significance of observed rate differences.

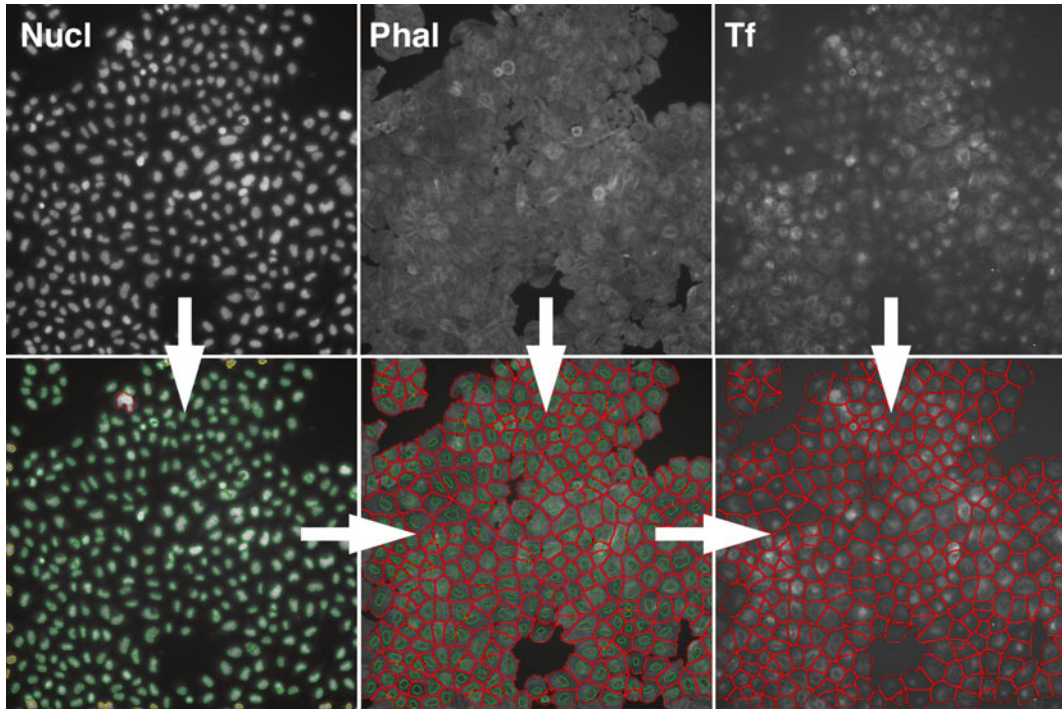


Fig. 1 Automatic identification of cells. In this example, HeLa cells were loaded for 45 min with Alexa-568-labeled Tf in a recycling experiment, fixed, stained with Hoechst 33342 and Alexa-488 phalloidin, and imaged in the corresponding three channels (Nucl, Phal, and Tf). The nuclei channel is used to identify nuclei (*green outlines*). Together with the phalloidin channel, this allows to delimit individual cells (outlined in *red*), which in turn is the basis to quantify the Tf signal per cell

2 Materials

2.1 Cell Culture

1. HeLa Kyoto α cells [12] cultured in Dulbecco's Modified Eagle's Medium containing 10 % fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma) at 37 °C with 7.5 % CO₂.
2. Assay medium consisting of Dulbecco's Modified Eagle's Medium containing 2 mM glutamine and buffered with 20 mM HEPES–NaOH, pH 7.2–7.5 (Invitrogen).
3. 96-well flat clear bottom black polystyrene TC-treated microplates (Corning Life Sciences).
4. FuGENE HD transfection reagent (Promega) for plasmid transfection.
5. Lipofectamine RNAiMAX transfection reagent (Invitrogen) for transfection of siRNA. Dilutions of transfection reagents and plasmids or siRNA prior to transfection are made with serum-free Dulbecco's Modified Eagle's Medium.

6. Tf from human serum, conjugated with Alexa Fluor 488 or 568 (Invitrogen), 5 mg/mL solution in H₂O with 2 mM sodium azide.
7. Stripping buffer: 150 mM NaCl, 50 mM NaAcetate, pH 3.5.
8. Deferoxamine mesylate (Sigma), 50 μ M solution in assay medium, prepared fresh for every assay.
9. PBS: 12.5 mM sodium phosphate buffer, pH 7.6, 125 mM NaCl.
10. Paraformaldehyde, 3 % solution in PBS. Aliquots are stored at -20°C .
11. Ammonium chloride solution, 50 mM in PBS.
12. 0.1 % Triton X-100 in PBS.
13. DY-647-Phalloidin (Dyomics), Alexa Fluor 488 or 568-conjugated Phalloidin (Invitrogen), 200 U/mL solution in methanol. Stored at -20°C protected from light.
14. Hoechst 33342, 10 mM solution in PBS with 25 % ethanol.
15. 12-channel multi-pipette.
16. Water bath or heating block for incubation at 37°C of the 96-well plates and $0-4^{\circ}\text{C}$ cooling bath or cooling plate (*see Note 1*).

2.2 Automated Image Acquisition

Image acquisition is performed with an automated wide-field screening microscope, such as the ImageXpress Micro System (Molecular Devices). The setup used here consists of the following components:

1. ImageXpress Micro automated cellular imaging microscope, equipped with a fast laser autofocus with a precision motorized Z-stage and a Lumencor Spectra X light engine.
2. A filter cube set (Semrock) to discriminate between Hoechst 33342 (BrightLine, DAPI-5060B), GFP (BrightLine, GFP-3035B), RFP (BrightLine, TXRED-4040B), Cy3 (BrightLine, CY3-4040C), and Cy5 (BrightLine, CY5-4040A).
3. CFI Super Fluor 10 $\times$ objective (Nikon).
4. CoolSNAP_{HQ} Monochrome CCD camera (Photometrics).
5. MetaXpress software controlling all components of the system.

2.3 Automated Image Analysis

Image analysis is performed with the CellProfiler cell image analysis software (www.cellprofiler.org) [13]. For the image analysis described here, version 2.0 of CellProfiler was used. MATLAB (MathWorks) can be used to read the output files and was used here together with a corresponding script to export the data into a Microsoft Excel spreadsheet. Other image analysis software may also be used.

3 Methods

When performing assays on 96-well plates, it is essential to directly compare different conditions (e.g., gene silencing vs. mock silencing) on the same plate, instead of using one plate per condition. This is to avoid overinterpretation of results due to interexperimental variation. Interexperimental variations can be caused by differences in the state of the cells at the time of the experiment (e.g., variation in seeding density or passage number).

3.1 Cell Culture and Transfection

Cells are grown in 96-well plates with eight rows and 12 columns. Every column represents a time point, except for the first one, in which the cells are not incubated with fluorescent Tf at all to determine background fluorescence. For siRNA silencing experiments, four rows are transfected with nontargeting siRNA (control) and four rows with siRNA against the gene of interest (*see Note 2*). For plasmid transfection experiments, six rows are transfected. Transfected cells are distinguished from untransfected cells by fluorescence intensity of a fluorescent reporter protein. For example, we use a dual expression plasmid (MultiLabel; [14]) for the simultaneous expression of the protein of interest and mCherry to detect the transfected cells. Alternatively, the protein of interest may be tagged with a fluorescent protein or it may be detected by antibody staining. Two rows are left untransfected in order to determine the background fluorescence in the mCherry channel.

For siRNA silencing, the reverse transfection method is used, whereby siRNA and transfection reagent are added to the microplate wells before the cells are seeded. Efficiency of gene silencing has to be determined individually. To test the silencing efficiency in a specific experiment, it is usually sufficient to use the lysate of four combined wells per condition for an immunoblot analysis.

1. To analyze HeLa cells without transfection, ~2,400 cells are seeded in 240 μ L medium per well and incubated at 37 °C with 7.5 % CO₂ for 72 h.
2. For siRNA transfection, transfection reagent and siRNA (final concentration: 20 nM) are mixed in serum-free DMEM according to the manufacturers' recommendations, and 90 μ L per well is added to the microplate. After an incubation at room temperature between 30 min and 60 min, 150 μ L of an appropriate HeLa cell dilution in DMEM containing 16 % FCS is added at a density of ~2,400 cells per well on top of the transfection mix. The cells are then incubated at 37 °C with 7.5 % CO₂ for 72 h (*see Note 3*).
3. For the analysis of cells transfected with plasmid DNA, ~5,000 cells per well are seeded in 240 μ L medium per well (*see Note 3*). After 24 h incubation at 37 °C with 7.5 % CO₂, transfection

reagent and plasmid DNA (final amount: approximately 100 ng per well) are mixed in serum-free medium according to the manufacturers' recommendations. After 15–20 min incubation at room temperature, the medium is removed from the cells, the transfection mix is diluted with DMEM containing 10 % FCS in a ratio of 1:20, and 100 μ L of this diluted transfection mix is added per well. The cells are then incubated for another 24 h at 37 °C with 7.5 % CO₂. The cells are then washed twice with DMEM containing 10 % FCS and are incubated in 240 μ L DMEM containing 10 % FCS for another 24 h at 37 °C with 7.5 % CO₂.

3.2 Continuous Endocytosis Assay

The first column of the microplate is used to determine background fluorescence (no Tf added). Columns 2–12 are allowed to internalize Tf for appropriate times, for example, for 1–11 min in 1-min intervals. To achieve this, Tf is first added to column 12, 1 min later to column 11, and so on, until Tf is finally added to column 2. One minute later, the plate is cooled rapidly to stop endocytosis in all wells. The result of a typical endocytosis experiment is shown in Fig. 2a. To determine the absolute rate of uptake, it may be necessary to perform a single-cycle endocytosis experiment (*see Note 4*).

1. All wells are washed once with 200 μ L/well assay medium and then incubated for 2 h at 37 °C with 200 μ L/well assay medium (*see Note 5*) in the incubator.
2. The microplate is transferred to the 37 °C water bath or heating plate.

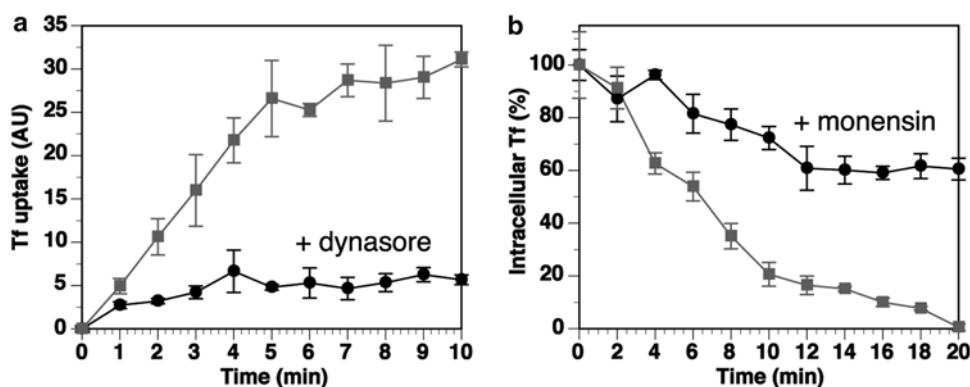


Fig. 2 Tf endocytosis and recycling. To analyze Tf endocytosis, (a) HeLa cells were incubated with fluorescent transferrin for 0–10 min in the absence or presence of 80 μ M dynasore, an inhibitor of dynamin-dependent endocytosis [15]. To analyze Tf recycling, (b) HeLa cells were loaded with fluorescent Tf for 45 min and then chased for 0–20 min in the presence or absence of 10 μ M monensin, an ionophore inhibitor of endosomal traffic [16]. The mean Tf intensity per cell was measured and averaged over all cells in a well. The average of three assay wells with standard deviation is plotted at each time point

3. Uptake of fluorescent Tf is initiated in columns 2–12 by removal of the medium and addition of 50 $\mu\text{L}/\text{well}$ of 50 $\mu\text{g}/\text{mL}$ Alexa Fluor-conjugated Tf in assay medium at appropriate time intervals starting with the wells for the longest incubation time.
4. At time 0 min, endocytosis is stopped by quickly transferring the microplate to 4 $^{\circ}\text{C}$ and adding 150 $\mu\text{L}/\text{well}$ ice-cold PBS. The cells are quickly washed twice with 200 $\mu\text{L}/\text{well}$ ice-cold PBS.
5. The cells are washed once with 200 $\mu\text{L}/\text{well}$ ice-cold stripping buffer and then four times with ice-cold PBS to remove bound Tf from the cell surface.
6. The cells are fixed by addition of 170 $\mu\text{L}/\text{well}$ of 3 % paraformaldehyde solution. The plate is removed from the cold, the bottom is cleaned with 70 % ethanol, and it is kept at room temperature for 10 min. The cells are then further processed as described in Subheading 3.4.

3.3 Recycling Assay

The first column of the microplate is used to determine background fluorescence (no Tf added). Columns 2–12 are allowed to internalize Tf into steady state. Column 2 is used to determine Tf intensity at steady state, and the cells in these wells are fixed without chase. Columns 3–12 are incubated with chase medium, and recycling is stopped at appropriate time points, e.g., at 2-min intervals. The result of a typical recycling experiment is shown in Fig. 2b.

1. The cells are washed once with 200 $\mu\text{L}/\text{well}$ assay medium and then incubated for 2 h at 37 $^{\circ}\text{C}$ with 200 $\mu\text{L}/\text{well}$ of assay medium in the incubator (*see Note 5*).
2. The cells in columns 2–12 are washed once with assay medium and then loaded with Tf by addition of 50 $\mu\text{L}/\text{well}$ of 50 $\mu\text{g}/\text{mL}$ Alexa Fluor-conjugated Tf in assay medium for 45 min in the 37 $^{\circ}\text{C}$ incubator.
3. The microplate is placed on the cold plate and washed twice with 200 $\mu\text{L}/\text{well}$ ice-cold PBS.
4. The cells are washed once with 200 $\mu\text{L}/\text{well}$ ice-cold stripping buffer and then four times with ice-cold PBS.
5. Columns 1 and 2 are fixed by addition of 170 $\mu\text{L}/\text{well}$ 3 % paraformaldehyde solution.
6. The microplate is placed at 37 $^{\circ}\text{C}$. To prevent rebinding of recycled Tf, 100 $\mu\text{L}/\text{well}$ assay medium containing 50 μM deferoxamine mesylate prewarmed to 37 $^{\circ}\text{C}$ is quickly added to columns 3–12.
7. Every 2 min, a column of wells is washed once with assay medium and then fixed by adding 170 $\mu\text{L}/\text{well}$ 3 % paraformaldehyde solution. After the last time point, the plate is removed from the water bath or heating plate, the bottom is

cleaned with 70 % ethanol, and it is kept at room temperature for an additional 5 min.

8. The cells are further processed as described in Subheading 3.4.

3.4 Staining of Cells

1. The cells fixed with 3 % paraformaldehyde are washed once with 200 μL /well of PBS at room temperature.
2. Residual paraformaldehyde is quenched by washing once with 200 μL /well 50 mM ammonium chloride solution and three times with 200 μL /well of PBS at room temperature.
3. The cells are then permeabilized with 100 μL /well 0.1 % Triton X-100 in PBS for 1 min at room temperature. 100 μL /well PBS is added to the cells, and they are washed four times with 200 μL /well of PBS. Experiments not requiring antibody staining should continue with **step 4**, experiments requiring antibody staining should continue with **step 5** (*see Note 6*).
4. The actin cytoskeleton and the nuclei are stained by the addition of 30 μL /well Alexa Fluor Phalloidin (final concentration 1 U/mL) and Hoechst 33342 (final concentration 10 μM) in PBS, respectively, for 30 min at room temperature. The cells are washed three times with 200 μL /well of PBS at room temperature. After the last wash, 200 μL /well of PBS is left on the plate, and it is stored protected from light at 4 °C until imaged. Plates may be stored for at least 1 week before imaging.
5. In case antibody staining is required, 200 μL /well 1 % BSA in PBS is added to the cells for 15 min at room temperature.
6. The primary antibody is diluted in 1 % BSA in PBS, and 30 μL is added per well. The cells are incubated at room temperature for 2 h. The appropriate antibody concentration has to be determined for every antibody individually.
7. The cells are washed four times with 200 μL 1 % BSA in PBS per well.
8. The fluorescent secondary antibody, Alexa Fluor Phalloidin (final concentration 1 U/mL), and Hoechst 33342 (final concentration 10 μM) are diluted in 1 % BSA in PBS, and 30 μL is added per well. The cells are incubated at room temperature for 30 min. The appropriate antibody concentration has to be determined for every antibody individually.
9. The cells are washed four times with 200 μL /well of PBS at room temperature, after which the plate is stored protected from light at 4 °C until imaged.

3.5 Automated Image Acquisition

1. The bottom of the microplate is cleaned with 70 % ethanol and loaded into the microscope. Before imaging, the parameters of the microplate (number of rows and columns, well diameter, column and row offset, column and row spacing, well depth,

well shape, and the length, width, and height of the plate) are entered or selected in the software operating the microscope.

2. For the assays described here, the 10× Super Fluor objective with a binning of two and a gain of two was always used. If high-resolution images are desired, binning can be set to one.
3. The wells and the number and spacing of the sites to be imaged within the wells are selected, e.g., 3 × 3 sites in the center of the well.
4. The number of channels to use is selected. For an siRNA experiment, three emission channels (for nuclei, phalloidin/actin, and Tf) are chosen; for plasmid transfected cells, four channels are selected (for nuclei, phalloidin/actin, Tf, and the fluorophore identifying transfected cells).
5. As the first channel, select the one showing the nuclei and adjust the *Post Laser Offset* until the nuclei are in focus.
6. Step five is repeated for the other channels until the images are in focus. The *Post Laser Offset* of subsequent channels is set with reference to the *Post Laser Offset* of the first (nuclei) channel.
7. Define where the images are saved on a remote server in real time during image acquisition using *Journals* (see **Note 7**).

3.6 Computer-Assisted Image Analysis

To analyze images with CellProfiler, a *Pipeline* is built defining a sequence of actions to be applied to the acquired images as outlined in Fig. 1.

In this section, a basic starting point for computer-assisted image analysis with CellProfiler is provided. Not all settings of every module are explained, since in many cases the default values are a good starting point. For a more detailed description of the modules and the algorithms used, refer to the CellProfiler manual.

1. Specify the input folder containing the images to analyze, the output folder specifying the location where the output file will be saved to, and the name of the output file. Add the following modules to the pipeline.
2. The *Load Images* module specifies the number of images (channels) to be loaded per analysis cycle and the parts of the filename that identifies them (e.g., DAPI, GFP, RFP, Cy5).
3. The *Correct Illumination Calculate* module is added twice to calculate the illumination correction function from all images for the nuclei and phalloidin channels (see **Note 8**). The illumination correction function is calculated once in the first analysis cycle (choose *All: First Circle for Calculate function for each image or based on all images?*). As *Smoothing Method* select *First Polynomial*.
4. The *Correct Illumination Apply* module corrects for uneven illumination by applying the illumination correction functions calculated in **step 3** in each cycle for nuclei and phalloidin channels.

5. The *Identify Primary Objects* module is used in the nuclei channel to mask nuclei as objects. *Otsu Adaptive Thresholding* algorithm works well for nuclei, since all nuclei have approximately the same intensity and shape. Since the Hoechst 33342 staining is usually strong with little background, a two-class thresholding can be used with a *Thresholding Correction Factor* of approximately 1.2. Select *Discard Objects Touching The Border Of The Image* and *Fill Holes In Identified Objects* (see **Note 9**).
6. The *Identify Secondary Objects* module is used in the phalloidin channel to identify the cells around the nuclei. Since the HeLa Kyoto cells have a rather compact morphology and usually grow in coherent patches, *Distance—B* can be used as a method to identify the secondary objects and *Otsu Adaptive* as the thresholding algorithm. For cells with a heterogeneous morphology, *Propagation* is a better method to identify the secondary objects. To improve object identification, *Otsu Per Object Thresholding* can also be used, but will prolong the analysis. Because of the variable fluorescence intensity of phalloidin staining within each cell, it is recommended to use *Three-Class Thresholding* and assign the middle intensity class to the foreground. The thresholding correction factor can be set below 1, since secondary objects will only be identified if a primary object is present. This can be useful when fluorescence intensity is low. *Fill Holes In Identified Objects* should be selected.
7. The *Measure Object Intensity* module is used to measure the fluorescence intensity of one or more channels within the identified objects, such as the intensity of Tf fluorescence within each cell.
8. The *Export To Spreadsheet* module will write the data into a Microsoft Excel spreadsheet for further data analysis. Alternatively, the final CellProfiler output file may be analyzed with MATLAB (MathWorks), from where it can also be exported to a Microsoft Excel spreadsheet using a corresponding script.
9. From the menu bar, select *Test* and *Start Test Run*. In *Test Run* mode, it is possible to manually cycle through the individual modules of the pipeline. The settings of the modules can be adjusted and their effect can be observed directly. When the settings are satisfactory, *Test Run* mode is ended by choosing *Test* and *Stop Test Run* from the menu bar.
10. To start the analysis, select *Analyze Images*.
11. The resulting output file contains all measured parameters including the total and the mean fluorescence intensities per cell, as well as further information acquired during the analysis (e.g., the number of objects identified in each image).

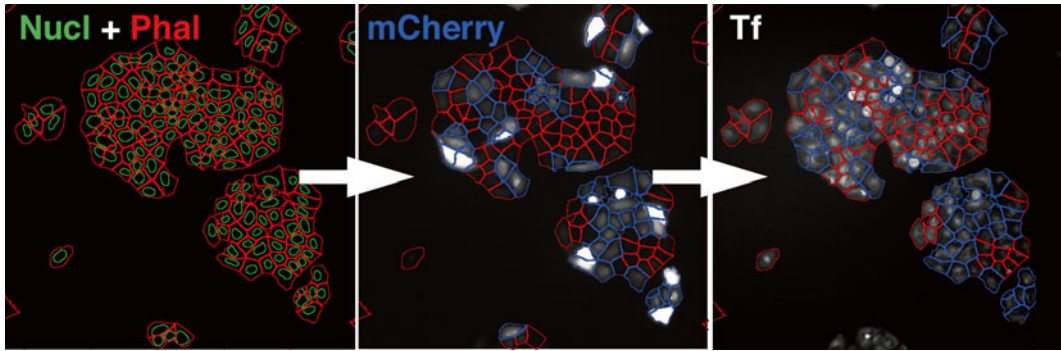


Fig. 3 Analysis of selected cell populations. In this example, HeLa were transfected with mCherry and the cells were loaded for 45 min with Alexa-488-labeled Tf in a recycling experiment, fixed, stained with Hoechst 33342 and DY-647 phalloidin, and imaged in the corresponding four channels (Nucl, Phal, mCherry, and Tf). The nuclei and the phalloidin channels are used to identify nuclei (*green outlines*) and cells (*red outlines*). The signal of mCherry is used to distinguish transfected (*blue outlines*) from untransfected cells (*red outlines*) for separate quantitation of transfected and untransfected cells. The mCherry signal is intentionally overexposed in order to visualize low expressing cells

In the examples for endocytosis and recycling in Fig. 2, the mean intensity of Tf fluorescence per cell was averaged for each well, and the mean values of three wells per time point are plotted.

3.7 Analysis of Selected Cell Populations

To analyze selected cell populations, two *Pipelines* are built, the first one to measure the parameters that are used to distinguish between cell populations, such as the fluorescence intensity of a fluorescent protein-tagged protein of interest transfected into the cells. For the example shown in Fig. 3, this is the intensity of a fluorescent reporter (mCherry) transfected together with the protein of interest on the same plasmid. To distinguish between transfected and untransfected cells, it is essential to have control wells without transfection on the same microplate. Cells can also be classified in more than two categories, for example, to distinguish between high and low expressing cells as well. The second *Pipeline* is built to measure Tf intensity.

1. The same *Pipeline* as in **steps 1–6** from Subheading 3.6 is set up.
2. The *Measure Object Intensity* module is used to distinguish cells based on their fluorescence intensity, for example, by transfecting the cells with a fluorescent reporter protein like mCherry.
3. The *Display Data On Image* module is used to display the single cell data of a certain measurement directly on the image that was measured during an ongoing analysis. The measurement to be displayed (e.g., mean fluorescence intensity), the channel from which the measurement is taken, and the channel on which the data will be displayed are selected.

4. In the menu of CellProfiler, *Test* and then the submenu *Start Test Run* are selected. To distinguish between the values of the two cell populations, it is necessary to manually go through several images from untransfected and transfected wells. The intensity values displayed for untransfected and transfected cells are noted as background and signal, respectively.
5. A new pipeline is constructed. First, the same pipeline as in **steps 1–6** from Subheading **3.6** is built.
6. The *Measure Object Intensity* module is added to the pipeline. The parameter that is used to distinguish between cell populations (e.g., fluorescent intensity of a reporter protein) is measured.
7. The *Filter Objects Module* is added to the pipeline twice. This module is used to filter and thereby distinguish cell populations by a measurable factor. Here, we will use the example of distinction by fluorescence intensity. The first of the two modules is used to filter for untransfected cells. *Intensity* is selected as the filter category and *Mean Intensity* as the measurement. As minimum value 0 is selected. As maximum value, the background fluorescence intensity determined in **step 3** is selected. The second of the two modules is used to filter for transfected cells. As minimum value, the minimal fluorescent intensity for a cell needed to qualify as transfected is chosen. As the maximum value, 1 or a maximum value above which cells should not be included anymore is selected.
8. The *Measure Object Intensity* module is added to the pipeline. Here, the intensity of the Tf fluorescence in the previously filtered cell populations is measured. Every object that should be measured has to be specified individually in this module.
9. In order to analyze the data, it is possible to use the *Export To Spreadsheet* module. This module will write the data into a Microsoft Excel spreadsheet. Alternatively, it is also possible to analyze the data with MATLAB (MathWorks). Furthermore, from MATLAB, the data can again be exported to a Microsoft Excel spreadsheet using a corresponding script.

4 Notes

1. Rapid and equal temperature changes are important. If a heating or cooling plate is used, it is important to have a sufficient amount of water on the plate to create an aqueous contact between the bottom of the culture plate and the heating or cooling surface. Rapid and equal temperature changes can also be achieved using water baths with a stable support for the plates just below the water surface.
2. In 96-well plates, it is possible that the cells in the outermost wells display a different behavior than the rest of the cells.

This so-called edge effects may be caused by a more extensive evaporation of cell culture medium at the outermost wells in comparison to the other wells of the 96-well plate. If edge effects are regularly observed, it is advisable to disregard the outermost wells. For example, in siRNA silencing experiments, this would mean that only three wells of a row are used for the same time point and condition instead of four.

3. After seeding the cells, leaving the microplate at room temperature for 10 min before incubating them at 37 °C can help to more evenly distribute the cells throughout the well.
4. A continuous endocytosis experiment measures the total uptake of Tf in fluorescence units per min. This apparent uptake rate and the steady-state level of intracellular Tf that will be reached also depend on the amount of surface Tf receptor, which is affected by changes in Tf receptor recycling and expression levels. To determine absolute internalization rates (% of surface-bound Tf internalized per minute), single-cycle endocytosis assays need to be performed. Surface receptors are first loaded with Tf at 4 °C. After removal of Tf in the medium, the cells are warmed to 37 °C to allow endocytosis for different times, after which they are cooled to 4 °C, and the remaining surface-bound Tf is stripped. The cells are then fixed and processed for microscopy. Each time point requires a separate plate.
5. Upon repeated complete removal of liquid from the wells, some cell types tend to detach because of surface tension. If this is the case, sufficient residual fluid should be left in the well to cover the cells. Approximately 30 µL of liquid is necessary to cover all cells in wells in 96-well plates. The resulting dilution of added solutions is negligible in most cases and can be further reduced by increasing the number of washing steps.
6. Permeabilization of the cells is necessary to stain the cells with fluorescently labeled phalloidin or with antibodies. Staining the cells with phalloidin is important for image analysis. Alternatively, fluorescently labeled wheat germ agglutinin can be used to avoid the need to permeabilize the cells.
7. Journals are software scripts used to manage a more complex sequence of hardware or software control features. Since automated image acquisition generates large datasets, it is useful to store these datasets on a remote server instead of the local storage on the computer that operates the microscope. By using journals, the images can be saved automatically and in real time to a remote server while image acquisition is ongoing.
8. Illumination correction should be applied separately for each wavelength used to identify objects, since object identification may be affected by uneven illumination. Illumination should not be corrected for the channels that are used to measure fluorescence intensity.

9. In order to find the best settings for object identification, it is advisable to use CellProfiler in test run mode. To do this, set up your pipeline, and then start the test run. While in test run mode, it is possible to change all parameters within a pipeline and then immediately visualize the changes in object identification.

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Part III

Omics Approaches

Identification of Factors Regulating MET Receptor Endocytosis by High-Throughput siRNA Screening

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Abstract

The tyrosine kinase MET, a receptor for hepatocyte growth factor, is a key regulator for normal development and organ renewal via stem cell maintenance. Dysregulated MET signaling contributes to tumor progression and metastasis and is considered a potent therapeutic target for a growing number of malignancies. Toward that goal it is critical to develop high-throughput assays to identify candidate regulators for the termination of MET signaling. We describe here a rapid and efficient method for identifying cellular factors required for MET ubiquitination, which utilizes high-throughput RNA interference screening (HT-siRNA) with a receptor internalization assay and an In-Cell ELISA in a 96-well format. The assay is amenable to a large array of cell surface proteins as well as genome-wide siRNA libraries, with high signal-to-background ratio and low well-to-well variability.

Key words RNA interference (siRNA), Hepatocyte growth factor (HGF), MET, HeLa cells, Ubiquitination, Ube3C, Receptor endocytosis

1 Introduction

Ubiquitination is a posttranslational modification by which ubiquitin is covalently attached to a target protein. Protein ubiquitination has been reported to regulate a myriad of cellular processes including DNA repair, chromatin rearrangement, enzymatic activation, and protein degradation [1–6]. In the case of cell surface receptors, ubiquitin functions as a tag for lysosomal degradation by mediating interactions between the ubiquitinated receptor and sorting machinery at the plasma membrane and endosomal compartments including early endosomes and multivesicular bodies [7, 8]. It is well established that ubiquitination of the hepatocyte growth factor (HGF) receptor/MET is critical for receptor trafficking in many cancer cell types [9–12]. Thus, a comprehensive understanding of MET interaction networks, particularly the proteins that ubiquitinate MET and terminate receptor signaling, remains an important goal in basic and pharmaceutical research.

Ubiquitination occurs in a three-step reaction, requiring three different classes of enzymes, namely, an E1 (ubiquitin-activating enzyme), an E2 (ubiquitin-conjugating enzyme), and an E3 (ubiquitin-ligating enzyme) [13]. The coordinated activity of these components may result in up to eight biochemically distinct inter-ubiquitin linkages through the seven internal lysine residues of ubiquitin or via its amino terminal methionine [14]. Several studies have demonstrated a strict requirement for the E3 ubiquitin ligase Cbl for the ubiquitination of HGF-activated MET [15–17]. Receptor ubiquitination by Cbl provides ubiquitin-recognition motifs for MET recruitment to multivesicular bodies for subsequent lysosomal degradation [16, 17]. Failure to ubiquitinate MET promotes PI3K/Akt/TOR signaling [18] and increases tumor growth in vivo [19]. The ubiquitin ligase activity of Cbl is also critical for clathrin-mediated MET endocytosis via interactions with the receptor binding partner Grb2 [16]. However, little is known regarding additional ubiquitination-related proteins critical for MET trafficking. This chapter describes a high-throughput RNA interference screen that identified a number of novel candidate regulators of MET ubiquitination. One of these candidates, the ubiquitin ligase E3C (Ube3C), was identified as a critical regulator of MET internalization. This finding is significant given that mutations in Ube3C are associated with increased tumor development and progression in patients with hepatocellular carcinoma. The study is an adaptation of a previously described protocol [20] using a commercially prepared siRNA library targeting ubiquitination proteins, demonstrating its general amenability to receptor tyrosine kinases.

2 Materials

All solutions were prepared in sterile, ultrapure H₂O (~18.2 MΩ cm; PURELAB Ultra; ELGA). When required, gloves and DEPC-treated H₂O were used. RNA- and DNA-free plastics were used at all times.

2.1 Growth and Preparation of HeLa Cells

1. HeLa cells: HeLa cells were routinely grown to a density of no more than 60–70 % in Dulbecco's Modified Eagle Medium (DMEM; cat# 11995; Life Technologies) supplemented with 4.5 g/l D-glucose, 10 % fetal bovine serum (FBS; HyClone), L-glutamine (2 mM; Life Technologies), penicillin (100 U/ml; Life Technologies), and streptomycin (100 µg/ml; Life Technologies). These cells moderately adhered to tissue culture plastics and were harvested by incubating with 5 mM EDTA, pH 8.0 in DPBS (Dulbecco's Phosphate-Buffered Saline, Life Technologies; Cat# 14190-250) for 5 min at 37 °C, to ensure that cell surface levels of receptor are not compromised by

protease treatments. To enhance cell attachment, plated cells were cooled on ice for 5 min prior to media changes. Immediately prior to transfections, HeLa cells were harvested and resuspended in DMEM supplemented with 10 % fetal bovine serum (FBS) at a concentration of 5,000 cells/50 μ l/well of the 96-well plate (*see* Subheading 3.1).

2. Recombinant human HGF: HGF (PeproTech; cat#100-39, Lot# 0109S201) was received as a lyophilized powder and was reconstituted in water to a concentration of 500 μ g/ml, aliquoted and stored at -20°C . For MET internalization, this solution was thawed and added to the cell media at a final concentration of 100 ng/ml.
3. Dynasore: GTPase inhibitor Dynasore was received as a lyophilized powder from Sigma-Aldrich (cat# D7693) and was reconstituted in DMSO to a concentration of 30 mM, aliquoted and stored at -80°C . For MET internalization studies, the Dynasore stock solution was thawed and added directly to the cell media at a final concentration of 50 μ M.
4. Cycloheximide: Cycloheximide (Sigma-Aldrich; C-7698) was received as a lyophilized powder and was reconstituted in water to a concentration of 10 mg/ml. To inhibit protein synthesis the stock solution was thawed and added to serum-free (SF) DMEM at a final concentration of 10 μ g/ml.

2.2 Cell Transfections and Analysis

1. DharmaFECT 2 transfection reagent: Transfection reagent DharmaFECT 2 (Thermo Fisher Scientific; cat# I-2002, Lot# 090917T) was used to deliver siRNA into cultured cells and in our hands gave the highest transfection efficiency with the lowest toxicity compared to DharmaFECT 1, 3, or 4 reagents. The transfection method was optimized using HeLa cells stably expressing a firefly luciferase transgene [21] and transfecting different numbers of cells with differing amounts of siRNA and over time. The best transfection efficiency was achieved using 5,000 cells/well in a 96-well plate and 0.1 μ l of DharmaFECT reagent with 40 pmol of siRNA combined with 9.9 μ l serum-free (SF) media after 48 h. For larger transfection volumes, DharmaFECT reagent amounts were scaled accordingly.
2. Antibodies: Normal goat IgG (Santa Cruz; sc-2028) and goat anti-human MET (R&D, AF276) were used at 0.2 μ g/ml for surface MET cell-based ELISA or 10 μ g/ml for flow cytometry analysis. Horseradish peroxidase (HRP)-conjugated bovine anti-goat IgG (Jackson ImmunoResearch Laboratories; 805-035-180) and R-phycoerythrin donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories; 705-116-147) were used as secondary antibodies. For western blot analysis, MET antibody c-28 (Santa Cruz, sc-161) was used at 1:5,000, Ube3C antibody (GeneTex; GTX119102) at 1:10,000, and

β -actin antibody (clone AC-15; Sigma-Aldrich; A5441) at 1:10,000. Appropriate HRP-conjugated secondary antibodies were used at 1:10,000 (Jackson ImmunoResearch Laboratories).

3. 4',6-Diamidino-2-phenylindole (DAPI): Nuclear counterstain DAPI was received as a lyophilized powder from Sigma-Aldrich (cat# D9542) and was reconstituted in H₂O to a stock concentration of 5 mg/ml, aliquoted and stored at -20 °C. For staining, the stock concentration was diluted to the final concentration of 5 μ g/ml in PBS.
4. Horse serum: Donor Equine Serum (HyClone; SH3007403).
5. siRNAs: Ubiquitin siRNA library (QIAGEN cat. No. 1027412; SO 0140368892) and siGenome Non-Targeting siRNA #5 (Thermo Fisher Scientific; D-001210-05-05). Nontargeting and control siRNAs provided in ubiquitin siRNA library: AllStars Negative Control (SI03650318), AllStars Hs Cell Death Control (SI04381048), Negative Control (SI03650325), GFP (SI04380467), Hs_Lamin A/C siRNA (SI03650332), Luciferase GL2 (SI03650353), Luciferase GL3 (SI03650360), Mm_Lmna_5 (SI02655450), MAPK1 (SI03650367), and GAPD_5 (SI02653266). Selected siRNA sequences from ubiquitin siRNA library include:

Hs_PSMD4_5	5'AACCATTACAGCCTAAATAAAA3'
Hs_PSMD4_6	5'TCAACATAGTTTGTTCATTCAA3'
Hs_PSMD4_10	5'AAGAGCAGATTGCTTATGCCA3'
Hs_Ube3C_1	5'CCCATAGAAGTTGTAGGTCAA3'
Hs_Ube3C_2	5'TACGGTGTGGTCACTTATTTA3'
Hs_Ube3C_3	5'CACGAGGATACTCACACTGCA3'

6. Phosphate-buffered saline (PBS): 10 $\times$ PBS stock solution was prepared by dissolving 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ in distilled water and adjusting pH to 7.4 and final volume to 1 l. Stock solution was autoclaved and stored at RT. 1 $\times$ PBS was prepared by diluting 10 $\times$ PBS stock in ultrapure water.
7. Fixative: 4 % paraformaldehyde was prepared fresh for every experiment. 2 g of paraformaldehyde (Sigma-Aldrich; cat# P-6148) was mixed with 50 ml of 1 $\times$ PBS pH 7.4 in 50 ml Falcon tube (Corning) and incubated at 60 °C with frequent mixing. Cells for flow cytometry were fixed in 2 % formaldehyde prepared by diluting 16 % formaldehyde solution (TED PELLA, cat # 18505) in 1 $\times$ PBS pH 7.4.
8. TGH lysis buffer: 50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA pH 8.0, 1 % Triton X-100, and

10 % glycerol were supplemented with 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM PMSF, 1 mM Na_3VO_4 , and 10 mM NaF prior to cell lysis.

9. Protein assay: BCA protein assay kit (Pierce; cat# 23227) was used to determine protein concentration in cell lysates.
10. QuantaRed Enhanced Chemifluorescent HRP Substrate (Thermo Fisher Scientific; cat # 15159): QuantaRed working solution was prepared by mixing QuantaRed Enhancer Solution, QuantaRed Stable Peroxide, and QuantaRed ADHP at 50:50:1 immediately before use. 100 μl of QuantaRed working solution was added to each well and incubated for 15 min at room temperature. The reaction was stopped with 10 μl of QuantaRed Stop solution per well and the plate mixed for 10–30 s. Plates were either immediately analyzed on a microplate reader or stored for later analysis at 4 $^{\circ}\text{C}$ for several days wrapped in foil with minimal effect on fluorescence intensity.
11. Chemiluminescence detection: ChemiGlow West Chemiluminescence Substrate Kit (ProteinSimple; cat # 60-12596-00) was used to visualize positive signals in immunoblots.
12. Instruments: Fluorescence was measured using a multimode microplate reader POLARstar Omega instrument (BMG LABTECH). For flow cytometry assay, BD FACSCantoTM instrument was used. Chemiluminescence was detected using a CCD camera (Alpha Innotech). Plates were routinely spun in a tabletop centrifuge at 2,000 rpm ($\sim 500\times g$, Eppendorf 5416) prior to reading.
13. 96-well plates: Standard tissue culture-treated 10 cm, 5 cm, 6-well, and 96-well plates were used for cell culture work (Corning). siRNAs were aliquoted and pooled in Microseal PCR working plates (Bio-Rad #MSS96011) and plates were sealed with Microseal “B” adhesive seal (Bio-Rad; MSB 1001). Cell transfection, HGF treatment, and MET staining and visualization were performed using solid black 96-well cell culture plates (Costar; cat # 3916). Cell staining and reading for flow cytometry was performed using round-bottom 96-well polypropylene microplates (Corning, cat# 3355).

2.3 siRNA Library: Handling and Storage

1. Ubiquitin siRNA library (0.25 nmol siRNA/well; QIAGEN cat. No. 1027412; SO 0140368892) comprises 129 genes distributed over six 96-well plates (named Ubi01–Ubi06). The library includes four different targeting siRNAs for each of 89 genes or three different targeting siRNAs for 40 genes. Each plate carried siRNAs for 24 genes and eight control siRNAs (Ubi01–Ubi05) with the exception of Ubi06, which carried siRNAs for nine genes and ten control siRNAs.
2. Prior to resuspension, the master siRNA plates were centrifuged at $500\times g$ for 5 min.

3. Each lyophilized siRNA was resuspended by gentle pipetting in 100 μ l of RNase-free water to create a 2.5 μ M stock, covered with new sealing film and left to dissolve for 30 min at 4 °C with occasional shaking or gentle vortexing. All plates were handled in a Class II Biosafety Cabinet without gloves to avoid static forces (*see Note 1*).
4. To avoid contamination of the master plates, two working copies (A and B) of each master plate were prepared by transferring 20 μ l of the resuspended mixture into the new Microseal PCR working plate leaving 60 μ l of the original 2.5 μ M siRNA stock in the master plates. Each master and working plate was covered with sealing film (Microseal B Film; Bio-Rad # MSB1001) and stored either at -20 °C or -80 °C.

2.4 Preparation of the siRNA Pools

1. Gloves should be worn for all subsequent steps unless indicated otherwise. Depending on the number of siRNAs/gene provided by the manufacturer, 3 μ l of each siRNA (4 siRNAs/gene) or 4 μ l of each siRNA (3 siRNAs/gene) was combined into a separate well of a new 96-well Microseal PCR working plate generating a total of 12 μ l siRNA pool per well.
2. 12 μ l of each control siRNA was pipetted into separate wells as required.
3. The resulting experimental siRNA plates were stored at -80 °C until use.

3 Methods

3.1 Reverse Transfection of HeLa Cells with Ubiquitin siRNA Library Using 96-Well Plates

1. Typically, each siRNA pool was evaluated using six separate wells in a 96-well Microseal PCR plate. Accordingly, all amounts and volumes are provided on a per siRNA pool basis. All of the solutions required for cell transfection were prepared in advance as required (*see Note 2*).
2. First, the appropriate experimental siRNA plates including control siRNAs were thawed at 4 °C. The plates were centrifuged at 500 $\times g$ for 5 min prior to opening (*see Notes 3 and 4*).
3. 48 μ l of serum-free DMEM (SF DMEM) was pipetted directly into each well containing test or control siRNAs and incubated for 5 min at room temperature.
4. 0.6 μ l DharmaFECT 2 was diluted with 59.4 μ l SF DMEM (transfection solution) per siRNA pool and incubated for 5 min at room temperature in a tube. 60 μ l of the transfection solution was added to each and incubated for 20 min at 37 °C. 180 μ l of DMEM supplemented with 10 % fetal bovine serum (FBS) was added to the tube (*see Note 5*).

5. 300 μ l of the siRNA/transfection mixture was distributed evenly over six wells (i.e., 50 μ l per well) of a solid black 96-well cell culture plate ensuring to exclude the outermost wells of the plate (*see Note 6*).
6. 50 μ l of the HeLa cell suspension prepared above in Subheading 2.1, item 1 was gently seeded into each well and mixed by pipetting to ensure equal cell distribution of the cells with the siRNA/transfection mixture. The plates were incubated at 37 °C in a CO₂ incubator (*see Note 7*).

3.2 HGF-Induced MET Internalization

1. 24 h following transfection, the cells were serum starved in SF DMEM. To remove media, the plates were flicked with medium force over a sink and 200 μ l of SF DMEM was then added to each well. The media change was repeated once more (*see Note 8*).
2. Following an additional 24 h (48 h following transfection), media were removed and replaced with 100 μ l of SF DMEM or SF DMEM supplemented with 100 ng/ml HGF prewarmed to 37 °C to the appropriate wells.
3. Cells were incubated for 15 min at 37 °C in a water bath.
4. For studies using Dynasore, appropriate wells were pretreated with 50 μ l of prewarmed SF DMEM containing 50 μ M Dynasore for 30 min at 37 °C (*see Note 9*).
5. Media were removed by flicking the plate over a sink, and then 100 μ l of prewarmed SF DMEM or SF DMEM supplemented with 100 ng/ml HGF, with or without 50 μ M Dynasore, was added to the appropriate wells. Plates were incubated at 37 °C for the required time (0–15 min).

3.3 Cell ELISA for MET Internalization

1. All immunostaining steps were performed in the absence of detergent to ensure the measurement of surface-associated receptor. All of the required solutions were prepared in advance.
2. Following HGF treatment, cells were fixed with freshly prepared 200 μ l of 4 % paraformaldehyde in PBS for 10 min at room temperature.
3. Cells were washed with PBS three times for 10 min each at room temperature and then incubated with 100 μ l of 0.2 μ g/ml goat anti-human MET antibody in PBS containing 10 % horse serum or 100 μ l of 0.2 μ g/ml of goat IgG in 10 % PBS containing 10 % horse serum for 1.5 h at room temperature.
4. Cells were washed with PBS 3 $\times$ 10 min at room temperature and then incubated with 100 μ l of secondary HRP-labeled bovine anti-goat IgG in 10 % (v/v) horse serum in PBS for 1 h.
5. Secondary antibody solution was discarded and replaced by 5 μ g/ml DAPI in PBS and incubated for 10 min at room temperature.

6. Cells were washed with PBS three times for 10 min each at room temperature.
7. 100 μ l of QuantaRed working solution was added to each well and the plates incubated at room temperature for 15 min.
8. 10 μ l of QuantaRed Stop solution was added to each well to stop the reaction and fluorescence signals measured using a POLARstar Omega microplated reader. QuantaRed was read at 544/590 and DAPI at 355/460 (top optic reading, ten flashes per well, 1,000 gain).
9. Relative cell number (CN_R) per well was determined as relative fluorescence intensity (RFI_{DAPI}) of DAPI stained cells divided by 10,000 ($CN_R = RFI_{DAPI}/10,000$). The relative fluorescence intensity for each sample (RFI_s) was normalized to the relative cell number/well (CN_R) to generate the RFI for normalized samples ($RFI_{sN} = RFI_s/CN_R$). The mean RFI of IgG only stained cells (RFI_{IgG}) was used as a baseline and after normalization was subtracted from the normalized sample RFI to establish MET fluorescence intensity [$MFI_M = (RFI_s/CN_R) - (RFI_{IgG}/CN_R)$]. To establish the percentage of internalized MET (I_{MET}) per triplicate wells ($MFI_{HGF(1-3)}$), HGF-treated values were subtracted from values of wells not treated with HGF (MFI_{UN}) and the resulting value was subtracted from 100 (e.g., $I_{MET(1)} = 100 - ((MFI_{UN(1)} - MFI_{HGF(1)}) + (MFI_{UN(1)} - MFI_{HGF(2)}) + (MFI_{UN(1)} - MFI_{HGF(3)}))/3$). For plate comparisons, I_{MET} for control sample $I_{MET(NT)}$ was set to 100 % and a conversion constant was determined k_c for each plate ($k_c = 100/(\Sigma I_{MET(NT)}/n)$; n = number of internalization values for NT siRNA). Internalization values of individual samples were then normalized to control sample on each plate ($I_{MET-N} = I_{MET} \times k_c$). All statistical analyses were performed using GraphPad Prism software (v4), using a one- or two-way analysis of variance (ANOVA) with a Bonferroni post hoc analysis to determine statistical significance between groups (*see Note 10*).
10. Each positive hit was validated as a siRNA pool and as individual component siRNAs in minimally two separate experiments. An example of a positive result with the E3 ubiquitin enzyme Ube3C for MET internalization is shown in Fig. 1a. Western blot analysis confirmed knockdown of Ube3C under these conditions (Fig. 1b).

3.4 Target Validation

1. Target validation is a critical component of any screen. As an alternative to cell-based ELISA, we confirmed the inhibitory effect of siRNA-mediated Ube3C knockdown on MET internalization from the cell surface and receptor downregulation using previously established flow cytometry and pulse-chase protocols [12, 22–24].

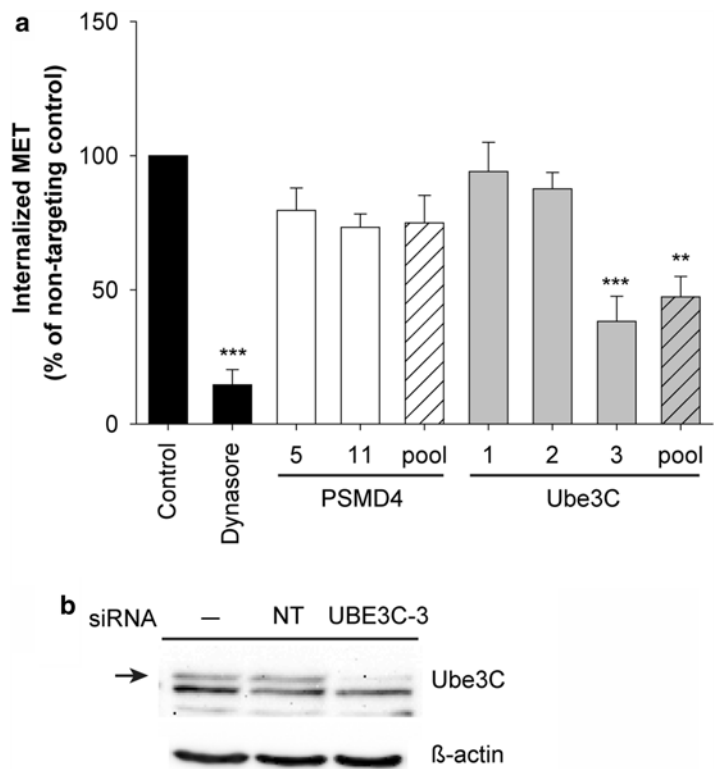


Fig. 1 High-throughput screen identifies Ube3C as a regulator of MET internalization. **(a)** MET internalization was blocked in HGF-treated HeLa cells reverse transfected with the siRNA Ube3C-3 and pooled Ube3C (1–3) siRNAs. The individual Ube3C-1 or Ube3C-2 siRNAs or siRNAs (5, 11, and pooled) directed against the non-ATPase regulatory subunit 4 (PSMD4) of the 26S proteasome had no effect on MET internalization. Control represents internalized MET in cells treated with nontargeting (NT) siRNA, which was set to 100 %. Pretreatment with Dynasore inhibited MET internalization. Values represent mean internalized MET levels $\pm$ SEM from triplicate experiments. **(b)** Western analysis confirmed knock-down of Ube3C in cells transfected with Ube3C-3 siRNA and not in untreated cells or cells treated with a nontargeting (NT) siRNA

2. For internalization studies, the transfection mixture of Ube3C-3 siRNA, NT siRNA, DharmaFECT 2, and 4×10^5 cells in 4 ml of DMEM containing 10 % FBS was left to adhere onto 60-mm plates. After 24 h incubation, the cells were serum starved for 24 h by incubation at 37 °C in DMEM only.
3. The following morning, the cells were incubated in prewarmed DMEM supplemented with 100 ng/ml HGF at 37 °C for 15, 30, or 60 min, respectively, to promote MET internalization. Controls included serum-starved cells that were treated with media lacking ligand.

4. The cells were rapidly placed on ice to halt receptor trafficking and then washed three times with fluorescence-activated cell sorting (FACS) buffer (PBS with 2 % FBS) immediately before use for 5 min each on a shaking platform to remove excess HGF. Cells were detached from the plate surface using 5 mM EDTA in DPBS, centrifuged and resuspended in FACS buffer to the final concentration of 1×10^6 cells/100 μ l.
5. Each sample was divided into three wells on 96-well plate and mixed with 40 μ l of FACS buffer containing 10 μ g/ml of goat anti-HGFR antibody and incubated for 40 min at 4 °C. After washing the cells two times with ice-cold FACS buffer, the cells were incubated with 2.5 μ g/ml R-phycoerythrin donkey anti-goat IgG secondary antibody in FACS buffer containing for 30 min on ice.
6. Following two washes in ice-cold FACS buffer, cells were fixed overnight in 2 % formaldehyde in PBS at 4 °C or at room temperature for 20 min. After three additional washes with FACS buffer, cells were analyzed by flow cytometry using a BD FACSCanto™ instrument and FACS DIVA software version 5.0.1 (BD Biosciences). Typically 20,000 cells are acquired for each time point. To quantify changes in surface MFI, the MFI of the cells at each time point (tx) following ligand treatment was compared to untreated cells (MFI_{unt}) and calculated using the following equation: $MFI = MFI_{tx} - MFI_{unt}$. An example of the results with SE is shown in Fig. 2a.
7. For degradation studies, the transfection mixture of Ube3C-3 siRNA or NT siRNA, DharmaFECT 2, and 1.4×10^5 cells in 2 ml of DMEM containing 10 % FBS was plated in individual wells of a six-well plate and incubated overnight at 37 °C.
8. 24 h later, the transfection mixture was replaced by SF DMEM and the cells were serum starved for an additional 24 h at 37 °C.
9. 48 h following the transfection at ~70 % confluency, cells were pretreated with 1 ml of 10 μ g/ml cycloheximide in SF DMEM for 1 h at 37 °C to inhibit protein synthesis.
10. Internalization and subsequent degradation of MET was initiated by treating HeLa cells with 100 ng/ml HGF and 10 μ g/ml cycloheximide in SF DMEM prewarmed to 37 °C for 0, 1, 2, 3, or 4 h. MET degradation was terminated by placing the plates on ice, washing them with prechilled PBS, and cells were incubated in TGH lysis buffer containing protease inhibitors overnight at 4 °C.
11. Protein lysates (40 μ g of protein per lane) were analyzed by SDS-PAGE and western blot analysis. MET was detected using 40 ng/ml MET c-28 antibody. As a loading control monoclonal antibody recognizing β -actin was used at dilution 1:20,000.

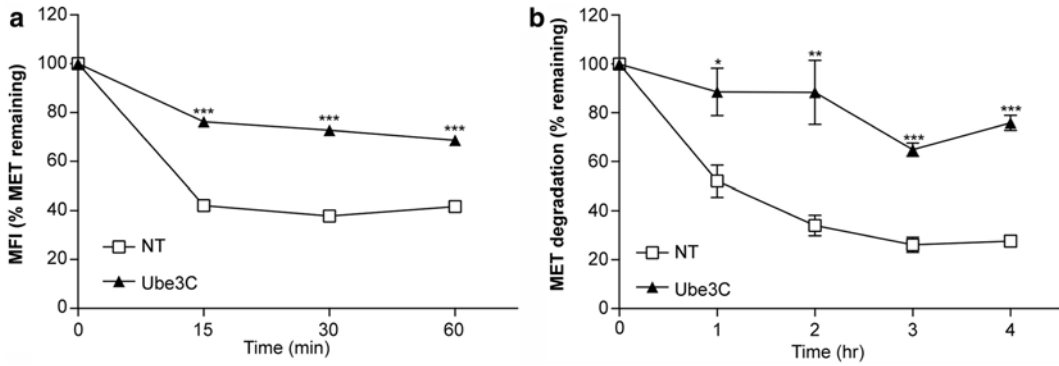


Fig. 2 siRNA-mediated knockdown of Ube3C blocks MET internalization and subsequent degradation. **(a)** HeLa cells were incubated in media containing 100 ng/ml of HGF for 15, 30, or 60 min at 37 °C. The cells were chilled to 4 °C, and MET remaining on the cell surface was specifically labeled with anti-MET antibody using non-permeabilized conditions and quantified by flow cytometry. Results represent the MET fluorescence intensity (MFI) normalized to untreated control cells under each experimental condition. Values represent the means for each data set from duplicate experiments $\pm$ SEM. **(b)** HeLa cells were pretreated in medium containing 10 μ g/ml of cycloheximide (37 °C/30 min) before stimulation with cycloheximide-containing media supplemented with HGF for the indicated times. The resulting cell lysates were examined by immunoblot analysis for total MET (MET) and actin. Band density was quantified using MetaMorph software and MET values were normalized to actin values in the corresponding sample. Mean MET protein levels $\pm$ SEM 2–3 separate experiments are shown

Positive signal was visualized using chemiluminescence and detected on CCD camera (Alpha Innotech). Resulting digitized blots were quantified using MetaMorph software as previously described [11, 12, 16, 23, 24]. An example of the results is shown in Fig. 2b.

4 Notes

1. To aliquot the siRNAs, we routinely used OneTouch tips (GeneMate; cat. # P-4170-303). These tips have a soft, easy-to-seal collar to ensure precise pipetting.
2. We routinely analyzed each siRNA pool using triplicate wells. Six wells are required to enable analysis in the presence or absence of HGF.
3. We routinely include at least two distinct nontargeting siRNAs per plate and one predetermined reference siRNA for blocking MET internalization as internal controls.
4. For unknown reasons, we noted off-target effects with some commercially available control siRNAs on MET cell surface levels, and therefore, precaution has to be taken when selecting

nontargeting siRNA as a reference point. We routinely used the provided siGENOME Non-Targeting siRNA #5 (Thermo Fisher Scientific) as we did not detect off-target effects on MET cell surface levels.

5. The choice of transfection reagents and the amount used for transfection must be empirically determined for each cell line, and one should factor in any possible effects on cell viability and cell surface receptor levels. We found that the most efficient approach to optimize transfection conditions was to use HeLa cells that were transformed to stably express low levels of firefly luciferase. This reporter has very high (6 logs) signal linearity as well as moderate cell turnover with a half-life of 3 h [21]. Most commercial siRNA against firefly luciferase behaved similarly. Using these cells and reagents, amounts of transfection reagent and siRNA could be rapidly evaluated for optimal performance and for peak of protein expression knock-down. We found DharmaFECT 2 (Thermo Fisher Scientific), Lipofectamine 2000 (Invitrogen), and HiPerFect (QIAGEN) to be routinely reproducible in our hands using this assay. Cell density is another critical factor that can affect transfection efficiencies. We have determined that 4,500–5,000 cells/well were optimal in our hands taking into consideration cell growth and spreading, as well as recovery times following transfections.
6. Plates from several manufacturers (Nunc, Corning, Costar, Greiner, BD Falcon all black, and Greiner black with clear bottom) were tested for signal strength and cell adherence during washes. All black plates tested provided the best signal/background ratio for this assay. However, we noted that the peripheral wells of 96-well plates are highly susceptible to temperature fluctuations that can affect the rate of receptor endocytosis. For this reason, we routinely excluded the outer wells in columns 1 and 12 and rows A and H on 96-well plates from the testing.
7. In reverse transfections, the complexes are prepared inside the wells, after which cells and medium are added. Typically reverse transfections are faster to perform than forward transfections and are therefore well suited to high-throughput assays.
8. HeLa cells easily detach from the surface of tissue culture-treated 96-well plates, particularly when media are being exchanged. To minimize cell loss, we routinely inverted the plates with a rapid flicking motion rather than aspirate media, which can dislodge cells. Unless stated otherwise, all medium changes were performed this way to avoid cell detachment. Maintaining the plates on ice promoted cell attachment, particularly when cells were serum starved, but should only be used following internalization incubations.

9. Dynasore is a GTPase inhibitor that targets dynamin-1 and dynamin-2. It acts as a potent inhibitor of endocytic pathways that require dynamin by rapidly blocking coated vesicle formation. MET is endocytosed through clathrin-coated pits, the scission of which requires dynamin GTPase activity. Thus cell treatment with Dynasore served as a positive control for the sensitivity and functionality of receptor internalization in this assay.
10. A high signal-to-background ratio is one the most important confounding values in this type of experiment. Antibody dilutions must be established to obtain the highest signal/background ratio, regardless of the level of the signal value itself. Other factors affecting this ratio can be the type of the plates used, HRP substrate, fixative, and basically design of the experiment itself.

5 Concluding Comments

Unbiased screens represent an objective approach for identifying novel components important for MET trafficking and signaling. The basic protocol here represents a flexible and adaptable approach that can be applied to future screens for identifying new targets for endocytic trafficking of cell surface receptors.

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Large-Scale Analysis of Membrane Transport in Yeast Using Invertase Reporters

Lauren Dalton, Michael Davey, and Elizabeth Conibear

Abstract

Transport of membrane proteins between cellular organelles requires the concerted action of many regulatory factors, which aid in cargo recognition and vesicle formation, targeting, and fusion. The yeast *Saccharomyces cerevisiae* is a useful model system for studying such regulators, due to the availability of genome-wide mutant collections and reporter proteins that provide sensitive biochemical readouts of individual transport pathways. Here, we describe an enzymatic invertase assay for evaluating endocytic recycling using a chimeric GFP-Snc1-Suc2 reporter. Cell surface levels of this reporter can be measured by a colorimetric assay that monitors sucrose hydrolysis at the plasma membrane, using two different methods. The first is a semiquantitative agar overlay assay followed by image densitometry that is suitable for high-throughput screening of arrayed yeast colonies. In the second, more quantitative assay, an enzymatic solution is added to yeast cultures in a multi-well plate and the absorbance is assessed by a plate reader. Furthermore, the modular nature of the chimeric reporter allows alternate transport signals to be introduced, thereby expanding the range of transport pathways that can be evaluated by this method. Together these techniques can be used to explore the function of genes involved in a variety of cellular trafficking pathways.

Key words Yeast, Deletion mutant collection, Phenotypic screening, Vesicle transport, Golgi, Endosome, Endocytosis, Snc1, GSS, Invertase

1 Introduction

Newly synthesized proteins, translocated into the ER, are transported to various membrane-bound organelles, where they perform their specific functions. These transport processes are tightly regulated and require the concerted action of vesicle coat proteins and other regulatory factors [1–3]. When a particular trafficking step is disrupted, proteins requiring that pathway are mislocalized. Thus, we can use protein mislocalization as a method to identify and study regulators of specific trafficking pathways.

Snc1, a membrane-associated protein related to mammalian synaptobrevin, is a well-established model protein used to study endocytic recycling in the yeast *Saccharomyces cerevisiae* [4]. After

synthesis, Snc1 transits the ER and Golgi and travels in secretory vesicles to the cell surface, where it aids in vesicle fusion with the plasma membrane. It is then rapidly internalized and transported back to the Golgi via an early endosomal recycling pathway, to participate in further rounds of transport [5]. Because Snc1 does not visit late endosomes, it can be used to identify genes required specifically for early endosome sorting, in addition to those involved in general endocytosis or exocytosis.

The localization of Snc1 in mutant strains is commonly monitored by green fluorescent protein (GFP) tagging and visualizing by fluorescence microscopy. While this approach has many advantages, phenotypic assays that provide biochemical readouts are typically more sensitive and quantitative and can lead to identification of mutants with relatively weak trafficking defects. Additionally, biochemical assays can be conducted on a large scale and therefore are well suited to screening yeast genome-wide mutant collections. To identify mutants with altered endocytosis and recycling through early endosomes, we created a Snc1-based chimeric reporter that can be used to systematically screen yeast colony arrays using an adapted invertase assay [6]. Biochemical approaches to study additional pathways, including trafficking through late endosomes, have been presented in the previous version of this chapter [7].

The GFP-Snc1-Suc2 (GSS) reporter contains Snc1 fused to GFP on the cytosolic side as well as the invertase enzyme (encoded by the *SUC2* gene) on the extracellular side (*see* Fig. 1). It is integrated into the *SUC2* locus which disrupts endogenous invertase expression [8]. Invertase activity can be measured by a colorimetric assay using membrane-impermeant reagents [8]. Thus, it provides a sensitive measure of the amount of the reporter molecule on the cell surface. Additionally, the modular nature of the construct means that Snc1 transmembrane or cytoplasmic sequences can easily be replaced by sequences encoding different sorting signals to create new chimeric reporters specific for other trafficking pathways.

Here we have provided a detailed methodology for introducing the GSS reporter into desired strains and handling mutant yeast arrays, followed by a description of two assays to quantitatively and qualitatively assess trafficking of the GSS reporter. These assays can be applied to study endocytosis, endosome recycling, as well as secretory pathways.

2 Materials

2.1 Introducing the GSS Reporter into Yeast Strains

2.1.1 Plasmid

pHB4 GSS reporter (Addgene, 53462).

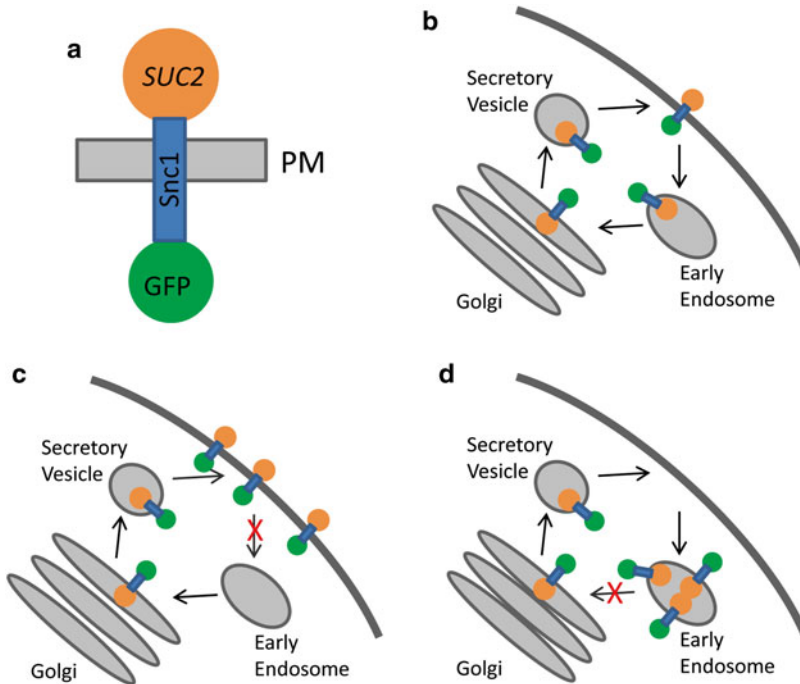


Fig. 1 Trafficking of the GSS reporter: **(a)** Schematic diagram of the GFP-Snc1-Suc2 chimera. Snc1 is tagged N-terminally with GFP which is on the cytosolic side of the membrane and C-terminally with Suc2 which is on the extracellular side of the membrane. **(b)** Trafficking of GSS in wild-type cells. The reporter is synthesized in the Golgi and is trafficked to the plasma membrane. It is then endocytosed and recycled back to the Golgi through an early endosome intermediate. **(c)** Trafficking of GSS in an endocytosis mutant. The reporter accumulates on the plasma membrane since it cannot be internalized. **(d)** Trafficking of GSS in a recycling mutant. The reporter is depleted from the plasma membrane and accumulates in intracellular vesicles. A block in secretion would also cause intracellular accumulation in Golgi or secretory vesicles

2.1.2 Yeast Strains

Many yeast strain backgrounds can be used for this assay including (see **Note 1**):

- BY4741 (GE Dharmacon, YSC1048). Genotype: MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (see **Note 2**).
- BY4742 (GE Dharmacon, YSC1049). Genotype: MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (see **Note 2**).
- Y7092: Starting strain for SGA query strain construction. Genotype: MATα *can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* (see **Note 3**).

2.1.3 Plates

- Nunc OmniTrays (Thermo Scientific, 242811).
- Nunc Petri Plates (Thermo Scientific, 150288): 100 mM round.
- Sealing tape (Sigma-Aldrich, T0197).

2.1.4 Manual Replicators The following items can be purchased from V&P Scientific, Inc. (San Diego, CA):

- 96-floating pin E-clip manual replicator (VP408FH).
- 384-floating pin E-clip manual replicator (VP384F).
- Replicator Templates: LIBRARY COPIER™ (VP381), COLONY COPIER™ (VP380).
- Pin Cleaning Supplies: Pin cleaning brush (VP425), water reservoirs (VP421), and alcohol reservoir with lid (VP420) (*see Note 4*).

2.1.5 Media and Stock Solutions

- ClonNat (WERNER BioAgents, Germany): 200 mg/mL in dH₂O (2,000× working concentration). Filter sterilize and store aliquots at −20 °C.
- G418 (Invitrogen, 118110031): 200 mg/mL in dH₂O (1,000× working concentration). Filter sterilize and store aliquots at −20 °C (*see Note 5*).
- 20 % D-Glucose (Sigma-Aldrich, G7021): 20 % v/v in dH₂O.
- YEP+Agar: Add 10 g yeast extract (Sigma-Aldrich, Y1625), 20 g peptone (Sigma-Aldrich, 70178), and 20 g Bacto agar (BD Difco, 214010) to 900 mL dH₂O in a 2 L flask and autoclave.
- YEPD+ClonNat+G418 OmniTrays: Add 100 mL of sterile 20 % D-glucose to 900 mL freshly autoclaved YEP+agar. Cool to 60 °C, and then add 0.5 mL G418 stock solution for a final concentration of 100 µg/mL G418 and 0.5 mL ClonNat stock solution for a final concentration of 200 µg/mL. Mix thoroughly and pour plates by pipetting 35 mL of media per OmniTray (*see Notes 6 and 7*).
- YEPD+ClonNat Petri Plates: Add 100 mL of sterile 20 % D-glucose to 900 mL freshly autoclaved YEP+agar. Cool to 60 °C, and then add 0.5 mL ClonNat stock solution for a final concentration of 100 µg/mL.
- SD-URA Petri Plates: Add 1.7 g yeast nitrogen base without amino acids (Sigma-Aldrich, Y0626), 5 g ammonium sulfate (Sigma-Aldrich, A4418), 2 g yeast synthetic dropout supplements without uracil (Sigma-Aldrich, 1501), and 400 mL dH₂O in a 2 L flask. In a 1 L flask, weigh 20 g Bacto agar (BD Difco, 214010) and add 500 mL dH₂O. In a 500 mL flask, weigh 20 g D-glucose (Sigma-Aldrich, G7021) and add 100 mL dH₂O. Autoclave all the components. When freshly autoclaved, combine all the ingredients and pour 25 mL per round petri dish.
- 100 % EtOH (Sigma-Aldrich, E7023).

2.2 Invertase Assays

2.2.1 Plate Overlay Assay

- 20 % D-Fructose (Sigma-Aldrich, F3510): 20 % v/v in dH₂O (*see Note 8*).
- YEPF OmniTrays: Add 100 mL of 20 % fructose to 900 mL freshly autoclaved YEP + agar (*see above*), and then cool to 60 °C. Mix thoroughly and pour plates by pipetting 35 mL of media per OmniTray.
- 4 % Bacto Agar (BD Difco, 214010): Make 250 mL of 4 % stock by adding 10 g Bacto agar to 250 mL dH₂O, in a 500 mL bottle, and autoclave.
- Ultra-pure Sucrose (Sigma-Aldrich, S7903): 1.72 g in 50 mL Falcon tubes (BD, 352070). Prepare one tube for every two OmniTrays to be assayed.
- Horseradish Peroxidase (HRP, Sigma-Aldrich, P6782): 1 mg/mL in dH₂O. Filter sterilize before use. Store at 4 °C.
- Glucose Oxidase (Sigma-Aldrich, G7141): 1,000 U/mL in dH₂O. Filter sterilize before use. Store at 4 °C (*see Note 9*).
- N-Ethylmaleimide (NEM, Sigma-Aldrich, E1271): 20 mM NEM in dH₂O. Prepare immediately prior to the assay.
- O-Dianisidine (Sigma-Aldrich, D3252): 10 mg/mL *o*-dianisidine in dH₂O. Prepare immediately prior to the assay.
- 1 M Sodium Acetate (NaOAc; Sigma-Aldrich, W302406): in dH₂O, pH 5.5.

2.2.2 Liquid Invertase Assay

- 96-well microtiter plates (Thermo Scientific, 2401).
- 20 % D-Fructose (Sigma-Aldrich, F3510): 20 % v/v in dH₂O (*see Note 8*).
- YEP Liquid: Add 10 g yeast extract (Sigma-Aldrich, Y1625) and 20 g peptone (Sigma-Aldrich, 70178) to 900 mL water in a 2 L flask and autoclave.
- YEPF Liquid: Add 100 mL of 20 % fructose to 900 mL autoclaved YEP liquid.
- Hydrogen Chloride (HCl, Sigma-Aldrich, 258148): 6 N.
- 0.1 M Sodium Acetate (NaOAc; Sigma-Aldrich, W302406): in dH₂O, pH 4.9.
- Glucose/NaOAc Solution: 10 mM D-glucose (Sigma-Aldrich, G8270) in 0.1 M NaOAc (Sigma-Aldrich, W302406), pH 4.9.
- Ultra-pure Sucrose (Sigma S7903): 0.5 M stock solution in dH₂O. Prepare right before use.
- Triton X-100 (Sigma-Aldrich, X100): 20 % v/v in water.
- Potassium Phosphate Buffer (K₂HPO₄): 0.1 M in dH₂O, pH 7.0. Make by combining 4.68 g/L KHPO₄ (Sigma-Aldrich, P0662) to 16.365 g/L KH₂PO₄ (Sigma-Aldrich, P3786) in dH₂O. Adjust pH as necessary.
- Glucose Oxidase (Sigma-Aldrich, G7141): 1,000 U/mL in dH₂O. Filter sterilize. Store at 4 °C (*see Note 9*).

- Horseradish Peroxidase (HRP, Sigma-Aldrich, P6782) 1 mg/mL in dH₂O. Filter sterilize. Store at 4 °C.
- *N*-Ethylmaleimide (NEM, Sigma-Aldrich, E1271): 20 mM in dH₂O. Prepare NEM immediately prior to the assay.
- *O*-Dianisidine (Sigma-Aldrich, D3252): 10 mg/mL in dH₂O. Prepare immediately prior to the assay.

3 Methods

3.1 Introducing the GSS Reporter and Handling Yeast Arrays

A schematic diagram of the GSS plasmid is shown in Fig. 2. The expression cassette consists of an ADH promoter driving the GFP-Snc1-Suc2 chimera, preceded by the ClonNat^R selectable marker, and flanked by sequences corresponding to 5' and 3' regions of *SUC2*. In order to perform the invertase assay, the GSS reporter is first introduced into strain(s) of interest that do not express endogenous *SUC2*, which would otherwise have background invertase activity. Because GSS is a centromere-based plasmid with both *URA3* and ClonNat^R selectable markers, it can be maintained at single copy in *suc2Δ* strains in media that either lacks uracil or contains Nat. Alternatively, the cassette containing the GSS reporter and ClonNat^R selectable marker can be excised and integrated at the *SUC2* locus by homologous recombination, thus disrupting the expression of wild-type *SUC2*.

An important feature of the plasmid is the presence of unique restriction sites that allow different parts of the chimera to be exchanged by homologous recombination. This makes it easy to create chimeras containing different transport signals or using different promoter sequences to drive expression. For example, we have created reporters in which Snc1's endocytosis signal has been mutated or where the entire Snc1 cytosolic domain has been replaced by that of the t-SNARE protein Sso1, which is internalized by different adaptor proteins [6].

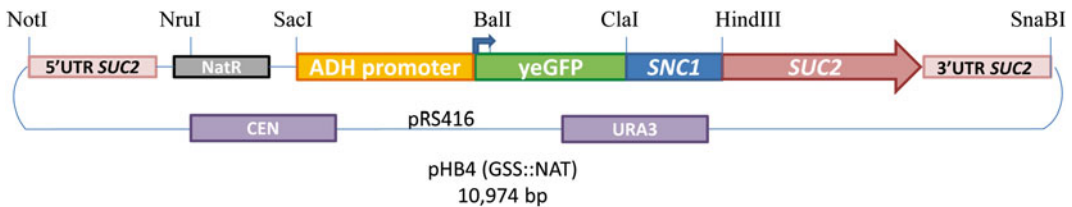


Fig. 2 GSS reporter plasmid map: The GSS reporter plasmid expresses a GFP-Snc1-Suc2 chimera driven by the ADH promoter. Surrounding the chimera are regions of flanking homology to *SUC2*. The chimera and reporter can be excised through a NotI/SnaBI double digest and integrated at the *SUC2* locus. Additionally, unique restriction sites allow the exchange of the promoter and chimera components by homologous recombination. Illustration is not to scale

In the methods described here, we assume that the reader is screening a collection of mutant strains containing the GSS reporter, which are arrayed on agar or in microtiter plates. The array format is advantageous even when working with a small number of strains as it allows multiple replicates to be assayed under identical conditions and provides highly sensitive, reproducible data. Genome-scale yeast mutant collections are commercially available from a variety of sources; the reporter can be introduced to a mutant array by conducting a large-scale genetic cross using a special query strain and synthetic genetic array (SGA) technology. For a detailed description of the SGA methodology, which is beyond the scope of this chapter, we refer the reader to Tong et al. [9]. Below we describe how to introduce the reporter into a single yeast strain, which could then be assayed directly or used as a starting point for introducing the reporter into mutant arrays by SGA.

3.1.1 Introducing the GSS Reporter into Yeast Strains

1. Double digest the GSS plasmid with NotI and SnaBI (*see Note 10*). This releases a 6,100 bp fragment containing the chimera as well as a 4,900 bp fragment corresponding to the backbone vector.
2. Transform 1–3 μ g digested plasmid into desired yeast strains (*see Note 11*).
3. Select for integration of the construct by plating yeast on YEPD + ClonNat (*see Note 12*).
4. Incubate for 2–3 days at 30 °C.
5. Streak resulting transformants to obtain single colonies on YEPD + ClonNat plates.
6. Test isolates for absence of growth on SD-URA plates to eliminate colonies that contain the uncut plasmid.
7. Confirm GSS has been integrated into the *SUC2* locus by colony PCR or sequencing.

3.1.2 Replicating 96- and 384-Density Arrays by Manual Pinning

Once a collection of yeast strains that contains the GSS reporter and mutations of interest is generated, a 96-colony array can be made by patching yeast onto media in an 8 $\times$ 12 grid in a random fashion. This 96-colony array will be the starting point for the assay. For the sake of the protocol, we assume the mutants contain a G418 resistance marker. If not, media containing G418 will have to be changed accordingly.

Yeast arrays should be maintained as frozen stocks in 96-well plates containing YPD +15 % glycerol. Liquid cultures frozen in 96-well plates should be pinned to agar in 96-density format before condensing to make 384 stock arrays (*see Note 13*). To access the array from frozen stocks, the array is pinned from the liquid to a solid agar plate. For ease of handling, the original 96-array plates are condensed fourfold, producing a set of 384-density arrays to give four biological replicates (*see Notes 14 and 15*).

Manual Pinning

1. Prepare a sufficient number of YPD + G418 + ClonNat plates and ensure they are clearly labeled.
2. The 96-pin manual replicator must be sterilized immediately prior to each pinning step to avoid contamination of the arrays. Set up five reservoirs of increasing depths (volumes of reservoirs may vary): three reservoirs of sterile water, one of 10 % bleach, and one of 100 % EtOH (*see* **Note 16**).
3. Place the replicator in each reservoir as follows: 13 mm water reservoir (1 min), 10 % bleach (20 s), 15 mm water reservoir (5 s), and 16 mm water reservoir (5 s). Finally, place the replicator in the 100 % ethanol reservoir (5 s). Allow the excess ethanol to drip from the pins, and then ignite with a flame while holding the replicator in a horizontal position. Allow the replicator to cool before contacting the colonies (*see* **Notes 17 and 18**).
4. Pin by gently lowering the pinhead onto the yeast. Then visit the destination plate and repeat (*see* **Note 19**).
 - (a) For every four 96-density arrays (or visit one plate four times if four replicates are desired) to make a single 384-density array with a sterile 96-pin replicator, use alignment holes A through D on the LIBRARY COPIER™ for successive pinnings.
 - (b) For pinning from liquid, lower the replicator pin head into the bottom of the well and gently agitate to ensure yeast are well mixed.
5. If desired, add appropriate control strains to empty positions of each 384-array plate by hand (*see* **Note 20**).
6. Incubate 384-density array plates for 2 days at 30 °C and store at 4 °C for up to 2 months.
7. Test arrays must be replicated to fresh media and grown for 2 days before use in phenotypic screens (*see* **Note 21**). Replicate the 384-density array onto fresh YPD + G418 + ClonNat plates using a sterile 384-pin replicator. Place the LIBRARY COPIER™ over an open OmniTray with the pair of nine-alignment holes on the front of the frame. Use the middle hole (E) to align the replicator with the yeast colonies.
8. Incubate the plates for 2 days at 30 °C. These 384-density “test” arrays are now ready for use in phenotypic screens.

Freezing an Array

1. Prepare selective yeast medium with 15 % (V/V) glycerol and add 150 µL per well to the desired number of 96-well microtiter plates.

2. Pin yeast from a 96-density solid agar array (as above) with a sterile replicator into 96-well plates with medium containing 15 % sterile glycerol.
3. Incubate 2 days at 30 °C.
4. Freeze at -80 °C.
5. Remove the frozen arrays briefly from freezer and apply sealing tape, and then return to freezer in order to minimize the risk of cross-contamination.

3.2 Invertase Assay

Invertase enzyme, encoded by the *SUC2* gene, hydrolyzes sucrose into glucose. The glucose produced is oxidized by glucose oxidase, which releases hydrogen peroxide as a by-product. Horseradish peroxidase (HRP) utilizes the free peroxide to oxidize the chromogen *o*-dianisidine, resulting in a change in color [8]. These reagents do not penetrate the cell and so the amount of sucrose hydrolysis depends on the amount of the GSS reporter that is located on the surface of the cell. Therefore, mutants that alter the surface levels of the GSS reporter can be detected [6].

To monitor GSS localization, sucrose hydrolysis is both initiated and monitored by the addition of an agar overlay or a liquid solution containing the necessary reagents. Glucose-containing media will give a high background. Therefore, yeast must be grown on media containing an alternative carbon source, such as fructose or galactose. For the agar overlay assay, normal plasma membrane localization results in a honey brown-colored colony, whereas mutants with enhanced localization of GSS to the plasma membrane will be much darker. In contrast, mutants that exhibit abnormal localization of GSS to internal compartments will remain white, indicating a lack of plasma membrane localized GSS (*see* Fig. 3). These plates can be imaged with a digital camera or scanned using a flatbed scanner, and then analyzed by densitometry.

While the invertase overlay assay lends itself to genome-wide analyses of yeast colony arrays, the liquid invertase assay is carried out in multi-well plates using a plate reader and is well suited for a medium-throughput follow-up. In the liquid invertase assay, the normal plasma membrane localization will result in a slightly pink color measured by the absorbance of light at 540 nm. Mutants with enriched plasma membrane GSS will have a higher absorbance value, whereas mutants with decreased plasma membrane GSS will have a smaller absorbance value. To convert absorbance values to glucose released from plasma membrane-bound GSS reporter, a standard curve is made with known glucose concentrations.

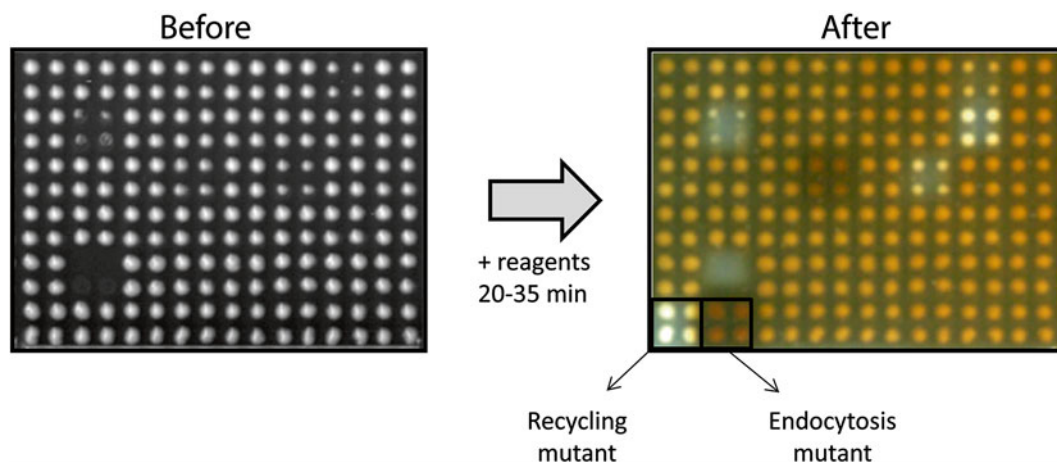


Fig. 3 Invertase overlay assay: a portion of a 1,536-colony array is shown before and after addition of the overlay reagents. Color development proceeds during a 20–35 min incubation period. Wild-type yeast become *honey brown*, while endocytosis mutants turn *darker brown* and recycling mutants remain *white*

3.2.1 Invertase Overlay Assay

1. Pin the array to be assayed onto YEPF OmniTrays (in 1536-density format if possible). Incubate at 30 °C for 24 h (*see* **Notes 21** and **22**).
2. To prevent washing away nearby colonies when pouring the agar overlay, cut a trough in the agar along the narrow side of the OmniTray between the plate rim and the first row of colonies (*see* **Note 23**).
3. Capture an image of the plates prior to performing the invertase assay using an imaging system or a flatbed scanner. This will provide a measure of colony growth (*see* **Note 24**).
4. If using refrigerated reagent stocks, allow them to equilibrate to room temperature before starting the assay.
5. Microwave the 4 % agar until it is near boiling. Add a stir bar and place the hot agar onto a hot plate set to ~200 °C (*see* **Note 25**).
6. Add 26 mL dH₂O to the 50-mL tube containing 1.72 g ultra-pure sucrose. Dissolve the sucrose by inverting the tube.
7. Add 4 mL 1 M NaOAc pH 5.5 and 0.8 mL NEM to the Falcon tube.
8. Add 0.4 mL HRP 1 mg/mL, 0.32 mL 1,000 U/mL glucose oxidase, and 2.4 mL 10 mg/mL *o*-dianisidine and invert twice.
9. Add 7 mL hot 4 % agar to the tube and invert twice. Pipette 20 mL of the reagent and agar mix rapidly into each of two OmniTrays by directing the flow into the middle of the cut trough.
10. Record the start time and allow 20–35 min reaction time before imaging. Leave lids off the plates to avoid heat-related artifacts (*see* **Notes 26** and **27**).
11. Repeat for the remaining arrays.

3.2.2 Liquid Invertase Assay Methods

1. Prepare one or more 96-density yeast arrays comprised of the double-mutant strains from the genome-wide screen.
2. Fill the appropriate numbers of 96-well microtiter plates with 150 μ L YEPF liquid.
3. Using a 96-pin hand pinner or a pinning robot, pin each 96-density array from agar plates to the YEPF containing the 96-well plate (*see* **Note 28**).
4. Incubate the 96-well plate overnight at 30 °C.
5. OD Measurement.
 - (a) Fill another microtiter plate for every plate of samples with 95 μ L YEPF solution.
 - (b) Add 5 μ L overnight culture to the wells containing YEPF.
 - (c) Take the absorbance measure measurement at 600 nm wavelength using a plate reader.
6. Setting up a glucose standard curve.
 - (a) Add 60 μ L 0.1 M NaOAc, pH 4.9 to two rows on the microtiter plate (two replicates).
 - (b) Add 5 μ L glucose/NaOAc solution to the first two wells. Then serially dilute to the next six wells by adding 120 μ L 0.1 M NaOAc to the first well and transferring to the subsequent wells. Discard the 120 μ L at the end.
 - (c) For the last well in the row, add 5 μ L YPF as a blank (*see* **Note 29**).
7. Sample preparation.
 - (a) Fill a microtiter plate for each plate of samples with 55 μ L NaOAc solution (*see* **Note 30**).
 - (b) Add 5 μ L o/n culture to the sample wells containing 55 μ L NaOAc.
8. Prepare the 0.5 M sucrose solution.
9. Prepare glucostat reagent immediately before use (enough for approximately 1 96-well plate): 13 mL K_2HPO_4 0.1 M pH 7.0, 26.7 μ L glucose oxidase, 1,000 U/mL, 33.3 μ L HRP 1 mg/mL, 66.7 μ L NEM 20 mM, and 200 μ L *o*-dianisidine 10 mg/mL.
10. Set timer. Then, add 13 μ L sucrose solution to each row 1–12 staggering 30 s between each row.
11. Incubate 5 min, and then add 100 μ L glucostat reagent.
12. Incubate the plate in glucostat reagent for approximately 2 min (*see* **Note 31**).

13. Add 100 μ L 6 N HCl to each well, row by row from A1 to H12 (again staggering timing one row for every 30 s).
14. The reaction is now stopped and the plate can be read at your leisure.
15. Measure OD540 using a 96-well plate reader.
16. Repeat for the remaining number of plates.

4 Notes

1. Mutant collections including homozygous and heterozygous diploid deletions, MATa haploid deletions, MAT α haploid deletions, temperature-sensitive mutants, and overexpression collections are available from commercial sources including GE Dharmacon (in North America) or Euroscarf (in Europe). These are provided as colony arrays on agar plates (in 96-array format) or as frozen stocks in 96-well plates. Colony arrays on solid agar are less expensive and are more convenient for immediate screening, but should be examined carefully for damage to the agar or cross-contamination of colonies during shipping. Agar-based arrays can be stored at 4 °C. However, for long-term storage, frozen glycerol stocks, stored at -80 °C, are the best. As many as 5 % of the knockout strains may have the incorrect genotype. Screening two or more independent deletion sets reduces error. Genotypes can be confirmed by PCR, and mutant phenotypes can be tested for linkage to the Kan^R marker and/or for complementation by a wild-type copy of the gene.
2. Yeast *suc2* Δ parental strains are available commercially from GE Dharmacon (Mat a, YSC6273-201925792; Mat α , YSC6272-201918995).
3. This strain is described in Tong and Boone [7].
4. Empty pipette box lids can also be used as reservoirs for pin cleaning.
5. G418 is sold by Invitrogen under the name GeneticinTM. Each batch of G418 varies in its activity, which is indicated on the bottle. The amount of G418 powder used to make the stock should be adjusted such that the final solution contains 200 mg/mL of *active* G418. Thawed G418 aliquots can be stored at 4 °C for long periods without noticeable loss of activity.
6. 35–40 mL agar is appropriate for OmniTrays. A thinner layer of agar is more likely to crack during incubation. Ensure plates are poured on a level surface and are completely dry prior to pinning (allow 2–3 days depending on ambient temperature and humidity). If agar is too moist, the pressure

from the pinning (especially using a robot) causes liquid to bead on the agar surface, disturbing colony growth and allowing cross-contamination.

7. Cooling YPD to 60 °C before adding G418 or ClonNat minimizes the possibility of thermal inactivation. 100 µg/mL G418 is preferred for maintaining Kan^R yeast deletion arrays because many Kan^R trafficking mutants show impaired growth at 200 µg/mL G418, the concentration typically used to for Kan^R selection.
8. Other non-glucose carbon sources such as galactose can be used (at final concentration of 2 %).
9. Each batch of glucose oxidase may have differing unit activity/gram.
10. Undigested plasmid results in background colonies that contain the intact plasmid and *SUC2* locus. Therefore, testing isolates to make sure they do not grow on SD-URA is essential because the *URA3* is contained on the backbone plasmid and not in the insert.
11. For a high-efficiency yeast transformation protocol, *see* Gietz and Woods [10].
12. This can be done on 100 mm petri plates, rather than OmniTrays, to reduce cost.
13. Yeast arrays frozen in 96-well plates are typically covered with cryogenic sealing tape that should be removed while plates are still frozen to prevent cross-contamination. Cover plates with lid and allow them to thaw at room temperature (approximately 15 min) before pinning. After pinning, reseal plates with fresh sealing tape and freeze. Yeast can survive several (at least three) freeze-thaw cycles without significant loss of viability.
14. Further condensing the 384 arrays to create 1,536 arrays results in more uniform colony size and reduces variability.
15. For better measurements we typically randomize positions around the plate to account for edge effects or nonuniform distribution of reagents.
16. We use the following depths when making frozen stocks, since the yeast can ride up the pins: 13, 15, and 16 mm for water reservoirs, 14 mm for 10 % bleach reservoir, and 17 mm for 100 % ethanol reservoir. When working with 96-density agar arrays, these depths can be reduced considerably, since the yeast is on the bottom of the pins. Any water reaching the pin support plate would prevent pins from floating freely. Thus it is important to reduce wash station depth where possible.
17. The wash reservoirs are set up to submerge pins in incremental depths in each step. For example, the first wash should sub-

merge the pins to the depth of a standard microtiter plate. The final ethanol wash should submerge the lower half of the pins. This prevents the dirtier initial wash liquid from dripping down the pins during later sterilization steps.

18. Check the pin head to ensure pins are freely floating by tilting the pin head upside down after sterilization and drying, as this ensures reliable and efficient transfer of yeast.
19. Robotic systems suitable for pinning yeast arrays include the Singer Rotor HDA benchtop robot (Singer Instruments, Somerset, UK); the QBot, QpixXT, and MegaPix (Genetix, Boston, MA); and the BM5 benchtop robot (S&P Robotics, Canada).
20. The MAT α and MAT α deletion arrays do not have strains at the H2 and H3 coordinates, respectively. Control strains placed at these positions are useful for quality control and plate normalization. We use one wild-type strain (with Kan^R marker) and two control strains for every experiment (*snx4 Δ* and *rvs167 Δ* mutants are appropriate controls for the GSS invertase assay), leaving the remaining position blank. Each 96-density plate has a unique blank position that is useful for tracking plates and assessing the effectiveness of sterilization procedures.
21. Using fresh source arrays (2 days old) results in more even patch size when constructing 1,536 arrays.
22. Synthetic media can be used, although we find rich media generally gives better discrimination. In order to use synthetic media, you will need 25 mL of overlay reagent because the overlay doesn't flow as effectively.
23. To make the trough, cut a line with a scalpel, insert a razor blade into the cut line, and then apply pressure toward the plate rim to pinch out a narrow (approx. 5 mm) strip of agar.
24. When using a scanner, inverting the plates on the flatbed scanner (i.e., imaging the tops of the colonies) gives the best data. Because this can produce fogging, plates should be cooled to room temperature (approx. 2 h) before scanning.
25. Agar must be boiling slightly or clumping will occur. Clumps of agar displace invertase reagents and can lead to misleading results. However, agar should be watched to prevent boiling over.
26. Leaving lids partially off the plates will create a temperature gradient, leading to uneven enzymatic reaction rates.
27. Many commercial and open-source image analysis programs designed for the analysis of microarray images can be used for densitometry of digital images (e.g., CellProfiler, GenePix, ImageJ).

28. When growing overnight cultures in a 96-well microtiter plate, we add a sterile 5 mm diameter glass bead (Thermo Scientific, 11-312C) to each well and put the plate in a shaking incubator to keep the cells agitated overnight. Do not add the bead before pinning with a robot as this may crash the robot.
29. This will give you 2×8 glucose standards of 33.3, 22.2, 14.8, 9.88, 6.58, 4.39, and 0 mM glucose, respectively (i.e., $2/3$ dilution down the plate).
30. It is best to do one plate at a time for the ease of timing all the steps in sequence.
31. Incubation times may vary as OD_{540} values need to be obtained in the 0.05–0.3 range. Do a test run with a few strains and controls to get the best incubation time.

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Chapter 28

RNAi Screens for Genes Involved in Golgi Glycosylation

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Abstract

RNAi screening has gained popularity in recent years, due to its usefulness in systematic investigations of biological pathways. Combined with high-content screening and advances in imaging and analysis methods, it can enable detailed genetic characterization of cellular processes such as protein glycosylation, a major function of the Golgi apparatus. Glycosylation concerns about one third of all human proteins and regulates various cellular behaviors. Yet the methods available to study it are limited and not easily accessible. In this chapter, we detail a step-by-step method to systematically and quantitatively investigate glycosylation using fluorescent lectin staining, following high-throughput RNAi-based downregulation of gene activities. We also provide a workflow for downstream analysis of the data generated.

Key words RNAi, High-throughput screening, High-content screening, Golgi, Glycosylation, Fluorescence imaging, Image analysis

1 Introduction

With the rapid development of genomic technologies in recent years and the discovery of RNAi as a useful tool in dissecting gene function, high-throughput RNAi screening has seen a soaring increase in use to systematically investigate a whole host of biological pathways [1–3]. Coupled with advances in imaging and analysis methods, a transition to high-content screening (HCS) is also emerging, which provides the screener subcellular information through more detailed and multiparametric data [4, 5]. HCS infrastructure may be costly to establish, both in terms of funding and time, but once in place, there is large potential for gathering extensive, quality data. RNAi screening offers the benefit of interrogating many genes relatively quickly and is applicable to a wide range of cellular processes.

The Golgi apparatus regulates posttranslational protein glycosylation, a modification important in modulating many biological interactions and functions [6]. Unlike DNA and protein, glycan synthesis does not follow a linear template, but involves multiple

enzymes whose activities depend on Golgi compartmentation. Most methods for glycan analysis such as mass spectrometry, nuclear magnetic resonance, and chromatography-based techniques [7], though potentially exhaustive, precise, and quantitative, are rather labor and time intensive and are not easily amenable to high throughput.

Lectins, proteins able to bind specific glycans, have long been used to detect changes in glycan expression [8]. Numerous lectins from various origins, from plants to invertebrates, have been characterized [9]. In addition, glycan-specific antibodies have also been developed. Using fluorescence staining and quantitative intensity analysis, these lectins and antibodies can be used to obtain a rapid readout of multiple glycosylation pathways for a large number of genes. In most cases, it is also possible to combine staining with multiple lectins targeting different glycan structures to obtain a simultaneous measurement of multiple glycans. Some lectins may bind to different glycans in vitro and in vivo, potentially complicating the interpretation of results. However, it is possible to assess the in vivo signal specificity by depleting relevant glycosylation enzymes or sugar transporters. We describe here some examples of lectins that can be used to probe a variety of structures in the mucin-type O-glycosylation and N-glycosylation pathways.

Helix pomatia lectin (HPL), *Vicia villosa* lectin (VVL), and peanut agglutinin (PNA) bind to the early structures in the biosynthetic pathway of mucin-type (O-linked GalNAc) O-glycans. HPL and VVL recognize terminal α -linked O-GalNAc (N-acetylgalactosamine), widely known as the Tn antigen [10, 11]. PNA recognizes terminal core 1 O-glycan, also widely known as the Thomsen–Friedenreich antigen (TF or T), Gal- β 1,3-GalNAc- α 1-Ser/Thr [12]. The Tn and TF antigens are both frequently associated with malignant transformation and their levels correlate with metastatic potential [13]. The TF structure is generated mostly by one enzyme, the core 1 galactosyltransferase (C1GALT1), and its knockdown almost completely abolishes PNA staining. In contrast, multiple enzymes can be involved in generating a specific glycan. The Tn antigen is generated by a family of enzymes called polypeptide N-acetylgalactosaminyltransferase (GALNTs) with overlapping substrate specificities [14]. While over 20 different enzymes have been identified, GALNT1 and 2 are the members that are ubiquitously expressed. We found that concurrent depletion of GALNT1 and 2 practically abolished HPL staining in HeLa cells (Fig. 1a).

For early N-glycan structures, concanavalin A (Con A) binds mainly α -D-mannosyl and α -D-glucosyl groups, having a high affinity for the N-glycan trimannosyl core; thus it reveals high-mannose N-glycans [15]. Mannosyl α 1,3-glycoprotein β 1,2-N-acetylglucosaminyltransferase (MGAT1) transfers the first N-acetyl-D-glucosamine (GlcNAc) residue onto the mannosyl core of N-glycans and is thus essential for the conversion of high-mannose to hybrid and complex N-glycans. MGAT1 knockdown

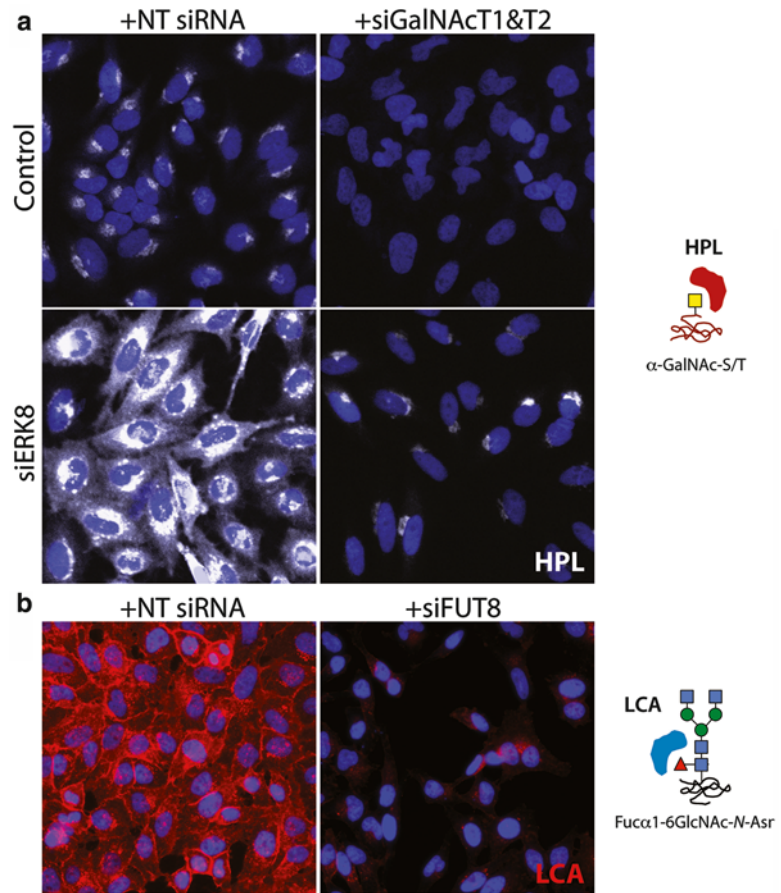


Fig. 1 Examples of lectin staining for *O*-GalNAc glycans and *N*-glycans and verification of lectin specificity by enzyme depletion. *Right*: schematic of the respective glycan structures bound by the indicated lectins. **(a)** HPL staining of the Tn antigen, α -GalNAc-Ser/Thr, in non-targeting siRNA control and GalNAcT1/2-depleted cells. Tn antigen expression is upregulated in ERK8 depletion, and the upregulation is rescued by GalNAcT1/2 co-depletion. Images are reproduced from ref. 27. **(b)** LCA staining of the core fucose, Fuc- α 1,6-GlcNAc-N-Asn, in non-targeting siRNA control and FUT8-depleted cells

results in more high-mannose glycan structures and thus increases Con A staining. *Lens culinaris* agglutinin (LCA) has high affinity for the core fucose on *N*-glycans, Fuc- α 1,6-GlcNAc-N-Asn [15], and knockdown of fucosyltransferase 8 (FUT8), which catalyzes its formation, decreases LCA staining (Fig. 1b). Fucosylation is known to increase in cancer cells and LCA has been used for cancer detection [16].

For the detection of later stage *N*- and *O*-glycans, *Erythrina cristagalli* lectin (ECA) has a preference for the disaccharide galactosyl- β 1,4-N-acetylglucosamine, therefore recognizing extended complex *N*- and *O*-glycans that are not capped by a Sialic acid (Sia or Neu5Ac) at the nonreducing end [17]. Knockdown of

Golgi UDP-GlcNAc transporter (SLC35A3) reduces the availability of UDP-GlcNAc substrate necessary for the addition of GlcNAc to extend both *N*- and *O*-glycans; knockdown of MGAT1 or C1GalT1 inhibits the extension of *N*-glycans or *O*-glycans, respectively, and thus Gal- β 1,4-GlcNAc structures. Thus knockdown of each of the three genes all leads to a reduction in ECA staining.

Phaseolus vulgaris leucoagglutinin (PHA-L) has specificity for tri- and tetra-antennary *N*-glycans, binding preferentially to GlcNAc in a β 1,6 linkage with the trimannosyl core [18, 19]. *Datura stramonium* lectin (DSL) has a relatively broad specificity for poly-LacNAc extended *N*- and *O*-glycans as well as tri- and tetra-antennary *N*-glycans [20–22]. To impair *N*-glycan synthesis, it is possible to co-knockdown STT3A and STT3B, subunits of the oligosaccharyltransferase complex, which initiates *N*-glycosylation by catalyzing the transfer of a lipid-linked high-mannose oligosaccharide to an asparagine residue on nascent polypeptide chains. This depletion inhibits both PHA-L and DSL binding.

Maackia amurensis lectin II (MAL-II) binds sialic acid residues, preferentially *O*-linked glycans containing the trisaccharide Sia- α 2,3-Gal- β 1,3-GalNAc [23]. Sialic acid (Or N-acetylneuraminic acid, Neu5Ac) is the terminal sugar added to glycan chains and cannot be further elongated; thus a decrease in sialylated structures may indicate incomplete glycosylation. Knockdown of synthesizing enzyme ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1) reduces MAL-II staining, as does depletion of the CMP-sialic acid transporter (SLC35A1), which reduces the availability of CMP-sialic acid substrate necessary for the capping. Alternatively, in vitro sialidase treatment, which cleaves off the sialic acid residues, also reduces MAL-II staining. These lectins, their binding specificities, and control genes to verify their specificities are summarized in Table 1.

Limitations to the technique, of course, exist. While the glycan structure recognized by a lectin may be well characterized, the full glycan on which the particular structure is expressed may not be known. What the binding can reveal is a change in synthesis of the particular structure, which could reflect such factors as enzyme expression/activity, substrate availability, enzyme localization, or protein cargo trafficking. Also, due to potential steric hindrance between glycans, which can be relatively large macromolecules, as well as the diversity of glycan structures bearing a lectin-binding motif, lectin binding cannot be considered fully quantitative. It should also be noted that some lectins exhibit cell density-dependent staining [24]. It is thus good practice to test a range of cell numbers to determine this dependency or to use the whole screen dataset to correct for it accordingly (Fig. 3).

Finally, while lectin specificity can be tested as in the above examples given, the researcher should be prepared to carefully

Table 1
Examples of lectins used for probing protein glycosylation

General reactivity	Lectin	Major carbohydrate structural motif	Reference	RNAi control and expected change in lectin binding
D-N-Acetylgalactosaminyl	<i>Helix pomatia</i> (HPA) <i>Vicia villosa</i> (VVL)	α -GalNAc	[10, 11]	• GALNTs, decrease • C1GALT1, increase
α/β -D-Galactosyl	<i>Arachis hypogaea</i> (peanut, PNA)	Gal β 1-3GalNAc	[12]	• C1GALT1, decrease • GCNTs, increase
D-Mannosyl	<i>Canavalia ensiformis</i> (Con A)	Branched N-linked α -D-mannosyl and α -D-glucosyl groups	[15]	• MGAT1, increase
D-Mannosyl	<i>Lens culinaris</i> (LCA)	Fuc α 1-6GlcNAc-N-Asn on N-linked glycans	[15]	• FUT8, decrease
α/β -D-Galactosyl	<i>Erythrina cristagalli</i> (ECA)	Gal β 1-4GlcNAc	[17]	• SLC35A3/C1GALT1/MGAT1, decrease
Complex	<i>Phaseolus vulgaris leucoagglutinating</i> (PHA-L)	N-linked tri-/tetra-antennary	[18, 19]	• STT3A+STT3B, decrease
D-N-Acetylglucosamine	<i>Datura stramonium</i> (DSA)	GlcNAc β 1-4, Gal β 1-4GlcNAc	[20–22]	• STT3A+STT3B, decrease
Sialic acid (Neu5Ac)	<i>Maackia amurensis II</i> (MAL-II)	Neu5Ac α 2-3Gal β 1-3GalNAc	[23]	• ST3GAL1/SLC35A1, decrease

Carbohydrate-binding specificities and suggested RNAi controls

interpret changes, or lack thereof, in lectin binding. For example, *Amaranthus caudatus* lectin (ACL) recognizes core 1 O-glycan, as PNA does, yet has distinct monosaccharide specificities and primary binding ligands [25]. Indeed, we have found that while knockdown of C1GALT1 in HeLa cells does abolish PNA staining, it does not reduce ACL staining, suggesting that this lectin's additional binding activity to other glycans becomes more prominent in the absence of core 1.

As a secondary validation screen, we suggest a double knock-down of candidate hit genes with a relevant glycosylation enzyme

to confirm that the increase in lectin staining is indeed attributable to the suspected glycosylation pathway. For example, knockdown of signaling genes such as ERK8 can result in a dramatic relocation of GALNTs from the Golgi to the endoplasmic reticulum (ER) [26, 27]. This relocation induces a marked increase in cellular Tn levels [27, 28]. A knockdown of GALNT1 and 2 together with ERK8 abrogates the dramatic increase in Tn staining observed with ERK8 depletion alone, confirming that the ERK8-dependent signal is due to GALNT activity (Fig. 1a).

It should be noted that as in all RNAi screens, hit validation to discard or reduce off-target results can involve various previously described methods, including using individual deconvoluted siRNA sequences to the gene, using a completely different siRNA library with different siRNA sequences, using shRNA depletion, or using overexpression of candidate hit genes [29]. Whatever the reagents chosen, the experimental and analysis methods are generally similar to the primary screen. We have thus focused here on validation of glycophenotypes as detected by lectin or antibody staining. Another consideration particular to RNAi screening in general is that knockdown penetrance and efficiency varies with siRNA sequence and many experimental factors; thus there will likely always be a fraction of false-negatives present in a screen.

To summarize, we provide a simple but robust and informative method using high-throughput RNAi downregulation of gene activities in 384-well plates to systematically and quantitatively investigate glycosylation using fluorescent lectin staining (Fig. 2). We begin with 384-well plates preprinted with siRNA libraries. Transfection complexes are made by adding transfection reagent to these siRNAs, after which the cells to be transfected are added and assayed 3–5 days later. In this case, the cells are fixed, stained, and imaged. An automated HCS imaging microscope is employed to ensure good and consistent image quality. We also provide a workflow for downstream analysis of the image data generated (Fig. 3). For secondary validation, we suggest a double knockdown of each candidate hit gene with the relevant glycosylation enzyme necessary for synthesis of the glycan structure being probed. This protocol can be adapted for other non-lectin screening projects. As is the case for a major experimental campaign, there are many variations possible on both the experimental and analysis methods. A simple and typical workflow is suggested in our protocol, with some variations mentioned in the Notes; others can further be explored in the References. Downstream analysis of the hits usually involves bioinformatics (e.g., protein–protein interactions, gene ontology analysis) to make conclusions or form further questions that are biologically meaningful. This is beyond the scope of this chapter and will not be covered here.

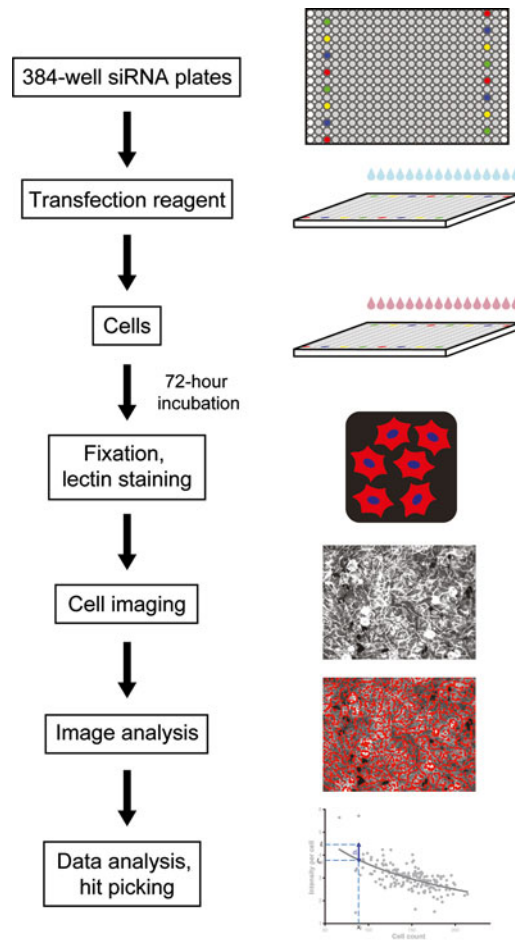


Fig. 2 High-content screening workflow. Microplates containing pre-seeded siRNAs are thawed. Transfection reagent is added and the plate incubated to allow transfection complex formation. Cells are then added and incubated for 72 h before fixation and staining with fluorescent lectins. The cells are then imaged on a high-throughput microscope, the images quantified, and the data analyzed (see Fig. 3)

2 Materials

Buffers and Reagents Triton-X

- HiPerFect transfection reagent (Qiagen).
- Opti-MEM I reduced serum medium (Life Technologies).
- DMEM, high glucose, pyruvate (Life Technologies).
- Phosphate-buffered saline (PBS).
- Trypsin.
- Fetal bovine serum (FBS) (Life Technologies).

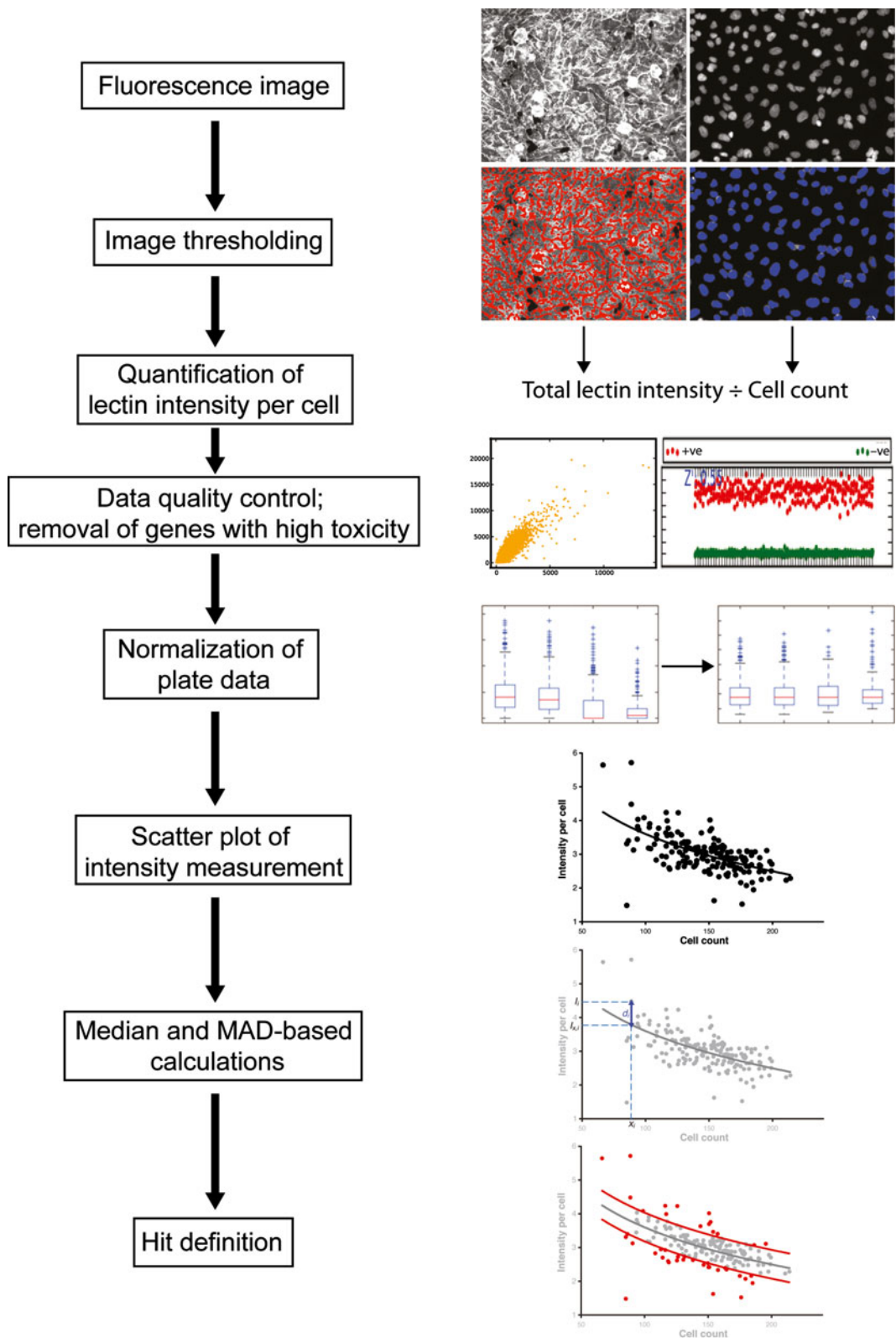


Fig. 3 Data analysis workflow. The number of cells in the image is determined by counting nuclei. In the lectin stain channel, a threshold is set using control fluorescent images such that cells but not background are covered

- Paraformaldehyde (PFA) (Sigma).
- Trypsin.
- Hoechst 33342 (Life Technologies).
- Fluorescent lectins (Vector Laboratories).

Equipment and Software

- CO₂ incubators for cell culture.
- Cell counter.
- 384-well cell culture microplate, black-walled, optical-quality clear bottom, with lid (e.g., Greiner Bio-One).
- Multi-well reagent dispenser (e.g., Multidrop Combi, Thermo Scientific).
- Multichannel electronic pipette (e.g., Biohit, Sartorius).
- Automated high-content screening microscope (e.g., ImageXpress Ultra, Molecular Devices).
- High-content image analysis software (e.g., MetaXpress, Molecular Devices).
- Data analysis and curve-fitting software (e.g., Prism, SigmaStat, MS Excel).

3 Methods

Apart from cell incubation, carry out all procedures at room temperature.

3.1 Assay Design and Optimization

1. Design the assay (*see Note 1*): decide on the fluorescent lectin reporters (*see Note 2*), cell line(s) (*see Note 3*), and RNAi library (*see Note 4*) to be used, and select multiple potential positive and negative controls for testing (*see Note 5*).
2. Using the same type of plates that the siRNA libraries are prepared in, optimize the siRNA transfection over a range of experimental factors: (a) cell density (we recommend testing in the range of 1,000–5,000 cells/well; also *see Note 6*), (b) type and (c) volume of transfection reagent (*see Note 7*), and (d)

←
Fig. 3 (continued) by the image mask. The total intensity of masked pixels is measured and divided by the number of nuclei to obtain the average intensity per cell of the image. After such analysis for all genes, data quality is assessed, overly toxic gene knockdowns or bad wells removed, and data normalized by plate if necessary. The intensity per cell (normalized or raw) is then plotted against the cell count for all genes to determine whether the lectin has cell density dependence. If so, it is fitted to a curve to obtain the expected intensity per cell for no siRNA knockdown, the median absolute deviation from the curve is determined, and a hit threshold chosen to define hit genes. If there is no cell density dependence, the median and median absolute deviation of lectin intensity per cell are determined and a hit threshold chosen to define hit genes

period of incubation after transfection (typically 3–5 days). Evaluate the transfection efficiency (such as the percentage of cells transfected with fluorescent siRNA, percent of cells with fluorescent reporter knocked down, or percent of cells with expected phenotype), cell toxicity (by staining and counting cells or a metabolic or redox assay kit such as CellTiter-Glo or WST-1/MTT), and dynamic range of the assay—good separation between positive and negative controls (*see* **Note 8**).

3. Once conditions are optimized, validate the complete protocol on at least one whole plate, including controls in the assigned wells, to assess the signal-to-noise ratio, as well as test for any potential artifacts introduced during the experimental procedure (*see* **Note 9**).
4. Acquire images of the plate using the same automated HCS microscope to be used for the screen. Decide on the objective and number of fields of view to be taken per well, to obtain a sufficient number of cells per knockdown condition. Optimize acquisition settings to obtain a good dynamic range, yet allow detection of perturbations, e.g., if an increase in signal is expected in target hit genes, ensure that the acquired images for the controls are not too near saturation.
5. Test the image analysis software to be used, which can be chosen from either open source (e.g., CellProfiler [30]) or commercial sources (e.g., Molecular Devices) (*see* **Note 10**). Follow this simple image analysis pipeline: measure the total lectin intensity of the field of view, count the number of cells using nuclei, and divide to get lectin intensity per cell. Software such as ScreenSifter [31], cellHTS [32], or MS Excel can be employed to visualize readouts by plate. Image analysis software themselves may also include the capability to display plate heatmaps (e.g., MetaXpress, CellProfiler).
6. (Optional but recommended) To optimize handling procedures, perform a “dry” run with substitute reagents (e.g., PBS) with the same number of plates planned for the actual screen. We recommend processing at most about 8–10 plates per batch.

3.2 Cell Seeding, Fixing, and Staining

1. Thaw previously prepared siRNA plates in the cell culture hood for 30 min.
2. Spin the plates down at 200×g for 1–2 min, before removing the plate seals.
3. Pipette control siRNAs into the desired wells (*see* **Note 11**).
4. Dilute HiPerFect transfection reagent into Opti-MEM and incubate for 5 min (*see* **Note 12**). Use a liquid dispenser such as the Multidrop Combi or a semiautomated pipette to dispense the transfection reagent uniformly into the plate wells.
5. Incubate the plates for 20 min at 200 rpm on an orbital shaker to allow transfection complex formation.

6. Trypsinize cells into a single-cell suspension and count (*see* **Notes 3** and **6**). Dilute an appropriate amount into the desired concentration (*see* **Note 12**) in a suitable sterile plastic container (e.g., 50 ml conical or a T75 tissue culture flask). Again, use a liquid dispenser such as the Multidrop Combi or a semiautomated pipette to dispense the cells uniformly into the plate wells.
7. Allow cells to settle in plates for 5 min. then visually check the cell seeding. Transfer plates to incubators and leave for the desired time period, without opening incubator doors unnecessarily so as to avoid evaporation and edge effects. Do not stack too many plates together; we recommend stacking at most two plates.
8. Remove plates from incubators and fix with 4 % PFA for 10 min, again using a liquid handler or semiautomated pipette. Wash with PBS, permeabilize with 0.2 % Triton for 10 min if required (*see* **Note 13**), wash again with PBS, stain with 5–20 $\mu\text{g}/\text{ml}$ lectin and 1 $\mu\text{g}/\text{ml}$ Hoechst 33342 diluted in 2 % FBS-PBS for 1–2 h, and wash 3 times with PBS for 5 min each. Removal of liquid in wells can be done by simply flicking the plates and then tapping them on paper towels, by aspiration with a multichannel adaptor, or with an automated liquid handler with aspirating functionality (e.g., BioTek plate washer). Store plates at 4 °C if not acquiring images immediately. It is best to acquire plates within a week after staining. Otherwise, re-stain the plate before acquisition to ensure good fluorescence signal.

3.3 Image Acquisition and Data Analysis

1. Image the stained cells on an automated HCS microscope. Before imaging the whole plate, test the previously optimized imaging settings on control wells.
2. Using the image analysis software of choice, measure the average lectin intensity per cell for all plates.
3. Export the data into a data analysis program with curve-fitting capability and assess data quality by:
 - (a) Calculating the *Z*-factor, given by $1 - 3(\sigma_p + \sigma_n)/|\mu_p + \mu_n|$, where σ and μ represent respectively the standard deviations and means of the positive ($_p$) and negative ($_n$) controls (*see* **Note 8**).
 - (b) Obtaining the correlation coefficient between replicates.
 - (c) Assessing the stability of control signals across different plates. If necessary, data can be normalized within plates to their respective plate controls to render data comparable between plates.
4. Eliminate highly toxic siRNA treatments by removing wells with too low cell counts (e.g., 20 % of the negative control).

5. Now determine a primary hit list of genes: for each lectin, combine data from all plates and plot lectin intensity per cell against cell count (*see* **Notes 14** and **15**).

If there is a dependent relationship between the two parameters, as occurs for many lectins:

- (a) Fit a curve to the plot and obtain the fitted equation (*see* **Note 16**).
- (b) For each data point (gene), calculate the absolute deviation (d_i , *see* Fig. 3) of the experimental y value from the fitted y value (determined by fit equation) for the same cell count (x_i). Then determine the median absolute deviation among all genes (*see* **Note 17**).
- (c) Decide on the cutoff value to define hit genes, such as fitted y value $\pm k$ median absolute deviation. A gene is then considered a hit if it has a value greater than fitted y value $+ k$ median absolute deviation or smaller than fitted y value $- k$ median absolute deviation (*see* **Note 18**).

If there is no dependent relationship between the two parameters:

- (a) Determine the median and the median absolute deviation of all genes (*see* **Note 17**).
- (b) Decide on the cutoff value to define hit genes, such as median $\pm k$ median absolute deviation. A gene is then considered a hit if it has a value greater than median $+ k$ median absolute deviation or smaller than median $- k$ median absolute deviation (*see* **Note 18**).

3.4 Secondary Assay: Double Knockdown for Lectin Signal Validation

1. Before beginning, test the double-knockdown protocol on a small scale and optimize if necessary. As a starting point, use an equal amount of glycosylation enzyme-targeting siRNA as the amount of hit gene siRNA and the same amount of HiPerFect transfection reagent as in the primary screen (*see* **Note 19**).
2. Thaw plates containing siRNAs targeting candidate hit genes in the cell culture hood for 30 min. Use duplicates of each plate.
3. *See* **step 2** of Subheading 3.2.
4. *See* **step 3** of Subheading 3.2.
5. In one replicate, dilute the siRNA targeting the relevant glycosylation enzyme into Opti-MEM such that the amount of siRNA dispensed is equal to that of the candidate hit gene siRNA in the well. The volume dispensed here should be about half that in **step 4** of Subheading 3.2. In the other replicate, use non-targeting siRNA instead.
6. *See* **step 4** of Subheading 3.2. The volume dispensed here, together with that in the previous step, should add up to the volume in **step 4** of Subheading 3.2.
7. *See* **steps 5–8** of Subheading 3.2.

8. Image and quantify the cell staining as in **steps 1–3** of Subheading 3.4. Compare the average lectin intensity per cell for each candidate hit gene with and without co-knockdown with the glycosylation enzyme, and determine the extent of rescue of lectin staining for each gene.

4 Notes

1. Design the assay as simple as possible (but not simpler), to answer a well-defined question. This may seem a trivial point but careful consideration of the assay readout and what the results may mean in the context of the biological question(s) will greatly aid experimental success as well as downstream analysis for high-content assays.
2. Factors to consider in choosing the reporters include the stability and sensitivity, specificity (some lectins are known to have wide binding specificities; choose those that show relatively specific binding and behave predictably under control conditions such as knockdown of a synthesizing enzyme), and costs (e.g., a stable fluorescent protein (FP)-expressing cell line is much more affordable and requires less post-transfection sample processing than antibody staining; plant lectins also cost less than antibodies).
3. Apart from its biological relevance to the question being studied, a key requirement for the cell line is that it is easily transfectable. It is also important that the cells are non-clumping for consistent cell counting and transfection (*see* also **Note 5**) and are stable for multiple passages if it is expressing a reporter. It is highly recommended to grow a large amount of healthy cells and freeze multiple tubes of cells, preferably in log phase of growth, at the same time.
4. RNAi libraries are available from different manufacturers and can be arrayed in multi-well plates as pools of distinct siRNA for each target, or individually. They can be obtained in quantities sufficient for making many assay plates or supplied as ready-to-transfect formats by some manufacturers. For the former, preparing the assay plates from the stock plate ideally requires a liquid handling robot. The choice of library for screening depends on the aim and scope of the screening project.
5. The importance of a positive control for quality data cannot be overstated: it is extremely useful for giving an indication of assay performance (the dynamic range and reproducibility) and also as a standard for image and data analysis (for use in optimization of image acquisition and analysis, data quality control, normalization, and quantitative assessment of hits). Ideally, for best comparability to test genes, the positive control chosen is a biological control that behaves as an

expected hit and is also RNAi mediated. This further helps in assessing transfection efficiency. Negative controls should be non-targeting, have little cytotoxicity and result in no change in phenotype from untransfected cells. There should be a sufficient number of control wells per plate for statistical analysis.

6. Another critically important experimental factor is the cell counting, as it impacts both transfection efficiency and cell behavior. Be scrupulously careful when counting cells. Perform replicates, and use the same counting method once established. Also try to count cells within a suitably optimal range of cell density, because different cell counters may have different optimal working ranges.
7. To minimize data variability, pick a transfection reagent whose performance is stable over time and across batches, if possible. It is best practice to test and then purchase a large batch of the same lot number of reagent.
8. A commonly used metric for assessing the quality of a high-throughput assay is the Z' -factor, given by $1 - 3(\sigma_p + \sigma_n)/|\mu_p - \mu_n|$, where σ and μ represent respectively the standard deviations and means of the positive ($_p$) and negative ($_n$) controls. It gives a measure of the variability and dynamic range between the positive and negative controls. A Z' -factor showing very good separation of controls should be between 0.5 and 1; however, for siRNA screens, a Z' -factor above 0 may be acceptable [33].
9. Common plate position artifacts that may be encountered include edge effects due to evaporation from assay plates and row and column artifacts due to liquid handling steps and/or chemical stability. These can be minimized by dedicating incubators for the screen (avoiding constant opening and closing of doors to maintain a consistent environment) small volumes of liquid, and careful handling of reagents (e.g., not leaving light-sensitive reagents exposed to light while in liquid dispenser tubing).
10. Cellular heterogeneity may require removal of artifacts wherever possible, such as out-of-focus fields of view, apoptotic and mitotic cells, or cells on the border of the image. Alternatively, averaging of a large number of cells may dilute and negate the effect of the artifacts.
11. Controls should be systematically distributed on the plate, to minimize skewing of readout due to plate positional artifacts.
12. Excess transfection reagent mix/cell suspension should be prepared, to account for both the liquid handler dead volume and general liquid loss during transfer. The amount of excess is best determined empirically during the optimization stage.

13. If the aim is to probe total cellular glycans, permeabilize the cells. If only cell surface glycans are to be probed, omit the permeabilization step.
14. Plotting other combinations of parameters can similarly give the screener an idea of any unexpected data dependencies influencing result interpretation and allow the opportunity to correct for them if necessary.
15. We recommend pooling the data from all plates (normalized if necessary) instead of obtaining the *z*-scores by plate, because the non-random organization of some libraries may cause large fluctuations of means across plates.
16. This method works well with a large screening set such as the whole genome since most genes are assumed to have little to no effect on the readout and offer a reliable measure of the expected unperturbed state.
17. The use of median absolute deviation instead of mean absolute deviation is increasingly encouraged for RNAi screens, as it is much more robust to outliers and is expected to result in fewer false-negatives [33, 34]. It may be especially useful for smaller screening sets, in which it may not be safe to assume that most genes have no effect on the measurement.
18. As a starting point, a suggested value for *k* is 3 [34].
19. A few hit genes from the primary screen can be tested here—in co-depletion with non-targeting or glycosylation enzyme-targeting siRNA, for use as potential controls. Otherwise, non-targeting and glycosylation enzyme-targeting siRNAs can be used as negative and positive controls, respectively, at the same total final concentration as the test wells.

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Proteomic Analyses of a Bi-Lobed Structure in *Trypanosoma brucei*

Ladan Gheiratmand and Cynthia Y. He

Abstract

The position and function of the Golgi apparatus are tightly coupled with the microtubule-organizing centers (MTOCs) that play important roles in cell growth and polarity. In the unicellular parasite *Trypanosoma brucei*, the single Golgi apparatus is adjacent to a novel, bilobed structure located at the proximal base of the flagellum, near to the basal body that nucleates the flagellar axoneme. The duplication and segregation of the bilobed structure are tightly coupled to the duplication and segregation of Golgi, ER exit site, basal bodies, and flagellum, suggesting a role of this unique structure in the precise positioning, biogenesis, and inheritance of these single-copied structures during the cell cycle of procyclic *T. brucei*. Here, we describe in details two different isolation and proteomic methods to characterize the protein components present on or associated with the bilobed structure, which allow further understanding of its function and association with other membranous and cytoskeletal organelles in the parasite.

Key words Immune isolation, Proteomics, *Trypanosoma brucei*, Bilobe, Flagellum

1 Introduction

The unicellular parasite *Trypanosoma brucei* contains a single Golgi stack, suitable for studies aiming to understand Golgi biogenesis and inheritance during the cell cycle [1]. The single Golgi apparatus is in close association to a bilobed structure located at the base of the single flagellum (Fig. 1). Two centrin proteins, TbCentrin2 and TbCentrin4, have been found present on the bilobe [2–4]; and *T. brucei* Plk1 is found transiently associated with the bilobe [5]. Functional analyses of the centrins and Plk1 suggest a possible regulatory role of the bilobe in the coordinated duplication and segregation of several single-copied organelles, including the Golgi, at least in the procyclic stage cells (a proliferative form residing in the insect vector). As a cytoskeletal component, the bilobe may provide an anchor to the Golgi apparatus, thus facilitating its accurate positioning, duplication, and segregation relative to other organelles.

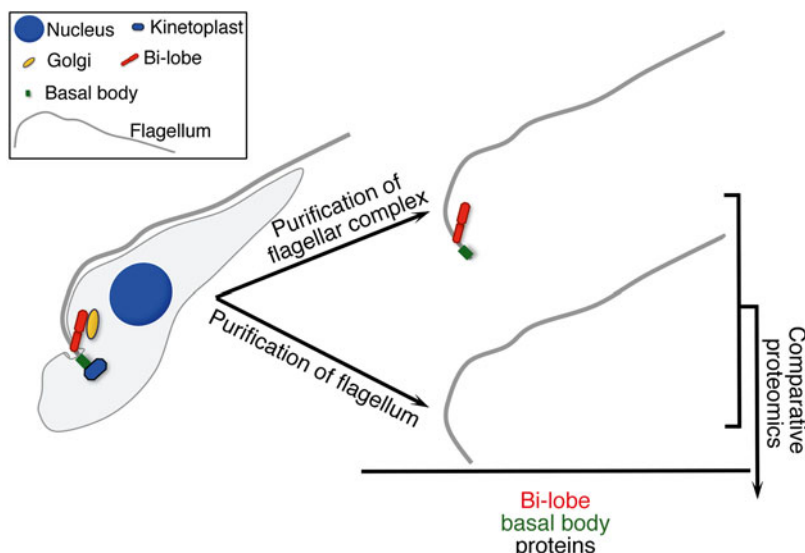


Fig. 1 A comparative proteomic approach to identify new bilobe proteins. A schematic presentation of the comparative proteomic approach. Each parasite cell contains a single copy each of the nucleus, the kinetoplast (condensed mitochondrial DNA), the basal bodies that seed the single flagellum, and the bilobe and the Golgi that are located near the base of the flagellum. Using different purification protocols, samples containing flagellum only, or flagellum in complex with basal bodies and bilobe, were prepared for comparative proteomic analyses by iTRAQ. Proteins that were found more abundant in the flagellar complex were deduced to be basal bodies or bilobe proteins, which were further verified and characterized experimentally

Apart from the centrins and Plk1, little was known about the bilobe composition and functions. Biochemical purification of the bilobe has been difficult, partly due to its extensive association with other cytoskeletal components [6]. We have therefore adopted two different isolation and proteomic methods that allowed identification of new bilobe and bilobe-associated proteins. In the first approach (Fig. 1), different protocols were used to purify *T. brucei* flagellum only or flagellum in complex with basal bodies and bilobe. Samples prepared using different protocols were then subjected to comparative proteomic analyses by the isobaric tag for relative and absolute quantitation (iTRAQ) method, which allowed identification of a new bilobe protein TbLRRP1 [7]. In the other approach (Fig. 2), YFP-TbLRRP1-labeled bilobe was immune isolated for proteomic analyses, allowing further identification of proteins and structures, membranous or cytoskeletal, associated with the bilobe [8]. Proteomic analyses, in combination with various cell fractionation methods, are powerful approaches toward understanding of organelle compositions and functions.

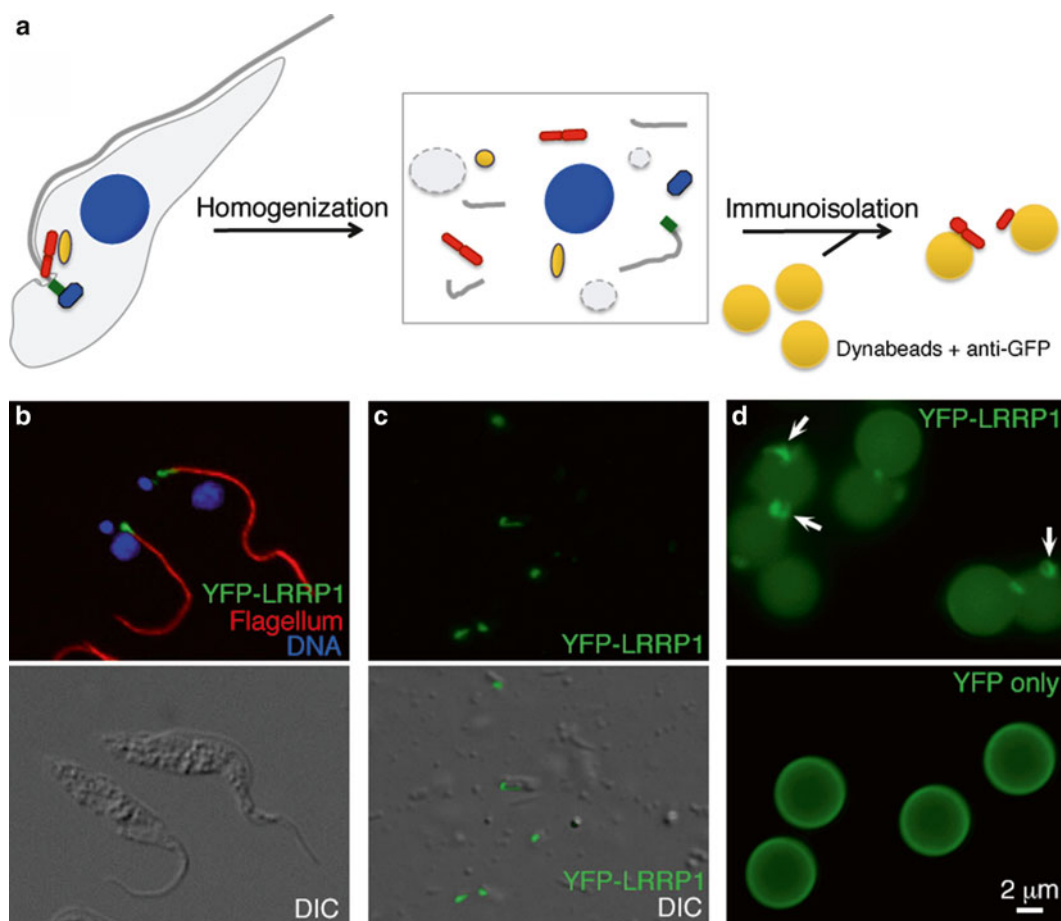


Fig. 2 Immune isolation of the bilobe. (a) A schematic presentation of the immune isolation procedure (b–d). YFP-TbLRRP1 (green) marks the bilobed structure at the proximal base of the flagellum (red) in between the nucleus and the kinetoplast (blue) (b). YFP-TbLRRP1 cells were sonicated in an isotonic buffer to release intracellular organelles (c). Cell lysates were then incubated with Dynabeads that were coated with anti-GFP. Cells expressing YFP only were used as negative control. Dynabeads incubated with YFP-TbLRRP1 (top panel) or YFP-only (bottom) cell lysates were visualized by fluorescence microscopy (d). YFP-labeled structure similar to the bilobe could be found bound to the beads (arrows, d)

2 Materials

All solutions were prepared using Milli-Q water. All reagents were kept refrigerated unless otherwise mentioned.

1. Homogenization buffer H (pH 7.4): 250 mM sucrose, 25 mM KCl, 50 mM HEPES-KOH, 4 mM MgCl₂ (or 5 mM MgSO₄).
2. Buffer A: 320 mM sucrose, 25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.2 mM EDTA, 12 mM beta-mercaptoethanol (see Note 1).

3. Protease inhibitor cocktails (PIs): Dissolve one complete protease inhibitor cocktail tablet (Roche) in 50 ml buffer where mentioned in the text. Prepare the PIs freshly when needed. For phenylmethanesulfonylfluoride (PMSF), prepare a 1 M stock solution in tissue culture grade DMSO and store in 200 μ l aliquots at -20°C . Use PMSF at the final concentration of 1 μM .
4. Sucrose gradients: To start, prepare a 2 M sucrose stock solution by dissolving 34.225 g ultrapure sucrose in ~ 30 ml of buffer A. Adjust the final volume with buffer A to 50 ml. Continue making the 1.84, 1.66, and 1.5 M sucrose solutions by diluting the 2 M stock solution with buffer A. For example, to prepare 10 ml of 1.84 M sucrose, dilute 9.2 ml 2 M sucrose solution with 0.8 ml buffer A. Mix well before use.
5. PBS (phosphate-buffered saline): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 . Adjust pH to 7.4 with HCl and sterilize by autoclaving.
6. PEM buffer: 100 mM PIPES (pH 6.9), 1 mM EGTA, 1 mM MgSO_4 , 0.1 mM CaCl_2 . Prepare 30 ml PEM buffer freshly before use by mixing 10 ml 300 mM PIPES stock solution (*see Note 2*), 60 μ l 0.5 M EGTA (pH 8.0) stock, 30 μ l 1 M MgSO_4 stock, and 3 μ l 1 M CaCl_2 stock solution. Adjust the final volume to 30 ml with Milli-Q H_2O . PEM supplemented with 1 % Nonidet P40 (NP40) and/or 1 M KCl was used to extract the cells. To prepare these, 3 ml 10 % NP40 stock and/or 15 ml 2 M KCl stock was included in the above PEM buffer, before adjusting the final volume to 30 ml with Milli-Q H_2O .
7. Dissolution buffer: 0.5 M triethylammonium bicarbonate (TEAB) and 1 % SDS.
8. Reducing reagent: 50 mM tris-(2-carboxyethyl) phosphine.
9. Cysteine blocking reagent: 200 mM methyl methanethiosulfonate in isopropanol.

3 Methods

3.1 A Comparative Proteomic Approach to Identify Bilobe Components

All the procedures were performed at 4°C unless otherwise indicated.

3.1.1 Purification of *T. brucei* Flagellar Complex

The flagellum and associated cytoskeletal structures including the basal bodies and the bilobe were prepared using a two-step extraction method as previously described [9]. These highly stringent extraction conditions allow the core cytoskeletal elements of flagellum and associated structures to be enriched from other cellular components.

1. Procyclic *T. brucei* cells (*see Note 3*) are cultivated in Cunningham's medium supplemented with 15 % heat-inactivated fetal calf serum at 28 °C, to near stationary phase ($\sim 1.0 \times 10^7$ /ml). A 300 ml cell culture ($\sim 3 \times 10^9$ cells) is harvested by centrifugation at $2,000 \times g$ for 10 min, in a tabletop centrifuge (Sorvall Legend RT+, Thermo Scientific with swinging buckets).
2. Wash the cells twice with 10 ml PEM buffer.
3. Resuspend the cells in 10 ml PEM buffer containing 1 % NP40 and protease inhibitors. Place on a rotation wheel to facilitate cell extraction for 15 min.
4. Centrifuge at $3,000 \times g$ for 10 min at 4 °C.
5. Repeat the extraction steps once with 10 ml PEM containing 1 % NP40 and twice with 10 ml PEM containing 1 % NP40 and 1 M KCl. Occasional mixing by vortex facilitates extraction and removal of non-flagellum-associated microtubules in the cells.
6. The final pellet containing extracted flagellum and associated structures [6, 7] was washed three times with PBS before further processing for mass spectrometry.

3.1.2 Purification and Extraction of *T. brucei* Flagellum

Purification of *T. brucei* flagellum only, without associated basal bodies or bilobe, was achieved following a previously published protocol [10] with modifications.

1. 1,000 ml procyclic *T. brucei* cells (*see Note 3*) are cultivated with Cunningham's medium supplemented with 15 % heat-inactivated fetal calf serum at 28 °C in a 2-L spinner bottle with gentle mixing at ~ 50 rpm, to near stationary phase ($\sim 1.6 \times 10^7$ /ml) (*see Note 4*). The cells are harvested by centrifugation at $2,000 \times g$ for 10 min.
2. Wash the cells twice with 30 ml buffer A.
3. Resuspend the cells in 30 ml buffer A supplemented with 1 % BSA, 0.1 mM CaCl_2 , and protease inhibitors.
4. Perform 5 sonication cycles, with each cycle containing 20 s pulse at 20 % of maximum output (Sonics Vibra-Cell, 130 W) and 40 s rest in between pulses, to detach the flagella from the cell bodies (*see Note 5*).
5. Centrifuge at $800 \times g$ for 10 min to remove unbroken cells.
6. Transfer the supernatant to a 30-ml polycarbonate tube. Centrifuge at $6,900 \times g$ for 15 min at 4 °C.
7. Remove the supernatant and resuspend the pellet in 4 ml buffer A supplemented with protease inhibitors.
8. Carefully load the resuspended pellet on top of a 6 ml 0.61 M sucrose cushion. Centrifuge at $1,200 \times g$ for 15 min in a swing-out rotor (such as SW 41 Ti, Beckman Coulter, Inc.).

9. Collect the top and the interface layers (~4.5 ml), mix well and load on top of another 0.61 M sucrose cushion, and repeat **step 8**. Transfer the top and the interface layers (~5 ml) into a 15-ml Falcon tube.
10. Set up the sucrose step gradient in two ultraclear SW 41 Ti centrifuge tubes as follows:
Place the tubes in a steady tube rack and avoid any disturbance throughout the procedure. Add 2 ml of 2 M sucrose solution at the bottom of each centrifuge tube. Gently overlay 2 ml from each of 1.84, 1.66, and 1.5 M sucrose solutions, respectively. To avoid mixing the different solutions, hold the pipette tip just above the meniscus of the solution in the tube and pipette the solutions by letting them run down the tube wall. Carefully load the material from **step 9**, ~2.5 ml to the top of each tube. Balance the two tubes to exact same weight (*see Note 6*).
11. Centrifuge overnight (16–20 h) at 4 °C in a SW 41 Ti rotor at 75,000 × *g* (28,000 rpm) (*see Note 7*).
12. Place the centrifuge tubes containing the sucrose gradient on a tube rack on ice. Flagella are enriched at the 1.66/1.84 M interface. Carefully remove all the top layers using a pipette to reach the 1.66 M layer.
13. Collect the 1.66/1.84 M interface. Pool the fractions together from the two tubes (~1 ml) and dilute 10× with buffer A. Centrifuge at 75,000 × *g* in a SW 41 Ti rotor for 30 min to concentrate the flagella.
14. Extract the purified flagella as described above in “Purification of *T. brucei* Flagellar Complex,” following **steps 3–6**.

3.1.3 Comparative Proteomics by Isobaric Tag for Relative and Absolute Quantitation (iTRAQ)

Solubilize the flagellum and flagellar complex samples prepared above in a small amount of dissolution buffer (*see Note 8*). Total protein concentration can be determined using a spectrophotometry-based protein assay (e.g., BCA assay, Pierce) or other appropriate quantification methods. Adjust total protein concentration to 5 µg/µl using 0.5 M TEAB. Further processing of the samples follows the iTRAQ protocols from Applied Biosystems and Sadowski et al. [11].

1. To each tube containing 20 µl protein sample (100 µg of protein each tube, two tubes of flagellum complex, and two tubes of flagellum samples), add 2 µl reducing reagent. Mix well and incubate at 60 °C for 1 h.
2. To each tube, add 1 µl of cysteine blocking reagent (*see Note 9*), mix well, and incubate at room temperature for 10 min.
3. Dilute each sample using 50 mM TEAB to reduce SDS concentration to 0.05 % or less.

4. Digest the protein samples with trypsin at 37 °C overnight (12–16 h). Monitor digestion using SDS-PAGE (*see* **Note 10**).
5. Dry the trypsin-digested samples with a speed vacuum concentrator.
6. Reconstitute each sample with 30 µl 0.5 M TEAB.
7. Label the samples with freshly prepared iTRAQ reagents. Label the flagellum samples with 114 and 116 reagents and the flagellar complex samples with 115 and 117 reagents, for 2 h at room temperature.
8. Pool the iTRAQ-labeled samples together, and clean up the mixture using a cation exchange column. Separate the peptides by reversed-phase high-performance liquid chromatography and analyze by MALDI TOF/TOF MS/MS (ABI 4800).

3.2 Proteomic Analysis of Immune-Isolated Bilobe

3.2.1 Immune Isolation of the Bilobe

T. brucei cells stably expressing YFP fusion to TbLRRP1 (Fig. 2a), a bilobe-resident protein [7], from an endogenous allele are used for immune isolation of the bilobed structure. Cells stably expressing the YFP reporter only are used as a negative control and processed side by side with the YFP-TbLRRP1 cells.

1. 100 ml each of YFP-TbLRRP1 and YFP log phase culture ($\sim 6\text{--}8 \times 10^6$ cells/ml) is harvested by centrifugation at $2,000 \times g$ for 10 min.
2. Wash the cells twice, with 50 and 25 ml lysis buffer H respectively.
3. Resuspend the cells in 1 ml cold buffer H supplemented with protease inhibitor cocktail (Roche) and 1 mM PMSF and transfer the cells to an Eppendorf tube. Lysing the cells in the isotonic buffer H allows protein components, either membrane or cytoskeleton associated, to be co-isolated with the bilobe.
4. Homogenize the parasite cells on ice by sonication for 6 cycles of 2 s pulses at 80 % output with 10 s interval between the pulses (Sonics Vibra-Cell, 130 W). Monitor cell lysis and bilobe integrity by light microscopy (Fig. 2).
5. Centrifuge the cell lysates at $2,500 \times g$ for 5 min at 4 °C to remove unbroken cells and large cell debris.
6. Add 20 µl DNase I (NEB, 2 U/ µl) to the supernatant, mix gently, and incubate on ice for 30 min (*see* **Note 11**).
7. Meanwhile, prepare anti-GFP (which cross-reacts to YFP)-coated beads as below:
 - (a) Completely resuspend Dynabeads® (Protein G, Invitrogen) by agitating the bottle on a shaker/roller.
 - (b) Transfer 200 µl (6 mg) of Dynabeads® suspension to an Eppendorf tube.

- (c) Place the tube on the magnet, allow Dynabeads® to sediment to one side of the tube, and remove the supernatant from the other side.
 - (d) Add ~75 µg anti-GFP diluted in 750 µl PBS to the tube containing Dynabeads® (*see Note 12*).
 - (e) Incubate the Dynabeads® anti-GFP mixture for 30 min at room temperature with gentle mixing.
 - (f) Wash the anti-GFP-coated Dynabeads® three times, with 750 µl PBS each time (*see Note 13*).
 - (g) After the final wash, resuspend the anti-GFP-coated Dynabeads® in 500 µl PBS and split the beads into two equal fractions of 250 µl each. One fraction is for the YFP-TbLRRP1 sample and the other for the YFP-only negative control.
8. To immune-isolate the YFP-TbLRRP1-labeled bilobe, add the DNase I-treated cell lysates from **step 6** to the anti-GFP-coated beads and mix well by pipetting gently. Mix the YFP cell supernatant with the other fraction of anti-GFP-coated beads.
 9. Incubate with gentle mixing for 4 h at 4 °C.
 10. Place the tubes on the magnet and remove the supernatant.
 11. Wash the Dynabeads® three times using 1 ml buffer H each time. Repeat the wash for another three times using 1 ml PBS (a total of six washes) (*see Note 14*). After the last wash, resuspend the beads in 50 µl PBS.
 12. To examine the magnetic beads by fluorescence microscopy, take 2 µl of the above bead suspensions, add 3 µl PBS, and put it on a glass slide. Place a coverslip on top and view it under the microscope (Fig. 2d) (*see Note 15*).
 13. Proteins bound to the beads are eluted by boiling in protein loading buffer, fractionated on 12 % SDS-PAGE, and processed for silver staining and immunoblotting using standard protocols.
 14. For further mass spectrometry analyses, gel regions containing the specific bands (found in YFP-TbLRRP1 sample but not in the YFP-only control) were excised, trypsinized, and further processed for LC/MS-MS following standard procedures.

4 Notes

1. Buffer A (without beta-mercaptoethanol) can be sterilized by autoclaving or filtration through 0.22 µm filters and stored at 4 °C for several months.
2. To prepare 100 ml of 300 mM PIPES stock, dissolve 9.07 g PIPES powder in 70 ml Milli-Q H₂O at room temperature and

adjust the pH to 6.9 with 5 M KOH. Once completely dissolved, adjust the volume and filter through a 0.22- μ m filter. Store at 4 °C for up to 2 months.

3. Procyclic form or the insect stage parasite that proliferates in the midgut of tsetse flies is used in these experiments.
4. With gentle mixing of the parasite culture, *T. brucei* cells can grow at higher density up to $\sim 2 \times 10^7$ cells/ml.
5. This gentle sonication protocol facilitates flagellum detachment from the cell body and fragmentation, with limited damage to the cell body.
6. It is helpful to mark the interfaces of the sucrose layers on the centrifuge tube before centrifugation. This makes it easier to recognize the interfaces later.
7. To avoid disturbance to the sucrose gradients, choose the “No brake” setting on the centrifuge.
8. It is important to use a small amount of dissolution buffer to solubilize the samples, so that in the final protein sample for iTRAQ labeling, SDS concentration can be diluted to 0.05 % or below, while protein content is not overdiluted.
9. Perform this step in a fume hood.
10. Complete trypsin digestion is crucial for further iTRAQ labeling. Incompletely digested samples shall be subjected to another round of trypsin treatment as described in **step 4**.
11. As the kinetoplast DNA is tightly associated with the basal bodies, which is also linked to the bilobed structure, the DNase I treatment helps to prevent co-isolation of the bilobe with other kinetoplast-associated proteins.
12. The binding capacity of Dynabeads® is ~ 8 μ g antibody/mg beads according to the product manual (Invitrogen). The binding of anti-GFP to the Dynabeads® had been optimized in small-scale binding experiments, where increasing amount of anti-GFP was incubated with 10 μ g beads until binding became saturated and unbound anti-GFP could be detected by SDS-PAGE and Coomassie blue staining.
13. The antibody can be cross-linked to the beads to reduce antibody elution in the next steps. However, the anti-GFP used in this experiment [12] appears sensitive to several cross-linkers tested and binds YFP less efficiently after the cross-linking treatments.
14. For each wash, resuspend the Dynabeads® in wash buffer, mixing gently on a rotator for 5 min at 4 °C. Remove the buffer from the beads by placing the tube on the magnet.
15. Images of immune-isolated bilobe on Dynabeads® are acquired using a ZEISS Axio Observer microscope equipped with a 63 $\times$ NA1.4 objective and a CoolSNAP HQ2 CCD camera (Photometrics).

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Application of the Proximity-Dependent Assay and Fluorescence Imaging Approaches to Study Viral Entry Pathways

Alex Lipovsky, Wei Zhang, Akiko Iwasaki, and Daniel DiMaio

Abstract

Virus entry into cells is a complex, multistep process that requires the coordinated activities of a large number of cellular factors and multiple membrane compartments. Because viruses can enter cells via one or more of a large number of preexisting pathways, understanding the mechanism of virus entry and transport between various intracellular compartments is a challenging task. The arrival of “omics” technologies such as genome-wide RNA interference screens has greatly advanced our ability to study the molecular intricacies of viral entry. Bioinformatics analyses of high-throughput screen data can identify enriched gene categories and specific individual genes required for infection, which can yield important insights into the cellular compartments that viruses traverse during infection. Although there are a variety of well-established genetic and biochemical approaches to validate genome-wide screen findings, confirmation of phenotypes obtained from RNA interference studies remains an important challenge. Imaging techniques commonly used to visualize virus localization to cellular organelles are often prone to artifacts that result from the necessity of using a high multiplicity of infection. Fortunately, recent advances in microscopy-based methods for studying protein location have improved our ability to accurately pinpoint virus localization within its host cell. Here we describe in detail one such technique—the proximity ligation assay (PLA)—as a tool to validate findings from a genome-wide loss-of-function genetic screen. In addition, we discuss a number of important considerations for the utilization of immunofluorescence microscopy and RNA interference to investigate the molecular mechanisms of virus entry.

Key words siRNA, Proximity ligation assay, Immunofluorescence, Virus entry, HPV, RNA interference

1 Introduction

High-throughput genetic screens have transformed our ability to identify the cellular factors required for all aspects of virus infection, including entry into cells, virus replication, and virus release from the infected cell. Loss-of-function screens based on RNA interference have found widespread use among researchers studying entry mechanisms by clinically relevant viral pathogens such as the human immunodeficiency virus (HIV), hepatitis C virus

(HCV), influenza virus, West Nile virus, and human papillomavirus (HPV) [1–5]. These studies have provided important insights into not only individual cell proteins but also pathways, compartments, and large protein networks that facilitate virus uptake. Genome-wide RNA interference screens will undoubtedly remain an important tool for virologists to study both existing and newly emerging pathogens for decades to come.

The increasing use of high-throughput genomics had also highlighted the need for careful and thorough hit validation during the follow-up stages of the screening process. Illustrating the importance of hit validation are the large and well-documented divergences in lists of required genes reported by investigators studying the same pathogen [6]. Although some of the discrepancies can be attributed to variations in assay design or inherent difficulties in interpreting the effects of siRNAs, the surprising lack of agreement among data from different laboratories further emphasizes the necessity for a multipronged experimental strategy to confirm the chief findings from each screen [7]. Here we briefly outline a number of experimental strategies to validate siRNA screen hits (Subheading 1) and describe in detail PLA- and flow cytometry-based approaches to track virus entry and quantitate the extent of virus disassembly (Subheadings 3.1 and 3.2, respectively).

1.1 Screen Hit Validation

Although transfection of siRNAs into cells (or expression of shRNAs) is an efficient method to downregulate target gene expression, some siRNAs bind to unintended mRNA transcripts and influence their stability or translation [8]. Knockdown studies that generate the same phenotype with more than one siRNA specific for non-overlapping sequences within the same target transcript can minimize the likelihood that a particular phenotype is due to off-target effects. Because it is unlikely that multiple sequence-divergent siRNAs will produce identical off-target effects, a phenotype (such as inhibition of virus infection) is likely to be valid when it is reproduced by two or more different siRNAs (Fig. 1).

In addition to testing a number of siRNAs to confirm the role of a gene in virus entry, validation of a phenotype can be achieved by the expression of an siRNA- or shRNA-resistant cDNA of the gene of interest (Fig. 2). In this approach, silent mutations are introduced into the cDNA of the targeted mRNA at the seed sequence recognized by the siRNA. If the delivery of this construct into cells eliminates the effect of the siRNA, this phenotype can be ascribed with confidence to target gene knockdown.

Pharmacological inhibition of protein function is a complementary approach to the use of genetic techniques to validate siRNA hits. The advantages of pharmacological inhibitors include the ease of use (no transfection or virus-mediated delivery is required), targeted inhibition of protein activity, and the ability to control the duration and dose of treatment. However, a major disadvantage of chemical compounds is their well-documented

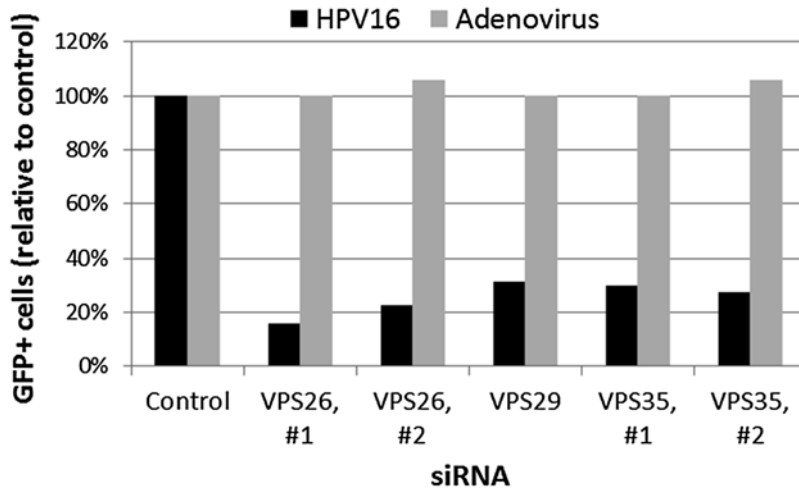


Fig. 1 Multiple subunits of retromer are required for HPV16 but not adenovirus infection. HeLa cells were transfected with a control siRNA or an siRNA against retromer subunits VPS26, VPS29, or VPS35. After 48 h, the cells were infected with HPV16-GFP (*black bars*) or adenovirus5-GFP (*gray bars*) at MOI of 0.5 and assayed for GFP expression 48 h later by flow cytometry, with normalization to cells transfected with control siRNA

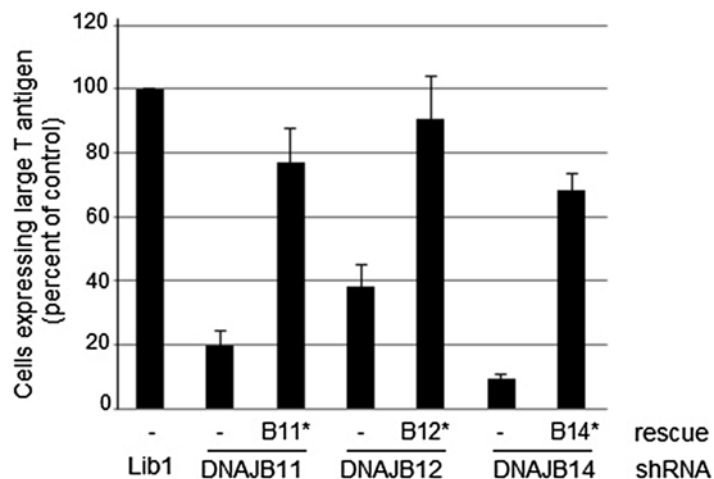


Fig. 2 Rescue of SV40 infection. Control (Lib1), DNAJB11, DNAJB12, and DNAJB14 knockdown HeLa cells were stably transduced with a DNAJB11*, DNAJB12*, or DNAJB14* gene containing a mutation in the shRNA binding site, as indicated, or empty vector (-). Forty-eight hours after SV40 infection, flow cytometry was used to measure the fraction of cells expressing SV40 large T antigen. Averaged results from independent experiments are shown. Reproduced from ref. 19 with permission from the American Society for Microbiology

tendency to produce wide-ranging off-target effects. Despite these limitations, a number of compounds have found widespread application in the studies of virus entry. The excellent review by Engel et al. lists chemicals that could target almost every step of virus entry [9].

1.2 Imaging and Biochemical Techniques to Study Virus Trafficking and Disassembly

Immunofluorescence (IF) microscopy remains one of the most common methods for studying virus entry. It is a powerful approach that can sometimes reveal virus within distinct cellular organelles without the need for complex and time-consuming biochemical analyses. When combined with live-cell imaging, IF-based imaging studies can shed important light on the spatiotemporal dynamics of virus internalization and trafficking. However, because efficient visualization of viruses usually requires a very high multiplicity of infection and because of the large particle-to-infectivity ratio for most viruses, IF is often unable to differentiate between infectious particles and those that enter nonproductive pathways. Moreover, subjecting cells to very high doses of pathogen can sometimes lead to nonphysiological, cytopathic effects. Therefore, although IF is a useful tool to study virus entry, it is far from ideal. A biochemical approach to study virus entry is immunoprecipitation (IP), which can detect physical association between the viral structural proteins and host cell proteins and therefore confirm the presence of virus within a particular subcellular compartment. And even though IP, often coupled to protein identification by mass spectrometry, is a powerful approach to identify individual components of a multi-subunit complex, non-interacting or many transiently associated proteins cannot be detected by IP, even when they are in close proximity to the virus.

1.3 Proximity Ligation and Flow Cytometry Assays to Track Virus Movement and Disassembly

The development of the proximity-dependent DNA ligation assay (PLA) has provided a sensitive tool to examine protein–protein interactions by taking advantage of two DNA aptamers, whose hybridization to a target probe produces an amplifiable DNA sequence [10]. Developed in part as an alternative to enzyme-linked immunosorbent assay (ELISA) for ultrasensitive detection of very small protein quantities in clinical samples, PLA was subsequently adapted to the study of protein–protein interactions in situ [11]. This was achieved by conjugating the DNA aptamers to antibodies specific for the two targets to be assayed. Hybridization of the aptamers to a common probe, which only occurs when the two target molecules are in close proximity, creates a template for rolling circle DNA amplification (RCA) by a polymerase chain reaction. The single-stranded RCA product is then incubated with fluorescent oligonucleotides and the resulting hybrid visualized as a single microscopic fluorescent spot. The number and fluorescence intensity of PLA signals within an image can be conveniently quantitated by dedicated imaging software. The PLA technology is especially powerful when combined with flow cytometry, since it allows for rapid quantification of protein–protein interactions within a large cell population [12].

Although in situ PLA was developed and most often applied to the study of proteins within a putative complex, it can detect targets that are not necessarily in direct physical contact [13].

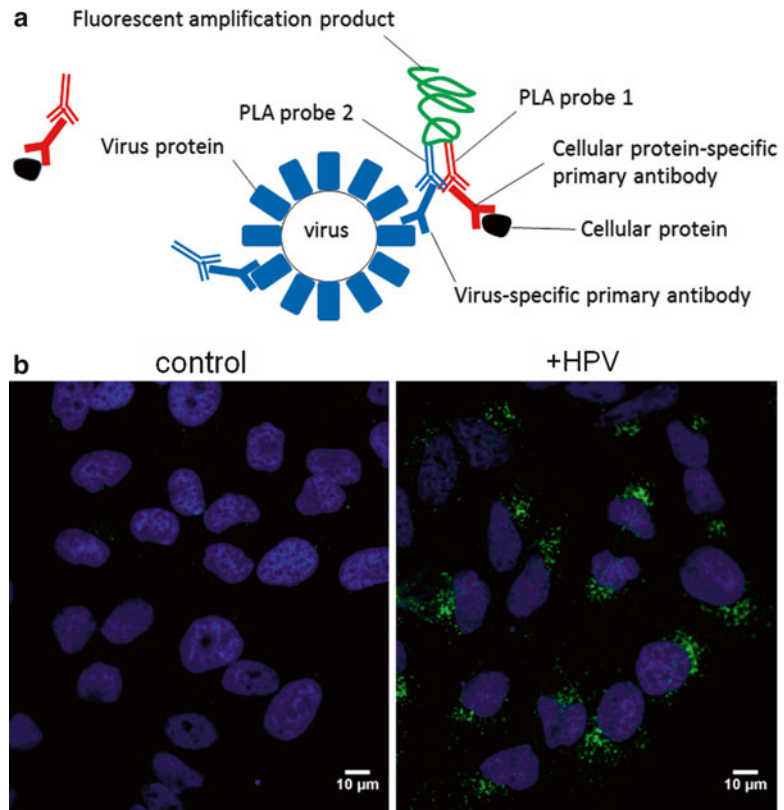


Fig. 3 Proximity ligation assay to study virus–host interactions. **(a)** A schematic diagram of the proximity ligation assay for the detection of virus localization to subcellular structures. **(b)** HeLa cells were infected with HPV16-GFP at MOI of 100 (*right panel*) or remained uninfected (*left panel*). Sixteen hours later, cells were stained with the L1 polyclonal antiserum and the GM130 Golgi-specific antibody and processed for PLA. A single confocal slice is shown in each panel. PLA signal is *green*. Nuclei are stained *blue* with DAPI. Reproduced from ref. 5 with permission from the Proceedings of the National Academy of Sciences, USA

In fact, commercially available kits based on PLA technology nominally allow the detection of proteins separated by 40 nm (approximately the diameter of a small virus capsid) [14]. PLA in effect converts antibody-mediated recognition of proximal epitopes into an amplifiable DNA sequence. Rather than being precipitated, the assembly of proteins, bound antibodies, nucleic acid aptamers, and amplification products is labeled in situ with a large number of fluorescent DNA probes, resulting in high sensitivity (Fig. 3a). Typically, to visualize incoming virus during infection studies, one antibody is directed against a virion protein and the other to a cellular protein localized in a particular compartment (or to a required hit on a siRNA screen). A successful proximity ligation assay for the presence of a virus in a particular

compartment will yield images with significant and reproducible differences in the average number of signals per cell between the infected sample and the uninfected negative control (Fig. 3b). The ever-lengthening list of publications using PLA technology is a testament to the unique combination of sensitivity, resolving power, and simplicity of this technique. Significantly, over the last few years, the interactions of a number of viral and bacterial pathogens with their host cells have been investigated with the aid of in situ PLA [5, 15–18].

A crucial step in the entry pathway of many viruses is the process of capsid disassembly. For a large number of viruses, uncoating of the viral genome is a complex sequence of capsid rearrangements and modifications that often occur in the context of low pH and proteolytic activities of the endolysosome compartments. Conformational changes in capsid structure are often transient and difficult to analyze and necessitate the application of both structural and imaging approaches. The use of specific antibodies that are reactive with capsid components only after conformational changes or partial capsid disassembly allows these events to be monitored in intact cells (Fig. 4).

When flow cytometry is used after staining with an antibody that recognizes an epitope that becomes exposed during viral disassembly, the number of cells in which disassembly has taken place can be conveniently plotted as a histogram with fluorescence and cell count values displayed along the x - and y -axis, respectively. Productive labeling of a disassembled virus with an epitope-specific antibody will produce a normal distribution whose peak is shifted to the right compared to the peak of a negative control sample (Fig. 5), providing a quantitative, objective measure of disassembly.

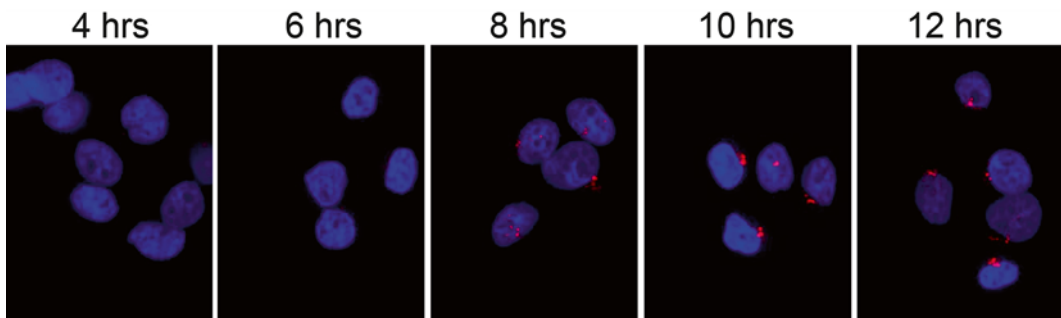


Fig. 4 Time course of exposure of the SV40 minor capsid protein VP2/3. HeLa cells were infected with SV40 at an MOI of 200 for the indicated times. Cells were then immunostained for VP2/3 (red) and visualized by confocal microscopy. Nuclei are stained blue. Individual slices were compressed along the z -axis to generate these images. Reproduced from ref. 19 with permission from the American Society for Microbiology

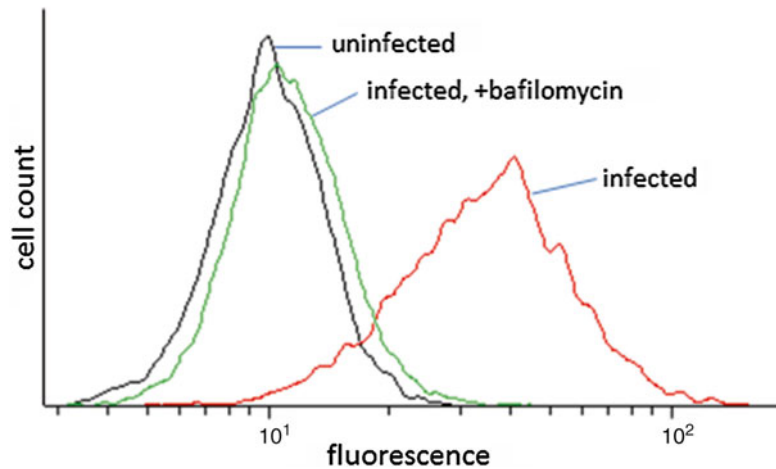


Fig. 5 Endosomal acidification is required for conformational change in HPV capsid. HeLa cells were infected with HPV16 at MOI of 20, and one infected sample was also co-treated with bafilomycin A. After 15 h, cells were stained with the 33L1-7 antibody (which recognizes a conformational change in the HPV L1 major capsid protein) and assayed by flow cytometry

2 Materials

2.1 Tissue Culture Reagents

1. Dulbecco's Modified Eagle's Medium-10 (DMEM-10): supplement DMEM medium (Sigma-Aldrich, Saint Louis, MO) with 10 % fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 1 % of penicillin–streptomycin (Gibco, Carlsbad, CA), and 2 mM L-glutamine (Gibco, Carlsbad, CA).
2. Phosphate buffered saline (PBS) pH=7.4 (1×): dilute 10× PBS pH=7.4 (Sigma-Aldrich, Saint Louis, MO) with sterile water.
3. 0.25 % trypsin-EDTA (Gibco, Carlsbad, CA).
4. Tissue culture plates and flasks (BD Falcon, San Jose, CA).

2.2 IF, PLA, and Flow Cytometry Reagents

1. Fixatives: formaldehyde-use fresh solution that is buffered (Fisher Scientific, Pittsburgh, PA) or 100 % methanol (Avantor, Center Valley, PA).
2. Permeabilization buffer: add Triton X-100 (Sigma-Aldrich, Saint Louis, MO) to PBS for a final concentration of 0.5 % (v/v).
3. Blocking/primary antibody buffer: add bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) to PBS for a final concentration of 5 % (v/v).
4. Duolink® In Situ PLA® probes (Sigma-Aldrich, Saint Louis, MO).
5. Duolink® In Situ Wash Buffer A: add 8.8 g of NaCl (Sigma-Aldrich, Saint Louis, MO), 1.2 g of Tris base (Sigma-Aldrich, Saint Louis, MO), and 0.5 mL of Tween 20 (Sigma-Aldrich,

Saint Louis, MO) to 1 L of high purity water, and adjust the pH to 7.4. Sterilize with a 0.22 μm filter and store at 4 °C.

6. Duolink® In Situ Wash Buffer B: add 5.84 g of NaCl (Sigma-Aldrich, Saint Louis, MO), 4.24 g of Tris base (Sigma-Aldrich, Saint Louis, MO), and 26 g of Tris-HCl (Sigma-Aldrich, Saint Louis, MO) to 1 L of high purity water, and adjust the pH to 7.5. Sterilize with a 0.22 μm filter and store at 4 °C.
7. Duolink® In Situ Detection Reagent (contains 5 $\times$ ligation buffer, 1 $\times$ ligase, 5 $\times$ amplification buffer, and 1 $\times$ polymerase) (Sigma-Aldrich, Saint Louis, MO).
8. Duolink® In Situ Mounting Medium with DAPI (Sigma-Aldrich, Saint Louis, MO).
9. Alexa Fluor Dye-conjugated secondary antibodies for IF and flow cytometry (Invitrogen, Carlsbad, CA).
10. ProLong® Gold Antifade Reagent with DAPI for IF (Invitrogen, Carlsbad, CA).

2.3 Microscopy Reagents, Equipment, and Software

1. Round glass coverslips, 12 mm diameter (#NC9227465, Fisher Scientific, Pittsburgh, PA).
2. Microscope slides (Fisher Scientific, Pittsburgh, PA).
3. ZEISS Axiovert 200 inverted fluorescent microscope or similar confocal microscopy imaging equipment.
4. IF image analysis: ImageJ (<http://imagej.nih.gov/ij/>).
5. PLA image analysis: BlobFinder (<http://www.cb.uu.se/~amin/BlobFinder>) or the Duolink® ImageTool (Sigma-Aldrich, Saint Louis, MO).

2.4 Flow Cytometry Equipment and Software

1. BD FACSCalibur platform or similar flow cytometry equipment.
2. Data analysis: FlowJo (Tree Star Inc., Ashland, OR).

3 Methods

3.1 Proximity Ligation Assay

1. Plate cells on glass coverslips in a 12-well plate and incubate overnight in 1 mL of tissue culture medium per well. Do not disturb the plate for at least 24 h to ensure complete cell adherence to the coverslip surface and maximal recovery of normal cell morphology. Infect cells with virus (*see Note 1*). Incubate cells at 37 °C to allow infection to proceed.
2. Slowly pipette 1 mL of chilled 4 % formaldehyde into the tissue culture medium, and incubate for at least 15 min at room temperature (*see Note 2*). Aspirate the fixative, pipette 0.5 mL volume of permeabilization buffer, and incubate for 15 min at room temperature.

3. Aspirate the permeabilization buffer, add 0.5 mL volume of blocking buffer, and incubate for 30 min at room temperature. Blocking can be done with either 5 % bovine serum albumin (BSA) in PBS or the supplied reagent.
4. Aspirate blocking buffer and wash cells twice with 50 μ L of PBS per coverslip. Carefully add 50 μ L of primary antibody mix diluted in blocking buffer onto the coverslips. Incubate for 1 h at 37 °C or overnight at 4 °C in a humidified chamber. Aspirate the primary antibody solution and wash cells three times with 50 μ L of PBS (*see Note 3*).
5. Prepare PLA probes diluted 1:5 in blocking buffer and allow the solution to sit for 20 min at room temperature. Add 30 μ L of diluted PLA probes to cells and incubate for 1 h at 37 °C.
6. Prepare the ligation mix immediately before use. Dilute 6 μ L of the concentrated ligation buffer with water for a total reaction volume of 30 μ L. Add 1 μ L of ligase to the diluted buffer and mix thoroughly. Aspirate the PLA probe solution and wash cells twice with 50 μ L of wash buffer A for 5 min under gentle agitation. Add ligation mix to cells and incubate for 0.5–1 h at 37 °C.
7. Prepare the amplification mixture immediately before use. Dilute 6 μ L of the concentrated amplification buffer with water for a total reaction volume of 30 μ L. Add 0.5 μ L of polymerase to the diluted buffer and mix thoroughly. Aspirate the ligation mix and wash cells twice with 50 μ L of wash buffer A for 2 min under gentle agitation. Add the amplification mix to cells and incubate for 2 h at 37 °C (*see Note 4*).
8. Aspirate the amplification mixture and wash cells twice with 50 μ L of wash buffer B for 10 min under gentle agitation. Repeat the washing once with 0.01 $\times$ of wash buffer B for 1 min. Let the coverslips dry completely at room temperature in the dark.
9. Mount the coverslips using 5 μ L of the Duolink® In Situ Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei (*see Note 5*). Seal the edges with nail polish to prevent leakage of the medium. Wait for at least 15 min prior to imaging the cells.
10. Visualize stained cells with a confocal fluorescence microscope using a fluorophore-specific filter.
11. Perform image analysis with either the Duolink® ImageTool or the BlobFinder program (*see Note 6*).

3.2 Flow Cytometry to Monitor Virus Disassembly

1. Plate $1\text{--}5 \times 10^5$ cells per well into a 6-well plate and incubate overnight (*see Note 7*).

2. Incubate cells with virus suspended in fresh medium appropriate for the target cells (*see Note 8*). Allow infection to proceed for the desired length of time.
3. Aspirate the virus-containing medium and wash infected cells once with 2 mL of PBS. Add 100 μ L of 0.25 % trypsin-EDTA and incubate at 37 °C for 5–10 min. Add 1 mL of medium to the trypsinized cells and pipette the cell mixture up and down several times to break up cell clumps. Transfer the resulting single cell suspension to a 5 mL polystyrene tube.
4. Centrifuge the suspension for 5 min at 1,000 rpm. Aspirate the supernatant and add 1 mL of cold 4 % formaldehyde to the pelleted cells. Vortex briefly and fix for 15 min at room temperature (*see Note 9*). If fixing with 100 % methanol, incubate cells for 15 min on ice and skip **step 5**.
5. Pellet the cells, aspirate the supernatant, and add 1 mL of permeabilization buffer. Vortex briefly and incubate for 15 min at room temperature.
6. Pellet the cells, aspirate the supernatant, and add 1 mL of blocking buffer. Vortex briefly and incubate for 15 min at room temperature.
7. Pellet the cells, aspirate the supernatant, and add 100 μ L of primary antibody diluted in blocking buffer. Incubate for 1 h at room temperature or overnight at 4 °C (*see Note 10*).
8. Wash the stained cells by first centrifuging at 1,000 rpm, then aspirating the antibody, and adding 1 mL of blocking buffer. Vortex briefly and repeat at least one more time.
9. Pellet the cells, aspirate the supernatant, and add 100 μ L of species-specific secondary antibody diluted in blocking buffer. Ensure the secondary antibody is conjugated with appropriate Alexa Fluor Dyes. Incubate for 30–60 min in the dark.
10. Wash the stained cells as above, then aspirate the antibody, and add 1 mL of blocking buffer. Vortex briefly and repeat at least one more time.
11. Analyze the sample with a flow cytometer using a fluorophore-specific channel. Analyze cytometry data with commercial software such as FlowJo or freeware such as WinMDI.

4 Notes

1. Proximity ligation assay reagents are expensive and substantial savings can be achieved by minimizing the surface area to be stained. We use 12 mm diameter round glass coverslips, which can be efficiently covered with as little as 30 μ L of solution. Proper humidification must be maintained throughout the staining procedure to prevent volume loss due to evaporation.

It is critical to include positive and negative controls with every PLA experiment, although the exact number and nature of controls will depend on the specific details of each experiment. Examples of essential negative controls include uninfected cells as well as infected cells processed for PLA in the absence of one or both primary antibodies. Another useful negative control may involve assaying for proximity between two proteins that are normally localized to distinct subcellular compartments. The best positive control is one that measures interaction between an abundant pair of proteins—one viral and one cellular—that are known to associate during infection. If such a positive control is unavailable, two interacting cellular proteins can be targeted instead. The measurement of proximity between two subunits of a protein complex may be a good internal control for testing optimal reagent performance over the time. Repeated assays of two proteins that demonstrate large fluctuations in signal intensity presumably indicate an issue with either a reagent or experimental technique.

Our experience with the proximity ligation assay has revealed a number of additional parameters that may affect data quality and reproducibility. One factor is cell confluency prior to infection. Highly confluent cultures generally exhibit a lower average number of PLA spots per cell compared to cells plated at low density. This appears to be a consequence of cell crowding and thus a decrease in cell size. Another factor is background fluorescence. Typically, the staining of cells with PLA reagents in the absence of primary antibodies results in only a few nonspecific signals per cell. If more than several spots per cell are observed for the negative control, it may indicate a need for further troubleshooting of either experimental conditions or assay implementation.

Because proximity ligation assays to measure virus colocalization with cellular structures rely on both host and microbial proteins, proper infection conditions are essential for robust assay performance. A number of experimental parameters can be adjusted to optimize signal strength and/or specificity. Conditions that can be adjusted include, but are not limited to, the specific virus strain and host cell used, viral multiplicity of infection (MOI), synchronization of viral entry by preincubation at 4 °C followed by shift to permissive temperature such as 37 °C, and selection of appropriate time points during infection.

2. It is recommended that fixation be done via gradual addition of the cold fixative directly into the cell culture medium. Such an approach minimizes cell stress and subsequent cell detachment from the coverslip surface during aspirations and washes. For convenience, the fixation, permeabilization, and blocking steps can be done directly in the tissue culture plate containing the

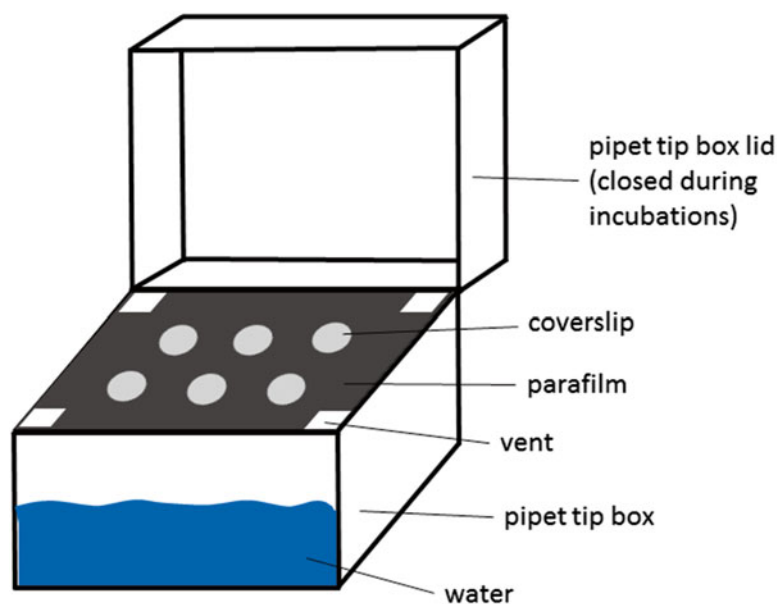


Fig. 6 Schematic diagram of a simple humidified chamber

coverslips with cells. Since subsequent steps involve very small solution volumes, the coverslips should then be removed from the culture plate and placed in a humidified chamber (Fig. 6).

Preheat the water in the humidified chamber to 37 °C prior to the first incubation. The use of the hydrophobic parafilm helps to immobilize the coverslips and prevents cross-contamination between adjacent coverslips due to unintended leakage of solution. Washing is carried out by positioning the aspirator tip at the coverslip rim and suctioning off the solution. Aspiration in this fashion efficiently removes nonspecific antibody binding and eliminates the need for time-consuming and laborious washes with specialized jars as recommended by the Duolink[®] manufacturer.

3. It is important to choose primary antibodies of different species when performing PLA to track virus entry, in order to generate a signal only when the two targets are in proximity. In cases when primary antibodies of the same species must be used, it is possible to conjugate the oligonucleotide arms of the PLA probes directly to the primary antibodies by using the Duolink[®] In Situ Probemaker kit. Ensure that each of the primary antibodies (or PLA probes, when following the standard protocol) is conjugated to either a PLUS or a MINUS oligonucleotide.

Proper dilution of primary antibody is critical for achieving the highest signal-to-noise ratio. If the antibodies had been used previously for IF, the same dilutions can be used as a starting point for PLA. Increasing the antibody concentration

can sometimes result in both brighter and more numerous PLA spots, although too much antibody will lead to high background fluorescence. However, antibodies that are too dilute will result in suboptimal assay performance. Choosing an abundant target is also helpful. A viral epitope that is sterically hindered or scarce may necessitate an increase in the MOI to compensate for suboptimal signal strength.

4. In some cases, extending the amplification time can improve assay performance. It is best to begin with 2 h of incubation and increase the amplification time if weak signal intensity is observed. We have used longer incubation time (up to 4 h) to obtain stronger PLA signals for weak antibody pairs.
5. Although theoretically a nonproprietary mounting medium such as ProLong[®] Gold Antifade Reagent with DAPI can be used for this step, our experience with various mounting media showed that the Duolink[®] version works well and consistently for long-term preservation of PLA signal intensity.
6. The Duolink[®] ImageTool can be purchased from Sigma-Aldrich, whereas BlobFinder can be downloaded free of charge from the Centre of Image Analysis at Uppsala University (<http://www.cb.uu.se/~amin/BlobFinder>). Both programs are easy to learn and use and utilize similar methods for the identification of PLA signals. It is recommended that the selection of images for capture and analysis be done in an unbiased manner using the DAPI channel. Also, cell populations to be compared must be roughly similar in terms of the number of nuclei as well as overall cell confluency. Such an approach will minimize variations in the average number of PLA spots per cell and background fluorescence.

It is essential to standardize the exposure time and threshold values for all images within a particular channel to obtain an accurate quantitative analysis. To reduce variability in signal values among cells and improve overall statistical significance, at least 100 cells must be imaged and analyzed. Two methods are available in BlobFinder to quantify signal levels: dot count and fluorescence intensity. In our experience, the dot count option works particularly well for low signal intensity, while the total fluorescence intensity option is best for highly abundant PLA spots.

7. Seed cells so that they are <50 % confluent on the next day. This will maximize efficiency of infection and minimize differences in virus entry among the infected cells. At least several hundred thousand cells must be plated to ensure the cell pellet is visible upon harvest and to prevent cell loss during the staining procedure.

As with any other technique, proper controls are crucial for correct data interpretation. Two key negative controls

are uninfected cells as well as cells incubated with fluorescent secondary, but not primary antibodies. A positive control typically includes cells stained for a normally inaccessible viral epitope that becomes increasingly exposed over the course of infection.

8. To achieve rapid binding of the virus to cells, the volume of tissue culture medium can be reduced by approximately twofold, as long as the infected cells are completely submerged in the medium. Alternatively, viruses could be absorbed to cells at 4 °C in a very low amount of growth medium with gentle agitation to produce more homogeneous infection kinetics upon subsequent temperature shift. In addition, since flow cytometry is a highly sensitive quantitative assay, the MOI required to achieve robust signal intensity is usually much lower than the MOI necessary to detect virus particles by IF microscopy or PLA. Decreasing the MOI often translates into increased ability of an assay to detect small changes in signal intensity. In our hands, flow cytometry-based assays for measuring virus capsid disassembly are superior to IF-based assays, because the former method eliminates the inherent bias of cell selection and is intrinsically quantitative and many more cells can be analyzed. Obviously, however, standard flow cytometry does not provide information regarding subcellular localization of the disassembly event.
9. All subsequent incubations can be carried out at room temperature and the centrifugations performed at 1,000 rpm for 5 min.
10. Do not vortex cells after addition of the primary (or secondary) antibody. Instead, gently pipette the cell suspension up and down several times to avoid splashing of the sample. Vortexing can be used again during the washing steps.

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