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Activity-Based Proteomics

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Activity-Based Proteomics

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Edited by

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Preface

Activity-based protein profiling has emerged in the past decades as an attractive strategy to at once simplify a proteome by selecting for a specific protein subset and providing functional data, in that proteins are selected based on their functioning, and not their mere expression. In a typical activity-based protein profiling experiment, a covalent and irreversible enzyme inhibitor is equipped with a visualization/identification tag (fluorophore, biotin) for analysis and enrichment by means of gel-based assays, affinity purification followed by mass spectrometry analysis, or a combination of the two. Covalent and irreversible inhibition can be affected by alkylation or acylation of an enzyme active site-residing nucleophile with an appropriate electrophilic species. In case such species are lacking photo-affinity labels can be used as a technology complementary to activity-based profiling and that has the added value that also non-enzyme protein families can be targeted. Activity-based protein profiling is a field that has benefited from bioorthogonal chemistry as a means to introduce an identification tag in two steps and altogether the field of activity-based protein profiling, or activity-based proteomics, brings together elements from analytical chemistry, biochemistry, and organic chemistry. In this *Methods in Molecular Biology* issue a concise overview of the different aspects of activity-based protein profiling will be presented in the form of specific protocol chapters. The issue opens with a concise review on the current state of the field and further consists of 14 chapters, each from experts in the field who unveil specific aspects of the subject based on their own findings. Part I *Explorative activity-based proteomics* focuses on the use of activity-based proteomics as a discovery tool to identify and annotate enzyme families as well as their target inhibitors. Part II *Biomedical applications of activity-based proteomics* represents examples on how the methodology is finding its way into biomedical research, whereas Part III *Chemical strategies in activity-based proteomics* gives a selection of chemical strategies by means of which activity-based proteomics can be further optimized.

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Chapter 1

Activity-Based Protein Profiling: From Chemical Novelty to Biomedical Stalwart

Sander I. van Kasteren, Bogdan I. Florea, and Herman S. Overkleeft

Abstract

Biological systems often respond to environmental changes by rapidly altering the activity of specific enzymes: for example through desequesterization of enzyme activities by dissociation from inhibitors, activation/deactivation through posttranslational modification, or relocation of the enzyme to different organelles. This means that expression levels of enzymes do not necessarily correlate with the activities observed for these enzymes. In this chapter we review some of the approaches used to selectively image only the active sub-populations of given enzymes, the so-called activity-based protein profiling. A focus lies on recent developments that are taking this approach from chemical novelty to biochemical stalwart.

Key words ABP, ABPP, Bio-orthogonal ligation

1 Introduction

Biological systems often respond to environmental changes by rapidly altering the activity of specific enzymes: for example through desequesterization of enzyme activies by dissociation from inhibitors, activation/deactivation through posttranslational modification, or relocation of the enzyme to different organelles. All these changes are far quicker than any changes in expression level of proteins. This means that enzyme activity, as opposed to its expression level, is an essential parameter in the understanding of biological processes.

Traditionally this information was obtained through for example the use of substrates of specific enzymes and enzyme classes. Changes in substrate turnover could then be correlated to changes in enzyme expression levels. The major downside to this approach is the lack of resolution and the lack of information it provided on the enzymes themselves: if different enzymes with overlapping function can convert a substrate, then only the sum of these enzymatic activities can be quantified.

A preferred method would be to disseminate and quantify this activity to the level of the individual enzyme, which is the aim

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underpinning the field of *activity-based protein profiling* (ABPP) [1, 2]. The basic approach is to probe enzyme function by using an inhibitor of the enzyme of interest—usually a covalent one—attached to a detectable group. The approach works by virtue that only catalytically active enzyme can be inhibited, allowing the detection of the enzyme in a manner proportional to its activity. The inhibitors linked to detectable groups are called *activity-based probes* (ABPs)

2 Early Examples of Enzyme Activity Profiling

A very early—if not the earliest—example of a proto-ABP for imaging was reported by Ostrowski and Barnard [3], who in 1961 described the use of a tritiated diisopropylfluorophosphonate to image esterase activity in rat tissue by autoradiography: the radioactive covalent inhibitor was incubated with rat tissue sections to react with the enzyme family of interest. After washing away unbound probe, the location of the enzyme could be imaged using autoradiography. This process is cumbersome as it is elegant: Synthetic approaches are severely restricted by the use of radionuclei and the resolution of detection is low. However, this is—to the best of our knowledge—the first example of a covalent inhibitor of an enzyme family (fluorophosphonate) being used to detect the active form of that enzyme in a complex biological sample using a detectable variant of the inhibitor. Other key examples of the use of radiolabeled ABPs were the use of tritiated lactacystin to confirm its covalent attachment of this inhibitor to the proteasome [4] and the radiolabelled analogs of the papain-like cysteine protease inhibitor E64, which formed the basis of later efforts to image this class of enzymes [5] Inhibition.

A major development in the evolution of ABPP came with the use of nonradioactive reporter groups. The first of such agents were biotin-modified inhibitors. Two early examples of this approach were reported by Bernstein et al. [6] and Steven et al. [7]. The former used non-covalent inhibitors attached to biotin via a short 11- or longer 19-carbon spacer to gain insights into the depth of the active site of angiotensin converting enzyme (ACE). They found that only biotin attached to the long spacer could be retrieved with streptavidin, showing the depth of the ACE active site pocket. Steven et al. used acdridin—a non-covalent inhibitor of guanidinobenzoatase—conjugated to biotin to label and image the activity of this protease on the surface of tumor cells using detectable variants of streptavidin. These are two very early examples of detectable inhibitor variants being used to gain insight into enzyme activities and localization.

Covalent inhibitors are the preferred scaffolds for the development of ABPs, as the covalent linkage between the enzyme and the

inhibitor allows the more robust retrieval and washing and broadens the scope of analysis. One of the first proto-ABPs using a covalent inhibitor of an enzyme—at the time unknown—came from the Crews laboratory [8]. They used a biotin-modified variant of the covalent angiogenesis inhibitor fumagillin to identify and pull down the target of the drug identify it by SDS-PAGE combined with tryptic digest and found it to be the metalloprotease methionine aminopeptidase. Other early examples of covalent inhibitor constructs came from the Ploegh laboratory [9], who used both a biotin-modified and a radioactive analog of the Z-Leu-Leu-Leu-vinyl sulfone inhibitor to show that this compound was actually a covalent proteasome inhibitor; rather than the cysteine protease inhibitor it was believed to be at the time.

The Ploegh-laboratory were also the first to report a different type of ABPP, namely those based on natural substrates, rather than inhibitors, of hydrolases modified with electrophilic traps [10]. They made and tested a series of ubiquitin-analogs carrying cysteine-reactive groups at their C-terminus to identify the cysteine-deubiquitinases from cell lysates and using this approach discovered the proteasomal association of USP-14. The application of ABPP to the discovery and identification of the hydrolases of ubiquitin and of the ubiquitin-like modifiers has expanded rapidly and has been the source of some exciting new developments, such as the use of highly *unreactive* alkyne warheads that make use of the high affinity of these hydrolases for their substrates [11, 12]. Other exciting developments in the field have been the use of the localization of the electrophilic traps between two ubiquitin chains to probe chain topology specificity of these enzymes [13–15] and in this volume [Chapter 9]. The labeling of hydrolases of other ubiquitin-like modifiers is also a rapidly expanding field, and is the subject of a chapter in this volume by Kessler et al. [Chapter 10].

A second paradigm shift occurred when Cravatt [16] switched from a drive to highly selective inhibitors as the basis for their ABPs to very broad-spectrum inhibitors that allowed the imaging of whole enzyme classes. In the paper that gave the field its name, Cravatt used a broad-spectrum fluorophosphonate group linked to either a biotin or fluorescein to image the activity of serine hydrolases after SDS-PAGE separation of the proteome in various rat organs. The authors identified tissue-specific serine hydrolase activity patterns that were purely dependent on folded, uninhibited proteins. The group of Bogyo [17] developed an ABP with an intermediate selectivity. By using the aforementioned broad spectrum cysteine-cathepsin inhibitor E64 conjugated via a spacer to a biotin moiety, this enzyme family could be imaged and retrieved in complex biological samples. Since these broad-spectrum inhibitors, the group of Bogyo has also worked on the development of highly specific cathepsin inhibitors for use in vivo. They elegantly applied the use of fluorogenic ABPs—probes that only become fluorescent upon ligation to

their target cathepsin [18]—to even the in vivo imaging of cathepsins [19] and other cysteine proteases [20]. In this volume, Edgington-Mitchell et al. describe the application of such quenched probes to the ABPP of cysteine proteases [Chapter 11].

3 The Architecture of an ABP

These papers highlight the essential components of an ABP: an electrophilic trap to react with an enzyme active site nucleophile, a—more or less selective—targeting moiety, a linker, and a detectable group. The chemical warhead is a group that reacts with the active site of the target enzyme families. When designing an ABP, it is essential that a warhead with suitable reactivity is chosen: If a selective ABP is required, warheads of modest reactivity towards their target nucleophile are required. If a very broad class of enzymes is to be labeled, a higher reactivity is useful. Two examples at either end of this activity spectrum are the alkyne warheads [11, 12] (very unreactive) and the iodoacetamides used for reactive cysteine profiling [21]. In the former examples, essentially unreactive groups (alkynes) were found to react with active-site cysteines when attached to very high affinity substrates, such as ubiquitin [11] (for the deubiquitinases), SUMO [12] (for the SUMOylases) and an interleukin-1 fragment (for the Caspases) [11]. These alkyne warheads were found to be so unreactive that they were unreactive for example during conjugation to resins. This allowed the covalent pulldown of deubiquitinases followed by their essentially background-free proteomic identification.

At the other end of the reactivity spectrum Weerapana et al. [21] reported the use of untargeted iodoacetamide probes. Rather than attempting the retrieval/identification of specific cysteine hydrolases, they used minute amounts of iodo-acetamide equipped ABPs to quantify cysteines based on their reactivity, rather than abundance. Combination of multiple probes with different mass tags, allowed the comparative ranking of this reactivity. This approach also allowed the retrieval of reactive cysteines that did not function as active site nucleophiles. A method describing this approach is included in this volume [Chapter 2].

In the more usual cases, a balance between warhead reactivity and selectivity of the targeting motif is to be found. This is best highlighted using an early example of deubiquitinase profiling. Borodovsky et al. used ubiquitin carrying different reactive groups at the ubiquitin C-terminus and found that different deubiquitinase families reacted differently with each of the warheads, with the OTU-DUBs only reacting with bromoethyl electrophiles [22].

Since its inception in the mid to late-90s, the field of ABPP has burgeoned, and this will result in increased acceptance by the "mainstream" biological and biomedical communities [23].

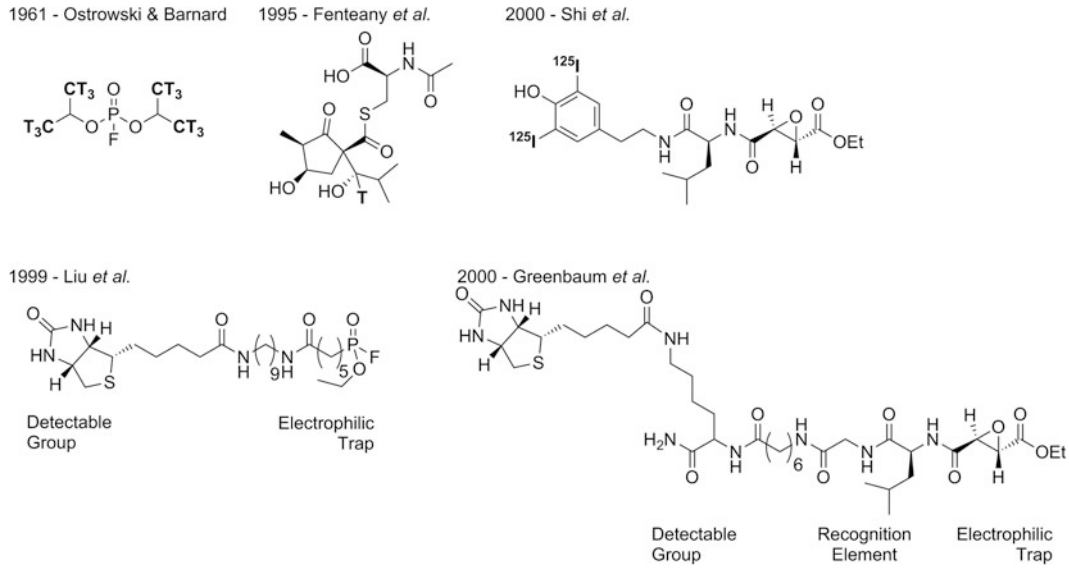

Fig. 1 Examples of activity-based probes. Early examples used radioacitivity as the detectable group (top row), whereas 2nd generation activity-based probes relied on retrievable biotin-modified probes. The design of all probes remains unaltered: a warhead capable of forming a covalent linkage is linked to a selectivity-inducing group, which is again attached to the detectable group

This volume covers a broad spectrum of developments in the field, that can be grouped in three main areas: development of ABPP strategies for difficult-to-target enzyme classes, new developments in the chemical versatility of the probes and new methodological advances that have allowed the increased application of ABPP to drug discovery, biological exploration; even in vivo(Fig. 1).

4 Development of New Classes of Enzyme Inhibitors

The development of new warheads has also switched focus to enzymes that do not carry as strongly nucleophilic residue in their active site. Traditionally, ABPP strategies have focused on targeting enzymes with highly nucleophilic active site residues, such as the aforementioned cathepsins and serine hydrolases. However, in recent years an increasing application of the approach to enzymes that are not so nucleophilic has been described. Examples are the recently reported ABPs for various glycosidases (reviewed in [24]). Methods for the ABPP of harder to target enzymes, such metalloproteases [Chapter 8], flavin-dependent oxidases [Chapter 7] and esterases [Chapter 6] are described in this volume, as are the modern equivalents of the use of inhibitors, namely natural product derived ABPs, to identify targets of these enzymes in human cell lysates [Chapter 3].

5 Development of Probe Chemistries

ABPs have traditionally consisted of an electrophilic trap capable of covalently attaching to an enzyme (family)-target, attached to a chemical group imbuing varying levels of selectivity on the warhead. These selectivity-imbuing groups are then attached via a linker to a group that can be used to image or identify the enzyme target through for example biotin-mediated affinity purification or fluorescent imaging combined with SDS-PAGE. However, many of these detectable groups and linkers rendered the probes cell impermeable. This has been overcome by the elegant application of bio-orthogonal ligation to ABPP using for example the copper-catalyzed Huisgen cycloaddition [25], or the Staudinger–Bertozzi ligation [26]. Instead of directly attaching the detectable group, the ABP was equipped with a bio-orthogonal reactive group, such as an azide or alkyne. After incubation of these bio-orthogonal probes that are cell permeable with live cells followed by lysis, the detectable group could be attached to the probe after the reaction. This approach has many advantages. Not just the increased cell permeability, but also the small size of the probe also impacts less on the binding properties of the warhead-targeting part of the ABP. The use of a bio-orthogonal handle also expands the versatility: a single probe can be used to either attach a retrievable group or an imaging group. In this volume, the 2-step labeling of the proteasome is described by Paniagua et al. [Chapter 15].

Another area that has been subject of intensive development has been the linker: the development of cleavable linker strategies to link the probes to the retrieval handle has greatly facilitated the analysis of targets of a probe or a particular drug by proteomics as—rather than attempting to break the very strong biotin-streptavidin interaction—targets can be released using selective photo, chemical or enzymatic release methods. In this volume, the use of cleavable linkers is described by the group of Verhelst [Chapter 14], as is the use of such cleavable linkers even in semi-permeabilized cells [Chapter 13]

6 Application Development

The developments regarding the breadth and depth of the probes available for ABPP have resulted in great strides in the application of ABPP to biological and medicinal questions. In the latter field it has allowed a paradigm shift in the way drug discovery can be done: from starting with a target in attempting to improve the selectivity for these targets, a drug can be used as the starting point for development. Using broad-spectrum ABPP in combination with a lead compound, the targets and off-targets of this

compound can rapidly be identified, as explored by for example Baggelaar et al. in this volume [Chapter 12] in the lipid metabolism pathway, and by Chandrasekar et al. to identify new enzyme inhibitors using ABPP [Chapter 4]. When combined with drug optimization, this can then be used to either correlate drug phenotype with inhibition of a particular (combination) of target enzyme(s). Combining this approach with a medicinal chemistry development allows the preclinical discovery and elimination of side effects which should result in a reduced late-stage dropout rate during pharmaceutical development [23]. This cannot just be done in lysates and mammalian cells, but can even be used to identify new drug target enzymes in prokaryotes and pathogens such as malaria [27]. In this volume Krysiak et al. describe the use of ABPP to image enzyme activities in bacteria [Chapter 5].

The early historic examples and these recent exciting developments mean that the field of ABPP is increasingly becoming a mainstream approach. This "Methods in Molecular Biology" volume is therefore a timely and exciting collection of some of the forerunning techniques.

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Part I

Explorative Activity-Based Proteomics

Chapter 2

A Quantitative Mass-Spectrometry Platform to Monitor Changes in Cysteine Reactivity

Yu Qian and Eranthie Weerapana

Abstract

Cysteine residues on proteins serve diverse functional roles in catalysis and regulation and are susceptible to numerous posttranslational modifications. Methods to monitor the reactivity of cysteines within the context of a complex proteome have facilitated the identification and functional characterization of cysteine residues on disparate proteins. Here, we describe the use of a cysteine-reactive iodoacetamide probe coupled to isotopically labeled, cleavable linkers to identify and quantify cysteine-reactivity changes from two biological samples.

Key words Cysteine-reactivity, isoTOP-ABPP, Cu + click, Iodoacetamide-alkyne, Chemically cleavable azobenzene linker, MudPIT

1 Introduction

Cysteine, one of the least abundant amino acids found in many organisms, is unique due to its intrinsically high nucleophilicity and sensitivity to oxidative modifications. These physicochemical properties enable cysteine to play critical roles in nucleophilic and redox catalysis, allosteric regulation, metal binding, and structural stabilization [1, 2]. Additionally, these functional cysteine residues are susceptible to a variety of posttranslational modifications (PTMs), including nitrosation, oxidation, palmitoylation, prenylation, and Michael additions to oxidized lipids [3–7]. The endogenous functions of a wide array of proteins (e.g., caspases, GAPDH, EGFR) [8–10] are known to be modulated through these oxidative and lipid-derived cysteine PTMs. To better understand the ubiquity of cysteine PTMs and unearth novel regulatory functions for cysteines, proteomic methods to monitor changes in cysteine reactivity are essential.

Several proteomic methods exist for specifically monitoring each of the cysteine PTMs mentioned above, including selective probes for sulfenic acids [11], the biotin-switch technique [12], and bioorthogonal lipid reporters [13]. Other more general

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techniques that quantitatively reports on global changes of cysteine reactivity are able to report on any PTM that alters the nucleophilicity of the cysteine thiol. One such technology, termed isoTOP-ABPP, applies chemical probes and quantitative mass spectrometry (MS) to identify and quantify the relative abundance of hundreds of reactive cysteines within a proteome [14]. An iodoacetamide-alkyne (IA) probe (Fig. 1a) is used to covalently tag reactive cysteines within a proteome, and these probe-labeled proteins are then enriched and analyzed by quantitative MS with the aid of an isotopically labeled, cleavable linker that is incorporated through copper-catalyzed azide-alkyne cycloaddition (CuAAC) [15]. This method has been successfully applied to rank cysteine residues in the human proteome by reactivity with an iodoacetamide electrophile [14], to identify bacterial proteins sensitive to peroxide-mediated oxidation [16] and to reveal cysteine residues susceptible to Michael addition reactions with lipid-derived electrophiles [17]. We recently reported a variation of the isoTOP-ABPP platform that utilizes chemically cleavable azobenzene (Azo) linkers (Fig. 1b) in place of the tobacco etch virus (TEV) protease-cleavable linkers in the original report [18]. The Azo linkers incorporate either light (Azo-L) or heavy (Azo-H) stable-isotope signatures that enable comparative and quantitative profiling by mass spectrometry. We have successfully applied the Azo platform to monitor cysteine-reactivity changes within a complex proteome

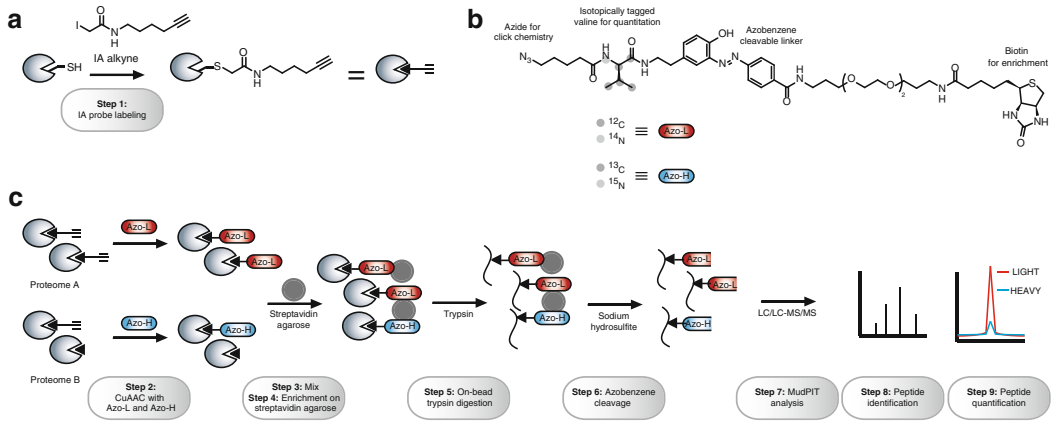

Fig. 1 The reagents and experimental steps of the Azo platform to identify and quantify reactive cysteines from two biological samples. (**a**) **Step 1** involves the addition of an iodoacetamide-alkyne (IA) probe to covalently modify reactive cysteines within a proteome. (**b**) The Azo tags contain an azide for click chemistry, an isotopically tagged valine for quantification, an azobenzene cleavable linker, and a biotin for enrichment. Azo-L and Azo-H have a mass difference of 6 Da. (**c**) The IA-labeled proteome is first subject to CuAAC to incorporate Azo-L and Azo-H (**Step 2**). The resulting samples are mixed together (**Step 3**) and subject to streptavidin enrichment (**Step 4**), on-bead trypsin digestion (**Step 5**), and sodium hydrosulfite treatment (**Step 6**). The resulting peptide mixture is analyzed by MudPIT (**Step 7**) and peptides are identified (**Step 8**) and quantified (**Step 9**)

[18], and identify cysteine residues that are regulated through Zn^{2+} binding [19]. Here, we provide the detailed protocol for applying this Azo platform to quantitatively monitor changes in cysteine reactivity in two biological samples.

The Azo platform (Fig. 1c) begins with labeling of two proteome samples (e.g., healthy and diseased mouse liver proteomes) with the IA probe (**Step 1**). Probe-labeled proteins are then conjugated to either Azo-L or Azo-H linkers using CuAAC (**Step 2**). The resulting proteomes are then mixed together (**Step 3**) and subjected to enrichment on streptavidin agarose beads (**Step 4**), on-bead trypsin digestion (**Step 5**) and chemical cleavage of the azobenzene linker with sodium hydrosulfite (**Step 6**). The resulting isotopically labeled peptides are analyzed by multidimensional protein identification technology (MudPIT) [20] on a high-resolution Orbitrap Mass Spectrometer (Thermo Scientific) (**Step 7**). Peptide identifications are obtained for every IA-labeled peptide using SEQUEST (**Step 8**) [21], and quantification of the relative abundance of each peptide in the two biological samples is achieved using CIMAGE [14] to generate a light–heavy ratio (R) (**Step 9**). In a typical experiment, approximately 1000 cysteines can be identified from a mammalian proteome and changes in the reactivity of these cysteines induced by a particular oxidative or electrophilic stress can be concurrently monitored.

2 Materials

2.1 Proteome Preparation, IA Labeling, and CuAAC Components

1. Phosphate-buffered saline (PBS) (Mediatech, Manassas, VA, USA).
2. Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA).
3. IA probe (10 mM solution in DMSO) (*see* **Note 1**).
4. Tris 2-carboxyethyl phosphine (TCEP) (50 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA) (*see* **Note 2**).
5. Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine ("ligand") (1.7 mM solution in DMSO:t-butanol 1:4) (Sigma-Aldrich, St. Louis, MO, USA).
6. Azo-L/Azo-H (5 mM solutions in DMSO) (*see* **Note 3**).
7. Copper (II) sulfate (50 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA).
8. Sodium dodecyl sulfate (SDS) (0.2 and 1.2 % solutions in PBS) (Bio-Rad, Hercules, CA, USA).

2.2 Streptavidin Enrichment Components

1. Streptavidin agarose beads (Thermo, Rockford, IL, USA).
2. Urea (2 M and 6 M solution in PBS) (Sigma-Aldrich, St. Louis, MO, USA).

3. Dithiothreitol (DTT) (200 mM solution in water) (Invitrogen, Carlsbad, CA, USA).
4. Iodoacetamide (400 mM solution in water) (Acros, NJ, USA).

2.3 On-Bead Trypsin Digestion and Sodium Hydrosulfite-Cleavage Components

1. Calcium chloride (100 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA).
2. Trypsin solution: 20 μg of sequencing-grade trypsin reconstituted in 40 μL of the trypsin buffer provided in package (Promega, Madison, WI, USA).
3. Bio-Spin columns (Bio-Rad, Hercules, CA, USA).
4. Formic acid (Fisher Scientific, Fair Lawn, NJ, USA).
5. Screw-top Eppendorf (Bio-Rad, Hercules, CA, USA).
6. Sodium hydrosulfite (50 mM solution in PBS) (Sigma-Aldrich, St. Louis, MO, USA).

2.4 Mass-Spectrometry Analysis

1. Fused-silica capillary tubing (100 and 250 μm internal diameter) (Agilent, Santa Clara, CA, USA).
2. Aqua C18 reverse-phase resin (Phenomenex, Torrance, CA, USA).
3. Partisphere strong cation exchange (SCX) resin (Phenomenex, Torrance, CA, USA).
4. Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid.
5. Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid.
6. Buffer C: 95% water, 5% acetonitrile, 0.1% formic acid, 500 mM Ammonium acetate.

3 Methods

3.1 Proteome Preparation, IA Labeling and CuAAC

1. Homogenize tissue or cell pellets (Samples A and B) in PBS, pH 7.4 and sonicate for 30 s. Centrifuge at 200,000 × *g* for 45 min to obtain soluble proteomes (Proteomes A and B) and measure protein concentrations using the Bio-Rad DC protein assay kit according to manufacturer's instructions.
2. Dilute Proteomes A and B to 2 mg/mL solutions in PBS (*see* **Note 4**). Add 500 μL of Proteomes A and B to each of two 1.5 mL Eppendorf tubes (4 tubes total). Add 5 μL of 10 mM IA probe solution to each 500 μL sample to achieve a final concentration of 100 μM. After addition of the probe, vortex and incubate the reaction at room temperature (RT) for 1 h.
3. Add 10 μL of 5 mM Azo-L (Proteome A) and Azo-H (Proteome B) stocks, 10 μL of fresh 50 mM TCEP solution (*see* **Note 2**), 30 μL of 1.7 mM "ligand" solution and 10 μL of 50 mM copper (II) sulfate to each tube and vortex. Incubate the reaction at RT for 1 h with vortexing every 15 min.

At this stage, the proteins will start to precipitate and the solution will turn cloudy.

4. After the one-hour incubation, combine the tubes pairwise (one Azo-L sample is combined with one Azo-H sample), and centrifuge for 4 min at 6500 × *g* at 4 °C. Remove the supernatant, add 500 μL cold methanol to each tube and sonicate for several seconds until the pellet is completely solubilized. Combine tubes pairwise again and centrifuge for 4 min at 6500 × *g*. Repeat methanol wash one more time (*see* **Note 5**).

3.2 Streptavidin Enrichment

1. Add 1 mL 1.2% SDS/PBS to the final pellet, sonicate for several seconds until the solution turns clear and heat to 80–90 °C for 5 min. Transfer the 1 mL sample to a 15 mL conical tube containing 5 mL PBS. The final concentration of SDS in the sample is 0.2%.
2. Aliquot ~100 μL of the streptavidin agarose beads into an Eppendorf tube and wash three times with 1 mL PBS. Transfer the washed beads to the conical tube containing the proteome sample and incubate at 4 °C overnight with constant rotation.

3.3 On-Bead Trypsin Digestion

1. After the overnight 4 °C incubation, warm the sample by rotating at RT over 1–2 h. Centrifuge at 1400 × *g* for 3 min and remove the supernatant. Wash the beads with 5 mL of 0.2% SDS/PBS, rotate for 10 min, spin at 1400 × *g* for 3 min and remove the supernatant.
2. Wash the beads with 5 mL PBS, gently shaking for several seconds to resuspend the beads, centrifuge at 1400 × *g* for 3 min, and follow by removal of the supernatant. Repeat the PBS wash two more times.
3. Wash the beads with 5 mL water three times as described in **step 2** (*see* **Note 6**).
4. Transfer the beads to a screw-capped Eppendorf tube using 500 μL of 6 M urea/PBS. Add 25 μL freshly made 200 mM DTT solution and incubate at 65 °C for 20 min with mixing every 10 min. Cool the tube for 1–2 min and add 25 μL of 400 mM iodoacetamide solution and incubate at 37 °C for 30 min with rotation (*see* **Note 7**).
5. Dilute the reaction by adding 950 μL of PBS, centrifuge at 1400 × *g* for 2 min and remove the supernatant. Add a premixed solution of 200 μL of 2 M urea/PBS, 2 μL of 100 mM calcium chloride, and 4 μL of trypsin solution. Incubate the reaction at 37 °C overnight with rotation (*see* **Note 8**).

3.4 Sodium-Hydrosulfite Cleavage

1. After overnight incubation, transfer the supernatant and beads to a Bio-Spin column and separate the beads by centrifugation at 1000 × *g* for 2 min. Wash the beads with 500 μL PBS 3 times and

500 μL water three times. Transfer the beads to a screw-capped Eppendorf tube using water, spin down the beads and remove the supernatant.

2. Add 75 μL 50 mM freshly made sodium hydrosulfite solution to the beads, rotate for 1 h at RT, centrifuge at 1400 × *g* for 3 min and collect the supernatant (*see* **Note 9**).
3. Repeat **step 2** two more times (*see* **Note 10**).
4. Wash beads with 100 μL of water and combine the wash with collected supernatant from **steps 2** and **3**. The final sample volume should be around 325 μL.
5. Add 17.5 μL of formic acid and store the sample at −20 °C until mass-spectrometry analysis.

3.5 Mass Spectrometry Analysis Using MudPIT

1. Cut ~45 cm of the 100 μm fused-silica capillary tubing, burn off ~3 cm of the polyimide coating in the middle and wipe the burnt coating with methanol to expose the underlying silica. Generate two 5 μm tips using a laser puller to afford the columns for MS analysis. Pack each column with 10 cm of Aqua C18 reverse-phase resin and 3 cm of strong cation exchange (SCX) resin.
2. Cut 12 cm of the 250 μm fused-silica capillary tubing and generate a desalting column by connecting the tubing with an inline microfilter assembly. Pack the desalting column with 4 cm of Aqua C18 reverse phase resin.
3. Equilibrate the tip and the desalting column from **steps 1** and **2** on an Agilent 1100 series HPLC using a gradient of 40% Buffer A; 60% Buffer B to 100% Buffer A; 0% Buffer B over 30 min. Flow rate is set at 0.1 mL/min and a tee splitter is used to reduce the flow rate to 300–400 nL/min.
4. Thaw the MS sample and pressure load onto the equilibrated desalting column. Combine the loaded desalting column with the equilibrated tip and align onto a nanospray stage attached to an Orbitrap XL mass spectrometer (Thermo Scientific).
5. Elute the peptides using five steps (Table 1). The flow rate through the column is set to ~0.25 μL/min and the spray voltage is set to 2.75 kV. One full MS scan (FTMS) (400–1800 MW) was followed by eight data dependent scans (ITMS) of the *n*th most intense ions with dynamic exclusion enabled (repeat count = 1; exclusion list size = 300; exclusion duration = 30s).

3.6 Data Analysis

1. The tandem MS data is searched against a protein sequence database using the SEQUEST algorithm for peptide identification [21].
2. A static modification of +57.02146 on cysteine is specified to account for iodoacetamide alkylation and differential

Table 1
Solvent systems utilized for MudPIT analysis

Step 1: 0% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
0	100	0	0
5	100	0	0
60	55	45	0
70	0	100	0
100	0	100	0

Step 2: 50% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
0	100	0	0
6	100	0	0
6.1	45	5	50
8	45	5	50
8.1	95	5	0
15	85	15	0
35	75	25	0
75	45	55	0
80	45	55	0

Step 3: 80% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
0	100	0	0
5	100	0	0
5.1	15	5	80
8	15	5	80
8.1	95	5	0
18	85	15	0
63	75	25	0
115	45	55	0

(continued)

Table 1
(continued)

Step 3: 80% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
120	45	55	0

Step 4: 100% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
0	100	0	0
4	100	0	0
4.1	0	0	100
20	0	0	100
20.1	93	7	0
25	85	15	0
100	70	30	0
184	0	100	0
194	0	100	0
195	100	0	0
200	100	0	0

Step 5: 100% Buffer C			
Time (min)	**% Buffer A**	**% Buffer B**	**% Buffer C**
0	100	0	0
4	100	0	0
4.1	0	0	100
14	0	0	100
14.1	93	7	0
30	70	30	0
50	0	100	0
55	0	100	0
56	100	0	0
60	100	0	0

modifications of +456.2849 (Azo-L modification) and +462.2987 (Azo-H modification) are specified on cysteine to account for probe modifications.

3. SEQUEST output files are filtered using DTASelect 2.0., where reported peptides are required to be fully tryptic and contain the desired probe modification. Discriminant analyses are performed to achieve a peptide false-positive rate below 5 %.
4. Quantification of light/heavy (Azo-L/Azo-H) ratios (R) is performed using the CIMAGE quantification package as previously described [14].

4 Notes

1. The IA probe is made in-house [14].
2. TCEP should be stored at 4 °C and the solution made fresh immediately prior to the CuAAC step.
3. Azo-L / Azo-H tags are made in-house [18].
4. When comparing cysteine reactivity in two different proteomes, ensure that the two proteome samples are accurately normalized in terms of total protein concentrations.
5. This step should be performed at 4 °C or on ice and the methanol should be prechilled at −20 °C.
6. In the last water wash, carefully remove the supernatant using a pipette. The beads should appear faintly yellow tinted at this stage due to enrichment of the Azo-tagged proteins.
7. All reagents used in this step should be made fresh. Avoid vortexing and flip the tube gently instead to resuspend the beads.
8. Dilute the freshly made 6 M urea/PBS to 2 M urea/PBS using PBS. The trypsin solution should be made directly before addition to the sample and is good for reuse within a week if stored at 4 °C.
9. 50 mM sodium hydrosulfite solution should be made fresh with PBS. Using water instead of PBS slightly reduces the cleavage efficiency.
10. After incubating with sodium hydrosulfite solution, the beads should turn white as an indication of successful cleavage of the azobenzene linker.

5 Results

To demonstrate the quantitative accuracy of the Azo platform, Azo-L and Azo-H tagged mouse liver proteomes were mixed in predetermined light–heavy ratios of 5:1, 2:1, 1:1, 1:2, and 1:5.

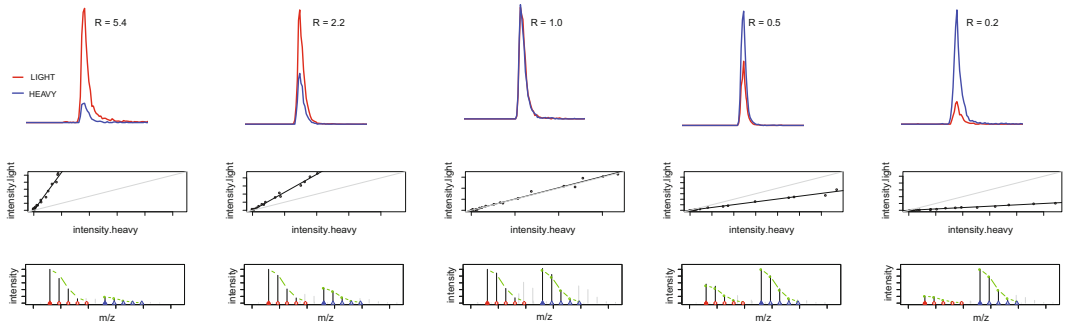

Fig. 2 Representative data for one cysteine-containing peptide (K.AYDATC*LVK.A from S-formylglutathione hydrolase (ESD)) identified in an experiment in which Azo-L and Azo-H labeled proteomes were mixed together in 5:1, 2:1, 1:1, 1:2, and 1:5 ratios. The top panel shows the extracted ion chromatograms for the light and heavy species (in *red* and *blue* respectively), with the corresponding calculated light–heavy ratios (R). The middle panel shows plots of the intensity of the light species against the intensity of the heavy species, showing ideal co-elution of the two isotopically labeled peptides. The bottom panel shows the isotope envelope of the light and heavy species, demonstrating an ideal match to the expected isotope pattern (in *green*)

These five samples were subject to enrichment and MS analysis according to the protocol described above. For each cysteine that is labeled by the IA probe, a light–heavy ratio was calculated in each of the five runs. Representative chromatography traces for one labeled peptide (K.AYDATC*LVK.A (where C* represents the cysteine modified by the IA probe) from S – formylglutathione hydrolase (ESD)) is shown in Fig. 2. The co-elution of the light and heavy species, the high signal-to-noise, and the characteristic isotopic peak pattern demonstrate that our methods represent an accurate platform for relative quantification of cysteine reactivity changes from two or more proteomes.

6 Discussion

The field of activity-based protein profiling applies active-site directed chemical probes to inform on the activity of a specific protein family. Here, we present a related technology in the area of "reactivity-based" profiling, where instead of grouping proteins by functional class; we globally profile the reactivity of a particular amino acid. We focus on cysteine due to the abundance of this amino acid in functional loci; as sites of nucleophilic and redox catalysis, metal binding and regulation. The reactivity state of these functional cysteines directly informs on protein activity, and thereby enables the concurrent profiling of hundreds of proteins from diverse functional classes. Due to the susceptibility of cysteine to a variety of oxidative and lipid-based posttranslational modifications, analyzing the abundance of proteins containing functional cysteines

does not often correlate with activity state. For this reason, we describe a chemical proteomic platform that is able to report on changes in cysteine reactivity as a means of identifying cysteine-mediated protein activity changes in two or more biological samples. This method couples the use of a general cysteine-reactive probe to a quantitative mass-spectrometry platform that utilizes an azobenzene cleavable linker for enrichment, selective release and quantification of probe-labeled peptides. Methods such as this, that globally quantify cysteine reactivity changes, can inform on targets of oxidative stress and the consequent protein-activity changes within a biological system.

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Chapter 3

Activity-Based Protein Profiling with Natural Product-Derived Chemical Probes in Human Cell Lysates

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Abstract

Bioreactive natural products represent versatile starting points for the development of structurally unique activity-based probes. In the present protocol, we describe the workflow for an activity-based protein profiling (ABPP) experiment with an alkyne-tagged natural product derivative. Our protocol includes experimental procedures for in vivo labeling, sample preparation and 2-step (click chemistry) visualization and sample preparation for mass spectrometry-based target identification.

Key words Activity-based protein profiling, Click probes, Bioreactive natural products, Target identification

1 Introduction

Bioreactive natural products have proven to be versatile activity-based probes [1–5]. For example, the epoxysuccinyl peptides commonly used to profile cysteine proteases and known as one of the first ABPs introduced to ABPP are derived from the natural product E64 [6, 7]. By now, many other natural product-derived ABPs have already been developed, such as probes for profiling the proteasome which have been derived from the natural products epoxomicin or syringolin [8–10] or PI3 kinase probes from wortmannin. [11] From a practical point-of-view, natural product-derived activity-based probes, i.e., bioreactive natural products that (1) covalently bind to the active site of enzymes, thus displaying activity-dependent labeling and (2) have been synthetically modified with a suitable reporter tag, can be used in a similar fashion as rationally designed probes. The advantage of bioreactive natural products over "classical" probes (derived for example from irreversible mechanistic enzyme inhibitors) is that their complex chemical structures often result in potent and more-or-less enzyme

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(class)-specific labeling [3–5]. Natural products, thus, allow the generation of chemical probes for which rational approaches fail. In addition, bioreactive substructures of natural products may serve as inspiring starting structures for the de novo design of chemically novel activity-based probes [12]. Their disadvantage, however, is the often cumbersome chemical synthesis which often requires extensive chemical manipulations.

The present protocol describes the workflow required to perform activity-based protein profiling of alkyne-tagged natural product-derived ABPs. As a model natural product-derived probe, we chose an alkyne-tagged version of Symplostatin 4, a bioreactive natural product derived from a marine species that has been reported to inhibit cysteine proteases in humans and Malaria (Fig.1) [13–17]. The protocol describes an exemplary in vivo labeling procedure in human cell cultures and subsequent target visualization and/or identification using a two-step labeling protocol involving click chemistry (CuAAC; Cu^{1+}-catalyzed alkyne–azide cycloaddition) [18]. Target identification is then performed by an optimized large scale protocol including purification steps that deliver samples suitable for MS analysis. The chemical synthesis of the alkyne-tagged natural product Symplostatin 4 is not reported here but can be found in ref. [17] as the chemical decoration of natural products with alkyne residues requires synthesis procedures tailored for the investigated natural product and thus cannot be captured in a general protocol. Of note, the provided protocol may not only be used to perform an activity-based protein profiling experiment, but may also serve as a blueprint to determine the direct targets of natural products via chemical proteomics methodologies [3–5, 19, 20].

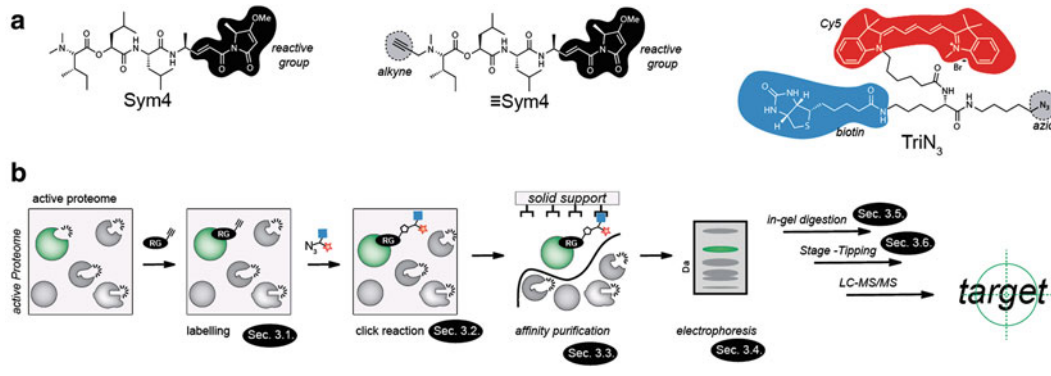

Fig. 1 Overview of the used chemical compounds and general workflow for target identification. (**a**). Chemical structure of the model bioreactive natural product Symplostatin 4 (Sym4), the alkyne-tagged derivative (≡Sym4) and the trifunctional reporter $TriN_3$. (**b**). Schematic overview of the steps involved in 2-step ABPP. The sections in which the different steps are described in detail are indicated (Subheadings 3.1 to 3.6)

2 Materials

2.1 In Vivo Labeling with Natural Com pound Derivative and Sample Preparation for the Click Chemistry Reaction

1. **RPMI 1640 (1×)** media (Gibco by Life Technologies, *see* **Note 1**): 50 mL heat inactivated FBS (Fetal Bovine Serum; Gibco by Life Technologies) and 5 mL Pen Strep (50,000 Units Penicillin, 50 mg Streptomycin; Gibco by Life Technologies) are added to 500 mL RPMI media.
2. Dulbecco's Phosphate Buffered Saline (**DBPS (1×)**; Gibco by Life Technologies).
3. Phosphate buffered saline (**10× PBS**): 30 mM Na_2HPO_4, 10.6 mM KH_2PO_4, and 1552 mM NaCl, pH 7.4. Weigh 1.44 g KH_2PO_4, 90.70 g NaCl, and 4.26 g Na_2HPO_4 and add 800 mL of MS water. After all the salts have been dissolved, adjust the pH (if necessary) to pH 7.4 by adding hydrochloric acid. Fill the solution in a graduated glass cylinder and adjust the volume to 1 L with MS water. Sterile filter the buffer using a 0.22 μM filter unit. The solution can be stored at RT for several months and is used as 1× PBS.
4. Sodium dodecyl sulfate (**10% w/v SDS**): 350 mM SDS. Dissolve 10 g SDS in 80 mL MS water. Stir gently until all SDS is dissolved (*see* **Note 2**) and the solution is clear. Transfer the solution to a graduated glass cylinder and adjust the volume to 100 mL. The solution can be indefinitely stored at RT.
5. E64-d (10 mM): E64-d is a membrane permeable inhibitor of papain-like cysteine proteases. Add 292 μL DMSO to 1 mg E64-d (342.43 g/mol) to obtain a 10 mM solution. Make aliquots and store them at −20 °C for up to half a year.
6. Triton X-100 is a non-denaturizing detergent that helps keep proteins in solution.
7. 7× EDTA-free Protease inhibitor cocktail (**PIC III**) (Roche Diagnostics). The protease inhibitor cocktail is provided in tablet form. Dissolve the tablet in ddH_2O following the manufacturer´s instructions. Aliquots of 20 μL are stored at −20 °C. Please make sure you use the EDTA-free version otherwise the click reaction will not work.
8. **Lysis buffer** (1× PBS, 0.1% w/v SDS, 1% v/v Triton X-100). Add 10 mL 10× PBS, 1 mL 10% w/v SDS stock solution, and 1 mL Triton X-100 to a 250 mL clean glass beaker and add 70 mL ddH_2O. When the solution is homogenous, transfer it to a graduated glass cylinder and adjust the volume to 100 mL with ddH_2O. Prepare 10 mL aliquots and store them at −20 °C. The PIC III is added after the labeling reaction to prevent undesirable inhibition of possible targets by the PIC components.
9. Suitable assay to determine the protein concentration (e.g. Bradford assay; use according to manufacturer's instructions).

10. Dimethyl sulfoxide (**DMSO**). This is a highly polar organic solvent used for dilution and dissolving of the natural small molecule-based probes. Additionally, it is used as negative control.
11. Natural small molecule-based probe modified by the addition of an alkyne group called Symplostatin 4 (≡**Sym4**): The working stock for large scale purification has a concentration of 1 mM in DMSO. All stocks are stored at −20 °C.
12. Activity-based fluorophosphonate probe (≡**FP**) containing an alkyne group. It is used as positive control. The working stock for large scale purification has a concentration of 1 mM in DMSO. All stocks are stored at −20 °C.
13. Solid urea in 400 mg portions.

2.2 Click Chemistry

1. pH indicator sticks (pH range from 1 to 14, e.g., from Merck).
2. Sodium dodecyl sulfate (**10% w/v SDS**): 350 mM SDS. *See* Subheading 2.1.
3. *t*-butanol–DMSO solution (t-**BuOH:DMSO 1:4**): Place the bottle with *t*-butanol (Sigma, 360538) in a water bath set to 40 °C. Over the next 20 min, agitate the bottle several times to help the melting process. Meanwhile, add 40 mL DMSO to a 50 mL Falcon tube. When all *t*-butanol has melted, transfer 10 mL of the liquid with a preheated 10 mL glass pipette (*see* **Note 3**) to the 50 mL DMSO-containing Falcon tube. Close the Falcon tube and mix by inverting the tube several times. Store the solution at room temperature.
4. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) solution (5 mM): Weigh 50 mg of TBTA (Sigma, 678937; solid white powder) into a 50 mL Falcon tube. Add 18.8 mL of the *t*-BuOH–DMSO 1:4 solution and mix thoroughly. It may take some time before everything is dissolved. Once the solution is clear, make 500 µL aliquots and freeze them at −20 °C. The dissolved compound is stable at −20 °C. However, do not use stocks older than 1 year.
5. Tris(2-carboxyethyl)phosphine hydrochloride (**TCEP**) solution (500 mM): Weigh 500 mg of TCEP (Sigma, C4706; solid white crystals) into a 4 mL brown glass screw cap bottle. Add 3.5 mL MS water and mix until all is dissolved. It may take some time before everything is dissolved. Once the solution is clear make 500 µL aliquots and freeze them at −20 °C. The dissolved compound should be stable at −20 °C. However, do not use stocks older than half a year.
6. Copper (II) sulfate (**CuSO4**) solution (50 mM): Weigh 200 mg of $CuSO_4$ (Sigma, C1297; blue crystals) into a 50 mL Falcon tube. Add 25 mL MS water and mix well. The salt will

dissolve quickly. Once the solution is clear (the color is pale blue) make 500 µL aliquots. The solution can be indefinitely stored at −20 °C.

7. Trifunctional azide (**TriN3**) dissolved in DMSO (5 mM). This reporter consists of three functional units: a fluorescent Cy5 group for easy in-gel detection of labeled targets, a biotin group for affinity purification of targets, and an azide group for the click reaction.
8. Ethylene diamine tetraacetic acid (**EDTA**) solution (0.5 M): weigh 46.5 g $Na_2EDTA \cdot 2H_2O$ and transfer it to a 500 mL glass beaker. Add 200 mL MS water and stir the solution vigorously with the help of a magnetic stirrer. When everything is dissolved, check the pH. It will be quite acidic. Start to add NaOH pellets while vigorously stirring until pH 8.0 is reached (*see* **Note 4**). Transfer the solution to a graduated glass cylinder and adjust the volume to 250 mL with MS water. Sterile filter the solution using a 0.22 µm filter unit. The solution can be stored at RT for several months.

2.3 Affinity Purification

1. Disposable **PD-10** desalting columns (GE Healthcare).
2. 1× PBS: Mix 10 mL 10× PBS solution with 90 mL ddH_2O.
3. 0.22 µm filter unit attached to a syringe.
4. Sodium dodecyl sulfate (10% w/v SDS): 350 mM SDS. *See* Subheading 2.1.
5. Equilibrated Avidin beads from Sigma Aldrich (*see* **Note 5**): We usually use 100 µL of the 50% slurry provided by the manufacturer per 1 mg protein or reaction. The beads tend to settle in the bottom of the glass bottle. Shake up the beads and transfer 100 µL of the beads with a cut 200 µL yellow tip to a low binding Eppendorf tube. Add 0.5 mL 1× PBS buffer. Gently mix the beads with the buffer by inverting the tube a couple of times. Spin down briefly (16,000 × *g*, 30 s, RT) and aspirate the supernatant (*see* **Note 6**). Repeat the PBS wash but discard the supernatant only immediately before adding the beads to the sample solution.
6. Equilibration buffer (0.2% w/v SDS in 1× PBS): Mix 300 µL 10% w/v SDS with 15 mL 1× PBS.
7. Washing buffer (1% w/v SDS in 1× PBS preheated to 40 °C): Mix 20 mL 10% w/v SDS with 20 mL 1× PBS and 160 mL MS water in a 250 mL blue capped bottle. Place the bottle in a preheated water bath at 40 °C.

2.4 Electrophoresis and Target Visualization

1. Thermomixer, e.g., HLC Ditabis MHR 23.
2. Bucket of ice.
3. Denaturing SDS Polyacrylamide gel (for protein separation): this can be either self-made or commercial. The percentage of

acrylamide depends on the molecular weight of the protein of interest. We recommend to generally use SDS gels with 10–13% acrylamide. Lower percentage gels are hard to handle, higher percentage gels cause problems during the in-gel digestion. We prefer to make our own gels. We cast the gels between low fluorescent glass plates which facilitates detection of fluorescence in the following step. It is also possible to use plastic cassettes. However, the gels have to be removed from these prior to detection on a fluorescence scanner. This requires more time as the gels must be excessively washed.

4. Electrophoresis buffer compatible with your denaturing gel.
5. Prestained protein ladder: Anyone suitable to your needs preferably with several differently colored bands (*see* **Note 7**).
6. SDS-PAGE gel-loading buffer (4× GLB): 280 mM SDS, 400 mM Tris, 40% glycerol, 1.4 M β-mercaptoethanol, 0.6 mM bromophenol blue, pH 6.8. Add the following components to a 50 mL Falcon tube: 2 g SDS, 10 mL 1 M Tris (pH 6.8), and 10 mL glycerol (*see* **Note 8**). Tumble this solution until all the components are dissolved. Then add 2.5 mL 14.2 M β-mercaptoethanol (*see* **Note 9**) and bring the volume up to 25 mL with water. Finally, add a pinch (~10 mg) of bromophenol blue (*see* **Note 10**). Make 1 mL aliquots and store these at –20 °C.
7. Gel loading tips.
8. KIMTECH paper towels (fluff-free).
9. Laser scanner or imaging camera with appropriate filter system to detect the fluorescent dye of the reporter. We use a Typhoon FLA 9000 Scanner from GE Healthcare. This scanner comes equipped with the filter settings and lasers for Cy2, Cy3, or Cy5 or similar dyes (*see* **Note 11**). Imaging systems are usually less sensitive and they do not allow to image large gels.
10. Transparent autoclave bag.
11. Disposable steel blades (*see* **Note 12**).
12. Typhoon Scanner (GE Healthcare).

2.5 In-gel Digestion

1. Ammonium bicarbonate solution (**ABC**), 100 mM: Dissolve 395 mg ammonium bicarbonate ($(NH_4)HCO_3$) in 50 mL MS-water.
2. TCEP solution (**TCEP**), 10 mM: Dissolve 28.7 mg Tris(2-carboxyethyl)phosphine (TCEP) in 10 mL MS-water (*see* **Note 13**). Prepare immediately before use.
3. Iodoacetamide (**IAA**), 55 mM: weigh 40.7 mg IAA in a 4 mL screw cap brown glass bottle (*see* **Note 14**) and add 4 mL MS-water. Mix until all IAA is dissolved. IAA is sensitive to light. Prepare immediately before use and store in a dark place.

4. Acetonitrile (**ACN**) (*see* **Note 15**).
5. ACN and 100 mM ammonium bicarbonate (50:50 solution): mix 25 mL 100 mM ammonium bicarbonate solution with 25 mL ACN.
6. Ammonium bicarbonate (25 mM): mix 1 mL 100 mM ammonium bicarbonate with 3 mL water.
7. Trypsin (100 ng/μL): Dissolve 20 μg lyophilised trypsin in 200 μL of trypsin resuspension buffer (provided by manufacturer, usually 50 mM acetic acid). Leave on the bench for 15 min to fully reconstitute trypsin. Make 10 μL aliquots in PCR tubes and store these at −80 °C for up to 6 months (*see* **Note 16**).
8. Trypsin (10 ng/μL): thaw a 10 μL aliquot of the 100 ng/μL Trypsin solution. Add 90 μL of the 25 mM ammonium bicarbonate solution and vortex briefly. Use this working stock immediately. Do not store this solution for longer periods (*see* **Note 17**).
9. Formic acid (**FA**, 5%): 1.33 mM FA. Mix 50 μL 100% FA (HCOOH) in 950 μL MS water.
10. Formic acid (**FA**, 0.1%): 0.27 mM FA. Mix 1 μL FA with 999 μL MS water.

2.6 Micro-purification of Samples on C18 StageTips

1. 200 μL C18-StageTips: self-made or commercial (Thermo).
2. Methanol (**MeOH**), 100%.
3. Acetonitrile (**ACN**), 100%. *See* Subheading 2.5.
4. Formic acid (**FA**), 100%.
5. Suitable centrifuge adapters that will hold 200 μL pipette tips (available from GL Sciences Cat.No. 5010 21514).
6. StageTip solution A (**STSA**): is 0.5% (v/v) FA in H_2O. Mix 10 μL FA and 1980 μL MS water in a 2 mL Eppendorf tube. Prepare immediately before use.
7. StageTip solution B (**STSB**): is 0.5% (v/v) FA in 80% (v/v) ACN/H_2O. Mix 10 μL FA, 1600 μL ACN and 390 μL MS water in a 2 mL Eppendorf tube. Prepare immediately before use.
8. LC-MS sample solution (**LCSS**): is 0.1% (v/v) FA in MS water. Mix 1 μL FA and 999 μL MS-grade H_2O in a 1.5 mL Eppendorf tube (*see* **Note 18**). This solution can be stored at room temperature for several weeks.
9. pH indicator sticks.

3 Methods

The methods in the following protocol detail how to perform a gel-based 2-step activity-based protein profiling experiment to determine putative in vivo targets of azide- or alkyne-tagged small

molecules, e.g., those of a natural compound derivative. As a case study, we chose an alkyne derivative of the natural product Symplostatin 4 (≡**Sym4**) as a probe. In order to classify ≡Sym4 targets and as Sym4 has previously been described as an inhibitor of papain-like cysteine proteases, our protocol also features a control reaction with E-64d. This compound is a membrane permeable inhibitor of papain-like cysteine proteases [6, 7] and thus blocks labeling of PLCPs by Sym4.

3.1 In Vivo Labeling with an Alkyne-Tagged Natural Product (Derivative) and Sample Preparation for the Subsequent Click Chemistry Reaction

1. HepG2 cell cultures are seeded in a 10 cm diameter culture dish with 15 mL complemented **RPMI** media and incubated for 16 h at 37 °C, 5 % CO_2.
2. The confluent cells are preincubated with either DMSO (Sample A) or only E64-d (Sample B) for 30 min. Subsequently, ≡Sym4 is added to a final concentration of 20 μM and incubated for 6 h.
3. The confluent cells (90 % confluence) are washed 3× with 5 mL sterile 1× DPBS and harvested with a cell scraper in 500 μL sterile 1× DPBS.
4. The cells are pelleted by centrifugation (1000 × *g*, 4 °C, 5 min) and the supernatant is discarded. In order to lyse the cells they are resuspended in 150 μL lysis buffer and placed on ice for 20 min.
5. The lysates are then cleared by centrifugation (17,000 × *g*, 4 °C, 5 min). The clear supernatant is carefully transferred to fresh Eppendorf tubes (labeled A or B for the two differently treated samples).
6. Next, the protein concentration of the cleared lysates is determined by a Bradford assay with BSA as standard (*see* **Note 19**).
7. Dilute the two protein lysates with 1× PBS to a final protein concentration of 1.0 μg/μL. You need 1 mg total protein extract for each large scale labeling reaction.
8. Add 1 mL of each 1 μg/μL lysate to a separate 4 mL brown glass screw cap vial (*see* **Note 20**).
9. Label the vials adequately (in our case A and B).
10. Add 400 mg solid urea to each reaction and mix until all the urea is dissolved (final urea concentration is around 6 M). The addition of urea denatures proteins and facilitates the subsequent click reaction. Urea is also required to keep proteins in solution during the following click reaction (*see* **Note 21** and Subheading 3.2).

3.2 Click Chemistry

1. Once the labeling reaction has been stopped by addition of urea, check the pH of the reaction solution. Use an indicator stick. The pH should be between 7 and 8 (*see* **Note 22**).

2. Add 20 μL of the 1 mM **TriN3** reporter (final concentration should be around ~20 μM) to each of the sample vials and vortex briefly.
3. Add 10 μL of the 100 mM **TCEP** solution (final concentration in this case is ~1 mM) and vortex briefly (*see* **Note 23**).
4. Add 20 μL of the 5 mM **TBTA** solution (final concentration in this case is ~100 μM) and vortex briefly.
5. Start the click reaction by adding 20 μL of the 50 mM **CuSO4** solution (final concentration is around 1 mM) (*see* **Note 24**) and vortex briefly. Incubate the reaction in the dark (*see* **Note 25**) at room temperature for 1 h, while gently shaking. Occasionally, you may observe the formation of a light cloudy precipitate (*see* **Note 26**) which will, however, vanish upon addition of EDTA in the next step.
6. Stop the reaction by adding 20 μL of the 0.5 M EDTA solution (final concentration ~10 mM) and vortex briefly (*see* **Note 27**).

3.3 Affinity Purification

1. Equilibrate PD-10 columns with 2× 15 mL 1× PBS (this can be done conveniently while the click reaction is running).
2. Once the click reaction has been stopped by adding EDTA (*see* Subheading 3.2.) dilute the samples with 1× PBS up to a final volume of 2.5 mL. The solution should be clear at this stage.
3. Apply the samples to the top of the equilibrated PD-10 column and let it enter by gravity. Discard the flow through.
4. Meanwhile add 183 μL of 10% w/v SDS solution to fresh 15 mL Falcon tubes. When all the reaction solution has entered the PD-10 columns, add 3.5 mL 1× PBS to the top of the PD-10 columns and collect the flow through in the SDS containing Falcon tubes (the final SDS concentration at this step is around 0.6% w/v SDS)
5. Boil 250 mL water in a 500 mL glass beaker (use a microwave oven). Take the beaker out of the microwave and place the Falcon tubes containing your diluted protein solutions in the boiling water (*see* **Note 28**) and incubate for 10 min (*see* **Note 29**). Invert the tubes from time to time.
6. Stop the incubation by placing the Falcon tubes on ice for 1 min. Do not place the tubes on ice for longer or SDS may precipitate.
7. Add to each tube 5 mL 1× PBS. The protein concentration is now around 0.1 mg/mL, the SDS concentration around 0.2% and the final volume around 8.5 mL.
8. Start the affinity purification by adding the equivalent of a 100 μL aliquot (50 μL bed volume) of 1× PBS equilibrated avidin beads to each of your samples.

9. Incubate for 1 h at RT by gently inverting the tube (*see* **Note 30**).
10. Collect the beads by centrifugation (400 × *g*, 3 min, 15 °C, swinging out rotor; *see* **Note 31**) and remove the supernatant with a 10 mL disposable pipette (*see* **Note 32**).
11. Add 10 mL washing solution (1 % w/v SDS in 1× PBS) to the beads and resuspend them by inverting the tube several times (*see* **Note 33**). Incubate at RT for 10 min by gently inverting the tube.
12. Collect the beads by centrifugation (400 × *g*, 3 min, 15 °C) and remove the supernatant with a 10 mL disposable pipette.
13. Repeat the washes with the washing solution (*see* **steps 11** and **12**) four more times.
14. Meanwhile label two fresh 1.5 mL Eppendorf tubes with "A" and "B".
15. Remove the last wash from the Falcon tubes. In each tube should be around 1 mL liquid and beads. Disperse the beads by pipetting up and down with a blue, cut-off pipette tip. Quickly transfer the beads to a fresh Eppendorf tube. Wash the Falcon tube with 500 µL 1× PBS and transfer the solution to the appropriate Eppendorf tube.
16. Collect the beads by centrifugation (16,000 × *g*, 1 min, 15 °C) and remove the supernatant carefully.
17. Wash the beads twice with water. This step is important as it will remove excess SDS from the samples. Too much SDS has adverse effects on the subsequent electrophoresis.

3.4 Electrophoresis and Target Visualization

1. Dilute an aliquot of the 4× GLB with an equal amount of MS water to a 2× GLB stock. Add 30 µL of the 2× GLB to your affinity purified samples and incubate for 10 min at 90 °C while vigorously shaking.
2. Place the samples 10 s on ice to cool them down (*see* **Note 34**).
3. Centrifuge the samples (16,000 × *g*, 1 min, RT). Insoluble matter and the beads will be concentrated at the bottom of the tubes.
4. Prepare your SDS PAGE gel and load 10 µL of a fluorescent marker in the first and the last lane of your gel.
5. Directly transfer the supernatant from **step 3** which contains the affinity purified and denatured proteins to your assembled SDS PAGE. Use a gel loader tip. Make sure that you do not transfer any beads. Then fill all empty lanes with 1× GLB.
6. Start the electrophoresis using the parameters suitable for your equipment (*see* **Note 35**). Cover the whole apparatus with a box to protect the fluorescent dye from bleaching.
7. When the electrophoresis is finished, take the gel out of the running chamber and rinse it with distilled water to remove buffer components, especially SDS and adhering gel splits and pieces.

8. Wipe the gel cassette dry with a KIMTECH paper towels (fluff-free) and proceed immediately with the in-gel detection of fluorescently labeled proteins.
9. Place the glass sandwich on the MultiStage of the Typhoon FLA 9000 scanner and scan the gel with the Cy5 settings (*see* **Note 36**). Make sure that you save the GEL and TIF image. The TIF image can be opened in for example Adobe Photoshop™ for further processing.
10. Open the saved TIF picture in Photoshop and enhance the contrast. This is necessary as often fluorescent bands are very faint. Adjust the intensity until you are satisfied with the visibility of your targets (e.g., Sym4 targets in Fig. 2a) and print the image without size compensation.
11. Mark the bands you want to cut out on the print-out. The lane where the sample A was loaded is labeled "A" and the bands are consecutively labeled, e.g., from 1 to 6 if you want to cut out 6 gel regions. Do the same for the samples loaded in lane "B".
12. Place the print-out on a solid support (bench-top) and mark the bands you want to cut out with a pencil or marker. Then cover the print-out with a new, transparent disposable autoclave bag.
13. Carefully pry the gel chamber open and remove the cover plate. The gel usually sticks on one gel plate. Take care that it is not moved during this operation to prevent gel breaking.

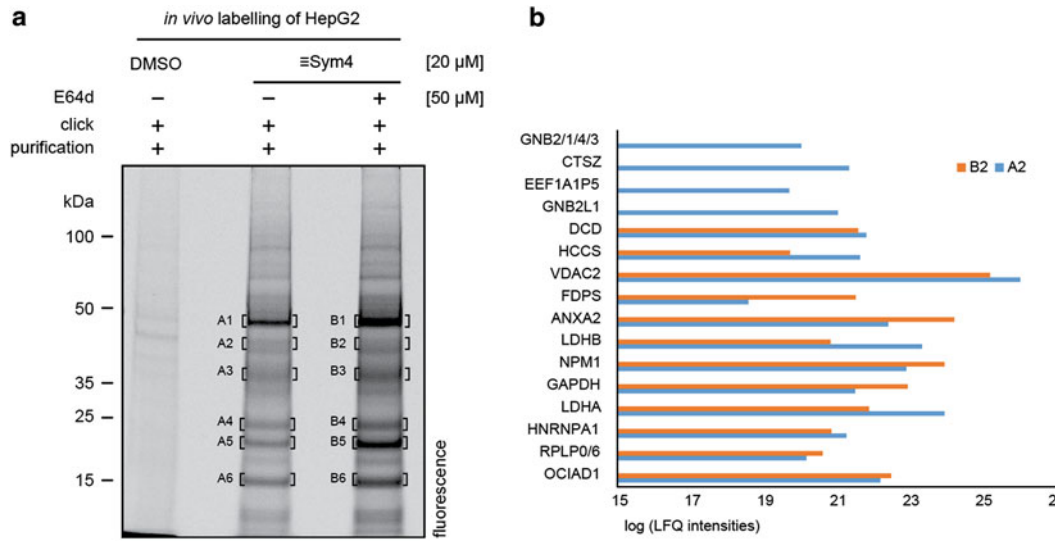

Fig. 2 Chemical proteomics analysis of the targets of ≡Sym4. (**a**) Fluorescent gel image after affinity purification and electrophoresis. The gel areas that were excised and subsequently prepared for MS analysis are indicated with *brackets*. (**b**) Overview of the logarithmic label-free quantification intensities (log (LFQ intensities)) reported by MaxQuant [33–35], exemplarily shown for samples A2 and B2. For many proteins only small intensity changes are observed. For some proteins LFQ values were not determined upon treatment with E64-d. This could indicate that the targets are PLCPs

14. Place the "opened" gel-cassette with the gel on top of the autoclave bag (gel looking up) and align the gel and the printout. Use the marker bands as a guide. Once gel and image are aligned, keep the contraption fixed.
15. Excise the marked bands of your gel with a new disposable steel blade (*see* **Notes 37** and **39**) and transfer the gel piece to an autoclave bag. Cut the gel slice in small pieces (*see* **Note 38**) and transfer these pieces to adequately labeled 1.5 mL Eppendorf tubes.

3.5 In-gel Digestion

1. Wash the gel pieces twice with 500 µL water for 15 min while vigorously shaking (*see* **Note 40**). Briefly centrifuge (10 s, 16,000 × *g*) and discard the supernatant.
2. Wash the gel pieces twice for 5–10 min with 500 µL 100 mM ammonium bicarbonate (*see* **Note 41**) while vigorously shaking. Briefly centrifuge (10 s, 16,000 × *g*) and discard the supernatant.
3. Add 200 µL 10 mM TCEP or an amount sufficient to completely cover the gel slices. Incubate at 62 °C for 30 min (*see* **Note 42**). Briefly centrifuge (10 s, 16,000 × *g*) and discard the supernatant.
4. Add 200 µL 55 mM IAA (*see* **Note 43**) to the gel pieces. The gel pieces have to be completely covered by the IAA solution. Tumble gently in the dark for 30 min at RT. Briefly centrifuge (10 s, 16,000 × *g*, RT) discard the supernatant.
5. Wash the gel slices three times 5–10 min with 500 µL 50:50 ACN: 100 mM ammonium bicarbonate (*see* **Note 44**) while vigorously shaking. Briefly centrifuge (10 s, 16,000 × *g*, RT) and discard the supernatant.
6. Add 50 µL 100% ACN (*see* **Note 45**) to dry gel slices (they become completely white). Remove ACN and place your samples with open lids in a vacuum concentrator (SpeedVac). Dry the samples for 5 min (*see* **Note 46**).
7. Add 20 µL of the 10 ng/µL trypsin solution to the gel slice and incubate for 10 min at RT (*see* **Note 47**).
8. Completely cover the slices with 25 mM ammonium bicarbonate solution and seal the tubes with Parafilm. Incubate overnight under constant shaking at 37 °C (*see* **Note 48**).
9. After 16 h, briefly centrifuge (10 s, 16,000 × *g*, RT) the samples. Transfer the supernatants from to fresh low binding Eppendorf tubes (or equivalent). Do NOT discard this solution as this is the major peptide fraction!
10. Add sufficient 5% formic acid to the gel slices to cover them (~100 µL) and incubate at RT for 15 min (*see* **Note 49**).
11. Carefully remove the supernatants and combine them with the fractions obtained from the overnight digestion (**step 9**).
12. Add around 100 µL ACN to the tubes with the gel pieces or an amount sufficient to cover them and incubate for 15 min at

RT while vigorously shaking. Carefully remove the supernatant and combine it with the supernatant from step 9 and 11.

13. Repeat **step 12** twice more until the gel slices have become opaque (e.g., use 70 µL ACN and finally 50 µL).
14. Indicate with a marker the initial liquid level on the outside of your tube (*see* **Note 50**). Reduce the volume of the combined supernatants in a vacuum concentrator (~3–5 h at 30 °C) to a final volume of 20–30 µL (*see* **Notes 51** and **52**).
15. This concentrated peptide solution is almost ready for MS. It contains all the tryptic peptides, undigested trypsin, salts and it may contain gel pieces. Therefore, this sample can still be considered as quite dirty and has to be further cleaned before MS.

3.6 Stage-Tip Purification

We describe here a protocol for one sample. Keep in mind that you have to use an individual StageTip per sample. You can process all the samples in parallel. This protocol is based on the excellent publication by Rappsilber et al. [21].

1. Place a StageTip in the opening of a 1.5 mL Eppendorf tube (*see* **Note 53**) and insert a C18 StageTip (*see* **Notes 54** and **55**).
2. Transfer 50 µL **MeOH** (*see* **Note 56**) with a pipette immediately on top of the white plug in the StageTip and centrifuge at 400–800 × *g* for 1 min (*see* **Notes 57** and **58**).
3. Add 50 µL **STSB** and centrifuge at 400–800 × *g* for 1 min (*see* **Note 59**).
4. Add 50 µL **STSA** and centrifuge at 400–800 × *g* for 1 min.
5. Add 90 µL **STSA** per 10 µL of your MS samples (*see* **Note 60**), vortex briefly and spin down 10 s 16,000 × *g*. Check with an indicator stick that the pH is around 3.
6. Screw the StageTip holder on a fresh 2 mL tube and place the conditioned StageTip in the hole. Load the peptide solution on the StageTip and centrifuge at 400–800 × *g* for 2 min (*see* **Note 61**).
7. Reapply the flow-through to the StageTip and centrifuge again at 400–800 × *g* for 2 min (*see* **Note 62**).
8. Screw the StageTip holder on a fresh 2 mL tube and place the StageTip with the immobilized peptides in the hole. Screw a clean lid onto the tube containing the flow through; label it properly and save it in case something went wrong with the binding of peptides.
9. Wash peptides by adding 50 µL STSA to the StageTip with immobilized peptides and centrifuge at 400–800 × *g* for 2 min. Repeat this step with another 50 µL STSA. Optional: repeat the washing step a third time with another 50 µL STSA (*see* **Note 63**).

10. Place the StageTip and holder on a clean 1.5 mL low binding Eppi. To elute the bound and washed peptides, add 50 µL STSB and centrifuge at 400–800 × *g* for 2 min and repeat this step a second time (*see* **Note 64**).
11. Place the Eppi with the eluted peptides in a SpeedVac and evaporate ACN until the remaining volume <20 µL (*see* **Note 65**). You can also remove most of the solution (<1 µL liquid left). Add 10–15 µL of LCSS to dilute the concentrated peptide solution.

4 Notes

1. 11875-RPMI-1640 media contains amino acids (L-configuration), vitamins, inorganic salts, d-glucose, glutathione and phenol red.
2. Do not stir vigorously as this will lead to excessive foam formation. This makes it hard to adjust the volume in the next step.
3. "Preheated" means that you should not use a cold pipette as this may lead to *t*-butanol solidification in your pipette. Just rapidly run the pipette a couple of times through a Bunsen burner flame or use an alternative means to slightly warm up your pipette.
4. You will need around 4 g of NaOH to reach a pH of 8.0. When the pH approaches pH 8.0, you should stop adding pellets. Wait until the remaining pellets are dissolved and continue with adding drops of a 1 M NaOH solution. When you take out the pellets from the NaOH bottle, take care. If possible wear a dust mask, at least open the bottle in a chemical hood. Especially new bottles of NaOH tend to form fine dust that represents a considerable health hazard if breathed in. When you add NaOH pellets to any aqueous solution, heat is generated. This can lead to boiling retardation, i.e., a sudden burst of liquid that may spray out of the beaker. To prevent boiling retardation, you must vigorously stir the solution when you add the pellets. Incidentally, this also helps to rapidly dissolve NaOH. Be careful when working with NaOH. Protect yourself; wear a lab coat, gloves and protective glasses at all times.
5. The protocol has been thoroughly tested with these beads. Beads from other manufacturers will also work if the avidin or streptavidin on the beads is able to bind biotinylated proteins at 0.2 % SDS and the interaction with the biotinylated proteins is not affected during washes with 1 % SDS and 6 M urea. You have to test this. Please note that Avidin beads from different suppliers have different storage requirements. Some beads are stored at 4 °C (e.g. Pierce), while others have to be stored at –20 °C (e.g. Sigma).

6. When you remove the supernatant from the beads, take care that you will not accidentally suck up some beads. This can be best achieved by not removing all solvents. For example, if the volume over the beads is 6 mL, remove only 5 mL. Add washing buffer again and remove everything except the last 1 mL. This 1 mL is your security buffer. Do not attempt to remove all the supernatant as any undesirable removal of beads will reduce your capture efficiency. Repeat this step once more.
7. If you plan to use the Typhoon scanner to detect your proteins by fluorescence, make sure you use a fluorescent protein ladder. This facilitates detection of your proteins. Fluorescent protein markers are usually quite expensive. Many of the regular protein ladders that contain red marker bands are fluorescent under the settings used to detect rhodamine-labeled proteins. The blue marker bands are often fluorescent when using the settings for Cy5. We usually use Spectra Multicolour Broad Range Protein Ladder from Thermo (Cat. Nr. 26623).
8. Glycerol is a viscous liquid that can be hard to handle. To dispense this liquid, always calculate the required weight (using the density of glycerol), place your tube on a balance and directly pour in the calculated amount. In this case, add ~12.5 g of glycerol (density of glycerole: 1.26 $g \cdot cm^{-3}$) directly to your Falcon tube.
9. β-Mercaptoethanol is a strong reducing agent. It is toxic [22] and smells bad. Wear a lab coat, gloves and protective glasses at all times when handling this chemical or samples containing it. Always work in a chemical hood especially when boiling samples containing β-mercaptoethanol.
10. Only tiny amounts of bromophenol blue are required. It is added as a pH indicator. As long as the pH is higher than 4.6, bromophenol blue containing solutions will be violet-blue. If the pH drops to a lower level, the color of the indicator will change to yellow [23].
11. Cy2: $\lambda_{ex} = 489$ nm, $\lambda_{em} = 506$ nm; Cy3: $\lambda_{ex} = 550$ nm, $\lambda_{em} = 570$ nm; Cy5: $\lambda_{ex} = 649$ nm, $\lambda_{em} = 670$ nm. Lasers: Blue 476 nm; Green 532 nm; Red 635 nm.
12. Steel blades are extremely sharp. Please take care when you handle steel blades. Only use steel blades at your bench. Never walk around with blank steel blades. If you need to relocate with your steel blade, place the blade in a 15 mL Falcon tube to assure a secure transport.
13. TCEP is stored at 4 °C. It is hygroscopic and water sensitive. Before you open the bottle containing TCEP, wait until it has reached room temperature. If you omit this step, water will condense in your TCEP bottle and TCEP will degrade over time. TCEP is a tricarboxylic phosphine. It is therefore a quite

corrosive compound. It causes burns on contact with tissues. Protect yourself; wear a lab coat, gloves, and protective glasses at all times.

14. IAA is stored at 4 °C. It is hygroscopic and water sensitive. Before you open the bottle containing IAA, wait until it has reached room temperature. If you omit this step, water will condense in your IAA bottle and IAA will degrade over time.
15. Acetonitrile is a highly flammable liquid. Keep and handle this liquid away from open fire and from other ignition sources. ACN is harmful to health when inhaled, swallowed or if brought in contact with the skin. It causes severe eye irritation. Keep away from heat, sparks, open flame, or hot surfaces. Be careful when working with ACN. Wear a lab coat, gloves and protective glasses at all times.
16. Trypsin has highest catalytical efficiency at a pH around 7.5–8.5. In the absence of a substrate, trypsin tends to cleave itself (autolysis). To prevent this, trypsin is stored in solutions at low pH and at low temperatures. There are alternative reconstitution protocols, e.g., you can also take up 20 μg trypsin in 40 μL 1 mM HCl (pH 2) and keep aliquots of this 0.5 μg/μL solution at −20 °C until needed. Keep in mind though that your final digestion pH must be around 7.5–8.5. The reaction buffer must be able to buffer out 50 mM acetic acid or HCl.
17. Calculate beforehand how much of the working solution you will need and prepare only this amount plus a little pipetting surplus. For example, if you have 20 in-gel digests and you need 20 μL of the working solution per digest, prepare $(20+2)\times 20\ \mu L = 440\ \mu L$ of the working solution. Discard the rest of the solution.
18. In order to reduce the surface tension of the LCSS solution you may add up to 3% ACN. This helps to reduce the pressure when loading the sample on the LC column. When you hand in your sample for LC-MS, please indicate the composition of your LCSS to the MS operator.
19. Use whatever assay is established in your lab and is compatible with SDS and Triton X-100. Expect 4-7 μg/μL.
20. After the click reaction (*see* Subheading 3.2), the samples will be diluted to a 2.5 mL final volume. Make sure that your reaction vessel can accommodate at least 3 mL. The brown glass vial has the advantage that it also protects the light-sensitive click reporter (Tri-N_3; *see* Subheading 3.2).
21. You may also use 1% SDS to stop the reaction, denature the proteins and keep proteins in solution in the subsequent steps. However, SDS is not compatible with the desalting step during affinity purification (*see* Subheading 3.3).

22. If this is not the case, add 1 μL of a 0.5 M phosphate buffer pH 8 or 1 M NaOH. Mix well and recheck the pH. Do not use Tris or other amine based buffers as they decrease the efficiency of the subsequent click reaction.
23. If you deal with extracts that are rich in phenolic compounds, e.g., certain plant proteomes (*N. benthamina*, tomato, …) increase the final TCEP concentration up to 2–3 mM.
24. The catalytically active compound in this click reaction is the Cu^{1+}-ion. When Cu^{2+} is added to the solution, it is reduced by the already present **TCEP** in the solution and Cu^{1+} is generated in situ. TBTA stabilizes the complex formed between Cu^{1+}, the azide (**TriN3**) and the alkyne (≡**FP**, ≡**Sym4**) [24, 25].
25. **TriN3** or rather the incorporated cyanine group is sensitive to light. Therefore, the reaction should be kept in the dark as much as possible. Store your reaction in a drawer and keep it shut or alternatively cover the whole reaction with an aluminum foil. If you used brown glass vials, no further protections are required.
26. The observed precipitate is caused by $CuSO_4$. The presence of this heavy metal causes protein misfolding which results in reduced protein solubility. Tests performed in the laboratory of Renier van der Hoorn in Cologne showed that in the presence of $CuSO_4$ >95% of proteins precipitate within the first 10 min (Kaschani and Van der Hoorn, unpublished data).
27. EDTA and Cu^{2+}-ions will form a strong complex [26]. Cu^{2+}-ions are therefore no longer available for reduction to Cu^{1+}. The reaction comes to a halt.
28. The tubes should stand upright or slightly inclined (e.g., leaning on the wall of a beaker).
29. The incubation at elevated temperature in the presence of SDS causes the proteins to denature. They will unfold and present protein complexes will break up. This step ensures that all biotin groups are displayed and accessible to avidin in the next step.
30. During incubation and washes it is important to constantly agitate the beads as they will otherwise quickly settle down. It does not matter how this is achieved (horizontal, by rolling or over-head), but it should be a gentle process.
31. It is important to pellet the beads at low g-values. If the centrifugal force is too high, the beads will stick together with precipitating proteins, thereby compromising the quality of the purification. For optimal settings, consult the manufacturer's instructions.
32. Remove only 90% of the supernatant (e.g. from 10 mL supernatant, remove only 9 mL). Do not attempt to remove all the supernatant as this may lead to accidental loss of beads which will reduce the yield of your purification.

33. You may also use preheated SDS (not more than 37 °C) to wash the beads. We noticed that the nonspecific background during subsequent MS analysis is reduced for some samples by this treatment. The biotin avidin interaction is stable under these conditions. Take however care that you do not use the washing solution at higher temperatures as this may have detrimental effects to capture efficiency. If you know that you are dealing with very sticky contaminants that are not removed by SDS treatment, you may consider washing the beads twice with 10 mL 6 M urea (Dissolve 36 g urea in 80 mL water. Then adjust the volume with water to 100 mL; do not preheat urea solutions). Wash first twice with 10 mL 1% w/v SDS, then twice with 10 mL 6 M urea and finally twice with 10 mL 1% w/v SDS.
34. Do not cool down the sample for too long. SDS and proteins may precipitate at low temperatures.
35. We recommend running a long 11% SDS gel (22 cm length, 16.5 cm width) at 55 V 550 mA, 70 W overnight, at RT in the dark. Cover the running chamber with a cardboard box or equivalent.
36. Choose the fluorescence mode and set the method for Cy5. The filter setting changes automatically when setting the method. For Cy5 scans, the LPR filter is used. Adjust the photomultiplier tube (PMT) to 1000. We use a pixel size of 50–100 μm. Select the fluorescence stage for the area option and define the area that has to be scanned according to where the gel(s) is/are placed. Then start the pre-scan at resolution 1000 μm to optimize the area settings. Then start the full scan at 100 μm resolution to obtain your gel pictures. 100 μm is more than sufficient for this application.
37. An alternative would be to use a stainless steel syringe needle and a suitable plunger (e.g. from Hamilton). Just punch several gel pieces out of the interesting gel region. Then push the pieces out into an Eppendorf tube. We rate this as the best method. It is however also possible to cut the tip of a 200 μL pipette tip off and use this as a puncher. Use a gel loader tip to push the excised gel pieces into a fresh Eppi. The disadvantage of this approach is, however, that your sample may be contaminated by plasticizers and may thus interfere with the downstream MS.
38. Do not squash the gel pieces. You do not want the gel pieces to be too small as this may cause problems in the subsequent experiments.
39. As the proteins are immobile in the gel, you can reuse the same steel blade or syringe needle for different gel regions if you take care that no gel pieces stick to the blade. Briefly wipe the blade with a KIMTECH paper towel.

40. It is necessary to remove all excess SDS from the gel. Small traces of residual SDS may inhibit the following alkylation reaction [27] and may later interfere with the separation of the peptides.
41. The following alkylation step requires a basic pH [28]. This is achieved by washing the gel slices with the ammonium bicarbonate solution.
42. TCEP will reduce disulfide-bonds (R1-S-S-R2 → R1-SH + HS-R2) which is essential for the subsequent cysteine alkylation [27]. You can also use other reducing agents like DTT and BME.
43. The IAA treatment is necessary to irreversibly alkylate the thiol-group of cysteins ($R\text{-}SH + I\text{-}CH_2\text{-}C(O)\text{-}NH_2 \rightarrow R\text{-}S\text{-}CH_2\text{-}C(O)\text{-}NH_2 + HI$) which are prone to oxidation [29]. This chemical reaction leads to a uniform a 57 Da carbamidomethyl modification of all thiol groups (-SH) which facilitates the automated detection of cysteine-containing peptides by MS. Make sure that you always use an excess of IAA in the alkylation reaction. IAA will also react with phosphines like TCEP (so called Michaelis-Arbuzov reaction [30]) and with other alkylating reagents like DTT or BME.
44. After each washing step the gel slices will become more opaque/white as acetonitrile extracts water from the gel slices. The remaining substance is solid polyacrylamide with your proteins trapped in the matrix.
45. When you pipet ACN you will notice that quickly a droplet will form at the end of the tip. ACN has a higher vapor pressure than water (9.7 kPa ACN, 2.3 kPa H_2O). It evaporates in the pipette (expansion) and presses the liquid out of the pipet. When you pipette ACN, be quick. Take care that you do not drop any ACN on the labels of your Eppendorf tubes.
46. It is beneficial to use a vacuum centrifuge. Alternatively, a desiccator attached to a vacuum pump can also be used to dry your samples although undesirable retardation of boiling may cause your gel pieces to jump out of their Eppendorf tubes. This may lead to a loss of your samples and cause contaminations.
47. The polyacrylamide flakes will start to swell once the trypsin mix is added.
48. It is advisable to seal the tubes with Parafilm which prevents accidental opening. In addition, it is advisable to put the tubes in a 200 mL plastic beaker and stuff the beaker with paper towels, thus preventing movement of the tubes. This beaker is then placed in a 37 °C shaker for bacteria and shaken overnight.
49. The formic acid treatment inactivates trypsin [31] and protonates all the peptides (the peptides will become positively charged). This is important for the subsequent MS analysis.

50. This will help you to keep track on how much of your solution has already been removed.
51. You start with ca. 500 µL solution. 40–50% of this is ACN. ACN has to be removed as it interferes with downstream analysis. At this percentage of ACN, peptides will not stick to the hydrophobic C18 material usually used in analytical columns or during StageTipping (*see* Subheading 3.6). ACN/water solutions will evaporate as an azeotrope. Consequently, a mixture of ACN/water will evaporate around the boiling point of ACN (81–82 °C). This mixture will contain 80% (w/w) ACN. If you reduce the volume in a SpeedVac from 500 to 50 µL, most of the ACN is removed and the solution is compatible with downstream applications.
52. Do not dry samples completely as this may cause loss of peptides. If the samples have dried accidentally, then add 20 µL 0.1% formic acid solution to bring the peptides back in solution and sonify for 5 min using an ultrasound bath.
53. To increase throughput, up to 96 StageTips can be placed in an ordinary tip container and spun in a centrifuge (e.g., Centrifuge 5810 with rotor A-2-DWP, Eppendorf) at 400–800 × g for 2–10 min depending on buffer volume and back pressure of the tips. Make sure that the tips will not touch the flow through and that the tip container resists ACN (or other organic solvents). If this is not the case the container may break in the centrifuge. Also make sure that you load the centrifuge properly. Check the centrifuge manual for proper loading instructions as this is not intuitive.
54. If you have only a few samples use a syringe or a similar device to press the liquid through. You can use a stepper pipet tip (e.g., Eppendorf Combitip plus) and cut the tip off so that it fits the StageTip. A syringe with an appropriate connector is also possible. After applying the solution press it through the StageTip.
55. You must wear protective glasses when you press the solutions through. All solutions are highly acidic and thus can cause severe chemical burns or upon eye contact may lead to loss of your eyesight.
56. This conditioning step is quite important. If you forget to condition the membrane with MeOH or EtOH, it will only poorly bind proteins.
57. The ideal centrifugation force has to be empirically determined. The C18 bedding must not run dry.
58. If you use a cutoff combitip to push the MeOH through, check the integrity of the StageTips afterwards. If the StageTips were not properly made the two C18 plugs lose contact. Discard tips if the C18 plugs lose contact.

59. We slowly change from organic to aqueous washing solution. MeOH to 80% ACN and in the next step only 0.1% FA. The C18 bedding must not run dry.
60. This step ensures that your sample will be >90% aqueous to assure that peptides stick to the StageTip. If your samples are already >90% aqueous, do not dilute them further. Take care that the pH is acidic (~pH 3). You can achieve this by adding FA to your sample to a final concentration of 0.1–0.5%. Minimal sample volume to load is ~10 μL.
61. When setting up the centrifugation conditions, aim at leaving a little film of STSA on top of the C18 material, thus ensuring that the tips will not get dry.
62. This step is not necessary if your sample contains large amounts of analyte. StageTips have a limited capacity (a two disk StageTip can bind 15 μg of peptides). If you load more sample than they can keep the excess analyte will elute in the flowthrough. Therefore, the StageTip procedure is ideal to prevent overloading of the nano LC column. Overloading of the nano LC leads to carry over effects that can make data interpretation difficult and therefore may spoil the results.
63. This step is only necessary if you suspect that your analyte is particularly dirty. Samples from in-gel digests are usually rather clean as the trypsin digestion is done in solutions containing little to no urea. If your sample contains high levels of phosphates or other salts, it is advised to wash a second time to remove these contaminants that may contaminate the mass spectrometer.
64. You can also increase the volume to 100 μL in the beginning. In extractions, it is important to use two small volumes (thereby following Nernst's distribution law). In this case, however, we are continuously eluting from the column; thus, it is not really relevant if you use twice 50 μL or once 100 μL.
65. The prepared LC/MS samples are ready for MS now. They can be kept at 4 °C for several days or almost indefinitely frozen at −20 °C. In cases where you expect a lot of peptides, dilute your samples, e.g. with LC-MS sample solution.

5 Discussion

The described protocol is robust and allows the routine identification of natural products when an alkyne (or azide) derivative is available. The procedure may even be coupled with photo affinity labeling to extend the applicability to non-covalently interacting natural compounds. In this case, the probe needs to be equipped with a photo cross-linker such as a benzophenone residue in addition to an alkyne (or azide) reporter [32].

In a case study, we used the protocol to perform an in vivo ABPP labeling experiment with ≡Sym4, the alkyne-tagged derivative of the natural product Sym4, in HepG2 cells (Fig. 2). As can be seen in Fig. 2a, labeling with ≡Sym4 leads to many different bands and subsequent MS analysis identified many putative ≡Sym4 targets some of which seem to be sensitive to pre-incubation with E64-d (Fig. 2b).

One of the targets of ≡Sym4 thereby seems to be cathepsin Z (CTSZ), a papain-like cysteine protease (Fig. 2b). This finding corroborates previous findings that described Sym4 as an inhibitor of cathepsins. Besides cathepsin Z, however, many other non-cysteine proteases are found, indicating that Sym4 has a rather broad chemical reactivity and addresses many different proteins. Thus, Sym4 is not a selective cathepsin inhibitor. These findings nicely illustrate the differences in target identification approaches: we have used a global approach, thereby allowing insight into the general bioreactivity pattern of Sym4 while in previous studies, the effect of Sym4 vs. selected protein targets was studied [13–17]. Nevertheless, it should be noted that from this point on, further studies are usually required to validate and better evaluate the target list from the chemical proteomics experiments.

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Chapter 4

Inhibitor Discovery by Convolution ABPP

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Abstract

Activity-based protein profiling (ABPP) has emerged as a powerful proteomic approach to study the active proteins in their native environment by using chemical probes that label active site residues in proteins. Traditionally, ABPP is classified as either comparative or competitive ABPP. In this protocol, we describe a simple method called convolution ABPP, which takes benefit from both the competitive and comparative ABPP. Convolution ABPP allows one to detect if a reduced signal observed during comparative ABPP could be due to the presence of inhibitors. In convolution ABPP, the proteomes are analyzed by comparing labeling intensities in two mixed proteomes that were labeled either before or after mixing. A reduction of labeling in the mix-and-label sample when compared to the label-and-mix sample indicates the presence of an inhibitor excess in one of the proteomes. This method is broadly applicable to detect inhibitors in proteomes against any proteome containing protein activities of interest. As a proof of concept, we applied convolution ABPP to analyze secreted proteomes from *Pseudomonas syringae*-infected *Nicotiana benthamiana* leaves to display the presence of a beta-galactosidase inhibitor.

Key words Activity-based protein profiling (ABPP), Convolution ABPP, Cyclophellitol-aziridine, Galactostatin, *Nicotiana benthamiana*, Secreted proteomes, Beta-galactosidase, *Pseudomonas syringae*

1 Introduction

Activity-based protein profiling (ABPP) involves chemical probes which label the active site residues in proteins [1]. This labeling displays the active state of the targeted proteins. Traditionally, ABPP is classified as either comparative or competitive ABPP [2]. Comparative ABPP involves the comparison of the active status of proteins in two or more biological samples (e.g., different treatments). In competitive ABPP, a proteome is preincubated with putative inhibitors and subsequently with the activity-based probes to label the non-inhibited proteins. Both of these approaches have emerged as powerful tools to study active proteins in crude proteomes. In this chapter we describe a simple method called convolution ABPP, which takes

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benefit from both the competitive and comparative ABPP. This method indicates if a reduced signal observed by comparative ABPP is due to the presence of inhibitors.

To illustrate the principle of convolution ABPP, two proteomes (A and B) are shown as example (Fig. 1). Labeling of proteomes A and B displays a signal that is reduced in sample B when compared to sample A. Convolution ABPP can be applied in this situation to determine if an excess of inhibitors in proteome B has caused the reduced labeling. Convolution ABPP is a simple, two-step protocol. The first step involves preparation of proteome D, which is a

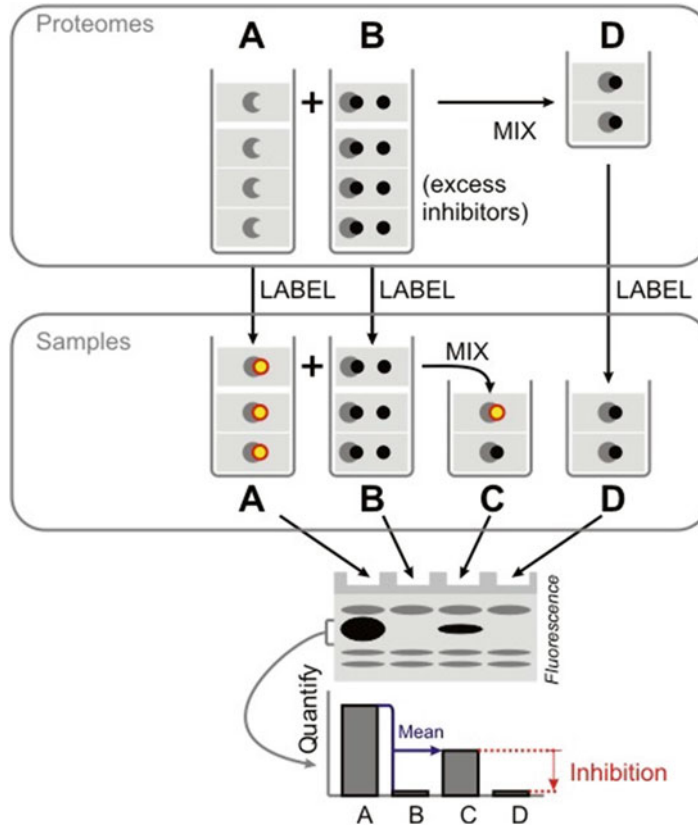

Fig. 1 Procedure of convolution ABPP. This approach can be used if a reduced labeling of a protein is observed in a sample (e.g., any biological treatment, Proteome B) compared to its control (Proteome A). To test if there is an inhibitor excess (*black dots*) in proteome B, one volume of proteome A and B are mixed together and the mixed proteome (Proteome D) is then labeled (mix-and-label Sample D). As a control for sample D, one volume of labeled samples A and B are mixed together (label-and-mix Sample C). Signals in Sample C should be the average of the signals in Samples A and B. If there is an excess inhibitor in Proteome B, then the signal intensity in Sample D will be lower than in Sample C. *Black dots* inhibitors, *yellow/red dots* activity-based probes, *grey moons* enzyme

mix of one volume of proteome A with one volume of proteome B. In the second step, proteomes A, B, and D are labeled with an activity-based probe. As a control to display the average signals of samples A and B, one volume of labeled sample A and one volume of labeled sample B are mixed together, resulting in sample C (label-and-mix). In short, the proteomes are mixed-and-labeled (sample D) or labeled-and-mixed (sample C). The fluorescent intensities of labeled proteins in sample C should be the mean of the labeling intensities in samples A and B. Reduced labeling of any protein in sample D when compared to sample C indicates the presence of an inhibitor excess in proteome B. We illustrate this method for detecting inhibitors in two related (biological) samples. However, this approach is broadly applicable for detecting inhibitors in extracts of interest against any proteome whose labeling profile is well characterized. One important note of caution is that reduced signals can also result from degradation, precipitation or enzymatic deactivation mechanisms other than inhibition. We use protease inhibitor cocktail to prevent degradation but additional experiments e.g., to detect the accumulation and molecular weight of the unlabeled protein is desired to exclude other inactivation mechanisms.

In this chapter, convolution ABPP is illustrated for glycosidases in secreted proteomes (apoplastic fluids) isolated from leaves of the wild tobacco plant *Nicotiana benthamiana*, using JJB70 (Fig 2a), a fluorescent activity-based probe for glycosidases [3]. JJB70 carries a cyclophellitol-aziridine reactive group and a fluorescent reporter tag. We have shown previously that JJB70 targets 19 different retaining glycosidases in apoplastic fluids of *Nicotiana benthamiana*, belonging to six different glycosyl hydrolase (GH) families [4]. Among the different labeled proteins, a 45 kDa signal was identified as a beta-galactosidase (BGAL) belonging to the GH35 family [4]. We have described two experiments to give proof of concept of convolution ABPP. In the first experiment we took two identical proteomes (A and B) and added an excess of galactosidase inhibitor to proteome B (Fig. 2c). In a second experiment we used convolution ABPP to detect an inhibitor in a biological sample isolated from a pathogen-infected leaf (Fig. 2d).

2 Materials

2.1 Infection of Nicotiana benthamiana Plants with Pseudomonas syringae

1. Bacterial stock: *Pto*DC3000 (Δ*hQ*) strain carrying GFP [5] frozen in 7% DMSO.
2. Rifampicin (25 mg/mL): dissolve 125 mg of rifampicin in 5 ml DMSO, vortex well and store at −20 °C in 500 µL aliquots.
3. Gentamicin (25 mg/mL): dissolve 125 mg of gentamicin in 5 L sterile water, filter-sterilize with 0.22 µM filter unit and store at −20 °C in 500 µL aliquots.

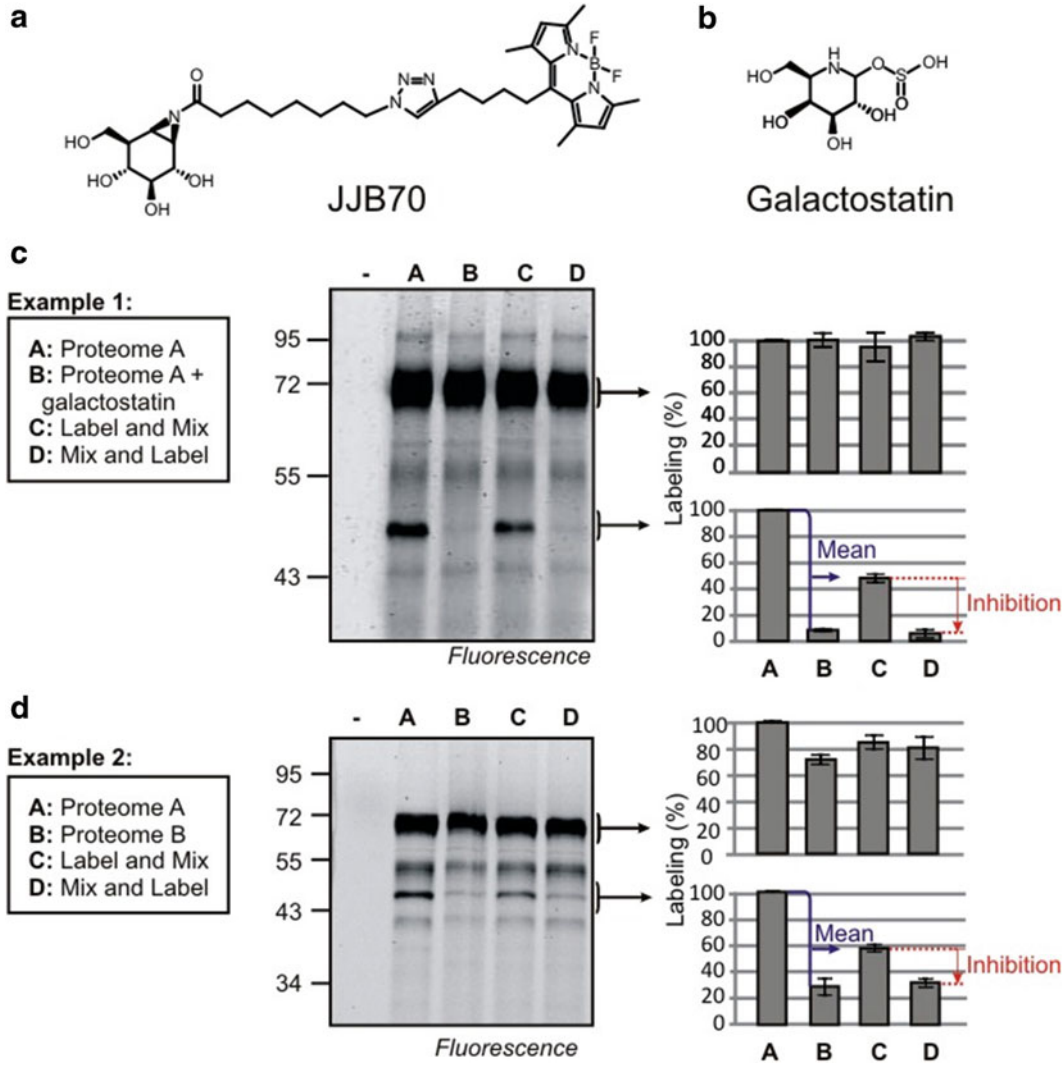

Fig. 2 Proof-of-concept for convolution ABPP. (**a**) Structure of JJB70 probe. JJB70 is a broad range glycosidase probe carrying cyclophellitol-aziridine and BODIPY as a reporter tag. (**b**) Structure of galactostatin bisulphite, a beta-galactosidase inhibitor. (**c**) Convolution ABPP with a proteome containing an excess of a known inhibitor. Proteome A is an apoplastic fluid from untreated *N. benthamiana* leaves and proteome B is the same proteome containing 10 μM galactostatin bisulphite. Equal volumes of proteome A and proteome B were mixed together, resulting in proteome D. Proteomes A, B, and D were labeled with 2 μM JJB70 for 1 h. After labeling, one volume of labeled samples A and B were mixed together (label-and-mix Sample C). The labeled samples A, B, C, D were separated on a protein gel, detected by in-gel fluorescent scanning and the labeling intensity was quantified. Error bars indicate the standard error of means from three independent experiments. (**d**) Convolution ABPP indicates the presence of inhibitors in *P. syringae*-infected *N. benthamiana* leaves. Proteome A represents apoplastic fluids from the mock-infiltrated *N. benthamiana* leaves and Proteome B represents apoplastic fluids from *P. syringae*-infiltrated *N. benthamiana* leaves. The labeled samples A, B, C (label-and-mix) and D (mix-and-label) were prepared and analyzed as in (c). Error bars indicate the standard error of means from three independent experiments

4. Liquid NYG medium: dissolve 5 g of Bacto™ peptone and 2 g of Bacto™ yeast extract in a 1 L beaker containing 800 mL of milliQ water. Use magnetic stirrer to dissolve. Add 20 mL of glycerol (2%) to the mixture and add milliQ water to a final volume of 1 L. Autoclave the medium at 121 °C and 100 kPa for 15 min.
5. Spectrophotometer and UV cuvettes for measuring optical density (OD) at 600 nM.
6. 1 mL syringe without needle.
7. *Nicotiana benthamiana* plants: *N. benthamiana* plants are grown at 22 °C and 60% relative humidity under a 12 h light regime. Leaves from 4-week-old *N. benthamiana* plants are used for the apoplastic fluid isolation and infection assays.

2.2 Apoplastic Fluid Isolation

1. Vacuum desiccator.
2. 1 L glass beaker, ice, and Styropor float.
3. 1 L MilliQ water.
4. Apoplastic fluid isolation apparatus [6].
5. Protease inhibitor cocktail: dissolve one cOmplete protease inhibitor tablet, (Roche, 04693116001) in 2 mL sterile water to give a 25× stock solution.

2.3 Proteomes for ABPP-based Mix and Label Approach

1. 10 mM galactostatin bisulfite: dissolve 1 mg of galactostatin bisulfite (Santa Cruz Biotechnology, sc-21855) in 411 µL DMSO.

2.4 General Procedure for Labeling the Proteomes

1. 500 mM (10×) MES buffer pH 5.0: dissolve 19.52 g of 2-(*N*-morpholino)ethanesulfonic (MES) in a 200 mL Duran bottle containing 190 mL of milliQ water. Adjust the pH to 5.0 by adding a few drops of 5 M NaOH. Add milliQ water to a final volume of 200 mL.
2. 100 µM JJB70: thaw the frozen 10 mM JJB70 stock at room temperature. Dilute this 10 mM stock in DMSO to obtain 100 µM JJB70 can be stored at −20 °C until needed.
3. SDS sample buffer (4×): Add 2 g SDS, 10 ml 1 M Tris (pH 6.8), and 10 mL glycerol in a 50 mL falcon tube. Tumble until the reagents are dissolved. Now add 2.5 mL 14.2 M β-mercaptoethanol and add water to a final volume of 25 mL. Finally, add a pinch of bromophenol blue and store in 1 mL aliquots at −20 °C.

2.5 Analysis and Detection of Samples

1. 1 M Tris(hydroxymethyl)aminomethane) (Tris) pH 6.8: dissolve 121.14 g of Tris in 1 L Duran bottle containing 800 mL of milliQ water. Adjust the pH to 6.8 with concentrated HCl. Add milliQ water to a final volume of 1 L.
2. 1.5 M Tris(hydroxymethyl)aminomethane) (Tris) pH 8.8: Dissolve 181.7 g of Tris in 1 L Duran bottle containing 800 ml

of milliQ water. Adjust the pH to 8.8 with concentrated HCl. Add milliQ water to a final volume of 1 L.

3. 10% ammonium persulfate (APS): dissolve 0.1 g of APS in 1 mL of milliQ water. Prepare the APS solution freshly each time while making protein gels.
4. 10% Sodium dodecylsulfate (SDS): Dissolve 20 g of SDS in 200 mL of milliQ water.
5. Ready to use 30% (w/v) acrylamide–bis solution (SERVA Electrophoresis GmbH, 10687).
6. TEMED (Sigma-Aldrich, T9281).
7. 12% resolving gel: mix the following components in a conical flask for making eight small protein gels: 18.5 mL water, 22.4 mL 30% (w/v) acrylamide–bis solution, 14 mL 1.5 M Tris pH 8.8, 560 µL 10% SDS, 560 µL 10% APS, and 22.4 µL TEMED. Pour the resolving gel solution into the gel cassettes and overlay with 2-butanol.
8. 6% stacking gel: mix the following components in a conical flask for making at least eight protein gels: 15.7 mL water, 4.8 mL 30% (w/v) acrylamide–bis solution, 3.024 mL 1.0 M Tris pH 6.8, 240 µL 10% SDS, 240 µL 10% APS, and 24 µL TEMED. Pour the stacking gel solution onto a polymerized resolving gel and insert the comb with desired well into the cassette.
9. 10X SDS running buffer (10×): dissolve 30 g Tris, 144 g glycine, and 10 g SDS in 1 L glass beaker with 800 mL water using a magnetic stirrer. Add water to a final volume to 1 L.
10. Typhoon scanner: Typhoon FLA 9000 scanner (GE Healthcare Life Sciences), or something similar.

2.6 Quantification of Fluorescent Signals

1. Quantification tool: ImageQuant Version 5.2, Molecular Dynamics, or something similar.

3 Methods

3.1 Infection of Nicotiana benthamiana Plants with Pseudomonas syringae (for Exp.2 Only)

1. Revive the *Pto*DC3000(Δ*hQ*) strain carrying GFP [7] from the glycerol stock by plating on LB agar medium containing 25 µg/mL rifampicin and 10 µg/mL gentamicin.
2. Incubate the plate for 48 h at 28 °C.
3. Pick a single bacterial colony from the plate and inoculate 10 mL liquid NYG medium containing 25 µg/mL rifampicin and 10 µg/mL gentamicin and grow the bacteria overnight at 28 °C.
4. Centrifuge the overnight grown bacterial culture at 2000×*g* for 15 min, remove the supernatant and resuspend the pellet in 4 mL sterile water.

5. Measure the OD at 600 nm and dilute the bacterial suspension with sterile water until $O.D_{600} = 1$ (*see* **Note 1**).
6. Dilute the bacterial suspension to a concentration of 10^6 bacteria/mL in sterile water.
7. As a control, infiltrate sterilized water into the leaves of 4-week-old *Nicotiana benthamiana* plant with a needleless syringe (*see* **Note 2**) and label the plant as "A".
8. Infiltrate the diluted bacteria into leaves of other *N. benthamiana* plants and label the plant as "B".
9. At 2 days post infiltration (2dpi), isolate apoplastic fluids from plants A and B and label the tubes as Proteome A and Proteome B (*see* Subheading 3.2).

3.2 Apoplastic Fluid Isolation (Exp.1 and Exp.2)

GMO warning: please be aware that infected leaves contain transgenic bacteria. Take appropriate precautions to sterilize the working area and equipment after the experiment.

1. Take four to six leaves from (infiltrated) *N. benthamiana* plants (*see* **Note 3**).
2. Rinse the leaves with water to remove any dirt from the surface of the leaves.
3. Add the leaves, 100 mL ice and 500 mL milliQ water to the 1 L beaker and submerge the leaves into the ice cold water with a support. The leaves can be submerged by pushing them down with a piece of Styropor that fits tightly in the beaker. In this way the leaves and Styropor remain under the water (*see* **Note 4**).
4. Place the beaker into a vacuum desiccator and apply about 25 in. Hg vacuum for at least 20 min (*see* **Note 5**).
5. Stop applying the vacuum and slowly release the vacuum (*see* **Note 6**). The water will now enter the leaves.
6. Place the water-infiltrated leaves on a tissue paper and carefully absorb the water droplets from the surface with tissue paper (*see* **Note 7**).
7. Roll 6–8 leaves up and carefully place them into the apoplastic isolation device [6] and centrifuge for 25 min at 2000 × *g*, 4° (*see* **Note 8**).
8. Collect the apoplastic fluids from the bottom of the apoplastic isolation device, transfer into a 15 mL falcon tube and keep it on ice until further analysis.
9. To an aliquot of the collected apoplastic fluids (e.g., 840 μL) add 25× protease inhibitor cocktail (e.g., 160 μL) to final concentration of 4× (*see* **Note 9**).

3.3 Proteomes for Convolution ABPP

Two experiments will be shown as a proof of concept:

	Proteome A (>100 µL)	Proteome B (>100 µL)
Exp.1 (Fig. 2c)	Apoplastic fluids from untreated plants	Apoplastic fluids from untreated plants + excess galactostatin
Exp.2 (Fig. 2d)	Apoplastic fluids from Mock-infiltrated plants	Apoplastic fluids from *P. syringae*-infiltrated plants.

1. Prepare proteome B of Exp.1 by adding galactostatin bisulphite (final concentration 10 µM) to the apoplastic fluids of untreated leaves.
2. Prepare proteome D by mixing 50 µL of proteome A with 50 µL of proteome B in a 1.5 mL eppendorf tube. Vortex the mixed proteome for 30–60 s and incubate at room temperature for 30 min.

3.4 General Procedure for Labeling the Proteomes

1. Start the labeling using the following schedule:

	No-probe-control	Sample A	Sample B	Sample D
10× MES buffer (500 mM, pH 5.0)	5 µL	5 µL	5 µL	5 µL
DMSO	1 µL	–	–	–
Probe (100 µM)	–	1 µL	1 µL	1 µL
Protein extracts	44 µL proteome D	44 µL proteome A	44 µL proteome B	44 µL proteome D
Total volume	50 µL	50 µL	50 µL	50 µL

2. After adding the proteomes, vortex the samples for 5 s and incubate at room temperature for 1 h. Meanwhile cover the samples with aluminum foil or place them in a drawer (*see* **Note 10**).
3. Stop the labeling reaction by adding 16 µl of 4× SDS sample buffer and heat the samples for 5 min at 95 °C in a heat block.
4. Briefly vortex and centrifuge the samples at 16,000 × *g* for 5 s.
5. For sample C, mix 5 µL of sample A and 5 µL of sample B in a new 1.5 mL eppendorf tube.

3.5 Analysis and Detection of Samples A, B, C, D

1. Load 10 µL of the no-probe-control and samples A-D and onto a 12% SDS-PAGE gel.
2. Separate the protein samples at 200 V. Meanwhile cover the tank with black plastic or cardboard box (*see* **Note 10**).
3. Take the gel from the cassette and place the gel in a clean box containing milliQ water.

4. Wash the gel three times with milliQ water for 5 min in the dark and immediately proceed to scan the gel with the Typhoon Scanner (*see* **Note 11**).
5. Detect the fluorescence of labeled proteins by scanning the gel with the Typhoon Scanner using the appropriate settings. For JJB70 we use the 472 nM laser and the BPB1 filter (515–545 nm) and 1000 V photo multiplier tube (PMT).

3.6 Quantification of Fluorescent Signals

1. Save the obtained image in .gel format.
2. To quantify the fluorescence of labeled proteins, open the gel image with ImageQuant quantification tool.
3. Draw a rectangle around the first signal of interest in sample A using the rectangle selection option.
4. Press "Ctrl + D" to create new rectangles with the same area as the previous one and place each rectangle on the corresponding band in the B, C, and D samples.
5. Select the "volume" option from the "analysis" menu and export the results in an excel file.
6. Subtract the values in the volume column with its corresponding background value and divide the obtained value by the area of the selected rectangle. The resulting volume values represent the average fluorescence intensity per unit area in the selected region.
7. Normalize the labeling intensities values to the fluorescence intensity in the sample A and express the values in percentage labeling by multiplying with 100.
8. Perform the **steps 4–9** for the other signals of the same gel.
9. Repeat entire assay (Subheadings 3.1–3.5) twice to obtain statistical data.
10. Calculate the standard deviation and standard error of the labeling percentages of three independent experiments and represent the errors bars on the bar graph drawn with the average labeling intensities against the samples.

4 Notes

1. Optical density (OD_{600}) of one represents 10^9 bacteria/mL.
2. Wear protective eye glasses during infiltration. Avoid air bubbles in the syringe during infiltration. Air bubbles in the syringe creates overpressure and causes splashing during infiltration. Air bubbles can also damage the leaf tissues and thereby cause cytoplasmic contaminations.
3. Make sure that the plant is watered well at least 30 min before apoplastic fluid isolation. Watering plants opens the stomata which increases access of water into the leaves under vacuum.

4. While submerging the leaves take precaution not to cause any damage to the leaves as this will lead to contamination with cytoplasmic proteins.
5. You will notice that air bubbles emerge after few minutes of applying the vacuum. These bubbles are the air present in the intercellular spaces.
6. Make sure that an entire leaf is completely infiltrated with water. If not, then repeat the vacuum process until the whole leaf is completely infiltrated.
7. Make sure that there are no water droplets left on the surface of the infiltrated leaves. This would dilute the apoplastic proteome.
8. Use slow acceleration and slow deceleration settings of the centrifuge to prevent leaf damage caused by centrifugation.
9. If the apoplastic fluids are isolated for protease activity profiling, the addition of protease inhibitor cocktail is not desired.
10. Labeling in the dark reduces photobleaching of the fluorophore.
11. Washing the gel before scanning prevents the formation of Newton-rings while scanning with a Typhoon scanner.

Acknowledgements

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Chapter 5

Activity-Based Protein Profiling in Bacteria

Joanna Krysiak and Stephan A. Sieber

Abstract

Understanding the molecular mechanisms of bacterial pathogenesis and virulence is of great importance from both an academic and clinical perspective, especially in view of an alarming increase in bacterial resistance to existing antibiotics and antibacterial agents. Use of small molecules to dissect the basis of these dynamic processes is a very attractive approach due to their ability for rapid spatiotemporal control of specific biochemical functions. Activity-based protein profiling (ABPP), employing small molecule probes to interrogate enzyme activities in complex proteomes, has emerged as a powerful tool to study bacterial pathogenesis. In this chapter, we present a set of ABPP methods to identify and analyze enzymes essential for growth, metabolism and virulence of different pathogens including *S. aureus* and *L. monocytogenes* using natural product-inspired activity-based probes.

Key words Activity-based protein profiling, Activity-based probes, γ-butyrolactones, ClpP, β-lactones, β-lactams, Natural products, Pathogenesis, *S. aureus*, Virulence

Abbreviations

ABPP	Activity-based protein profiling
BCA	Bicinchoninic acid
CC	Click chemistry
ClpP	Caseinolytic protein protease P
DMSO	Dimethyl sufoxide
DTT	1,4-dithio-D-threitol
HMP	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate
Hyd	Hydrolase CocE/NonD family
KAS II	β-Ketoacyl acyl carrier protein synthase II
KAS II	I β-Ketoacyl acyl carrier protein synthase III
Lip	Lipase
LLO	Listeriolysin L
LPL	Lysophospholipase
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
Mur1/2	UDP-*N*-Acetylglucosamine 1-carboxyvinyltransferases ½
PBP	Penicillin-binding protein

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PBS	Phosphate buffered saline
PI-PLC	Phosphatidylinositol-specific phospholipase C
PL	Pyridoxal
SDS-PAGE	Sodium dodecyl polyacrylamide electrophoresis
TAMRA	5(6)-carboxytetramethylrhodamine
TBE	Tributyrin esterase
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine
TCEP	Tris(2-carboxyethyl)phosphine
TF	Trigger factor

1 Introduction

Bacterial growth, metabolism, and virulence are multifaceted processes which are spatiotemporally controlled by global regulatory networks [1–3]. However, our understanding of these complex systems is still very limited. Therefore, identification, in-depth functional analysis of enzymes responsible for bacterial pathogenesis and virulence, as well as dissection of their regulatory networks and host–pathogen interactions is of the utmost interest to both the scientific community and pharmaceutical industry. Eventually, this knowledge could help combat bacteria-mediated diseases and overcome antibiotic resistance issues [4, 5].

In our lab, we employ a powerful combination of methods to elucidate specific molecular mechanisms and their functional implications in bacterial pathogenesis and virulence. Among other techniques, we widely apply activity-based protein profiling (ABPP) [6, 7], since small molecule activity-based probes (ABPs) are simple but powerful tools to map protein activity during bacterial growth and infection in real time, surpassing in that respect genetic tools [8].

In recent years we have developed a diverse repertoire of irreversible activity-based probes (ABPs) including β-lactones [9–15], γ-butyrolactones and α-alkylidene-γ-butyrolactones [16–18], β-lactams [19, 20], β-sultams [21], Michael-acceptors [22–25], epoxides [26], and other protein-reactive compounds [27] in order to profile enzyme activities and their functions across different dangerous human pathogens, especially clinically relevant strains of *Staphylococcus aureus* or *Listeria monocytogenes*. We have also designed and applied probes based on vancomycin or γ-butyrolactones that are equipped with a photocrosslinker to elucidate non-covalent targets of these compounds [16, 28]. Prominent examples of our probe collection are shown in Fig. 1.

ABPs are typically based on biologically active natural products or are inspired by natural products (biomimetics). Natural products, considered as privileged structures due to their biosynthetic origin and evolutionary purpose to address protein targets to exert their biological role [29], very often show inhibition of bacterial growth or attenuation of virulence factors (e.g., α-hemolysin). Hence, they

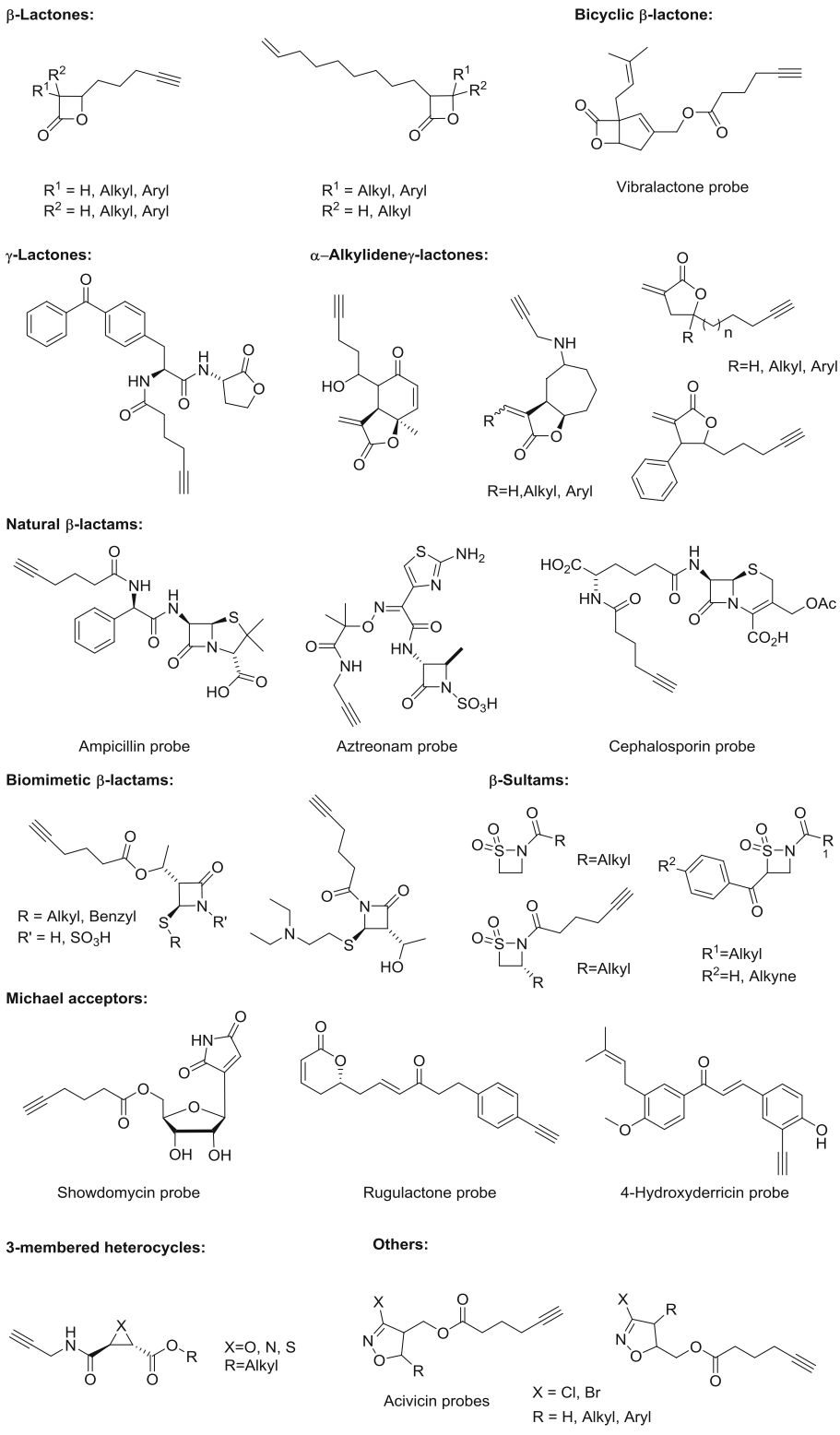

Fig. 1 Library of ABPP probes used for profiling enzymes in bacteria

were and still are a precious reservoir for new antibiotics, antibacterial and antivirulence agents as well as probes for ABPP [30–32]. Such biological activities (antibiotic, antivirulence) are a desired prerequisite for our ABPs as the molecular basis of the observed biological effect can be then unraveled and characterized by a combination of ABPP, mass spectrometry, genetic tools, and biochemical assays. A typical workflow for bacterial ABPP is depicted on Fig. 2.

ABPP profiling across different pathogenic bacteria with β-lactones identified a set of mechanistically distinct enzymes, involved in metabolism, antibiotics resistance, or virulence (Fig. 3) [9]. Identified proteins included caseinolytic protein protease (ClpP), a highly conserved serine protease which is responsible for cell homeostasis and is an important virulence regulator. Chemical knockout of ClpP with improved β-lactone inhibitors subsequently demonstrated significant reduction of secreted toxins and enzymes in MRSA strains [10–12] as well as downregulation of virulence factors listeriolysin L (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC), in *Listeria monocytogenes* [13].

β-Lactones are thus new antivirulence lead structures for the treatment of resistant bacteria. Moreover, vibralactone, a bicyclic β-lactone ABPP probe, allowed light to be shed on the activity and structure of the ClpP1P2 heterooligomeric complex in *L. monocytogenes* [14, 15].

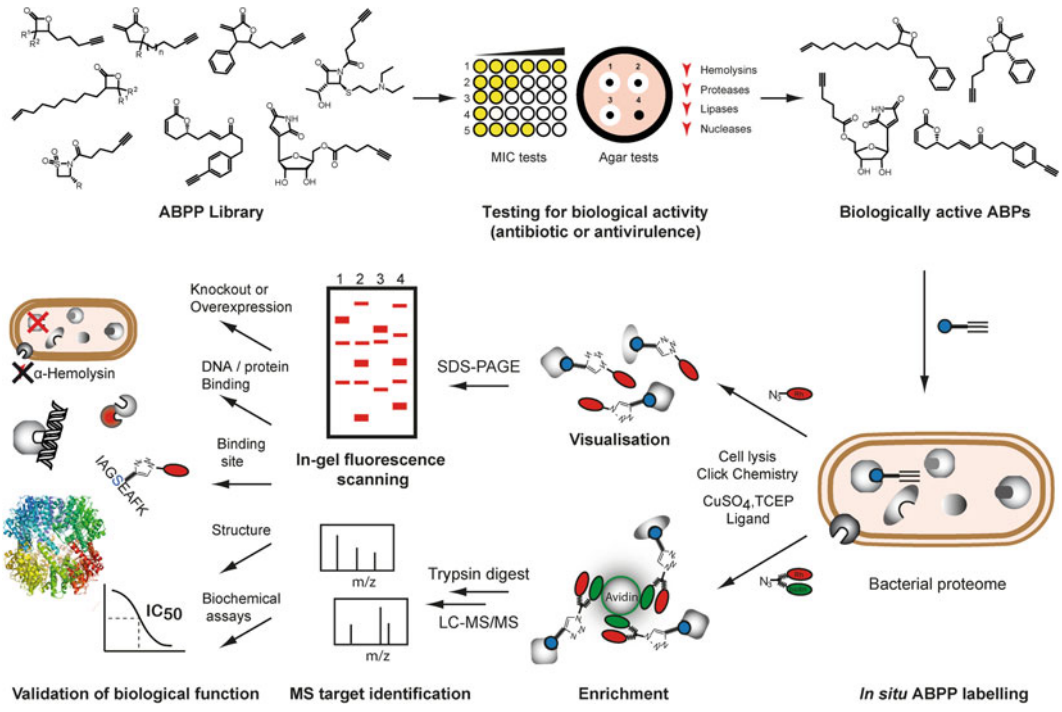

Fig. 2 ABPP workflow for profiling and validation of enzymes in bacteria

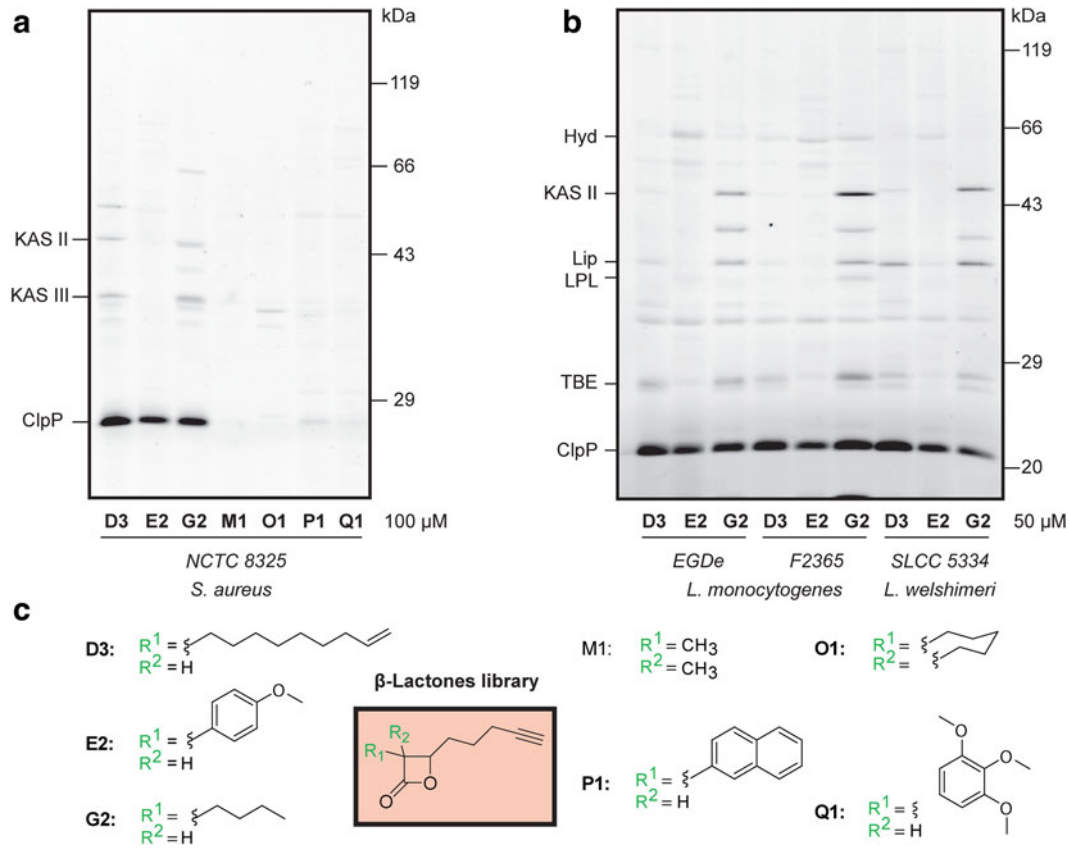

Fig. 3 Fluorescent SDS-PAGE analysis. Labeling profile of living *S. aureus* (**a**), *L. monocytogenes* and *L. welshimeri* (**b**) with β-lactone probes (**c**). Protein targets identified by gel-based LC-MS/MS analysis are assigned to the corresponding gel band (for full names of proteins *see* Abbreviation list). Adapted with permission from refs. [10] and [13]. Copyright (2009) American Chemical Society and (2009) Wiley and Sons, respectively

γ-Butyrolactone and α-alkylidene-γ-butyrolactone structures provided an excellent scaffold for affinity- and activity-based probes with fine-tuned reactivity and allowed identification of several enzymes (trigger factor (TF), formate acyltransferase, dihydrolipoamide dehydrogenase, 6-phosphofructokinase, MurA1, and MurA2 (the latter two enzymes being essential for bacterial cell wall biosynthesis)) in bacterial proteomes [16]. Xanthanine inspired α-alkylidene-γ-butyrolactone ABPs revealed that a specific c2450 enzyme is only expressed and active in uropathogenic *E. coli* strains [17]. Application of a carefully selected α-alkylidene-γ-butyrolactone ABPP library to a *S. aureus* strain resulted in a significant attenuation of its virulence and reduced invasion into human cells by inhibition of several important transcription factors (SarA, SarR, MgrA), providing additional scaffolds for future anti-virulence medications [18].

Equipping clinically relevant β-lactam antibiotics with an alkyne tag delivered a set of ABPs which were successfully used to identify a series of penicillin-binding proteins (PBPs) in different pathogenic and nonpathogenic strains [19]. On the other hand, synthetic β-lactam probes based on the monocyclic aztreonam structure showed a preference towards other enzymes such as β-ketoacyl acyl carrier protein III (KAS III), a β-lactamase, a lipase acylhydrolase, an antioxidant (AhpC), and the virulence-associated protein ClpP [19]. In an MRSA strain these probes additionally targeted resistance associated enzymes such as PBP2 and PBP2′ as well as serine proteinase D_0 and dipeptidase PepV [20].

β-Sultams based ABPP probes, although structurally related to β-lactones and β-lactams, labeled quite a different spectrum of protein targets within bacterial proteomes (e.g., adenosylhomocysteinase (AHC) and thiol disulfide interchange proteins DsbC and DsbA) and showed unusual inhibition of several azoreductase enzymes, known mostly for their biotechnological application in the detoxification of azo dyes [21].

Michael acceptor containing probes based on showdomycin, rugulactone, and 4-hydroxyderricin proved very useful for elucidating the molecular mechanism underlying the antibiotic effects of these natural products in pathogenic bacteria [22–24]. For instance, ABPP disclosed ThiD enzyme as the major protein target of rugulactone in *S. aureus* and in *L. monocytogenes*. This enzyme was subsequently characterized by in-depth biochemical and structural studies [24] which revealed its dual nature as 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP) and pyridoxal (PL) ribokinase involved in thiamine biosynthesis and PL salvage, respectively, which are essential for bacterial viability.

In summary, ABPP in bacteria using small molecule probes based on antibiotics, natural products or inspired by natural compounds has hugely expanded our understanding of enzyme targets essential for different cellular processes as well as for viability of nonpathogenic and pathogenic bacteria and shed light on molecular mechanism of virulence and mechanisms for its attenuation. Many ABPP probes proved to be efficient enzyme inhibitors and could be used as lead structures for future antibacterial or antivirulence treatment, thereby overcoming the problem of antibiotic resistance.

Here, we present well-established methods for in vitro and in situ ABPP labeling of bacteria using both irreversible probes and reversible compounds equipped with a photocrosslinker for covalent linkage to targets. We provide a protocol for the visualization of protein targets by click chemistry and fluorescence scanning as well as a procedure for gel-based MS identification of protein targets.

2 Materials

2.1 Recipes for Media, Buffers, Solutions

1. B Medium: Dissolve in 1 L deionized H_2O: 5.0 g yeast extracts, 10.0 g tryptic peptone, 5.0 g NaCl, 1.0 g K_2HPO_4.
2. Brain heart infusion broth (BHB): Dissolve in 1 L deionized H_2O: 17.5 g brain heart infusion, 2.5 g Na_2HPO_4, 2.0 g glucose, 5.0 g tryptic peptone, 5.0 g NaCl.
3. Lysogeny broth (LB): Dissolve in 1 L deionized H_2O: 5.0 g yeast extracts, 10.0 g tryptic peptone, 5.0 g NaCl.
4. Casein soya broth/tryptic soy broth (CASO): Dissolve in 1 L deionized H_2O: 17.0 g casein peptone, 3.0 g soya peptone, 2.5 g glucose, 2.5 g K_2HPO_4, 5.0 g NaCl.
5. PBS (phosphate buffered saline): Dissolve in 1 L deionized H_2O: 8.00 g (136.9 mM) NaCl, 1.44 g (10.1 mM) Na_2HPO_4, 0.20 g (2.7 mM) KCl, 0.24 g (1.8 mM) KH_2PO_4. Adjust pH to 7.4 with conc. HCl.
6. 2× SDS loading buffer: Mix 2.5 mL 1 M Tris–HCl pH 6.8 (125 mM), 4 mL glycerol (20% (v/v)), 0.8 g SDS (4% (w/v)), 2 mL β-mercaptoethanol (10% (v/v)), 1 mg bromophenol blue (0.005% (w/v)) and fill up to 20 mL with deionized H_2O.
7. 1× SDS-PAGE running buffer: Dissolve in 1 L deionized H_2O: 14.4 g glycine (192 mM), 3.0 g Tris-base (25 mM), 1.0 g SDS (0.1% (w/v)). Adjust pH to 8.3.
8. Coomassie staining solution: Dissolve in 1 L deionized H_2O: 2.5 g Coomassie Brilliant Blue R250 (0.25% (w/v)), 92 mL acetic acid (concentrated) (9.2% (v/v)), 454 mL ethanol (absolute) (45.4% (v/v)).
9. Coomassie destaining solution: Dissolve in 1 L deionized H_2O: 100 mL acetic acid (concentrated) (10.0% (v/v)), 200 mL ethanol (absolute) (20% (v/v)).

2.2 Specialized Equipment

1. Incubator: New Brunswick Scientific programmable Innova 42 incubator shaker or an equivalent.
2. Laminar-flow hood: Thermo Scientific Herasafe KS12 safety cabinet or an equivalent.
3. UV-VIS photometer: Eppendorf BioPhotometer or an equivalent.
4. Centrifuge: Thermo Scientific Sorvall RC6 or an equivalent.
5. Ultrasonic homogenizer: Bandelin Sonoplus HD2070 or an equivalent.
6. Peqlab Precellys® 24 homogenizer or an equivalent.
7. Microplate reader: TECAN Infinite® M200 Pro or an equivalent.

8. Electrophoresis apparatus: Peqlab PerfectBlue Dual Gel System Twin or an equivalent.
9. Fluorescence in-gel scanner: Fujifilm Life Science Las-4000 Luminescent Image Analyzer equipped with a Fujinon VRF43LMD3 Lens and a 575DF20 Cy3 Filter or an equivalent.
10. Mass spectrometer: LTQ-Orbitrap XL or an equivalent.

3 Methods

3.1 In Situ Analytical ABPP Labeling

1. Prepare overnight bacterial culture (*see* **Note 1**) by inoculating 5 mL sterile culture medium with 5 μL glycerol cryostock 1:1000 (v/v) in a 10 mL plastic culture tube (*see* **Note 2**).
2. Incubate bacteria at 37 °C, 200 rpm, overnight (ca. 14–16 h) (*see* **Note 3**).
3. Dilute overnight culture 1:100 (v/v) with a fresh medium and incubate at 37 °C, 200 rpm (*see* **Note 4**).
4. Allow bacterial subculture to grow to the time point at which the labeling experiment is carried out (*see* **Note 5**). As a starting point, let bacteria grow to the stationary phase (*see* **Note 6**).
5. Calculate volume of the culture for centrifugation using the following formula:
   [40 × (0.2 mL × number of samples)]/measured OD_{600} (*see* **Note 7**).
6. Pellet cells at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
7. Resuspend cells in the same volume PBS, then centrifuge at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
8. Resuspend cells in the same volume of fresh PBS at RT.
9. Divide prepared bacterial suspension in PBS into 200 μL aliquots in 1.5 mL Eppendorf tubes.
10. Add 2 μL ABPP probe (from 100×DMSO stock) or DMSO (vehicle control) to 200 μL bacterial suspension in PBS (1:100 (v/v)), vortex gently (*see* **Notes 8–10**).
11. Incubate for 1–2 h at RT with an occasional vortexing (*see* **Note 8**).
12. Pellet the cells at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
13. Wash the cell pellet with 2 × 1 mL PBS to remove excess of the probe. Then, centrifuge at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.

    PAUSE POINT: The cell pellet can be shock-frozen in liquid N_2 and stored at −80 °C until use.

14. Resuspend the cells in 200 µL PBS.
15. Lyse bacterial cells by sonication (4 × 15 s or 3 × 20 s, 80% intensity) under ice cooling (*see* **Note 11**).
16. Separate the insoluble (pellet) from soluble fraction (supernatant) by centrifugation at the maximum speed (e.g., 21,000 × *g*) for 45–60 min at 4 °C (*see* **Note 12**).
17. Transfer supernatant (soluble fraction) into new 1.5 mL Eppendorf tubes.
18. Wash pellet (insoluble fraction) by resuspending it in 200 µL PBS by sonication (1 × 10 s, 10% intensity) under ice cooling and pelletizing at 21,000 × *g* for 10 min at 4 °C. Discard supernatant.
19. Resuspend pellet in 200 µL PBS by sonication (1 × 10 s, 10% intensity) under ice cooling.

    PAUSE POINT: The cell lysates can be shock-frozen in liquid N_2 and stored at −80 °C until use.
20. Follow Protocol *Click-chemistry* (*CC*) *reaction with TAMRA-azide* (*TAMRA-N3*), *protein electrophoresis* (*SDS-PAGE*), *and in-gel fluorescence scanning* (*see* Subheading 3.6).

3.2 In Situ Analytical ABPP Labeling with a Photoprobe

1. Follow **steps 1–10** from Protocol *In situ analytical ABPP labeling* (*see* Subheading 3.1).
2. Incubate for between 15 min and up to 1 h at RT with an occasional vortexing.
3. Transfer samples into 24-well plates and irradiate for 15 min with UV light (350 nm for diazirines and 360 nm for benzophenone photocrosslinker) under ice cooling (*see* **Notes 13** and **14**).
4. Transfer samples back into 1.5 mL Eppendorf tubes.
5. Follow **steps 12–20** from Protocol *In situ analytical ABPP labeling* (*see* Subheading 3.1).

3.3 In Situ Preparative ABPP Labeling for Gel-Based MS Target Identification

1. Follow **steps 1–4** from Protocol *In situ analytical ABPP labeling* (*see* Subheading 3.1).
2. Pellet 2 × 10 mL bacterial culture at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
3. Resuspend each cell pellet in 5 mL PBS, then centrifuge at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
4. Resuspend each cell pellet in 500 µL fresh PBS at RT.
5. Add 1 µL ABPP probe (from 500 × DMSO stock) or 1 µL DMSO (vehicle control), vortex gently (*see* **Notes 9** and **15**).
6. Incubate for 2 h at RT with an occasional vortexing.
7. Pellet the cells at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.
8. Wash the cell pellet with 2 × 1 mL PBS to remove excess of the probe. Then, centrifuge at 6000 × *g*, for 10 min at 4 °C. Discard supernatant.

PAUSE POINT: The cell pellet can be shock-frozen in liquid N_2 and stored at −80 °C until use.

9. Resuspend cells in 500 µL PBS.
10. Lyse cells by sonication (4 × 15 s or 3 × 20 s, 80% intensity) under ice cooling (*see* **Note 11**).
11. Separate the insoluble (pellet) from soluble fraction (supernatant) by centrifugation at the maximum speed (e.g., 21,000 × *g*) for 45–60 min at 4 °C (*see* **Note 12**).
12. Transfer supernatant (soluble fraction) into new 2.0 mL Eppendorf tubes.
13. Wash pellet (insoluble fraction) by resuspending it in 200 µL PBS by sonication (1 × 10 s, 10% intensity) under ice cooling and pelletizing at 21,000 × *g* for 10 min at 4 °C. Discard supernatant.
14. Resuspend pellet in 500 µL PBS by sonication (1 × 10 s, 10% intensity) under ice cooling.
15. Add 5 µL trifunctional TAMRA-biotin-N_3 (10 mM stock in DMSO) and vortex.
16. Add 10 µL tris(carboxyethyl)phosphine (TCEP) (50 mM stock in water) and vortex.
17. Add 30 µL TBTA ligand (1.7 mM stock in DMSO–*tert*-BuOH 1:4 (v/v)) and vortex.
18. Add 10 µL $CuSO_4$ (50 mM or 12.5 mg $CuSO_4 \cdot 5\ H_2O$/mL stock in water) to initiate click chemistry reaction and vortex gently. Final concentrations are as follows: 100 µM TAMRA-biotin-N_3, 1 mM TCEP, 100 µM TBTA and 1 mM $CuSO_4$.
19. Incubate samples at RT for 1 h in the dark under constant mixing.
20. Precipitate proteins by adding four volumes of prechilled acetone. Vortex thoroughly (*see* **Note 16**).
21. Incubate samples for 1 h up to overnight at −21 °C.
22. Pelletize precipitated proteins at 21,000 × *g* for 20 min at 4 °C. Discard supernatant.
23. Wash the pellet 2 × 200 µL prechilled methanol and resuspend by sonication (5–10 s, 10% intensity) under ice cooling. Discard supernatant.
24. Air-dry the pellet and let it warm up to RT (*see* **Note 17**).
25. Dissolve the pellet in 1 mL 0.2% SDS in PBS by sonication (2–3 × 10 s, 10–20% intensity) until clear solution (*see* **Note 18**).
26. Incubate the sample under gentle mixing with 50 µL of pre-equilibrated avidin-agarose beads for 1 h at RT under continuous mixing (*see* **Note 19**).
27. Centrifuge beads down for 2 min at 380–400 × *g* at RT.

28. Thereafter, wash beads with: 0.2% SDS in PBS (3×1 mL), 6 M urea (2×1 mL, 6 M in water) (*see* **Note 20**) and PBS (3×1 mL). After each washing, spin beads down for 1–2 min at 2000 rpm and remove supernatant carefully (*see* **Note 21**).
29. Add 50 µL 2× SDS loading buffer to the beads and release the proteins from the beads for the preparative SDS-PAGE by incubation for 6 min at 96 °C.
30. Centrifuge the beads down for 2 min at maximum speed (e.g., 21,000×*g*) at RT.
31. Transfer the supernatant containing released proteins to new Eppendorf tubes.

    PAUSE POINT: The samples can be stored at −20 °C until preparative SDS gel running.
32. Resolve proteins by SDS-PAGE on 10% polyacrylamide gels (20×20 cm) (50 µL protein sample loading) using 300 V voltage (ca. 3–4 h) (*see* **Note 22**) or on any other type of SDS gels (10–15%).
33. Record fluorescence on a Fujifilm LAS-4000 Luminescent Image Analyzer with a Fujinon VRF43LMD3 Lens and a 575DF20 filter (TAMRA-N_3 excitation maximum: Ex = 546 nm, emission maximum: Em = 579 nm) or an equivalent fluorescence scanner.
34. Excise gel bands of interest along with the corresponding regions from DMSO control.
35. Wash, reduce, alkylate, in-gel digest and extract proteins and run LC-MS/MS to identify protein targets as described elsewhere (*33*).

3.4 In Vitro Analytical ABPP Labeling

1. Follow **steps 1–4** from Protocol *In situ analytical ABPP labeling* (*see* Subheading 3.1).
2. Pellet cells at 6000×*g*, for 10 min at 4 °C. Discard supernatant.
3. Wash cells with fresh PBS, then centrifuge at 6000×*g*, for 10 min at 4 °C. Discard supernatant.
4. Resuspend cells in cold PBS (*see* **Note 23**).
5. Lyse bacterial cells by sonication (4×15 s, 80% intensity) under ice cooling (*see* **Note 10**).
6. Separate the insoluble (pellet) from soluble fraction (supernatant) by centrifugation at the maximum speed (e.g., 21,000×*g*) for 45–60 at 4 °C (*see* **Note 12**).
7. Transfer supernatant (soluble fraction) into new 1.5 mL Eppendorf tubes.
8. Wash pellet (insoluble fraction) by resuspending it in PBS by sonication (1×10 s, 10% intensity) under ice cooling and pelletizing at 21,000×*g* for 10 min at 4 °C. Discard supernatant.

9. Resuspend pellet in PBS by sonication (1 × 10 s, 10% intensity) under ice cooling (*see* **Note 23**).
10. Assign protein concentration using a Bradford or BCA assay (*see* **Note 24**) and adjust total protein concentration to 1–2 mg/mL by dilution with PBS.

    PAUSE POINT: The cell lysates can be shock-frozen in liquid N_2 and stored at −80 °C until use.
11. Dispense 43 µL soluble and insoluble fraction of cell lysate into new Eppendorf tubes. For heat controls, denature 41 µL proteome with 2 µL 21.5% SDS (2% SDS final concentration) for 6 min at 96 °C and cool samples down to RT.
12. Add 1 µL probe from 50× DMSO stock (*see* **Notes 8** and **9**).
13. Incubate for 1 h at RT with an occasional vortexing (*see* **Note 8**).
14. Follow Protocol *Click-chemistry* (*CC*) *reaction with TAMRA-azide* (*TAMRA-N3*), *protein electrophoresis* (*SDS-PAGE*) *and in-gel fluorescence scanning* (*see* Subheading 3.6).

3.5 In Vitro Analytical ABPP Labeling with a Photoprobe

1. Follow **steps 1–13** from Protocol *In vitro ABPP labeling* (*see* Subheading 3.3).
2. Transfer samples into 24-well plates and irradiate for 15 min with UV light (350 nm for diazirines and 360 nm for benzophenone photocrosslinker) under ice cooling (*see* **Notes 13** and **14**).
3. Transfer samples back into 1.5 mL Eppendorf tubes.
4. Follow Protocol *Click-chemistry* (*CC*) *reaction with TAMRA-azide* (*TAMRA-N*^{3}), *protein electrophoresis* (*SDS-PAGE*), *and in-gel fluorescence scanning* (*see* Subheading 3.6).

3.6 Click-Chemistry (CC) Reaction with TAMRA-azide (TAMRA-N_3), Protein Electrophoresis (SDS-PAGE), and In-Gel Fluorescence Scanning

1. Dispense 44 µL soluble and insoluble fraction of probe-bound cell lysate into new Eppendorf tube.
2. Add 1 µL TAMRA-N_3 (5 mM stock in DMSO) and vortex gently (*see* **Note 25**).
3. Add 1 µL TCEP (50 mM or 15 mg/mL stock in water) and vortex gently.
4. Add 3 µL TBTA ligand (1.7 mM stock in DMSO–*tert*-BuOH 1:4 (v/v)) and vortex gently (*see* **Note 26**).
5. Add 1 µL $CuSO_4$ (50 mM or 12.5 mg $CuSO_4 \cdot 5\ H_2O$/mL stock in water) to initiate click chemistry reaction and vortex gently. Total reaction volume is 50 µL and final concentrations are as follows: 100 µM TAMRA-N_3, 1 mM TCEP, 100 µM TBTA, and 1 mM $CuSO_4$.
6. Incubate samples at RT for 1 h in the dark.
7. Quench the reaction by mixing the sample with 50 µL 2 × SDS loading buffer (*see* **Note 27**).

8. Denature samples for 5 min at 95 °C.
   PAUSE POINT: The samples can be stored at −20 °C until SDS gel running.
9. Resolve proteins by SDS-PAGE on 10% polyacrylamide gels (20×20 cm) (50 µL protein sample loading, ca. 50 µg protein/lane) using 300 V voltage (ca. 3–4 h) (*see* **Note 22**).
10. Record fluorescence on a Fujifilm LAS-4000 Luminescent Image Analyzer with a Fujinon VRF43LMD3 Lens and a 575DF20 filter or an equivalent fluorescence scanner (*see* **Note 28**).
11. Subject gels to Coomassie Brilliant Blue staining to verify equivalent protein loading.

4 Notes

1. The following bacterial strains have been used for ABPP experiments. All the strains were grown at 37 °C with shaking at 200 rpm. Appropriate media are given in parentheses. Biosafety level S1: *Escherichia coli* K12 ATCC 10798 (ATCC, USA) (LB medium), *Bacillus subtilis* 168 ATCC 23857 (ATCC, USA) (LB medium), *Bacillus licheniformis* 46 ATCC 14580 (ATCC, USA) (LB medium), *Listeria welshimeri* SLCC 5334 serovar 6b (DSMZ, Germany) (BHB medium), *Enterococcus faecalis* OF1RF ATCC 47077 (LGC Standards, Germany) (BHB medium), *Burkholderia thailandensis* E264 ATCC 700388 (ATCC, USA) (CASO medium). Biosafety level S2: *Escherichia coli* 536 (clinical isolate from pyelonephritis) *Escherichia coli* CFT073 (clinical isolate from urosepsis) *Escherichia coli* UTI89 (clinical isolate from cystitis) (LB medium), *Pseudomonas aeruginosa* PAO1 (Institute Pasteur, France) (LB or CASO medium), *Staphylococcus aureus* NCTC 8325 (Institute Pasteur, France) (B or BHB medium), *S. aureus* Mu50 ATCC 700699 (Institute Pasteur, France) (B or BHB medium), *S. aureus* DSM 18827 (DSMZ, Germany) (clinical isolate from the tracheal secretion) (B or BHB medium), *S. aureus* DSM 19041 (DSMZ, Germany) (an highly toxin producing isolate from a human furuncle) (B or BHB medium), *S. aureus* USA300 FPR3757 ATCC BAA1556 (LGC Standards, Germany) (B or BHB medium), *Listeria monocytogenes* EGDe (Institut Pasteur, France) (BHB medium), *L. monocytogenes* F2365 (BCCM/LMG Bacteria Collection) (BHB medium), *Enterococcus faecalis* V583 ATCC 700802 (LGC Standards, Germany) (BHB medium), *Burkholderia cenocepacia* J2315 ATCC BAA245 (ATCC, USA) (CASO medium).
2. Glycerol stocks are used to store bacterial strains at low temperatures (−20 and −80 °C). In order to prepare glycerol stock harvest bacterial culture from the late exponential or stationary

phase at 6000 g, for 10 min at 4 °C. Remove supernatant completely. Concentrate bacteria by resuspending the cell pellet in ¼ volume fresh culture medium. Add approximately the same volume of a sterile glycerol to 50% (v/v). Vortex, aliquot and freeze at −80 °C. Using aliquots of the same glycerol stock guarantee the reproducibility of follow up experiments.

3. Bacteria which are grown overnight are in the late stationary phase.
4. Adjust the size of the culture to the amount of bacterial culture needed for further labeling experiments. As a rule of thumb, use 100 mL medium in 500 mL glass culture flask for a single labeling experiment up to 50 individual samples. Ratio 1:5 (v/v) culture volume to flask volume is optimal for a proper aeration of the culture. Scale up/down if necessary.
5. In order to choose an appropriate time point for labeling as well as to achieve reproducible results, it is recommended to assign first the growth curves by plotting optical density (OD_{600}) of the bacterial culture versus time on a logarithmic scale in at least three independent biological experiments. The selection of the bacterial growth point needs to match the biological process in question.
6. In order to assign actual state of the bacterial culture measure its optical density (OD_{600}). Dilute 1:9 (v/v) 100 μL bacterial culture with 900 μL fresh medium, mix well, measure in triplicate. When OD_{600} is no longer changing significantly, the bacterial culture has reached stationary phase.
7. Add to the calculation ca. 10–20% excess.
8. Concentration of an ABPP probe and time of the labeling need to be optimized and adjusted to a biological question being studied. As a rule of thumb, 50 μM and 1 h incubation is a good starting point.
9. DMSO content in medium should not exceed 1% (v/v), therefore at least 100 × DMSO probe stock or higher should be applied.
10. For a competitive ABPP labeling experiment, cells are preincubated with an excess (typically 10 to 100-fold) of a competitor (usually natural product or a specific inhibitor) for 15 min up to 1 h. This is then followed by addition of an ABPP probe which is further incubated with the sample.
11. Cells can be alternatively lysed using a homogenizer (5400 rpm, 3 × 20 s) with 2 min intermittent cooling on ice. For this, cell suspension is transferred to 500 μL Precellys® vials filled with 0.5 mm glass beads. After the lysis, the beads and cell debris are separated by centrifugation at 2000 rpm, for 2 min at 4 °C. Cell disruption by bead mill homogenization is especially recommended for Gram-positive bacterial strains.

12. For a better separation of the cytosolic fraction from membrane fraction, the ultracentrifugation at 100,000 × *g*, for 45 min at 4 °C is recommended.
13. The photoirradiation can be done directly on a cell suspension with an excess of the probe. However to reduce unspecific photocrosslinking, the cells can be first pelletized, supernatant with excess of a probe removed, cells washed in PBS and resuspended again in PBS and then irradiated with UV light.
14. Photoirradiation time may also have to be optimized to minimize unspecific background labeling.
15. Concentration of an ABPP probe for protein target identification should be chosen in such a way that specific labeling is strong while unspecific background is minimized.
16. Alternatively to acetone protein precipitation, methanol–chloroform method can be used.

    Use 150 μL samples. Add 4 volumes of MeOH (600 μL), followed by addition of one volume of chloroform (150 μL). Vortex well and then check whether there is only one phase. Add three volumes of distilled water (450 μL), vortex thoroughly. Centrifuge for 1 min at maximum speed at RT in a table centrifuge. Remove upper organic phase without disturbing interphase (which contains proteins). Add three volumes of MeOH (450 μL), vortex thoroughly. Spin for 1–2 min at maximum speed at RT. Remove supernatant carefully and air-dry pellet, Dissolve the pellet in PBS or an appropriate buffer.
17. Avoid adding 0.2 % SDS in PBS before the pellet reached ambient temperature, as precipitation of SDS can happen.
18. The complete dissolution of protein pellet is crucial for a successful enrichment procedure. If dissolving turns difficult, it can be facilitated by a short incubation at elevated temperature (e.g., 10 min at 30–37 °C) or by increasing SDS content to 0.4 % (w/v).
19. Equilibrate avidin-agarose beads (avidin-agarose from egg white, 1.1 mg/mL in aqueous glycerol suspension) by resuspension in 1 mL 0.2 % SDS in PBS, centrifugation for 1–2 min at 2000 rpm and careful removal of supernatant. Repeat the procedure two times.
20. Prepare urea solution freshly by dissolving 7.2 g urea in 20 mL distilled water.
21. Careful removal of the supernatant from above the beads is important; otherwise the beads can be partially removed. Use a yellow pipette tip (200 μL) stacked on a blue tip (1000 μL) and remove supernatant slowly from above going down to the beads level.

22. SDS-PAGE is performed Peqlab PerfectBlue Dual Gel System Twin L (45-2020-I) (big gels 20 × 20 cm) or Peqlab PerfectBlue Dual Gel System Twin S (45-1010-I) (small gels 10 × 10 cm).
23. Volume of PBS used for resuspending cells depends on the volume of the bacterial culture. The volume should be reduced to ca. 1/10 to 1/50 of the original culture.
24. For Bradford or BCA protein concentration assignment Bio-Rad Protein Assay Kit II or Carl Roth Roti®-Quant universal BCA Protein Assay Kit is used, respectively. Assays are performed according to manufacturer's procedure. Bovine serum albumin (BSA) standards (0–400 μg/mL range: 0, 25, 50, 100, 200, 400 μg/mL) is prepared from the 2.0 mg/mL stock solution in distilled water in order to establish protein concentration standard curve. Absorbance is recorded by a TECAN Infinite® M200 Pro. All samples are measured in triplicate.
25. Alternatively, a master mix can be prepared directly before click chemistry reaction. In this case to each sample add 5 μL freshly prepared master mix containing 1 μL TAMRA-N_3 (5 mM stock in DMSO), 1 μL TCEP (50 mM stock in water), and 3 μL TBTA ligand (1.7 mM stock in DMSO–*tert*-BuOH 1:4 (v/v)). 3. Vortex samples gently.
26. 1× TBTA ligand has concentration 1.667 mM and is prepared by mixing 800 μL *t*-BuOH, 180 μL DMSO, and 20 μL 50× TBTA ligand. 50× TBTA ligand has concentration 83.35 mM and is prepared by dissolving 8.85 mg ligand in 200 μL DMSO.
27. Addition of ethylenediaminetetraacetic acid disodium salt (EDTA) solution to final concentration of 10 mM can also quench click chemistry reaction.
28. When fluorescence signal is weak or unspecific background high, it is recommended to precipitate proteins and remove excess of the fluorescent cocktail (by any common method (e.g., acetone or methanol–chloroform precipitation), then resuspend sample in SDS loading buffer before running an SDS gel.

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Chapter 6

Activity-Based Lipid Esterase Profiling of *M. bovis* BCG at Different Metabolic States Using Tetrahydrolipstatin (THL) as Bait

Madhu Sudhan Ravindran and Markus R. Wenk

Abstract

This chapter provides a step-by-step protocol using activity-based protein profiling (ABPP) as a chemical-proteomic tool to survey the antibiotic properties of a small molecule. Here, we investigate the molecular mechanism behind the bactericidal activity of tetrahydrolipstatin (THL). ABPP relies on small molecule probes that target the active site of specific enzymes in complex proteomes. These probes in turn are equipped with a reporter tag that allows capturing, visualization, enrichment, identification, and quantification of its targets either in vitro or in situ. THL possesses bactericidal activities, but its precise spectrum of molecular targets is poorly characterized. Here, we used THL analogs functionalized to enable Huisgen-base cycloaddition, commonly known as "click chemistry," to identify target proteins after enrichment from mycobacterial cell lysates obtained from different physiological conditions.

Key words Activity-based protein profiling, Cycloaddition reaction, Lipid esterases, Lipases, Tetrahydrolipstatin

1 Introduction

Tetrahydrolipstatin (also known as Orlistat) (Fig. 1a) is an FDA-approved antiobesity drug with potential bactericidal and antitumor properties [1–5]. The target of this β-lactam containing structure is often associated with the lipid-catabolizing enzymes [6, 7], but its detailed target spectrum is poorly characterized. Here we attempt to provide a detail methodology to profile the THL enzymatic targets in complex bacterial proteome.

M. bovis BCG is an attenuated relative of pathogenic *M. tuberculosis*, with high genome and proteome sequence homology [8–11]. Both these species are known to persist under different pathophysiological states [12–14]. Under these physiological states the bacteria are known to survive by utilizing self and host lipids as a carbon source via action of lipases and/or esterases [15–17]. Comparative sequence analysis has revealed the involvement of more than 250

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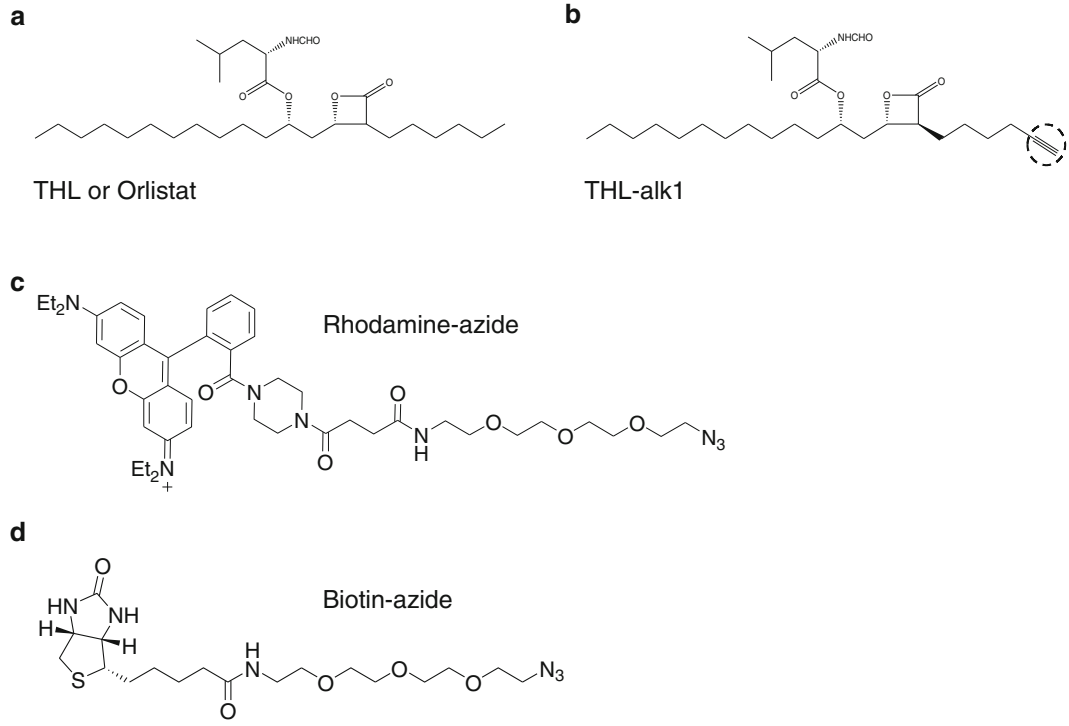


Fig. 1 Chemical structure of THL analog and ABPP reagents. (**a**) and (**b**). Chemical structure of THL (**a**) and its analog (THL-alkyne) (**b**) containing alkyne group (*dotted circle*). (**c**) and (**d**). Chemical structure of rhodamine-azide (**c**) and biotin-azide (**d**). Adapted from Ravindran MS, et al. MCP, 2014 [34]

lipid metabolizing genes, emphasizing the contribution of these genes in bacterial survival and pathogenicity [18, 19]. Among these annotated genes, few have been functionally characterized and/or associated to its life cycle [20–23]. In the current chapter, we describe, a step-by-step high-throughput and robust protocol for lipid-esterase profile of *M. bovis* BCG culture extracts obtained at three different physiological states (exponential growth under aerobic conditions (logarithmic), non-replicating persistent state (NRP), and regrowth from NRP state) using THL as bait.

ABPP is a functional proteomics technique that relies on the irreversible-ligand formation targeting the active sites of a specific family or class of enzymes in complex proteomes [24, 25]. The target(s) bound ligands are often equipped with reactive groups that can be loaded with fluorescent or affinity tags for visualization and/or enrichment. The enriched proteins are then accurately identified by means of liquid chromatography–mass spectrometry (LC-MS)-based proteomics, thereby providing a quantitative readout of the functional state of individual enzymes in the family [26, 27]. In this chapter, we use THL-alkyne analog (Fig. 1b) to quantify the lipid esteraseactivity in *Mycobacterium bovis* BCG at different metabolic state. The seven-step (Fig. 2) high-resolution profiling entails: (1) preparation of cell lysates, (2) incubation with

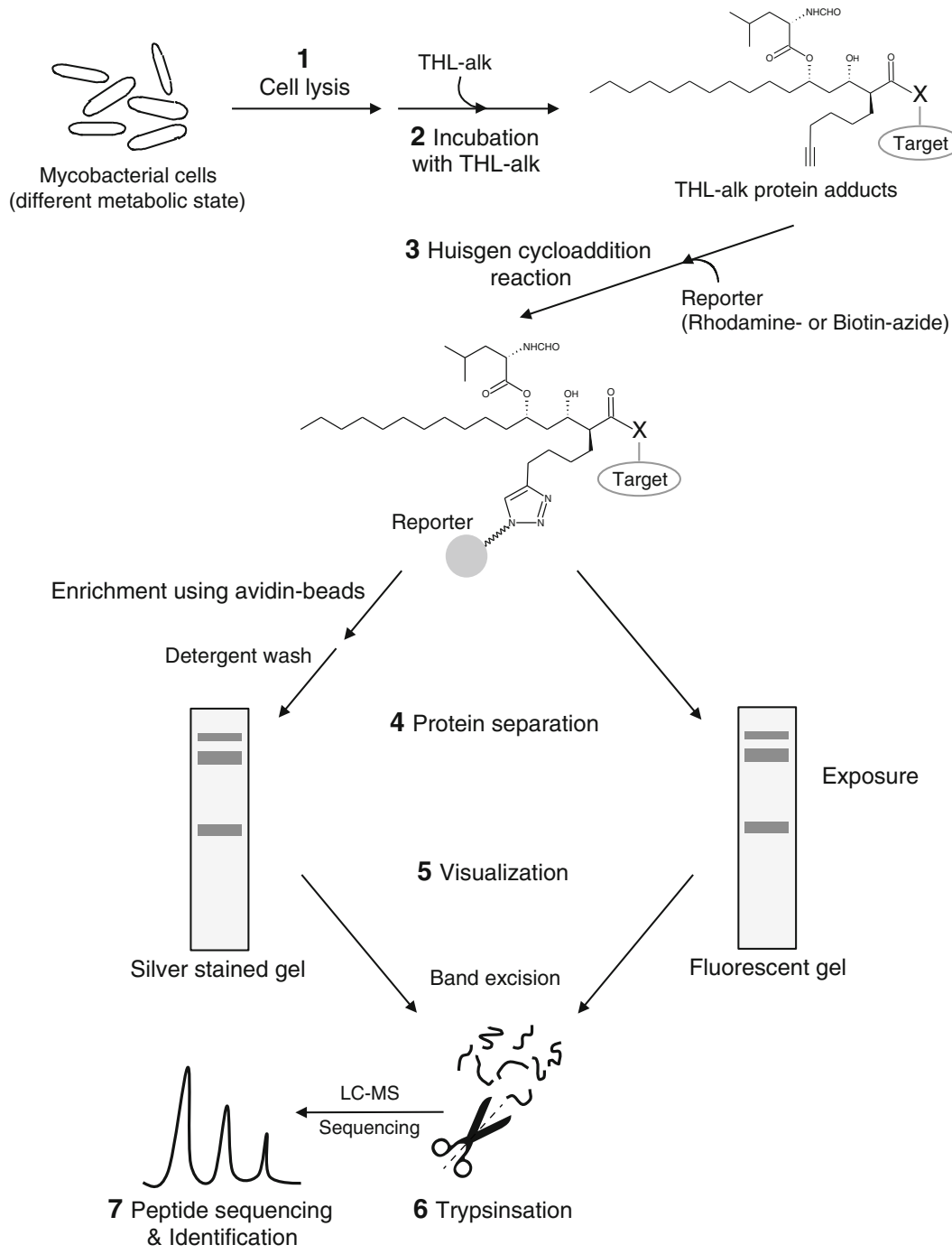


Fig. 2 Schematic representation of the workflow and steps involved in the identification of THL targets. *M. bovis* BCG was grown in three different physiological states in vitro, cells lysed by sonication (**step 1**) and total cell extracts incubated with THL-alk (**step 2**). The alkyne moiety was then used to either tag (via the Huisgen cycloaddition reaction) protein-THL adducts with rhodamine-azide or biotin-azide (**step 3**). Whole cell lysates (in the case of rhodamine tagged adducts) or enriched fractions (in the case of biotin tagged adducts) were separated by SDS-PAGE (**step 4**) and visualized using fluorescent scan (rhodamine tagged) or silver staining (enriched fractions) (**step 5**). Specific protein band of interests are excised and trypsinized (**step 6**), prior to their identification via LC-MS (**step 7**). The identified proteins were compared with visualized protein patterns (**step 5**) to derive the THL target list. Adapted from Ravindran MS, et al. MCP, 2014 [34]

alkyne-containing ligand, (3) installation of azide-containing reporter warhead, (4) visualization or enrichment, (5) separation on protein gel, (6) trypsinization, and (7) identification by LC-MS.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise specified). Carefully follow all waste disposal regulations when disposing waste materials. Always wear gloves to prevent keratin contamination.

2.1 Mycobacterial Cultures

1. *Mycobacterium bovis* BCG Pasteur strain (ATCC 35734).
2. 7H11 agar plates: Resuspend 19 g of the 7H11 agar medium (Difco, Detroit, MI) in 900 ml water containing 5 ml of glycerol (Sigma-Aldrich, St. Louis, MO). Resuspend the contents well, heat to boiling if required. Autoclave at 121 °C for 10 min at 15 psi, cool to 40–50 °C (*see* **Note 1**). Aseptically add 100 ml of OADC (Difco) and pour 25 ml of this mixture on to 10 cm petri dishes (Corning, Tweksbury, MA). Once the agar solidifies, the plates are sealed with Parafilm and stored at 4 °C.
3. Dubos liquid medium: Weigh 6.5 g of broth media base (Difco) and transfer to a 1 l glass bottle. Add 900 ml water, mix and autoclave at 121 °C for 15 min at 15 psi. Cool the media (*see* **Note 2**) and aseptically add 20 ml of medium albumin–dextrose supplement (Difco) (*see* **Note 3**) and 0.05% Tween 80 (*see* **Note 4**). Tighten caps and store the media indefinitely at 4 °C.
4. 500 ml volume roller bottles (Corning) and roller culture apparatus (Fisher Scientific, Waltham, MA).
5. 1 l clear threaded glass bottles (Duran, Mainz, Germany) with rubber-seal lids (Sigma-Aldrich) containing 2-in. magnetic bead (*see* **Note 5**). Autoclave for 15 min at 15 psi, 121 °C and cool to room temperature.
6. Multipoint magnetic stir platform (Thermo Scientific, Rockford, IL).
7. 0.5% methylene blue (w/v) (Sigma-Aldrich) indicator (1000× stock) prepared in sterile water.
8. Culture pellet wash buffer: Phosphate buffered saline (PBS) containing 0.05% tween 80.
9. Probe sonicator: Qsonica Q125 sonicator (Newtown, CT) standard probe.

2.2 ABPP Reagents

1. THL-alkyne, rhodamine-azide, and biotin-azide (Fig. 1) are synthesized as described previously [28, 29].
2. 1 mM THL-alkyne (200× stock) (Fig. 1b) prepared in DMSO and aliquots are stored at −20 °C.

3. 1 mM rhodamine-azide (10× stock) (Fig. 1c) prepared in DMSO. Protect from light and store at −20 °C.
4. 1 mM biotin-azide (10× stock) (Fig. 1d) prepared in DMSO. Store at −20 °C.
5. 100 mM $CuSO_4$ (100× stock) (Sigma-Aldrich) prepared in water. Protect from light and store at room temperature.
6. 100 mM *tris*-(benzyltriazolylmethyl)amine (TBTA) (10× stock) (Sigma-Aldrich) prepared in DMSO and aliquots are stored at −20 °C.
7. 1 mM tris(2-carboxyethyl)phosphine (TCEP) (10× stock) (Sigma-Aldrich) prepared in water and aliquots are stored at −20 °C.

2.3 Protein Separation and Identification Reagents

1. 4× sodium dodecyl sulfate (SDS) loading buffer: 200 mM Tris–HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, 4% β-mercaptoethanol, 40% glycerol. Leave one aliquot at 4 °C for current use and store remaining aliquots at −20 °C (*see* **Note 6**).
2. Pre-cast 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) with 1.0 mm thickness containing 10 wells.
3. In-gel fluorescence scanner: Typhoon 9410 Variable Mode Imager scanner (GE Amersham, USA) with scanning wavelength for rhodamine (560–650 nm).
4. Coomassie R-250 stain and destain: 0.25% Coomassie R-250 (Bio-Rad) is dissolved in glacial acetic acid–methanol–water (v/v/v) 1:4.5:4.5. The contents are stored in dark. The solution without R250 is used as a destaining solution (*see* **Note 7**).
5. Ice-cold acetone and methanol (*see* **Note 8**).
6. Avidin agarose beads: NeutrAvidin agarose beads (Thermo Scientific, USA) are pre-washed thrice with 100 mM Tris pH 8 followed by PBS.
7. Beads washing buffers: Buffer A (8 M urea, 200 mM NaCl, 2% SDS, 100 mM Tris pH 8); buffer B (8 M urea, 1.2 M NaCl, 0.2% SDS, 100 mM Tris pH 8, 10% ethanol, 10% isopropanol); buffer C (8 M urea, 100 mM Tris pH 8).

3 Methods

3.1 Preparation of Whole Cell Lysates of *M. bovis* BCG Cultured at Different Metabolic States

1. *M. bovis* BCG preculture is prepared by inoculating a single colony picked from Middlebrook 7H11 agar plates. Propagate the culture in Dubos liquid medium in roller bottles rotated at 50 rpm for 3 days with an initial optical density at 600 nm ($OD_{600\ nm}$) of 0.05 (*see* **Note 9**).
2. NRP BCG cultures are cultivated by subjecting bacilli to the slow withdrawal of oxygen; the protocol was adapted from

[30, 31]. Briefly, aerobic cultures (780 ml), with an initial $OD_{600\,nm}$ of 0.005, are expanded in 1 l glass bottles, achieving an optimal headspace ratio (0.5 HSR) of air volume to liquid volume. The sealed cultures are stirred gently at 80 rpm for 18 days to allow the bacilli to enter into NRP. Monitor oxygen depletion in these cultures by the use of the 0.5 μg/ml methylene blue as an oxygen indicator (*see* **Note 10**).

3. For regrowth of NRP cultures, reintroduce fresh oxygenated air into cultures by loosening the caps and culture further for 3 days. This exposure of air allows NRP bacilli to regrow.
4. Growth and survival for different time periods is monitored by inventory of culture forming units (CFU) on Middlebrook 7H11 agar after plating of appropriate dilutions (10^{-4} till 10^{-7}) (*see* **Note 11**). Plates are sealed with Parafilm and incubated at 37 °C for 4 weeks.
5. Harvest mycobacterial cultures by centrifugation at 3000 × *g* for 20 min at 4 °C (*see* **Note 12**). Wash cell pellets thrice with 25 ml of PBST and store pellets at −80 °C.
6. Prepare the total cell lysates by disrupting cells in PBS using sonication on ice for 10 min at 50% amplitude (with a 10 s pulse and 10 s interval) (*see* **Note 13**). Centrifuge the lysed cells at 20,000 × *g* for 15 min at 4 °C and snap-freeze the supernatant aliquots in liquid nitrogen and store at −80 °C (*see* **Note 14**).

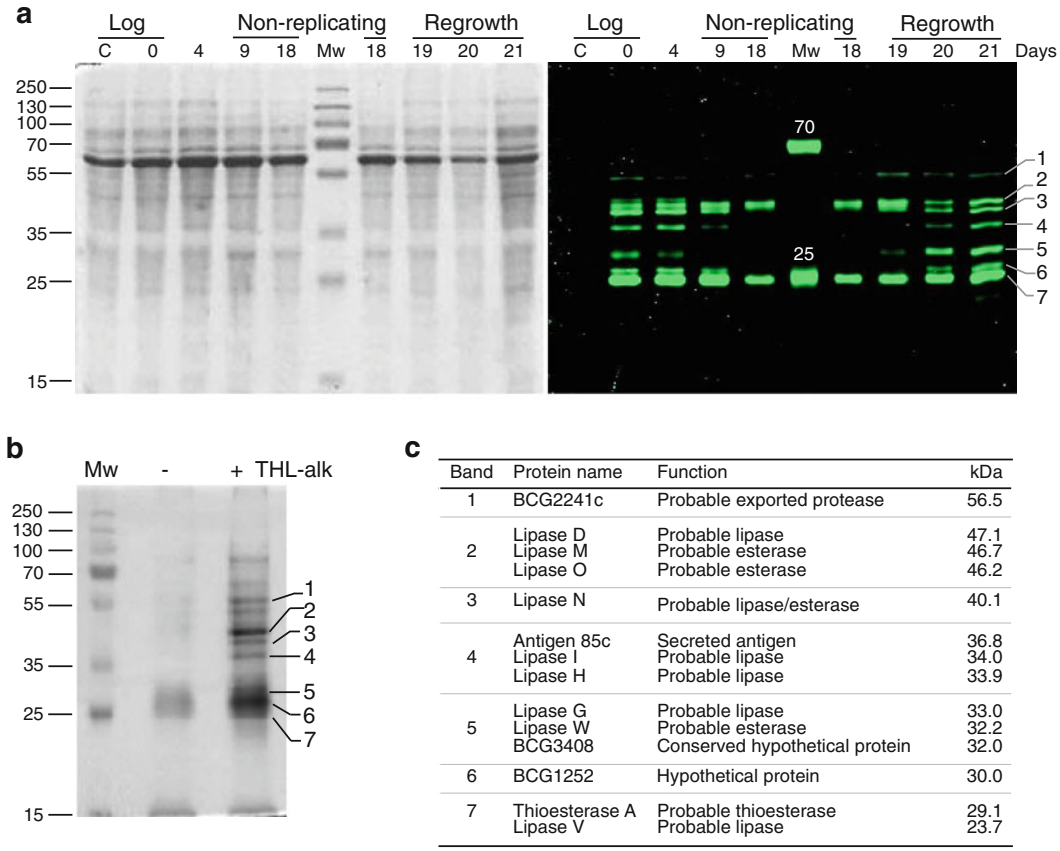
3.2 Huisgen Cycloaddition Reaction

1. Determine the *M. bovis* BCG total protein concentration and adjust the concentration to 1 μg/μl using PBS. Incubate 100 μg of total protein with 5 μM of THL-alkyne at room temperature for 1 h. Another reaction with 0.5% DMSO (without THL-alkyne) is used as a control.
2. A freshly premixed solution containing 1 mM $CuSO_4$, 1 mM TBTA, 100 μM TCEP, and 100 μM rhodamine-azide is prepared from stock solutions. Add 20 μl of this mixture to the tubes containing cell lysates incubated with THL-alkyne (or DMSO control) to initiate the Huisgen cycloaddition reaction. Stir the reaction at room temperature for 2 h in the dark and precipitate the proteins by adding 600 μl of ice-cold acetone followed by overnight incubation at −20 °C (*see* **Note 15**).
3. Pellet the overnight precipitated proteins at 20,000 × *g* for 15 min at 4 °C. Wash protein pellets twice with 500 μl of methanol by vortexing and centrifugation. Air-dry the pellet for few minutes and add 50 μl of 1× SDS loading buffer. Boil at 96 °C for 15 min. Separate 5 μl (~10 μg) of protein 12% SDS-PAGE using the following settings: 120 V (constant), 60 mA and 120 min. Wash the gel twice with distilled water,

followed by in-gel fluorescence scanning and visualize total protein using Coomassie R-250 staining.

4. For the enrichment of THL targets, use 2 mg of the total protein to perform the Huisgen cycloaddition reaction. The cycloaddition reaction mixture (as mentioned in Subheading 3.2, **step 2**) contains 100 μM biotin-azide instead of rhodamine-azide. Stir the reaction at room temperature for 4 h and precipitate the proteins by the addition of 12 ml of ice-cold acetone followed by overnight incubation at −20 °C.
5. Wash the acetone-precipitated pellets with methanol and air-dry. Resuspend the pellet in 1 ml of 0.2% SDS in PBS and briefly sonicate (*see* **Note 16**). Centrifuge the resuspended pellet at 20,000 × *g* for 5 min. Incubate the supernatants with 25 μl of avidin agarose beads overnight at 4 °C in a mechanical stirrer.
6. Collect beads by centrifugation and wash thrice with 1 ml of wash buffer A, buffer B, buffer C and finally with PBS (*see* **Note 17**). Elute proteins from beads with 25 μl of 2× SDS-loading buffer and incubate at 96 °C for 20 min.
7. Separate 20 μl of elute on a 12% SDS-PAGE using the settings mentioned in Subheading 3.2, **step 3**. Wash the gel twice with distilled water post-electrophoresis (*see* **Note 18**) and visualize proteins by Vorum and Mann silver staining protocol adapted from [32]. Excise protein bands corresponding to both DMSO-treated (control) (Fig. 3a, b) and THL-alkyne treated samples as single bands (*see* **Note 19**) followed by in-gel trypsin digestions as described previously [33]. Dry the eluted peptides were dried under vacuum and store at −20 °C until analysis.
8. Resuspend the tryptic-digested dried peptides in 1% formic acid and separate and analyze on a liquid chromatography system coupled to an mass spectrometry (LC-MS) as described in [34].
9. Manually filter the proteins from DMSO-treated (control) list and identify based on their scores, number of unique matching peptides, protein abundance and molecular weight. MASCOT Protein scores greater than ($p < 0.05$), 2 or more numbers of unique matching peptides and protein abundance (emPAI) score greater than 0.1 correspond to confident identifications (Fig. 3c).

Overall, we used a THL analog and activity-based protein profiling to identify target proteins of *Mycobacterium bovis* BCG cultured under replicating and non-replicating conditions. Our results define the target spectrum of THL in a biological species with particularly diverse lipid metabolic pathways. We furthermore derive a conceptual approach that demonstrates the use of such THL probes for the characterization of substrate recognition by lipases and related enzymes.



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Band	Protein name	Function	kDa
1	BCG2241c	Probable exported protease	56.5
2	Lipase D Lipase M Lipase O	Probable lipase Probable esterase Probable esterase	47.1 46.7 46.2
3	Lipase N	Probable lipase/esterase	40.1
4	Antigen 85c Lipase I Lipase H	Secreted antigen Probable lipase Probable lipase	36.8 34.0 33.9
5	Lipase G Lipase W BCG3408	Probable lipase Probable esterase Conserved hypothetical protein	33.0 32.2 32.0
6	BCG1252	Hypothetical protein	30.0
7	Thioesterase A Lipase V	Probable thioesterase Probable lipase	29.1 23.7

Fig. 3 THL target spectrum in *M. bovis* BCG largely comprises lipid esterases. (**a**) Cell lysates prepared from *M. bovis* BCG cultured in different physiological states were incubated with THL-alkyne (and DMSO as a control). Huisgen cycloaddition reactions were performed using rhodamine-azide dye. Equal amounts of proteins were separated by SDS-PAGE and visualized by in-gel fluorescence (*left panel*), followed by Coomassie staining (*right panel*). (**b**) THL-alk bound targets were enriched using biotin–avidin affinity chromatography. Enriched fractions were separated on SDS-PAGE and stained with silver. The bands indicated on the *right side* were excised and subjected to tandem MS analysis. (**c**) THL targets in *M. bovis* BCG total cell extracts derived from logarithmically growing cultures. Only proteins found consistently in at least 2 out of 3 three replicate experiments, not present in control incubations (DMSO in the absence of THL-alk), and, with matching, co-migrating fluorescent targets (*Panel* **b**), were included in this list. *This research was originally published in Molecular and Cellular Proteomics Journal*. Ravindran et al. (2014) [34]. © the American Society for Biochemistry and Molecular Biology

4 Notes

1. Prevent prolong cooling of the agar at room temperature to avoid agar clumping, solidification and frothing.
2. Add albumin–dextrose supplement to the autoclaved media once the media has cooled to below 50 °C (hot to the touch, but bearable).

3. Occasionally the albumin–dextrose supplement contains some precipitate, hence, it is recommended to filter sterile the broth–supplement–Tween 80 mixture via 0.22 μM vacuum filtration system.
4. Prepare 0.05% tween 80 by adding tween 80 directly into water in a flask and sonicated to mix the content (avoid vigorous shaking to prevent frothing). Once dissolved completely, filter sterile the content using 0.22 μM vacuum filtration system and store at 4 °C.
5. The 1 l culture bottles rims are prone to fracture during washing and handling, therefore it is recommended to manually check for broken rims to prevent leakage of air into hypoxic cultures.
6. SDS precipitates at 4 °C. Therefore, the lysis buffer needs to be warmed prior to use.
7. Gels can be destained faster by replacing the destain solution frequently until the protein band of interest is visible and background gel is completely transparent.
8. 50 ml of acetone and methanol are aliquoted into glass bottles and stored in −20 °C for at least an hour before use.
9. Precultures were tested for possible contamination from non-mycobacterial microorganisms using Gram and acid-fast (Zeil-Neelsen) staining (Merck-Millipore) following manufacturer's protocol.
10. The culture tested using methylene blue indicator cannot be processed further for biological experiments.
11. To determine the CFU, 100 μl of culture is serially diluted in 900 μl of 7H9 media and mixed thoroughly. 100 μl is plated on 7H11 agar plates.
12. Occasionally, post-centrifugation, layers of bacterial cells are observed floating on top of the supernatant. These cells can also be collected and pooled with the bacterial pellets, the cells are buoyant due to lipid enriched bacillus.
13. The bacterial pellets are resuspended in PBS by vortexing or pipetting before probe sonication.
14. The lysates obtained after sonication should be immediately snap-frozen in liquid nitrogen and smaller aliquots are stored in −80 °C. Freeze–thaw cycle should be avoided.
15. The protein precipitation in acetone can be done for 2 h to overnight.
16. To resuspended the air-dried pellet entirely, the protein pellets are broken by tapping or vortexing before adding 0.2% SDS containing buffer.
17. After each buffer wash, the contents are completely removed using micro-pipette.

18. Use distilled (or ultrapure) water for silver staining. Presence of contaminant (like various ions) and differing pH might interfere with staining protocol.
19. Gel bands are excised using a fresh scalpel; wear gloves and face mask to avoid keratin contamination.

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Chapter 7

Comparative Activity-Based Flavin-Dependent Oxidase Profiling

Joanna Krysiak and Rolf Breinbauer

Abstract

Activity-based protein profiling (ABPP) has become a powerful chemoproteomic technology allowing for the dissection of complex ligand–protein interactions in their native cellular environment. One of the biggest challenges for ABPP is the extension of the proteome coverage. In this chapter a new ABPP strategy dedicated to monoamine oxidases (MAO) is presented. These enzymes are representative examples of flavin-dependent oxidases, playing a crucial role in the regulation of nervous system signaling.

Key words Activity-based protein profiling, Click chemistry, Flavoproteins, Mitsunobu reaction, Monoamine oxidase, Oxidoreductase, Pargyline, Target identification

Abbreviations

ABPP	Activity-based protein profiling
CC	Click chemistry
DMSO	Dimethyl sulfoxide
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
GC-MS	Gas chromatography mass spectrometry
HRMS	High-resolution mass spectrometry
MAO	Monoamine oxidase
MS	Mass spectrometry
PBS	Phosphate-buffered saline
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TAMRA	5(6)-carboxytetramethylrhodamine
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine
TCEP	Tris(2-carboxyethyl)phosphine
TLC	Thin-layer chromatography
THF	Tetrahydrofuran

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1 Introduction

Activity-based protein profiling (ABPP) has become a powerful chemical proteomic technology allowing the dissection of complex ligand–protein interactions in their native cellular environment [1, 2]. Thus, one important challenge in ABPP is expanding the pool of probe molecules to enzyme classes with more complex catalytic activities to extend the proteome coverage. Recently, we have introduced an activity-based probe for an important group of oxidoreductases, namely flavin-dependent oxidases [3]. Flavin-dependent enzymes catalyze a diverse set of reactions encompassing oxidations, monooxygenations, dehydrogenations, reductions, and halogenations, making them indispensable for many cellular processes. Among them, flavin-dependent oxidases represent a complex subgroup which oxidizes a broad spectrum of molecules by the employment of molecular oxygen as electron acceptor [4]. Their intrinsic structural diversity, low level of sequence identity, multiplicity of accepted substrates, and lack of conserved motifs and nucleophilic residues in the active site make them elusive to functional annotation via established genomic, structural, or proteomic analyses [5]. In contrast ABPP could serve as a powerful and simple alternative for global profiling of these enzymes. We envisioned that selective activity-based probes could be built on the simple principle of binding affinity of the oxidatively activated probes towards the flavin cofactor, the only common and intrinsic feature of flavin-dependent oxidases (Fig. 1).

The designed ABPP methodology was examined using a monoamine oxidase enzyme, a representative example of flavin-dependent oxidases, to validate the new labeling mechanism on a well-known target. Monoamine oxidases [6] (MAO, EC 1.4.3.4) are FAD-containing enzymes, localized in the mitochondrial outer membrane, which catalyze the oxidative deamination of several

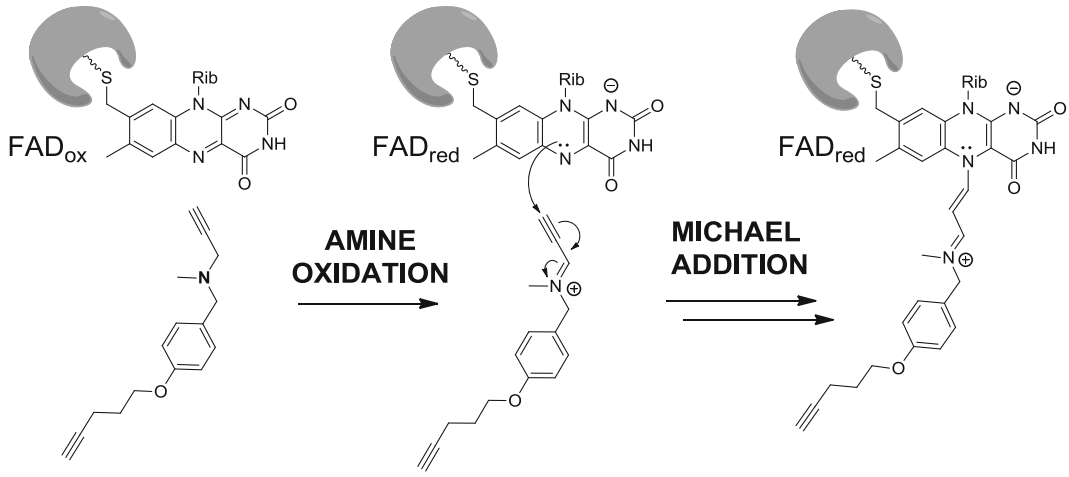

Fig. 1 Mechanism for the activation of the probe used for monoamine oxidases

important neurotransmitters in the central nervous system (CNS), including serotonin, norepinephrine, and dopamine as well as xenobiotic amines. In humans, monoamine oxidases exist in two different isoforms designated MAO A and MAO B [7], which are encoded by two distinct genes on the X chromosome and display unique substrate selectivities and inhibitor sensitivities although they share a high level of sequence identity (70%). We designed and synthesized a novel activity probe **P1** (Fig. 2a) based on the structure of the known irreversible MAO A and MAO B inhibitor pargyline (Fig. 2b), which after activity-based protein modification can be visualized through Cu(I)-catalyzed click coupling with TAMRA-Azide (Fig. 2c).

This inhibitor features a *N*-propargylamine group, which is essentially involved in irreversible enzyme inhibition and formation of a stable covalent adduct [8]. The probe has been found useful in profiling the MAO A and MAO B activity in different cells under in vivo inhibition conditions. It showed a remarkable selectivity towards MAO A and MAO B in a glioblastoma multiforme cell line called RAEW as well in mouse brain tissue lysates (Fig. 3a, b, respectively). The identity of the protein targets was revealed by a combination of gel-based mass spectrometry and Western blotting and validated by in vitro studies with recombinant enzymes (Fig. 3c) [3].

Very recently, the concept has been adapted to design a MAO B-specific probe for imaging purposes [9]. Also, a small-molecule probe for simultanous profiling and imaging of monoamine oxidase B in models of Parkinson's disease has been introduced demonstrating a power of this approach [10]. In this chapter the synthesis of the probe compound **P1** and its application in ABPP is described.

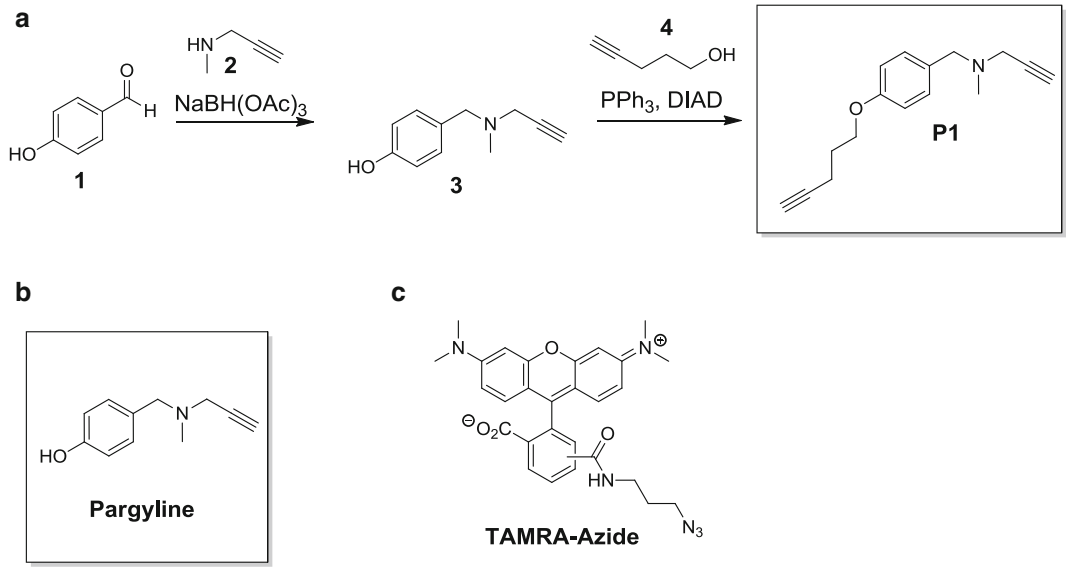

Fig. 2 Synthesis of MAO-probe **P1** (**a**) and the structure of Pargyline (**b**) and the fluorescent click-probe TAMRA-Azide (**c**)

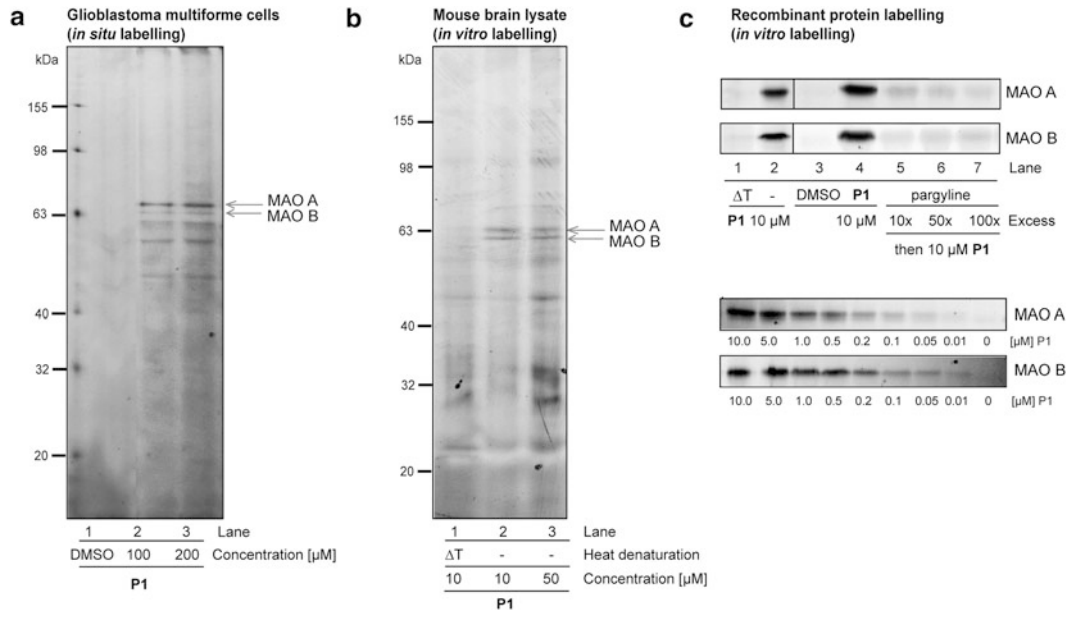

Fig. 3 Labeling of different proteomes with probe **P1**. (**a**) In situ labeling of multiblastoma multiforme cell line RAEW. (**b**) In vitro labeling of mouse brain tissue lysate. (**c**) Labeling of recombinant enzymes with probe **P1** for target validation. Labeling of MAO A and MAO B after heat (*lane 1*) and without heat denaturation (*lane 2*) and competitive labeling of MAO A and MAO B with pargyline (used in 10–100-fold excess, *lanes 5–7*) and P1 (*lane 4*)

2 Materials

All chemical reagents have been purchased from Aldrich. Thin-layer chromatography (TLC) was carried out on Merck TLC silica gel 60 F254 aluminum sheets and spots visualized by UV light ($\lambda = 254$ nm). Flash column chromatography was performed on silica gel 0.035–0.070 mm, 60 Å (Acros Organics).

2.1 Recipes for Buffers, Solutions

1. PBS (phosphate buffered saline): Dissolve in 1 L deionized H_2O: 8.00 g (136.9 mM) NaCl, 1.44 g (10.1 mM) Na_2HPO_4, 0.20 g (2.7 mM) KCl, 0.24 g (1.8 mM) KH_2PO_4. Adjust pH to 7.4 with conc. HCl.
2. 2× SDS loading buffer: Mix 2.5 mL 1 M Tris–HCl pH 6.8 (125 mM), 4 mL glycerol (20% (v/v)), 0.8 g SDS (4% (w/v)), 2 mL β-mercaptoethanol (10% (v/v)), 1 mg bromophenol blue (0.005% (w/v)) and fill up to 20 mL with deionized H_2O.
3. 1× SDS-PAGE running buffer: Dissolve in 1 L deionized H_2O: 14.4 g glycine (192 mM), 3.0 g Tris-base (25 mM), 1.0 g SDS (0.1% (w/v)). Adjust pH to 8.3.
4. Coomassie staining solution: Dissolve in 1 L deionized H_2O: 2.5 g Coomassie Brilliant Blue R250 (0.25% (w/v)), 75 mL

acetic acid (concentrated) (7.5% (v/v)), 500 mL ethanol (absolute) (50% (v/v)).

5. Coomassie destaining solution: Dissolve in 1 L deionized H_2O: 75 mL acetic acid (concentrated) (7.5% (v/v)), 200 mL ethanol (absolute) (20% (v/v)).
6. Fixing solution: Dissolve in 1 L deionized H_2O: 70 mL acetic acid (concentrated) (7.0% (v/v)), 100 mL ethanol (absolute) (10% (v/v)).

2.2 Specialized Equipment

1. Humidified CO_2 incubator: Autoflow Nuaire IR Direct Heat CO_2 incubator or an equivalent.
2. Laminar-flow hood: Thermo Scientific Heraeus KS12 safety cabinet or an equivalent.
3. Ultrasonic homogenizer: Bandelin Sonoplus HD2070 or an equivalent.
4. Microplate reader: TECAN Infinite® M200 Pro or an equivalent.
5. Electrophoresis apparatus: Peqlab PerfectBlue Dual Gel System Twin or an equivalent.
6. Fluorescence in-gel scanner: Fujifilm Life Science Las-4000 Luminescent Image Analyzer equipped with a Fujinon VRF43LMD3 Lens and a 575DF20 Cy3 Filter or an equivalent.

3 Methods

3.1 Synthesis of 4-((methyl(prop-2-yn-1-yl)amino)methyl) phenol (3)

1. Charge an oven-dried, evacuated, and argon-purged 100 mL Schlenk flask with 610 mg 4-hydroxybenzaldehyde (5.0 mmol, 1.0 eq) (**1**) (*see* **Note 1**).
2. Dissolve **1** by addition of 20 mL dry and degassed THF at RT.
3. Add to the resulting clear yellowish solution 380 mg *N*-methylpropargylamine (464 μL, 5.5 mmol, 1.1 eq) (**2**) in one portion.
4. Stir the resulting bright yellow solution for 30 min at RT.
5. Add 1.483 g sodium triacetoxyborohydride (7.0 mmol, 1.4 eq) in one portion. Through addition of the reducing agent the mixture becomes more viscous and cloudy (*see* **Note 2**).
6. Stir the reaction mixture for 5 h at RT until complete consumption of the aldehyde is indicated by GC-MS and TLC.
7. Quench the reaction mixture by a slow addition of 25 mL saturated $NaHCO_3$ (*see* **Note 3**).
8. Pour reaction mixture into a separation funnel and extract the aqueous phase with ethyl acetate (4 × 20 mL).

9. Wash the combined organic layers with water (2 × 20 mL) and brine (1 × 20 mL).
10. Dry organic phase over anhydrous Na_2SO_4 until the organic phase appears clear.
11. Filter the solution through a fritted funnel.
12. Remove the volatiles under reduced pressure on a rotary evaporator.
13. Purify the crude via flash column chromatography (SiO_2, cyclohexane–ethyl acetate = 7:3 (v/v), R_f = 0.16)
14. Concentration of the fractions furnishes 790 mg (90%) 4-((methyl(prop-2-yn-1-yl)amino)methyl)phenol (**3**) as a pale yellowish solid (*see* **Note 4**).

3.2 Synthesis of Mechanistic Probe N-methyl-N-(4-(pent-4-yn-1-yloxy)benzyl) prop-2-yn-1-amine (P1)

1. Charge an oven-dried, evacuated, and argon-purged 10 mL Schlenk flask with 87.6 mg 4-((methyl(prop-2-yn-1-yl)amino) methyl)phenol (**3**) (0.5 mmol, 1.0 eq).
2. Dissolve **3** by addition of 1 mL dry and degassed THF.
3. Cool the resulting clear yellowish solution in an ice-water bath to 0 °C.
4. Add 157 mg triphenylphosphine (0.6 mmol, 1.2 eq) and wait until it is dissolved.
5. Add in a counterflow of argon 121 mg diisopropyl azodicarboxylate (DIAD) (118 μL, 0.6 mmol, 1.2 eq) with a syringe over the course of 5 min.
6. Add 50.5 mg 4-pentyn-1-ol (56 μL, 0.6 mmol, 1.2 eq) in one portion.
7. Stir the reaction mixture in the warming up ice bath.
8. Remove the water bath and stir the reaction mixture under inert atmosphere at RT for 22 h (*see* **Note 5**).
9. Transfer solution in a round bottom flask
10. Remove solvent under reduced pressure on a rotary evaporator.
11. Purify the crude product via flash column chromatography (SiO_2, cyclohexane–ethyl acetate = 95:5 to 90:10 (v/v)) (*see* **Note 6**).
12. Concentrate the fractions on a rotary evaporator and yield 102 mg (85%) *N*-methyl-*N*-(4-(pent-4-yn-1-yloxy)benzyl) prop-2-yn-1-amine (probe **P1**) as a yellowish oil (*see* **Note 7**).

3.3 In Situ ABPP Experiments with Probe P1

1. Grow cells (*see* **Note 8**) to ca. 80–90% confluency in a complete medium (*see* **Note 9**) on petri dishes (150 × 25 mm) (*see* **Note 10**).
2. Aspirate culture medium, wash cells with 10 mL PBS, remove PBS by gentle suction.

3. Prepare complete medium containing probe **P1** at the desired concentration (10–200 μM) by diluting 1000× DMSO stock (10–200 mM) with the medium.
4. Prepare vehicle control by diluting DMSO with the medium 1:1000 (v/v).
5. Add 10 mL medium containing DMSO or probe **P1** to cells (*see* **Notes 11** and **12**).
6. Incubate cells for 2 h at 37 °C in a humidified 5 % CO_2 incubator (*see* **Note 13**).
7. Carefully aspirate medium, wash cells with PBS (2 × 10 mL) to remove the excess of the probe.
8. Harvest cells in 10–20 mL fresh cold PBS by scraping.
9. Isolate cell pellets by centrifugation (800 × *g*, 5 min, RT).
10. Resuspend cells in 500 μL cold PBS.
11. Lyse cells by sonication under ice cooling (2 × 10 s, 10–20 % intensity).
12. Separate soluble and insoluble fractions by centrifugation at 21,000 × *g* for 60 min at 4 °C. Alternatively, perform ultracentrifugation at 1,00,000 × *g* for 30–60 min at 4 °C.
13. Resuspend insoluble pellets in 500 μL cold PBS by sonication under ice cooling (2 × 5 s, 10–20 % intensity).
14. Determine protein concentration using Bradford or BCA protein concentration assay (Bio-Rad or Carl Roth, respectively) (*see* **Note 14**) and adjust total protein concentration to 2 mg/mL in PBS.
15. Follow Protocol 3.5.

3.4 In Vitro ABPP Experiments with Probe P1

1. Prepare overexpressed enzyme suspension (*see* **Note 15**), tissue (*see* **Note 16**), or cell lysate (*see* **Note 17**) and dilute it with PBS or a suitable buffer to an appropriate total protein concentration (typically 1–2 mg/mL).
2. Dispense 43 μL soluble and insoluble fraction of enzyme/tissue/cell lysate into new 1.5 mL Eppendorf tubes. For heat controls, denature 43 μL proteome for 10 min at 95 °C and cool samples down to RT, then resuspend samples well by vortexing (*see* **Note 18**).
3. Add 1 μL DMSO (vehicle control) or 1 μL probe **P1** (from 50× stock in DMSO) at a given concentration (*see* **Note 19**).
4. Incubate for 1 h at RT with gentle mixing at 400–500 rpm.
5. Follow Protocol 3.5.

3.5 Visualization of Labeled Protein Targets Via Click Chemistry (CC) with TAMRA-azide, Protein Electrophoresis (SDS-PAGE) and In-Gel Fluorescence Scanning

1. 44 μL probe-bound lysate (2 mg/mL protein concentration) is used for attachment of a fluorescent reporter tag via click chemistry (CC) reaction.
2. Add 5 μL freshly prepared master mix containing 1 μL TAMRA-azide (5 mM stock in DMSO), 1 μL tris(carboxyethyl) phosphine (TCEP) (50 mM stock in water) and 3 μL TBTA ligand (1.7 mM stock in DMSO–*tert*-BuOH 1:4 (v/v)).
3. Vortex samples gently.
4. Add 1 μL $CuSO_4$ (50 mM stock in water) to initiate the 1,3-cycloaddition and vortex gently. Total reaction volume is 50 μL and final concentrations are as follows: 100 μM TAMRA-azide, 1 mM TCEP, 100 μM TBTA, and 1 mM $CuSO_4$.
5. Incubate samples at RT for 1 h at 500 rpm in the dark.
6. Quench the reaction by mixing the sample with 50 μL 2× SDS loading buffer (*see* **Note 20**).
7. Denature samples for 5 min at 95 °C.
8. Resolve proteins by SDS-PAGE on 10% polyacrylamide gels (20 × 20 cm) (50 μL protein sample loading, ca. 50 μg protein/lane) using for first 30 min 150 V, then 300 V voltage (ca. 3–4 h) (*see* **Note 21**) or any other type of SDS gel.
9. Record fluorescence on a Fujifilm LAS-4000 Luminescent Image Analyzer with a Fujinon VRF43LMD3 Lens and a 575DF20 filter or an equivalent fluorescence scanner (*see* **Note 22**).
10. Subject gels to Coomassie Brilliant Blue staining to verify equivalent protein loading.

4 Notes

1. Reactions are carried out using classical Schlenk techniques under an inert atmosphere of argon; however, nitrogen gas will be sufficient as well. When applying Schlenk technique all solvents used were dry and degassed before use and kept under an inert atmosphere of argon or nitrogen.
2. As sodium triacetoxyborohydride is a moisture-sensitive compound, for good yields of the reaction it is important to use fresh or properly stored (under inert gas atmosphere) reducing agent.
3. Quenching is an exothermic process, so cooling in ice-water bath is recommended.
4. Characterization data for **3**: ^{1}H NMR (300 MHz, MeOD-d_4) δ: 7.14 (d, J = 8.7 Hz, 2H, arom.), 6.74 (d, J = 8.7 Hz, 2H, arom.), 3.50 (s, 2H, CH_2NR_2), 3.24 (d, J = 2.1 Hz, 2H, $CH_2C{\equiv}CH$), 2.69 (t, J = 2.4 Hz, 1H, $CH_2C{\equiv}CH$), 2.30 (s,

3H, CH_3). ^{13}C NMR (75 MHz, MeOD-d_4) δ: 158.06, 131.93, 129.14, 116.09, 78.77, 75.37, 60.20, 44.98, 41.69. R_f = 0.35 (cyclohexane–ethyl acetate = 1:1 (v/v)), 0.16 (cyclohexane–ethyl acetate = 7:3 (v/v)). m.p. 105–107 °C (pale yellowish solid). HRMS (EI) calcd. for $C_{11}H_{13}NO$ 175.0997 [M+], found 175.0998.

5. Alternatively, the reaction can be carried out in an ultrasound bath which shortens the reaction time to only 30–60 min.
6. Before the crude is purified by flash column chromatography, it also can be triturated with cold cyclohexane in order to precipitate side products: triphenylphosphine oxide and diisopropyl hydrazodicarboxylate. The precipitate is then filtrated off, washed with cold cyclohexane while the filtrate is concentrated in vacuo and applied to flash column chromatography.
7. Characterization data for Probe **P1**: ^1H NMR (300 MHz, $CDCl_3$) δ: 7.24 (d, *J* = 8.7 Hz, 2H, arom.), 6.85 (d, *J* = 8.7 Hz, 2H, arom.), 4.05 (t, *J* = 6.0 Hz, 2H, OCH_2), 3.51 (s, 2H, Ph-CH_2N), 3.29 (d, *J* = 2.4 Hz, 2H,—$NCH_2C{\equiv}CH$), 2.40 (td, *J* = 7.0 Hz, 2.7 Hz, 2H, $CH_2C{\equiv}CH$), 2.33 (s, 3H, CH_3), 2.27 (t, *J* = 2.4 Hz, 1H, $NCH_2C{\equiv}CH$), 1.99 (quint, *J* = 6.6 Hz, 2H, $CH_2CH_2CH_2$), 1.96 (t, *J* = 2.7 Hz, 1H, $CH_2C{\equiv}CH$). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 158.31, 130.55, 130.50, 114.47, 83.66, 78.72, 73.45, 68.96, 66.29, 59.41, 44.73, 41.77, 28.37, 15.33. R_f = 0.36 (cyclohexane–ethyl acetate = 7:3 (v/v), 0.13 (cyclohexane–ethyl acetate = 9:1 (v/v)). HRMS (EI) calcd. for $C_{16}H_{19}NO$ 241.1467 [M+], found 241.1463.
8. Any eukaryotic cell line of interest (adherent or suspension cells) can be used for in situ labeling. The following cell lines have been tested: DBTRG-05MG (glioblastoma multiforme (GBM) model, treated with local brain irradiation and multidrug chemotherapy, ATCC no. CRL2020™), U373 MG (glioblastoma-astroglioma model, ATCC no. HTB-17™ or ICLC HTL99014), RAEW (glioblastoma multiforme model, cell line established from a surgical specimen and analyzed in early passage number), HeLa (cervix adenocarcinoma, ATCC no. CCL-2™), MDA-MB-231 (breast adenocarcinoma, ATCC no. HTB-26™), H460 (large cell lung carcinoma, ATCC no. HTB-177™). and HepG2 (liver hepatocellular carcinoma, ATCC no. HB-8065™) All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell line RAEW was generously donated by Prof. Walter Berger (Institute of Cancer Research, Medical University of Vienna, Austria).
9. Cells are cultured in an appropriate medium at 37 °C in a humidified atmosphere containing 5 % CO_2.
10. Glioblastoma multiforme cells RAEW or DBTRG-05MG and H460 cells are cultured in RPMI 1640 medium containing

2 mM l-glutamine, 100 μM penicillin/streptomycin supplemented with 10% FCS. Cell line U373 MG is maintained in MEME medium (Minimum Essential Medium with Earle's salts) containing 2 mM l-glutamine, 1% (v/v) nonessential amino acids (NEAA), 1 mM sodium pyruvate, 100 μM penicillin/streptomycin supplemented with 10% FCS. HeLa cells are cultured in DMEM (Dulbecco's Modified Eagle Medium) medium containing 2 mM l-glutamine, 100 μM penicillin/streptomycin supplemented with 10% FCS. MDA-MB-231 cells are cultured in Leibovitz's l-15 medium containing 2 mM l-glutamine, 100 μM penicillin/streptomycin supplemented with 10% FCS in a humidified 5% CO_2 incubator at 37 °C. HepG2 cell line is grown in DMEM medium (high glucose (4.5 g/L)) containing 4 mM l-glutamine, 1 mM sodium pyruvate, 100 μM penicillin/streptomycin, supplemented with 10% FCS.

11. For an analytical scale labeling, cells can be cultured and labeled on a 6-well plate in 1 mL medium.
12. The volume of the medium should be adjusted so that the cell monolayer is covered completely.
13. Incubation time is dependent on a cell line and should be optimized.
14. For Bradford or BCA protein concentration assignment Bio-Rad Protein Assay Kit II or Carl Roth Roti®-Quant universal BCA Protein Assay Kit is used, respectively. Assays are performed according to manufacturer's procedure. Bovine serum albumin (BSA) standards (0–400 μg/mL range: 0, 25, 50, 100, 200, 400 μg/mL) is prepared from the 2.0 mg/mL stock solution in distilled water in order to assign protein concentration standard curve. Absorbance is recorded by a plate reader. All samples are measured in triplicate.
15. Human recombinant MAO A and MAO B were generously provided by Prof. Dale E. Edmondson (Department of Biochemistry and Chemistry, Emory University, Atlanta, USA), aliquoted and stored at −80 °C until use. *Pichia pastoris* membrane preparations overexpressing human monoamine oxidase MAO A and MAO B were then diluted with 50 mM potassium phosphate buffer, pH 7.5, up to the concentration of ca. 1 mg/mL.
16. Cell lysates are prepared as follows: grow cells to ca. 80–90% confluency in a complete medium on petri dishes (150 × 25 mm), aspirate medium, wash cells with chilled PBS (10 mL), and remove PBS by suction. Then, harvest cells in 20 mL cold PBS by scraping, pelletize cells by centrifugation (140 × *g*, 10 min, 4 °C), resuspend in 500 μL PBS and lyse by sonication (10 s, 10–20% intensity) under ice cooling. Separate soluble and

insoluble fraction by centrifugation at 21,000 × *g* for 60 min at 4 °C or ultracentrifugation at 1,00,000 × *g* for 30 min at 4 °C. Resuspend insoluble pellets in 500 μL PBS by sonication (5 s, 10% intensity) under ice cooling. Determine protein concentration using Bradford or BCA protein concentration assay. The lysates can be shock-frozen in liquid N_2 and stored at −80 °C until use.

17. The tissue lysates are prepared as follows: Cut frozen (−80 °C) mouse tissues (brain, liver) into ca. 50–80 mg fragments and then slice them into smaller pieces. Subsequently, transfer the tissue pieces into a chilled 2.0 mL vial containing cold ceramic beads and add ca. 700 μL chilled PBS. Lyse the tissue using a homogenizer (3000 × *g* , 2 × 15 s) with 2 min intermittent cooling on ice. The tissue lysate can be additionally sonicated under ice cooling (5–10 s, 10–20% intensity) depending on tissue and homogenization state. Separate soluble and insoluble fraction by centrifugation at 21,000 × *g* for 60 min at 4 °C or ultracentrifugation at 1,00,000 × *g* for 30 min at 4 °C. Resuspend the insoluble fraction in ca. 800 μL chilled PBS buffer by sonication under ice cooling. Determine protein concentration using Bradford or BCA protein concentration assay. The lysates can be shock-frozen in liquid N_2 and stored at −80 °C until use.
18. In competitive ABPP labeling experiments 42 μL protein sample were incubated for 30 min at RT with 1 μL given MAO inhibitor (pargyline, deprenyl, or clorgyline) (10–100-fold probe excess), followed by addition of 1 μL ABPP probe.
19. For in vitro labeling the concentration range between 1 and 50 μM is recommended. For labeling of overexpressed enzyme concentration of 10 μM is optimal.
20. When fluorescence signal is weak or unspecific background high, it is recommended to precipitate proteins after click chemistry reaction to remove excess of the fluorescent cocktail (by any common method (e.g., acetone, trichloroacetic acid (TCA), or methanol–chloroform precipitation), then resuspend sample in SDS loading buffer before running an SDS gel.

    Acetone precipitation: after click chemistry reaction, add 200 μL prechilled acetone (−21 °C), vortex samples and incubate at −21 °C for 1 h up to overnight. Then, centrifuge samples at 21,000 × *g* for 20 min at 4 °C, discard supernatant and wash pellets twice with 200 μL prechilled methanol, resuspend by sonication, centrifuge at 21,000 × *g* for 15 min at 4 °C and remove supernatant, air-dry pellets for 5 min. Dissolve samples in SDS-PAGE loading buffer.

    Methanol–chloroform method: Add PBS to adjust volume of the samples to 150 μL. Add four volumes of MeOH

(600 µL), followed by addition of one volume of chloroform (150 µL). Vortex well and then check whether there is only one phase. Add three volumes of distilled water (450 µL), vortex thoroughly. Spin down for 1 min at maximum speed at RT in a table centrifuge. Remove upper organic phase without disturbing interphase (which contains proteins). Add three volumes of MeOH (450 µL), vortex thoroughly. Spin for 1–2 min at maximum speed at RT. Remove supernatant carefully and air-dry pellet. Dissolve the pellet in PBS, an appropriate buffer or SDS-PAGE loading buffer.

TCA method: Dilute samples up to volume 400 µL with PBS, add 100 µL (1/4 volume) 50 % (w/w) aqueous solution of trichloroacetic acid (TCA), gently vortex samples, and incubate on ice for 1 h. Then, centrifuge samples at 21,000 × *g* for 15 min at 4 °C. Discard supernatant and wash the protein pellets with 2 × 200 µL prechilled acetone. Air-dry samples for 5–10 min. Dissolve the pellet in PBS, an appropriate buffer or SDS-PAGE loading buffer.

21. SDS-PAGE is performed Peqlab PerfectBlue Dual Gel System Twin L (45-2020-I) (big gels 20 × 20 cm) or Peqlab PerfectBlue Dual Gel System Twin S (45-1010-I) (small gels 10 × 10 cm).
22. The ratio signal-to-noise on a fluorescent gel can be enhanced by incubating the gel in a fixing solution for 30–60 min in the dark on an orbital shaker.

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Part II

Medical Applications of Activity-Based Proteomics

Chapter 8

Activity-Dependent Photoaffinity Labeling of Metalloproteases

Laurette Prely, Theo Klein, Paul P. Geurink, Krisztina Paal, Herman S. Overkleeft, and Rainer Bischoff

Abstract

Metalloproteases, notably members of the matrix metalloprotease (MMP) and A Disintegrin And Metalloprotease (ADAM) families play crucial roles in tissue remodeling, the liberation of growth factors and cytokines from cell membranes (shedding) and cell–cell or cell–matrix interactions. Activity of MMPs or ADAMs must therefore be tightly controlled in time and space by activation of pro-enzymes upon appropriate stimuli and inhibition by endogenous tissue inhibitors of metalloproteases (TIMPs) or α_2-macroglobulin to prevent irreversible tissue damage due to excessive degradation or uncontrolled release of potent inflammatory mediators, such as tumor necrosis factor-α (TNF-α).

Although there is a wide range of methods to measure the amount of metalloproteases based on immunological approaches, relatively little is known about the activation status of a given enzyme at any given time and location. This information is, however, critical in order to understand the function and possible implication of these enzymes in disease. Since metalloproteases use an active-site bound water molecule to cleave the peptide bond, it is not possible to apply known active-site-directed labeling approaches with electrophilic "warheads." We therefore developed novel metalloprotease inhibitors that contain a photoactivatable trifluoromethylphenyldiazirine group and show that such inhibitors are suitable for activity-dependent photoaffinity labeling of MMPs and ADAMs.

Key words Metalloprotease, Photoaffinity labeling, Matrix metalloprotease (MMP), A Disintegrin And Metalloprotease (ADAM), Trifluoromethylphenyldiazirine

1 Introduction

The analysis of hydrolases and notably proteases using activity-dependent affinity probes (ABPs) was initiated about 10 years ago and is finding more widespread use due to the availability of ABPs for serine and cysteine proteases [1–5]. All of these probes are based on an electrophilic "warhead," which reacts with the nucleophilic hydroxyl or thiol groups in the protease active site. Such an approach is, however, not feasible in the case of metalloproteases, which activate an active-site-bound water molecule to effectuate

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cleavage of the peptide bond. It is thus necessary to find other ways of covalently labeling MMPs and ADAMs one of which is through photoaffinity labeling [6–11]. Photoaffinity labeling is a priori based on chemical reactions with little selectivity due to the reactivity of the generated radicals (nitrenes or carbenes). It is thus of utmost importance to assure active-site selectivity through the peptide-like moiety of the inhibitor, to which the photoactivatable group is attached, in combination with the Zn^{2+}-chelating group that assures effective inhibition of enzyme activity. Combinatorial chemistry approaches have delivered libraries of metalloprotease inhibitors [12], that can be further modified with photoactivatable groups, such as a benzophenone [13]. Despite significant efforts from both pharmaceutical industry and academic laboratories, it has remained elusive to synthesize inhibitors with single-enzyme specificity, so that most presently available MMP and ADAM inhibitors target at least a group of related enzymes.

In this chapter we described a method for the activity-dependent photoaffinity labeling of MMPs and ADAMs based on a peptide-like, hydroxamate-containing inhibitor containing a trifluoromethylphenyldiazirine group.

2 Materials

2.1 Recombinant Metalloproteases and Tissue Inhibitors of Metalloproteases

1. ADAM-8, -9, -10, and -17 (ectodomain) and recombinant human TIMP-1 (Tissue Inhibitor of Metalloproteases-1) and TIMP-3 were purchased from R&D systems (Minneapolis, MN, USA). ADAM-8 was autocatalytically activated by incubation at 37 °C for 5 days according to the manufacturer's instructions.
2. Recombinant catalytic domains (CD) of human MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-10, MMP-11, and MMP-13 were from Biomol International (Butler Pike, PA, USA).
3. Recombinant CD of human MMP-12 CD and MMP-9 without fibronectin type II inserts, expressed in *E. coli* as described [14, 15], were a gift from AstraZeneca R&D (Lund & Moelndal, Sweden).
4. Alkaline phosphatase-conjugated streptavidin was from Sigma-Aldrich (Zwijndrecht, The Netherlands). 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were from Duchefa (Haarlem, The Netherlands).
5. The photoactivatable inhibitor-probe 1 (HAPhe(Tmd) AhxLys(Bio)NH2) was synthesized as described earlier [9]. Control probe 2 was synthesized in the same manner without addition of the biotin moiety (*see* Fig. 1).
6. Fluorogenic substrate Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2 for measuring recombinant ADAM-10 and -17 activity (Mca:

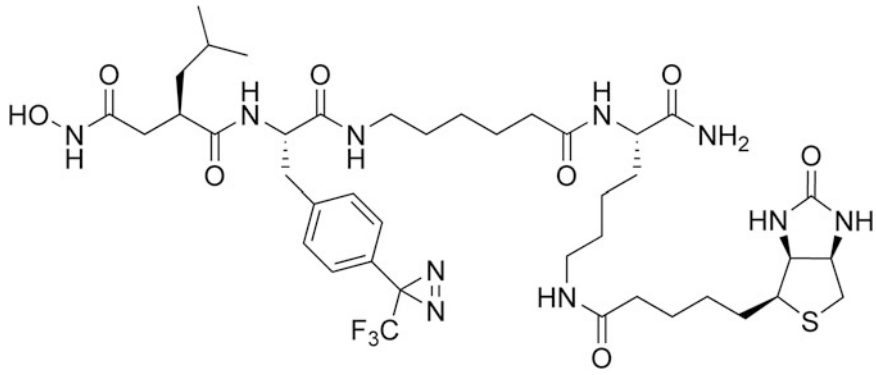

Fig. 1 Peptide hydroxamate-based activity-dependent metalloprotease probe (ABP) with the photoactivatable group in position P2′ targeting the S2′ pocket of MMPs and ADAMs [9]

(7-Methoxycoumarin-4-yl)acetyl;Dpa:N-3-(2,4-Dinitrophenyl)-L-2,3-diaminopropionyl)) was from (R&D Systems).

7. Fluorogenic substrate Mca-P-L-G-L-Dpa-A-R-NH_2 for measuring recombinant MMP-9 and -12 activity was from (Bachem, Bubendorf, Switzerland).
8. All other chemicals were from Sigma-Aldrich.

2.2 Determination of IC_{50} Values

1. ADAM assay buffer (25 mM Tris–HCl, pH 9.0, 2.5 μM $ZnCl_2$, 0.005 % w/v Brij-35) (*see* **Note 1**).
2. MMP assay buffer (50 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 2.5 μM $ZnCl_2$, 0.05 % (v/v) Brij-35) (*see* **Note 1**).

2.3 Photoaffinity Labeling

Buffers for labeling of MMPs and ADAMs are as described in Subheading 2.2.

2.4 SDS-Polyacrylamide Gel Electrophoresis

All the buffers are prepared with ultrapure water (18.2 MΩ x cm).

1. Separating buffer (4×): 1.5 M Tris–HCl pH 8.8, 0.4 % SDS (w/v). Store at room temperature for up to 3 months.
2. Stacking buffer (4×): 0.5 M Tris–HCl pH 6.8, 0.4 % SDS (w/v). Store at room temperature for up to 3 months.
3. Acrylamide–bisacrylamide solution: 30 % acrylamide–bis solution (Bio-Rad, Veenendal, The Netherlands) 37.5:1. Store according the manufacturer's recommendations (for up to 1 year at 4 °C).
4. *N,N,N,N′*-tetramethyl-ethylenediamine (TEMED, Bio-Rad, Veenendal, The Netherlands) store at room temperature in dark stored up to 6 months.
5. Laemmli buffer (Pierce Biotechnology, Rockford, USA): 0.3 M Tris–HCl pH 6.8, 5 % SDS (w/v), 50 % glycerol (w/v) and proprietary pink tracking dye. Store at room temperature for up to 1 year.

6. APS (ammonium persulfate) (Bio-Rad, Veenendal, The Netherlands): stock solution of 10% (w/v) in water in 100 μL aliquots. Store at −20 °C (single use).
7. Running buffer (10×): 0.25 M Tris–HCl, 34 mM SDS, 1.9 M glycine, pH 8.3 (no need to adjust pH). Store at room temperature for up to 3 months. The actual running buffer (1×, 1 L) is sufficient for three runs.
8. Prestained molecular weight markers (Bio-Rad, Veenendal, The Netherlands) 10–250 KDa.

2.5 Western Blotting

1. Blotting buffer (10×): 0.25 M Tris–HCl, 1.9 M glycine, pH 8.3 (no need to adjust pH); store at room temperature for up to 3 months.
2. Blotting buffer (1×): 10% blotting buffer(v/v) (10×), 20% methanol (v/v), 70% water (v/v) (single use).
3. Tris-buffered saline (TBS) (10×): 250 mM Tris–HCl, pH 7.5, 1.5 M NaCl; store at room temperature for up to 3 months.
4. Tris-buffered saline with Tween (TBST) (1×): dilute TBS (10×) and add 0.05% Tween 20 (v/v) (single use).
5. Blocking buffer: 5% (w/v) nonfat dry milk (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) in TBST-T prepared fresh before use.
6. Alkaline phosphatase buffer: 0.1 M Tris–HCl, pH 9.5, 5 mM $MgCl_2$; store at room temperature for up to 3 months.
7. Nitroblue tetrazolium (NBT) stock: 61 mM of NBT in 70% (v/v) dimethylformamide (DMF), 30% water (v/v). Store in a polypropylene tube at 4% in the dark for up to 3 months.
8. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 0.1 M of BCIP in water. Store in a polypropylene tube at 4% in the dark for up to 3 months.
9. Alkaline phosphatase: Sigma-Aldrich (Zwijndrecht, The Netherlands); store at −20 °C.

2.6 Competition with TIMPs

Buffers for labeling of MMPs and ADAMs as described in Subheading 2.2.

2.7 Competition with Non-biotinylated ABPs

Buffers for labeling of MMPs and ADAMs as described in Subheading 2.2.

3 Methods

First it is necessary to assess the inhibitory efficiency of newly developed ABPs by measuring the IC_{50} value against a range of target metalloproteases to assure that incorporation of the rather

bulky photoactivatable trifluoromethylphenyldiazirine group does not lead to a drastic loss of inhibitory efficiency. Table 1 shows that placing the trifluoromethylphenyldiazirine group at the P2′ position of the inhibitor results in nM IC_{50} values.

Successful photoaffinity labeling is assessed by SDS-PAGE and subsequent Western blotting against the incorporated biotin. While this is a sensitive way of detecting labeling, it is not very quantitative, meaning that it is only possible to estimate the percentage of labeling. Figure 2 shows the successful labeling of ADAMs-9, 10, and 17 with the ABP. Denaturing the enzymes by boiling in 2% SDS prior to labeling alleviates photoaffinity labeling, indicating that labeling is activity-dependent. Further support for the fact that labeling with the described ABP is activity-dependent was obtained by competition experiments with non-biotinylated ABPs (Fig. 3).

3.1 Determination of IC_{50} Values

1. IC_{50} values for MMP-9 and MMP-12: All measurements are performed in Costar White 96-well plates (Corning, Schiphol-Rijk, The Netherlands) by measuring fluorescence (excitation 320 nm; emission 440 nm) increase over 15 min using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) at 37 °C in the presence of increasing concentrations of the ABP. This assay can be easily adapted to other fluorescent plate readers having the appropriate excitation and emission wavelengths. Six-point inhibition curves are plotted in Origin 7.0 (Micronal) and IC_{50} values determined by sigmoidal fitting. Inhibition of MMP proteolytic activity is determined with 10 ng of MMP-9 or MMP-12 per well with a final concentration of 2 μM substrate (Mca-P-L-G-L-Dpa-A-R-NH_2) in 100 μL MMP assay buffer (50 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 2.5 μM $ZnCl_2$, 0.05% (v/v) Brij-35).
2. IC_{50} values for ADAM-10 and ADAM-17: All measurements are performed as described above except that 10 ng ADAM-10 and 100 ng ADAM-17 are used per well. The final concentration of substrate (Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2) is 10 μM in a final volume of 100 μL ADAM assay buffer (25 mM Tris–HCl, pH 9.0, 2.5 μM $ZnCl_2$, 0.005% w/v Brij-35).

Table 1
IC_{50} values (in nM) of the photoactivatable probe shown in Fig. 1

MMP-9	MMP-12	ADAM-10	ADAM-17
25.1	**3.60**[a]	**114**[a]	**20.6**[a]

[a]From Ref. [9]

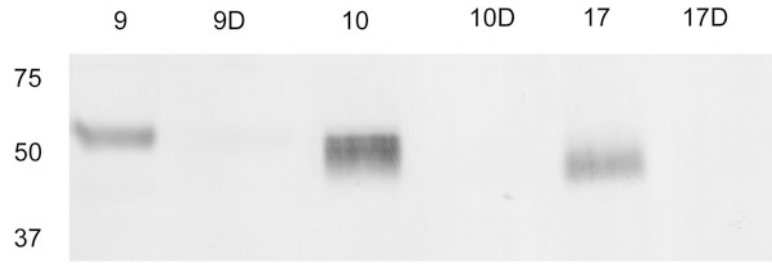

Fig. 2 SDS-PAGE analysis followed by anti-biotin Western blotting after photoaffinity labeling of ADAMs-9, 10 and 17 with the ABP (*see* Fig. 1). The "D" signifies that the respective enzyme was denatured by boiling for 5 min in the presence of 2 % SDS prior to labeling

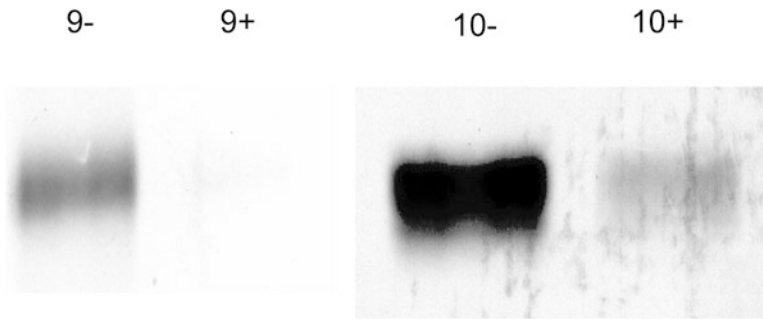

Fig. 3 Anti-biotin Western blot analysis of ADAM-9 and ADAM-10 (4 pmol) photolabeled with 250 nM ABP (*see* Fig. 1) without (–) and with (+) preincubation with the non-biotinylated, photoactivatable control probe

3.2 Photoaffinity Labeling

Labeling of MMPs and ADAMs: Recombinant MMPs and ADAMs are incubated with the photoactivatable inhibitor probe in 96-well plates (Costar White). Each well (final volume 30 µL) contains 300 ng protein and 250 nM inhibitor in assay buffer (25 mM Tris–HCl, pH 9.0, 2.5 µM $ZnCl_2$, 0.005 % w/v Brij-35). The plate is irradiated at 366 nm (Camag universal UV lamp, 20 W, distance to plate 4 cm) for 30 min on ice (*see* **Notes 2–6**). The reaction is stopped by adding 10 µL 5-times concentrated non-reducing SDS-PAGE sample buffer (Pierce Biotechnology, Rockford, USA).

3.3 SDS-PAGE and Western Blotting

1. SDS-PAGE is performed on 0.75 mm thick 12.5 % polyacrylamide gels in a mini-Protean III electrophoresis system (Bio-Rad, Veenendal, The Netherlands) at 20 mA. The procedure can be easily adapted to other electrophoresis systems. The 12.5 % polyacrylamide separating gel was prepared by mixing 1.3 mL of separating buffer, 2.1 mL of acrylamide–bis (30 %) stock solution, 1.6 mL of water, 50 µL of APS (10 %), 2 µL of TEMED. The gel is poured into clean plates (wash extensively in the following order: tap water, 0.1 % SDS added to ultrapure

water, ultrapure water and dry with clean paper). The separating gel is overlaid with isobutanol until polymerization (around 30 min). Isobutanol is then removed and the top of the separating gel washed extensively with ultrapure water and dried with filter paper. The stacking gel is prepared by initiating polymerization of 1.14 mL of water, 0.33 mL of acrylamide–bis (30%) stock solution, 0.5 mL of stacking buffer through addition of 20 μL of APS (10%) and 2 μL TEMED after inserting the appropriate combs. After approximately 30 min, the combs are removed and the wells washed with running buffer (1×). The cassettes are assembled as follows: the inside chamber (cathode chamber) and the outside chamber (anode chamber) are filled with running buffer (1×) covering the top and the bottom of the gel totally. The gel is run until the tracking dye reaches the bottom of the gel.

2. Separated proteins are transferred to an Immun-Blot PVDF membrane (0.2 μm) manually cut to the gel's dimensions. The membrane is wetted in methanol before to immersion in blotting buffer for a couple of minutes. Blotting is performed in a mini Trans-blot cell (Bio-Rad) in 25 mM Tris, 190 mM glycine with 20% v/v methanol at 350 mA for 60 min. Membranes are blocked overnight at 4 °C in TBST (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% v/v Tween 20) supplemented with 5% w/v nonfat dried milk (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) followed by incubation for 1 h in a 1:1500 dilution of streptavidin-alkaline phosphatase (0.67 μg/mL; Sigma-Aldrich (Zwijndrecht, The Netherlands)) in TBST supplemented with 1% nonfat dried milk. Biotinylated proteins are visualized by staining with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Duchefa, Haarlem, The Netherlands) in 0.1 M Tris–HCl, pH 9.5, 5 mM $MgCl_2$, 0.15 mg/mL BCIP, 0.30 mg/mL NBT. To avoid background staining, we recommend to wash the membranes (twice 10 min) with TBST before applying the alkaline phosphatase substrates. For the same reason we advise to avoid extended incubation with the BCIP/NBT solutions. Revelation is done until clear bands are visible above a faint background. At this step the membrane is rinsed with UP water, dried amongst two sheets of clean paper (dark) and stored at room temperature in the dark.

3.4 Competition with TIMPs

MMP-9 and MMP-12 (4 pmol) are incubated overnight at 4 °C with equimolar equivalents of TIMP-1. ADAM-10 and ADAM-17 (4 pmol) are incubated overnight at 4 °C with equimolar equivalents of recombinant TIMP-3 in assay buffer. Control aliquots are kept at 4 °C overnight without TIMPs. The photoactivatable inhibitor is added to TIMP-incubated and control solutions at a final concentration of 250 nM. Labeling and analysis are performed as described above.

3.5 Competition with Non-biotinylated ABPs

The non-biotinylated photoactivatable probe, which is structurally identical to the biotinylated inhibitor except for the lack of the biotin moiety (*see* Fig. 1), is preincubated with MMP-9 and MMP-12 (4 pmol in 30 μL final volume) for 15 min at a concentration of 400 nM under irradiation at 366 nm as described in Subheading 3.2. The biotinylated ABP is subsequently added at a final concentration of 200 nM followed by a second round of irradiation. ADAM-9 and -10 (4 pmol in 30 μL final volume) are preincubated for 15 min with 2.5 μM of the control probe under irradiation after which the biotinylated ABP is added at a final concentration of 250 nM. Photolabeled proteins are analyzed by SDS-PAGE and Western blotting as described in Subheading 3.3 (*see* Fig. 3).

4 Notes

1. The surfactant Brij35, that is added to the enzyme buffers, is crucial to preserve activity. This surfactant also reduces adsorption of the enzymes to the well plate.
2. We recommend to use a small incubation buffer volume (30 μL works fine for us) to be able to work with small amounts of enzyme and inhibitor at elevated concentrations 130 and 250 nM respectively.
3. Even though we assume complete inhibition of enzyme activity under the conditions of photolabeling, we advice to perform photolabeling with the samples on ice to avoid autodegradation.
4. The distance between samples and the light source should be minimized to increase the power of the light reaching the samples.
5. Make sure that enzyme and ABP are well mixed prior to irradiation.
6. We recommend to maintain slow shaking of the samples during irradiation (well plate and UV source are placed on a flatbed mixer).
7. We recommend 10 min preincubation of enzyme and ABP in the dark at room temperature prior to irradiation, to assure complete binding of the inhibitor to the active site according to [7].
8. Since the Camag light source used in our experiments is not focused and the output in W/cm^2 at best approximate, investigations with a light source with more defined properties (light source with an aperture and a series of lenses focusing the polychromatic light on a monochromator followed by a series of lenses that focus the monochromatic light on the samples) (*see* Ref. [7]) is recommended.
9. It is highly recommended to keep the ABP in the dark prior to photolabeling to avoid photodegradation prior to the experiment.

10. To avoid degradation of the ABP during storage at −20 °C, it is recommended to store it dry in aliquots of an appropriate size for a single labeling experiment.
11. As most proteases, MMPs and ADAMs autodegrade easily when in pure form. It is thus recommended to store enzymes at −80 °C in aliquots for single use. Check enzymatic activity from time to time using the profluorescent substrate assay and check homogeneity by SDS-PAGE.

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Chapter 9

Profiling the Activity of Deubiquitinating Enzymes Using Chemically Synthesized Ubiquitin-Based Probes

Yves Leestemaker, Annemieke de Jong, and Huib Ovaa

Abstract

Deubiquitinating enzymes (DUBs) are of interest as potential new targets for pharmacological intervention. Active-site-directed probes can be used for the accurate profiling of DUB activity as well as the identification of DUBs and DUB inhibitor selectivity. Previously, active-site directed DUB probes have been obtained using intein-based methods that have inherent limitations. Total chemical synthesis of ubiquitin allows for easy incorporation of different tags, such as fluorescent reporters, affinity tags, and cleavable linkers. Here, we describe the total chemical synthesis of a fluorescent active-site directed DUB probe, which facilitates fast, in-gel detection of active DUBs and circumvents the use of Western blot analysis. In addition, an in-gel activity-based DUB profiling assay is described in detail, in which the fluorescent DUB probe is used to visualize active DUBs in cell lysates. Finally, an inhibition assay is described in which the fluorescent probe is used to determine the specificity and potency of a small molecule DUB inhibitor.

Key words Activity-based protein profiling, Deubiquitinating enzymes, Fluorescent probes, Solid-phase synthesis, Ubiquitin

1 Introduction

Ubiquitin (Ub) is a 76-amino acid regulatory protein involved in the regulation of many cellular processes, such as proteasomal protein degradation, DNA repair, and cell cycle regulation. Posttranslational modification of proteins with ubiquitin is performed by the consecutive actions of Ub ligases from three different classes [1]. Mono-ubiquitination or poly-ubiquitination can affect proteins in many different ways, such as tagging proteins for degradation by the proteasome, altering protein localization, affecting protein activity, and promoting or preventing protein–protein interactions [1]. In contrast to ubiquitin ligases, deubiquitinating enzymes (DUBs) remove Ub from substrate proteins. Approximately 100 DUBs are encoded in the human genome and these can be divided into five distinct classes: four classes of cysteine proteases, and one metalloprotease class [2]. DUBs are key

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regulators of important cellular functions [3]. The inhibition of a specific DUB might highly selectively affect the function, localization, or stability of a specific set of proteins, which may be of interest for therapy of human diseases in which ubiquitin-dependent physiological processes are deregulated [4]. Therefore, suitable assay reagents to study DUB activity are valuable research tools and can contribute to identifying DUB inhibitors, which could possibly have future therapeutic applications [5, 6].

Current assay reagents that are commonly used to study DUB activity include activity-based probes [7–11], fluorogenic substrates [12–14] such as Ub-7-amino-4-methylcoumarin and Ub-rhodamine, fluorescence polarization reagents [15–17], ubiquitinated proteins/peptides [16], and hydrolyzable and non-hydrolyzable [18, 19] ubiquitin linkage-specific reagents, such as diubiquitin or polyubiquitin [17, 20, 21]. The advantage of activity-based DUB probes over the other methods is the ability to monitor the activity of multiple DUBs separately in a single experiment. In addition, DUB probes have successfully been used to identify novel DUBs in both eukaryotes and in a wide range of pathogens directly from lysates [7, 14, 22, 23]. Classical activity-based DUB probes are based on the sequence of Ub as the DUB-targeting motif and comprise a reactive C-terminal warhead such as vinyl methyl ester (VME), and an N-terminal epitope tag (Fig. 1a) [7, 24, 25]. These probes react with the active-site cysteine residue that is present in most DUBs, thereby forming a covalent bond between the probe

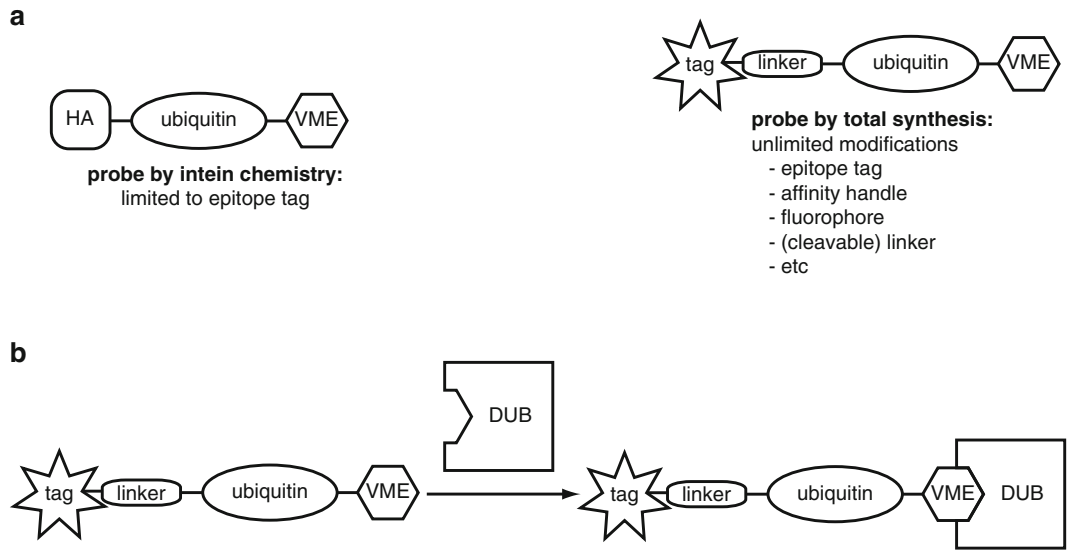

Fig. 1 (**a**) The differences between classically prepared DUB probes versus chemically synthesized probes. Classically prepared probes are largely limited to incorporation of natural amino acids, whereas chemically synthesized probes, a large variety of building blocks can be incorporated. (**b**) Ub-based probes react with the cysteine present in the active site of the majority of DUBs, forming a covalent bond

and the DUB (Fig. 1b). After probe labeling, antibodies against the HA epitope tag are used for the detection of labeled DUBs by western blot analysis or for the immunoprecipitation of labeled DUBs for identification purposes [7]. Despite their usefulness, classical activity-based probes are made using intein-based expression methods, which have inherent limitations, such as the difficulty to obtain the probes in scalable amounts and the limitation to versions with an expressed epitope tag, although recent publications show modification of the Ub-sequence by genetic code expansion methods [26]. In contrast, when Ub-based activity probes are chemically synthesized, these limitations can be overcome. Using the previously reported total linear synthesis of Ub [27], convenient control is allowed over additional moieties that can be incorporated in the Ub protein sequence, such as fluorescent dyes, affinity handles (such as epitope tags or biotin), chemical spacers, and cleavable linkers, while on the C-terminal an active-site directed moiety can be selectively coupled (Figs. 1a and 2).

To demonstrate that DUB probes prepared by total synthesis can be successfully used for labeling DUBs, we chemically synthesized HA-tagged UbVME, making use of the previously reported total linear synthesis of Ub [27]. Subsequently, we incubated EL4 (Murine Thymic Lymphoma) cell extract with increasing concentrations of both chemically synthesized and classically prepared DUB probe HAUbVME. DUB activity was visualized by immunoblotting, showing that the DUB labeling profile for labeling cell extract using chemically synthesized HAUbVME was almost identical to that obtained with the classically prepared probe. This demonstrates that DUB probes prepared by total synthesis can be

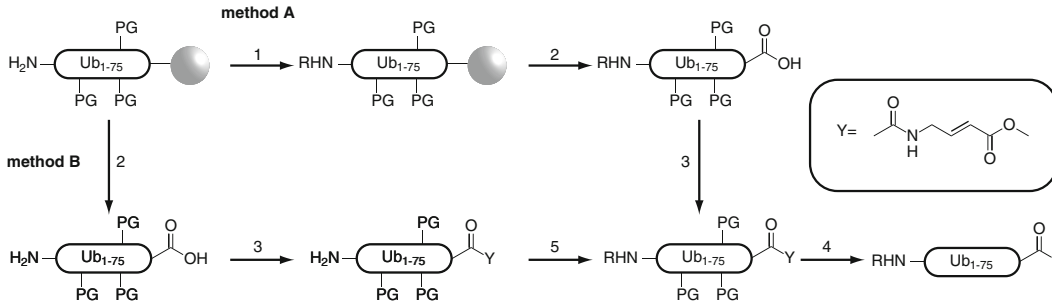

Fig. 2 Synthesis scheme: two reaction pathways to chemically synthesize active-site directed Ub-based DUB probes. The ubiquitin sequence is built up on solid-phase using Fmoc-based solid-phase synthesis. Using method A, building blocks of choice, with side-chains protected, are coupled to the N-terminus of ubiquitin on solid-phase, after which the C-terminal VME warhead is coupled in solution. When the building block to be coupled to the N-terminus of Ub contains an extra free carboxylic acid, as in 5-carboxytetramethylrhodamine (TMR) for the synthesis of TMRUbVME, method B should be followed. Using this method, the VME warhead is coupled to the C-terminus of Ub in solution first, followed by coupling of TMR (or another building block of choice) to the N-terminus of Ub in solution, so that the VME warhead is not coupled to the other carboxylic acid of TMR

successfully used for labeling DUBs in vitro [8, 24]. Subsequent replacement of the HA-epitope tag by the fluorophore 5-carboxytetramethylrhodamine (TMR), yielded the fluorescent DUB activity probe TMRUbVME, allowing for direct in-gel scanning of the SDS-PAGE gel for fluorescence emission of labeled DUBs. Direct fluorescence imaging of SDS-PAGE gels is a more rapid method compared to immunoblotting and is not accompanied with unspecific background labeling signal caused by antibody cross-reactivity. A typical SDS-PAGE-based activity profiling experiment is shown in Fig. 3, in which EL4 cell extract was incubated with increasing concentrations of TMRUbVME and proteins were subsequently resolved by SDS-PAGE. The resulting gel was imaged for fluorescence emission using a fluorescence scanner to visualize fluorescently labeled DUBs. Similar DUB labeling profiles were obtained using the fluorescent probe, compared to the earlier reported HAUbVME probe, demonstrating that similar reactivity towards the DUBs in cell extract was observed for both HAUbVME and TMRUbVME. Compared to HAUbVME, higher resolution results were obtained using TMRUbVME, although resolution could be increased when fluorescent secondary antibodies for western blotting were used. Furthermore, compared to HAUbVME, additional bands were observed for TMRUbVME, indicating the greater sensitivity provided by the latter reagent [8].

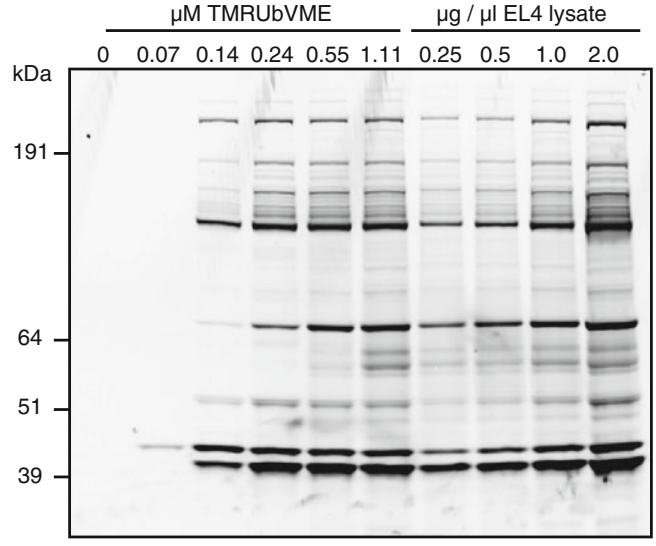

Fig. 3 EL4 lysate was incubated with the indicated concentrations of TMRUbVME (*left*) or different amounts of EL4 lysate were incubated with 1 μM TMRUbVME (*right*). Proteins were separated by SDS-PAGE and the residual DUB activity was visualized by in-gel fluorescence scanning (λ (ex/em) = 550/590). When increasing concentrations of TMRUbVME probe are used, more DUBs are labeled. Using increasing lysate concentrations do not seem to influence the number of labeled DUBs; however, the DUBs that are labeled are better visible

DUB activity profiling can be used to visualize a subset of the most active or abundant DUBs present in a cell extract simultaneously. Therefore, DUB activity probes can be used to test the potency and selectivity of DUB inhibitors [10, 28] in a competition assay, which can find application in the identification of specific DUB inhibitors. A typical DUB inhibitor-profiling assay is shown in Fig. 4. Lysates of EL4 cells were incubated with a series of concentrations of the known DUB inhibitor WP1130 [28, 29] ranging from 1.6 to 200 μM and subsequently incubated with TMRUbVME to label active DUBs. Inhibition of the activity of a DUB results in a disappearance of the respective fluorescent band on the gel, as the probe can no longer bind to this DUB. Since there is a lot of variety between the active sites of DUBs, DUB inhibitors can have different selectivity for the different DUBs present in a cell. Using this probe, the selectivity and potency of DUB inhibitors can be investigated. Cell biological and genetic manipulation of DUBs, such as knockdown or overexpression of specific DUBs, can also be monitored using DUB activity probes [8]. Using western blot analysis of specific DUBs or their tags, the proportion of reacted enzyme can be determined quantitatively In addition, the probe can be used to

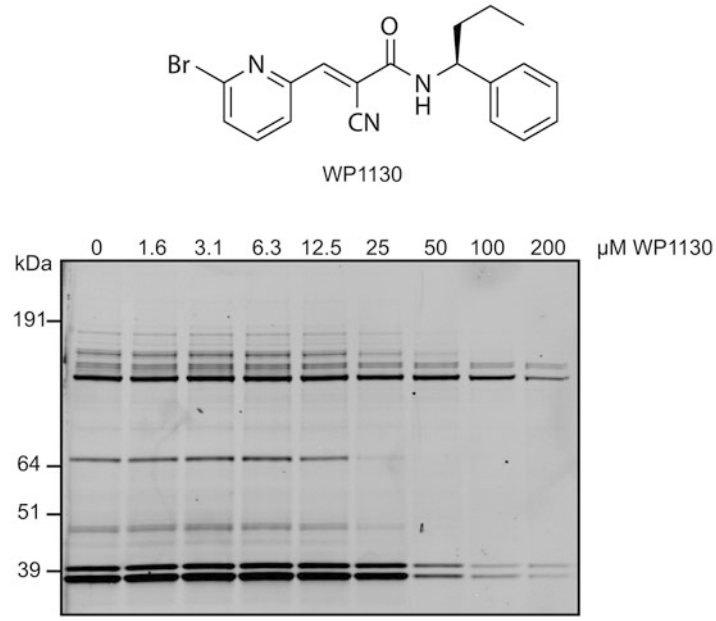

Fig. 4 *Top*: chemical structure of WP1130. *Bottom*: EL4 lysate was incubated with the indicated concentrations of small-molecule DUB inhibitor WP1130 [19, 20]. Subsequently, lysate was labeled with DUB activity probe. Proteins were separated by SDS-PAGE and the residual DUB activity was visualized by in-gel fluorescence scanning (λ (ex/em) = 550/590). Increasing concentrations of WP1130 results in less visible bands, indicating inhibition of these DUBs by WP1130

visualize differential DUB activity profiles in a variety of cell lines, which is very cell line dependent [8]. When an affinity tag, such as an epitope tag, poly-histidine tag or biotin tag, in combination with a cleavable moiety is incorporated in the DUB probe, the resulting probe enables the affinity catch-and-release of DUBs of interest, facilitating identification [8].

This chapter covers two methods for the total chemical synthesis of activity-based DUB probes, dependent on the reactive moieties present in the building blocks of choice. In the first method the building blocks of choice are coupled to the N-terminus of Ub on solid phase, after which the VME warhead is coupled to the N-terminus of Ub in solution. When the building block of choice to be coupled to the N-terminus of Ub contains an extra free carboxylic acid, such as in TMR, a different method should be used. In this method both the VME warhead and the desired N-terminal tag are coupled sequentially in solution. In addition, optimized SDS-PAGE procedures for profiling DUB activity in cell lysate using the fluorescent DUB activity probe TMRUbVME are described, including the assessment of small-molecule DUB inhibitor specificities.

2 Materials

2.1 General Materials

All reagents used in this protocol were purchased from Biosolve (Valkenswaard, The Netherlands) or Sigma Aldrich (Zwijndrecht, The Netherlands), unless otherwise indicated, at the highest commercially available grade. All chemicals and solvents were used as received. Peptide building blocks were all l-stereoisomers and purchased from Novabiochem (EMD Millipore).

2.2 Total Chemical Synthesis of Ubiquitin-Based DUB Activity Probes

2.2.1 Synthesis of Ub (1–75) (Scheme 1, See Notes 1 and 2)

1. TentaGel R TRT-Gly Fmoc (Rapp Polymere, Tübingen, Germany); Fmoc-protected and side-chain protected natural amino acids: Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Tyr(tBu)-OH; pseudoproline building blocks: Fmoc-L-Leu-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Ile-L-Thr(ψMe, Mepro)-OH, Fmoc-L-Leu-L-Ser(ψMe,Mepro)-OH, Fmoc-L-Ser(tBu)-L-Thr(ψMe,Mepro)-OH; Dmb dipeptides: Fmoc-L-Ala-(Dmb)Gly-OH, Fmoc-L-Asp(OtBu)-(Dmb) Gly-OH; piperidine; *N*-methyl-2-pyrrolidone (NMP); *N*,*N*-diisopropylethylamine (DIPEA); benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop).
2. Diethyl ether.

2.2.2 Method A (Scheme 1, See Note 1)

1. *See* Subheading 2.2.1.
2. Piperidine, NMP, DIPEA, Pybop, peptide building blocks of choice (*see* **Note 2**).
3. Dichloromethane (DCM), 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP).
4. (E)-methyl-4-aminobut-2-enoate (glycine vinyl methyl ester, GlyVME, synthesized according to established procedures [25, 30], PyBOP, triethyl amine, DCM.
5. $KHSO_4$.
6. Na_2SO_4.
7. Trifluoroacetic acid, triisopropylsilane, MilliQ water (MQ).
8. Pentane, diethyl ether.
9. MQ, acetonitrile, acetic acid.

2.2.3 Method B (Scheme 1, See Note 1), for the Synthesis of TMRUbVME

1. *See* Subheading 2.2.1.
2. DCM, HFIP.
3. GlyVME, DCM, PyBOP, triethyl amine.
4. $KHSO_4$.
5. Na_2SO_4.
6. 5-carboxytetramethylrhodamine (TMR, synthesized according to established procedures [31]) or other building block of choice containing free carboxylic acids, PyBOP, DIPEA, DCM.

2.3 Profiling of DUB Activity Using SDS-PAGE Based Assays

2.3.1 Cell Harvesting and Lysis

1. Cell line of choice, cultured in appropriate medium, e.g., RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines and DMEM (Dulbecco's modified Eagle's medium) for adherent cell lines, supplemented with fetal calf serum (FCS).
2. Stock solution of inhibitor of choice, dissolved in dimethyl sulfoxide (DMSO), aqueous buffer or medium, in the appropriate concentration.
3. Phosphate-buffered saline (PBS), trypsin solution (0.05%, Gibco) for adherent cells, appropriate cell culture medium.
4. HR lysis buffer (*see* **Note 3**): 50 mM Tris–HCl (pH 7.4), 5 mM MgCl2, 250 mM sucrose. Optional supplements (*see* **Note 4**): 0.5% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 0.1% NP40, 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors (e.g., cOmplete protease inhibitor cocktail, Roche). Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4 °C.
5. Sonication equipment (e.g., Bioruptor, Diagenode).
6. Protein concentration determination assay reagents (e.g., Bio-Rad protein assay).

2.3.2 In Vitro Profiling of DUB Activity in Cell Lysates

1. Cell lysate obtained in Subheading 2.3.1, HR lysis buffer (*see* **Notes 4** and **5**).
2. Stock solution of 0.25 mg/mL TMRUbVME probe in 50 mM sodium acetate (pH 4.5, 5% DMSO) (*see* **Note 6**).
3. 50 mM NaOH.
4. 3× reducing sample buffer (*see* **Note 7**): 4× NuPAGE® LDS Sample Buffer, 2-Mercaptoethanol, MQ (75:17.5:7.5). Store at room temperature.

2.3.3 Assessment of DUB Inhibitor Potency in Cell Lysates Using TMRUbVME

1. Cell lysate obtained in Subheading 2.3.1, HR lysis buffer, supplemented with 0.5% CHAPS and 0.1% NP40 (*see* **Notes 4** and **5**). Optional supplements (*see* **Note 4**): 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors (e.g., cOmplete protease inhibitor cocktail, Roche). Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4 °C.
2. 20× stock solution of inhibitor of choice, dissolved in DMSO, aqueous buffer or medium.
3. Stock solution of 0.125 mg/mL TMRUbVME probe in 50 mM sodium acetate (pH 4.5, 5% DMSO) (*see* **Note 6**).
4. 50 mM NaOH.
5. 3× reducing sample buffer (*see* **Note 7**).

2.3.4 Gel Electrophoresis and In-Gel Fluorescence Scanning

1. Precast gel system (NuPAGE, Invitrogen), 4–12% NuPAGE® Novex® Bis-Tris precast protein gel (1.0 mm) (Invitrogen).
2. NuPAGE® MOPS SDS Running buffer (Invitrogen).
3. NuPAGE® Antioxidant (Invitrogen).
4. SeeBlue® Pre-Stained Standard (Invitrogen) (*see* **Note 8**).
5. 3× Reducing sample buffer.
6. Power supply, e.g., PowerPac Basic Power Supply (Bio-Rad).
7. ProXPRESS 2D Proteomic imaging system (Perkin Elmer).
8. TotalLab analysis software.

3 Methods

3.1 General Methods

1. Perform solid phase peptide synthesis on a Syro II MultiSyntech Automated Peptide synthesizer.
2. Perform preparative reverse-phase HPLC purifications on a Prominence HPLC system (Shimadzu) equipped with an Atlantis T3 column, using the following mobile phases: A (TFA (0,1%) in water) and B (formic acid (0,1%) in acetonitrile), the following gradient: 0–5 min 5% B, 5–8 min 5–25%

B, 8–30 min 25–60% B, 30–33 min 60–95% B, 33–35 min 95% B, and the following settings: column temperature: 40 °C; flow-rate: 7.5 mL/min; run-time: 35 min.; UV-detection at 230 and 254 nm.

3. Perform analytical HPLC on a 1525EF Binary HPLC pump (Waters) equipped with a 2487 Dual λ Absorbance Detector. Samples were run over an Atlantis DC18 column (6.4 × 50 mm, 10 mm; Waters) with the following two mobile phases: A (TFA (0.05%) in water) and B (TFA (0.05%) in acetonitrile) using the following gradient: 0–1 min 1% B, 1–13 min, 1–90% B, 13–16 min, 90% B; 16–17 min, 90–1% B, 17–25 min, 1% B; or 0–5 min 5% B, 5–30 min 5–95% B, 30–35 min 95% B, 35–40 min 95–5% B, 40–45 min 5% B.
4. Perform LC-MS measurements on a system equipped with an Alliance 2795 Separation Module (Waters), 2996 Photodiode Array Detector (190–750 nm, Waters) and LCT Orthogonal Acceleration Time of Flight Mass Spectrometer. Run samples over a Kinetex C18 column (2.1 × 50 mm, 2.6 μM, Phenomenex, Torrence, CA), at 0.8 mL/min, for 6 min, at a column temperature of 40 °C, using the following two mobile phases: A (acetonitrile(1%) and formic acid (0.1%), in water) and B (water (1%) and formic acid (0.1%) in acetonitrile), and the following gradient: 0–0.5 min 5% B, 0.5–4 min, 5–95% B, 4–5.5 min, 95% B.
5. Perform data processing using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function, Waters).
6. Optional: Perform preparative cation-chromatography using an ÄKTA Unichromat 1500-"PRO" system (15 × 185 mm column packed with Workbeads 40 S) at 4 °C, using the following two mobile phases: A (50 mM NaOAc, pH 4.5) and B (1 M NaCl in 50 mM NaOAc, pH 4.5) using a flow-rate of 5 mL/min.

3.2 Total Chemical Synthesis of Ubiquitin-Based DUB Activity Probes

3.2.1 Synthesis of Ub (1–75) (Scheme 1, See Note 1)

1. Fmoc-Gly functionalized trityl resin (0.14–0.2 mmol/g) is subjected to standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide synthesis to synthesize Ub (1–75) using an automated peptide synthesizer at 25 μmol scale.
2. *Cycles 1–30*: Deprotect the Fmoc group with 20% piperidine in NMP (1 × 5 min., 1 × 10 min., 1 × 5 min.) and wash the resin five times with NMP couple the next amino acid of the Ub sequence (counting from the C-terminus) using Fmoc protected amino acid (4 eq., *see* **Note 2**), DIPEA (8 eq.), and PyBop coupling reagent (4 eq.), for 45 min. and repeat the deprotection, and coupling for every next amino acid in the Ub sequence. After each step, wash the resin two times with NMP.

   Cycles 31–61: Extend the coupling time to 60 min.
   Cycles 62–68: Perform double couplings for all amino acids. Decrease coupling time to 30 min.

Use pseudoproline building blocks Fmoc-L-Leu-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Ile-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Leu-L-Ser(ψMe,Mepro)-OH, Fmoc-L-Ser(tBu)-L-Thr(ψMe,Mepro)-OH and Dmb dipeptides Fmoc-L-Ala-(Dmb)Gly-OH and Fmoc-L-Asp(OtBu)-(Dmb)Gly-OH to replace Leu8-Thr9, Ile13-Thr14, Leu56-Ser57, Ser65-Thr66, Ala46-Gly47, and Asp52-Gly53, respectively, and use single couplings for 120 min. to couple these building blocks.

3. Wash resin three times with DCM, three times with diethyl ether and dry the resin under high vacuum. Store in a dry environment.

3.2.2 Method A (Scheme 1, See Note 1)

1. Synthesize Ub (1–75) as described in Subheading 3.2.1.
2. Perform double couplings to couple the building blocks of choice (e.g., amino acids of the HA tag sequence YPYDVPDYA, 6His-tag, photocleavable or chemocleavable building blocks, fluorophores, and/or biotin) to the N-terminus of the Ub sequence on the resin by using (Fmoc) building block (4 eq. *see* **Note 2**), PyBOP (4 eq.) and DIPEA (8 eq.) in NMP at ambient temperature for 16 h. and similar deprotection reagents and conditions as described in Subheading 3.2.1.
3. Cleave the Ub conjugates from the trityl resin by treating the resin with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filter the resin. Wash the resin with 3–5 mL DCM, and combine and concentrate the filtrates.
4. Couple GlyVME to the C-terminus of protected Ub conjugate in solution using GlyVME (10 eq.), PyBOP (5 eq.), and triethyl amine (20 eq.) in DCM and stir for 16 h at ambient temperature.
5. Remove excess GlyVME by washing the DCM solution two times with 1 M $KHSO_4$.
6. Dry the organic layer with Na_2SO_4 and concentrate the organic layer to dryness in vacuo.
7. Remove the side chain protecting groups by taking the residue up in 5 mL trifluoroacetic acid–triisopropylsilane–water (95:2.5:2.5) and stir the solution for 3 h. at ambient temperature.
8. Add the mixture to a 50 mL falcon tube containing 40 mL ice-cold pentane/diethyl ether (1:3) to precipitate the Ub conjugate. Isolate the precipitate by centrifugation (1500 × *g*, 6 min. 4 °C) and wash the precipitate by three cycles of resuspension in ice-cold diethyl ether and centrifugation.
9. Take the pellet up in water–acetonitrile–acetic acid (65:25:10), freeze the pellet, and lyophilize the frozen pellet. Purify the activity probe by preparative HPLC. Perform LC-MS measurements to verify the right mass of the activity probe and to check for purity. In Fig. 5, the LC-MS analysis profile is shown for Ub-based DUB probe TMRUbVME.

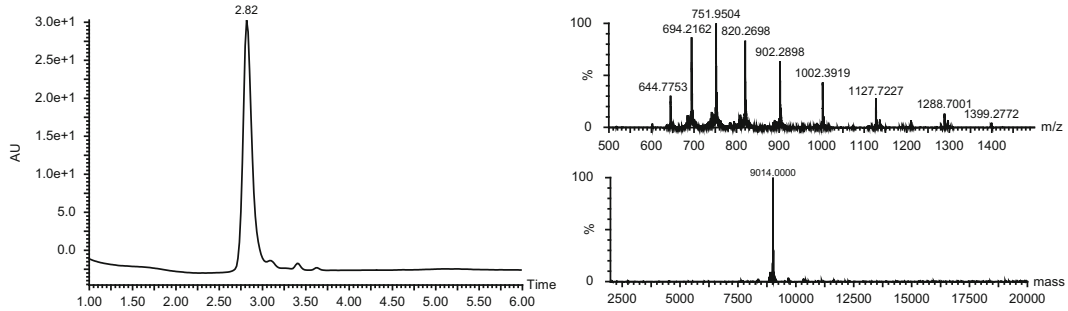

Fig. 5 LC profile of purified TMRUbVME by HPLC (*left*). MS analysis of purified TMRUbVME probe (*top right*) and deconvoluted spectrum (bottom right). Calculated $[M+H]^+$ 9012.8 Da, observed $[M+H]^+$ 9014.0 Da

3.2.3 Method B (Scheme 1, See Note 1), for the Synthesis of TMRUbVME

1. Synthesize Ub (1–75) as described in Subheading 3.2.1.
2. Cleave the Ub conjugates from the trityl resin by treating the resin with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filter the resin. Wash the resin with 3–5 mL DCM, and combine and concentrate the filtrates.
3. Couple GlyVME to the C-terminus of protected Ub conjugate in solution using GlyVME (10 eq.), PyBOP (5 eq.), and triethyl amine (20 eq.) in DCM and stirred for 16 h at ambient temperature.
4. Remove excess GlyVME by washing the DCM solution two times with 1 M $KHSO_4$.
5. Dry the organic layer with Na_2SO_4 and concentrate the organic layer to dryness in vacuo.
6. Couple TMR (or any other building block of choice containing free carboxylic acids) to the N-terminus of protected Ub conjugate in solution using TMR (4 eq.), PyBOP (4 eq.), and DIPEA (10 eq.) in DCM and stir for 16 h at ambient temperature. Concentrate the organic layer to dryness in vacuo. Proceed with **step 7** of Subheading 3.2.2.

3.3 Profiling of DUB Activity Using SDS-PAGE Based Assays

3.3.1 Cell Harvesting and Lysis

1. Culture cell line of choice in appropriate medium and under appropriate culture conditions. Suspension cells should be cultured until log-phase and adherent cells should be passaged when approximately 80% confluency is reached.
2. Seed cells in a multi well tissue culture plate and allow the cells to attach. Add the compounds to be tested, dissolved in DMSO (or medium if the compounds are water-soluble), in the desired concentrations to the cells. Make sure to have enough wells available to include all of the appropriate controls.
   Critical: the final concentration of DMSO should not exceed 0.5% as this can interfere with the assay.

3. To harvest adherent cells by trypsinization, aspirate the medium, wash cells with PBS and aspirate. Add sufficient trypsin and wait for cells to detach. After the cells have detached, add medium supplemented with 10% FCS to the cells to inactivate the trypsin. Collect cells and pellet by centrifugation at 1300 × *g* for 5 min at 4 °C. To harvest suspension cells, pellet cells by centrifugation at 1300 × *g* for 5 min at 4 °C. Wash cells using 10–20 pellet volumes of PBS and pellet cells again centrifugation at 1300 × *g* for 5 min at 4 °C. Discard the supernatant.
   Pause point: at this time, cell pellets can be snap-frozen in liquid nitrogen and stored at −20 °C until further use (*see* **Note 9**).
4. Resuspend cell pellets in two pellet volumes of cold HR buffer (*see* **Note 3**). Optional (*see* **Note 4**): Supplement the HR lysis buffer with 0.5% CHAPS, 0.1% NP40, 1 mM DTT (add freshly from a 1 M stock solution before use), 2 mM ATP (add freshly from a 0.5 M stock solution before use), and/or protease inhibitors (add freshly, e.g., cOmplete protease inhibitor cocktail, Roche). Keep samples on ice.
5. Lyse cells by sonication using for example a Bioruptor (five cycles of 30 s on and 30 s off).
6. Centrifuge cells at maximum speed for 15 min at 4 °C to remove cell debris. Transfer the supernatant to a fresh Eppendorf tube and determine the protein concentration using for example the Bio-Rad protein assay or a comparable protein assay according to the manufacturer's instructions.
   Pause point: At this point, lysates can be snap-frozen in liquid nitrogen and stored at −20 °C until further use.

To label DUBs directly proceed to Subheading 3.3.2. To determine the effect of DUB inhibitors prior to DUB labeling, proceed to Subheading 3.3.3.

3.3.2 In Vitro Profiling of DUB Activity in Cell Lysates Using Ub-Based DUB Probe TMRUbVME (See **Note 10***)*

1. Add 25 µg of cell lysate to an eppendorf tube and adjust the volume to 22 µL with HR buffer (a final volume of 25 µL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH, **steps 2** and **3**) (*see* **Notes 4** and **5**).
2. Add 1 µL of a 25 µM TMRUbVME solution in sodium acetate buffer (50 mM NaOAc, 5% DMSO, pH 4.5, *see* **Note 6**) to the lysate.
3. Add 2 µL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (*see* **Notes 5** and **6**). Vortex and spin samples briefly. Incubate for 30 min at 37 °C.
4. Add 12,5 µL of a 3× reducing sample buffer (e.g., 4× Invitrogen NuPAGE® LDS Sample Buffer, supplemented with 2-mercaptoethanol and MQ) to the reaction mixture and heat the

samples for 10 min at 70 °C (*see* **Note 7**). Centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

Pause point: At this point, reduced and heated samples can be snap-frozen in liquid N2 and stored at −20 °C until further use.

Proceed to Subheading 3.3.4.

3.3.3 *In Vitro Assessment of DUB Inhibitor Potency in Cell Lysates Using TMRUbVME*

1. Add 25 μg of cell lysate to an Eppendorf tube and adjust the volume to 20.75 μL with HR buffer (*see* **Notes 4** and **5**) supplemented with 0,5 % CHAPS, and 0,1 % NP40 (for improved solubility of DUB inhibitors, *see* **Note 4**), so that a final volume of 25 μL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH (**steps 2** and **3**).
   Critical: The presence of 1 mM DTT can improve inhibitory effect. Check beforehand whether the DUB inhibitor of choice is stable in the presence of DTT (*see* **Note 4**).
2. Add 1.25 μL of a 20× stock solution of the desired inhibitor in DMSO. Include a reference sample to which 1.25 μL DMSO, but no inhibitor is added. Vortex and incubate the samples for the desired time period at 37 °C. Typically, samples are incubated for 1 h.
   Critical: The quality of labeling will decrease if more than 5 % DMSO is present in the reaction mixture.
3. Add 1 μL of a 12.5 μM TMRUbVME solution in sodium acetate buffer (50 mM NaOAc, 5 % DMSO, pH 4.5, *see* **Note 6**) to the lysate.
   Critical: When non-covalent inhibitors are used, the use of a lower concentration of TMRUbVME solution and lower probe incubation temperatures are preferred (*see* **Note 11**), compared to standard DUB labeling (Subheading 3.3.2).
4. Add 2 μL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (*see* **Notes 5** and **6**) Vortex and spin samples briefly. Incubate for 5 min at ambient temperature.
5. Add 12.5 μL of a 3× reducing sample buffer to the reaction mixture and heat the samples for 10 min at 70 °C (*see* **Note 7**). Centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

   Proceed to Subheading 3.3.4.

3.3.4 *Gel Electrophoresis and In-Gel Fluorescence Scanning*

The following instructions assume the use of the NuPAGE precast gel system and precast protein gels from Invitrogen.

1. Assemble the NuPAGE gel unit using a precast NuPAGE 4–12 % Bis-Tris gel according to the manufacturer's instructions.

2. Add 1× MOPS SDS running buffer to both the inner and outer chamber of the gel unit.
3. Add 125 mL antioxidant to the inner gel chamber to keep the samples in a reduced state.
4. Load 10–30 μL (depending on of the size of the wells) of the reduced and heated samples into the wells of the gel.
   When reduced samples were frozen for storage, heat the samples again for 10 min at 70 °C, centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets, and gently vortex the sample.
   Keep one well free and load this well with 10 μL of prestained protein molecular weight marker (e.g., SeeBlue® Pre-Stained Standard from Invitrogen, *see* **Note 8**).
5. Load 3× reducing sample buffer to remaining empty wells (use a volume of 1/3 of the sample volume).
6. Run the gel at 170–180 V for appropriate time (at least until the blue loading front is no longer visible) using a Power Supply (e.g., PowerPac Basic Power Supply, Bio-Rad).
7. Gently take the gel out from the cassette and image the gel using a fluorescence imager containing appropriate filter settings (λ (ex/em) = 550/590 nm for TMR). For imaging the bands of the protein molecular weight marker SeeBlue® Pre-Stained Standard, image the gel once more using the following filter settings: λ (ex/em) = 625/680 nm.
8. Analyze images using appropriate software.

4 Notes

1. Depending on the reactive moieties present in the building blocks that will be coupled to the N-terminus of Ub, different methods can be used to synthesize Ub-based probes. The Ub sequence is built up from the C-terminus on solid phase using Fmoc-based solid phase synthesis. Method A describes the coupling of building blocks to the N-terminus of Ub directly on solid-phase using Fmoc-based solid phase synthesis, after which the C-terminal warhead is coupled in solution and protecting groups are removed. This applies in case the building blocks that will be coupled to the N-terminus of Ub contain only one free carboxylic acid and other reactive moieties, such as other carboxylic acids and amines, are protected. Method B describes the coupling of the C-terminal warhead to Ub in solution, after which N-terminal building blocks are coupled in solution, followed by deprotection of reactive moieties. Method B applies when the building blocks that will be

coupled to the N-terminus of Ub contain an extra unprotected carboxylic acid. For the synthesis of TMRUbVME method B is used, since 5-carboxytetramethylrhodamine (TMR) contains two unprotected carboxylic acids. If method A would be used, the free-amine containing warhead that should only be coupled to the C-terminus of Ub will be coupled to the other carboxylic acid of TMR as well.

2. Dry all Fmoc-protected amino acid building blocks overnight under high vacuum. Drying removes moisture, as well as traces of acetic acid (or other acids) that are present, which are detrimental for peptide synthesis.
3. Other lysis buffers en methods can be used. Nonetheless, DUB labeling efficiency should be determined experimentally using other lysis buffers and methods.
4. The use of non-supplemented HR lysis buffer in combination with sonication should be sufficient for lysis of the cells. However, the use of additives can increase lysis efficiency and/or labeling efficiency:
   - *Detergents*: Detergents CHAPS and NP40 will improve lysis of cells and increases solubility of DUB inhibitors. However, the use of detergents can decrease the quality of DUB labeling using DUB probe TMRUbVME and should be determined experimentally. Though, the use of 0.5% CHAPS and 0.1% NP40 does not decrease labeling efficiency.
   - *DTT*: DUB inhibitory effect can be increased when DTT is used. However, the effect of DTT on the DUB inhibitor should be examined beforehand. The use of DTT does not affect labeling of DUBs in cell lysates. Add DTT freshly before use, since DTT is not stable in solution.
   - *ATP*: The effect of ATP on labeling is not thoroughly investigated and should be established experimentally.
   - *Protease inhibitors*: The addition of protease inhibitors is recommended, to protect deubiquitinating enzymes in cell lysate from degradation. Labeling efficiency in the presence of protease inhibitors should be tested beforehand. The use of cOmplete protease inhibitor cocktail from Roche did not seem to negatively affect labeling efficiency. Add protease inhibitors freshly, e.g., from a 50× stock solution, before use. Manufacturer's instructions should be checked for storage conditions of stock solutions.
5. Different buffers than HR lysis buffer can be used for the incubation of cell lysate with DUB inhibitors and probe. When stronger incubation buffers are used, possibly the addition of

NaOH, subsequent to the addition of the acidic probe buffer, becomes redundant. The effect of incubation buffers on the inhibition, labeling of DUBs and solubility of DUB inhibitors should be determined experimentally, however. In addition, other reaction volumes and other concentrations can be used. Changing probe and lysate protein concentrations will affect DUB labeling efficiency. We advise to use different conditions at first, to determine optimal labeling conditions.

6. Other buffers can be used to dissolve Ub-based DUB probes. When a buffer of neutral pH is used, the addition of 50 mM NaOH, after the addition of the acidic probe solution, becomes redundant. Probe solubility and labeling efficiency using other buffers should be experimentally established.
7. Heating samples at 70 °C for 10 min is optimal for Invitrogen NuPAGE® LDS Sample Buffer containing buffers. When the NuPAGE precast gel system is used, also NuPAGE LDS Sample buffer (or other recommended buffers) should be used to prepare the 3× reducing sample buffer. The use of a different reducing sample buffer can result in improper running of the gel.
8. Other protein molecular weight marker than SeeBlue® Pre-Stained Standard from Invitrogen can be used. However, molecular weight markers that possess similar fluorescence properties to the dyes used in the DUB probe (in case of TRM: λ (ex/em) = 550/590) could contribute to high signal intensities of these markers during fluorescence imaging.
9. Freezing cell pellets could give rise to differential DUB labeling profiles compared to freshly lysed cells. This should be experimentally determined. Frozen cells cannot be taken into culture again. They will not survive, unless frozen in proper freezing medium.
10. Similar labeling conditions can be used for Ub-based DUB probes containing other dyes (e.g., Cy5) or visualization handles (e.g., HA-tag). Use appropriate fluorescence settings for other dyes or other appropriate visualization methods for other handles (e.g., Western Blotting for HA-tag or Biotin).
11. Since the covalent binding of the TMRUbVME probe to DUBs is very efficient, competition with a non-covalent DUB inhibitor is challenging. When non-covalent inhibitors are used, the use of a lower concentration of TMRUbVME probe, shorter probe incubation times, and lower probe incubation temperatures are preferred, compared to the conditions used for standard DUB labeling (Subheading 3.3.3). In addition, a DUB inhibitor incubation temperature of 37 °C, and long DUB inhibitor incubation times are beneficial.

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Chapter 10

Biochemical and Mass Spectrometry-Based Approaches to Profile SUMOylation in Human Cells

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Abstract

Posttranslational modification of proteins with the small ubiquitin-like modifier (SUMO) regulates protein function in the context of cell cycle and DNA repair. The occurrence of SUMOylation is less frequent as compared to protein modification with ubiquitin, and appears to be controlled by a smaller pool of conjugating and deconjugating enzymes. Mass spectrometry has been instrumental in defining specific as well as proteome-wide views of SUMO-dependent biological processes, and several methodological approaches have been developed in the recent past. Here, we provide an overview of the latest experimental approaches to the study of SUMOylation, and also describe hands-on protocols using a combination of biochemistry and mass spectrometry-based technologies to profile proteins that are SUMOylated in human cells.

Key words Small ubiquitin-like modifier, SUMO, Posttranslational modification, Isopeptide bond, Mass spectrometry, SUMOylation

1 Introduction

Reversible protein modifications by ubiquitin and ubiquitin-like molecules are fundamental for the regulation of protein function in normal physiology. In particular, DNA repair mechanisms, apoptosis, and gene transcription are controlled by specific ubiquitylation and SUMOylation events [1, 2]. Similar to ubiquitin, the 12 kDa small ubiquitin-like modifier (SUMO) can be attached to the (ε)-NH_2 side chain of lysine residues in protein substrates via an isopeptide bond and can also form polymers and mixed chains [3]. Covalent attachment of SUMO to its substrates requires prior activation by the E1 complex SAE1-SAE2 and interactions with the E2 enzyme UBE2I, which is promoted by a SUMO E3 ligase such as PIAS1-4, RANBP2, or CBX4 [4]. SUMO modifications are reversible as they can be cleaved by proteases such as SENP1 [5]. Whereas protein ubiquitylation has widespread cellular functions, protein SUMOylation has hitherto been predominantly linked functionally

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to targeting proteins in the nucleus, and it seems to play a role in the cell cycle [6], DNA replication stress [1], and hypoxia [7]. SUMO exists in three forms in human cells, SUMO-1, SUMO-2, and SUMO-3. In some instances, SUMO-2 and -3 are involved in targeting proteins for degradation by the ubiquitin–proteasome system, in particular after induction of cell stress, such as DNA damage [8, 9]. Polymeric SUMO2 chains are particularly susceptible to polyubiquitination [10, 11]. However, SUMO-3 may also act as an antagonist of ubiquitin/SUMO-2 in the degradation process.

There are several examples of cross talk between SUMO and ubiquitin modifications. For instance, ubiquitin-dependent degradation of SUMO-targets can be promoted by ubiquitin E3 ligases (STUbl) that recognize poly-SUMO-protein substrates. Moreover, several proteins contain both Ub and SUMO recognition motifs [12]. Also, lysine residues compete for different modifications. For instance, in the case of USP25, modification by ubiquitin on Lys99 activates the enzyme, whereas SUMOylation at the same residue is inhibitory [13]. Related to this, USP28 N-terminal SUMOylation is linked to the inhibition of its deubiquitinating (DUB) activity [14].

The biology of SUMOylation has been studied with the aid of antibodies specific for SUMO-1/-2/-3, but it is the application of mass spectrometry that has led to an acceleration in the discovery of SUMOylated proteins at the proteome level [1]. For mapping ubiquitylation sites on proteins, the conventional proteomics approach using a trypsin digestion step generates a Gly–Gly tag at Lys side chains bearing a ubiquitin which can be readily detected in MS/MS experiments [15]. This approach is not directly applicable to the mapping of SUMO-1/-2/-3 modifications as their C-termini do not have trypsin cleavage sites [16]. Trypsin digestion of SUMO-1 would generate a 27-amino acid and SUMO-2 a 32-amino acid long branched peptide attached to the target peptide derived from the SUMOylated substrate (Fig. 1). To overcome this, trypsin/Lys-C/elastase combined digestion strategies were developed and shown to be useful for the mapping of SUMOylation sites in proteins, at least in low complexity samples [17, 18]. However, for very complex samples such as cell extracts, a more successful strategy has been to first introduce specific trypsin cleavage sites into the C-terminal region of SUMO, such as the T105R mutation for human SUMO-1 (SUMO-1^{T105R}), Q87R or T91R mutations for SUMO-2 (SUMO-2^{Q87R} or SUMO-2^{T91R}), and or F96R or T100R for SUMO-3 (SUMO-3^{F96R} or SUMO-3^{T100R}) (Figs. 1 and 2). Tagged and mutated SUMO variants are then expressed in mammalian cell lines, enriched from total cell extracts and subsequently used for MS/MS based mapping for the characterization of SUMOylation sites (Fig. 2) [8, 9]. Alternatively, in the case of the SUMO-1^{T105R}/SUMO-2^{T91R}/SUMO-3^{T100R} mutations, digestion with trypsin generates a Gly–Gly tag on peptide fragments derived from SUMOylated substrates, and these target peptides can be enriched using specific anti-Gly–Gly

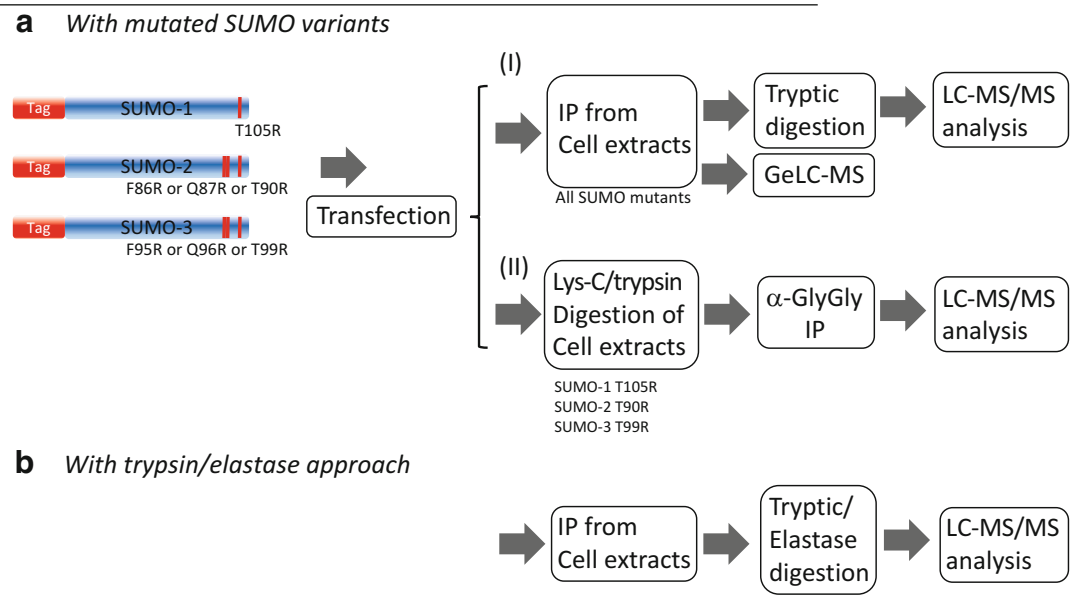

Fig. 1 SUMO mutation strategies for mass spectrometry based studies. Human protein sequences of SUMO-1 (Sprot accession nr P63165-1), SUMO-2 (Sprot accession nr P61956-1), and SUMO-3 (Sprot accession nr P55854-1) and the most commonly used mutation strategies (**a**) to introduce small peptide mass tags generated by trypsin digestion that are suitable for tandem mass spectrometry analysis (**b**) Initial methionine residues are not accounted for in the numbering of amino acid positions

antibodies (Fig. 2) [15, 19]. It is noteworthy that SUMOylation of protein substrates by SUMO-2 or SUMO-3 cannot be distinguished using these methods. The biology of SUMOylation has attracted wide interest beyond DNA repair and cellular stress [20]. In order to facilitate access to experimental approaches that define protein SUMOylation events, we here provide an overview of systematic workflows and describe a protocol that enables the study of protein SUMOylation by mass spectrometry and proteomics.

2 Materials

2.1 Generation of Tagged Human SUMO Mutant Expression Constructs

Human SUMO sequences can be amplified using the polymerase chain reaction (PCR) from the I.M.A.G.E. consortium or the mammalian gene collection (MGC) clones as described [8]. The NCBI references for DNA sequences are NM_003352 (SUMO-1), NM_006937 (SUMO-2), and NM_006936 (SUMO-3). For SUMO2, the PCR amplified product was cloned into the pcDNA4/T0-Strep-HA bacterial expression vector, and the Strep-HA-SUMO-2 cDNA then subcloned into the pcDNA3.1(+) mammalian expression vector. SUMO mutants were generated via the QuickChange site-directed mutagenesis approach (Stratagene) using the SUMO plasmid as a template [9]. The most common mutations introduced in human SUMO-1, -2, or -3 are described and summarized in Fig. 1.

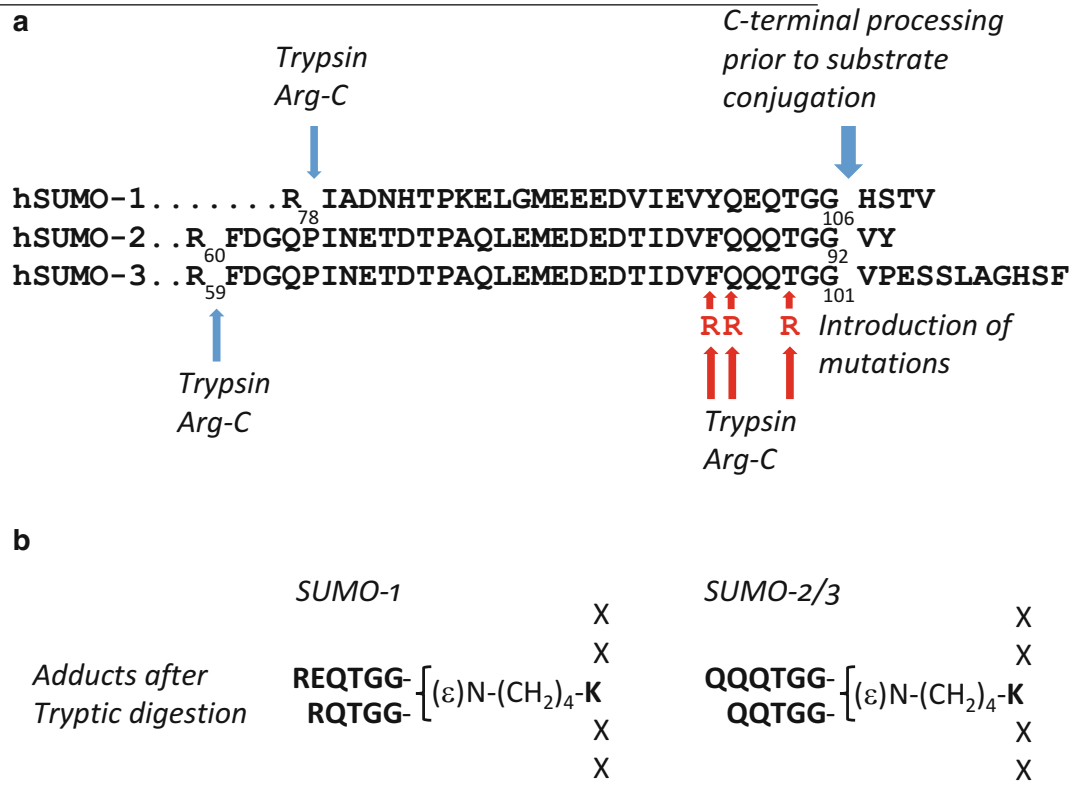

Fig. 2 Workflows for protein SUMOylation analysis by mass spectrometry. Different approaches have been developed to study protein SUMOylation, based on SUMO mutagenesis (**a**) combined with ectopic expression in mammalian cells followed by SUMOylated protein enrichment at the protein/peptide level and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). (**b**) Alternatively, SUMOylated protein material can be digested using a multi-protease digestion approach that renders the mapping of SUMOylation sites amenable to analysis by LC-MS/MS

2.2 Cell Culture, SILAC Labeling, and Transfection

HEK293T or U2OS cells (mostly used for such studies, *see* also **Note 1**) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml). For stable isotope labeling of amino acids in cell culture (SILAC) (*see* **Note 2**), cells were propagated in DMEM medium containing l-arginine and l-lysine (light), l-arginine-$^{13}C_6$ and l-lysine-D_4 (medium) or l-arginine-$^{13}C_6$-$^{15}N_4$ and l-lysine-$^{13}C_6$-$^{15}N_2$ (heavy) (Cambridge Isotope Laboratories) supplemented with dialysed FBS and penicillin/streptomycin as described above. Cells were passaged through at least 6–8 doublings in order to achieve >95% incorporation of labeled amino acids into proteins. For a typical transient transfection experiment, ~70 µg pcDNA3.1-Strep-HA-SUMO-2 plasmid was transfected into ~10^8 U2OS cells (ten 10 cm tissue culture dishes) using FuGENE 6 transfection reagent

(Promega). Alternatively, transfection can be done at a much smaller scale to economize DNA/transfection reagent (10–50-fold less), and G418 resistant clones expressing Strep-HA-SUMO-2 protein selected and expanded in light, medium or heavy medium [9].

3 Methods

3.1 Protocol for Enrichment of SUMOylated Proteins

The light, medium, and heavy cell populations are exposed to different physiological situations/stimuli, followed by harvesting cells on ice using a cell scraper into ice-cold PBS.

1. Cells are washed twice in ice-cold PBS, counted and mixed at a 1:1:1 ratio prior to cell lysis with 1 ml lysis buffer 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM EDTA protease/phosphatase inhibitor cocktail (Roche, 1 tablet per 10 ml buffer) and 20 mM N-ethylmaleimide (NEM).
2. Protein concentration is determined using a BCA assay (Thermo Scientific Pierce). Typically, cell lysate with 2-3 mg of protein for each cell population, representing ~$0.5–1 \times 10^8$ cells in total, is used per experiment and incubated with Strep-tactin (IBA) resin (50 µl of slurry per sample) for 2 h at 4 °C with rotation (10 rpm). Alternatively, anti-HA coupled agarose beads can be used (*see* **Note 3**).
3. Purified SUMOylated protein material is washed four times with 1 ml lysis buffer by repeated steps of centrifugation for 1 min at 14,000 × *g* at room temperature, and the supernatant removed, fresh lysis buffer added, and mixture vortexed. Protein material is eluted by boiling at 95 °C in reducing SDS sample buffer (4% SDS, 50 mM Tris–HCl, 10% glycerol, 2% ß-mercapto-ethanol, and bromophenol blue), followed by centrifugation for 5 min at 14,000 × *g* at room temperature and supernatant being collected. Samples are stored at –20 °C until analysis.

3.2 Sample Preparation and Analysis by Mass Spectrometry

Eluate material from immunoprecipitations in reducing SDS-sample buffer are analyzed using either a GeLC-MS or in-solution digestion approach (*see* also **Note 4**).

3.3 GeLC-MS Method

1. Samples (typically ~50 µg material) were separated by SDS-PAGE and SUMOylated bands visualized by Coomassie blue staining using a Colloidal Blue staining kit (Life Technologies, for an example, *see* also ref. [9], Fig. 3b).
2. Gel lanes are cut into ten individual gel bands which were then subjected to in-gel trypsin digestion as described previously [21]. Gel bands are cut into pieces of ~1 mm in size and washed

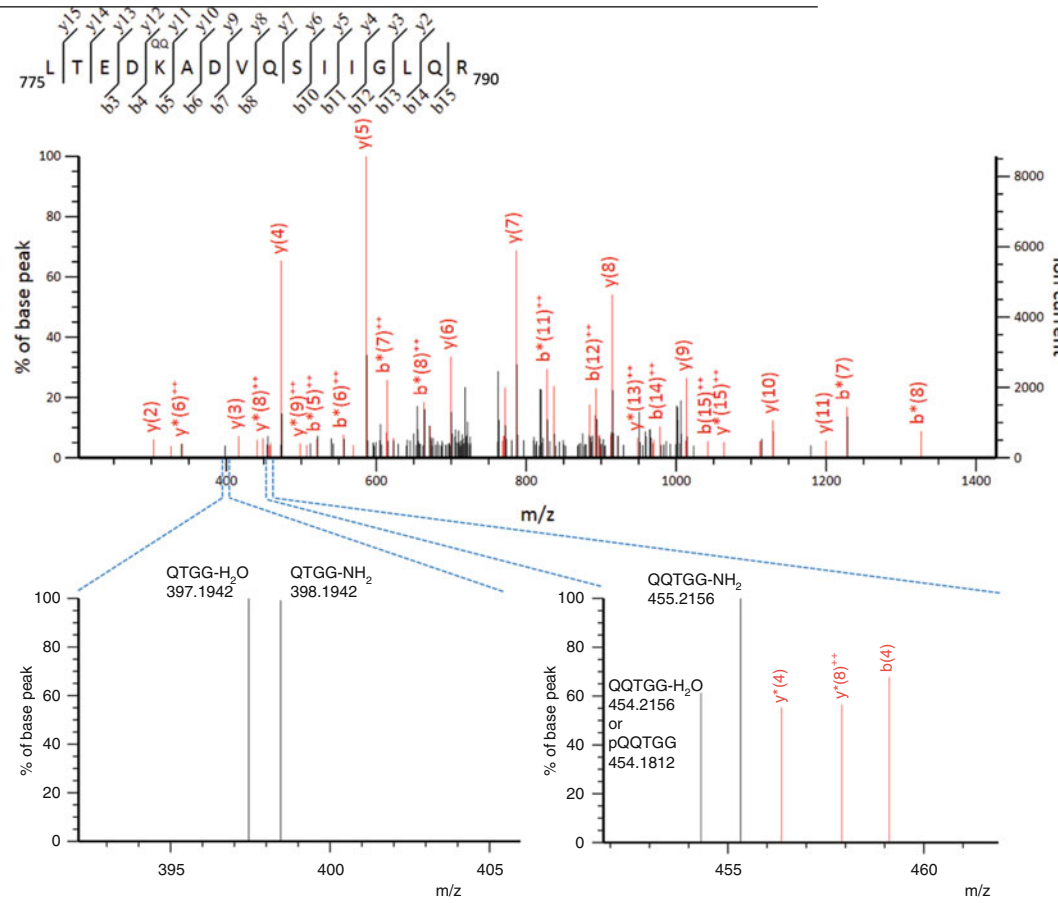

Fig. 3 Mass spectrometry-based protein SUMOylation mapping. MS/MS spectrum of the precursor ion *m/z* 753.0655 $[M+3H]^{3+}$ matching the tryptic peptide 775–790 derived from the transcription intermediary factor 1-beta/TRIM28 (Sprot accession nr Q13263), calculated mass $[M+H]^{+}$ 2256.1710. Fragment ion analysis reveals the presence of diagnostic ions for the QQTGG tag such as QQTGG and QTGG with loss of H_2O or NH_2 or Pyro-QQTGG (pQQTGG) [29], indicating the presence of a SUMOylation site that was mapped to Lys779. These diagnostic ions were not present in the MS/MS spectrum of the corresponding unmodified peptide (data not shown)

with a solution of 50 % methanol, 5 % acetic acid in water overnight at room temperature on a shaker (Eppendorf, 20 rpm).

3. After removing excess liquid, 200 µl acetonitrile is added to dehydrate the gel pieces for 5 min at room temperature.
4. Excess liquid is removed, and the step repeated.
5. Thirty µl of 10 mM DTT in 100 mM ammonium bicarbonate pH ~8 is added and samples are incubated for 30 min at room temperature.
6. Excess liquid is removed, 30 µl of 50 mM iodoacetamide (IAA) is added, and samples are incubated for 30 min at room temperature in the dark.

7. Excess liquid is removed, and 200 µl acetonitrile is added, followed by incubation of the samples for 10 min.
8. Excess liquid is removed, and the step is repeated.
9. After the removal of excess acetonitrile, 30 µl trypsin solution (20 ng/µl in 50 mM ammonium bicarbonate) is added while the samples are kept on ice, and the samples are then incubated to allow for rehydration for 10 min with occasional gentle mixing.
10. Gel pieces are then centrifuged shortly, and excess trypsin solution was removed.
11. Five µl of 100 µM ammonium bicarbonate buffer is then added, and samples are incubated at 37 °C overnight.
12. Fifty µl of 100 mM ammonium bicarbonate buffer is then added, and the samples are incubated for 10 min with occasional gentle vortex mixing, followed by the collection of supernatant in a separate 1.5 ml tube.
13. Fifty µl of Extraction Buffer 1 (50% acetonitrile, 5% formic acid in H_2O) is added to the gel pieces, incubated for 10 min with occasional gentle mixing, and supernatants are transferred to the 1.5 ml collection tube.
14. The same step is repeated with Extraction Buffer 2 (85% acetonitrile, 5% formic acid in H_2O). Collected supernatant material is dried at room temperature using a vacuum centrifuge to dryness, subsequently resuspended in Buffer A (98% H_2O, 2% acetonitrile, 0.1% formic acid) and kept at –20 °C until further analysis.

3.3.1 Buffers Used for This Procedure

100 mM ammonium bicarbonate buffer: 79 g/mol; 0.79 g NH_4HCO_3 in 100 ml H_2O (Milli-Q quality of similar). For 50 mM ammonium bicarbonate buffer, dilute with H_2O at a 1:1 ratio.

1 M and 10 mM DTT solutions: 154 g/mol; 1.54 g dithiothreitol (DTT) in 10 ml H_2O. Keep aliquots at –20 °C. For a 10 mM DTT solution, dilute stock in H_2O at a 1:10 ratio.

50 mM iodoacetamide: 185 g/mol; 0.0092 g iodoacetamide in 1 ml H2O. For best results, this solution should be freshly prepared for each experiment.

Extraction Buffer 1: 50% acetonitrile, 5% formic acid in H_2O

Extraction Buffer 2: 85% acetonitrile, 5% formic acid in H_2O

Buffer A: 98% H_2O, 2% acetonitrile, 0.1% formic acid

In-solution digestion method: Alternatively to the gel-based digestion method, eluted samples in reducing SDS-sample buffer were subjected to protein precipitation by chloroform/methanol [22] prior to carrying out the protocol below (*see* also **Note 5**). Of note is that the digestion procedure is work-

ing more efficiently when the reduction and alkylation steps are carried out before precipitation (*see* also **Note 6**).

1. Samples are resuspended in 200 µl 6 M urea in Tris buffer pH 7.8. Vortexing and sonication (sonication bath) was used when necessary.
2. Five µl of 200 mM DTT in 100 mM Tris pH 7.8 (final concentration ~5 mM) is added and samples are incubated for 30–60 min at room temperature.
3. Twenty µl of 200 mM iodoacetamide (in 100 mM Tris–HCl pH 7.8) is added and samples are incubated for 30–60 min at room temperature (*see* also **Note 6**).
4. To remove as much SDS/deoxycholate detergent as possible, pellet material is resuspended in 200 µl of water, and the chloroform–methanol extraction step is repeated (*see* also **Note 7**).
5. Protein pellets are resuspended in 50 µl 6 M urea buffer by vortexing and sonicating the samples, and ~250 µl of H_2O is added to reduce urea concentrations to <1 M. Trypsin is then added at a 1:50 ratio (weight–weight) regarding protein content in the samples (*see* also **Note 8**), and samples are incubated overnight at 37 °C.
6. Samples are subsequently subjected to desalting using SEP-PAK C18 cartridges (Waters) according to the manufacturer's protocol (*see* **Note 9**), and samples dried by vacuum centrifugation. Samples are resuspended in 20 µl of Buffer A (98% H_2O, 2% acetonitrile, 0.1% formic acid) and kept at −20 °C until further analysis.

3.3.2 Buffers Used for This Procedure

400 mM Tris solution: Dissolve 12.1 g of Tris base in 200 ml H_2O. Adjust pH to pH 7.8 with 6 M HCl. Add H_2O to a final volume of 250 ml, store a 4 °C.

6 M urea in Tris buffer pH 7.8: Dissolve 2.0 g of urea in a 15 ml falcon tube. Add 1.25 ml of 0.4 Tris stock solution. Adjust the total volume to 5 ml with H_2O.

200 mM DTT in 100 mM Tris buffer pH 7.8. Dissolve 0.031 g of DTT in 750 µl of H_2O. Add 250 µl of 0.4 M Tris stock solution and vortex.

200 mM iodoacetamide in 100 mM Tris buffer pH 7.8. Dissolve 0.037 g of iodoacetamide in 750 µl of H_2O. Add 250 µl of 0.4 M Tris stock solution and vortex. For optimal results, this solution should be made fresh for each experiment.

Trypsin solution. Add 25 µl of ice-cold 0.4 M Tris stock solution and 75 of ice-cold H_2O to 20 µg of sequencing grade modified trypsin (Promega) and resuspend carefully. The final concentration is 0.2 µg/µl. Keep on ice until use.

3.4 Mapping SUMOylation Sites

1. Raw MS data is processed using MaxQuant (version 1.0.14.3) or processed with ProteoWizard (v 3.0.4743) to generate Mascot generic files (mgf). The following parameters are used for data analysis (*see* also **Note 10**): peptide mass accuracy 10 ppm; MS/MS mass accuracy 0.5 Da (for Orbitrap Velos MS instrument in CID mode); missed cleavages 1; fixed modification—cysteine carbamidomethylation; variable modifications—methionine oxidation, N-acetylation, serine/threonine and tyrosine phosphorylation.
2. For the detection of SUMOylation sites, a mass addition of 471.2078 (QQTGG signature tag remnant of $SUMO2^{Q87R}$) is used. For the $SUMO1^{T104R}$, $SUMO2^{T90R}$, and $SUMO3^{T99R}$ mutants, the 114.0429 mass tag representing GG on lysine residues can be used (*see* also Fig. 1 and **Note 11**). The Sprot/Uniprot nonredundant database (human sequences—it is recommended to use the most recent one—can be downloaded from http://www.uniprot.org/downloads) is used for Mascot (Matrixscience, version 2.5) and MaxQuant based searches. For SILAC based data, the quant options for medium (R6K4) and heavy (R10K8) Lys/Arg are used.
3. MS/MS spectra indicating SUMOylated proteins are manually inspected using the viewer module in Mascot, verifying the presence of diagnostic ions for SUMO tags (Fig. 3) and investigating localization probabilities using Mascot MD-score (calculated in % per site) [23]. Alternatively, the PTM Score algorithm can be used in MaxQuant/Andromeda [24].

4 Notes (Tips and Tricks)

1. *Choice of cell lines for SUMOylation studies.* Cell lines that can easily be transfected have been shown to be advantageous for SUMOylation studies. In addition, U2OS cells do have large nuclei relative to their total cell volume that is suitable as SUMO-related biology involves processes predominantly localized in the nucleus.
2. *Design of quantitation experiments. SILAC versus LFQ.* Mass spectrometry based quantitative proteomics studies have mostly relied on heavy isotope labeling techniques such as SILAC, which in the context of PTM studies have the advantage of generating multiple MS/MS spectra for one peptide/PTM, leading to increased confidence of identification. Alternatively, label-free quantitation (LFQ) methods have recently established solid workflows thanks to advancements in LC-ESI technologies and software such as LC-Progenesis [25, 26] and MaxQuant Andromeda [24].

3. *Anti-HA agarose beads for pull-down experiments.* We have used Strept-tactin (IBA) resin in the context of our studies [8, 9]. Alternatively, anti-HA coupled to agarose beads (Sigma) can also be used using similar experimental conditions.
4. *GeLC-MS versus in-solution based sample preparation.* Front-end fractionation based on liquid chromatography (LC) or sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), also referred as to multidimensional protein identification technology (MudPIT) can simplify biological sample complexity prior to LC-MS/MS analysis.
5. *Tips and tricks for chloroform–methanol precipitation of proteins.* Desalting of protein material can be achieved using different precipitation protocols based on acetone, trichloroacetic acid, or chloroform–methanol. The latter seems to be most effective in protein recovery in part because the protein pellets can be dissolved more easily [22]. For this protocol, to a 200 µl of protein solution, 600 µl methanol and 150 µl chloroform are added followed by vigorous vortexing. 450 µl H_2O is then added and samples vortexed again followed by centrifugation at 20,000 × *g* for 1 min at room temperature. The upper phase layer is carefully removed using a pipet tip without disrupting the interphase representing precipitated proteins. 450 µl methanol is then added, samples are vortexed and centrifuged at 20,000 × *g* for 2 min at room temperature. Supernatant liquid is carefully removed and the protein pellets are allowed to air-dry. Samples are kept at −20 °C until further processing.
6. *Reduction and alkylation prior to precipitation improve trypsin digestion efficiency.* Our observation is that the efficiency of trypsin digestion as judged by the number of missed cleavages was increased when the reduction and alkylation steps are performed prior to protein precipitation. This removes excess alkylation agent that can interfere with trypsin digestion [27].
7. *How to deal with SDS-containing samples.* SDS-containing samples can be subjected to two rounds of methanol–chloroform precipitation to make them compatible with in-solution digestion and LC-MS/MS analysis. To this end, pellets from the first precipitation step are resuspended in 200 µl H_2O, and the procedure is repeated as described in **Note 5**.
8. *Optimizing trypsin amounts for sample digestion.* The recommended enzyme to substrate ratio is 1:50–1:100 (weight–weight). For in-gel digestion procedures, we found that reducing trypsin concentration from 20 ng/µl to 2 ng/µl works as efficiently and reduced trypsin generated mass peaks in LC-MS/MS analyses.
9. *Tips and tricks for desalting.* Desalting and enrichment of protein can be achieved using solid-phase extraction (SPE) cartridges from a number of vendors (e.g., Waters and Thermo). In

essence, C18 based SPEs have been mostly used for the desalting of peptide material (after digestion steps). The dimension of the SPE column should be adapted to the sample amount. For instance, Waters C18 Sep-Pak cartridges WAT020515 are suitable for protein amounts up to 1 mg, and the "Light" version for up to 100 mg. For samples with less than 5 μg, a zip-tip (Millipore) procedure might be more adequate to minimize sample loss. Most protocols include an (i) equilibration step by flushing the column with >3 column volumes of Buffer B (65% acetonitrile, 35 % H_2O, 0.1% trifluoroacetic acid (recommended) or 0.1% formic acid), followed by flushing with >3 column volumes of buffer A (98% H_2O, 2% acetonitrile, 0.1% trifluoroacetic acid (recommended), or 0.1% formic acid). Samples are then loaded onto the column followed by flushing with 3–5 column volumes of Buffer A (98% H_2O, 2% acetonitrile, 0.1% formic acid), followed by elution with ~1 column volume of buffer B (65% acetonitrile, 35% H_2O, 0.1% trifluoroacetic acid (recommended), or 0.1% formic acid). Recommended buffers for this procedure: Buffer A: 98% H_2O, 2% acetonitrile, 0.1% trifluoroacetic acid (recommended), or 0.1% formic acid. Buffer B: 65% acetonitrile, 35% H_2O, 0.1% trifluoroacetic acid (recommended), or 0.1% formic acid.

10. *Adapting search parameters for the analysis of proteomics data.* Analysis of proteomics data sets depends on the type of mass spectrometer that was used. In the case of high-resolution instruments such as the orbitrap ELITE (HCD mode) or Q Exactive (classic, plus or HF), mass tolerances for precursor ions and fragment MS/MS ions can be set as <10 ppm. Alternatively, for example for data acquired on orbitrap Velos/Elite in CID mode, the MS/MS fragment tolerance should be set to 0.5 Da.
11. *Mass tags for SUMOylation analysis by mass spectrometry.* Despite the improvements of PTM localization scores available through a number of search algorithms, the mapping of SUMOylation sites has been most reliable when MS/MS spectra are examined on an individual basis. For instance, using $SUMO2^{Q87R}$ mutants that can generate the QQTGG tag after trypsin digestion, the fragment ions for the tag can provide further confidence for the presence of SUMOylation within this peptide sequence (an example is shown in Fig. 3).

5 Results and Discussion

For the study of protein SUMOylation, a number of different experimental workflows have been developed and applied (Fig. 1). In general, the nature of the SUMO posttranslational protein modification is not readily compatible with a classical proteomics workflow consisting of protein isolation, trypsin digestion and analysis by liquid

chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). To overcome this, site-directed mutagenesis has been used to introduce trypsin/Arg-C cleavage sites into SUMO-1 (SUMO-1^{T105R}), SUMO-2 (SUMO-2^{F86R}, SUMO-2^{Q87R}, SUMO-2^{T90R}), and SUMO-3 (SUMO-3^{F95R}, SUMO-3^{Q96R}, SUMO-3^{T99R}) (Fig. 1a) [9, 28]. Tagged versions of SUMO variants can be ectopically expressed in cells, and endogenous SUMOylation targets isolated through immunoprecipitation followed either by SDS-PAGE based separation or direct in-solution digestion of eluate material prior to LC-MS/MS analysis. Alternatively, a trypsin/elastase combined digestion approach has also been demonstrated to be compatible with MS based SUMO PTM mapping, but has so far been used on isolated protein substrates in proof-of-principle experiments (Fig. 1b) [18]. Trypsin (or Arg-C) based proteolytic cleavage of proteins modified by mutated SUMO variants result in short amino acid tags that can serve as "SUMOylation signatures" present in branched peptides (Fig. 2). The most commonly used example of this is SUMO-2^{Q87R} that yields a QQTGG-tag with a mass addition of 471.2078 and also characteristic fragment ions (Fig. 3) [9]. An example is shown for the characterization of the SUMO-2ylation site on the TRIM28 protein on Lys779 (Fig. 3). The MS/MS spectrum for the tryptic peptide 775–790 shows some fragment ions typical of the orthogonal QQTGG tag next to the b and y ion fragments generated from the substrate peptide backbone (Fig. 3 inserts). Further confirmation was provided by the absence of these diagnostic ions in MS/MS spectra of the corresponding unmodified peptide (data not shown). It has now become more common to specifically examine MS/MS spectra for these signature ions to ascertain the presence and localization of a SUMOylation site. Methodological improvements for accurate determination of SUMOylation events at the endogenous level will continue to boost the number of identifiable proteins labeled by SUMO [29], providing the framework for studying the underlying biology of this posttranslational modification.

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Conflicts of Interest

B.M.K. is associated with Cancer Research Technologies and Forma Therapeutics.

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Chapter 11

Live Cell Imaging and Profiling of Cysteine Cathepsin Activity Using a Quenched Activity-Based Probe

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Abstract

Since protease activity is highly regulated by structural and environmental influences, the abundance of a protease often does not directly correlate with its activity. Because in most of the cases it is the activity of a protease that gives rise to its biological relevance, tools to report on this activity are of great value to the research community. Activity-based probes (ABPs) are small molecule tools that allow for the monitoring and profiling of protease activities in complex biological systems. The class of fluorescent quenched ABPs (qABPs), being intrinsically "dark" and only emitting fluorescence after reaction with the target protease, are ideally suited for imaging techniques such as small animal noninvasive fluorescence imaging and live cell fluorescence microscopy. An additional powerful characteristic of qABPs is their covalent and irreversible modification of the labeled protease, enabling in-depth target characterization. Here we describe the synthesis of a pan-cysteine cathepsin qABP **BMV109** and the application of this probe to live cell fluorescence imaging and fluorescent SDS-PAGE cysteine cathepsin activity profiling.

Key words Activity-based probe, Protease, Cysteine cathepsin, Live cell imaging, Fluorescent SDS-PAGE

1 Introduction

Cysteine cathepsins are a family of proteases that govern many cellular processes, including antigen presentation, apoptosis, and tissue homeostasis [1]. Dysregulation of their proteolytic activity is a key step in the pathogenesis of cancer, atherosclerosis, and pancreatitis, among other inflammatory diseases [2–9]. Cysteine cathepsins are most abundantly expressed in the lysosome, where the acidic environment triggers their activation. In some circumstances, these proteases can be found in the nucleus, cytoplasm, or bound to the plasma membrane, and they can also be secreted [10–15]. In cancer, where hypoxia creates an acidic extracellular environment, secreted cathepsins are likely active and can mediate the migration and invasion of tumor cells through degradation of extracellular matrix components [16].

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The activity of cysteine cathepsins is regulated through numerous mechanisms. Zymogen activation occurs at low pH, where conformational changes make removal of the propeptide more energetically favorable [17]. Cleavage by upstream proteases such as legumain has also been implicated in their activation [18]. Several cysteine cathepsins are largely unstable and inactive at neutral pH (cathepsin S is still proteolytically active at neutral pH); however, binding to sulfated glycosaminoglycans (GAGs) such as heparin can have a stabilizing and activating effect [19, 20]. In other settings, GAGs can have inhibitory effects on proteolytic activity [21, 22]. Endogenous inhibitors, such as cystatins and some serpins, can also bind to cysteine cathepsins, competing with substrates for access to the active site [23]. Furthermore, cathepsin activity can be chemically modulated through introduction of exogenous inhibitors. Blocking the function of cathepsins with small-molecule inhibitors has been used as a therapeutic strategy in preclinical models of cancer and inflammation [3, 24].

Because cathepsins are highly regulated at the posttranslational level, total protein expression is rarely indicative of proteolytic activity. To directly monitor *active* enzymes, the field of activity-based proteomics has emerged [25–27]. Activity-based probes (ABPs) are small molecules containing an electrophilic moiety (warhead) that covalently binds to the active-site cysteine in an activity-dependent manner. Quenched ABPs (qABPs) for proteases contain a fluorophore–quencher pair [28, 29]. When an active protease performs a nucleophilic attack on the electrophile, the protease is covalently and irreversibly labeled and the quenching group is released, resulting in the emission of fluorescence (Fig. 1). The fact that qABPs are

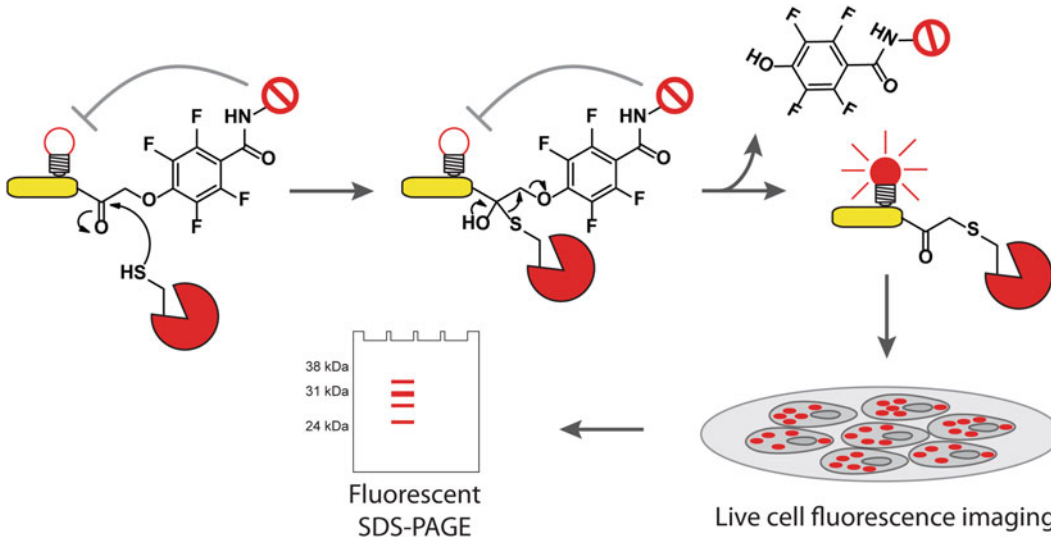


Fig. 1 Schematic presentation of the mechanism of action of quenched activity-based probes and their application in live cell fluorescence imaging and fluorescent SDS-PAGE

intrinsically "dark" (nonfluorescent) means that they are perfectly suited for live cell fluorescence confocal imaging. The use of qABPs enables fluorescence microscopy studies without the need for long-term probe washout periods or extensive washing after permeabilization of fixed cells, which is often required when using non-quenched ABPs. Because probe-binding is covalent and irreversible, labeled target proteases can be monitored by fluorescent SDS-PAGE. The proteins of probe-labeled samples are resolved by standard SDS-PAGE methods, after which the gel is scanned for fluorescence using a flat-bed laser scanner. This way fluorescence detected by microscopy can be linked to probe binding and unquenching by specific protease species.

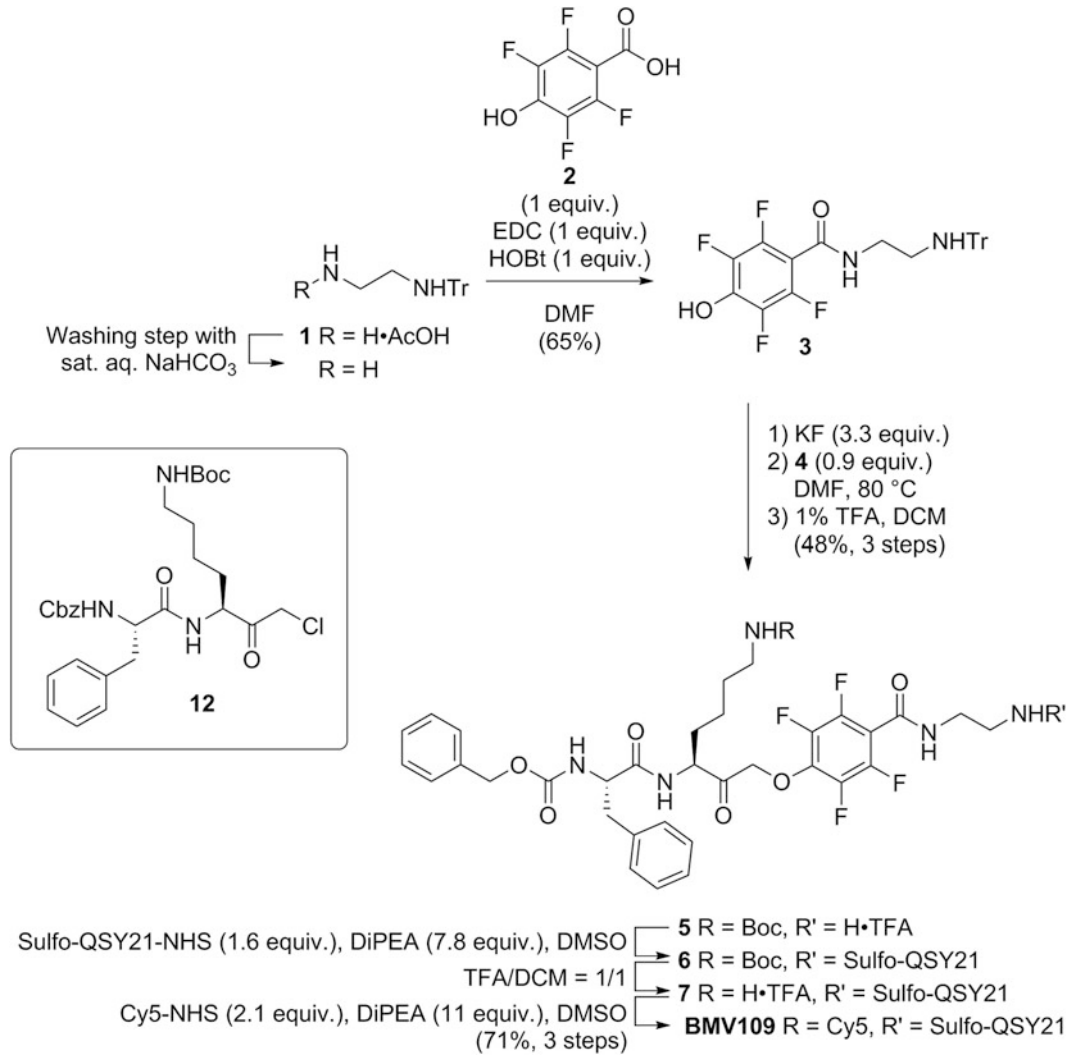
An optimized broad-spectrum qABP for cathepsins, **BMV109**, has recently been shown to have enhanced efficacy over previously described cathepsin probes [30]. **BMV109** has broad reactivity within the cysteine cathepsin family, allowing for simultaneous monitoring of the activities of cathepsins X, B, S, and L within the same experiment. This qABP also has improved solubility and yields brighter signal than other probes, most likely due to increased cathepsin reactivity of the phenoxymethyl ketone (PMK) warhead and enhanced cellular uptake. **BMV109** also demonstrated remarkable in vivo properties in a mouse model of breast cancer, yielding a tumor-specific signal that was 25 times brighter than the first-generation qABPs.

In this chapter, the synthesis of **BMV109** will be described (Subheadings 2.1 and 3.1) along with protocols for in vitro applications of the probe. The use of **BMV109** for live-cell fluorescence microscopy will be discussed (Subheadings 2.2 and 3.2), along with a protocol for fluorescent SDS-PAGE analysis of probe-labeled proteins (Subheadings 2.3 and 3.3). Finally, a method for validating the identity of probe-labeled cysteine cathepsin species by immunoprecipitation will be described (Subheadings 2.4 and 3.4). This experimental set up provides information about the cellular localization of the labeled pool of active cysteine cathepsins, which can directly be analyzed by fluorescent SDS-PAGE analysis and subsequently validated by immunoprecipitation (*see* **Note 1**).

2 Materials

2.1 Synthesis of BMV109

1. All solvents (dichloromethane [DCM], dimethylformamide [DMF], ethyl acetate, hexane, acetonitrile [CH_3CN], dimethylsulfoxide [DMSO], trifluoroacetic acid [TFA], toluene) were purchased from Fisher Scientific (HPLC grade).
2. Cbz-Phe-Lys(Boc)-CMK (chloromethyl ketone **4**, Scheme 1) was synthesized as previously reported (28) (*see* **Note 2**).
3. Sulfo-QSY21 *N*-hydroxysuccinimide (NHS) was synthesized as previously reported [31].



Scheme 1 Synthesis of the quenched activity-based probe **BMV109**

4. Sulfo-Cy5-NHS was synthesized in house, but can be purchased from a variety of vendors.
5. All other reagents (2,3,5,6-tetrafluoro-4-hydroxybenzoic acid, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride [EDC], potassium fluoride, triisopropylsilane, were purchased from Sigma Aldrich, except mono-trityl ethylenediamine acetic acid salt (Novabiochem) and HOBt monohydrate (Advanced Chemtech).
6. Saturated aqueous (sat. aq.) $NaHCO_3$ solution.
7. Anhydrous Na_2SO_4.
8. Reactions were analyzed by LC-MS using an API 150EX single-quadrupole mass spectrometer (Applied Biosystems).

9. Reverse-phase HPLC was conducted with an AKTA explorer 100 (Amersham Pharmacia Biotech) using C18 columns.
10. NMR spectra were recorded on a Varian 400 MHz (400/100), Varian 500 MHz (500/125) or a Varian Inova 600 MHz (600/150 MHz) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz.

2.2 Live Cell Imaging of Cysteine Cathepsin Activity and Lysosomal Staining

1. Nunc™ Lab-Tek™ II Chambered Coverglass, 4 Chamber (Thermo Scientific).
2. (Adherent) cell type of interest (here mouse bone marrow-derived dendritic cells are used (*see* **Note 3**).
3. Appropriate culture media (phenol red-free if available).
4. Humidified 37 °C incubator (5% CO2 in air).
5. Cysteine cathepsin inhibitor (here a 20 mM JPM-OEt solution in DMSO is used. *See* **Note 4**).
6. Cysteine cathepsin qABP: 5 mM **BMV109** solution in DMSO.
7. LysoTracker Green DND-26 (1 mM in DMSO, Invitrogen, L7526), 1 in 10 dilution (100 μM) in DMSO.
8. Inverted (confocal) fluorescence microscope (here an epi-fluorescence Leica DMI6000 microscope with a 63× oil 1.4 NA objective, a metal halide EL6000 lamp for excitation, a DFC365FX CCD camera [Leica] and GFP and Y5 filter sets [for lysotracker green and Cy5, respectively; all from Leica] was used).

2.3 Fluorescent SDS-PAGE Analysis

1. Refrigerated microcentrifuge.
2. Lysis buffer: 50 mM Citrate [pH 5.5], 5 mM DDT, 0.5% CHAPS, and 0.1% Triton X.
3. Ice bucket.
4. 4× sample buffer: 0.8 g SDS, 1 mL 2 M Tris pH 6.8, 4 mL glycerol, 0.4 mL 2-mercaptoethanol, 8 mg bromophenol blue, 4.6 mL water.
5. Eppendorf tube heating block at 95 °C.
6. SDS-PAGE gel 0.75 mm or 1.5 mm. 15% polyacrylamide running gel and 4% stacking gel.
7. Amersham ECL Plex Fluorescent Rainbow Markers (GE Healthcare).
8. Fluorescent flat-bed scanner (here the Typhoon Trio+, GE Healthcare was used).
9. Coomassie staining solution: for 1 L, add 100 mL of glacial acetic acid to 500 mL of distilled water. Add 400 mL of methanol and mix. Add 1 g of Coomassie R250 dye and mix. Filter to remove particulates.

10. Microwave.
11. Orbital shaker.
12. Destaining solution: for 1 L, add 100 mL of glacial acetic acid to 700 mL of distilled water. Add 200 mL of methanol and mix.
13. Flat-bed scanner.

2.4 Validation of Probe-Labeled Species by Immunoprecipitation

1. **BMV109**-labeled protein lysate (leftover from 3.3 or prepared fresh).
2. IP Buffer: PBS (pH 7.4), 0.5% Nonidet P-40 (NP-40; v,v), 1 mM EDTA.
3. Anti-Cathepsin Antibodies:

   Goat anti-mouse Cathepsin B (R&D, AF965).

   Goat anti-mouse Cathepsin X (R&D, AF1033).

   Goat anti-mouse Cathepsin L (R&D, AF1515).

   Goat anti-human Cathepsin S (Abcam, 18822).
4. Protein A/G agarose beads (Santa Cruz, sc-2003).
5. Nutator or rocker.
6. 2× Sample Buffer (dilute the 4× sample buffer prepared in Subheading 2.3 two times with distilled water).
7. 0.9% Sodium Chloride.
8. Insulin syringe.
9. Materials required for SDS-PAGE analysis (*see* Subheading 2.3).

3 Methods

3.1 Synthesis of BMV109

In this section, the synthesis of the cysteine cathepsin qABP **BMV109** is described (Scheme 1). All chemical reactions should be performed in a chemistry fume hood using laboratory glassware unless stated otherwise. All water-sensitive reactions are performed in anhydrous solvents under positive pressure of argon.

1. To remove the acetic acid, take up the mono-trityl ethylenediamine acetic acid salt (**1**) (100 mg, 0.28 mmol) in DCM and washed with sat. aq. $NaHCO_3$ and dry the organic phase over Na_2SO_4 and concentrate in vacuo.
2. Dissolve the amine in DMF (5 mL) and add HOBt monohydrate (43 mg, 0.28 mmol, 1 equiv.), EDC (54 mg, 0.28 mmol, 1 equiv.) and 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid (**2**) (59 mg, 0.28 mmol, 1 equiv.). Stir the reaction mixture overnight under argon atmosphere and concentrate in vacuo.
3. Isolate 2,3,5,6-tetrafluoro-4-hydroxy-*N*-(2-(tritylamino)ethyl) benzamide (**3**) by silica gel flash column chromatography puri-

fication (20%→35% ethyl acetate in hexane). Expected isolated yield: 0.18 mmol, 65% (*see* **Note 5**).

4. Suspend potassium fluoride (6.3 mg, 108 μmol, 3.3 equiv.) in DMF by sonication for 5 min. under argon atmosphere. Add phenol **3** (19.4 mg, 39 μmol) and stir the reaction mixture for 10 min.
5. Add chloromethyl ketone **4** (20 mg, 36 μmol, 0.9 equiv.) and stir the reaction mixture in an oil bath at 80 °C for 3 h. Concentrate the reaction mixture in vacuo.
6. Take up the crude product in 1% TFA in DCM (the solution will turn yellow) and stir for 30 min. Quench the carbocations (yellow color) by the addition of triisopropylsilane until the solution turns colorless. Add toluene to the reaction mixture and concentrate in vacuo.
7. Isolate intermediate **5** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 20:80–60:40 over 20 min; 5 mL/min) and lyophilize the product containing fractions. Expected isolated yield: 15.4 mg, 17.3 μmol, 48% from CMK **4**, as a white powder (*see* **Note 6**).
8. Dissolve intermediate **5** (5.8 mg, 6.5 μmol) in DMSO (100 μl) in a 1.5 mL eppendorf tube and add Sulfo-QSY21-NHS (9.75 mg, 10.39 μmol, 1.6 equiv.) and DiPEA (8.4 μl, 50.5 μmol, 7.8 equiv.). Vortex to dissolve all components and react overnight in the dark.
9. Isolate Sulfo-QSY21 amide-intermediate **6** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 25:75–55:45 over 20 min; 5 mL/min), followed by lyophilization of the product containing fractions to afford a dark blue powder.
10. Take up the Sulfo-QSY21 amide-intermediate **6** in a 50% TFA solution in DCM and react for 30 min. to remove the Boc protective group. Coevaporate the solvents with toluene (3×) and dissolve the residue (intermediate **7**) in DMSO (250 μl).
11. Add Sulfo-Cy5-NHS (10.5 mg, 13.9 μmol, 2.1 equiv.) and DiPEA (12 μl, 72 μmol, 11 equiv.) and after 4–20 h isolate **BMV109** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 25:75–45:55 over 20 min; 5 mL/min), followed by lyophilization of the product containing fractions. Expected isolated yield: 7.74 mg, 4.61 μmol, 71% over three steps as a dark blue powder (*see* **Note 7**).

3.2 Live Cell Imaging of Cysteine Cathepsin Activity and Lysosomal Staining

This protocol can be used to image the localization of cysteine cathepsin activity within an adherent cell. When **BMV109** is added to the culture media, it freely enters the cell, largely by endocytosis. When reacting with active cysteine cathepsins, the probe is unquenched and fluorescence can be detected by fluorescence (confocal) microscopy. To ensure that the fluorescent signal is cathepsin-dependent, a control experiment should be performed

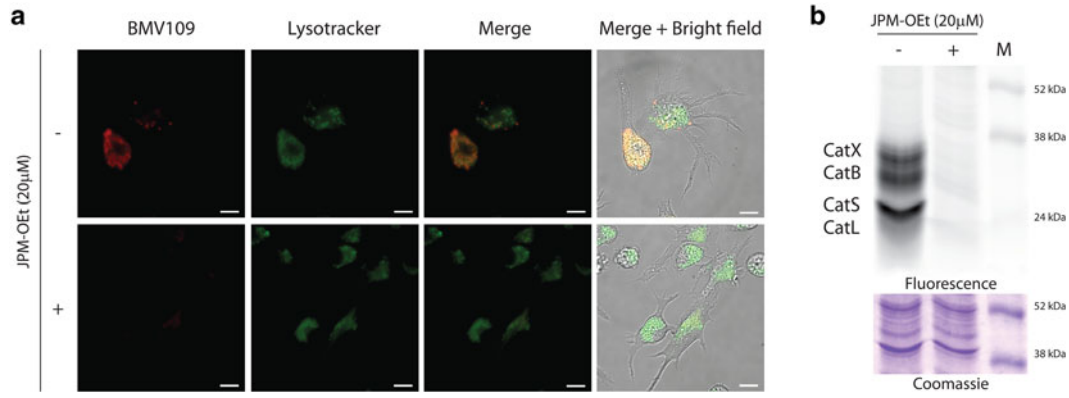


Fig. 2 Live cell fluorescence microscopy of **BMV109** labeled cells and fluorescent SDS-PAGE of the labeled protease targets. (**a**) Living mouse bone marrow-derived dendritic cells were labeled with 5 μM **BMV109** (*red*) and 100 nM Lysotracker (*green*) and imaged for probe fluorescence. In the cysteine cathepsin inhibitor (JPM-OEt) pretreated sample (lower panels) minimal **BMV109** fluorescence is observed. Scale bar represents 10 μm. (**b**) After imaging, the cells were harvested and lysed and the proteins were resolved on SDS-PAGE (15 %). The probe labeled protease bands were visualized using a fluorescent flat-bed scanner. The cysteine cathepsin inhibitor (JPM-OEt) pretreated sample (center lane) confirms **BMV109** target protease inhibition. Equal protein loading was confirmed by Coomassie blue staining (*lower panel*)

in parallel, in which a broad-spectrum cysteine cathepsin inhibitor is applied 30 min prior to **BMV109** addition. This inhibitor irreversibly inactivates the cysteine cathepsins, rendering them unable to unquench **BMV109**. As observed in Fig. 2a, inhibitor-treated cells have little fluorescent signal by microscopy compared to non-inhibited cells, which yield increasing fluorescence over time. In order to verify that the localization of the cathepsin signal is lysosomal, LysoTracker Green may be added. This dye is fluorescent specifically in acidic compartments of the cell. Co-localization of green and red signal indicates a lysosomal distribution of cathepsins, as shown in Fig. 2a. If signal in other organelles is detected (i.e., nucleus, plasma membrane), other cellular markers may be required. All steps of the following protocol need to be performed using sterile materials in a cell culture flow cabinet.

1. Harvest and count the cells of interest and resuspend them in culture media at a concentration of 150,000 cells/mL (*see* **Note 8**).
2. Seed the cells by gently pipetting 1 mL of the cell suspension in each well of the chambered coverglass. Culture the cells for 1–12 h to allow the cells to adhere (*see* **Note 9**).
3. For the inhibitor pretreatment control well, pipet 0.5 μl of the 20 mM JPM-OEt solution in DMSO in a sterile 1.5 mL eppendorf tube. Transfer 499 μl of conditioned culture media from the appropriate well to the eppendorf tube containing the inhibitor (final JPM-OEt concentration 20 μM).

4. Carefully aspirate off the remaining culture media from the well. Quickly to prevent the cells from running dry, pipet the inhibitor-containing conditioned culture media from the 1.5 mL eppendorf tube back onto the cells gently. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 30 min.
5. For the rest of the wells, repeat **steps 3** and **4** using 0.5 μl DMSO instead of 0.5 μl of the 20 mM JPM-OEt solution in DMSO.
6. After the 30 min. inhibitor (or DMSO) pre-incubation, pipet 0.5 μl of the 5 mM **BMV109** solution in DMSO in a sterile 1.5 mL eppendorp tube (*see* **Note 10**). Transfer the culture media from the well to the eppendorf tube containing the probe (final **BMV109** concentration 5 μM).
7. Gently pipet the probe-containing conditioned culture media back onto the cells. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 1 h (*see* **Note 10**).
8. After 1 h probe incubation time, pipet 0.5 μl of the 100 μM Lysotracker solution in DMSO in a sterile 1.5 mL eppendorf tube. Transfer the culture media from the well to the eppendorf tube containing the Lysotracker (final Lysotracker concentration 100 nM) (*see* **Note 11**).
9. Gently pipet the Lysotracker-containing conditioned culture media back onto the cells. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 1 h.
10. After 1 h of Lysotracker incubation (and 2 h of total probe incubation time), remove the Lysotracker containing media from the cells and replace it with pre-warmed fresh (phenol red-free) culture media.
11. Image the cells in each well for Lysotracker Green and Cy5 fluorescence on an inverted fluorescence (confocal) microscope (Fig. 2a).

3.3 Fluorescent SDS-PAGE Analysis

In this section, a biochemical analysis of the cells that were imaged in the 4-chambered coverglass wells (Subheading 3.2) is described. The cells are lysed and analyzed by fluorescent SDS-PAGE. The detected fluorescent bands correlate with the fluorescence that is observed by fluorescence microscopy, providing a biochemical readout of cysteine cathepsin activity. As observed in Fig. 2b, the JPM-OEt-treated inhibitor control yields dramatically decreased labeling intensity compared to the non-inhibited sample, which has several bands corresponding to the sizes of cathepsin X, B, S, and L. This labeling pattern may vary depending on the cell type and conditions used. To ensure that the lanes are loaded with equal protein amounts, a Coomassie stain may be performed.

This protocol can be applied to cells (or lysates) labeled with **BMV109** in any experimental setting. Treatments aimed to modulate cathepsin activity (i.e., cytokines such as IL-4, serum

deprivation, apoptotic agents, etc.) may be added to the cells prior to the addition of **BMV109**. A densitometry analysis can be performed on the fluorescent bands, allowing for a quantitative readout of relative cathepsin activities.

1. Carefully aspirate off culture media from the well and wash the cells by gently pipetting 1 mL of PBS onto the cells. Aspirate off the PBS and pipet 500 μl PBS into the well.
2. Remove the cells from the bottom of the well using the blunt end of a 200 μl pipet tip by placing the tip perpendicular to the bottom of the well and gently scraping in a circular fashion. Transfer the PBS cell suspension to a sterile 1.5 mL eppendorf tube and place on ice. Repeat **steps 1** and **2** for each well.
3. Centrifuge the cell suspension for 30 s at 9,500 × *g* and remove the supernatant. Resuspend the cell pellet in 9 μl lysis buffer and incubate the cells on ice for 15 min. Centrifuge at 4 °C for 20 min. at 21,000 × *g*.
4. Transfer the supernatants to new 1.5 mL eppendorf tubes and add 3 μl 4× sample buffer to each sample (*see* **Note 12**). Heat the samples to denature and reduce the proteins for 5 min. at 95 °C. Centrifuge the samples for 10 s. at full speed (*see* **Note 13**).
5. Load the samples and the fluorescent molecular weight marker (5 μl) on the SDS-PAGE gel and run the first 15 min. on 80 V to run the proteins through the stacking gel. Increase the voltage to 130 V and stop the gel when the dye front has run off (*see* **Note 14**).
6. Remove the gel from the glass and place it in a tray with water for transfer to the Typhoon Imager (*see* **Note 15**). Place the wet gel slab on the scanner surface (*see* **Note 16**) and select the area to be scanned with the software. Choose the excitation and emission parameters for the Cy5 dye and scan the gel. If the fluorescent signal is saturated, lower the PMT value and repeat the scan (*see* **Note 17**).
7. To control for equal protein loading per lane, Coomassie staining can be performed (*see* **Note 18**). After scanning, transfer the gel to a microwave proof tray. Add enough Coomassie staining solution to submerge the gel completely. Place the tray in the microwave and heat for 10–15 s (prevent boiling of the solution). Place the tray on the orbital shaker and shake for 5 min.
8. Dispose the staining solution and wash the gel with water. Add enough destaining solution to submerge the gel completely. Place the tray in the microwave and heat for 10–15 s (prevent boiling of the solution). Roll up a tissue (Kimwipes work well—the tissue will soak up the Coomassie), wet it with water and place it along the edge of the tray and shake until the gel has destained to satisfaction (*see* **Note 19**). Scan the gel on a flat-bed scanner.

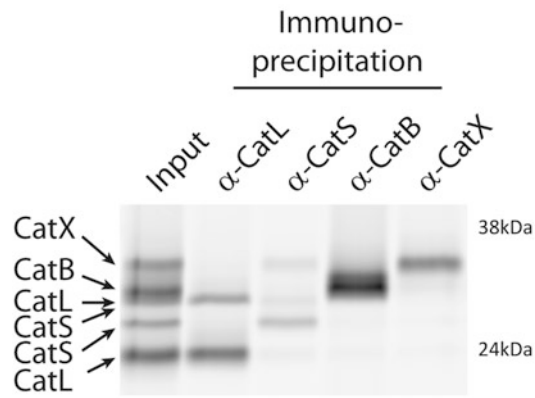


Fig. 3 Cysteine cathepsin immunoprecipitation. After labeling with **BMV109**, target cysteine cathepsin annotation can be validated by immunoprecipitation and analyzed by fluorescent SDS-PAGE

3.4 Validation of Probe-Labeled Species by Immunoprecipitation

Different cell types may express different forms of active cathepsins, and the identities of the labeled proteins may be difficult to determine based on size and banding pattern alone. To definitively assign each signal to the correct cathepsin family member, an immunoprecipitation experiment may be performed. This involves incubating probe-labeled lysate with cathepsin-specific antibodies in the presence of Protein A/G agarose beads. The precipitated proteins are then analyzed by fluorescent SDS-PAGE alongside an input sample. In the example shown (Fig. 3), lysates from RAW cell macrophages labeled with **BMV109** were immunoprecipitated with cathepsin-specific antibodies, and the labeled proteins were assigned to multiple species of cathepsins X, B, S, and L.

1. Prepare **BMV109**-labeled lysate according to Subheading 3.3. After boiling the lysate in sample buffer, set aside 30 μg total protein for the input sample. Then aliquot 100 μg protein into four microcentrifuge tubes for immunoprecipitation of the cathepsin X, B, S, and L.
2. To the four tubes, add 500 μl IP buffer and the indicated antibody: 10 μl for X, B, and L (2 μg) or 20 μl for S (4 μg) (*see* **Note 20**). Incubate the tubes on ice for 10 min.
3. Meanwhile, prepare the Protein A/G agarose beads. Aliquot 160 μl bead slurry (40 μl for each immunoprecipitation) into a microcentrifuge tube. Add 1.3 mL IP buffer to wash the beads and invert the tube once. Centrifuge for 30 s at high speed in a table-top centrifuge and then remove the buffer, taking care not to disturb the bead pellet. Add 160 μl of IP buffer, resuspend the slurry, and then aliquot 50 μl to each of the 4 immunoprecipitation tubes prepared in **step 2**. Nutate or rock the samples overnight at 4 °C.

4. The next morning, centrifuge for 30 s at high speed in a tabletop centrifuge and aspirate supernatant. Wash the beads four times with 1 mL IP buffer, and 1 time with 0.9% sodium chloride, centrifuging and removing the supernatant between each wash. After the final wash, remove all traces of liquid with an insulin syringe, and resuspend the beads with 20 μl 2× sample buffer. Boil for 10 min. and then load the immunoprecipitations along with the input sample on a 15% gel as described in Subheading 3.3. To analyze, scan the gel for fluorescence, also according to the method in Subheading 3.3.

4 Notes

1. For in vivo applications of **BMV109**, refer to [30].
2. **CAUTION!** The method involves the preparation and use of diazomethane. This is a **HIGHLY EXPLOSIVE** reagent! Special glassware and safety precautions are needed. If not experienced in performing these reactions, seek advice from a chemistry department. Diazomethane is prepared as described in the Aldrich technical Bulletin (AL-180), which is available online.
3. For the generation of bone marrow-derived dendritic cells *see* for example [32, 33].
4. E-64d or leupeptin are commonly used commercially available cysteine cathepsin inhibitors that could be used instead of JPM-OEt.
5. ^{1}H NMR (400 MHz, DMSO) δ = 8.77 (t, *J* = 6.0, 1H), 7.39 (d, *J* = 7.8, 6H), 7.27 (t, *J* = 7.7, 6H), 7.17 (t, *J* = 7.2, 3H), 3.40–3.35 (m, 2H), 2.86–2.77 (m, 1H), 2.14–2.04 (m, 2H).
6. ^{1}H NMR (400 MHz, CD_3OD) δ = 7.36–7.12 (m, 10H), 5.05 (s, 2H), 4.86–4.81 (m, 2H), 4.42–4.37 (m, 2H), 3.64 (t, *J* = 6.5, 2H), 3.14 (t, *J* = 6.5, 2H), 3.08 (dd, *J* = 13.9, 7.2, 1H), 2.99 (t, *J* = 6.5, 2H), 2.91 (dd, *J* = 13.9, 8.4, 1H), 1.90–1.78 (m, 1H), 1.62–1.48 (m, 1H), 1.41 (s, 9H), 1.46–1.20 (m, 4H).
   HRMS (ESI): calc. for $C_{38}H_{46}F_4N_5O_8^+$ 776.3277, found 776.3286, $C_{38}H_{45}F_4N_5O_8Na^+$ 798.3096, found 798.3104.
7. 7. ^{1}H NMR (600 MHz, CD_3CN) δ 8.12–8.08 (m, 1H), 8.01–7.93 (m, 2H), 7.89–7.85 (m, 2H), 7.75 (dd, *J* = 12.0, 1.5 Hz, 2H), 7.72 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.69 (dd, *J* = 8.3, 1.2 Hz, 1H), 7.66 (s, 2H), 7.62–7.57 (m, 2H), 7.51 (dd, *J* = 8.4, 5.1 Hz, 2H), 7.46 (d, *J* = 9.4 Hz, 2H), 7.41–7.35 (m, 3H), 7.24 (s, 1H), 7.22 (s, 1H), 7.21–7.14 (m, 6H), 7.13–7.09 (m, 6H), 7.05 (dd, *J* = 8.8, 4.6 Hz, 1H), 6.39 (t, *J* = 12.8 Hz, 1H), 6.11 (t, *J* = 12.6 Hz, 1H), 4.87 (q, *J* = 12.7 Hz, 2H), 4.83 (dd, *J* = 39.7, 14.1 Hz, 2H), 4.23–4.12 (m, 4H), 3.93 (q, *J* = 7.2 Hz, 2H), 3.86 (t, *J* = 7.4 Hz, 2H), 3.34 (dd, *J* = 6.7, 4.1 Hz, 2H), 3.28–3.15 (m, 9H), 3.04–2.92 (m, 3H), 2.80–2.74 (m, 1H), 2.45 (t, *J* = 11.9 Hz, 2H), 2.15–2.09 (m, 1H), 2.09–2.03 (m, 2H), 1.74–1.58 (m, 7H),

1.57 (s, 6H), 1.55 (s, 6H), 1.49 (dd, J=15.1, 7.4 Hz, 4H), 1.35–1.22 (m, 7H), 1.20 (t, J=7.3 Hz, 3H), 1.16–1.12 (m, 4H).

HRMS (ESI): calc. for $C_{107}H_{106}F_4N_{10}O_{23}S_5^{2-}$ 1067,2991, found 1067,2961, $C_{107}H_{107}F_4N_{10}O_{23}S_5^{-}$ 2135.6056, found 2135.5990, $C_{107}H_{106}F_4N_{10}O_{23}S_5Na^{-}$ 2157.5875, found 2157.5831.

8. The optimal amount of cells and seeding density needed for microscopy and subsequent fluorescent SDS-PAGE needs to be determined for every new cell type to be analyzed. The amount of active cathepsins differs per cell type.
9. Optimize this step for every new cell type to be analyzed.
10. Probe concentration and labeling time should be optimized to achieve the desired labeling saturation for every new cell type to be analyzed.
11. Lysotracker concentration and incubation time should be optimized for every new cell type to be analyzed.
12. In this example, the lysate from all the cells per well (equal cell numbers) is loaded on the SDS-PAGE gel. When using larger cell numbers the protein concentration of the lysate could be determined. Typically 30 μg of total protein is sufficient for detection of cathepsin labeling by fluorescent SDS-PAGE analysis, but this differs per cell type and should be optimized. Overloading of the gel will result in poor gel scans.
13. At this point the samples could be frozen for continuation at a later stage. Before the next step, heat the samples for 5 min at 95 °C, followed by centrifugation for 10 s. at full speed.
14. For a more detailed protocol on standard SDS-PAGE methods, *see* Ref. [34].
15. Depending on the plates being used, it may also be possible to scan the gel while still in the plates. This option ensures that the gel does not tear during handling and that it stays flat for the scan. In this case, choose "+3" for the focal plane. Otherwise, use the default "platen" option.
16. Place the gel on the Typhoon with the side of the gel (the short end) at the bottom to reduce scanning time (the Typhoon scans from left to right).
17. After saving the .GEL file it can be analyzed and saved as a .TIFF file using the open source image processing software ImageJ (http://imagej.net/).
18. Alternatively, the gel may be transferred to a membrane and blotted with cathepsin antibodies to evaluate total protein expression. Other proteins such as actin or GAPDH may be assessed as loading controls.
19. To speed up the process, the destaining solution can be replaced and **step 8** can be repeated.
20. If antibodies other than those recommended are used, the amount of antibody will need to be optimized.

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Chapter 12

Competitive ABPP of Serine Hydrolases: A Case Study on DAGL-Alpha

Marc P. Baggelaar and Mario Van der Stelt

Abstract

Competitive activity-based protein profiling is a highly efficient chemical biology technique to determine target engagement and selectivity profiles of enzyme inhibitors in complex proteomes. Fluorophosphonate-based fluorescent inhibitors are widely used as broad-spectrum probes for serine hydrolases. However, diacylglycerol lipase-α is not labeled by fluorophosphonate-based probes. To overcome this problem, we have developed a tailor-made activity-based probe that reacts with diacylglycerol lipase-α. Here we describe a case study in which we apply competitive activity-based protein profiling using a broad-spectrum and a tailor-made activity-based probe to establish selectivity and activity profiles of inhibitors targeting diacylglycerol lipase-α in the mouse brain proteome.

Key words Diacylglycerol lipase-alpha, Competitive ABPP, Serine hydrolase, Membrane protein, Selectivity profile

1 Introduction

Competitive activity-based protein profiling (ABPP) is emerging as an important tool for hit discovery and profiling small molecule inhibitors that can perturb and modulate enzyme activity [1, 2]. Competitive ABPP takes advantage of the ability of activity-based probes (ABPs) to bind directly and covalently in the active site of targeted enzymes, enabling measurement of affinity of small molecule inhibitors competing for these active sites. The technique involves pretreatment of a proteome with small molecule inhibitors followed by labeling with an ABP; comparison of labeling patterns of the untreated proteome with the inhibitor-treated proteome allows for detection of inhibitory activity. Compared to conventional substrate-based assays, competitive ABPP has several advantages. The technique does not rely on recombinant expressed or purified native proteins, instead competitive ABPP can be used in complex proteomes against native enzymes with post-translational modifications representative for the tissue of interest [3]. This also allows for

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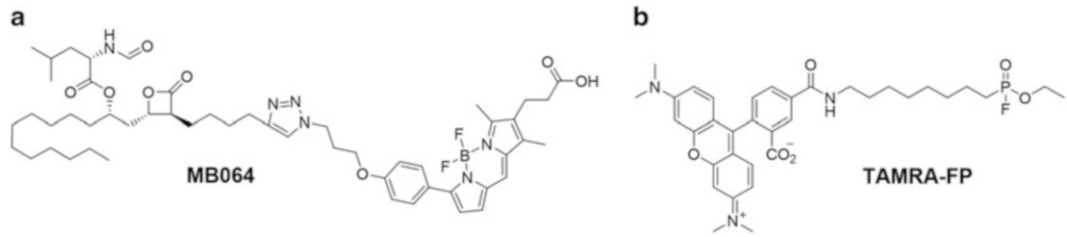

Fig. 1 Structures of both activity-based probes. (**a**) DAGL-α tailored activity-based probe (MB064). (**b**). Broad-spectrum serine hydrolase probe (TAMRA-FP)

inhibitor identification of proteins having unknown substrates and /or function. Furthermore, employing broad range ABPs does not only facilitate optimization of potency but also allows rapid selectivity profiling against multiple proteins in parallel.

The ability of competitive ABPP to rapidly determine the selectivity and activity of small molecule inhibitors in complex proteomes is especially important for studying inhibitors targeting members of the serine hydrolase family. This is a large family containing more than 200 members with various physiological functions [4, 5]. Many members of this family contain the classical *GXSXG* motif in the active site and a large number share a highly conserved α/β hydrolase fold.

Routinely, Fluorophosphonate based probes (e.g., TAMRA-FP; Fig. 1) are employed in competitive ABPP experiments to determine selectivity and activity of serine hydrolase inhibitors in complex proteomes. However, the enzyme that is subject of this case study diacylglycerol lipase-α (DAGL-α) does not react well with fluorophosphonate-based probes [6]. Therefore, we used a complementary and tailored ABP (MB064) (Fig. 1) that labels DAGL-α [7]. The combination of these two probes can be used to determine the activity of inhibitors against DAGL-α, and establish selectivity profiles of DAGL-α targeting inhibitors against multiple serine hydrolases in parallel.

2 Materials

1. For 5 mL pH 7.2 Lysis buffer: 100 μL 1 M Hepes pH 7.2 (final concentration 20 mM), 10 μL 1 M DTT (final concentration 2 mM), 5 μL 1 M $MgCl_2$ (final concentration 1 mM), 0.5 μL (Benzonase® Nuclease, ≥250 U/μL, ≥90% (SDS-PAGE)), recombinant, expressed in E. coli, buffered in aqueous glycerol solution; Sigma-Aldrich (*see* **Note 1**) and 4883.5 μl H_2O.
2. For 5 mL pH 7.2 storage buffer: 100 μL 1 M Hepes pH 7.2 (final concentration 20 mM), 10 μL 1 M DTT (final concentration 2 mM), and 4.9 mL H_2O (*see* **Note 2**).

3. For 5 mL 3× sample buffer: 1.5 mL 10% SDS, 0.75 mL 0.625 M Tris–HCl, 1.575 mL 87% glycerol, 0.3 mL β-mercaptoethanol (Sigma-Aldrich), 0.075 mL 10% (wt/vol) bromophenol blue solution in H_2O, and 0.85 mL H_2O.
4. Coomassie staining mix: 1.25 g coomassie brilliant blue, 100 mL acetic acid, 450 mL MeOH, and 450 mL H_2O.

3 Methods

3.1 Mouse Brain Proteome Preparation

1. Thaw the mouse brain on ice (*see* **Note 3**).
2. Add 1.5 mL pH 7.2 lysis buffer and mechanically homogenize the brain tissue (3 × 5 s) using a silent crusher S (Heidolph) or a dounce homogenizer.
3. Incubate the homogenized mouse brain for 15 min on ice (*see* **Note 4**).
4. Centrifuge the homogenate at 2500 g (4 °C, 3 min) and transfer the supernatant to an ultracentrifuge tube (on ice). Resuspend the pellet in 1 mL pH 7.2 lysis buffer and repeat the centrifuge step.
5. Subject the combined supernatants to ultracentrifugation (100,000 × *g*, 45 min. 4 °C, Beckman Coulter, Type Ti70 rotor) to yield the cytosolic fraction in the supernatant and the membrane proteins in the pellet.
6. *Cytosolic fraction*: Collect the supernatant, aliquot in 100 µl portions, flash freeze in liquid nitrogen, and store at −80 °C until use (*see* **Notes 5** and **6**).
7. *Membrane fraction*: Dissolve the pellet in pH 7.2 storage buffer, aliquot in 100 µL portions, flash freeze in liquid nitrogen, and store at −80 °C until use.
8. Determine the total protein concentration of both the cytosolic and the membrane fraction with Quick Start Bradford assay (Bio-Rad).

3.2 Competitive ABPP for Selectivity Studies

1. Slowly thaw the mouse brain proteome (cytosolic or membrane fraction) on ice.
2. Dilute the proteome with cold (4 °C) pH 7.2 storage buffer to a total protein concentration of 2.5 mg/mL.
3. Load 19.5 µL of diluted proteome in a 1.5 mL Eppendorf vial for each individual experiment (*see* **Note 7**).
4. Add 0.5 µL of inhibitor solution (from an 800 µM stock; final concentration 20 µM) in DMSO or DMSO (vehicle) to the proteome. Vortex the samples and incubate for 30 min at room temperature (ca 20 °C).

5. Add 0.5 μL MB064 (10 μM stock; final concentration 250 nM) or 0.5 μL TAMRA-FP (20 μM stock; final concentration 500 nM). Vortex the samples and incubate for 15 min at rt (*see* **Note 8**).
6. Quench the reaction by adding 10 μL of 3× sample buffer and vortex.
7. Allow the samples to stand for 30 min at rt (*see* **Note 9**).
8. Centrifuge the samples at 10,000 × *g* for 3 min (*see* **Note 10**).
9. Load the samples on a 10% SDS-PAGE gel and resolve the proteins by electrophoresis at 200 V for 90 min.
10. Scan the gel with a Typhoon Variable Mode Imager (Amersham Biosciences) using Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm).
11. Stain the scanned gels by gentle shaking for 20 min in coomassie brilliant blue staining mix (*see* **Note 11**).
12. Remove the coomassie staining mix and wash the gel with water and destain overnight by gently shaking the gel in water (*see* **Note 12**).
13. Scan the coomassie stained gel with a Biorad GS800 densitometer.

3.3 Data Analysis for Selectivity Profiling

1. Normalize enzyme loading using the coomassie gel scan. Determine the integrated optical intensity per lane by using ImageJ software, set the optical intensity of the vehicle treated lane at 100%.
2. Measure the optical intensity of the fluorescent protein bands using ImageJ software, set the band intensity of the vehicle treated proteins at 100% and correct for the total protein loading per lane as determined in step 1 (Tables 1 and 2). Reduction of the intensity of the protein band compared to the corresponding protein in the vehicle lane indicates off-target activity of the inhibitor (*see* **Note 13** and Fig. 2).

3.4 Competitive ABPP for Activity

1. The first steps of this assay follow a similar procedure as the first three steps of the selectivity assay.
2. Add 0.5 μL DMSO (vehicle) or 40× the final concentration of inhibitor in 0.5 μL DMSO to the proteome and vortex. Allow the inhibitor to incubate for 30 min at rt.
3. Add ABP MB064 (0.5 μL from a 10 μM stock; final concentration 250 nM) and vortex. Incubate the ABP for 15 min at rt.
4. Follow the same **steps 6–13** as for the selectivity assay.

3.5 Data Analysis for Activity Assays

1. Normalize enzyme loading using the coomassie gel scan. Determine the integrated optical intensity per lane by using ImageJ software, set the optical intensity of the vehicle treated lane at 100%.

Table 1
Relative band intensities of the proteins labeled by MB064 ($N = 3$)

Band	Vehicle (%)	THL (%)	LEI103 (%)	LEI104 (%)
1	100 ± 4	1 ± 3***	56 ± 2***	1 ± 3***
2	100 ± 24	7 ± 0***	7 ± 6***	132 ± 7
3	100 ± 8	1 ± 3***	4 ± 4***	95 ± 4
4#	100 ± 9	-5 ± 3***	54 ± 4***	4 ± 4***
5	100 ± 6	0 ± 1***	2 ± 5***	99 ± 5
6	100 ± 4	0 ± 3***	85 ± 7	109 ± 7
7	100 ± 2	40 ± 6***	116 ± 9	92 ± 10
8	100 ± 4	0 ± 5***	17 ± 8***	88 ± 6
9	100 ± 16	117 ± 8	104 ± 15	98 ± 7
10	100 ± 5	84 ± 35	28 ± 21***	103 ± 9

Means ± stdev, $N = 3$, normalized to the intensity of the protein bands from the samples treated with vehicle. Values are corrected for protein loading per lane as determined with coomassie staining. Statistical analysis: 2-way ANOVA with Bonferroni's posttest (*** = $p < 0.001$ vs. vehicle)

2. Measure the optical intensity of the fluorescent protein bands using ImageJ software, set the band intensity of the vehicle treated proteins at 100%, and correct for the total protein loading per lane as determined in step 1
3. Transfer the normalized corrected band intensity data to a Graphpad prism data table.
4. Fit an IC_{50} curve using the LOG [inhibitor] vs. normalized response—variable slope settings (*see* **Note 14**). Results are given in Fig. 3.

4 Conclusions

This case study demonstrates that two complementary ABPs can be used to rapidly prioritize DAGL-α inhibitors. A competitive ABPP assay for the assessment of inhibitor selectivity revealed that THL and LEI103 cross-reacted with multiple proteins in the mouse brain membrane proteome, whereas LEI104 had only one off-target. The activity assay showed that LEI104 was ~40-fold more active against native DAGL-α than LEI103. These combined data show that LEI104 displays superior properties compared to LEI103, and is the best candidate for further inhibitor optimization. Furthermore, the process of inhibitor optimization can be guided by competitive ABPP using the same assays and probes.

Table 2
Relative band intensities of the proteins labeled by TAMRA-FP (*N* = 3)

	Vehicle (%)	THL (%)	LEI103 (%)	LEI104 (%)
1	100 ± 2	98 ± 1	101 ± 4	102 ± 3
2	100 ± 7	93 ± 6	37 ± 5***	97 ± 5
3	100 ± 19	99 ± 15	61 ± 38**	102 ± 16
4	100 ± 19	77 ± 5	60 ± 17**	82 ± 13
5	100 ± 16	18 ± 20***	22 ± 9***	73 ± 24
6	100 ± 5	94 ± 10	-13 ± 2***	99 ± 4
7	100 ± 6	97 ± 2	68 ± 11*	95 ± 7
8	100 ± 2	96 ± 2	109 ± 5	102 ± 5
9	100 ± 8	89 ± 9	24 ± 1***	12 ± 1***
10	100 ± 12	91 ± 9	40 ± 6***	99 ± 5
11	100 ± 7	92 ± 11	44 ± 2***	99 ± 7
12	100 ± 16	76 ± 10	80 ± 6	107 ± 8
13	100 ± 10	131 ± 18	118 ± 11	111 ± 12
14	100 ± 28	83 ± 20	64 ± 37**	83 ± 37
15	100 ± 12	90 ± 5	42 ± 2***	101 ± 7
16	100 ± 9	68 ± 3*	52 ± 2***	94 ± 6
17	100 ± 8	76 ± 11	43 ± 8***	85 ± 10
18	100 ± 3	91 ± 5	94 ± 4	90 ± 1
19	100 ± 7	96 ± 4	58 ± 5***	96 ± 7
20	100 ± 3	88 ± 2	31 ± 1***	94 ± 2

Means ± stdev, $N = 3$, normalized to the intensity of the protein bands from the samples treated with vehicle. Values are corrected for protein loading per lane as determined with coomassie staining. Statistical analysis: 2-way ANOVA with Bonferroni's posttest (*** = $p < 0.001$; ** = $P < 0.01$; * = $p < 0.05$ vs. vehicle)

5 Notes

1. Benzonase is added to reduce sample viscosity.
2. No protease inhibitor cocktail is added in the lysis and storage buffer; protease inhibitor cocktails may inhibit the serine hydrolases, which prevents their labeling by activity-based probes.
3. Use precooled buffers and keep every sample on ice to minimize protein degradation.
4. Incubate at least 15 min to allow the benzonase to degrade DNA.

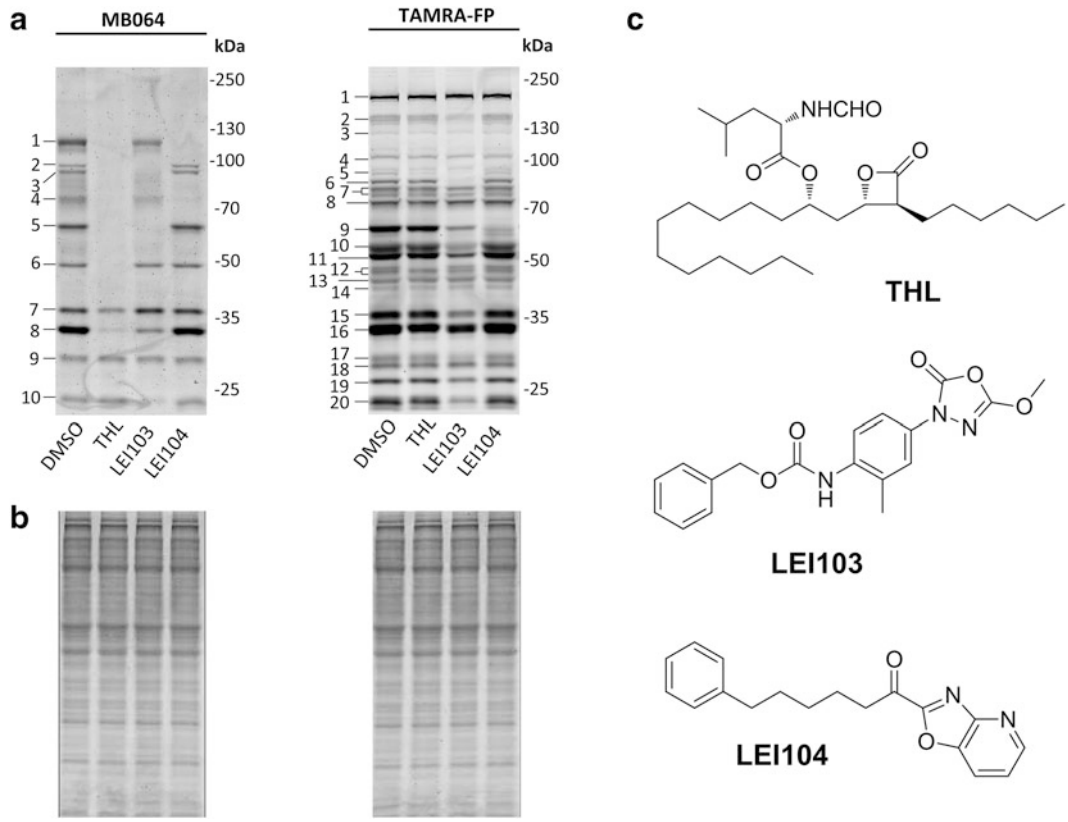

Fig. 2 Gel images of the SDS-PAGE gels for selectivity studies in the mouse brain membrane proteome. (**a**) Fluorescent gel images after preincubation with 20 μM of indicated inhibitor and labeling with MB064 or TAMRA-FP. Decreased labeling compared to the DMSO (vehicle) treated lane indicates enzyme inhibition. (**b**) Coomassie gel images of the above gels to control total protein loading per lane. (**c**) Structures of DAGL-α inhibitors THL, LEI103, and LEI104

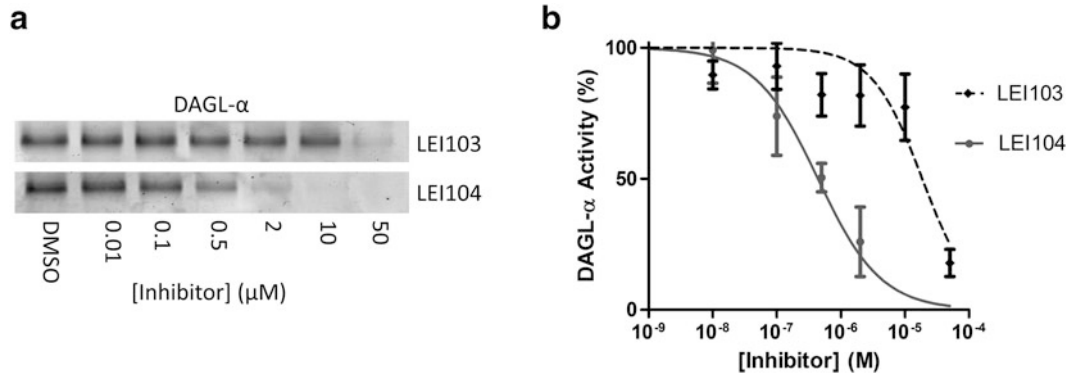

Fig. 3 (**a**) Concentration-dependent inhibition of DAGL-α in the mouse brain membrane proteome by LEI103 and LEI104 using ABP MB064. (**b**) Dose response curve for DAGL-α inhibition by LEI103 and LEI104 in the mouse brain membrane proteome as measured by competitive ABPP with ABP MB064 (±SEM, $n = 3$)

5. Both the MB064 and TAMRA-FP probe target a large number of enzymes. Separation of the membrane and the cytosolic fraction minimizes overlap of protein bands and thereby simplifies interpretation of enzyme activity profiles.
6. At this point save a few microliters for determination of the total protein concentration in step 8, while the samples are already stored at −80 °C.
7. Low volumes can be used because the samples were lysed in the presence of benzonase, decreasing viscosity and thereby increasing pipetting accuracy.
8. The probe incubation time is kept short and at room temperature to keep the assay in kinetically controlled conditions, because some compounds (e.g., LEI-104) are reversible inhibitors.
9. As opposed to many ABPP protocols, the sample is not boiled prior to loading on SDS-PAGE gel. Hydrophobic membrane proteins can aggregate upon boiling, reducing resolving efficiency with SDS-PAGE electrophoresis.
10. The samples are subjected to centrifugation prior to loading on gel to pellet any undissolved proteins or debris.
11. Add enough coomassie staining mix to cover the gel by at least 1 cm.
12. Add enough water for effective destaining; a filter paper can be added to increase destaining efficiency.
13. Perform the experiments in triplicate and analyze with 2-way ANOVA with Bonferroni's posttest using GraphPad prism software to determine which band intensity is significantly reduced compared to vehicle.
14. The obtained IC_{50} is an apparent IC_{50} which is very useful to compare inhibitors tested in the same assay using the same settings; however this value is dependent on various assay parameters, e.g., protein concentration, affinity of the probe for the enzyme, incubation times, and temperature.

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Part III

Chemical Strategies in Activity-Based Proteomics

Chapter 13

A Protocol for Protein Profiling Using Chemoselective Cleavable Linker Probes in Semi-permeabilized Cells

Jasper H.L. Claessen and Martin D. Witte

Abstract

Activity-based protein profiling using activity-based probes (ABPs) resulted in the identification of various enzymes that are involved in the onset and progress of diseases. Detection of such proteins, often expressed at low abundance, is greatly enhanced by incorporating chemically cleavable linkers in the ABP of choice. Initial affinity purification, followed by tailored chemical cleavage of the linker, allows for specific release of the captured enzymes and their interaction partners. When the ABPs are delivered directly to semi-permeabilized cells, in contrast to a crude cell lysate, the sensitivity and efficacy of cell impermeable probes can be enhanced even further.

Key words Activity-based probes, Cleavable linkers, Chemical cleavage, Delivery, Semi-permeable cells, Perfringolysin O

1 Introduction

The identification of factors that regulate biological processes, such as disease onset or cellular development, increases the understanding of these processes and will lead to novel methods to modulate them. Protein profiling has become an important means to identify such factors. This technique exploits activity-based probes (ABPs) and affinity-based probes (AfBPs) to visualize and compare the activity of a specific enzyme (or class of enzymes) in cell lysates, living cells, or whole animals. These probes are typically obtained by equipping known (reversible) inhibitors with a reactive group, to covalently bind the enzyme of interest, and a label, such as a fluorophore or an affinity-handle, which can be used for immediate on gel detection or to isolate the labeled proteins, respectively [1, 2]. Depending on the conditions used during affinity purification, either protein complexes or only the labeled proteins will be isolated [3]. Analysis of retrieved material by gel electrophoresis and possibly mass spectrometry enables identifying (novel) enzymes that are involved in the biological process of interest.

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Using such an experimental setup in aggressive cancer cell lines with fluorophosphonate probes led to the discovery that monoacylglycerol lipase activity is elevated in these cells [4]. ABPs have also been used to monitor expression and activity of target enzyme(s) in cells that are challenged by a pathogen. Direct comparison of infected versus noninfected cells resulted in the discovery of several enzymes encoded by pathogens, such as the chlamydial deubiquitylating enzyme 1 (ChlaDUB1) and Epstein Barr Virus deubiquitylating enzyme (EBV-DUB) [5, 6].

Low abundance proteins may escape detection in protein profiling experiments, despite the affinity purification step prior to analysis of the proteins. The predominant reasons for failing to detect these proteins are nonspecific binding of proteins to the affinity resin (high background), and failure to deliver cell impermeable probes to the required cellular compartment (inefficient labeling of the target) [7]. The former becomes especially apparent when targeting protein complexes. Here, affinity purification is performed under native conditions. The stringent washing conditions (e.g., 4 M urea) that are normally used to remove proteins that are nonspecifically bound to the affinity matrix cannot be used in this experimental setup as they would disrupt the protein complexes under scrutiny [8]. Incorporation of a chemically cleavable linker into the probe decreases the background signal, as it allows the specific release of the probe-captured material [9–13]. Inefficient labeling of the target results from the current practice to apply the probe to an inherently dilute cell lysate. This can be resolved by direct delivery of the cell impermeable probes in a semi-permeable cell system [14]. We have used a combination of these strategies to demonstrate the expression of ChlaDUB2, a chlamydial deubiquitylating enzyme whose expression was predicted based on homology screening of the chlamydial genome, but previously escaped detection [15]. We here describe a detailed protocol as used for protein profiling with cleavable linker containing ABPs in semi-permeabilized cells (Fig. 1). The notes of this protocol contain alternative options to allow the researcher to tailor the experiment with the desired outcome in mind.

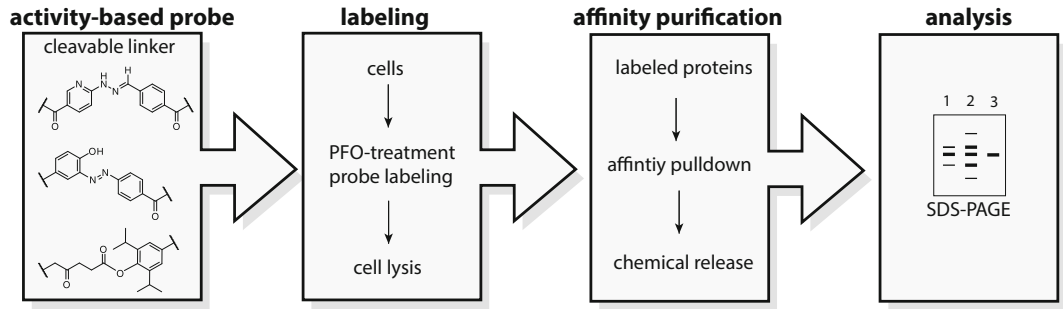

Fig. 1 Flowchart of the described protocol. Cleavable linker probes are added to semi-permeabilized cells (Subheading 3.1). The labeled proteins are, after cell lysis, purified by affinity purified and released by chemical cleavage (Subheading 3.2). The retrieved material is analyzed by SDS-PAGE (Subheading 3.3)

2 Materials

2.1 Components for the Activity-Based Labeling in Semi-permeabilized Cells

1. Activity-Based Probes containing azobenzene, Chromalink™ or levulinoyl cleavable linkers connected to biotin. (For synthesis of these cleavable linkers *see* Refs. [9, 11, 12, 15] (*see* **Note 1**).
2. HEK293T (American Type Culture Collection, ATCC® CRL-11268™) or HeLa cells (American Type Culture Collection, ATCC® CCL-2™).
3. [^{35}S] labeled L-methionine, [^{35}S] labeled L-cysteine, protein labeling mix (perkin elmer).
4. Methionine/cysteine-free Dulbecco's modified Eagle's medium (DMEM).
5. Dulbecco's modified Eagle's medium (DMEM).
6. Dialyzed inactivated fetal serum.
7. Perfringolysin-O (PFO) pore forming toxin (for preparation of recombinant PFO *see* Ref. [14]).
8. Phosphate buffered saline (PBS).
9. Trypsin.
10. Hanks' balanced salt solution (HBSS).
11. 0.5 times Hanks' balanced salt solution. Dilute 5 mL HBSS with 5 mL milliQ H_2O.
12. 10 cm cell culture dishes.
13. Disposable pipettes (5 mL, 10 mL).
14. Pipet-aid.
15. Micropipettes.
16. Centrifuge tubes, 1.5 mL.
17. Test-tube racks.
18. Humidified CO_2 regulated incubator.
19. Cooled tabletop centrifuge for microfuge tubes.
20. Nonidet-P40 or Nonidet-P40 substitute (NP40).
21. 20% NP40: Dilute 20 mL NP40 with milliQ H_2O to a final volume of 100 mL.
22. NP40-lysis buffer: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% nonidet-P40 substitute. Mix 84 µL 1 M Tris–HCl, 16 µL 1 1 M Tris, 375 µL 4 M NaCl in H_2O, 25 µL 2 M $MgCl_2$ in H_2O, 250 µL 20% NP40 and add milliQ H_2O to obtain a final volume of 10 mL.

2.2 Components for the Pull Down of the Probe-Labeled Proteins

1. Hydroxylamine hydrochloride.
2. Hydrazine monohydrate.
3. Sodium dithionite, 90%.
4. Aniline.

5. Magnesium chloride hexahydrate.
6. Tris hydrochloride.
7. Tris free base.
8. Hydrochloric acid solution, 6 M HCl.
9. Sodium hydroxide.
10. Acetic acid.
11. Methanol.
12. Ethanol.
13. Sodium Chloride.
14. Complete Mini, protease inhibitor cocktail tablets.
15. Streptavidin agarose.
16. Nonidet-P40 or Nonidet-P40 substitute (NP40).
17. Sodium dodecylsulfate (SDS).
18. 200 mM sodium phosphate buffer, pH 6.0: Dissolve 2.42 g sodium phosphate monobasic monohydrate and 0.659 g sodium phosphate dibasic heptahydrate in final volume of 100 mL milliQ H_2O.
19. 1 M Tris base: Dissolve 1.21 g Tris free base in a final volume of 10 mL milliQ H_2O.
20. 1 M Tris–HCl: Dissolve 1.57 g Tris–HCl in a final volume of 10 mL milliQ H_2O.
21. 2 M sodium hydroxide: Dissolve 0.799 g sodium hydroxide in a final volume of 10 mL milliQ H_2O.
22. 4 M sodium chloride: Dissolve 2.34 g sodium chloride in 10 mL milliQ H_2O.
23. 20% NP40: Dilute 20 mL NP40 with milliQ H_2O to a final volume of 100 mL.
24. NP40-lysis buffer: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40. Mix 84 µL 1 M Tris–HCl, 16 µL 1 M Tris, 375 µL 4 M NaCl in H_2O, 25 µL 2 M $MgCl_2$ in H_2O, 250 µL 20% NP40 and add milliQ H_2O to obtain a final volume of 10 mL. Store at 4 °C.
25. Levulinoyl washing buffer: 20 mM Tris–HCl, pH 8, 0.1% NP40. Mix 100 µL 1 M Tris free base, 100 µL 1 M Tris–HCl, and 50 µL 20% NP40. Add milliQ H_2O to obtain a final volume of 10 mL. Store at 4 °C.
26. Azobenzene washing buffer: 100 mM Tris–HCl, pH 8, 100 mM NaCl, 0.1% NP40. Mix 500 µL 1 M Tris free base, 500 µL 1 M Tris–HCl, 250 µL 4 M NaCl, and 50 µL 20% NP40. Add milliQ H_2O to obtain a final volume of 10 mL. Store at 4 °C.

27. Hydrazone washing buffer: 100 mM sodium phosphate buffer, pH 6.0, 0.1% NP40. Take 5 mL of 200 mM sodium phosphate and adjust the pH to 6.0 with 2 M NaOH. Add 50 μL 20% NP40 and dilute to a final volume of 10 mL with milliQ H_2O. Store at 4 °C.
28. Levulinoyl cleavage buffer: 20 mM Tris–HCl, 100 mM hydrazine, pH 7.7, 0.1% NP40. Mix 100 μL 1 M Tris free base, 100 μL 1 M Tris–HCl, 50 μL hydrazine monohydrate, and 165 μL 6 M HCl. Check the pH and adjust to pH 7.7 with 6 M HCl. Add 50 μL 20% NP40 and add milliQ H_2O to obtain a final volume of 10 mL. Store at 4 °C.
29. Azobenzene cleavage buffer: 100 mM Tris–HCl pH 8, 100 mM NaCl, 50 mM sodium dithionite, 0.1% NP40. Mix 500 μL 1 M Tris free base, 500 μL 1 M Tris–HCl, 250 μL 4 M NaCl, 96 mg sodium dithionite, and 50 μL 20% NP40. Adjust volume to 10 mL by adding milliQ H_2O. Store at 4 °C.
30. Hydrazone cleavage buffer: 100 mM sodium phosphate buffer, 100 mM aniline, 100 mM hydroxylamine hydrochloride, pH 6.0, 0.1% NP40. Take 5 mL of 200 mM sodium phosphate and add 91 μL aniline and 69.5 mg hydroxylamine hydrochloride. Adjust the pH to 6.0 with 2 M NaOH. Add 50 μL 20% NP40 and dilute to a final volume of 10 mL with milliQ H_2O. Store at 4 °C.
31. Laemmli sample loading buffer (5×).

2.3 Analysis of the Retrieved Proteins

1. Acrylamide/Bis solution (30%:0.8% acrylamide:Bis).
2. Ammonium persulfate (APS): 10% in water (wt/v). Store aliquots at −20 °C.
3. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED).
4. 5× SDS electrophoresis buffer: Tris base (0.125 M), glycine (0.96 M), 0.5% SDS. Dilute to 1× before using.
5. 4× Stacking buffer: 0.5 M Tris–HCl (pH 6.8) containing 0.4% SDS.
6. 4× Resolving buffer: 1.5 M Tris–HCl (pH 8.8) containing 0.4% SDS.
7. 2,5-diphenyloxazole (PPO).
8. Dimethylsulfoxide (DMSO).
9. 20% PPO in DMSO. Dissolve 20 g PPO in 100 mL DMSO.
10. Carestream Kodak X-OMAT XAR-5 Autoradiography films.
11. Exposure cassette Carestream Kodak BioMax.
12. Kodak X-OMAT film processor.

3 Methods

Perform all steps at room temperature unless stated otherwise.

3.1 Activity-Based Labeling in Semi-permeabilized Cells (See Note 2)

1. Culture HEK293T or Hela cells in DMEM supplemented with 10% IFS in 10 cm dishes until 70% confluent in a humidified regulated CO_2 incubator at 37 °C (*see* **Notes 3** and **4**).
2. Remove the medium and add 10 mL fresh methionine/cysteine-free DMEM supplemented with 10% dialyzed IFS and 100 μCi of [^{35}S]methionine/cysteine per milliliter of medium. Culture the cells overnight at 37 °C in humidified CO_2 incubator to achieve steady-state [^{35}S] protein labeling.
3. Remove the medium and wash the cells with 5 mL of PBS buffer (*see* **Note 5**).
4. Detach the cells from the cell culture dish by incubating with 1 mL of PBS buffer containing 0.05% trypsin for 2 min at 37 °C (*see* **Note 6**).
5. Collect the cells by centrifugation at 800 × *g* for 5 min at 4 °C. Wash the cells with 1 mL of cold PBS, and repeat the centrifugation step to collect a cell pellet (*see* **Note 5**). Keep the cell pellet on ice.
6. Wash the pellet with 100 μL Hanks' balanced salt solution on ice and collect the cells by again centrifuging at 800 × *g* for 5 min at 4 °C. Resuspend the pellet in 100 μL ice-cold 0.5 times Hanks' balanced salt solution, containing 100 nM purified PFO and 10 μM ABP on ice (*see* **Notes 7** and **8**).
7. Transfer the cell suspension to 37 °C to induce pore formation and incubate for 30 min (*see* **Note 9**).
8. Return the cell suspension to 4 °C and separate the cytosolic fraction from the organelles by centrifuging the cell suspension at 800 × *g* for 5 min. Collect the supernatant (cytosolic fraction) and transfer it to a separate Eppendorf tube. Dilute the cytosolic fraction with ice-cold NP40 lysis buffer to a final volume of 1 mL and keep this solution on ice (*see* **Notes 10–12**).
9. Gently wash the pellet with 1 mL ice-cold HBSS and centrifuge the resulting suspension at 800 × *g* for 5 min to collect the remainder of the permeabilized cells. Resuspend the pellet in 1 mL NP40 lysis buffer (*see* **Notes 10** and **13**).

3.2 Affinity Purification

1. Add 30 μL of a streptavidin-agarose suspension to the labeling reaction solutions and gently agitate the resulting slurry for 3 h at 4 °C (*see* **Note 14**).
2. Pellet the agarose beads by centrifuging the suspension at 10,000 × *g* for 1 min. Remove and discard the supernatant. Wash the beads with 1 mL NP40 lysis buffer to remove nonspecifically bound proteins. Repeat this step three times (*see* **Note 15**).

3. Equilibrate the beads with 1 mL of the appropriate washing buffer for the cleavable linker used. Centrifuge at 10,000 × *g* for 1 min and remove and discard the washing buffer (*see* **Note 16**).
4. Subject the affinity matrix for 5 h to 100 μL of the appropriate cleavage buffer at 37 °C to elute the probe-bound proteins (*see* **Note 17**).
5. Centrifuge at 10,000 × *g* for 1 min, retrieve the supernatant, and dilute the eluted sample with 100 μL Laemmli sample loading buffer (2×). Boil the sample for 5 min at 100 °C (*see* **Note 18**).

3.3 Analysis of [^{35}S] Labeled Polypeptides by Fluorography [16]

1. Mix 20 mL resolving buffer (4×), 26.67 mL 30% acrylamide solution, and 32.5 mL milliQ H_2O and add 800 μL 10% APS and 80 μL TEMED (amount required for a large 10% SDS-PAGE gel). Pour the resolving gel and cover the solution with *n*-butanol. Allow the mixture to polymerize for approximately 20 min.
2. Remove the *n*-butanol when the acrylamide has polymerized. Briefly wash the resolving gel with water to remove traces of *n*-butanol. Discard the water and remove remaining water with a piece of filter paper.
3. Mix 3.66 mL stacking buffer (4×), 1.5 mL 30% acrylamide solution, and 9.5 mL milliQ H_2O and add 150 μL 10% APS and 6.5 μL TEMED. Pour the stacking gel and carefully place the comb. Allow polymerizing for 15 min.
4. Place the gel in the gel running system. Fill the chambers with 1× running buffer and remove the comb. Load 100 μL protein sample into the well and run the gel at 150 V till the dye front reaches the stacking gel and at 200 V till the dye front reaches the bottom of the gel (*see* **Note 19**) [17].
5. Remove the SDS-PAGE from the cassette, fix the gel with methanol, and subsequently remove water from the gel by washing the gel twice with DMSO for 30 min (*see* **Note 20**).
6. Impregnate the gel with 2,5-diphenyloxazole (PPO) by agitating the gel in a solution of 20% PPO in DMSO (w/v) for 1 h. Agitate the gel in water for 30 min to precipitate the PPO and to remove DMSO (*see* **Note 21**). Replace the water repeatedly during this period.
7. Place the gel on a filter paper and transfer it to a standard gel drying system. Cover the back of the gel with Saran wrap and dry the gel at 80 °C under vacuum for 1.5 h.
8. Check if the gel has been dried thoroughly and remove the gel from the gel drying system if this is the case.
9. Place the gel in an exposure cassette and add an autoradiography film on top. Seal the cassette and store at −80 °C (*see* **Note 22**).
10. Develop the film in a kodak film processor (*see* **Note 23**).
11. Compare the retrieved proteins in the different lanes by eye (*see* Fig. 2).

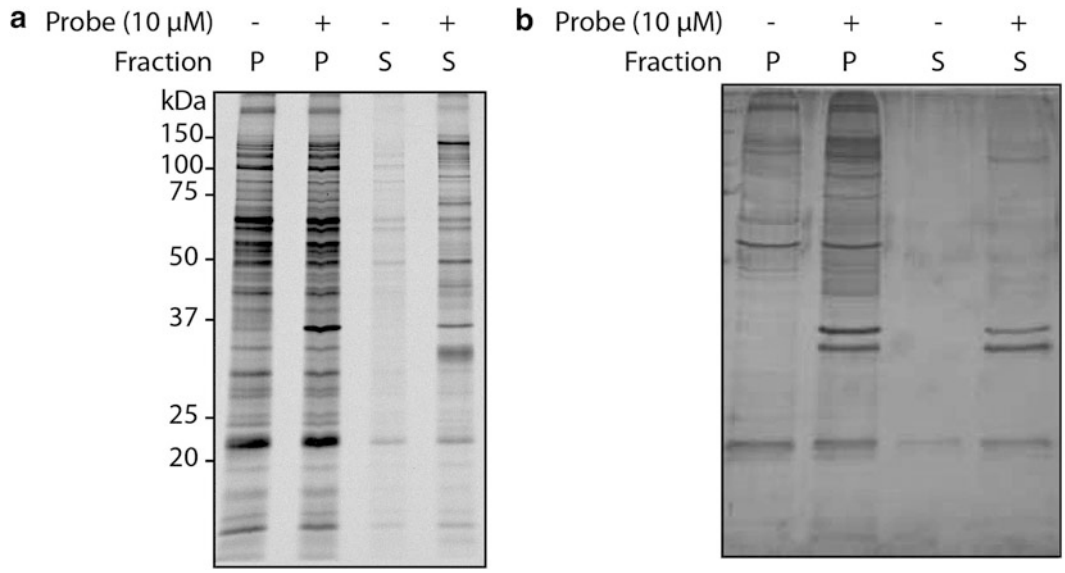

Fig. 2 A typical result obtained by labeling semi-permeabilized cells with cleavable linker containing UbVME probes. HEK293T cells were treated with PFO and an azobenzene containing ubiquitin vinyl methyl ester probe (10 μM). After 30 min at 37 °C, the cytosolic fraction (supernatant (S)) and the organelle fraction (pellet (P)) were separated, loaded on a gel, and analyzed by fluorography (Fig 2a: Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Ref. [15]) for [^{35}S] labeled proteins or silver staining (Fig 2b) for non [^{35}S] labeled proteins

4 Notes

1. Each cleavable linker has its advantages. The bisarylhydrazone linker (Chromalink) is commercially available, but showed the slowest cleavage in our hands. The azobenzene cleavable linker revealed to cleave most efficiently, but the used conditions may inflict damage on the protein and it is therefore not suitable for subsequent immunoprecipitations (IP). Material that is obtained from the levulinoyl linker can be used in subsequent IPs.
2. Semi-permeabilized cells facilitate ABP labeling of enzymes at higher concentrations, which improves labeling efficiency compared to labeling in cell lysates, and facilitates subsequent fractionation of the sample, which may give additional information about the subcellular localization of the ABP labeled proteins. *Alternative 1*: ABP labeling can be performed in cell lysates, which is straightforward and does not require additional reagents. In this case it is crucial that the cell lysates are prepared with a mild nondenaturing buffer as stronger detergents, such as SDS, will unfold the proteins and will inhibit labeling by the ABP. *Alternative 2*: cell permeable ABPs can be used to directly label the target enzymes in cells and do not require permeabilization of the cells with PFO prior to labeling.

3. The here-described protocol employs autoradiography of the retrieved [^{35}S] labeled proteins for the head to head comparison of the different cleavable linker containing ABPs. Steady-state labeling with [^{35}S]methionine/cysteine was the most sensitive method in our hands. As *alternative* other protein staining methods can be used. Proceed to **step 3** if the probe-bound proteins and their interacting partners will be analyzed with silver staining or another general protein staining method alike (*see* Fig. 2b for an example of a result obtained with silver staining).
4. It is preferable to omit **step 2** when the affinity-purified proteins are analyzed by mass spectrometry to prevent contamination of the mass spectrometer with radioactive material.
5. The removed solutions contain radioactive waste and should be disposed of accordingly.
6. Monitor the trypsinization by eye. Increase the incubation time with trypsin or the amount of trypsin if incomplete detaching of the cells is observed.
7. Diluting Hanks' balanced salt solution two times is essential to introduce the probe into the semi-permeabilized cells. The mild hypotonic buffer creates a mild osmotic shock upon pore formation at 37 °C, which introduces the probes into the cells.
8. The probe concentration depends on the potency of the used probe and should be adjusted accordingly.
9. Transferring the cells to 37 °C induces pore formation and allows entry of the probe into the cells. It is therefore important to keep the cells on ice to prevent preliminary pore formation.
10. Resuspending the fractions in NP-40 lysis buffer results in the retrieval of both the probe-bound proteins and their interacting partners. *Alternative*: SDS lysis buffer can be used to exclusively retrieve probe-bound proteins during the affinity purification step. Add 100 μL SDS lysis buffer to the fractions and dilute to 1 mL with NP40 buffer prior to affinity purification.
11. Whole lysates may be prepared by adding 1 mL of NP40 lysis buffer to the semi-permeabilized cells immediately after **step 7** when separation of the cytosolic fraction from the organelles is not required.
12. Disturbing the pellet while removing the supernatant leads to contamination of the cytosolic fraction and should be avoided. Western blot analysis of the cytosolic protein GAPDH and the ER-resident PDI helps detecting if the pellet and supernatant fraction have been separated correctly.
13. The pellet fraction should be carefully washed to remove residual amounts of cytosolic proteins. Western blot analysis of the cytosolic protein GAPDH and the ER-resident PDI helps detecting if the pellet and supernatant fraction have been separated correctly.

14. The amount of affinity resin depends on the amount of probe that has been added. Higher amounts of streptavidin-agarose resin need to be added when more probe is used. The approximate amount needed can be calculated using available binding sites.
15. These mild washing conditions are necessary to maintain the protein complexes. More stringent washing conditions (4 M urea) can be used if only interested in probe-bound proteins.
16. We observed that washing with the appropriate washing buffer prior to cleavage is essential to obtain optimal cleavage of the cleavable linker.
17. Longer cleavage times increase the yield of the recovered proteins from the bisaryl hydrazone linkers.
18. The efficiency of the chemical cleavage can be controlled by boiling the affinity matrix in Laemmli sample buffer after chemical cleavage, separating the released proteins by SDS-PAGE and analyzing the released proteins by autoradiography.
19. For an extensive protocol on gel electrophoresis *see* Ref. [17].
20. Alternatively, the retained proteins can be visualized by silver staining. For a detailed protocol on silver staining of SDS-PAGE *see* Refs. [18, 19]. *See* Fig. 2b for an example of a result obtained using this visualization method.
21. Contact of the skin with the 20% PPO in DMSO solution readily results in subcutaneous precipitation of PPO. Proper personal protective equipment should be used at all times to prevent contact with the skin.
22. A rough estimation for the exposure time can be obtained by monitoring the radioactivity with a Geiger counter.
23. Films may be developed manually as well.

5 Anticipated Results

Typical results obtained using this protocol in steady-state [^{35}S] labeled cells or nonlabeled cells are depicted in Fig. 2a, b respectively. Azobenzene containing ubiquitin vinyl methyl ester (AzoUbVME) probe (10 μM) was delivered to the cytosol of the HEK293T cells by treating the cells with PFO (100 nM) in 0.5 times HBSS for 30 min at 37 °C. The cytosol (supernatant (S)) and the organelles (pellet (P)) were separated by centrifugation and the fractions diluted with NP40-lysis buffer. Probe-labeled proteins and their interaction partners were isolated using streptavidin-agarose as affinity matrix. The retrieved material was released from the affinity matrix by chemical cleavage of the linker. The proteins were separated on SDS-PAGE and visualized by fluorography (Fig. 2a) or silver staining (Fig. 2b). As a negative control

(−), cells were treated with PFO (100 nM) in 0.5 times HBSS for 30 min at 37 °C without AzoUbVME. Comparing the lanes that received the probe (+) with those that did not receive the probe (−) reveals the retrieval of a specific set of proteins using the AzoUbVME probe. While the background signal is still considerable in the pellet fraction despite the chemical cleavage step, hardly any background is observed in the cytosolic fraction. Figure 2 also shows that in this case steady-state protein labeling using [^{35}S] methionine/cysteine (Fig. 2a) is considerably more sensitive than silver staining (Fig. 2b).

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Chapter 14

Cleavable Linkers in Chemical Proteomics Applications

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Abstract

The discovery of the protein targets of small molecule probes is a crucial aspect of activity-based protein profiling and chemical biology. Mass spectrometry is the primary method for target identification, and in the last decade, cleavable linkers have become a popular strategy to facilitate protein enrichment and identification. In this chapter, we provide an overview of cleavable linkers used in chemical proteomics approaches, discuss their different chemistries, and describe how they aid in protein identification.

Key words Activity-based probes, Chemical proteomics, Cleavable linkers, Click chemistry, Enrichment, Mass spectrometry, Protein identification, Target discovery

1 Introduction

Classical shotgun proteomics aims to identify as many proteins as possible from a given sample, creating a snapshot of the expressed proteins in a cell or tissue at a certain point in time. Technical developments in mass spectrometry (MS) now allow the identification of thousands of proteins from lower and lower amounts of sample [1]. Chemical proteomics focuses on the identification of special traits of proteins using chemical techniques. These traits can vary from the activity state of enzymes to post-translational modifications, such as glycosylation, acetylation, and lipidation. While some techniques use noncovalent small molecules [2], covalent chemical probes are especially popular. Covalent probes (Fig. 1) include activity-based probes (ABPs) for activity-based protein profiling (ABPP), reactivity-probes (RPs), affinity-based probes (AfBPs; often using photocrosslinkers), and probes introduced by metabolic labeling (e.g., alkyne- or azide-labeled fatty acids). By optimizing probe structure and reactivity, it is now possible to make broad-spectrum probes as well as highly selective probes [3]. Although this volume is dedicated to ABPP, the tagging and detection strategies for other covalent chemical probes are very similar. They include the use of biotin, isotope labels, and fluorophores [4].

Herman S. Overkleeft and Bogdan I. Florea (eds.), *Activity-Based Proteomics: Methods and Protocols*, Methods in Molecular Biology, vol. 1491, DOI 10.1007/978-1-4939-6439-0_14,

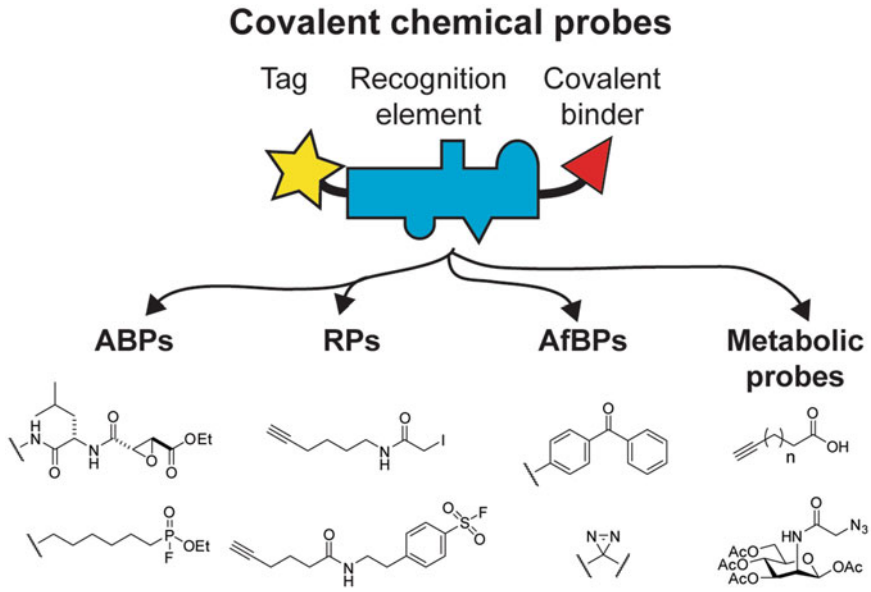


Fig. 1 Covalent chemical probes rely on different mechanisms of covalent attachment to their target proteins. Activity-based probes (ABPs) undergo a mechanism-based reaction with the active site machinery of specific enzymes. Probe selectivity is caused by a combination of the reactive electrophile and an adjacent recognition element. Reactivity probes (RPs) lack recognition elements and only contain a reactive group and a detection tag. Photocrosslinkers can be appended to small molecules for photoaffinity labeling of protein targets. In metabolic labeling, azide or alkyne derivatives of building blocks found in post-translational modifications are incorporated into proteins by the cellular metabolic machinery

Biotin and fluorophore tags can be bulky and may influence the probe reactivity or cell permeability. Bioorthogonal chemistries, in particular the Cu(I)-catalyzed azide alkyne cycloaddition (CuAAC), have become popular alternatives for labeling proteins in chemical biology [5, 6]. In this tandem labeling process, the probe is only modified with a small azide or alkyne "mini-tag" and the tagging moiety is introduced in a second stage. Click chemistry has therefore become the method of choice when performing ABPP in cell culture or in in vivo models.

The detection of probe targets by MS starts with a purification step to separate labeled from unlabeled proteins. Target purification mostly relies on the biotin-streptavidin interaction due to its near diffusion limited binding (Kd ~ 10^{-15} mol/L), which allows enrichment of even highly dilute proteins. Avidin and streptavidin immobilized on agarose, polyacrylamide, or magnetic beads are available from multiple commercial sources. In order to release the biotinylated target proteins, harsh, denaturing conditions are required for disruption of the biotin-streptavidin interaction. As a consequence, nonspecifically bound proteins, endogenously biotinylated proteins, and streptavidin will be simultaneously released and will contaminate the true probe targets. To circumvent the

elution problem, streptavidin mutants and several different biotin analogs such as 2-iminobiotin [7], 2′-thiobiotin [8], desthiobiotin [9], and N3′-ethyl biotin [10] have been developed in order to reduce the affinity between streptavidin and biotin. The lower binding affinity allows the immobilized proteins to be released under milder conditions. For example, N3′-ethyl biotin allows release of target proteins by incubation with 2 mM of biotin. However, the lower binding affinity of these derivatives may lead to the inefficient enrichment of low abundant target proteins and loss of targets during stringent washing steps. The usage of cleavable linkers forms an elegant alternative that allows selective elution of probe targets.

In this chapter, we discuss the currently available cleavable linkers for target enrichment and identification. While we will focus on ABPP, we will also mention linkers used in other areas of chemical proteomics, as they may be readily used in ABPP applications.

2 Cleavable Linkers

Cleavable linkers have been developed for a variety of applications including solid phase synthesis and chemical biology [11]. A substantial amount of cleavable linkers in chemical proteomics has been inspired by protecting groups (PGs) developed for selective protection and deprotection of functional groups in organic synthesis. Examples include the *t*-butyloxycarbonyl PG (Boc) for primary amines and the levulinoyl ester PG (Lev) for alcohols.

The general workflow of protein identification using cleavable linkers is depicted in Fig. 2. Probes may either contain a cleavable linker between the reactive group and the biotin moiety, or a bioorthogonal handle, such as an alkyne, which is subsequently labeled with an azide biotin derivative containing a cleavable linker. After (tandem) labeling, targets are captured onto immobilized streptavidin, washed to remove most nonspecifically bound proteins, and subjected to the conditions for selective chemical release. Targets can then be digested and analyzed by tandem MS (Fig. 2, path a). Alternatively, proteins may first be digested "on bead," after which the modified peptide can be selectively released and analyzed by MS for analysis of the modification site (path b).

In the next paragraphs, we provide an overview of the different cleavable linker chemistries and discuss their advantages and limitations.

2.1 Acid-Labile Linkers

Since peptides and proteins are generally stable to treatment with acid (even strong acids such as neat trifluoroacetic acid (TFA)), acid-labile protecting groups and linkers are extensively used in peptide synthesis protocols. They are also applicable as cleavable linkers in chemical proteomics. For example, cleavable

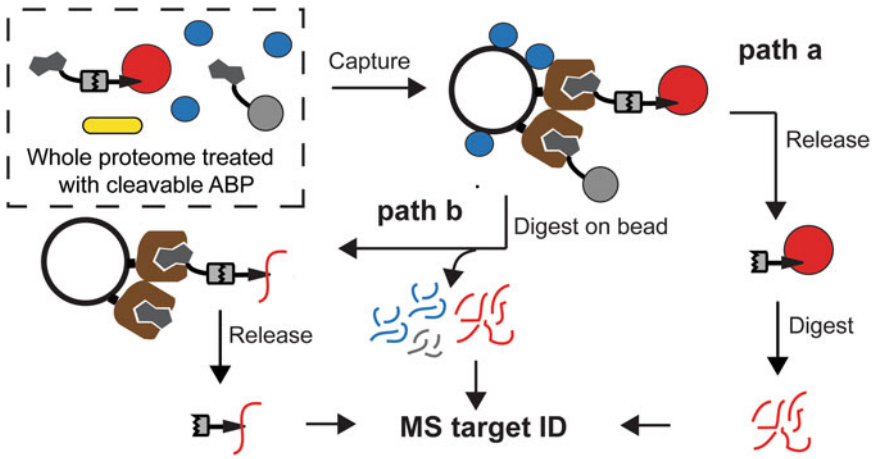
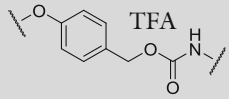
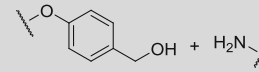
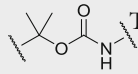
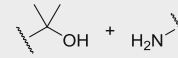
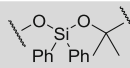
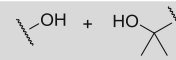
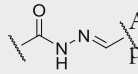
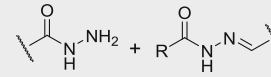
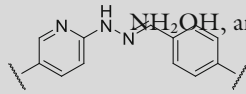
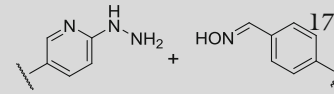
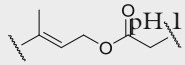
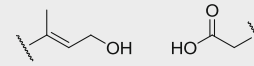
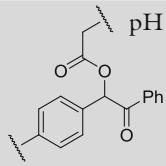
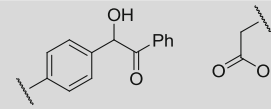
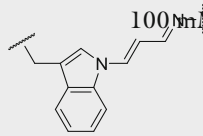
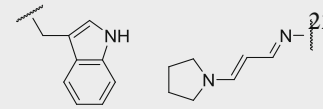
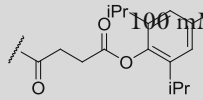
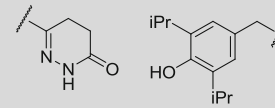
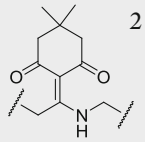
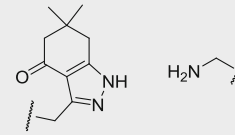
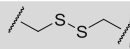


Fig. 2 Target identification using cleavable linkers. A probe-labeled proteome may be obtained by treating a cell lysate with a cleavable probe or by tandem labeling, in which cells are labeled in situ or in vivo and a cleavable biotin tag is introduced after lysis by click chemistry. Capture on immobilized streptavidin enriches targets, but may co-purify endogenously biotinylated proteins and highly abundant proteins. *Path a* elutes probe targets directly using the release conditions of the cleavable linker. Subsequent digest allows protein identification by MS. *Path b* first digests proteins "on bead." The resulting peptides can be analyzed, but may contain peptides from contaminating proteins. A subsequent elution with the release conditions of the cleavable linker specifically isolates the probe-modified peptide, which can then be analyzed for the modification site

linkers based on the Wang linker (4-hydroxy-benzylalcohol; Table 1, entry 1) [12] and the Boc (*tert*-butyl-oxycarbonyl) group (Table 1, entry 2) [13, 14] have been used in the enrichment of peptides labeled with ICAT (isotope-coded affinity tag) reagents. The main purpose here is the elimination of biotin, leading to less complex fragmentation patterns during tandem MS. However, these acid-cleavable linkers still need strong acid (high concentrations of TFA) to be cleaved, which can result in the denaturation of proteins and may lead to the release of non-specifically bound proteins.

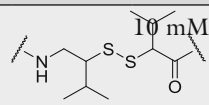
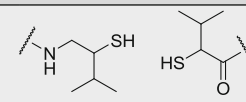
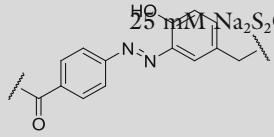
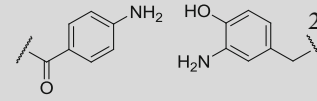
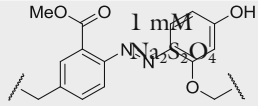
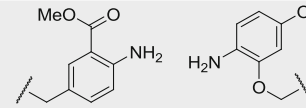
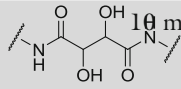
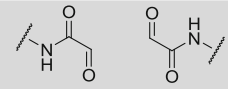
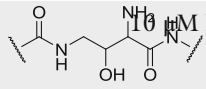
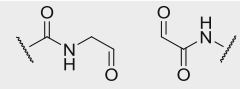
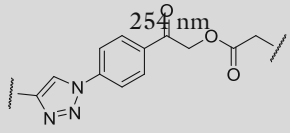
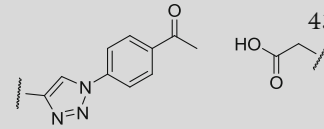
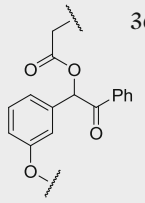
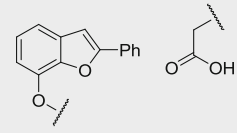
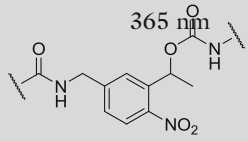
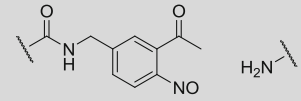
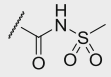
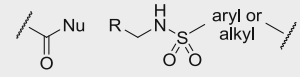
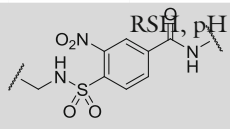
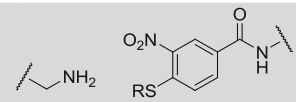
Linkers based on dialkoxydiphenylsilane (Table 1, entry 3) are much more labile and can be efficiently cleaved under milder conditions (10% formic acid). The Tirrell laboratory built this linker into a cleavable azide-containing biotin derivative for the labeling and enrichment of alkyne-derivatized biomolecules [15]. Hydrazones can also be cleaved under mild acidic conditions. An azido-biotin reagent with an acyl hydrazone was developed by Kohn and coworkers (Table 1, entry 4) [16]. Acyl hydrazones are stable at basic pH (pH 8–10), but hydrolyze or undergo hydrazone exchange with hydrazides under slightly acidic conditions (pH 4–5). However, they tend to prematurely hydrolyze at neutral pH. The Dawson laboratory developed a bisaryl hydrazone formed from a benzaldehyde and a hydrazinopyridine [17]. The resulting

Table 1
Overview of cleavable linkers

Entry	Linker	Cleavage conditions	Cleavage products	Ref.
1		TFA		12
2		TFA		13,14
3		10% HCOOH		15
4		Acyl hydrazine pH 4–5		16
5		NH_2OH, aniline pH 4–5		17
6		pH 11		20
7		pH 8		21
8		100 mM pyrrolidine		22
9		100 mM NH_2NH_2		23
10		2% NH_2NH_2		19
11		DTT TCEP or BME		19,15

(Continued)

Table 1
(continued)

Entry	Linker	Cleavage conditions	Cleavage products	Ref.
12		10 mM TCEP		27,28
13		25 mM $Na_2S_2O_4$		29
14		1 mM $Na_2S_2O_4$		31
15		10 mM $NaIO_4$		38
16		10 mM $NaIO_4$		40
17		254 nm		43
18		365 nm		44
19		365 nm		15
20	–ENLYFQG–	TEV protease	–ENLYFQ-OH NH_2-G–	48,49
21	–5'-GTAACGATCCAGCTGTCACT-3'–	Hoch endonuclease	–5'-GTAACGATCCAG-3' 5'-CTGTCACT-3'–	53
22		1. ICH_2CN pH 9, NH_4OH or pH 5 ICH_2COO^-		54
23		RSH, pH 9.2		57

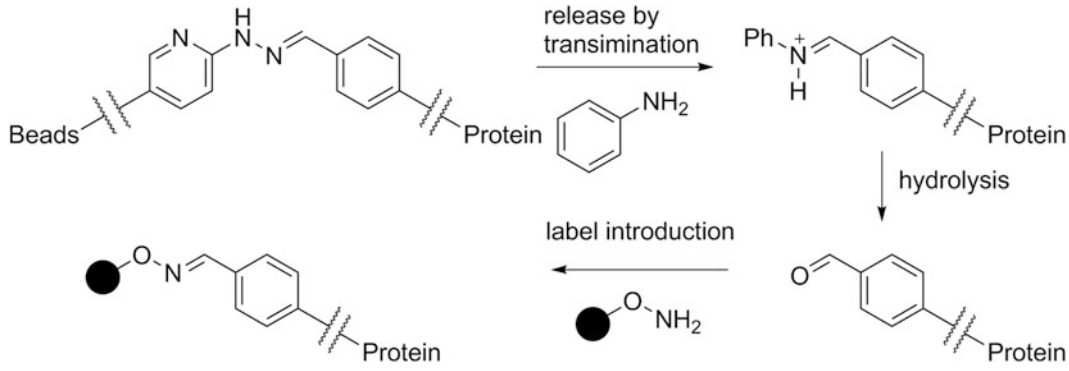


Fig. 3 Cleavage of a bisaryl hydrazone linker with subsequent label introduction. Transimination with aniline as a catalyst increases the release efficiency of immobilized target proteins. Hydrolysis liberates a benzaldehyde function, which can be labeled by an amino-oxy derivative

hydrazone is stable at neutral pH and undergoes—under catalysis of aniline—transimination with hydroxylamines at acidic pH (Table 1, entry 5). However, high concentrations of aniline (100 mM) are required and full cleavage is not always achieved [18, 19]. A beneficial feature of the bisaryl hydrazone linker is the possibility to reintroduce a label to the released biomolecule, since any amino-oxy compound can be used for the transimination (Fig. 3). This was shown for amino-oxy derivatives of Alexa Fluor 488 and of a FLAG peptide, applied at 10 mM concentration [17].

2.2 Base-Labile Linkers

Several cleavable linkers that are sensitive to basic conditions have been reported. In general, esters can be irreversibly hydrolyzed at high pH. However, ester linkages can also be cleaved by esterases, which may hamper the use of probes containing ester cleavable linkers in cells or lysates. Rando and coworkers used a chloroacetate ester of a biotin-functionalized retinol to capture retinoid binding proteins. The chloroacetate ester acted not only as a covalent electrophilic trap for nucleophilic residues in the retinoid binding pocket but also as a base-labile cleavable linker that can be hydrolyzed at pH 11 (Table 1, entry 6) [20]. When designing a photocleavable benzoin ester, Liebler and coworkers found that the ester bond was susceptible to cleavage under mild basic conditions (Table 1, entry 7) [21]. The linker turned out to be stable at pH 4.5 but labile at higher pH, with full cleavage achieved overnight at pH 7.5 and 8 in sodium phosphate or ammonium bicarbonate. These very mild cleavage conditions, however, prevent the usage under physiological conditions or during a tryptic digest.

Sturm et al. developed a linker based on a condensation product of an indole and malondialdehyde (Table 1, entry 8), a reaction used before for reversible tagging of tryptophan residues [22]. Cleavage up to 80% was achieved by incubation with 50 mM pyrrolidine. The authors used this linker to create an

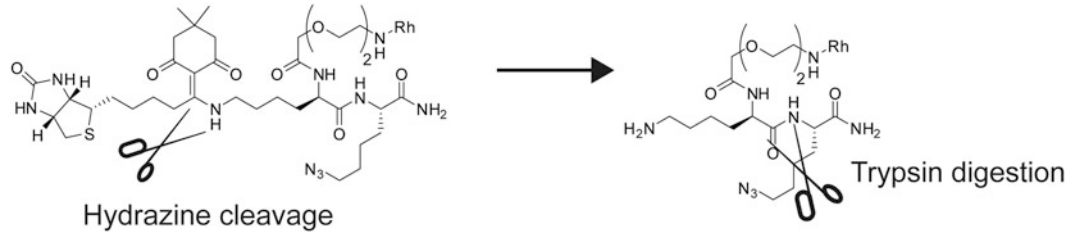


Fig. 4 A hidden trypsin digestion site. The depicted trifunctional tag, which can be clicked on any alkynylated protein, exposes a free lysine residue upon cleavage of the Dde-type linker. Next, treatment with trypsin does not only digest the protein, but also eliminates the majority of the remaining tag

affinity resin with immobilized bosutinib, a noncovalent tyrosine kinase inhibitor. Up to 31 kinases were found within a single run using this cleavable linker, but the degree of protein background reduction remained low, as more than 400 other proteins were identified as well [22].

Hydrazine-labile PGs have been used in carbohydrate and peptide chemistry due to their compatibility with a variety of chemical reagents and other PGs. The Overkleeft laboratory developed a cleavable linker based on the levulinoyl ester (Lev) [23]. The Lev group is a PG for alcohols that is used in carbohydrate chemistry orthogonally to other esters such as acetyl and benzoyl esters. For usage as a cleavable linker, the intrinsic base lability of the Lev ester was overcome by choosing an electron-rich and sterically congested alcohol for attachment to the levulinolate part (Table 1, entry 9). As a result, the linker is compatible with a variety of common buffers. It has been used for capture and identification of proteasome subunits of an epoxomicin-based ABP [23]. Verhelst and coworkers used a derivative of N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl), abbreviated Dde, a PG for amines used in peptide chemistry, which can also be cleaved by hydrazine (Table 1, entry 10). Built into a trifunctional cleavable reagent with an azide, a biotin, and a fluorophore, this linker results in a fluorophore-labeled protein after chemical elution (Fig. 4). This trifunctional reagent has the additional feature that it exposes a trypsin digestion site upon cleavage, allowing the majority of the tag to be eliminated before MS (Fig. 4) [19].

2.3 Reductively Cleavable Linkers

Cleavable linkers which utilize mild reductive conditions were reported in the literature over 30 years ago. The first applications of the disulfide cleavable linker (Table 1, entry 11) included the use of cleavable biotinylated nucleotides for the identification of protein-DNA complexes [24, 25]. At present, disulfides remain among the most commonly used cleavable linkers in biochemistry and are available in a variety of commercial protein biotinylation kits. For elution, the disulfide linker is usually cleaved with a mild reducing agent such as dithiothreitol (DTT),

tris(carboxyethyl)phosphine (TCEP), or β-mercaptoethanol. Because of these sensitive cleavage conditions, the disulfide linker is incompatible with the intracellular environment, reducing buffers, and CuAAC with TCEP as a reductive agent. Disulfide linkers are also prone to disulfide exchange which causes premature release and nonspecific labeling of proteins containing free thiol groups [15, 19, 26].

In ABPP, disulfides have been used in comparative analyses of cleavable linkers, but suffered from disulfide exchange and inefficient release of target proteins [15, 19]. An interesting approach to improve the properties of the disulfide linker was reported by the Gygi laboratory, which used a different carbon framework that introduces sterical hindrances and stabilizes the disulfide bond. The linker is now better resistant to DTT but still retains susceptibility to cleavage by phosphines (Table 1, entry 12) [27]. It was used in isotope-coded reagents for global labeling of cysteine residues [27] as well as in ABPP for the profiling of serine hydrolases [28].

The general shortcomings of the disulfide cleavable linker were addressed by the development of a diazobenzene linker (Table 1, entry 13) which is efficiently cleaved by sodium dithionite and enables selective elution under mild reductive conditions [29]. Incorporated in epoxide-based ABPs, the diazobenzene cleavable linker was successfully used for proteomic profiling of cysteine proteases, showing its compatibility with reductive conditions and target identification by tandem MS [30]. In the first versions of the linker, several elutions with 25 mM sodium dithionite were needed for effective elution. A few years later, an optimization of the diazobenzene scaffold was reported, resulting in a linker with a different substitution pattern (Table 1, entry 14) that can be effectively cleaved with much lower concentration of dithionite (1 mM) [31]. Because of their specific and efficient elution, diazobenzene linkers were quickly applied in the development of CuAAC affinity tags for bioorthogonal proteomics [15, 19, 32–36]. A special need for a mild cleavable linker was reported by Battenberg et al. who analyzed the targets of a 4-hydroxyderricin-based probe by clicking a biotin-rhodamine tag followed by enrichment and gel analysis [36]. Due to the labile nature of the probe-protein linkage, traditional heat-assisted protein elution resulted in loss of the probe and the fluorescent tag. Application of a diazobenzene linker with mild reductive elution left the covalent probe-protein complex intact and gave clear fluorescent protein bands, which were identified by in-gel digestion and tandem MS. For relative quantification of enriched proteins, isotope-labeled versions of the diazobenzene cleavable linker have been developed by Weerapana and coworkers [37].

2.4 Oxidatively Cleavable Linkers

Lengthy syntheses, low yields, or difficult purifications of cleavable linker building blocks can be a hurdle for cleavable linker application

in ABPP. Verhelst and coworkers introduced a vicinal diol cleavable linker in ABPP, which can be easily made on a large scale from inexpensive tartrate by only two protecting group manipulation steps [38]. The tartrate-based vicinal diol is cleaved at neutral pH with 10 mM periodate (Table 1, entry 15). Although periodate can lead to some side reactions with proteins, such as oxidation of glycans, methionines, and N-terminal serine and threonine residues, these processes did not negatively influence identification of cysteine cathepsins by an E-64-based ABP [38] and of aspartic proteases by a noncovalent statin-based affinity resin [39]. The usage of the vicinal diol cleavable linker also led to 80–90% reduction in background protein identifications compared with an "on bead" digestion, illustrating the benefit of the cleavable linker approach [38]. Within a trifunctional tag (*see* also Fig. 4) the diol linker led to efficient enrichment and release of target proteins [19].

1,2-Amino alcohols are much more sensitive to periodate cleavage than vicinal diols. Recently, such a linker was used in the release of cleavable peptides from MHC molecules induced by only 10 μM periodate (Table 1, entry 16) [40]. The application of a vicinal amino alcohol in ABPP could be beneficial as it may prevent some of the oxidative side reactions.

2.5 Photocleavable Linkers

Photocleavable linkers are generally stable under a variety of conditions, yet they become labile when irradiated with light of a specific wavelength. Therefore, they have both been used as protecting groups in synthesis and as linkers in solid phase chemistry [41, 42]. For protein enrichment by chemical proteomics or ABPP, several photocleavable linkers have been used. Sieber and coworkers used a biotinylated phenacyl ester (Table 1, entry 17) and demonstrated up to 70% of release of cargo upon irradiation at 254 nm [43]. However, higher wavelengths are desirable in order to prevent photodamage of proteins. Porter and colleagues applied a benzoin ester (Table 1, entry 18, similar to entry 7), incorporated into a clickable biotin tag [44]. This linker can be cleaved with 365 nm light. It was used for the enrichment of proteins labeled by an alkyne version of 4-hydroxy-2-nonenal (HNE), a toxic product of lipid oxidation that not only forms mutagenic DNA adducts, but can also covalently react with proteins. In a model system using human plasma, the authors were able to map several modification sites, which included lysine, histidine, and cysteine residues on a variety of proteins [44]. In a comparative analysis of several cleavable linkers, the Tirrell lab used an *ortho*-nitro-benzyl derivative (Table 1, entry 19) built into an azido-biotin tag [15]. This linker gave near quantitative cleavage after irradiation at 365 nm for 30 min.

2.6 Enzymatically Cleavable Linkers

Compared with chemical elution, linker cleavage by enzymes presents several new challenges. Besides introducing an additional protein to the system, the cleavage efficiency strongly depends

on the activity and stability of the enzyme. Proteases are the most commonly used enzymatic agents for linker cleavage. Since proteases can in principle also degrade labeled proteins during the elution step, a narrow cleavage specificity of the used protease is crucial for successful application. One such enzyme is the TEV protease (Tobacco Etch Virus nuclear inclusion A peptidase) [45], which has a unique recognition motif: it specifically cleaves between the Gln-Gly residues of the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly, which makes it highly appropriate for specific enrichment of proteins. The TEV specific linker has, for example, been used in affinity purification approaches for enrichment and proteomic identification of protein complexes [46, 47]. In ABPP, the TEV cleavable linker was first applied by Cravatt and coworkers who developed an azidebiotin click tag with an incorporated TEV cleavable sequence (Table 1, entry 20) [48, 49]. This approach showed efficient enrichment of targets labeled by a phenyl sulfonate alkyne probe, with low presence of background proteins. Although this method can generally identify the exact labeling sites (*see* Fig. 2, path b), in some cases site annotation can be problematic due to the structural complexity of the probe [49].

Trypsin-specific cleavable linkers are another type of proteolytic linker reported in proteomic applications. As a part of a click chemistry tag, two examples of its use are enrichment of newly synthesized proteins in the BONCAT methodology (bioorthogonal noncanonical amino acid tagging) [50] and identification of N-myristoylated and GPI-anchored proteins in *P falciparum* [51]. Trypsin is fully compatible with proteomic analysis, since it is commonly used in "bottom-up" approaches. In comparison to the TEV linker, the main disadvantage of the trypsin cleavable linker is the lack of specificity during the elution step. Trypsin cleaves proteins after lysine and arginine residues and therefore releases labeled as well as nonspecifically bound and natively biotinylated background proteins from the affinity resin. The use of a trypsin cleavable linker can therefore produce the same background protein contamination issues as "on bead" digestion approaches [30]. Cleavable linkers which utilize other proteases commonly used in proteomics, such as the endoproteinase Glu-C, have also been reported [52].

Proteases are not the only enzymes used for specific elution of labeled proteins. Recently, a DNA oligo-based cleavable linker was reported by Zheng and coworkers, who developed a biotin-DNA-azide linker which can be cleaved by a restriction endonuclease (Table 1, entry 21) [53]. This linker was used for the enrichment of alkyne-tagged glycoproteins from mammalian cell lysates and showed efficient and specific elution of target proteins. In the future, DNA-based cleavable linkers could become an interesting alternative to proteolytically cleavable linkers.

2.7 Others

In this paragraph, we discuss some linkers that do not easily fit the categories of the previous sections. Park et al. used an acylsulfonamide [54], which has previously been used as "safety-catch" linker in solid phase synthesis [55]. The acylsulfonamide is stable under acidic and basic conditions. However, upon N-alkylation, attack of a nucleophile on the acyl moiety leads to cleavage of the acylsulfonamide group (Table 1, entry 22). The authors built this linker into a photoaffinity probe for lectins. In a proof-of-principle study, cleavage was performed by alkylation with iodoacetonitrile in a 0.1 M pH 9 borate buffer followed by addition of 20 mM ammonia, which led to approximately 75% target release [54]. As the acylsulfonamide proton has a low pK_a, alkylation can also take place at pH 5 [56]. Yokoshima et al. developed a cleavable linker based on the 2-nitrobenzenesulfonamide group [57], a protecting group for amines that can be removed by nucleophilic attack by a thiolate (Table 1, entry 23) [58]. Within a photoaffinity probe for γ-secretase, the linker led to enrichment of presenilin-1 (the catalytically active subunit of γ-secretase), although cleavage did not occur in a quantitative manner [57].

The groups of Brown and Porter utilized selective complexation of dicobalt octacarbonyl to alkyne groups in organic solvents as a method to tag and enrich alkyne-modified lipids from membrane preparations isolated from cells fed with alkyne-derivatized fatty acids (Fig. 5a) [59, 60]. The cobalt complex cannot only be used for quantification using reverse-phase HPLC with UV detection at a specific wavelength (349 nm) but also for capture and release using an immobilized phosphine (capture) followed by oxidation of the cobalt complex by Fe(III) (release) (Fig. 5a). The application of cobalt chemistry allows the use of internal alkynes, whereas CuAAC is restricted to terminal alkynes. In addition, cleavage regenerates the unmodified alkyne and leaves no residual tagging moiety behind. In order to circumvent the use of organic solvents and the oxidative release conditions, the laboratory of Sodeoka pre-formed a cobalt-phosphine complex on solid support for the direct capture of propargyl carbamate-functionalized molecules (Fig. 5b) [61]. In a proof-of-principle study with dipeptide models, they showed compatibility with aqueous conditions [62]. Acidic conditions (5% TFA) cleaved the carbamate and released the cargo from the solid support. These cobalt-mediated enrichments may be used in future ABPP capture and release using alkyne-containing probes.

3 Methods

Below, we illustrate cleavable linker mediated enrichment and identification of protein targets by a detailed protocol for the capture and release of cathepsin proteases using a diazobenzene containing probe. Since their advent as cleavable linkers [29, 30], diazobenzenes have been optimized and used in several different

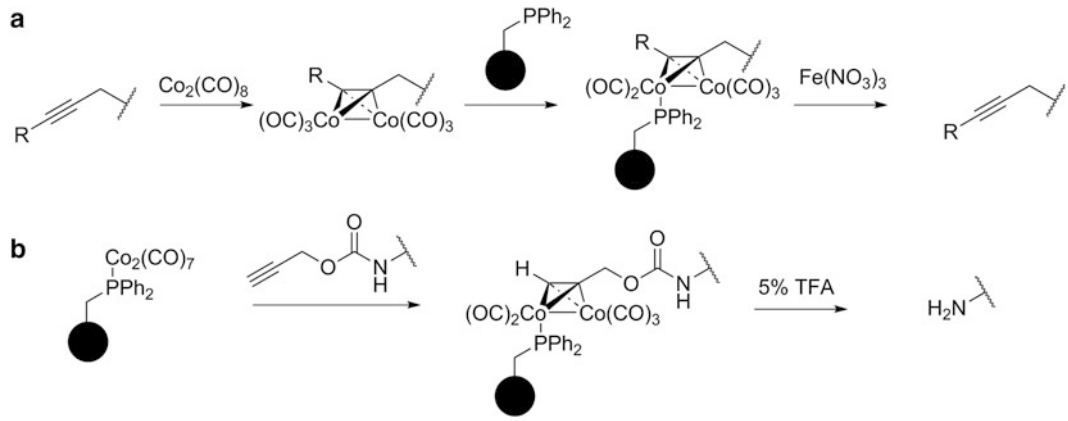


Fig. 5 Cobalt-alkyne complexes for enrichment. (**a**) An internal or terminal alkyne within a lipid or fatty acid is reacted with cobalt octacarbonyl in methanol or another organic solvent. Enrichment takes place by incubation with a phosphine-functionalized resin. Oxidation of cobalt releases the alkyne again. (**b**) A pre-formed cobalt-containing phosphine resin can be used for direct enrichment of propargyl carbamate-conjugated peptides from aqueous environment. Treatment with acid induces a Nicholas reaction and releases a free amine

studies, including bioorthogonal ligation with click chemistry [19, 32, 34–36] and development of isotope-coded tags for relative quantification [38].

3.1 Materials

1. PD-10 gel filtration column (Amersham Biosciences).
2. Ultralink immobilized streptavidin (Pierce).
3. 10× PBS (phosphate buffer saline) (Fisher Scientific).
4. Elution buffer: 100 mM ammonium hydrogen carbonate, 25 mM sodium dithionite ($Na_2S_2O_4$; Sigma-Aldrich, 85%, technical grade. Also called sodium hydrosulfite). The elution buffer should be freshly prepared.
5. Denaturation solution: 50 mM ammonium hydrogencarbonate, 6 M urea.
6. Reduction solution: 50 mM ammonium hydrogencarbonate, 200 mM DTT.
7. Alkylation solution: 50 mM ammonium hydrogencarbonate, 200 mM iodoacetamide.
8. Sequencing grade trypsin (Promega), 12.5 ng/μL solution in 25 mM ammonium hydrogencarbonate (*see* **Note 1**).
9. Vivapure C_{18} spin columns (Sartorius).
10. Mobile phase A: 0.1% formic acid, 99.9% water (MS-grade, Burdick & Jackson).
11. Mobile phase B: 0.1% formic acid, 99.9% acetonitrile (MS-grade, Burdick & Jackson).
12. BioBasic Picofrit C_{18} capillary column (New Objective).

3.2 Methods

1. Diazobenzene cathepsin ABPs are synthesized as described [29]. Tissue or cell lysate is prepared at pH 5.5 (for labeling of cathepsins in lysate), followed by incubation with a stock solution of ABP (1–10 μM final concentration) at room temperature for 30–60 min. After the labeling step, dilute the sample to 2.5 ml with PBS and load it on a PD-10 column to remove free probe. Alternatively, for labeling in whole cells, cells are first labeled with a clickable ABP, such as alkyne-E-64 [19], then lysed at pH 7.4 and click chemistry is performed with an azido-diazobenzene tag. Again, the sample is diluted to 2.5 mL with PBS and loaded on a PD-10 buffer exchange column to remove free click probe. Depending on the sensitivity of the mass spectrometer, 5 mg or less total protein is taken for a single labeling experiment.
2. Wash 50 μL of streptavidin beads with PBS (3 washes with 1 ml of PBS). Between washes, centrifuge streptavidin beads for 1 min at 10,000 × *g*. After each wash discard the supernatant. Mix the protein sample with 50 μL of washed streptavidin beads and incubate with shaking for 1 h at room temperature (*see* **Note 2**). Centrifuge the streptavidin beads in order to separate them from the unbound supernatant (1 min at 10,000 × *g*). Wash streptavidin beads consecutively first with PBS buffer, then with PBS containing 1 M NaCl and with PBS containing 10% EtOH (each wash is done three times with 1 mL of buffer). Stringent washing should remove the majority of nonspecifically bound background proteins. Finally, wash beads with 1 mL of 100 mM ammonium hydrogencarbonate buffer.
3. Elution of labeled proteins is performed in the presence of sodium dithionite. Resuspend streptavidin beads in 20 μL of elution buffer and incubate it at room temperature for 15 min. For complete elution of labeled proteins, repeat the elution step two times and pool the eluted aliquots (total volume is 60 μL).
4. Add an equal amount of 12 M urea solution to the eluted sample (60 μL). Prepare the urea solution in water and heat it until it is completely dissolved.
5. Add 6 μL of reduction solution and incubate the sample for 1 h at room temperature.
6. The sample is Alkynylated by addition of 20 μL of alkylation solution and incubation in the dark for 1 h.
7. Neutralize excess iodoacetamide by addition of an equal amount of reduction solution. Incubate the sample for another hour at room temperature.
8. Decrease the urea concentration by diluting the sample with water (1 ml final volume) and add 10 μL of trypsin solution. Incubate overnight at 37 °C and stop trypsin digestion by addition of formic acid (1% final concentration).

9. Purify the digested peptides on a C_{18} spin column (Sartorius) and concentrate the eluted sample on a Speedvac to a final volume of approximately 10 μL.
10. Set up a chromatography method with a 40 min gradient from 3 to 50 % acetonitrile, with a mobile phase flow of 350 nL/min (*see* **Note 3**). For the separation of tryptic peptides use a C_{18} reverse phase capillary column. Set the MS/MS acquisition method so that the three most intense base peaks in each scan will be chosen for MS/MS fragmentation with dynamic exclusion at repeat count 2 with duration of 2 min.

3.3 Notes

1. Dissolve 20 μg of sequence grade trypsin (Promega) in 40 μL of trypsin resuspension buffer (buffer is supplied with trypsin). Prepare 1 μL aliquots of trypsin and store them at −20 °C until needed. Prior to trypsin digestion, add 40 μL of ice-cold 25 mM ammonium hydrogencarbonate to a trypsin aliquot and store it on ice. Trypsin solution has to be used the same day.
2. With some tissue lysates we have noticed a precipitate formation during the streptavidin binding step. This problem could be alleviated by addition of SDS up to 0.1 % final concentration. The presence of SDS did not interfere with binding of labeled targets to streptavidin
3. LC-MS/MS analysis should be performed with a nanoflow HPLC unit coupled to a mass spectrometry capable of acquiring tandem mass spectra. Typical protein identification is done by a Sequest algorithm using an NCBInr protein database. Peptides with XCorr values over 1.5 (+1 charge), 2 (+2 charge), and 2.5 (+3 charge) and ΔCn value over 0.1 are considered for further evaluation. Final statistical evaluation is done by peptide and protein prophet algorithms. Protein identifications are accepted if they have an over 99 % probability score with at least two identified peptides.

4 Conclusions

The isolation and identification of probe targets plays an important role within ABPP. Cleavable linkers have been developed in order to facilitate this process. The benefits of using a cleavable linker strategy are threefold. First, the elution can take place under milder conditions and with better efficiency than the traditional heat-induced denaturation of (strept)avidin. Second, the covalent modification on the protein is smaller, since part of the probe is cleaved off. This is primarily an advantage for MS-based identification. In tandem MS experiments, biotin can fragment, which may prevent proper identification of the attached peptide. The smaller the covalent modification, the lower the likelihood that complex fragmentation patterns

will occur. Third, the mild cleavage will prevent contamination of the sample by background proteins that are nonspecifically bound to streptavidin or the resin material. This will reduce the number of false-positive protein identifications and simplify further validation of labeled targets.

The cleavable linkers discussed in this chapter are based on a wide range of chemistries. Their implementation into probes may depend on the availability of lab equipment (for synthesis and/or cleavage) and on the specific application. When minor oxidative by-products are nonproblematic, a periodate cleavable linker may be the method of choice. If a new label is to be introduced after elution, a bisaryl hydrazone linker may be appropriate. Very mild conditions can be found within the reductively and enzymatically cleavable linkers. For a small residual tag, a digestible linker or the reversible cobalt-alkyne linkage may be used. Overall, we expect that a variety of these linkers will continue to find new application in chemical proteomics and ABPP.

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Chapter 15

Two-Step Activity-Based Protein Profiling with the Proteasome System as Model of Study

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Abstract

Activity-based protein profiling (ABPP) is a method to highlight enzymatic activities in a biological sample, which uses chemical probes that react covalently with the catalytic nucleophile of the enzyme. To circumvent disadvantages associated with the presence of reporter tags on chemical probes, the probe is equipped with a ligation handle to which the reporter can be reacted at the desired time and place in the ABPP workflow. This chapter demonstrates the power of a triple bioorthogonal ligation strategy which addresses the three activities of the proteasome: the β5-subunit selective norbornene-tagged probe is reacted with fluorescent tetrazine, the β1-selective azide-functionalized probe was addressed with a biotinylated phosphine, followed by an alkyne-substituted pan-reactive probe to label the remaining β2 activity to which an azide-coupled fluorophore was ligated. The result of the triple ligation was similar to each reaction performed separately demonstrating the value of the triple ligation strategy for a single experiment.

Key words Activity-based protein profiling (ABPP), Bioorthogonal chemistry, Proteasome, Staudinger-Bertozzi ligation, Copper(I)-catalyzed azide-alkyne cycloaddition (click reaction), Inverse-electron-demand Diels-Alder reaction

1 Introduction

Activity-based protein profiling (ABPP) is a proteomics technique used for identification, quantification, and enrichment of enzymatic activities. It is widely applied to study a broad range of biological systems in vitro, in cell cultures, and even in animal models [1]. This technique makes use of activity-based probes (ABPs) as tools, which are chemical probes designed to be recognized by the target enzyme(s) and to perform a covalent and irreversible reaction with the active site residue thus labeling only active enzymes. A typical ABP can be divided into three parts, a warhead, a linker, and a reporter tag. The function of the warhead is to react with the enzyme's active site in a covalent and irreversible manner while the linker is designed to target the probe to the desired enzyme(s) thus conferring specificity to the probe normally by mimicking the

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enzyme substrates. Finally an ABP is equipped with a means to identify its target protein. This reporter tag can be a fluorophore, a biotin, an epitope tag, or a combination of these.

ABPP has some advantages compared to other proteomics techniques; the experiments are fast and can be performed with high throughput. It also offers the possibility of doing competitive or comparative studies with different inhibitors. In case of cell permeable ABPs, imaging of live cells in vivo or even in animals makes its applications broader by allowing localization and dynamic studies of the target enzymes.

However, ABPP also has some limitations. The covalent bond between the probe and the target enzyme does not allow the recovering of enzymatic activity in a sample treated with ABP and thus making it difficult to examine the enzyme kinetics. With this technique absolute quantification becomes challenging and only relative quantification is possible by comparing the test samples to control ones [2]. Another possible disadvantage of the so-called one-step ABPP is that the reporter tag is normally a large moiety which might affect the probe properties, like cell permeability, selectivity, affinity, or bioavailability. To overcome these problems a new strategy has become increasingly popular in the last years, named two-step ABPP. In this approach the ABP's reporter tag has been replaced by a ligation handle which will be coupled with the reporter group after the attachment of the probe to its target enzyme. This tactic allows researchers to decide in every condition which reporter group to use depending on the desired method of analysis, which might be an advantage compared to direct labeling by a tagged coupled, one-step ABP. The reaction between the ABP ligation handle and the reporter group needs to be fast and selective, with ideally no side reactions [3].

Since bioorthogonal chemistry has become an important tool in chemical biology research by allowing the performance of selective chemical modifications in complex biological samples, it is suitable for two-step ABPP. The term bioorthogonal stands for a chemoselective reaction that ideally can take place in the aqueous environment of a biological system without any side reactions. The most used reactions include the Staudinger-Bertozzi ligation in which an azide ABP is reacted with a phosphine reagent equipped tag, the copper(I)-catalyzed click reaction between an azide and an alkyne, the copper-free strain-promoted azide-alkyne cycloaddition, and the inverse-electron-demand Diels-Alder reaction of tetrazine with strained alkenes [4–7]. Each of them has some pros and cons. The Staudinger-Bertozzi ligation is found to be selective but the use of the large phosphine moiety may give problems especially in native conditions where the ABP is found inside an enzyme pocket. The copper(I)-catalyzed click reaction is a versatile ligation but the presence of Cu does not allow its application in living cells

or organism. In case of His-tagged proteins, Cu ions might bind to the His-tag and cause precipitation of the protein. Copper may also precipitate other proteins thus losing sample material. The copper-free cycloaddition is a fast and efficient ligation method but, due to the relatively high reactivity of the tags, yields strong side reactions in our lab and it is omitted from this study. The inverse-electron-demand Diels-Alder reaction is very selective under native conditions; it does show side reactions in denaturing conditions. All the ligations can yield side reactions with amino groups or with the thiols groups of cysteines; that's why we recommend exploring each method in your own system and choosing the ligation strategy that fits better your purpose.

Bioorthogonal reactions have been initially used for the study of cell surface glycoproteins [4]; however in the present day they are applied to study a broad range of biological systems, such as post-translational modifications [8] among others. Here we have summarized the main bioorthogonal reactions used in two-step ABPP (activity-based protein profiling) taking the proteasome as model system. Bioorthogonal chemistry can also be applied in tandem ligation strategies, when multiple targets need to be labeled simultaneously. Here we show that labeling of the different proteasome active sites in a tandem ligation strategy is feasible.

2 Materials

Prepare all solutions using ultrapure water (prepared by deionization of water to obtain a resistance of 18 M Ω cm^{-1} at 25 °C) and analytical grade reagents. Dilutions should be made with ultrapure water unless indicated otherwise.

2.1 Cell Culture

1. Culture cells in their specified medium supplemented with 10% Fetal Calf Serum, 10 U/mL penicillin, and 10 μg/mL streptomycin in a 5% CO_2 humidified incubator at 37 °C.
2. Harvest cells, wash them with PBS (2×), and lyse in lysis buffer for 30 min on ice.
3. After centrifugation of the cells for 20 min at 16,000 × *g* and 4 °C, collect the supernatant and determine the protein concentration by Bradford assay.

2.2 Chloroform/Methanol Precipitation (c/m)

Inspired by [9].

1. Add first 400 μL methanol and second 100 μL chloroform to the sample. Vortex vigorously after each addition for few seconds. Add 300 μL water to the mixture to induce phase separation (sample will turn turbid because of protein precipitation) and vortex strongly.

2. Centrifuge the sample for 2 min at 9000×*g*. A protein membrane interphase should be observed at this point between the two liquid phases (the upper phase is the aqueous solution, water, and methanol, and the lower phase is chloroform). Discard the upper phase carefully and add 300 μL methanol. Vortex gently but thoroughly (without disrupting the membrane) until the protein membrane reaches the bottom of the tube.
3. Centrifuge the sample for 2 min at 9000×*g*. Discard the supernatant and dry the protein membrane for max 5 min.

   NOTE: too dry pellets are hard to dissolve

2.3 Buffer Composition

1. Tris buffer stock: 1 M Tris–HCl. For 50 mL solution weight 3.025 g Tris (MW 121.14) in a measuring cylinder and add water up to 40 mL. Dissolve the powder to completion. Adjust the pH to 6.8, 7.5, or 8 (depending which buffer is needed) with 10 M HCl. Adjust the total volume to 50 mL with water.
2. Tris buffer 50 mM: Dilute the 1 M stock with ultrapure water to a final concentration of 50 mM by adding 250 μL 1 M Tris to 4.75 mL water.
3. 3× Laemmli's Buffer: 6% (w/v) SDS, 30% (v/v) glycerol, 150 mM Tris pH 6.8, 0.05% (w/v) bromophenol blue, and 3% (v/v) 2-mercaptoethanol.
4. Lysis buffer: 50 mM Tris–HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 0.025% (w/v) digitonin
5. 8 M urea in 100 mM NH_4-HCO_3: For 25 mL solution weight 12.01 g urea (MW 60.06), add 2.5 mL 1 M NH_4-HCO_3 and adjust volume to 25 mL with MilliQ water. Urea might be a bit hard to dissolve in this volume due to its high concentration. Warming a bit the solution will help dissolving urea.
6. Click cocktail: 50 mM Tris–HCl pH 8.0, 1 mM $CuSO_4$, 100 μM TBTA, and 1 mM ascorbic acid (or 1 mM TCEP). First add $CuSO_4$ to the buffer (solution will turn blue due to the Cu (II)), afterwards add ascorbic acid or TCEP (reducing agents) and vortex the mixture. Solution should turn yellow or colorless due to the reduction of Cu (II) to Cu (I). Add TBTA as a ligand to help the stabilization of the Cu (I) state (make sure the color has changed before adding it to the samples; Cu (I) is the catalyst of the reaction and not Cu (II)).

   NOTE 1: Basic pH (8.0) enhances the click reaction

   NOTE 2: Copper (I) salts (e.g., CuBr or CuI) can also be used, anyway the generation of Cu (I) in situ eliminates the need of a base and of a reducing agent will also react that might get consumed by the oxygen gas dissolved in the solution.

3 Methods

3.1 Staudinger-Bertozzi Ligation

3.1.1 In Vitro

Native Conditions

1. Per experiment, add lysis buffer to 20 μg (total protein) cell lysate to a total volume of 9 μL. Expose the samples to 1 μM azide-ABP (1) (1 μL 10 μM in DMSO) for 1 h at 37 °C.
2. Bring the sample volume up to 50 μL with lysis buffer without digitonin, add 200 μM phosphine (3) (5.5 μL 10× solution in DMSO), and incubate for 1 h at 37 °C.
3. Remove excess reagents by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on a 12.5% SDS-PAGE gel. For biotinylated phosphines detect the labels by strep-HRP hybridization western blotting.

Denaturing Conditions

1. Per experiment, take 50 μg (total protein) cell lysate in a total volume of 18 μL lysis buffer. Expose the samples to 5 μM azide-ABP (1) (2 μL 50 μM in DMSO) for 1 h at 37 °C.
2. Denature the proteins by adding 2 μL 10% (w/v) SDS to a final concentration of 1% and boil the samples for 5 min at 100 °C
3. Perform a c/m precipitation to remove excess reagents.
4. Dissolve the pellet in 15 μL 8 M urea in 50 mM Tris pH 8.0.
5. Bring the sample volume up to 100 μL with lysis buffer without digitonin and add 400 μM phosphine (3) (11 μL 10× solution in DMSO) and incubate for 1 h at 37 °C.
6. Clean by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on a 12.5% SDS-PAGE gel. For biotinylated phosphines, detect the labels by strep-HRP hybridization western blotting.

3.2 Copper(I)-Catalyzed Click Ligation

3.2.1 In Vitro

Native Conditions

1. Per experiment, take 20 μg (total protein) cell lysate in a total volume of 9 μL lysis buffer. Expose the samples to 1 μM azide-ABP (1) (1 μL 10 μM in DMSO) for 1 h at 37 °C.
2. Bring the sample volume to 50 μL with lysis buffer and add 50 μL click cocktail and 400 μM alkyne (4) (5.5 μL 10× solution in DMSO). Incubate the mixture for 1 h at 37 °C. (Note that proteins might precipitate due to Cu(I) but they should solubilize again in 1× Laemmli's buffer (next step)).
3. Clean by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on 12.5% SDS-PAGE. For fluorescent alkynes visualize the labeling in the wet gel slabs using a fluorescent scanner; in the case of biotinylated alkynes detect the labels by strep-HRP hybridization western blotting.

Denaturing Conditions

1. Per experiment, take 50 μg (total protein) cell lysate in a total volume of 18 μL lysis buffer. Expose the samples to 5 μM azide-ABP (1) (2 μL 50 μM in DMSO) for 1 h at 37 °C.
2. Add 2 μL 10% (w/v) SDS to a final concentration of 1% and boil the samples for 5 min at 100 °C followed by a c/m precipitation.
3. Dissolve the pellet in 15 μL 8 M urea in 50 mM Tris pH 8.0.
4. Bring the sample volume to 50 μL with lysis buffer and add 50 μL click cocktail and 400 μM alkyne (4) (5.5 μL 10× solution in DMSO). Incubate the mixture for 1 h at 37 °C.
5. Clean by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on 12.5% SDS-PAGE. For fluorescent alkynes visualize the labeling in the wet gel slabs using a fluorescent scanner; in the case of biotinylated alkynes detect the labels by strep-HRP hybridization western blotting.

3.3 Anticipated Results: Click and Staudinger-Bertozzi Ligation Techniques

The chemical tools used in this study are shown in Fig. 1. A competitive ABPP experiment was performed on Hek293T lysates. Active proteasome subunits were first labeled with the azide-containing ABP (1) and then incubated with fluorescent ABP (2) for residual activity labeling before the bioorthogonal ligations were performed. In either native or denaturing conditions (Fig. 2), both biotin-alkyne (4) (lane A) and biotin-phosphine (3) (lane C) could label ABP1, although click chemistry ligation seems to label

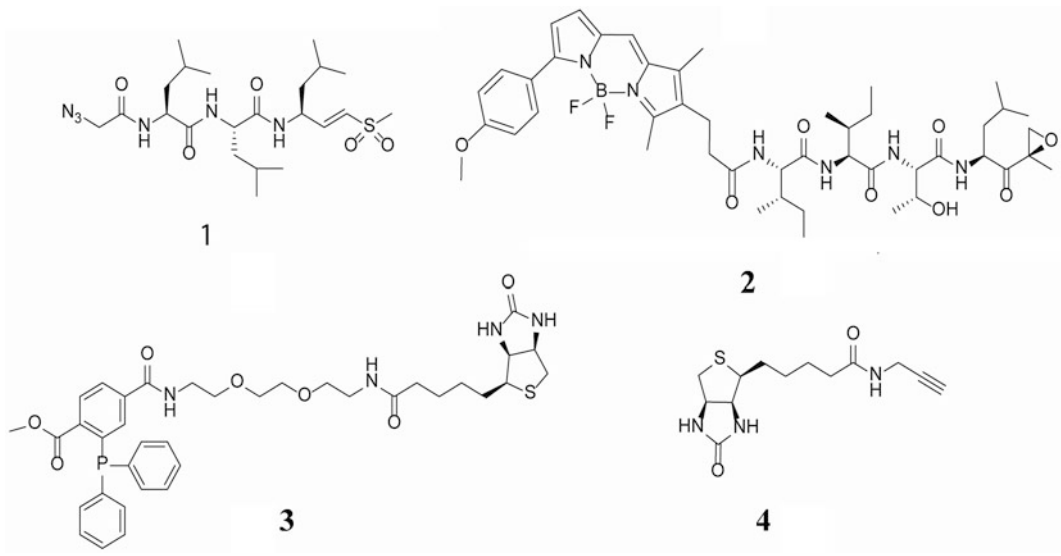

Fig. 1 ABPs and ligation handles used in the Staudinger-Bertozzi ligation and in the Copper(I)-catalyzed Click ligation both for native and denatured conditions. **1**: Azide-Leu_3-vinyl sulfone; **2**: MVB003 (Bodipy-TMR epoxomicin) [10]; **3**: biotin-phosphine [11]; **4**: biotin-alkyne

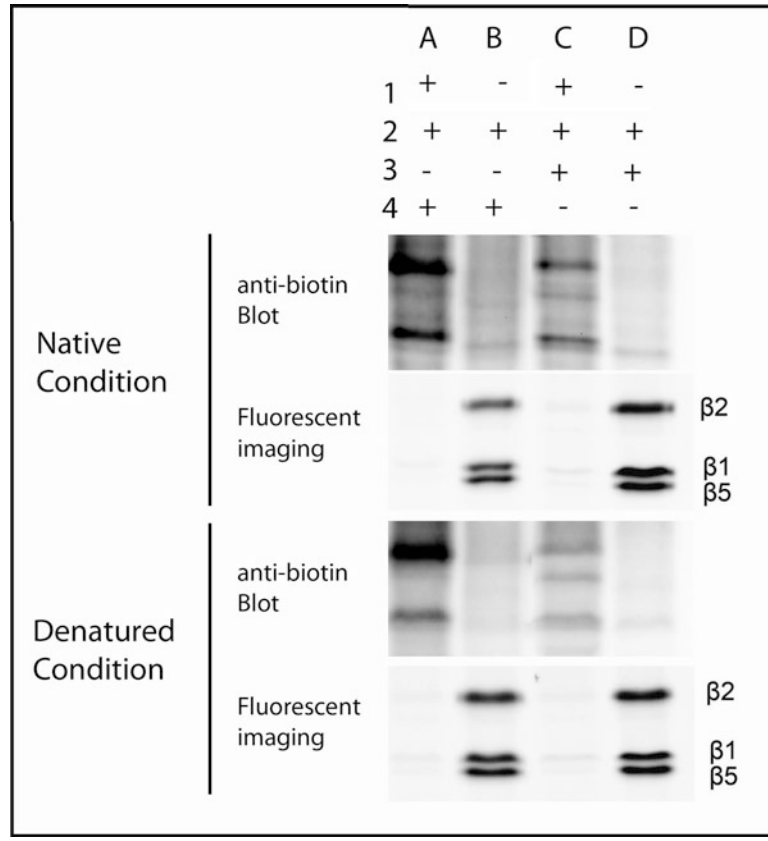

Fig. 2 Anti-biotin blots and in-gel fluorescent imaging to visualize the proteasome labeling patterns. The bioorthogonal ligations were performed on either native proteins (shown in *upper panels*) or denatured proteins (*lower panels*)

the proteasome subunits more efficiently than Staudinger-Bertozzi reaction comparing lane A to C. However a difference in background is observed when only bioorthogonal reagents, but no azide-equipped probes, were added (lane B and D).

3.4 Tetrazine Ligation

3.4.1 In Situ

1. Per experiment, seed around 1×10^6 cells in a 6 cm petri dish and let them grow overnight (only for adherent cells, to let them attach to the coated surface; suspension cells can be treated directly), afterwards remove the medium and expose the cells to 10 μM norbornene-ABP (6) (2 μL 10 mM in DMSO) in 2 mL fresh medium for 3 h at 37 °C.
2. After exposure remove the medium, wash the cells with 3 mL fresh medium for 30 min at 37 °C and then expose them to 50 μM tetrazine (8) (2 μL 50 mM solution in DMSO) in 2 mL fresh medium for 1 h at 37 °C.
3. Next, harvest the cells by scrapping, centrifuge for 5 min at $300 \times g$ and wash with PBS (twice). Flash freeze the cell pellets

in liquid nitrogen (pellets can be stored at −80 °C), lyse in 50 μL lysis buffer, and determine protein concentration.

4. Boil 20 μg (total protein) of cell lysate for 5 min at 100 °C in 1× Laemmli's buffer and resolve on 12.5% SDS-PAGE. For fluorescent tetrazines visualize the labeling in the wet gel slabs using a fluorescent scanner; in the case of biotinylated tetrazines, detect the labels by strep-HRP hybridization western blotting.

3.4.2 In Vitro

1. Per experiment, take 20 μg (total protein) cell lysate in a total volume of 9 μL lysis buffer. Expose the samples to 1 μM norbornene-ABP (6) (1 μL 10 μM in DMSO) for 1 h at 37 °C followed by the addition of 50 μM tetrazine (8) (1.1 μL 500 μM in DMSO) and incubate for 1 h at 37 °C.
2. Clean by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on a 12.5% SDS-PAGE gel. For fluorescent tetrazines visualize the labeling in the wet gel slabs using a fluorescent scanner; in the case of biotinylated tetrazines, detect the labels by strep-HRP hybridization western blotting.

3.5 Triple Ligation

3.5.1 In Vitro

1. Per experiment, take 30 μg (total protein) cell lysate up in 9 μL lysis buffer and expose it to both 1 μM azide-ABP (7) and 5 μM norbornene-ABP (6) (0.5 μL 20× solution in DMSO each) for 1 h at 37 °C followed by incubation with 1 μM alkyne-ABP (5) (0.5 μL 20 μM in DMSO) for 1 h at 37 °C.
2. Dilute the samples with an additional 9 μL lysis buffer followed by addition of 25 μM BodipyTMR-tetrazine (8) and 100 μM biotin-phosphine (3) (0.5 μL 40× solution in DMSO each) and incubate for 1 h at 37 °C.
3. Remove the excess of reagent by buffer exchange to 50 mM Tris pH 7.5 using Millipore Amicon centrifugal filters (3 kDa MWCO). Wash the samples twice with 300 μL 50 mM Tris buffer pH 7.5 and concentrate to a final volume of 60 μL.
4. Add 1 mM $CuSO_4$, 1 mM TCEP, and 100 μM TBTA (0.6 μL 100× solution each) and let the mixtures react with 25 μM BodipyFL-azide (9) (1.5 μL 1 mM in DMSO) for 1 h at 37 °C.
5. Clean by c/m precipitation, take the proteins up in 10 μL 1× Laemmli's buffer, boil for 5 min at 100 °C, and resolve on a 12.5% SDS-PAGE gel. For fluorescent tetrazines visualize the labeling in the wet gel slabs using a fluorescent scanner; in the case of biotinylated tetrazines, detect the labels by strep-HRP hybridization western blotting (Fig. 3).

3.6 Anticipated Results: Triple Ligation Strategy

The chemical tools used in this strategy are shown in Fig. 4. The three active proteasome subunits were labeled with a different tag via one of the different ligation reactions. The β5-subunit selective norbornene-tagged ABP (6) together with the β1-selective

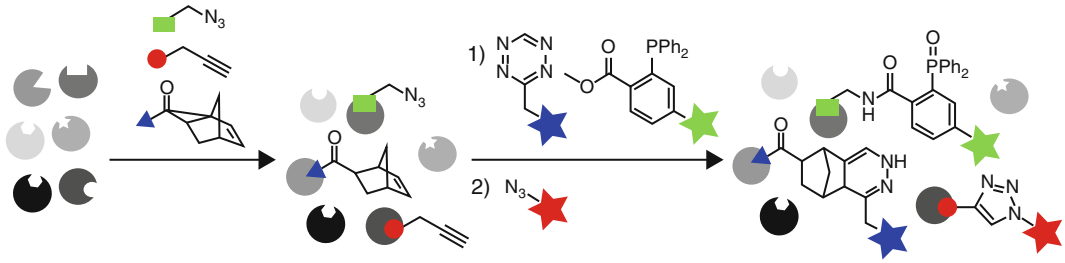

Fig. 3 Schematic workflow of the triple ligation strategy involving copper(I)-catalyzed click reaction, Staudinger-Bertozzi ligation, and tetrazine ligation. Briefly, each subunit is labeled with a different equipped-tag ABP. Next, tetrazine and Staudinger-Bertozzi ligations are performed. Afterwards the click reaction can take place after removal of previous reagents to prevent side reactions. Finally each subunit can be independently visualized due to the different reporter groups on each of the ligation handles

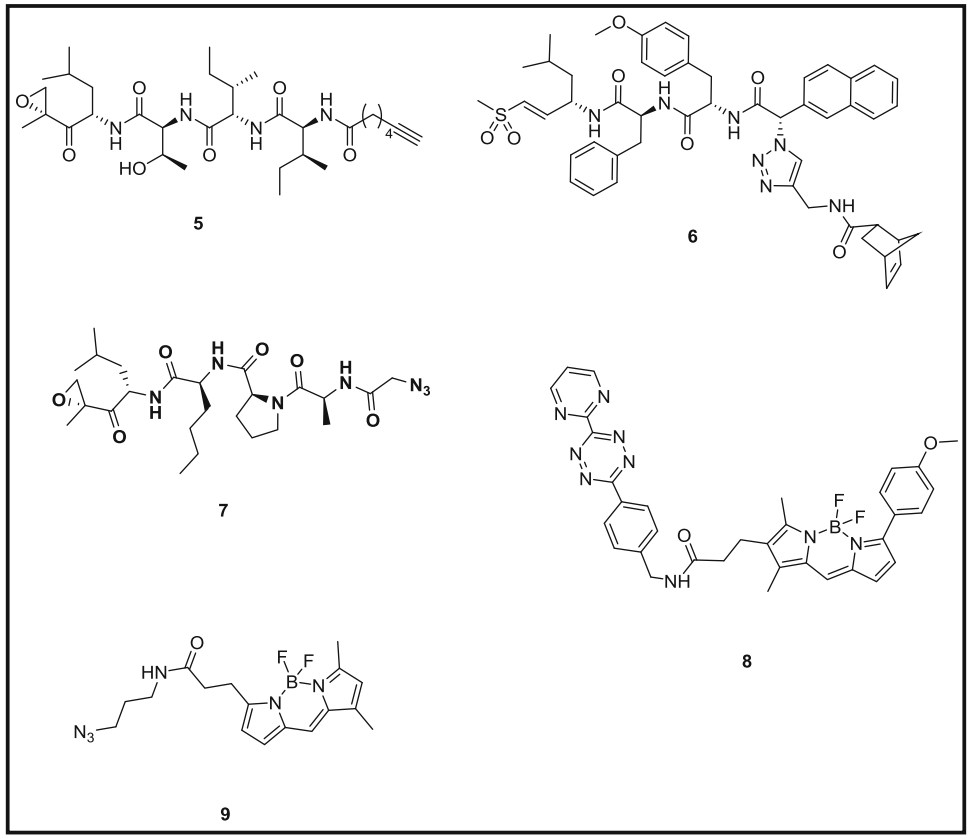

Fig. 4 ABPs and ligation handles used in the triple ligation strategy. **5**: pan-reactive alkyne-epoxomicin; **6**: β5/β5i selective norbornene-equipped vinyl sulfone; **7**: β1/β1i selective azide-equipped epoxyketone; **8**: Tetrazine-BodipyTMR; **9**: Azido-Bodipy FL

azide-functionalized ABP (7) was incubated with HEK293T lysates in order to enable separate labeling of the β-subunits. After exposure to (6) and (7), pan-reactive ABP (5) was added, which could only label the nonoccupied β2-subunits. Using this addition sequence allows us to selectively tag the β5-subunits with a norbornene, the β1-subunits with an azide, and the β2-subunits with an alkyne. Next, the lysates were exposed to tetrazine (8) and phosphine (3) for 1 h, after which the excess of reagents was removed. Afterwards the copper(I)-catalyzed click ligation was performed with azide (9). The washing step in between the ligation reactions is necessary to remove both the excess of tetrazine and phosphine which might react with the cooper catalyst and the azide reagent, respectively. By using two different fluorophores and a biotin tag we can visualize the proteins labeled by each of the ligation reactions.

As it can be seen in Fig. 5, the three ligation methods labeled selectively a single proteasome subunit. The results obtained by performing separate ligation reactions (last three lanes in Fig. 5) are similar to those of the simultaneous triple ligation, showing the value of the triple ligation strategy for a single experiment.

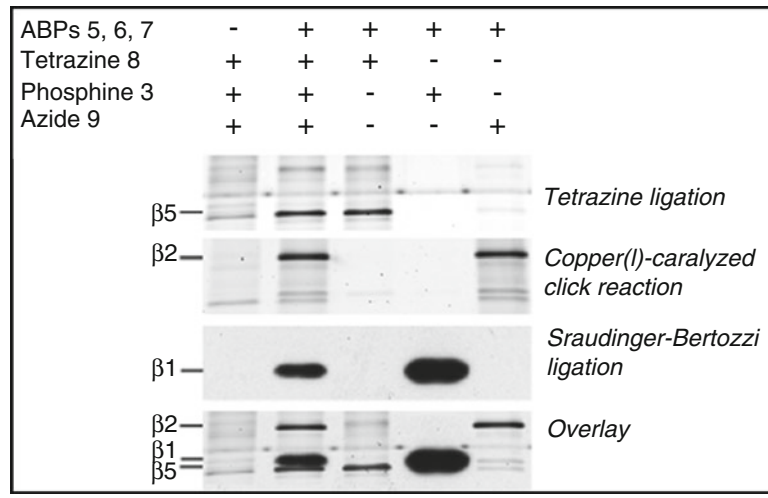

Fig. 5 Anti-biotin blots and in-gel fluorescent imaging to visualize the proteasome labeling patterns in each single reaction and for the simultaneous triple ligation

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