Methods in Molecular Biology 1664

Springer Protocols

Kay Ohlendieck *Editor*

Difference Gel Electrophoresis

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springer.com/series/7651

Difference Gel Electrophoresis

Methods and Protocols

Edited by

Kay Ohlendieck

Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland

Humana Press

Editor
Kay Ohlendieck
Department of Biology
Maynooth University, National University of Ireland
Maynooth, Co. Kildare, Ireland

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-7267-8 ISBN 978-1-4939-7268-5 (eBook)
DOI 10.1007/978-1-4939-7268-5

Library of Congress Control Number: 2017952363

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature
The registered company is Springer Science+Business Media LLC
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

The aim of this new edition of *Difference Gel Electrophoresis* is to provide a comprehensive update of this key method of gel-based proteomics. Two-dimensional gel electrophoresis is one of the most frequently used protein separation techniques in modern biochemistry and proteomics. *Difference Gel Electrophoresis* employs the direct labeling of proteomes with fluorescent dyes prior to large-scale gel electrophoretic separation enabling high-throughput comparative analyses. Labeling of two or more samples with different dyes followed by separation on the same gel system eliminates gel-to-gel variations, making this advanced technique a robust and highly suitable method for proteomic profiling surveys with diverse biological applications. *Difference Gel Electrophoresis* presents an introduction into the development of this method and outlines the principles of differential protein labeling and two-dimensional gel electrophoresis. The bioanalytical integration of the *Difference Gel Electrophoresis* technique into optimized proteomic workflows using advanced mass spectrometry for protein identification is provided, as well as detailed step-by-step protocols for the application of this method in basic biological research and applied biomarker discovery. Essential bioinformatics tools used routinely in systems biology and standard biochemical and cell biological approaches for the independent verification of DIGE-based data sets are described. Detailed protocols of *Difference Gel Electrophoresis* applications are provided for a wide range of systematic proteomic applications in basic biology, pathobiology, and applied biochemistry.

Maynooth, Ireland ***Kay Ohlendieck***

Contents

Contributors

Doreen Ackermann • *Interdisziplinäres Zentrum für Klinische Forschung, IZKF Core Unit Proteomics, University of Münster, Münster, Germany*

Heinrich Brinkmeier • *Institute of Pathophysiology, University Medicine Greifswald, Karlsburg, Germany*

Daniele Capitanio • *Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy*

Andrzej Ciereszko • *Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland*

Diksha Dani • *Physical Biochemistry, Department of Chemistry, Technische Universität Darmstadt, Darmstadt, Germany*

Norbert A. Dencher • *Physical Biochemistry, Department of Chemistry, Technische Universität Darmstadt, Darmstadt, Germany*

Mariola A. Dietrich • *Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland*

Alessio Di Luca • *Department of Agricultural and Food Sciences, School of Agriculture and Veterinary Medicine, University of Bologna, Bologna, Italy*

Andrew Dowd • *Monaghan Biosciences, Tyholland, Co., Monaghan, Ireland*

Paul Dowling • *Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland*

Giuliano Elia • *Philochem AG, Otelfingen, Switzerland*

Cecilia Gelfi • *Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy; U.O. Proteomica clinica, IRCCS Policlinico San Donato, San Donato, Milan, Italy; Istituto di Bioimmagini e Fisiologia Molecolare, CNR, Segrate, Milan, Italy*

Ruth Hamill • *Teagasc, Food Research Centre, Ashtown, Dublin, Ireland*

Abduladim Hmmier • *Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland*

Ashling Holland • *Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK*

Lukas Alfons Huber • *Division of Cell Biology, Biocenter, Innsbruck Medical University, Innsbruck, Austria*

Isao Ishii • *Laboratory of Health Chemistry, Showa Pharmaceutical University, Tokyo, Japan*

Shotaro Kamata • *Laboratory of Health Chemistry, Showa Pharmaceutical University, Tokyo, Japan*

Simone König • *Interdisziplinäres Zentrum für Klinische Forschung, IZKF Core Unit Proteomics, University of Münster, Münster, Germany*

Martin Landsberger • *Institute of Pathophysiology, University Medicine Greifswald, Karlsburg, Germany*

Emilio Marengo • *Department of Sciences and Technological Innovation, University of Piemonte Orientale, Alessandria, Italy*

Paula Meleady • *National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland*

Anne Maria Mullen • *Teagasc, Food Research Centre, Ashtown, Dublin, Ireland*

Rustam R. Mundegar • *Institut für Physiologie II, Rheinische Friedrich-Wilhelms-Universität Bonn, Bonn, Germany*
Sandra Murphy • *Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland*
Jarlath E. Nally • *Infectious Bacterial Diseases, National Animal Disease Center-USDA-ARS, Ames, IA, USA*
Joanna Nynca • *Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland*
Kay Ohlendieck • *Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland*
Elisa Robotti • *Department of Sciences and Technological Innovation, University of Piemonte Orientale, Alessandria, Italy*
Simone Schuller • *Division of Small Animal Internal Medicine, Department of Clinical Veterinary Medicine, University of Bern, Bern, Switzerland*
Taras Stasyk • *Division of Cell Biology, Biocenter, Innsbruck Medical University, Innsbruck, Austria*
Dieter Swandulla • *Institut für Physiologie II, Rheinische Friedrich-Wilhelms-Universität Bonn, Bonn, Germany*
Margit Zweyer • *Institut für Physiologie II, Rheinische Friedrich-Wilhelms-Universität Bonn, Bonn, Germany*

Part I

Introduction

Chapter 1

Two-Dimensional Gel Electrophoresis and 2D-DIGE

Paula Meleady

Abstract

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) continues to be one of the most versatile and widely used techniques to study the proteome of a biological system. In particular, a modified version of 2D-PAGE, two-dimensional difference gel electrophoresis (2D-DIGE), which uses differential labeling of protein samples with up to three fluorescent tags, offers greater sensitivity and reproducibility over conventional 2D-PAGE gels for differential quantitative analysis of protein expression between experimental groups. Both these methods have distinct advantages in the separation and identification of thousands of individual proteins species including protein isoforms and post-translational modifications. This review will discuss the principles of 2D-PAGE and 2D-DIGE including limitations to the methods. 2D-PAGE and 2D-DIGE continue to be popular methods in bioprocessing-related research (particularly on recombinant Chinese hamster ovary cells), which will also be discussed in the review chapter.

Key words Two-dimensional gel electrophoresis, Two-dimensional difference gel electrophoresis, Protein expression profiling, Proteomics

1 General Introduction to Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the oldest and most highly useful methods for protein separation of complex biological mixtures [1]. The technique is based on the ability to separate proteins based on physiochemical properties such as isoelectric point and molecular weight of proteins. In the first dimension, proteins are separated according to their isoelectric point (p*I*) along a pH gradient using immobilized pH gradient (IPG) strips. In the second dimension, denatured proteins are separated according to their molecular weights using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The IPG strips from the first-dimension IEF separation are aligned along the top of a polyacrylamide gel and once charge is applied, proteins migrate from the strip into the gel and are separated based on their size using SDS-PAGE. The result of this process is a 2D gel which, when stained, produces a series of spots, with each spot

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_1,

corresponding to a protein. Commonly used protein stains include Coomassie Brilliant Blue and silver nitrate, both of which are global protein stains. This results in the simultaneous separation of thousands of distinct protein species on the same gel where a protein is resolved based on individual p*I* and molecular weight properties. Comparison of gels allows relative protein quantitation between different samples. This is one of the main reasons why 2D gels are still in use despite the advances in LC-MS-based approaches as 2D gels exhibit the highest resolution at the protein level (often referred to as 'top-down' proteomics) [2]. The method is also highly successful for the separation of protein isoforms and post-translational modifications; these isoforms and modifications are displayed through changes in spot position in the p*I* direction [2].

Advances made in LC-MS/MS instrumentation for protein identification have resulted in a relatively limited technique becoming hugely popular and successful for quantitative proteomic studies [3, 4]. The introduction of 2D difference gel electrophoresis (2D-DIGE) was also a major advancement in the field of gel-based proteomics which resulted in an increase in the reliability of qualitative and quantitative results from 2D gels [5]. 2D-DIGE involves pre-labeling the protein samples with different spectrally resolvable fluorescent dyes (e.g., Cy2, Cy3, and Cy5) so that they can be mixed together, co-separated, and visualized on a single 2D-PAGE gel in order to examine quantitative differential expression of proteins between experimental conditions with statistical confidence [6], while controlling for gel-to-gel variation and other variations of non-biological origin [7]. 2D-DIGE will be discussed in further detail in Subheading 2.

A form of two-dimensional electrophoresis which has gained in popularity over the last few years in functional proteomics is the analysis of membrane proteins and protein complexes using Blue Native Electrophoresis [8] followed by SDS-PAGE [9, 10]. This method involves the binding of the anionic dye Coomassie Brilliant Blue G-250 to the hydrophobic areas of membrane proteins and protein complexes solubilized with mild detergents such as dodecyl maltoside and digitonin. Separation of the negatively charged dye–protein complexes is carried out under native conditions at a neutral pH and these can stay intact during electrophoresis in a polyacrylamide gradient gel. After the run the separation lanes are cut from the gels and applied to an SDS electrophoresis gel where the individual subunits of the complexes migrate and separate according to their size. After staining the gel, the composition of the respective complexes is revealed from the two-dimensional pattern [2]. The method has been used to analyze protein complexes of mitochondria [9], chloroplast membrane protein complexes [10], and integrin and histone complexes in placenta [11].

To date, 2D-PAGE and 2D-DIGE have been successfully applied to the differential proteomic analysis of many types of

biological samples including tissue [4, 12] and biofluids such as serum from cancer patients [13] to better understand the molecular basis of the pathogenesis of disease to identify new biomarkers or new therapeutic targets of disease. The technique has also been applied to try to improve our understanding of the biology of recombinant Chinese hamster ovary cells used in the production of biotherapeutics such as monoclonal antibodies [14]. In order to enhance the production capabilities and efficiency of the recombinant CHO cell line, an increased understanding of cellular physiology of CHO cells using 'omic approaches is of critical importance [15]. Applications of 2D-PAGE and 2D-DIGE in bioprocessing will be discussed in Subheading 3.

2 Two-Dimensional Differential Gel Electrophoresis (2D-DIGE)

2D-DIGE has greatly improved the quantitative analysis of protein changes compared to traditional 2D gels due to increased throughput, ease of use, reproducibility, and accurate quantitation of protein expression differences [5]. This system enables the separation of two or three fluorescently labeled protein samples (Cy2, Cy3, and Cy5) on the same gel, therefore minimizing gel-to-gel variation. The DIGE fluorescent dyes (Cy2, Cy3, and Cy5) are matched for mass and charge but possess distinct excitation and emission spectra [6].

Experimental samples are differentially labeled with up to three of these spectrally resolvable fluorescent dyes and co-resolved on a single 2D gel for direct quantitation. For example, the Cy3 dye can be used to label one sample group and the Cy5 dye to label a second sample. A pool of all samples can then be created and labeled with Cy2 which acts as an internal standard and is used on all gels. All gels can be normalized against the Cy2 standard which results in accurate reproducibility and protein abundance comparisons between gels [6].

2.1 Sample Preparation

It is generally recommended that sample preparation methods should be as simple as possible in order to reduce technical variability. A suitable sample preparation is critical for high-quality accurate and reliable 2D-PAGE results. To reduce the possibility of artificial spots and streaks appearing on the 2D gel, interfering substances (e.g., salts, lipids, etc.) should be removed and protein modifications during sample preparation should be avoided. However, there is no unique way to prepare samples; therefore, each protocol differs depending on the type of sample used. Similarly, sample selection and handling are of utmost importance and should be standardized for each sample used; for example, rapid changes in the phosphoproteome have been reported in tissue samples following collection [16, 17]. The sample preparation method chosen

should be effective in solubilization of all proteins, prevent protein aggregation during focusing, prevent chemical modification of the protein, remove contaminating nucleic acids and other interfering molecules, and yield proteins of interest at detectable levels. The method may also remove high abundant proteins that may mask low abundant proteins which may be of more clinical interest, for example, the removal of high abundant proteins such as serum albumin and immunoglobulins from blood using immuno-depletion techniques [13]. Protein solubilization is achieved by the use of chaotropes, detergents, and reducing agents [18].

2.1.1 Common Chemicals Required for 2D-PAGE

For 2D-PAGE, proteins need to be denatured and solubilized. Urea is a popular uncharged chaotrope used in 2D-PAGE, disrupting hydrogen bonding thereby leading to protein unfolding and denaturation. It is typically used at high concentrations, typically in the range 5–8 M. Thiourea (at 2 M concentrations), when used in conjunction with high concentrations of urea, adds to the power of the solubilization solution. Surfactants are also included in the solubilization buffer as they act synergistically with chaotropes. They prevent hydrophobic interactions, which might occur via chaotrope-generated exposure of hydrophobic domains. These hydrophobic stretches must be protected or protein loss can occur through aggregation and adsorption. Non-ionic or zwitterionic surfactants are commonly used in 2D-PAGE buffers. It has been shown that zwitterionic surfactants such as CHAPS show superior efficiency over non-ionic detergents such as NP-40 and TritonX-100 [19]. Thiol-reducing agents that can break intra- and inter-molecular disulfide bonds are critical for protein solubilization. The most commonly used reducing agents are dithiothreitol (DTT) and dithioerythritol (DTE). Iodoacetamide is subsequently used to remove excess DTT, which is responsible for causing "point streaking" in 2D gels [20]. Carrier ampholytes are also added to help reduce the problem of protein–matrix interactions and in maintaining the pH gradient during IEF. The various buffer components that are recommended for 2D gel analysis are described in detail by Gorg et al. [18].

2.1.2 Sample Disruption and Enrichment of Protein Classes

The sample disruption method used depends on the type of sample being prepared. For example, homogenization techniques may be required for optimal disruption of tissue samples which can be achieved by sample grinding using a pestle and mortar, a dounce homogenizer, or a blade homogenizer.

It may also be required to enrich for certain classes of proteins such as nuclear, membrane, mitochondrial, or proteins with post-translational modifications of interest such as phosphorylation, glycosylation, etc. Subcellular fractionation can enrich cells for analysis and reduce the complexity of the protein mixture [21–23]. Subcellular fractionation often requires large quantities

of biopsy material and therefore may not be applicable to some types of tumors where material can be limiting.

Subcellular fractionation of proteins can be achieved in a number of ways including:

- Isolating the cell compartments and/or organelles (ribosomes, mitochondria, or plasma membrane) by high-speed ultracentrifugation.
- Sequential extraction procedures with increasingly stronger solubilizing buffers (e.g., aqueous buffers, ethanol or chloroform/methanol, and detergent-based extraction solutions).

Human biofluids, including plasma or serum from blood, are an excellent source of candidate biomarkers for various diseases; however, the enormous dynamic range of protein concentrations in biofluids represents a significant analytical challenge for detecting low abundant proteins which may be of clinical benefit. This is due to the high concentration of a number of proteins in serum including albumin and immunoglobulins which contribute to ~90% of the protein concentration of serum. These high abundant proteins therefore have the potential to mask lower abundant proteins that may be of more clinical relevance [24]. Immunoaffinity chromatography techniques have been developed to deplete/remove high abundant proteins when analyzing serum proteomes [13, 25].

2.2 Sample Labeling

2D-DIGE sample labeling with fluorescent Cy dyes is carried out using two principal methods: minimal and saturation labeling.

2.2.1 Minimal Labeling

The 'minimal' labeling 2D-DIGE method is a highly sensitive 2D-gel-based technique with a detection limit of less than 1 fmol per spot and a linear dynamic range of over four orders of magnitude [26, 27]. In comparison, the limit of detection with silver stain is in the region of 1 ng of protein with a dynamic range of no more than two orders of magnitude [28]. This allows for the quantification and detection of minor changes in abundance of proteins between samples [29]. The labeling reaction involves the nucleophilic substitution reaction of the dye-attached N-hydroxysuccinimidyl ester with the epsilon amino group of lysine side chains [26]. The optimal ratio of protein to dye is 50 μg protein:200 pmol dye and should be carried out at pH 8.5 [26]. The labeling reaction is optimized such that the stoichiometry of protein to Cy dye results in only 2–5% of the total number of lysine residues being labeled. The Cy dyes carry an intrinsic charge of +1, such that the p*I* of the protein is preserved upon labeling. The Cy dyes are also mass matched with each labeling event adding approximately 500 Da to the mass of the protein [26].

2.2.2 Saturation Labeling

Saturation labeling is optimally designed for use where sample abundance is limited. In contrast to minimal labeling, saturation labeling dyes react via maleimide with free SH (thiol) groups. Saturation labeling is much more sensitive than minimal labeling where an order of magnitude increase in sensitivity over the original minimal dyes has been reported [30]. This allows for small sample amounts to be used (~5 μg), which is especially useful for scarce samples such as clinical material [30]. Whilst the added sensitivity that these dyes provide is desirable, their use is technically more challenging [31]. For example, the reaction conditions have to be carefully optimized for each type of sample to ensure complete reduction of cysteine residues and a protein:dye ratio sufficient for stoichiometric labeling. Sub-stoichiometric labeling will lead to multiple spots in the second dimension, whereas the use of too much dye may lead to unwanted addition reactions with lysine residues, resulting in the formation of charge trains in the first dimension [31].

2.3 Experimental Design

The most commonly used method for 2D-DIGE analysis involves the use of minimal labeling as described in Subheading 2.2.1. In these experiments, up to three Cy dyes are used: Cy2, Cy3, and Cy5. The Cy3 dye is used to label one sample group and Cy5 to label a second sample group. A pool of all samples can then be created and labeled with Cy2 which acts as an internal standard and is used on all gels. This is commonly referred to as the 'three-dye' approach [29]. All gels can be normalized against the Cy2 standard which results in accurate reproducibility and protein abundance comparisons between gels. As the dye:protein ratio is optimized so that only 3–5% of the protein is labeled the method ensures that proteins with a single dye molecule are visualized, resulting in co-migration of proteins originating from separate samples. Consequently, the same protein labeled with any of the dyes will migrate to the same position on the 2D gel. By using different dyes to separately label proteins isolated from normal or treated cells, proteins can be co-separated and quantitated by scanning gels at three different set of wavelengths, i.e., Cy2 at 488 nm, Cy3 at 532 nm, and Cy5 at 633. The inclusion of a pooled internal standard (Cy2), containing every protein from all samples, is often used to match protein patterns across all of the gels. This feature reduces inter-gel variation, allows normalization of individual experiments and accurate quantification of differences between samples with statistical significance [5, 6]. By including the internal standard on each gel in the experiment with the individual biological samples means that the abundance of each protein spot on a gel can be measured relative to its corresponding spot from the internal standard present on the same gel [32]. This increases the confidence that differences found between samples are due to real

changes, rather than inherent biological variation or experimental variability [33].

It has been demonstrated that one of the drawbacks of the 'three-dye' approach as described above using the Cy2 internal standard is a skewing of p-values toward 1, suggesting that standardized volumes are not truly independent [29, 34]. In this 'two-dye' approach, there is one sample labeled (usually with Cy3) and a pooled internal standard co-resolved (usually labeled with Cy5) [29].

2.4 Data Analysis

Gels are scanned at the appropriate excitation and emission wavelengths using fluorescent scanners or imagers, and the acquired images are imported into a relevant software package for the differential and statistical analysis of the resultant gels from the experimental groups. 2D-DIGE is capable of reliably detecting as low as 0.5 fmol of protein, and protein differences down to ±15%, over a >10,000-fold protein concentration range [27]. Commercially available software for analysis of 2D gels includes PDQuest (BioRad), Decyder (GE Healthcare), Delta2D (Decodon), and Progenesis Samespots (NonLinear Dynamics). These programs accurately detect statistically significant protein abundance changes between the gels and effectively minimize gel-to-gel variation. Statistical evaluation of the data is possible within these software packages, with ANOVA and Student's t-test the most commonly used approaches applied [29]. Differentially expressed proteins of interest are then excised from the gel, subjected to 'in-gel' digestion and identified using mass spectrometry.

2.5 Limitations of 2D-PAGE

The following are the most frequently encountered limitations associated with 2D-GE methodology:

- Low abundant proteins can be very difficult to investigate using 2D-PAGE approaches due to the inherent lack of sensitivity of the technique [35, 36]. It has been shown that only 40% of differentially expressed proteins identified using 2D-DIGE of mouse liver were abundant enough to be identified using mass spectrometry [26]. Poor detection of low abundant proteins can also be due to the presence of higher abundant soluble proteins obscuring their detection. There are mechanisms to try to enrich for low abundant proteins, by utilizing fractionation methods to enrich for protein classes/organelles of interest prior to running out on a 2D gel, e.g., immuno-depletion of high abundant serum proteins [13, 24].
- Poor separation and resolution of hydrophobic proteins. The under-representation of membrane proteins on a 2D gel is caused by their poor solubility in the aqueous buffers used in standard 2D-PAGE [37, 38]. This can prevent the membrane proteins from entering the IEF gel or aggregating at their

isoelectric points [39]. In certain cases, membrane proteins can be identified by using mild detergents such as oligooxyethylene, sulfobetaine, dodecyl maltoside, and decaethylene glycol mono hexadecyl, as the use of strong detergents like SDS interfere with the isoelectric focusing of proteins [40].

- There are difficulties associated with separating and detecting larger proteins with molecular masses >150 kDa using 2D-PAGE methods [35].
- Proteins with extremes of pH are difficult to separate using 2D-PAGE, in particular, those with p*I* values <4 or >9 [35].
- Co-migration of proteins to the same spot is also a major issue in 2D-PAGE, with proteins of similar isoelectric points and denatured molecular weight becoming focused at the same position of the gel. This makes it impossible to accurately determine the relative abundance of an individual protein within a mixed spot [35, 41].
- Automation and high-throughput analysis. Despite improvements in the field of 2D-PAGE resulting in increased high-throughput and reproducibility the method remains time-consuming, labor-intensive, and a poorly automated technology. For example, for an optimized 2D-DIGE workflow applied to serum proteomics it still takes at least 1 week from serum sample preparation to the software analysis of resultant 2D gel images, which is not compatible with acute diagnostics in a clinical setting [13].

3 Application of 2D Gel Electrophoresis in Bioprocessing

Chinese hamster ovary (CHO) cells are the most commonly used host cell line for the production of biopharmaceuticals because of their ability to correctly fold and post-translationally modify recombinant proteins that are compatible with human use. In order to enhance the production capabilities and efficiency of the host cell line, an increased understanding of cellular physiology of CHO cells is of critical importance. Our group and others have used 2D-PAGE and 2D-DIGE approaches to study recombinant Chinese hamster ovary cells in order to gain a greater understanding of the biology of these cells in bioprocess-relevant conditions relating to growth and productivity [14, 42–49]. Such studies may give insights for genetic intervention to possibly create better host cell lines for more efficient protein production or even to provide clues to more rational strategies for cell line and process development [15]. Figure 1 shows a representative 2D-DIGE gel of a Cy3-labeled CHO-K1 cell lysate.

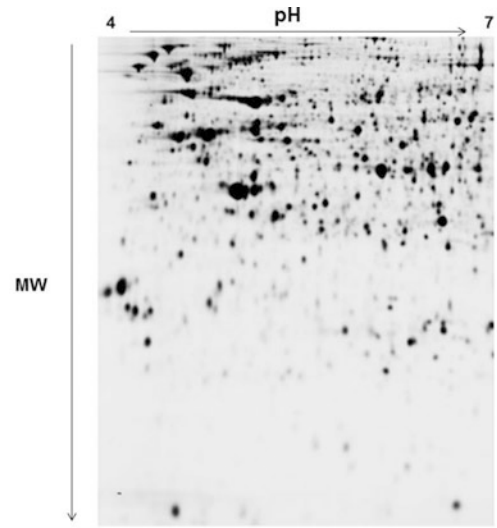

Fig. 1 Representative 2D-DIGE gel from a CHO-KI cell lysate separated in the first-dimension IEF using IPG 4–7 strips

An interesting application of 2D-PAGE and 2D-DIGE in bioprocessing is in the analysis of host cell protein clearance during downstream purification of recombinant biotherapeutics such as monoclonal antibodies [50–54]. Recovery of recombinant protein products that have been produced from cultured mammalian cell systems such as Chinese hamster ovary cells starts with harvest of the cell culture fluid followed by a series of downstream processing steps to purify the recombinant product of interest but also to remove process-related impurities such as host cell proteins (HCPs), DNA, and lipids. These impurities have the potential to be immunogenic in the patient [55, 56]. Enzyme-linked immunosorbent assays (ELISA) are the most commonly used assays for detecting HCPs during downstream processing and in the final product; however, a key limitation of this technique is that non-immunoreactive or weakly immunoreactive proteins will not be detected using this method [57].

During downstream processing, 2D-PAGE has been used for monitoring and identification of HCPs post-protein A chromatography [54]. 2D-PAGE in conjunction with shotgun proteomics has also been used to analyze specific difficult-to-remove HCPs from non-affinity chromatographic resins which are commonly used in the polishing steps of monoclonal antibody purification following Protein A chromatography [58]. In this study, eight HCPs were identified that interacted with two monoclonal antibodies in hydrophobic interaction chromatography (HIC) conditions [58].

2D-PAGE and 2D-DIGE have also been used to investigate changes to the HCP proteome during upstream cultivation of

recombinant CHO cells. The impact of upstream process parameters such as cell culture media, bioreactor control strategy, feeding strategy, and cell culture duration/cell viability on the HCP profile was investigated and it was found that cell viability had the biggest effect on HCP profile [59]. 2D-PAGE and LC-MS/MS were also used to investigate HCP over culture duration and it was shown that the majority of HCPs in the supernatant of the cell lines was through lysis or breakage of cells, again associated with a loss in cellular viability [60].

4 Conclusion

Despite a decrease in popularity over the past few years due to the advances made in LC-MS-based quantitative proteomics, 2D-PAGE (particularly 2D-DIGE) continues to be a widely used technique to study the proteome of many cellular systems [2, 39] and should continue to be for future years to come.

References

1. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021
2. Westermeier R (2016) 2D gel-based proteomics: there's life in the old dog yet. Arch Physiol Biochem 122:236–237
3. Rabilloud T (2002) Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics 2:3–10
4. Murphy S, Dowling P, Ohlendieck K (2016) Comparative skeletal muscle proteomics using two-dimensional gel electrophoresis. Proteomes 4(3):E27
5. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
6. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3:36–44
7. Kondo T, Hirohashi S (2009) Application of 2D-DIGE in cancer proteomics toward personalized medicine. Methods Mol Biol 577:135–154
8. Schägger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 199(2):223–231
9. Sunderhaus S, Eubel H, Braun HP (2007) Two-dimensional blue native/blue native polyacrylamide gel electrophoresis for the characterization of mitochondrial protein complexes and supercomplexes. Methods Mol Biol 372:315–324
10. Kikuchi S, Bédard J, Nakai M (2011) One- and two-dimensional blue native-PAGE and immunodetection of low-abundance chloroplast membrane protein complexes. Methods Mol Biol 775:3–17
11. Wang F, Wang L, Xu Z, Liang G (2013) Identification and analysis of multi-protein complexes in placenta. PLoS One 8(4):e62988
12. Linge A, Kennedy S, O'Flynn D, Beatty S, Moriarty P, Henry M, Clynes M, Larkin A, Meleady P (2012) Differential expression of fourteen proteins between uveal melanoma from patients who subsequently developed distant metastases versus those who did not. Invest Ophthalmol Vis Sci 53:4634–4643
13. Strohkamp S, Gemoll T, Habermann JK (2016) Possibilities and limitations of 2DE-based analyses for identifying low-abundant tumor markers in human serum and plasma. Proteomics 16:2519–2532
14. Meleady P, Doolan P, Henry M, Barron N, Keenan J, O'Sullivan F, Clarke C, Gammell P, Melville MW, Leonard M, Clynes M (2011) Sustained productivity in recombinant Chinese hamster ovary (CHO) cell lines: proteome

analysis of the molecular basis for a process-related phenotype. BMC Biotechnol 11:78

15. Kildegaard HF, Baycin-Hizal D, Lewis NE, Betenbaugh MJ (2013) The emerging CHO systems biology era: harnessing the 'omics revolution for biotechnology. Curr Opin Biotechnol 24:1102–1107
16. Mertins P, Yang F, Liu T, Mani DR, Petyuk VA, Gillette MA, Clauser KR, Qiao JW, Gritsenko MA, Moore RJ, Levine DA, Townsend R, Erdmann-Gilmore P, Snider JE, Davies SR, Ruggles KV, Fenyo D, Kitchens RT, Li S, Olvera N, Dao F, Rodriguez H, Chan DW, Liebler D, White F, Rodland KD, Mills GB, Smith RD, Paulovich AG, Ellis M, Carr SA (2014) Ischemia in tumors induces early and sustained phosphorylation changes in stress kinase pathways but does not affect global protein levels. Mol Cell Proteomics 13:1690–1704
17. Espina V, Edmiston KH, Heiby M, Pierobon M, Sciro M, Merritt B, Banks S, Deng J, VanMeter AJ, Geho DH, Pastore L, Sennesh J, Petricoin EF 3rd, Liotta LA (2008) A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. Mol Cell Proteomics 7:1998–2018
18. Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685
19. Molloy MP (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal Biochem 280:1–10
20. Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037–1053
21. Lee YH, Tan HT, Chung MC (2010) Subcellular fractionation methods and strategies for proteomics. Proteomics 10:3935–3956
22. Drissi R, Dubois ML, Boisvert FM (2013) Proteomics methods for subcellular proteome analysis. FEBS j 280:5626–5634
23. Kumar A, Baycin-Hizal D, Shiloach J, Bowen MA, Betenbaugh MJ (2015) Coupling enrichment methods with proteomics for understanding and treating disease. Proteomics Clin Appl 9:33–47
24. Dowling P, Meleady P, Henry M, Clynes M (2010) Recent advances in clinical proteomics using mass spectrometry. Bioanalysis 2:1609–1615
25. Wu C, Duan J, Liu T, Smith RD, Qian WJ (2016) Contributions of immunoaffinity chromatography to deep proteome profiling of human biofluids. J Chromatogr B Analyt Technol Biomed Life Sci 1021:57–68
26. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396
27. Viswanathan S, Unlü M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358
28. Lilley KS, Razzaq A, Dupree P (2002) Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. Curr Opin Chem Biol 6:46–50
29. Arentz G, Weiland F, Oehler MK, Hoffmann P (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9:277–288
30. Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, Tonge R (2003) Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. Proteomics 3:1181–1195
31. Arnold GJ, Fröhlich T (2012) 2D DIGE saturation labeling for minute sample amounts. Methods Mol Biol 854:89–112
32. Lilley KS, Friedman DB (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 1:401–409
33. Tannu NS, Hemby SE (2006) Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. Nat Protoc 1:1732–1742
34. Karp NA, McCormick PS, Russell MR, Lilley KS (2007) Experimental and statistical considerations to avoid false conclusions in proteomics studies using differential in-gel electrophoresis. Mol Cell Proteomics 6:1354–1364
35. Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc Natl Acad Sci U S A 97:9390–9395
36. Bunai K, Yamane K (2005) Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives. J Chromatogr B Analyt Technol Biomed Life Sci 815:227–236
37. Santoni V, Molloy M, Rabilloud T (2000) Membrane proteins and proteomics: un amour impossible? Electrophoresis 21(6):1054–1070
38. Rabilloud T (2009) Membrane proteins and proteomics: love is possible, but so difficult. Electrophoresis 30(Suppl 1):S174–S180
39. Westermeier R (2014) Looking at proteins from two dimensions: a review on five decades of 2D electrophoresis. Arch Physiol Biochem 120:168–172

40. Luche S, Santoni V, Rabilloud T (2003) Evaluation of nonionic and zwitterionic detergents as membrane protein solubilizers in two-dimensional electrophoresis. Proteomics 3:249–253
41. Wu WW, Wang G, Baek SJ, Shen RF (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. J Proteome Res 5:651–658
42. Meleady P, Henry M, Gammell P, Doolan P, Sinacore M, Melville M, Francullo L, Leonard M, Charlebois T, Clynes M (2008) Proteomic profiling of CHO cells with enhanced rhBMP-2 productivity following co-expression of PACEsol. Proteomics 8:2611–2624
43. Kumar N, Gammell P, Meleady P, Henry M, Clynes M (2008) Differential protein expression following low temperature culture of suspension CHO-K1 cells. BMC Biotechnol 8:42
44. Doolan P, Meleady P, Barron N, Henry M, Gallagher R, Gammell P, Melville M, Sinacore M, McCarthy K, Leonard M, Charlebois T, Clynes M (2010) Microarray and proteomics expression profiling identifies several candidates, including the valosin-containing protein (VCP), involved in regulating high cellular growth rate in production CHO cell lines. Biotechnol Bioeng 106:42–56
45. Baik JY, Lee GM (2010) A DIGE approach for the assessment of differential expression of the CHO proteome under sodium butyrate addition: effect of Bcl-x(L) overexpression. Biotechnol Bioeng 105:358–367
46. Kim JY, Kim YG, Lee GM (2012) Differential in-gel electrophoresis (DIGE) analysis of CHO cells under hyperosmotic pressure: osmoprotective effect of glycine betaine addition. Biotechnol Bioeng 109:1395–1403
47. Blondeel EJ, Ho R, Schulze S, Sokolenko S, Guillemette SR, Slivac I, Durocher Y, Guillemette JG, McConkey BJ, Chang D, Aucoin MG (2016) An omics approach to rational feed: enhancing growth in CHO cultures with NMR metabolomics and 2D-DIGE proteomics. J Biotechnol 234:127–138
48. Darja O, Stanislav M, Saša S, Andrej F, Lea B, Branka J (2016) Responses of CHO cell lines to increased pCO2 at normal (37 °C) and reduced (33 °C) culture temperatures. J Biotechnol 219:98–109
49. Wingens M, Gätgens J, Schmidt A, Albaum SP, Büntemeyer H, Noll T, Hoffrogge R (2015) 2D-DIGE screening of high-productive CHO cells under glucose limitation–basic changes in the proteome equipment and hints for epigenetic effects. J Biotechnol 201:86–97
50. Tscheliessnig AL, Konrath J, Bates R, Jungbauer A (2013) Host cell protein analysis in therapeutic protein bioprocessing–methods and applications. Biotechnol J 8:655–670
51. Grzeskowiak JK, Tscheliessnig A, Toh PC, Chusainow J, Lee YY, Wong N, Jungbauer A (2009) Two-dimensional fluorescence difference gel electrophoresis for comparison of affinity and non-affinity based downstream processing of recombinant monoclonal antibody. J Chromatogr A 1216:4902–4912
52. Grzeskowiak JK, Tscheliessnig A, Toh PC, Chusainow J, Lee YY, Wong N, Jungbauer A (2009) 2-D DIGE to expedite downstream process development for human monoclonal antibody purification. Protein Expr Purif 66:58–65
53. Grzeskowiak JK, Tscheliessnig A, MW W, Toh PC, Chusainow J, Lee YY, Wong N, Jungbauer A (2010) Two-dimensional difference fluorescence gel electrophoresis to verify the scale-up of a non-affinity-based downstream process for isolation of a therapeutic recombinant antibody. Electrophoresis 31:1862–1872
54. Hogwood CE, Tait AS, Koloteva-Levine N, Bracewell DG, Smales CM (2013) The dynamics of the CHO host cell protein profile during clarification and protein A capture in a platform antibody purification process. Biotechnol Bioeng 110:240–251
55. Bailey-Kellogg C, Gutiérrez AH, Moise L, Terry F, Martin WD, De Groot AS (2014) CHOPPI: a web tool for the analysis of immunogenicity risk from host cell proteins in CHO-based protein production. Biotechnol Bioeng 111:2170–2182
56. Hogwood CE, Bracewell DG, Smales CM (2014) Measurement and control of host cell proteins (HCPs) in CHO cell bioprocesses. Curr Opin Biotechnol 30:153–160
57. Champion K, Madden H, Dougherty J, Shacter E (2005) Defining your product profile and maintaining control over it, part 2. Bioprocess Int 3:52–57
58. Levy NE, Valente KN, Lee KH, Lenhoff AM (2016) Host cell protein impurities in chromatographic polishing steps for monoclonal antibody purification. Biotechnol Bioeng 113:1260–1272
59. Jin M, Szapiel N, Zhang J, Hickey J, Ghose S (2010) Profiling of host cell proteins by two-dimensional difference gel electrophoresis (2D-DIGE): implications for downstream process development. Biotechnol Bioeng 105:306–316
60. Tait AS, Hogwood CE, Smales CM, Bracewell DG (2012) Host cell protein dynamics in the supernatant of a mAb producing CHO cell line. Biotechnol Bioeng 109:971–982

Part II

Difference Gel Electrophoresis

Chapter 2

Comparative DIGE Proteomics

Kay Ohlendieck

Abstract

Gel-based proteomics has been widely used for the systematic cataloging of the protein constituents of defined biofluids, purified organelles, individual cell types, heterogeneous tissues and isolated organs, as well as being applied to comparative biochemical and biomedical analyses of complex biological specimens. Of the many electrophoretic techniques used in modern biochemical approaches, large-scale protein separation by difference gel electrophoresis (DIGE) has established itself as the most powerful analytical tool in comparative proteomics. Both 2-dye and 3-dye fluorescence systems with minimal or saturation labeling are routinely used. This chapter briefly describes the technical advantages of the pre-electrophoretic fluorescent labeling technique and discusses the bioanalytical usefulness of this highly successful electrophoretic method.

Key words CyDye, Difference gel electrophoresis, Difference in-gel electrophoresis, DIGE, Fluorescence labeling, Gel electrophoresis, Mass spectrometry, Proteomics, Two-dimensional gel electrophoresis

1 Introduction

The successful isolation, identification, and detailed characterization of individual protein species, complex protein assemblies, organellar subproteomes, or entire proteomes by analytical biochemistry rely heavily on the appropriate combination of a variety of standardized techniques. The routine workflow in many protein biochemical and proteomic studies includes: (1) the efficient extraction of proteins with widely differing physicochemical properties from complex biological samples, (2) the careful protection of sensitive proteins from degradation during lengthy isolation procedures, (3) the basic separation of structurally or functionally related protein populations by relatively crude subcellular fractionation methods such as differential or density gradient centrifugation, (4) the refined separation of different protein classes by the systematic application of large-scale methods such as gel

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_2,

electrophoresis, liquid chromatography, and/or affinity methods, (5) the reliable identification of representative peptides, individual protein fragments or intact proteins by mass spectrometry or antibody-based techniques, (6) the swift determination of protein concentration and isoform expression patterns, and (7) the comprehensive determination of post-translational modifications [1–3].

Over the last few decades of bioresearch, gel electrophoresis has played a central role in both preparative and analytical terms [4] and has been widely employed for efficient peptide and protein separation in the more recently established field of mass spectrometry-based proteomics [5–7]. Although a variety of liquid chromatographic methods are now frequently incorporated in proteomic studies, gel electrophoretic techniques are at the core of modern proteomics and routinely employed in the systems biological analysis of proteome-wide changes and adaptations in health and disease [8–10]. Many different electrophoretic approaches are employed in analytical biochemistry, whereby difference gel electrophoresis (DIGE) presents one of the most powerful comparative techniques in modern proteomics [11]. Using particular DIGE-based methods, the efficient gel electrophoretic separation of proteins is mostly based on natural or modified differences in overall charge between individual polypeptide chains, as well as dissimilarities in molecular size under native or denatured conditions. This chapter briefly outlines the development, technical advances, and applications of DIGE in modern proteomics.

2 Gel-Based Proteomics and DIGE Analysis

In general, gel electrophoretic techniques can be distinguished based on the dimensionality of the gel system and the main labeling procedure for the visualization of protein bands or spot patterns. Routinely used one-dimensional gel electrophoretic methods for the separation of proteins include isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis [4]. A combination of both techniques in two-dimensional gel electrophoresis is significantly more efficient for large-scale protein separation approaches due to its huge capacity and high resolution, making it a frequently used method in high-throughput proteomic surveys [8]. Depending on the particular gel electrophoretic technique and the analyzed samples, several hundred to thousands of individual protein spots can be visualized by standardized two-dimensional gel electrophoresis [5]. Besides optimizing the electrophoretic procedure, protein labeling is a crucial step in gel-based analytical proteomics. Standard post-electrophoretic staining approaches include Coomassie Brilliant Blue, silver, and a variety of fluorescent dyes. For the efficient pre-electrophoretic comparative labeling of

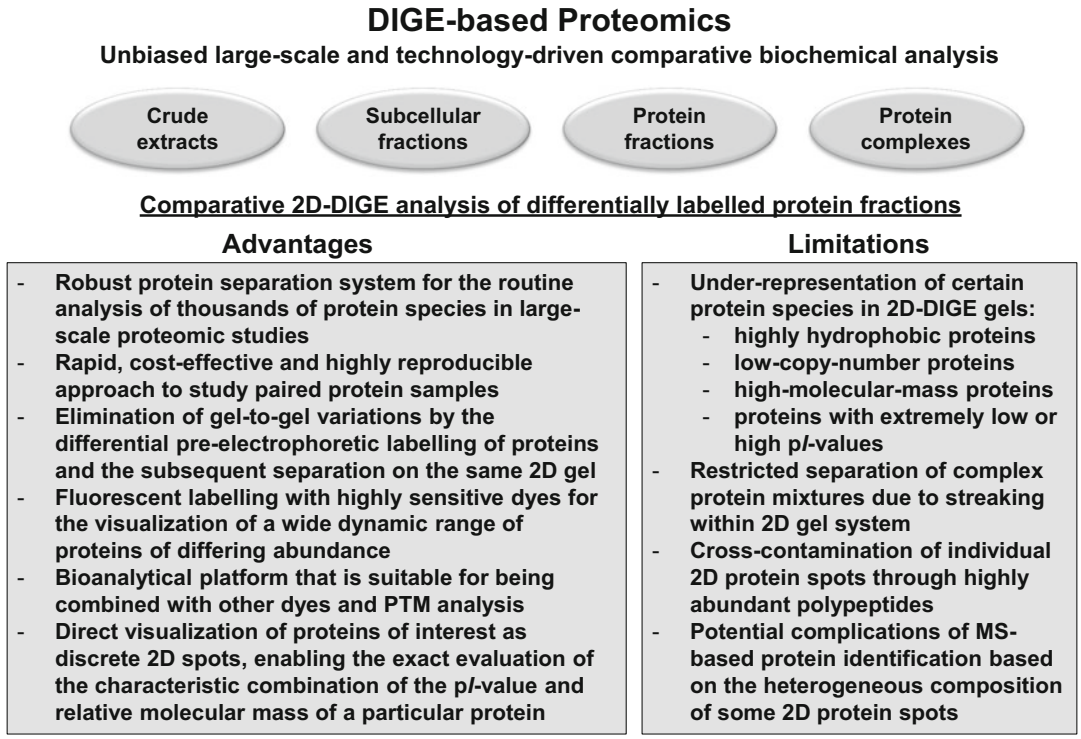

Fig. 1 Overview of the advantages versus technical limitations of DIGE analysis. Listed are advantages versus technical limitations of the comparative difference gel electrophoresis (DIGE) analysis of pre-electrophoretically labeled protein fractions

different protein samples, fluorescence DIGE analysis represents one of the most powerful methods [12]. The DIGE method can be carried out with 2-CyDye or 3-CyDye systems to differentially label proteins belonging to dissimilar protein populations [13].

General advantages versus technical limitations of DIGE analyses are summarized in Fig. 1. The advantages of DIGE over other protein separation methods are its robustness for the routine analysis of thousands of protein species, the cost-effective and highly reproducible nature of fluorescence gel electrophoresis, the elimination of gel-to-gel variations and the direct visualization of a wide dynamic range of proteins of differing abundance, as well as the capability of being combined with other protein staining methods and post-translational modification analysis. Potential limitations are presented by the possible under-representation of certain protein species in DIGE gels, including highly hydrophobic proteins, low-copy-number proteins, high-molecular-mass proteins and protein species with extremely low or high p*I*-values, the restricted separation of complex protein mixtures, and the cross-contamination of individual protein spots through highly abundant polypeptides [14].

3 Proteomic DIGE Workflow

A typical DIGE-based proteomic workflow for the comparative analysis of differing proteomes involves several critical steps, including (1) efficient protein extraction from samples derived from defined biological specimens, such as biofluids, tissue extracts, or subcellular fractions, (2) the pre-electrophoretic fluorescent labeling employing 2-dye or 3-dye fluorescence systems with minimal or saturation labeling, (3) effective protein separation by two-dimensional gel electrophoresis using optimized combinations of isoelectric focusing gels and second-dimension slab gels, (4) densitometric scanning of fluorescent images using computer-assisted analysis for the generation of meaningful proteomic maps, (5) optimized protein digestion for the generation of representative peptide signatures, such as the consecutive application of the enzymes Lys-C and trypsin for the controlled and highly reproducible production of peptide populations, and (6) the proteomic identification of individual proteins of interest by sensitive mass spectrometric analysis [15]. Figure 2 outlines the usage of 2-dye versus 3-dye systems in

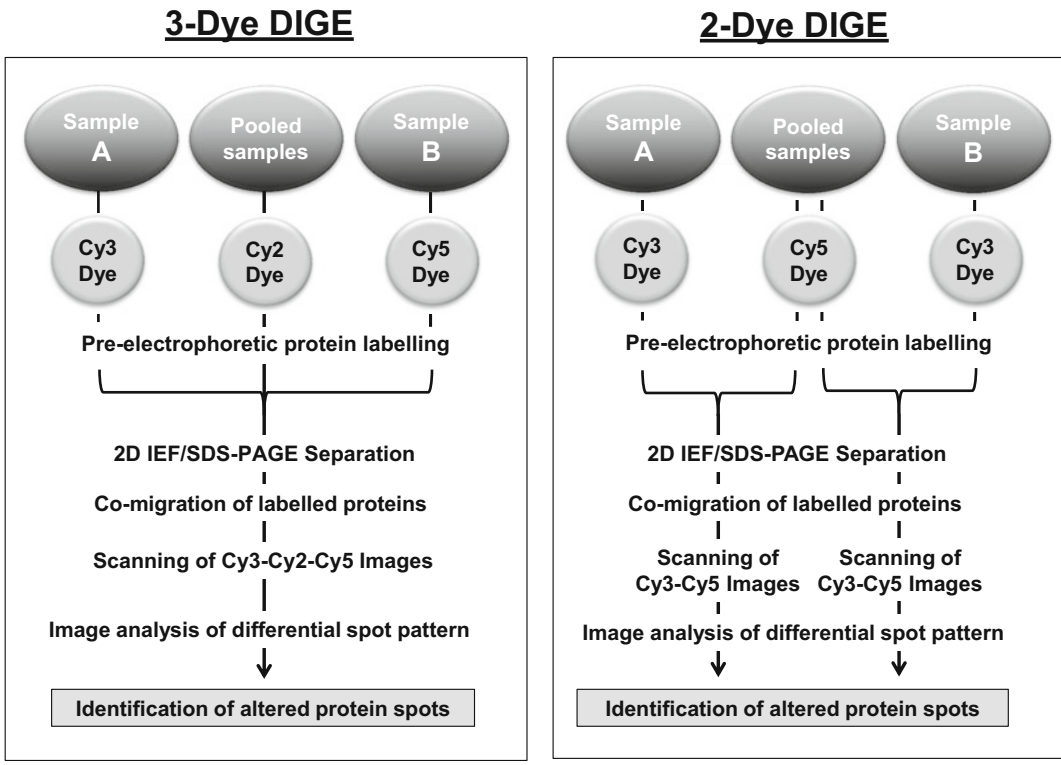

Fig. 2 Outline of 2-dye versus 3-dye DIGE analysis. Shown are flowcharts of routine comparative difference gel electrophoresis (DIGE) analyses of pre-electrophoretically labeled protein fractions using fluorescent Cy2, Cy3, and Cy5 dyes

comparative DIGE studies. Often DIGE-generated data are independently verified by routine biochemical, cell biological or biophysical assays, including immunoblotting, enzyme-linked immunosorbent assays, enzyme testing, binding assays, physiological measurements, and/or immunofluorescence microscopy.

4 DIGE Labeling Approaches

The comparative DIGE method has been applied in a variety of biological and biomedical research areas, including microbiology, environmental sciences, plant science, animal science, biomedicine, pharmaco-proteomics, and biomarker research, to improve diagnostics, prognostics, therapy-monitoring, and the evaluation of potentially harmful side effects [14]. Routine DIGE analyses are usually carried out with two different approaches, i.e., minimal or saturation protein labeling employing the pre-electrophoretic labeling with fluorescent 2-dye or 3-dye systems [16–18]. The method was originally developed by Unlü et al. [19] and is now one of the most frequently used gel-based techniques of comparative proteomics. Sophisticated 2D-DIGE software analysis tools are available to study paired protein samples [20] and are extremely useful for the routine quantitative analysis of multiple fluorescently labeled protein populations following high-resolution and two-dimensional gel electrophoretic separation [21].

Comparative DIGE experiments are routinely carried out with two different dye labeling approaches, i.e., minimal labeling versus saturation labeling. Major differences in the two methods are diagrammatically summarized in Fig. 3. Minimal labeling chemistry is based on N-hydroxy succinimidyl ester dye reagents that form sub-stochiometric bonds with the ε-amine groups of assessable lysine side chains. In contrast to the minimal dye coupling process that involves a nucleophilic substitution reaction, saturation labeling is performed with maleimide dye reagents and the complete labeling of all cysteine sulfhydryls [14]. A 3-sample comparison is shown in Fig. 4, summarizing the labeling approach with the least number of gels to achieve the evaluation of reverse labeling, sample pairing, and biological repeats. If two sets of six DIGE gels are run in parallel, this 12-gel DIGE system can be electrophoresed in the same buffer tank and achieve optimum results in relation to biological repeats ($n = 4$), sample pairing ($n = 2$), technical repeats ($n = 2$), and reverse fluorescent dye labeling ($n = 2$), as previously described for the comparison of normal, diseased, and therapeutically treated diaphragm muscle specimens [22].

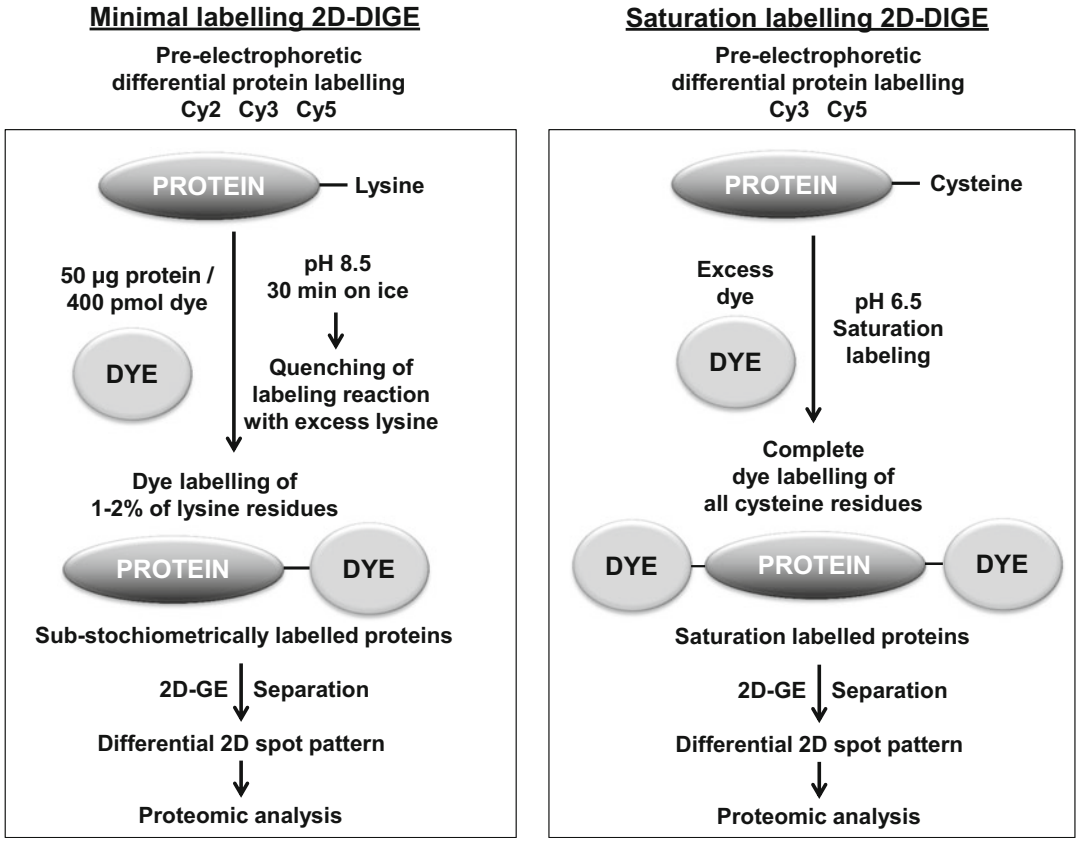

Fig. 3 Overview of minimal versus saturation DIGE labeling. Shown are flowcharts that compare the minimal labeling difference gel electrophoresis (DIGE) approach with pre-electrophoretic differential protein labeling using Cy2, Cy3, and Cy5 dyes, and the saturation labeling DIGE approach with pre-electrophoretic differential protein labeling using Cy3 and Cy5 dyes

5 Conclusions

The many successful applications of the DIGE technique in combination with advanced mass spectrometry emphasize the analytical power of this comparative proteomic approach. DIGE has been employed in many areas of biological research, biotechnology, biomedicine, and biomarker discovery. The bioanalytical robustness of the DIGE technique makes this biochemical method an ideal comparative tool for the comprehensive analysis of large and complex proteomes. The elimination of gel-to-gel variations and the capability of using DIGE in combination with the analysis of post-translational modifications established this fluorescent gel-based method as a key technique of comparative proteomics. Future applications of comparative DIGE analyses in combination with sensitive protein identification approaches promise to further improve our biochemical understanding of complex proteomes in a variety of biological systems.

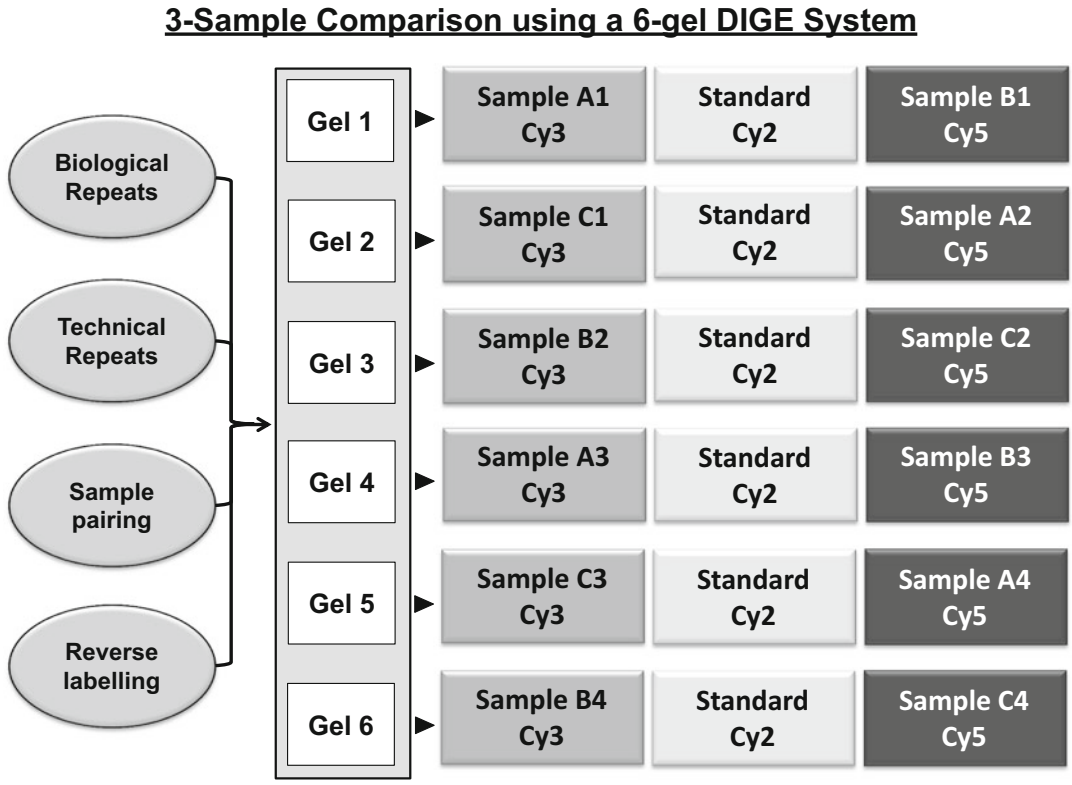

Fig. 4 Outline of 3-sample DIGE analysis. Shown is the combination of 3 different biological samples (A1-A4, B1-B4, and C1-C4) and 3 fluorescent dyes (Cy2, Cy3, and Cy5) for the routine comparative difference gel electrophoresis (DIGE) analyses of pre-electrophoretically labeled protein fractions using a 6-gel system. If two sets of six DIGE gels are run in parallel, a 12-gel DIGE system can achieve optimum results in relation to biological repeats ($n = 4$), sample pairing ($n = 2$), technical repeats ($n = 2$), and reverse fluorescent dye labeling ($n = 2$)

Acknowledgements

Research in the author's laboratory has been supported by project grants from the Irish Higher Education Authority, the Irish Health Research Board, and Muscular Dystrophy Ireland.

References

1. Brewis IA, Brennan P (2010) Proteomics technologies for the global identification and quantification of proteins. Adv Protein Chem Struct Biol 80:1–44
2. Angel TE, Aryal UK, Hengel SM et al (2012) Mass spectrometry-based proteomics: existing capabilities and future directions. Chem Soc Rev 41:3912–3928
3. Zhang Z, Wu S, Stenoien DL et al (2014) High-throughput proteomics. Annu Rev Anal Chem 7:427–454
4. Friedman DB, Hoving S, Westermeier R (2009) Isoelectric focusing and two-dimensional gel electrophoresis. Methods Enzymol 463:515–540

5. Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685
6. Carrette O, Burkhard PR, Sanchez JC, Hochstrasser DF (2006) State-of-the-art two-dimensional gel electrophoresis: a key tool of proteomics research. Nat Protoc 1:812–823
7. Rabilloud T, Lelong C (2011) Two-dimensional gel electrophoresis in proteomics: a tutorial. J Proteomics 74:1829–1841
8. Rabilloud T, Chevallet M, Luche S, Lelong C (2010) Two-dimensional gel electrophoresis in proteomics: past, present and future. J Proteomics 73:2064–2077
9. Oliveira BM, Coorssen JR, Martins-de-Souza D (2014) 2DE: the phoenix of proteomics. J Proteomics 104:140–150
10. Murphy S, Dowling P, Ohlendieck K (2016) Comparative skeletal muscle proteomics using two-dimensional gel electrophoresis. Proteomes 4:27
11. Timms JF, Cramer R (2008) Difference gel electrophoresis. Proteomics 8:4886–4897
12. Viswanathan S, Unlü M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358
13. Alban A, David SO, Bjorkesten L et al (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3:36–44
14. Arentz G, Weiland F, Oehler MK et al (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9:277–288
15. Minden JS, Dowd SR, Meyer HE et al (2009) Difference gel electrophoresis. Electrophoresis 30:S156–S161
16. Karp NA, Kreil DP, Lilley KS (2004) Determining a significant change in protein expression with DeCyder during a pair-wise comparison using two-dimensional difference gel electrophoresis. Proteomics 4:1421–1432
17. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5:3105–3115
18. Carberry S, Zweyer M, Swandulla D et al (2013) Application of fluorescence two-dimensional difference in-gel electrophoresis as a proteomic biomarker discovery tool in muscular dystrophy research. Biology (Basel) 2:1438–1464
19. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
20. Tonge R, Shaw J, Middleton B et al (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396
21. Marouga R, David S, Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382:669–678
22. Doran P, Wilton SD, Fletcher S et al (2009) Proteomic profiling of antisense-induced exon skipping reveals reversal of pathobiochemical abnormalities in dystrophic mdx diaphragm. Proteomics 9:671–685

Chapter 3

2D-DIGE and Fluorescence Image Analysis

Elisa Robotti and Emilio Marengo

Abstract

2D-DIGE is still a very widespread technique in proteomics for the identification of panels of biomarkers, allowing to tackle with some important drawback of classical two-dimensional gel-electrophoresis. However, once 2D-gels are obtained, they must undergo a quite articulated multistep image analysis procedure before the final differential analysis via statistical mono- and multivariate methods. Here, the main steps of image analysis software are described and the most recent procedures reported in the literature are briefly presented.

Key words DIGE, Image analysis, Warping, Image preprocessing, Spot detection and quantification, Spot matching, Differential analysis

1 Introduction

Two-dimensional differential gel electrophoresis (2D-DIGE) [1–4] represents an evolution of two-dimensional gel electrophoresis (2DE), where complex protein mixtures are separated in single proteins according to their isoelectric point (p*I*) and molecular weight. In 2D-DIGE, different samples, labeled with different fluorescent dyes, are run simultaneously on the same gel; the detection of the spots belonging to the different samples present on the gel can be accomplished by scanning the final image at the different wavelengths characteristic of the different dyes used for labeling.

2D-DIGE allows to address the main drawback of 2DE, namely the large sample-to-sample variability due to the low reproducibility of the complex overall experimental procedure. In 2D-DIGE in fact gel-to-gel variations are minimized and the large variability usually affecting 2D gel-electrophoresis is greatly reduced [5, 6].

Notwithstanding the recent widespread use of liquid chromatography (LC)-based proteomics [7, 8], 2D-DIGE is yet an important tool in the clinical field due to its significant ability in visualizing a great number of proteins: differential analysis

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_3,

accomplished with 2D-DIGE can identify changes of the p*I* and/or the molecular weight of proteins that could be due to events such as truncations, degradations, alternative splicing, post-translational modifications (PTMs), or variations in the genetic code. Therefore, the future is still bright for 2D-DIGE, as evidenced by the numerous studies present in literature, above all in the field of biomarkers identification [7–16].

All experimental setups are based on protein labeling by a fluorescent dye before the gel-based separation. From an experimental point of view, a great distinction can be done between minimal and saturation labeling.

In minimal labeling [2, 17], proteins are labeled with N-hydroxy succinimidyl ester derivatives (Cy2, Cy3, and Cy5), that are covalently tagged to the ε-amino group of the lysine residues. The labeling is minimal since only one lysine residue per protein is labeled in order to prevent protein precipitation. Minimal labeling is characterized by low detection limits (about 1 fmol per spot) and four orders of magnitude of the linear dynamic range [2, 18, 19]. Labeling with a sub-stoichiometric dye:lysine ratio avoids artificial mass changes and vertical artifacts on the final gel [18, 20]. Saturation labeling [21] is instead based on the saturation of cysteine residues by the dyes molecules. Certainly, saturation labeling provides a higher sensitivity (one order of magnitude higher than minimal labeling) and the sample needed to quantify protein changes is smaller [2, 21].

For what regards the experimental setup, two main procedures are available (Fig. 1):

- Two-dye approach: one sample and a pooled internal standard are labeled with two different dyes and run simultaneously on the same gel.
- Three-dye approach: two samples and a pooled internal standard are labeled with three different dyes and run contemporarily on the same gel.

In general, the two-dye approach should be preferred since the three-dye approach suffers from a smaller signal-to-noise (S/N) ratio and the standardized spot volumes have been proved not to be independent [2, 22].

Once 2D-gels are obtained, they are scanned via a fluorescent densitometer in order to acquire the image at each wavelength specific for each of the two or three dyes used as labeling agents. The final purpose of gel-based proteomics is to accomplish a differential analysis where classes of samples (e.g., control vs. diseased) are compared to identify panels of biomarkers. Therefore, 2D-gels images must undergo a quite articulated multistep procedure of data analysis which involves [2, 23–25]:

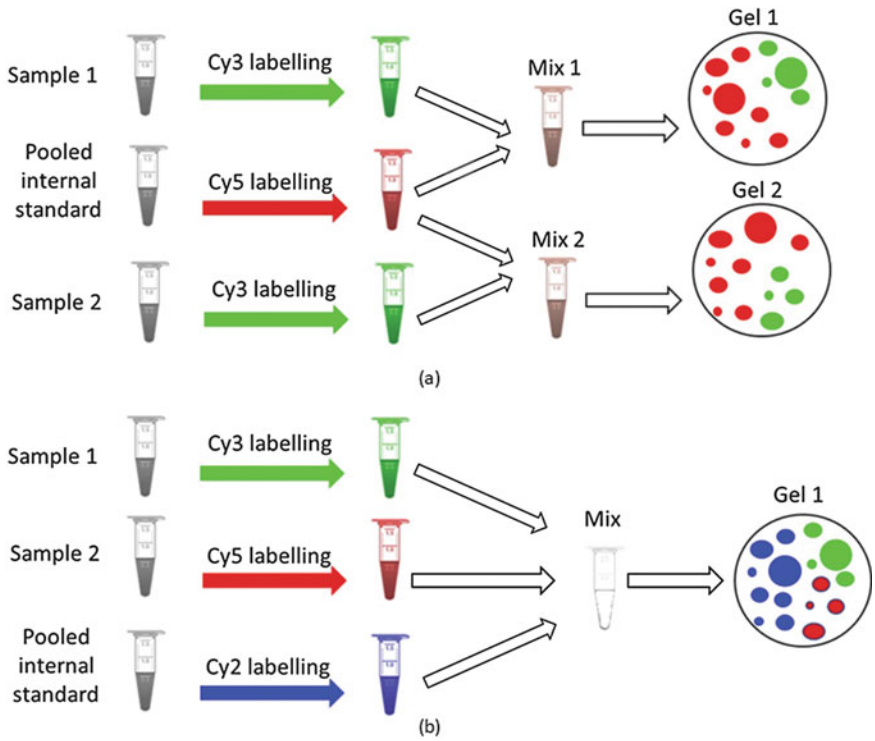

Fig. 1 Two- (**a**) and three- (**b**) dye approaches

- Image preprocessing, involving background and noise subtraction [26].
- Spot detection and quantitation [27–35].
- Spot filtering, removing spots not satisfying specific criteria.
- Image warping and alignment [23, 24, 27, 36–48].
- Spot matching [28, 42, 43, 49–51].
- Differential analysis. In a more classical approach, this step is carried out on spot volume datasets, where each sample is described by the volumes of the spots detected on its surface [52–59]. Another approach is based on the comparison of 2D-gels directly at the pixel level [60–64]. Statistically significant up- and down-regulations can be detected by classical statistics (Student's *t*-test, Mann-Whitney test, etc.) [65] or by multivariate methods (pattern recognition and classification tools [66–68]).

Classical or last-generation commercially available software packages differ essentially for the order in which the different steps are accomplished, reflecting in advantages or drawbacks that affect the final differential analysis. Three workflows are possible (Fig. 2):

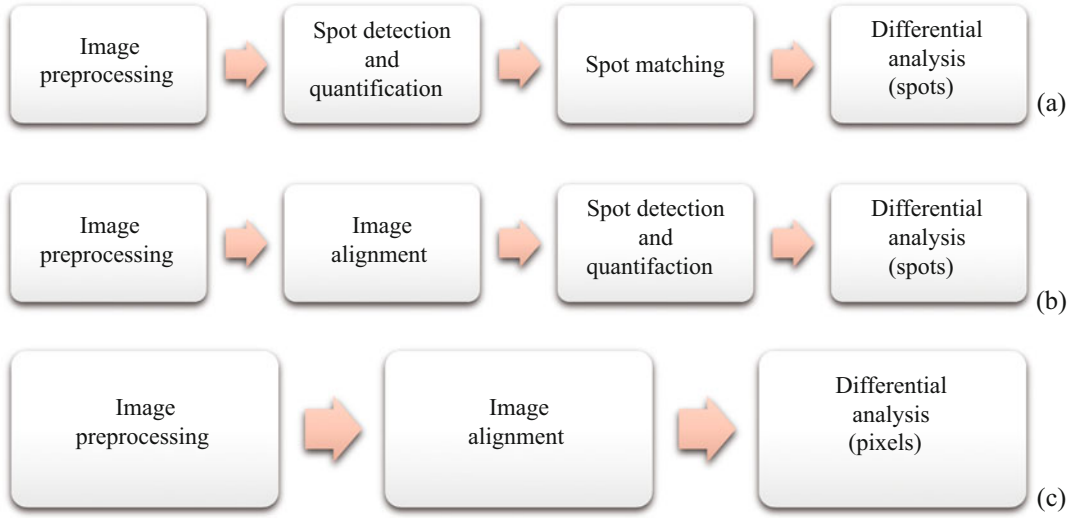

Fig. 2 2-DE image analysis strategies

- Image preprocessing, followed by spot detection, spot matching, and differential analysis (accomplished on protein spot volumes);
- Image preprocessing, followed by image alignment, spot detection, and differential analysis (accomplished on protein spot volumes);
- Image preprocessing, followed by image alignment and differential analysis (accomplished on pixel intensities).

In the first two approaches, the final differential analysis is accomplished on spot volume data. In classical software packages (first approach), spot detection is usually performed before gel matching and differential analysis, while warping is carried out before spot detection across all gels, in last-generation software packages (second approach). The first approach can be applied with or without image preprocessing and alignment [28, 29, 35]: preprocessing can improve spot identification while alignment is usually bypassed by spot matching. In the second approach, the alignment is accomplished at the pixel level; spot detection is carried out on a master or reference gel [39, 46, 69, 70]. Clark and Gutstein [71] proved that the first approach suffers from a drawback, i.e., the proportion of accurately matched spots decreases with larger sample sizes, while the second one avoids missing data [72]. The third approach avoids spot detection and quantification since it compares sets of images directly on pixel intensities [73–76].

The most widespread commercially available software packages for the analysis of 2-DE images are: DeCyder 2D Differential Analysis and Image master 2-D, from GE Healthcare (Little

Chalfont, UK); Progenesis SameSpots and Phoretix 2-D Advanced, from Nonlinear Dynamics (Newcastle Upon Tyne, UK); Delta2D, from Decodon (Greifswald, Germany); Dymension, from Syngene (Cambridge, UK); PDQuest and Proteomweaver, from Bio-Rad Laboratories (Hercules, CA, USA); Melanie, from Geneva Bioinformatics (Geneva, Switzerland); Redfin Solo, from Ludesi (Malmoe, Sweden). The specialized literature offers also some less widespread academic packages [77–79]. Kang et al. [80] compared three commercial software packages for 2D-DIGE image analysis and described their general advantages and disadvantages.

Here, we discuss the main steps of image analysis and the advantages and disadvantages characteristic of each procedure.

2 Image Pre-Processing

Image preprocessing is a fundamental step in image analysis since it allows to tackle with some important drawbacks of the use of gel-based approaches, increasing variability: the presence of noise and image artifacts (e.g., streaks and spurious spots) and the presence of background effects. The purpose of preprocessing is the general improvement of image quality and removal of artifacts, to provide more reliable spot detections and quantifications and therefore a more robust identification of panels of biomarkers during the final differential analysis. McNamara et al. [81] proposed methods for preventing and treating artifacts in 2D-DIGE. Notwithstanding the basic concept that these steps should not alter the final differential analysis, it has been proved that both noise filtering and background removal can affect spot quantification and analysis variability [82, 83]. Noise can be present in 2-DE images in two main forms [36, 84]:

- "Gaussian noise", generally related to the acquisition step;
- "Spike noise", mainly related to the experimental steps.

Noise is characterized by high-frequency features that should be removed and separated from low-frequency features representing spots [36, 78, 85]. Commercial software packages usually exploit spatial filtering [82, 84, 86] based on local nxn kernel/window filters [36, 37, 87, 88]. These filters are based on the calculation of a new value for the pixel at the center of the kernel from the values of the pixels within the kernel; to this purpose, the researcher can choose among different approaches: median filter, adaptive filters, Gaussian filters, polynomial filters. These approaches usually tend to erode the spot edges and to alter the intensity values of the spot pixels [77, 84]. Other approaches for the efficient removal of Gaussian noise are based on spatial-frequency domain filters, as wavelet filters and contourlet denoising

[84, 87]. Contour denoising was developed to address the main drawback of wavelet denoising, i.e., its directionality, since this approach captures noise along the horizontal, vertical, and diagonal directions. The main disadvantage of these filters with respect to spatial filtering is the quite complex setting of the filtering parameters hampering their use in the standard laboratory practice when a great number of gels has to be processed simultaneously. Cannistraci et al. [89] developed a nonlinear adaptive spatial filter, named median modified Wiener filter (MMWF), for eliminating different kinds of noise simultaneously (small-noise-features, spikes, Gaussian noise).

Background subtraction is a mandatory step to eliminate from the images contributions due to not homogeneous gel staining or variability in image acquisition. Several methods are available for calculating the background level but almost all the procedures belong to one of the following categories:

- Methods based on an estimation of the background based on the intensity of the pixels just outside the spot boundary. Two methods belonging to this category are the most widespread where background is estimated: (a) by the minimum value of intensity detected on the boundary of the spot; (b) by the average of the intensities detected on all the spot boundary. These methods are suitable when the background is homogeneous on all the gel [90].
- Methods based on a mathematical model representing the background on all the gel surface [90]. The pixels used for modeling the background are those not contained in identified spots, therefore these methods are usually applied after spot detection. These methods are exploited in several commercial software (PDQuest, Delta 2D, Progenesis PG240, SameSpot).

Silva et al. [91] stated that last-generation software packages introduce a smaller variability, since identical spot boundaries are drawn across all gels images.

In 2D-DIGE, a fundamental step of image preprocessing is image normalization, since the intensities measured both at the spot or pixel level suffer from important dye effects [92–94]. Normalization parameters can depend on pixel intensities and/or on the spatial localization in the gel [36, 95]. In such cases, normalization operators can be applied to sub-areas of the gels even if the computational effort increases. Normalization operators have to be chosen with extreme care since they affect the final identification of biomarkers. In 2D-DIGE, the so-called *M-A* plots can be employed to compare different normalization methods [95]. In this approach, M values are calculated as $\log_2(\text{Cy5}/\text{Cy3})$ (when a pool channel is not used) or $\log_2(\text{Cy5}/\text{Cy2})/(\text{Cy3}/\text{Cy2})$ (when a pool channel is used). Then, m-values are calculated as: $\log_2(\text{Cy5}/$

Cy2) or $\log_2$(Cy3/Cy2). The average $\log_2$ intensity (A) is given by: ($\log_2$Cy5 + $\log_2$Cy3)/2. Different methods can be compared by means of box plots of M and m-values or scatterplots of M versus A-values [95].

Other methods depend on specific software: for example, DeCyder [96] provides two methods for data normalization:

- "DeCyder no pool" is used when the pool channel is not used. The method performs channel-specific shifts of the log intensities (log volumes), so that the distribution of the log intensities is centered on zero for each dye channel.
- "DeCyder pool" is similar but shifts each of the two series of log ratios: log Cy5/Cy2 and log Cy3/Cy2, for each gel.

Another approach is the Locally Weighted Regression (Loess or Lowess) Normalization [97], and recently applied to DIGE [95, 98] but originally developed for microarrays [99–102]. In Loess normalization, experimental data are fitted by a normalization curve; the procedure combines multiple regression and a k-nearest-neighbors approach. M-A plots of comparable spot intensities are exploited as starting point for the fitting procedure. At each point in the M–A plot, Loess fits a low-degree polynomial to a subset of the data with values near the point whose response is being estimated. The subsets of data used are selected by a nearest-neighbors approach. Weighted least squares regression is used to give more importance to points close to the point whose response is being estimated. The weight is assigned to each sample iteratively, in order to limit the influence of potential outliers: in this case, at each new estimation of the Loess curve, the weight of the sample is reduced based on its residual with respect to the previous estimation. Once the estimation is completed, pixel intensities across all gels are normalized by the Loess curve obtained. Notwithstanding the fact that the method is quite computationally intensive, it has the great advantage of a small user intervention: the user has just to set the window and the degree of the local polynomial. Variants of the Loess Normalization are also available [95, 96, 98, 99, 103]. Other methods exploited in 2D-DIGE are based on quantile normalization [95–104].

3 Spot Detection and Quantitation

The step of spot detection allows the identification of the spots on the gel image and provides a list of spots located on the gel that will be further matched across all gels. Spot detection usually implies: spot center detection; spots segmentation to identify gel areas each containing a single spot; model each area by a function to extract spots characteristics and identify co-migrations.

Image segmentation algorithms can be based on edge detection (e.g., Laplacian filtering), even if this approach could produce spurious spots if noise and artifacts are still present in the gel [74, 105]. Recent approaches make use of geometric algorithms [78], parametric spot models [106], the pixel value collection method [30, 74], and slice tree with confidence evaluation [29]. However, the most widespread method is certainly the watershed transformation algorithm [28, 33, 91]. Watershed in geosciences is the boundary of a catchment basin in a landscape where all waters drain to. Using this parallel, a gel is considered as a topographic relief and the spots are depressions. The algorithm therefore identifies the basins, while spots characteristics are determined by iterative fitting methods [33, 107].

The algorithm for spots detection and quantitation can be parametric or nonparametric [107]:

- Parametric algorithms are exploited in classical software and are based on spot modeling: actual spots are transformed into ideal elliptic spots by means of 2D Gaussian fitting.
- Nonparametric algorithms are exploited in last-generation software and are not based on spots modeling so that each spot maintains its shape.

Notwithstanding the fact that Gaussian modeling is effective for small spots, it is not a good choice for larger ones, showing in general more irregular shapes, nor for saturated spots, while it shows better results with overlapping spots [82].

Classical software packages usually suffer from the necessity of a larger user intervention to correct spot detections, e.g., remove spurious spots, add faint spots or split overlapping ones. Last-generation packages, instead, carry out alignment at the pixel level, so that spots are detected simultaneously across all the gels, superimposing on all gels one spot map: spot editing is therefore less time-consuming since it is performed just once on the fusion image and then propagated to all single images [39]. Even if last-generation software avoids missing data, they tend to merge together close spots, so that overlapping problems can increase with respect to classical software [6, 71].

4 Gel Warping and Matching

Differentially expressed proteins can be effectively identified if the images are properly registered. Gel matching performances are deeply influenced by image warping. The aim of warping is to adjust systematic geometric distortions. Matching and warping algorithms allow to overcome the difficulties due to variations in the migration positions of spots among gels; this is particularly true

for 2-DE, while in 2D-DIGE intra-gel variations are minimized. Registration of two images consists in deforming one image (source) into another (template), so that similar structures in the images match one to each other [108]. The available warping procedures for 2-DE and 2D-DIGE can be divided into two categories [109], according to the information used to guide the registration process:

- Methods based on the selection of landmarks [86, 110–112]. In these methods, after the image segmentation, to identify the spots, image registration is carried out, consisting in aligning the landmarks and computing the image transformation function.
- Methods based on intensities. These methods register the two images optimizing an intensity similarity index calculated between the raw images (e.g., the sum of squared intensity differences or the cross-correlation).

Intensity-based methods [23, 44, 45, 113, 114] are nowadays the most widespread since they do not need segmentation and exploit image information to a larger extent. To overcome the limitation of both procedures, the combination of intensity and landmark information has now become more popular [108, 115]: Kang et al. [80] reported that using five landmarks, Progenesis SameSpots outperformed DeCyder and Dymension. In general, last-generation software exploit warping procedures reducing analysis time and improving accuracy and subjectivity of the subsequent matching.

In spot-based methods, the software input is a list of detected spots (or landmarks) that are successively considered by the algorithms as individual points [116]. Traditional methods exploit polynomial functions to align the spots in different images [117]. Where low-order polynomials cannot correct the geometric distortions, new methods have been developed: thin plate splines (TPS) [117], which allow nonlinear bending of the gel coordinates, and hierarchical grid transformation, both characterized by a superior warping efficiency [41, 44, 118, 119]. Other tested methods make use of the Iterative Closest Point procedure [109] and of deformed graphs [50].

In pixel-based methods, warping is performed directly at the pixel level [23]. The warping based on the pixel values exploits numerous additional features like spot shape and intensity spread, that otherwise are lost in the spot detection approach. The general idea is the maximization of the correlation between two images. Different procedures are available in literature [120]: the pixel-based analysis of multiple images (PMC) [73] and fuzzy warping [23–25, 38, 44, 47, 60, 61, 76, 85, 121, 122].

References

1. Knowles MR, Cervino S, Skynner HA, Hunt SP, de Felipe C, Salim K, Meneses-Lorente G, McAllister G, Guest PC (2003) Multiplex proteomic analysis by two-dimensional differential in-gel electrophoresis. Proteomics 3:1162–1171
2. Arentz G, Weiland F, Oehler MK, Hoffmann P (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9(3–4):277–288
3. Mahnke RC, Corzett TH, McCutchen-Maloney SL, Chromy BA (2006) An integrated proteomic workflow for two-dimensional differential gel electrophoresis and robotic spot picking. J Proteome Res 5:2093–2097
4. Marouga R, David S, Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382:669–678
5. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5:3105–3115
6. Karp NA, Feret R, Rubtsov DV, Lilley KS (2008) Comparison of DIGE and post-stained gel electrophoresis with both traditional and SameSpots analysis for quantitative proteomics. Proteomics 8:948–960
7. Mitulovic G (2015) New HPLC techniques for proteomics analysis: a short overview of latest developments. J Liq Chromatogr Relat Technol 38(3):390–403
8. Denoroy L, Zimmer L, Renaud B, Parrot S (2013) Ultra high performance liquid chromatography as a tool for the discovery and the analysis of biomarkers of diseases: a review. J Chromatogr B 927:37–53
9. Pendharkar N, Gajbhiye A, Taunk K, Roy-Choudhury S, Dhali S, Seal S, Mane A, Abhang S, Santra MK, Chaudhury K, Rapole S (2016) Quantitative tissue proteomic investigation of invasive ductal carcinoma of breast with luminal B HER2 positive and HER2 enriched subtypes towards potential diagnostic and therapeutic biomarkers. J Proteomics 132:112–130
10. Schubert KO, Föcking M, Cotter DR (2015) Proteomic pathway analysis of the hippocampus in schizophrenia and bipolar affective disorder implicates 14-3-3 signaling, aryl hydrocarbon receptor signaling, and glucose metabolism: potential roles in GABAergic interneuron pathology. Schizophr Res 167(1–3):64–72
11. Casanova-Salas I, Masiá E, Armiñán A, Calatrava A, Mancarella C, Rubio-Briones J, Scotlandi K, Vicent MJ, López-Guerrero JA (2015) MiR-187 targets the androgen-regulated gene ALDH1A3 in prostate cancer. PLoS One 10(5) art. no. e0125576
12. Davalieva K, Kostovska IM, Kiprijanovska S, Markoska K, Kubelka-Sabit K, Filipovski V, Stavridis S, Stankov O, Komina S, Petrusevska G, Polenakovic M (2015) Proteomics analysis of malignant and benign prostate tissue by 2D DIGE/MS reveals new insights into proteins involved in prostate cancer. Prostate 75 (14):1586–1600
13. Juste C, Kreil DP, Beauvallet C, Guillot A, Vaca S, Carapito C, Mondot S, Sykacek P, Sokol H, Blon F, Lepercq P, Levenez F, Valot B, Carré W, Loux V, Pons N, David O, Schaeffer B, Lepage P, Martin P, Monnet V, Seksik P, Beaugerie L, Ehrlich SD, Gibrat J-F, Van Dorsselaer A, Doré J (2014) Bacterial protein signals are associated with Crohn's disease. Gut 63(10):1566–1577
14. Wu J-Y, Cheng C-C, Wang J-Y, Wu D-C, Hsieh J-S, Lee S-C, Wang W-M (2014) Discovery of tumor markers for gastric cancer by proteomics. PLoS One 9(1), art. no. e84158
15. Gilabert M, Launay S, Ginestier C, Bertucci F, Audebert S, Pophillat M, Toiron Y, Baudelet E, Finetti P, Noguchi T, Sobol H, Birnbaum D, Borg J-P, Charafe-Jauffret E, Gonçalves A (2014) Poly(ADP-Ribose) polymerase 1 (PARP1) overexpression in human breast cancer stem cells and resistance to olaparib. PLoS One 9(8), art. no. e104302
16. Timms JF, Arslan-Low E, Kabir M, Worthington J, Camuzeaux S, Sinclair J, Szaub J, Afrough B, Podust VN, Fourkala E-O, Cubizolles M, Kronenberg F, Fung ET, Gentry-Maharaj A, Menon U, Jacobs I (2014) Discovery of serum biomarkers of ovarian cancer using complementary proteomic profiling strategies. Proteomics Clin Appl 8(11–12):982–993
17. Van den Bergh G, Arckens L (2003) Fluorescent two-dimensional difference gel electrophoresis unveils the potential of gel-based proteomics. Curr Opin Biotechnol 15:38–43
18. Tonge R, Shaw J, Middleton B, Rowlinson R et al (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396
19. Viswanathan S, Unlu M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358
20. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel

method for detecting changes in protein extracts. Electrophoresis 18:2071–2077

21. Shaw J, Rowlinson R, Nickson J, Stone T et al (2003) Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. Proteomics 3:1181–1195
22. Karp NA, McCormick PS, Russell MR, Lilley KS (2007) Experimental and statistical considerations to avoid false conclusions in proteomics studies using differential in-gel electrophoresis. Mol Cell Proteomics 6:1354–1364
23. Veeser S, Dunn MJ, Yang GZ (2001) Multiresolution image registration for two-dimensional gel electrophoresis. Proteomics 1:856–870
24. Dowsey AW, English J, Pennington K, Cotter D et al (2006) Examination of 2-DE in the human proteome organisation brain proteome project pilot studies with the new RAIN gel matching technique. Proteomics 6:5030–5047
25. Dowsey AW, Dunn MJ, Yang GZ (2008) Automated image alignment for 2D gel electrophoresis in a high-throughput proteomics pipeline. Bioinformatics 24:950–957
26. Rye M, Fargestad EM (2012) Preprocessing of electrophoretic images in 2-DE analysis. Chemom Intel Lab Syst 117:70–79
27. Moller B, Posch S (2009) Robust features for 2-DE gel image registration. Electrophoresis 30:4137–4148
28. Srinark T, Kambhamettu C (2008) An image analysis suite for spot detection and spot matching in two-dimensional electrophoresis gels. Electrophoresis 29:706–715
29. Liu YS, Chen SY, Liu RS, Duh DJ, Chao YT, Tsai YC, Hsieh JS (2009) Spot detection for a 2-DE gel image using a slice tree with confidence evaluation. Math Comput Modell 50:1–14
30. Cutler P, Heald G, White IR, Ruan J (2003) A novel approach to spot detection for two-dimensional gel electrophoresis images using pixel value collection. Proteomics 3:392–401
31. Kazhiyur-Mannar R, Smiraglia DJ, Plass C, Wenger R (2006) Contour area filtering of two-dimensional electrophoresis images. Med Image Anal 10:353–365
32. Wu Y, Lemkin PF, Upton K (1993) A fast spot segmentation algorithm for two-dimensional gel electrophoresis analysis. Electrophoresis 14:1351–1356
33. Bettens E, Scheunders P, VanDyck D, Moens L, VanOsta P (1997) Computer analysis of two-dimensional electrophoresis gels: a new segmentation and modeling algorithm. Electrophoresis 18:792–798
34. Tsakanikas P, Manolakos ES (2011) Protein spot detection and quantification in 2-DE gel images using machine-learning methods. Proteomics 11:2038–2050
35. Langella O, Zivy M (2008) A method based on bead flows for spot detection on 2-D gel images. Proteomics 8:4914–4918
36. Dowsey AW, Dunn MJ, Yang GZ (2003) The role of bioinformatics in two-dimensional gel electrophoresis. Proteomics 3:1567–1596
37. Conradsen K, Pedersen J (1992) Analysis of 2dimensional electrophoretic gels. Biometrics 48:1273–1287
38. Woodward AM, Rowland JJ, Kell DB (2004) Fast automatic registration of images using the phase of a complex wavelet transform: application to proteome gels. Analyst 129:542–552
39. Luhn S, Berth M, Hecker M, Bernhardt J (2003) Using standard positions and image fusion to create proteome maps from collections of two-dimensional gel electrophoresis images. Proteomics 3:1117–1127
40. Schultz J, Gottlieb DM, Petersen M, Nesic L, Jacobsen S, Sondergaard I (2004) Explorative data analysis of two-dimensional electrophoresis gels. Electrophoresis 25:502–511
41. Salmi J, Aittokallio T, Westerholm J, Griese M, Rosengren A, Nyman TA, Lahesmaa R, Nevalainen O (2002) Hierarchical grid transformation for image warping in the analysis of two dimensional electrophoresis gels. Proteomics 2:1504–1515
42. Panek J, Vohradsky J (1999) Point pattern matching in the analysis of two-dimensional gel electropherograms. Electrophoresis 20:3483–3491
43. Kaczmarek K, Walczak B, de Jong S, Vandeginste BG (2002) Feature based fuzzy matching of 2D gel electrophoresis images. J Chem Inf Comput Sci 42:1431–1442
44. Gustafsson JS, Blomberg A, Rudemo M (2002) Warping two-dimensional electrophoresis gel images to correct for geometric distortions of the spot pattern. Electrophoresis 23:1731–1744
45. Smilansky Z (2001) Automatic registration for images of two-dimensional protein gels. Electrophoresis 22:1616–1626
46. Sorzano CO, Arganda-Carreras I, Thevenaz P, Beloso A, Morales G, Valdes I, Perez-Garcia C, Castillo C, Garrido E, Unser M (2008) Elastic image registration of 2-D gels for differential and repeatability studies. Proteomics 8:62–65

47. Daszykowski M, Faergestad EM, Grove H, Martens H, Walczak B (2009) Matching 2D gel electrophoresis images with Matlab 'image processing toolbox'. Chemom Intel Lab Syst 96:188–195
48. Potra FA, Liu X, Seillier-Moiseiwitsch F, Roy A, Hang Y, Marten MR, Raman B, Whisnant C (2006) Protein image alignment via piecewise affine transformations. J Comput Biol 13:614–630
49. Rogers M, Graham J (2007) Robust and accurate registration of 2-D electrophoresis gels using point-matching. IEEE Trans Image Process 16:624–635
50. Noma A, Pardo A, Cesar RM (2011) Structural matching of 2D electrophoresis gels using deformed graphs. Pattern Recogn Lett 32:3–11
51. Xin HM, Zhu Y (2009) Multiple information based spot matching method for 2-DE images. Electrophoresis 30:2477–2480
52. Marengo E, Robotti E, Cecconi D, Hamdan M, Scarpa A, Righetti PG (2004) Identification of the regulatory proteins in human pancreatic cancers treated with Trichostatin-A by 2DPAGE maps and multivariate statistical analysis. Anal Bioanal Chem 379 (7–8):992–1003
53. Marengo E, Robotti E, Bobba M, Liparota MC, Rustichelli C, Zamo A, Chilosi M, Righetti PG (2006) Multivariate statistical tools applied to the characterisation of the proteomic profiles of two human lymphoma cell lines by two-dimensional gel electrophoresis. Electrophoresis 27(2):484–494
54. Marengo E, Robotti E, Bobba M, Milli A, Campostrini N, Righetti SC, Cecconi D, Righetti PG (2008) Application of partial least squares discriminant analysis and variable selection procedures: a 2D-PAGE proteomic study. Anal Bioanal Chem 390(5):1327–1342
55. Robotti E, Demartini M, Gosetti F, Calabrese G, Marengo E (2011) Development of a classification and ranking method for the identification of possible biomarkers in two-dimensional gel-electrophoresis based on principal component analysis and variable selection procedures. Mol Biosyst 7 (3):677–686
56. Negri AS, Robotti E, Prinsi B, Espen L, Marengo E (2011) Proteins involved in biotic and abiotic stress responses as the most significant biomarkers in the ripening of pinot noir skins. Funct Integr Genomics 11(2):341–355
57. Polati R, Menini M, Robotti E, Millioni R, Marengo E, Novelli E, Balzan S, Cecconi D (2012) Proteomic changes involved in tenderization of bovine Longissimus dorsi muscle during prolonged aging. Food Chem 135(3):2052–2069
58. Marengo E, Robotti E, Bobba M (2008) 2DPAGE maps analysis. In: Vlahou A (ed) Clinical proteomics: methods and protocols, Methods in molecular biology, vol vol 428. Humana, Totowa, NJ, pp 291–325
59. Marengo E, Robotti E, Righetti PG, Campostrini N, Pascali J, Ponzoni M, Hamdan M, Astner H (2004) Study of proteomic changes associated with healthy and tumoral murine samples in neuroblastoma by principal component analysis and classification methods. Clin Chim Acta 345(1–2):55–67
60. Marengo E, Robotti E, Gianotti V, Righetti PG, Cecconi D, Domenici E (2003) A new integrated statistical approach to the diagnostic use of proteomic two-dimensional maps. Electrophoresis 24(1–2):225–236
61. Marengo E, Robotti E, Righetti PG, Antonucci F (2003) New approach based on fuzzy logic and principal component analysis for the classification of two-dimensional maps in health and disease. Application to lymphomas. J Chromatogr a 1004(1–2):13–28
62. Marengo E, Bobba M, Liparota MC, Robotti E, Righetti PG (2005) Use of legendre moments for the fast comparison of twodimensional polyacrylamide gel electrophoresis maps images. J Chromatogr A 1096 (1–2):86–91
63. Marengo E, Cocchi M, Demartini M, Robotti E, Bobba M, Righetti PG (2011) Investigation of the applicability of Zernike moments to the classification of SDS 2D-PAGE maps. Anal Bioanal Chem 400(5):1419–1431
64. Marengo E, Robotti E, Bobba M, Demartini M, Righetti PG (2008) A new method for comparing 2D-PAGE maps based on the computation of Zernike moments and multivariate statistical tools. Anal Bioanal Chem 391(4):1163–1173
65. Carpentier S, Panis B, Swennen R, Lammertyn J (2008) Finding the significant markers: statistical analysis of proteomic data. In: Vlahou A (ed) Clinical proteomics, Methods in molecular biology, vol vol 428. Humana, Totowa, NJ, pp 327–347
66. Jacobsen S, Grove H, Jensen KN, Sorensen HA, Jessen F, Hollung K, Uhlen AK, Jorgensen BM, Faergestad EM, Sondergaard I (2007) Multivariate analysis of 2-DE protein patterns—practical approaches. Electrophoresis 28:1289–1299
67. Faergestad EM, Rye MB, Nhek S, Hollung K, Grove H (2011) The use of chemometrics to

Normalization for 2-DE and 2-D-DIGE analyse protein patterns from gel electrophoresis. Acta Chromatogr 23:1–40

68. Grove H, Jorgensen BM, Jessen F, Sondergaard I, Jacobsen S, Hollung K, Indahl U, Faergestad EM (2008) Combination of statistical approaches for analysis of 2-DE data gives complementary results. J Proteome Res 7(12):5119–5124
69. Bandow JE, Baker JD, Berth M, Painter C, Sepulveda OJ, Clark KA, Kilty I, VanBogelen RA (2008) Improved image analysis workflow for 2-D gels enables large-scale 2-D gel-based proteomics studies—COPD biomarker discovery study. Proteomics 8:3030–3041
70. Rye MB, Faergestad EM, Alsberg BK (2008) A new method for assigning common spot boundaries for multiple gels in two dimensional gel electrophoresis. Electrophoresis 29:1359–1368
71. Clark BN, Gutstein HB (2008) The myth of automated, high-throughput two-dimensional gel analysis. Proteomics 8:1197–1203
72. Morris JS, Clark BN, Wei W, Gutstein HB (2010) Evaluating the performance of new approaches to spot quantification and differential expression in 2-dimensional gel electrophoresis studies. J Proteome Res 9:595–604
73. Faergestad EM, Rye M, Walczak B, Gidskehaug L, Wold JP, Grove H, Jia X, Hollung K, Indahl UG, Westad F, van den Berg F, Martens H (2007) Pixel-based analysis of multiple images for the identification of changes: a novel approach applied to unravel proteome patterns [corrected] of 2-D electrophoresis gel images. Proteomics 7:3450–3461
74. Rye MB, Faergestad EM, Martens H, Wold JP, Alsberg BK (2008) An improved pixel-based approach for analyzing images in two-dimensional gel electrophoresis. Electrophoresis 29:1382–1393
75. Van Belle W, Anensen N, Haaland I, Bruserud O, Hogda KA, Gjertsen BT (2006) Correlation analysis of two-dimensional gel electrophoretic protein patterns and biological variables. BMC Bioinformatics 7:198
76. Daszykowski M, Stanimirova I, BodzonKulakowska A, Silberring J, Lubec G, Walczak B (2007) Start-to-end processing of twodimensional gel electrophoretic images. J Chromatogr A 1158:306–317
77. Cecconi D (2016) Comparative evaluation of software features and performances. Methods Mol Biol 1384:69–78
78. Morris JS, Clark BN, Gutstein HB (2008) Pinnacle: a fast, automatic and accurate method for detecting andquantifying protein spots in 2-dimensional gel electrophoresis data. Bioinformatics 24(4):529–536
79. Wu Y, Zhang L (2011) Comparison of two academic software packages for analyzing two-dimensional gel images. J Bioinform Comput Biol 9(6):775–794
80. Kang Y, Techanukul T, Mantalaris A, Nagy JM (2009) Comparison of three commercially available DIGE analysis software packages: minimal user intervention in gel-based proteomics. J Proteome Res 8:1077–1084
81. McNamara LE, Kantawong FA, Dalby MJ, Riehle MO, Burchmore R (2011) Preventing and troubleshooting artefacts in saturation labelled fluorescence 2-D difference gel electrophoresis (saturation DiGE). Proteomics 11:4610–4621
82. Rogers M, Graham J, Tonge RP (2003) Using statistical image models for objective evaluation of spot detection in two-dimensional gels. Proteomics 3(6):879–886
83. Tsakanikas P, Manolakos ES (2009) Improving 2-DE gel image denoising using contourlets. Proteomics 9(15):3877–3888
84. Tsakanikas P, Manolakos I (2007) Effective denoising of 2D gel proteomics images using contourlets. Image Processing, 2007, ICIP 2007. IEEE International Conference on 2, pp. 1–22
85. Marengo E, Robotti E, Antonucci F et al (2005) Numerical approaches for quantitative analysis of two-dimensional maps: a review of commercial software and home-made systems. Proteomics 5:654–666
86. Appel RD, Palagi PM, Walther D et al (1997) Melanie II–a third-generation software package for analysis of two-dimensional electrophoresis images: I. Features and user interface. Electrophoresis 18:2724–2734
87. Kaczmarek K, Walczak B, deJong S et al (2004) Preprocessing of two-dimensional gel electrophoresis images. Proteomics 4:2377–2389
88. Appel RD, Vargas JR, Palagi PM et al (1997) Melanie II–a third-generation software package for analysis of two-dimensional electrophoresis images: II. Algorithms. Electrophoresis 18:2735–2748
89. Cannistraci CV, Montevecchi FM, Alessio M (2009) Median-modified Wiener filter provides efficient denoising, preserving spot edge and morphology in 2-DE image processing. Proteomics 9:4908–4919

90. Robotti E, Marengo E, Quasso F (2016) Image pretreatment tools II: normalization techniques for 2-DE and 2-D DIGE. Methods Mol Biol 1384:91–107
91. Silva E, O'Gorman M, Becker S, Auer G, Eklund A, Grunewald J, Wheelock AM (2010) In the eye of the beholder: does the master see the SameSpots as the novice? J Proteome Res 9(3):1522–1532
92. Krogh M, Liu Y, Waldemarson S, Valastro B, James P (2007) Analysis of DIGE data using a linear mixed model allowing for proteinspecific dye effects. Proteomics 7:4235–4244
93. Kreil DP, Karp NA, Lilley KS (2004) DNA microarray normalization methods can remove bias from differential protein expression analysis of 2D difference gel electrophoresis results. Bioinformatics 20:2026–2034
94. Engelen K, Sifrim A, Van de Plas B, Laukens K, Arckens L, Marchal K (2010) Alternative experimental design with an applied normalization scheme can improve statistical power in 2D-DIGE experiments. J Proteome Res 9:4919–4926
95. Kultima K, Scholz B, Alm H, Skold K, Svensson M, Crossman AR, Bezard E, Andren PE, Lonnstedt I (2006) Normalization and expression changes in predefined sets of proteins using 2D gel electrophoresis: a proteomic study of L-DOPA induced dyskinesia in an animal model of Parkinson's disease using DIGE. BMC Bioinformatics 7(1):475
96. Keeping AJ, Collins RA (2011) Data variance and statistical significance in 2D-gel electrophoresis and DIGE experiments: comparison of the effects of normalization methods. J Proteome Res 10:1353–1360
97. Jacoby WG (2000) Loess: a nonparametric, graphical tool for depicting relationships between variables. Elect Stud 19:577–613
98. Fodor IK, Nelson DO, Alegria-Hartman M, Robbins K, Langlois RG, Turteltaub KW, Corzett TH, McCutchen-Maloney SL (2005) Statistical challenges in the analysis of twodimensional difference gel electrophoresis experiments using DeCyder(TM). Bioinformatics 21:3733–3740
99. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30:e15
100. Cleveland WS (1979) Robust locally weighted regression and smoothing scatterplots. J Am Stat Assoc 74(368):829–836
101. Cleveland WS, Devlin SJ (1988) Locally-weighted regression: an approach to regression analysis by local fitting. J Am Stat Assoc 83(403):596–610
102. Berger JA, Hautaniemi S, Jarvinen AK, Edgren H, Mitra SK, Astola J (2004) Optimized LOWESS normalization parameter selection for DNA microarray data. BMC Bioinformatics 5:194
103. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article 3
104. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185–193
105. Pleissner KP, Hoffmann F, Kriegel K, Wenk C, Wegner S, Sahlstrom A, Oswald H, Alt H, Fleck E (1999) New algorithmic approaches to protein spot detection and pattern matching in two-dimensional electrophoresis gel databases. Electrophoresis 20:755–765
106. Berth M, Moser FM, Kolbe M, Bernhardt J (2007) The state of the art in the analysis of two-dimensional gel electrophoresis images. Appl Microbiol Biotechnol 76:1223–1243
107. Rogers M, Graham J, Tonge RP (2003) Statistical models of shape for the analysis of protein spots in two-dimensional electrophoresis gel images. Proteomics 3:887–896
108. Rohr K, Cathier P, Worz S (2004) Elastic registration of electrophoresis images using intensity information and point landmarks. Pattern Recogn 37:1035–1048
109. Shi G, Jiang T, Zhu W, Liu B, Zhao H (2007) Alignment of two-dimensional electrophoresis gels. Biochem Biophys Res Commun 357:427–432
110. Raman B, Cheung A, Marten MR (2002) Quantitative comparison and evaluation of two commercially available, two-dimensional electrophoresis image analysis software packages, Z3 and Melanie. Electrophoresis 23:2194–2202
111. Lemkin PF (1997) Comparing twodimensional gels across the internet. Electrophoresis 18(3–4):461–470
112. Efrat A, Hoffmann F, Kriegel K, Schultz C, Wenk C (2002) Geometric algorithms for the analysis of 2D-electrophoresis gels. J Comput Biol 9(2):299–316
113. Baker M, Busse H, Vogt M (2000) An automatic registration and segmentation algorithm for multiple electrophoresis images. In: Hanson KM (ed) Medical Imaging 2000—Image Processing (MI'2000), Proceedings of the SPIE International

Symposium, vol 3979, 14–17 Feb 2000, San Diego, CA, pp 426–436

114. Josso B, Zindy E, Aldemir H (2000) Automatic 2D gel registration using distance minimisation of image morphing. Proceedings of the IEEE International Conference on Information Visualization (IV'00), London, England, 19–21 Jul 2000, pp 357–361
115. Worz S, Winz M-L, Rohr K (2009) Geometric alignment of 2D gel electrophoresis images. Methods Inf Med 48:320–323
116. Lemkin P, Lipkin LE (1981) GELLAB: a computer system for 2D gel electrophoresis analysis. II. Pairing spots. Comput Biomed Res 14:355–380
117. Bookstein FL (1989) Principal warps: thin-plate splines and the decomposition of deformation. IEEE Trans Pattern Anal Mach Intell 11:567–585
118. Marengo E, Cocchi M, Demartini M, Robotti E, Cecconi D, Calabrese G (2012) GENOCOP algorithm and hierarchical grid transformation for image warping of two dimensional gel eletrophoretic maps. Mol Biosyst 8(4):975–984
119. Robotti E, Marengo E, Demartini M (2016) GENOCOP algorithm and hierarchical grid transformation for image warping of two-dimensional gel electrophoretic maps. Methods Mol Biol 1384:165–184
120. Glasbey CA, Mardia KV (2001) A penalized likelihood approach to image warping. J R Statist Soc B 63:465–514
121. Rodriguez A, Fernandez-Lozano C, Dorado J, Rabun ˜a JR (2014) Two-dimensional gel electrophoresis image registration using block-matching techniques and deformation models. Anal Biochem 454:53–59
122. Lin D-T (2010) Autonomous sub-image matching for two-dimensional electrophoresis gels using MaxRST algorithm. Image Vis Comput 28:1267–1279

Chapter 4

DIGE Analysis Software and Protein Identification Approaches

Abduladim Hmmier and Paul Dowling

Abstract

DIGE is a high-resolution two-dimensional gel electrophoresis method, with excellent dynamic range obtained by fluorescent tag labeling of protein samples. Scanned images of DIGE gels show thousands of protein spots, each spot representing a single or a group of protein isoforms. By using commercially available software, each protein spot is defined by an outline, which is digitized and correlated with the quantity of proteins present in each spot. Software packages include DeCyder, SameSpots, and Dymension 3. In addition, proteins of interest can be excised from post-stained gels and identified with conventional mass spectrometry techniques. High-throughput mass spectrometry is performed using sophisticated instrumentation including matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), MALDI-TOF/TOF, and liquid chromatography tandem mass spectrometry (LC-MS/MS). Tandem MS (MALDI-TOF/TOF or LC-MS/MS), analyzes fragmented peptides, resulting in amino acid sequence information, especially useful when protein spots are low abundant or where a mixture of proteins is present.

Key words DeCyder, SameSpots, Dymension 3, MALDI-TOF/TOF, LC-MS

1 Introduction

Two-dimensional difference gel electrophoresis (2D–DIGE) is a powerful proteomics technology for the study of differential protein expression when comparing samples under different conditions [1]. DIGE has a high sensitivity of 0.2 ng/spot allowing users to run smaller amounts of protein on 2D DIGE gels, resulting in significantly better spot resolution [2]. This high spot resolution enables accurate software-aided spot quantitation and protein expression comparison between samples under investigation [3]. Since the protein expression patterns from three different samples are compared in the same gel (Cy2, Cy3, and Cy5), fewer gels are required to examine all samples. Software aided in-gel analysis enables researchers to generate results quickly post gel running and scanning. The inclusion of an internal standard pooled from

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_4,

aliquots of all samples under investigation in the experiment, usually labeled with Cy2, improves the matching of intra- and inter-gel images and allows normalization across all gels [4]. Scanned images of DIGE gels display thousands of protein spots, each spot representing a single protein or a group of protein isoforms, having a particular p*I* and molecular weight. The benefit of DIGE can only be realized by analyzing gel images with software packages dedicated to DIGE analysis. Three commercially available DIGE analysis software tools that are widely used to analysis raw data include DeCyder V7.2 (GE Healthcare), SameSpots (Totallab), and Dymension 3 (Syngene) [3, 5, 6].

2 DIGE Analysis Software

2.1 DeCyder 2-D Differential Analysis Software

DeCyder 2-D Differential Analysis Software exploits the properties of CyDye DIGE Fluor dyes. It is a fully automated analysis platform, which uses the three gel images obtained from a single gel to perform detection, quantitation, positional matching, and analysis of the images to identify differentially expressed protein spots. The DeCyder 2-D software comprises of four modules: Image loader, Differential In-gel Analysis (DIA), Biological Variation Analysis (BVA), and Extended Data Analysis (EDA).

The image loader takes the scanned images from the imager (Typhoon) and imports them into the analysis software database either as .tiff or customized .gel files. The image loader modules can also be utilized to crop the gel images, removing unnecessary areas to further streamline the analysis. The three images per gel generated because of the different CyDye labels are grouped together with each image containing the respective dye chemistry tag when the samples are named by the user [7].

Images must be processed in the DIA module before subsequent analysis using BVA. This module uses a co-detection algorithm generated by combining image derived from merging individual images associated with each gel. This co-detection algorithm facilitates the establishment of identical spot boundaries for each gel image, which increase the accuracy for individual protein detection across all gels in the project. Background subtraction, gel artifact removal, in-gel normalization, and quantitation for the amount of protein present in each spot are performed after spot detection [8]. DIA expresses abundance values as a ratio, for examples between control (Cy3) and test (Cy5), using the in-gel internal standard reference image (Cy2) for each protein spot. This process is repeated across the gel for each protein spot, providing information on protein abundances for potentially thousands of proteins in the respective gels. This analysis is then saved within the DIA

workspace and can be carried forward to the BVA module for multi-gel analyses [9].

The BVA module uses information already processed in DIA to perform gel to gel matching of all protein spots (within the cropped areas) across all gels in the experiment. This matching across all gels facilitates the quantitative comparison of protein expression associated with the phenotypic comparison under investigation, control versus test for example. A single master gel can be selected automatically or as defined by the user, which can subsequently be used to match all other gels and associated protein spots in the experiment to [10]. A range of statistical tests can be performed in the BVA module to identify significant proteins of interest between comparisons [11].

The EDA module uses the data already processed and contained within the BVA module. EDA can perform a number of different analyses approaches, including Principal Component Analysis (PCA), hierarchical clustering, K-means clustering, and discriminant analysis [12]. PCA facilitates use in the identification of variation within the experimental set-up, providing information on how well the comparison groups will be separated (and consequently cluster together) based on a series of statistically significant proteins [13]. PCA is especially important in the identification of outliers within the gels, possibly the result of poor matching to the master gel or a contaminated sample. Two types of unsupervised clustering approaches are also contained in EDA, Hierarchical clustering and K-means clustering. Hierarchical clustering generates a heat map with associated dendrograms, identifying if different subsets exist within the data set [14]. K-means clustering is useful for visualizing clusters of proteins with similar expression patterns. Discriminant analysis identifies potential biomarkers and based on their expression levels creates a classifier using a number of predefined proteins of interest by the user (Fig. 1). This classifier can then be used to assign unknown samples in respective groups, based on the expression levels of this pre-defined panel of candidate biomarkers. Many different classifiers can be developed, each with its own group of specific proteins. Classifiers can then be compared to each other, based on their ability to correctly assign unknown samples into the correct groups. A potential biomarker panel can be refined using this process, providing very valuable information and the initial development of a diagnostic test [15].

The BVA module also contains the functionality to allow users to perform spot picking from preparative gels by generating a pick list(s). A number of individual preparative gels can be used to perform spot picking, with many users preferring the ability to run the same protein spot multiple times from different gels by mass spectrometry, improving the chances of positively identifying the protein of interest. When using the minimal dye labeling, a Coomassie brilliant blue or fluorescently stained (LavaPurple or

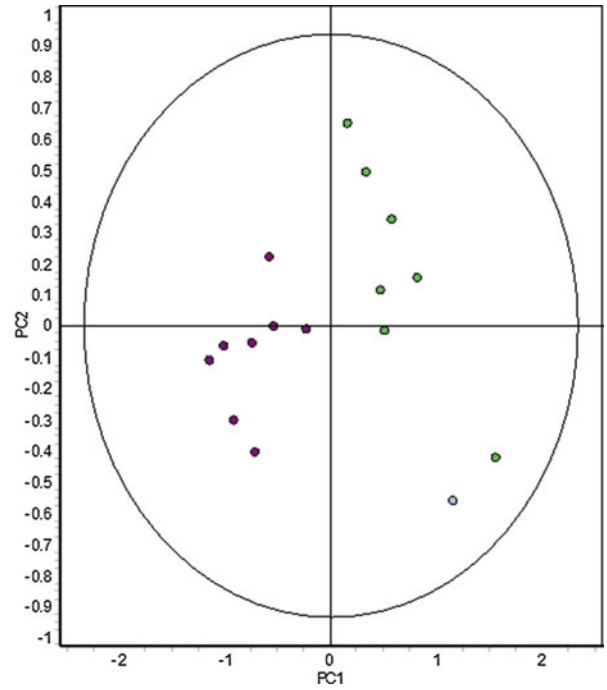

Fig. 1 Principal component analysis (PCA) showing the position of control (*purple*) compared to cancer (*green*) patient samples. This PCA is based on 10 serum proteins that were found to be significantly changed in abundance levels between the two cohorts using DeCyder software

SYPRO Ruby) pick gel is required [16–18]. This is because the unlabeled protein migrates slightly differently to the <5% labeled protein, therefore making spot picking for protein identification difficult as only small amounts of protein would be captured in the gel plugs. The LavaPurple or SYPRO Ruby stained gel image is matched to the analytical set using a series of landmark proteins, which are normally very distinct across all gels and distributed evenly across the gels in terms of their locations. Using reference markers applied to the back of the preparative gels, every protein is tagged with a unique x and y coordinate [19]. This x and y coordinate, associated with the proteins of interest from the pick lists is transferred via a .txt file to the automated Ettan Spot Picker or Ettan Spot Handling Workstation for spot excision, washing, digestion, and mass spectrometry analysis [20].

Spike Protein Normalization (SPN) is a feature added in the DIA and the BVA modules. In conventional DIGE experiments, normally only a small percentage of proteins change significantly in expression levels between the samples groups under investigation. Sample normalization is then based on the large percentage of proteins that show little difference between groups. However, this normalization protocol is not useful for samples that have few

protein spots per gel or samples comparisons when the majority of proteins detected are changing significantly in expression levels (non-normal distribution). In these situations, it is possible to normalize the proteins in an experiment to user defined spiked-in proteins. Spike proteins are included prior to the CyDye labeling step and must reside in an area on the 2D gel that does not of contain any interfering protein spots from the analytical samples. These spiked-in proteins should also be of other origin/species than the analytical samples to help confirm their identification by mass spectrometry. The spiked-in proteins are located during image processing in the DIA or BVA modules and are used by the software to normalize spot volumes for all other proteins in the analysis.

2.2 SameSpots and Dymension 3 Software Packages for DIGE Analysis

Progenesis SameSpots software for 2D gel electrophoresis image analysis, originally sold by Nonlinear Dynamics (now part of the Waters Corporation) was transferred to a sister company, TotalLab in 2013. Image import into SameSpots supports .tiff, .img, .gel and .mel images, with the ability to add new images to existing experiments at any stage [6]. Image quality control automatically highlights many common problems with image quality so the user can decide to remove these gels from the analysis. Images can be cropped, flipped, rotated, and inverted after importation. Alignment of images is performed at the pixel level before image analysis [21]. This feature allows the software to generate a common spot outline map across every image. Filtering and a review of normalization allow the user to remove any artifacts and/or damaged areas from the analytical gels prior to statistical analysis. The software with correction of dye-related offsets in DIGE generates a common spot outline map across every image. The next set is to set up the experimental design, either "between-subject design" or "within-subject design". The "within-subject design" is especially useful for samples from clinical trial as an example, where patient samples may be investigated over a time course associated with treatment.

Differentially expressed protein spots are highlighted by significance based on ANOVA p-value [22]. By using specific tags, protein spots with shared characteristics can be grouped together, based on significant p-values or fold-change associated with protein expression. The software also displays coefficient of variation (CV), to check experimental variation is within the limits defined by the user. Subsequently proteins of interest can be selected for further statistical analysis, picking, and reporting [23].

Statistical packages available in SameSpots include false discovery rate (q-values), PCA, correlation analysis, and power analysis [24]. Using these additional measures of significance, the list of protein of interest can be refined to establish a robust panel of proteins that are significantly changed across experimental groups [25]. Proteins of interest can be highlighted on the imported preparative gels and high precision pick lists send forward to spot

cutting robots including GelPix, ProPic™, and Ettan™ as well as manual picking [26–29].

The Dymension 3 software package from Syngene automatically detects and assigns statistical confidence to each detected protein spot and identifies proteins of interest from Cy2, Cy3, and Cy5 labeled gels [30]. Results of the analysis are displayed as tables, with 3D spot profiles, bar charts together with correlation and scatter plots [31]. The Dymension 3 software package also has different background correction protocols, including a SYPRO Ruby filter, which can detect and automatically remove spot artifacts, created by crystals sticking to the gel during the staining procedure. The statistical analysis available in Dymension 3 allows users to compare expression profiles simultaneously across multiple samples sets and accurately pick proteins of interest for mass spectrometry analysis [3].

3 Protein Identification

3.1 Protein Identification by Peptide Mass Fingerprinting

Identification of proteins is completed by in-gel digestion of protein spots followed by mass spectrometry analysis of the extracted peptides from the gel plugs. Excised gel plugs are destained, washed a number of times, dehydrated and enzymatically digested with trypsin. Other enzymes are available such as Arg-C (clostripain), Asp-N and Glu-C; however, trypsin is used in the majority of experiments. Among the available mass spectrometric methods for protein identification of proteins of interest analyzed by DIGE, peptide mass fingerprinting (PMF) by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS), MALDI tandem MS using MALDI-TOF/TOF mass spectrometry and liquid chromatography tandem mass spectrometry (LC-MS/MS) using reversed-phase chromatography coupled online to the mass spectrometer.

In MALD-TOF, samples are spotted, manually or robotically, onto a metal target plate and mixed with a suitable aromatic organic acid that forms the matrix (e.g., cyano-4-hydroxy cinnamic acid—CHCA) to facilitate ionization of the peptide by donating protons to the analyte. The sample plate is inserted into a vacuum lock and transferred into a vacuum chamber in the mass spectrometer [32]. Some of the sample is used up initially by repeated blasts from a nitrogen laser (337 nm) fired at the spot containing peptides and matrix molecules. The samples is vaporized (desorption) into the gas phase which move away from the target rapidly. The ions produced 'fly' up a tube to the mass analyzer as charged matrix molecules collide with analyte molecules. Light ions reach the detector faster than heavier ions and their masses (mass-to-charge ratio - m/z) are determined by their 'time-of-flight'.

Mass detection must be performed after calibration of the spectrum with internal standards, by using auto-proteolytic peptide masses of trypsin or spiked-in peptides. The list of peptide masses can be transferred into the PMF search program, such as Mascot. The experimentally determined peptide masses may be compared against the NCBI database for a specific organism. Common setting to include when comparing experimental masses to the predetermined mass available in the databases are missed cleavages (allow up to one missed cleavage is normally specified), carbamidomethyl as a fixed modification if iodoacetamide was used to modify cysteines and oxidation of methionines. The peptide tolerance on experimental peptide mass values is based on the type of MS instrument used and the accuracy of the calibration. The results of a peptide mass search are probability-based (MOlecular Weight SEarch (MOWSE) algorithm) meaning the protein scores should be as high as possible to provide the user with a high degree of confidence in the positive protein identification as opposed to a random hit [33].

3.2 Tandem Mass Spectrometry Analysis of Fragmented Peptides

Time-of-flight/time-of-flight (TOF-TOF), or tandem TOF mass spectrometry (MS), consists of two TOF accelerations on the most abundant peptide ions (precursor ions) present in the sample. The first TOF selects, isolates, and fragments a precursor ion of interest using ion-gas collisions to induce fragmentation, a process known as collision-induced decay (CID). The second TOF reaccelerates the precursor ion and fragments, establishing precise masses for sequence ions of the selected peptide, which will be used for database searching [34]. Proteins are then identified by matching the experimental fragmentation patterns of a select peptide with the in-silico fragmentation patterns of all peptides in a specified database that generates an identification score, providing the user with information on the confidence that the particular identified protein is a significant positive hit [35].

In some cases, when only a limited number of peptides are observed for a protein or when there are interferences in the MALDI-TOF spectra from other contaminating proteins, the PMF analysis can fail to provide any positive identification or produce equivocal results resulting in low confidence. In this situation, LC-MS/MS is a very valuable approach to increase confidence in results when low protein scores are achieved. Nano LC-MS/MS is a sensitive and reliable method for protein identification at low fmol level. The peptide mixture from a proteolytic digestion is applied directly into the instrument from an in-line high-performance liquid chromatography (HPLC) system that separates peptides using reversed phase chromatography columns [36]. As peptides elute from the reverse-phase column, fragmentation by CID is performed with a neutral gas and a MS/MS spectra is acquired for each fragmented peptide. B-ions (charge is retained by the

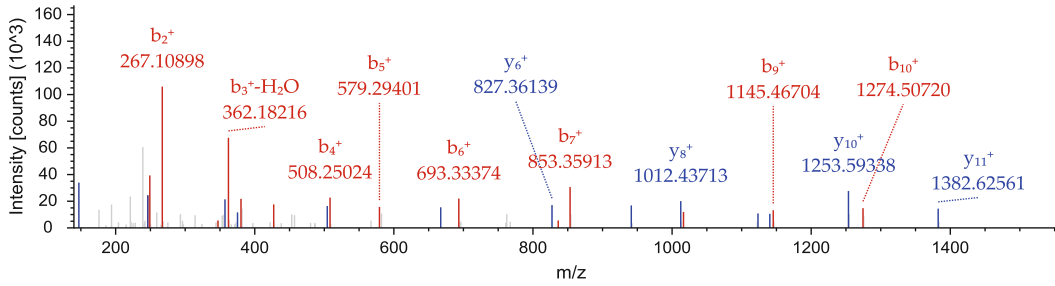

Fig. 2 Example of b- and y- ion series of fragment ions for peptide 1519.68193 (MH+) from Cofilin-1

amino-terminal fragment) and y-ions (charge is retained by the carboxy-terminal fragment) are formed through breakage of the amide bond along the peptide backbone [37]. The MS/MS spectra containing specific peptide sequence information is used to search protein database for matched peptides. The b- and y- ion series represent fragmentation at consecutive peptide bonds and are the foundations to reconstruct the associated peptide sequence (Fig. 2). Database searching algorithms are then used to compare acquired experimental CID spectra to theoretical spectra generated in-silico from a specified protein database, and identify any resulting matches [38]. Electron transfer dissociation (ETD) fragmentation-based analysis has been described as a preferred approach to identify peptides carrying specific post-translational modifications (PTMs). ETD produces c- and z-ions and leaves side-chains intact [39]. By combining data from CID and ETD fragmentation, an array of complementarity fragment ions can be produced, which in some cases can lead to increased amino acid sequence coverage and the identification of more proteins.

References

1. Dowling P, O'Driscoll L, O'Sullivan F, Dowd A, Henry M, Jeppesen PB, Meleady P, Clynes M (2006) Proteomic screening of glucose-responsive and glucose non-responsive MIN-6 beta cells reveals differential expression of proteins involved in protein folding, secretion and oxidative stress. Proteomics 6(24):6578–6587. doi:10.1002/pmic.200600298
2. Gupta S, Ghulmiyyah J, Sharma R, Halabi J, Agarwal A (2014) Power of proteomics in linking oxidative stress and female infertility. Biomed Res Int 2014:916212. doi:10.1155/2014/916212
3. Kang Y, Techanukul T, Mantalaris A, Nagy JM (2009) Comparison of three commercially available DIGE analysis software packages: minimal user intervention in gel-based proteomics. J Proteome Res 8(2):1077–1084. doi:10.1021/pr800588f
4. Malm C, Hadrevi J, Bergström SA, Pedrosa-Domellöf F, Antti H, Svensson M, Frängsmyr L (2008) Evaluation of 2-D DIGE for skeletal muscle: protocol and repeatability. Scand J Clin Lab Invest 68(8):793–800. doi:10.1080/00365510802277464
5. Doran P, Martin G, Dowling P, Jockusch H, Ohlendieck K (2006) Proteome analysis of the dystrophin-deficient MDX diaphragm reveals a drastic increase in the heat shock protein cvHSP. Proteomics 6(16):4610–4621. doi:10.1002/pmic.200600082
6. Missous G, Thammavongs B, Dieuleveux V, Houssin M, Henry J, Panoff JM (2012) Proteomic expression of microfungal ripening starter Geotrichum candidum submitted to cold stress is strain-dependent: studies using 2d-dige technology and samespots software analysis. Cryo Letters 33(4):289–298

7. Donoghue P, Staunton L, Mullen E, Manning G, Ohlendieck K (2010) DIGE analysis of rat skeletal muscle proteins using nonionic detergent phase extraction of young adult versus aged gastrocnemius tissue. J Proteome 73 (8):1441–1453. doi:10.1016/j.jprot.2010.01.014
8. Soares AJ, Santos MF, Trugilho MR, Neves-Ferreira AG, Perales J, Domont GB (2009) Differential proteomics of the plasma of individuals with sepsis caused by Acinetobacter Baumannii. J Proteome 73(2):267–278. doi:10.1016/j.jprot.2009.09.010
9. Doran P, Wilton SD, Fletcher S, Ohlendieck K (2009) Proteomic profiling of antisense-induced exon skipping reveals reversal of pathobiochemical abnormalities in dystrophic mdx diaphragm. Proteomics 9(3):671–685. doi:10.1002/pmic.200800441
10. Ferrín G, Rodríguez-Perálvarez M, Aguilar-Melero P, Ranchal I, Llamoza C, Linares CI, González-Rubio S, Muntané J, Briceño J, López-Cillero P, Montero-Álvarez JL, de la Mata M (2015) Plasma protein biomarkers of hepatocellular carcinoma in HCV-infected alcoholic patients with cirrhosis. PLoS One 10 (3):e0118527. doi:10.1371/journal.pone.0118527
11. Doran P, O'Connell K, Gannon J, Kavanagh M, Ohlendieck K (2008) Opposite pathobiochemical fate of pyruvate kinase and adenylate kinase in aged rat skeletal muscle as revealed by proteomic DIGE analysis. Proteomics 8 (2):364–377. doi:10.1002/pmic.200700475
12. Davalieva K, Kiprijanovska S, Komina S, Petrusevska G, Zografska NC, Polenakovic M (2015) Proteomics analysis of urine reveals acute phase response proteins as candidate diagnostic biomarkers for prostate cancer. Proteome Sci 13(1):2. doi:10.1186/s12953-014-0059-9
13. Eustace AJ, Dowling P, Henry M, Doolan P, Meleady P, Clynes M, Crown J, O'Donovan N (2011) 2D-DIGE analysis of phospho-enriched fractions from dasatinib-treated melanoma cell lines. J Proteome 74(4):490–501. doi:10.1016/j.jprot.2010.12.011
14. Korte J, Fröhlich T, Kohn M, Kaspers B, Arnold GJ, Härtle S (2013) 2D DIGE analysis of the bursa of Fabricius reveals characteristic proteome profiles for different stages of chicken B-cell development. Proteomics 13 (1):119–133. doi:10.1002/pmic.201200177
15. Davalieva K, Kostovska IM, Kiprijanovska S, Markoska K, Kubelka-Sabit K, Filipovski V, Stavridis S, Stankov O, Komina S, Petrusevska G, Polenakovic M (2015) Proteomics analysis of malignant and benign prostate tissue by 2D DIGE/MS reveals new insights into proteins involved in prostate cancer. Prostate 75 (14):1586–1600. doi:10.1002/pros.23034
16. Karp NA, Feret R, Rubtsov DV, Lilley KS (2008) Comparison of DIGE and post-stained gel electrophoresis with both traditional and SameSpots analysis for quantitative proteomics. Proteomics 8(5):948–960. doi:10.1002/pmic.200700812
17. Tokutomi Y, Araki N, Kataoka K, Yamamoto E, Kim-Mitsuyama S (2007) Oxidation of Prx2 and phosphorylation of GRP58 by angiotensin II in human coronary smooth muscle cells identified by 2D-DIGE analysis. Biochem Biophys Res Commun 364(4):822–830. doi:10.1016/j.bbrc.2007.10.095
18. Tewari AK, Popova-Butler A, El-Mahdy MA, Zweier JL (2011) Identification of differentially expressed proteins in blood plasma of control and cigarette smoke-exposed mice by 2-D DIGE/MS. Proteomics 11 (10):2051–2062. doi:10.1002/pmic.201000159
19. Ignjatovic V, Lai C, Summerhayes R, Mathesius U, Tawfilis S, Perugini MA, Monagle P (2011) Age-related differences in plasma proteins: how plasma proteins change from neonates to adults. PLoS One 6(2):e17213. doi:10.1371/journal.pone.0017213
20. Zurawel A, Moore EE, Peltz ED, Jordan JR, Damle S, Dzieciatkowska M, Banerjee A, Hansen KC (2010) Proteomic profiling of the mesenteric lymph after hemorrhagic shock: differential gel electrophoresis and mass spectrometry analysis. Clin Proteomics 8(1):1. doi:10.1186/1559-0275-8-1
21. Bailey MJ, Shield-Artin KL, Oliva K, Ayhan M, Reisman S, Rice GE (2013) Stage-specific analysis of plasma protein profiles in ovarian cancer: difference in-gel electrophoresis analysis of pooled clinical samples. J Carcinog 12:10. doi:10.4103/1477-3163.114216
22. Vélez P, Izquierdo I, Rosa I, García Á (2015) A 2D-DIGE-based proteomic analysis reveals differences in the platelet releasate composition when comparing thrombin and collagen stimulations. Sci Rep 5:8198. doi:10.1038/srep08198
23. Bouvet M, Turkieh A, Acosta-Martin AE, Chwastyniak M, Beseme O, Amouyel P, Pinet F (2014) Proteomic profiling of macrophages by 2D electrophoresis. J Vis Exp 93:e52219. doi:10.3791/52219
24. Heywood WE, Madgett TE, Wang D, Wallington A, Hogg J, Mills K, Avent ND (2011) 2D DIGE analysis of maternal plasma for potential biomarkers of down syndrome. Proteome Sci 9:56. doi:10.1186/1477-5956-9-56

25. Choudhary MK, Nomura Y, Shi H, Nakagami H, Somers DE (2016) Circadian profiling of the Arabidopsis proteome using 2D-DIGE. Front Plant Sci 7:1007. doi:10.3389/fpls.2016.01007
26. Jiang YJ, Sun Q, Fang XS, Wang X (2009) Comparative mitochondrial proteomic analysis of Rji cells exposed to adriamycin. Mol Med 15 (5–6):173–182. doi:10.2119/molmed.2008.00129
27. Pressey JG, Pressey CS, Robinson G, Herring R, Wilson L, Kelly DR, Kim H (2011) 2D-difference gel electrophoretic proteomic analysis of a cell culture model of alveolar rhabdomyosarcoma. J Proteome Res 10(2):624–636. doi:10.1021/pr1008493
28. Ramadoss J, Magness RR (2011) 2-D DIGE uterine endothelial proteomic profile for maternal chronic binge-like alcohol exposure. J Proteome 74(12):2986–2994. doi:10.1016/j.jprot.2011.07.029
29. Kultima K, Scholz B, Alm H, Sköld K, Svensson M, Crossman AR, Bezard E, Andrén PE, Lönnstedt I (2006) Normalization and expression changes in predefined sets of proteins using 2D gel electrophoresis: a proteomic study of L-DOPA induced dyskinesia in an animal model of Parkinson's disease using DIGE. BMC Bioinform 7:475. doi:10.1186/1471-2105-7-475
30. Penque D (2009) Two-dimensional gel electrophoresis and mass spectrometry for biomarker discovery. Proteomics Clin Appl 3 (2):155–172. doi:10.1002/prca.200800025
31. Dowsey AW, Morris JS, Gutstein HB, Yang GZ (2010) Informatics and statistics for analyzing 2-d gel electrophoresis images. Methods Mol Biol 604:239–255. doi:10.1007/978-1-60761-444-9_16
32. Della Corte A, Maugeri N, Pampuch A, Cerletti C, de Gaetano G, Rotilio D (2008) Application of 2-dimensional difference gel electrophoresis (2D-DIGE) to the study of thrombin-activated human platelet secretome. Platelets 19(1):43–50. doi:10.1080/09537100701609035
33. Chen J, Wu W, Chen L, Zhou H, Yang R, Hu L, Zhao Y (2013) Profiling the potential tumor markers of pancreatic ductal adenocarcinoma using 2D-DIGE and MALDI-TOF-MS: up-regulation of complement C3 and alpha-2-HS-glycoprotein. Pancreatology 13(3):290–297. doi:10.1016/j.pan.2013.03.010
34. Sikulu MT, Monkman J, Dave KA, Hastie ML, Dale PE, Kitching RL, Killeen GF, Kay BH, Gorman JJ, Hugo LE (2015) Mass spectrometry identification of age-associated proteins from the malaria mosquitoes Anopheles gambiae s.S. And Anopheles Stephensi. Data Brief 4:461–467. doi:10.1016/j.dib.2015.07.007
35. Lu J, Zhou Z, Zheng J, Zhang Z, Lu R, Liu H, Shi H, Tu Z (2015) 2D-DIGE and MALDI TOF/TOF MS analysis reveal that small GTPase signaling pathways may play an important role in cadmium-induced colon cell malignant transformation. Toxicol Appl Pharmacol 288 (1):106–113. doi:10.1016/j.taap.2015.07.020
36. Weeks ME (2010) Urinary proteome profiling using 2D-DIGE and LC-MS/MS. Methods Mol Biol 658:293–309. doi:10.1007/978-1-60761-780-8_18
37. Sinclair J, Metodieva G, Dafou D, Gayther SA, Timms JF (2011) Profiling signatures of ovarian cancer tumour suppression using 2D-DIGE and 2D-LC-MS/MS with tandem mass tagging. J Proteome 74(4):451–465. doi:10.1016/j.jprot.2010.12.009
38. Hammer E, Bien S, Salazar MG, Steil L, Scharf C, Hildebrandt P, Schroeder HW, Kroemer HK, Völker U, Ritter CA (2010) Proteomic analysis of doxorubicin-induced changes in the proteome of HepG2cells combining 2-D DIGE and LC-MS/MS approaches. Proteomics 10(1):99–114. doi:10.1002/pmic.200800626
39. Burniston JG, Kenyani J, Gray D, Guadagnin E, Jarman IH, Cobley JN, Cuthbertson DJ, Chen YW, Wastling JM, Lisboa PJ, Koch LG, Britton SL (2014) Conditional independence mapping of DIGE data reveals PDIA3 protein species as key nodes associated with muscle aerobic capacity. J Proteome 106:230–245. doi:10.1016/j.jprot.2014.04.015

Part III

DIGE Applications

Chapter 5

Native DIGE: Efficient Tool to Elucidate Protein Interactomes

Diksha Dani and Norbert A. Dencher

Abstract

Protein–protein interactions and multi-protein assemblies are inherent features of proteomes, involving soluble and membrane proteins. This imparts structural and functional heterogeneity to the proteome. One needs to consider this aspect while studying changes in abundance or activities of proteins in response to any physiological stimulus. Abundance changes in components of a given proteome can be best visualized and quantified using electrophoresis-based approaches. Here, we describe the method of Blue Native Difference Gel Electrophoresis (BN DIGE) to quantify abundance changes in proteins in the context of protein–protein interactions. This method confers an additional advantage to monitor quantitative changes in membrane proteins, which otherwise is a difficult task.

Key words Polyacrylamide gel electrophoresis, Proteomics, Native PAGE, Protein–protein interactions, DIGE

1 Introduction

Detection and quantification of abundance as well as activity changes in proteins is imperative to understand the impact of a physiological stimulus, disease-mediated changes, an altered metabolic status or even for identification of biomarkers. Two-dimensional (2D) gel-based techniques like isoelectric focusing (IEF), Blue/Colorless Native (BN/CN) or benzyldimethyl-n-hexadecylammonium chloride (16-BAC)-PAGE [1] in association with several pre-electrophoretic labeling (fluorescence, radio- or stable isotope) [2], or post-electrophoretic staining methods (e.g., Sypro family stains) offer an excellent platform for quantitative proteomic studies. Among these, the recently developed methodology of Difference Gel Electrophoresis (DIGE) is at the forefront, with high sensitivity, accuracy and a wide linear dynamic range comparable to stable isotope labeling [2–4]. DIGE has been essentially evolved as a method in conjunction with 2D–IEF, though it can be efficiently integrated with the alternate approach of Native PAGE [5, 6]. The major reason behind this deviation is the inability

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_5,

of IEF to deal with proteins which are highly hydrophobic (in particular the membrane proteins) as well as those having extreme isoelectric points (highly acidic or basic proteins).

2D BN/SDS gel electrophoresis has been proved to be an efficient system to resolve very basic or highly hydrophobic membrane proteins [7–10], while CN/SDS PAGE is specifically effective for highly acidic proteins as well as hydrophobic membrane proteins, like respiratory complexes in mitochondrial membranes [11–15]. Not only this, the native gel-based techniques also take care of delicate multi-protein/multimeric assemblies, giving a unique insight for heterogeneity of proteomes caused by protein-protein interactions. It is possible to run three-dimensional gels as well. This involves first dimension native gel (BN/CN), subsequently followed by an orthogonal dimension of semi-native gel, where assemblies of proteins are perturbed by using slightly harsher detergents to determine the stoichiometry of protein complexes forming supercomplexes. The third dimension is of denaturing SDS PAGE to resolve and identify subunits of individual protein complexes involved thereby [12, 16]. The native gels can be used to measure in-gel activities of proteins or protein complexes resolved [17]. The Coomassie dye can be removed from first dimension BN gel, if necessary [18]. Even large multimeric/multi-protein assemblies, as huge as 3×10^6 Da can be successfully eluted from preparative BN/CN gels to study structural and functional aspects [19]. These features of native PAGE make the analysis of proteomes much more versatile and meaningful, though one has to compromise with resolution efficiency to some extent. BN-DIGE integrates advantages of DIGE fluorescence labeling with those of BN PAGE to detect abundance changes in soluble as well as membrane proteins, occurring simultaneously as individual species and/or as components of multi-protein/multimeric assemblies that may differ in function and stability [5, 20]. CN PAGE is not commonly used in combination with DIGE, though the absence of Coomassie dye in the first dimension may help sustain the higher labeling efficiency by minimizing any possible interference by Coomassie dye [11] as well as to preserve delicate protein–protein interactions [8]. Thus one has the choice to select BN or CN PAGE to integrate with DIGE methodology, depending on experimental system and focus of investigation.

Given protocol describes the methodology of BN DIGE as an efficient tool for interaction proteomics studies. Migration of proteins and protein complexes in the first dimension BN gels is according to their size (molecular mass). This is also partly influenced by their native shape. While combining with DIGE, the fluorescence labeling of solubilized samples is done just prior to first dimension blue native gel electrophoresis. In the second dimension (denaturing SDS gel), the labeled protein complexes dissociate into constituent subunits that resolve vertically below the position of native protein species in the first dimension (Fig. 1).

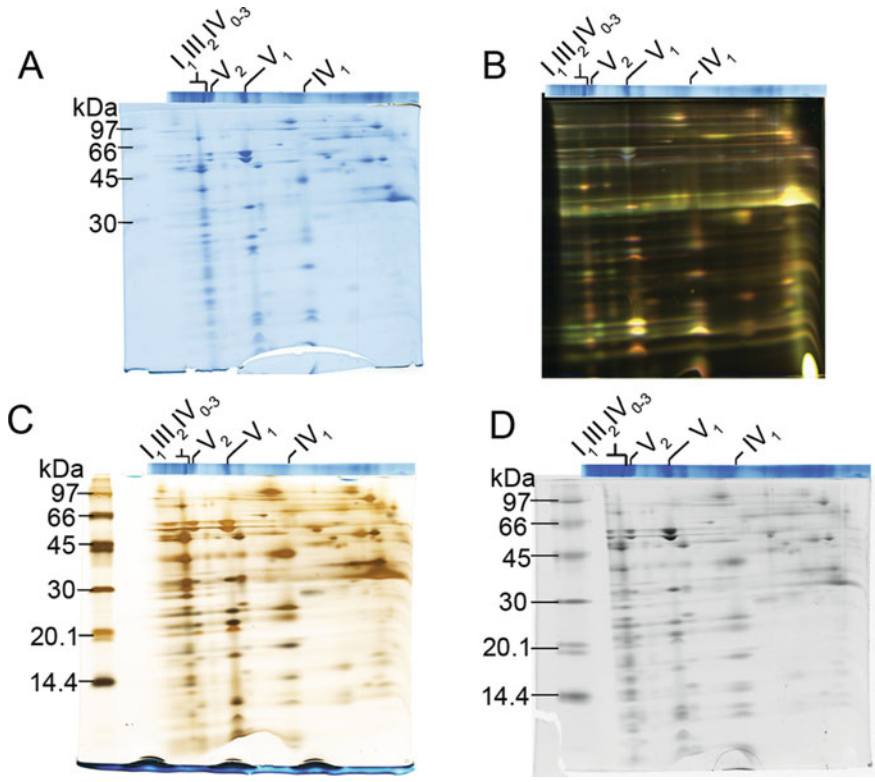

Fig. 1 BN/SDS PAGE of bovine heart mitochondria. The figure represents 2D gel images (differentially stained and G-dye labeled) of bovine heart mitochondrial proteome, as resolved by BN/SDS PAGE. (**a**) Coomassie blue stained; (**b**) G-dye labeled; (**c**) Silver stained; (**d**): SYPRO Ruby stained. Mitochondria were solubilized with the mild detergent digitonin (3 g/g detergent/protein). Positions of some complexes and supercomplexes of mitochondrial respiratory chain proteins are marked on the first-dimension BN gel strip, placed on top of each second-dimension gel image resolving respective protein subunits. As reference, the approximate mass of native OxPhos complexes (IV_1: 200 kDa, V_1: 750 kDa, V_2: 1500 kDa, $I_1III_2IV_{0-3}$: about 1600–2100 kDa) are indicated. To the left of the separated OxPhos supercomplexes $I_1III_2IV_{0-3}$, the trimer of the proton ATP-synthase V_3 (in (**a**) the tetramer V_4 too) is resolved, characterized by the prominent alpha and beta subunits

2 Materials

Use Milli-Q grade, double distilled or equivalent pure, deionized water for all reagents and protocol steps. Follow the specific temperatures as mentioned, for working and storage conditions. Recalculate the volumes for gel mixtures, sample loading, etc., according to the specificities of electrophoresis instrument used (*see* **Note 1**).

2.1 Sample Solubilization and Labeling

1. Sample solubilization buffer pH 7.4: Prepare the sample solubilization buffer by dissolving 150 mM potassium acetate, 30 mM HEPES, 10% glycerol, protease inhibitor cocktail and 5% detergent digitonin (digitonin/protein ratio of 2–8 g/g) in water (*see* **Notes 2** and **3**; refs. 15, 16). Adjust pH to 7.4 using NaOH. Store this buffer frozen in suitable aliquots.

2. Solubilization buffer pH 9.0: Dissolve 150 mM potassium acetate, 30 mM HEPES, 10% glycerol in water. Adjust pH to 9.0 using NaOH. Store this buffer frozen in suitable aliquots.
3. Fluorescent dyes: Use three different, spectrally resolvable fluorescent dyes (CyDyes or G-Dyes). Prepare the stock solutions as per instructions of manufacturer and the working solution of desired concentration (200 or 400 pmoles/μL, *see* **Note 4**, refs. 4, 18). Store the stock and working solutions frozen.
4. Quenching solution: Dissolve 10 mM lysine in water. This solution can be stored frozen in small aliquotes (500 mL) for about 2 months.
5. Sample loading buffer**:** Add 105 mg Bis-Tris (50 mM final), 656 mg 6-aminohexanoic acid (500 mM final), 500 mg Serva Blue G Coomassie brilliant blue G250 (5% w/v final) to 9 mL water. Adjust pH 7.0 with 1 N HCl. Bring total volume to 10 mL with water. Store the buffer up to 2 months at room temperature.

2.2 First-Dimension Blue Native Gel

1. Gel A: 30% (w/v) acrylamide solution. Store up to 2 months at 4 °C (*see* **Note 5**).
2. Gel A/B: 40% (w/v) acrylamide/N,N′-methylene bis-acrylamide solution 29:1. Store up to 2 months at 4 °C.
3. Gel B: 2% (w/v) N,N′-methylene bis-acrylamide solution. Store up to 2 months at 4 °C
4. TEMED: Use 99% pure N, N, N′, N′-tetramethylethylenediamine (TEMED) solution, stored at 4 °C in small aliquots.
5. 10% (w/v) ammonium persulfate (APS): Prepare a 50 mL stock solution, dispense into 1 mL aliquots, and store up to 2 months at −20 °C. Always use freshly thawed solution.
6. Light BN gel buffer**:** 1.27 g imidazole (75 mM final), 49.19 g 6-aminohexanoic acid (1.5 M final). Dissolve in 180 mL water. Adjust to pH 7.0 with 5–6 N HCl. Add water to 250 mL total volume. Filter the solution through a 0.45-μm filter and store up to 1 month at 4 °C (*see* **Note 6**).
7. Heavy BN gel buffer: 1.27 g imidazole (75 mM final), 49.19 g 6-aminohexanoic acid (1.5 M final), 150 g glycerol (60% (w/v) final). Dissolve in 50 mL water. Adjust to pH 7.0 with 5 to 6 N HCl. Add water to 250 mL total volume. Filter the solution through a 0.45-μm filter and store up to 1 month at 4 °C.
8. Anode buffer: Dissolve 3.404 g imidazole (25 mM final) in 1 L water. Adjust to pH 7.0 with 5 to 6 N HCl. Add water to 2 L and store up to 1 month at 4 °C.
9. Cathode buffer clear: Dissolve 1.02 g imidazole (7.5 mM final) and 17.92 g tricine (50 mM final) in 1 L water. Do not adjust

pH; it should be approximately 7. Add water to 2 L and store up to 1 month at 4 °C.

10. Cathode buffer (dark blue): Dissolve 0.4 g Coomassie Blue G-250 (0.02% w/v final) in 2 L of cathode buffer clear. Stir this mixture overnight to ensure complete dissolution of Coomassie and filter through analytical grade paper filter. Store up to 1 month at room temperature.
11. Cathode buffer (light blue): Dilute 1 vol cathode buffer dark blue with 9 vol of cathode buffer clear. Store up to 1 month at 4 °C.
12. *3% gel mixture*: Mix 1438 µL Gel A solution, 667 µL Gel B solution, 4.94 mL light BN gel buffer 3× without glycerol, 7.7 mL water. Stir well and pour this mixture in respective chamber of gradient maker. Start polymerization by adding 6.8 µL TEMED and 68 µL APS.
13. *13% gel mixture*: Mix 6.23 mL Gel A solution, 2.89 mL Gel B solution, 4.94 mL heavy BN gel buffer 3× with glycerol. Make volume to 14.78 mL with water. Stir well and pour this mixture in the respective chamber of gradient maker. Start polymerization by adding 4.1 µL TEMED and 41 µL APS.
14. In-gel denaturation solution: Dissolve 2% SDS (w/v), 2% beta-mercaptoethanol (BME, v/v) and 66 mM $NaHCO_3$ in 10 mL water. Store the solution (up to 3 weeks) at 4 °C (*see* **Note 7**).

2.3 Second-Dimension Tricine-SDS Gel

1. Tricine-SDS buffer 3× (glycerol): Dissolve carefully 90.86 g Tris (3.0 M final), 0.375 g SDS (0.15% final), 75 g glycerol (30% final) in 210 mL water (final volume) and 5 mL of 30% HCl. Adjust to pH 7.0 with 30% HCl. Add water to 250 mL total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at room temperature.
2. Tricine-SDS buffer 3×: Dissolve 90.86 g Tris (3.0 M final), 0.375 g SDS (0.15% final) carefully in 210 mL water (final volume) and 5 mL of 30% HCl. Adjust to pH 7.0 with 30% HCl. Add water to 250 mL total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at room temperature.
3. Anode buffer: Dissolve 12.11 g Tris (100 mM final) in 900 mL water. Adjust to pH 8.9 with 5–6 N HCl. Make total volume to 1000 mL with water. Store up to 1 month at 4 °C.
4. Cathode buffer: Dissolve 12.11 g Tris (100 mM final), 17.96 g tricine (100 mM final), 0.5 g SDS (0.05% final) in 900 mL water. Make total volume to 1000 mL. Do not adjust the pH, the final pH is approximately 8.25. Store the buffer up to 1 month at 4 °C.
5. 13% separating gel mixture (total volume 30 mL): Mix 1.3 mL Gel A solution, 8.8 mL Gel A/B solution, 10 mL SDS gel

buffer 3× (with glycerol). Make total volume to 29.8 mL with water. Start polymerization by adding 15 μL TEMED and 150 μL APS.

6. 5% denaturing stacking gel mixture (total volume 10 mL): Mix 1.62 mL Gel A solution, 0.75 mL Gel B solution, 3.3 mL SDS gel buffer 3× (without glycerol). Make volume to 9.95 mL with water. Start polymerization by adding 5 μL TEMED and 50 μL APS.
7. 5% semi-native stacking gel mixture (total volume 10 mL): Mix 1.62 mL Gel A solution, 0.75 mL Gel B solution, 3.3 mL light BN gel buffer 3× (without glycerol), 100 μL of 20% SDS solution. Make volume to 9.9 mL by adding water. Start polymerization by adding 7 μL TEMED and 70 μL APS.

2.4 Gel Documentation System

Typhoon gel scanner is best recommended for DIGE; however, any other digital gel documentation system with possibility to select variable wavelengths for image acquisition (according to different fluorescent dyes with varying absorption/excitation and emission maxima) can also be employed.

2.5 Image Analysis Software

Image analysis and statistical tests for fluorescence DIGE-labeled samples can be performed using integrated packages like DeCyder (GE Healthcare) and Delta2D (Decodon) or combination of individual packages like Delta Vision (Applied Precision, WA), IPLab (Scanalytics), and SExtractor (http://sourceforge. Net/projects/sextractor).

2.6 Other Accessories

1. Electrophoresis apparatus: Suitable slab-gel electrophoresis apparatus with clamps, separate gel-casting unit and buffer chambers (*see* **Note 8**).
2. 1.5-mm spacers.
3. Normal and low-fluorescence glass plates.
4. Peristaltic pump with 2.79-mm PVC tubing.
5. 19-G needle (1.00 mm × 200 mm).
6. Gradient maker with two chambers (250 mL volume).
7. Magnetic stirrer and magnetic bars.
8. Teflon combs (10-, 15-, or 20-tooth comb).

3 Methods

3.1 Sample Solubilization and Labeling

1. Solubilize the samples (equal protein basis before solubilization; 100 μg protein for mitochondria as case example) separately, by incubation with solubilization buffer pH 7.4 containing detergent and protease inhibitor cocktail (1:200

dilution), on ice for 30 min. Centrifuge the samples for 10 min at 4 °C (at least 20,500 × *g*) to remove any insoluble material. Collect the supernatant solubilizate (*see* **Note 9**).

2. Prepare internal standard for DIGE by pooling all samples under comparison and solubilizing the pooled sample as described above. Store the internal standard aliquots (corresponding to 50 μg protein after solubilization in given case example of mitochondria) frozen and use these for all the gels to facilitate the normalization and overcome the error by gel to gel variation.
3. Adjust the pH of solubilizate (actual samples and internal standard) to 8.5 by adding two volumes of same solubilization buffer set to pH 9.0, without detergent and protease inhibitors (*see* **Note 9**).
4. Add 1 μL of fluorescent dye working solution to the solubilizate (200–400 pmoles of dye/50 μg of protein after solubilization) adjusted to pH 8.5 (*see* **Note 10**). Mix the contents by brief vortexing, followed by a quick low-speed centrifugation using a table-top centrifuge. Continue the labeling in dark on ice for 30 min.
5. Perform dye swapping (*see* **Note 11**) for the samples with a pair of fluorescent dyes so that each sample gets labeled with each dye. Keep the dye for internal standard separate (usually the one with lowest absorption/excitation maxima) and do not include it for dye swapping with rest of the samples.
6. Quench the labeling process by adding 10 μL of 10 mM lysine. Vortex the contents briefly and keep the samples on ice, in dark for 10 min.
7. Mix the samples (in suitable pairs and with internal standard on equal protein basis) to be loaded in the same well for first-dimension gel (*see* **Note 12**).
8. Add desired quantity (about 10–12 μL) of sample loading buffer to achieve 4:1 detergent:dye ratio (*see* **Note 13**).

3.2 First-Dimension Blue Native Gel Assembly

The gradient gel for first dimension electrophoretic run is prepared and kept ready prior to solubilization of samples.

1. Preparing the gel: For casting a single gel (3–13% gradient) with 1.5-mm × 14-cm × 16-cm dimensions, prepare 15 mL volume of each solution to give 30 mL of total volume for gradient separating gel. Stacking gel solution (3%) is prepared separately in 10 mL volume.
2. Assemble the gel-casting unit for one gel with 1.5-mm spacers, using normal glass plates.
3. Set up the peristaltic pump by inserting tubing. Connect one end of tubing to the 19-G needle. Using water, adjust the flow

rate so that the whole volume of the gradient solution (30 mL) is poured in approximately 10–15 min.

4. Place the gradient maker on a magnetic stirrer. Connect it to peristaltic pump. Carry out the further procedures at 4 °C in refrigerator or a cold room. Use pre-cooled gel solutions and buffers.
5. Place magnetic bars in both chambers of the gradient maker and close all valves. Place the gel-casting unit near to the peristaltic pump at 4 °C and insert the needle between the glass plates of the gel-casting unit, placing it vertically along one of the spacers. The opening of the needle should just touch the bottom of plates ensuring that 3% gel solution enters first.
6. Prepare the 3% and 13% mixtures for gradient separating gel as described in Materials section and pour in respective chambers of gradient mixer. 3% gel mixture should be the first to enter gel-casting unit, hence placed in chamber adjacent to the outlet tubing.
7. While stirring the solution thoroughly in the gradient maker, switch on the peristaltic pump and simultaneously open the interconnection between both the chambers of gradient maker. Pump the gel mixture into the gel-casting unit, leaving behind a small portion in tubing before stopping the pump, in order to avoid air bubbles entering the gel.
8. Carefully remove the needle from the gel chamber while preventing air bubbles. Move the gel casting unit out of the refrigerator/cold room and let the gel polymerize at room temperature.
9. Prepare the stacking gel solution by mixing 1455 μL of Gel A solution, 675 μL of Gel B solution, 5 mL of BN gel buffer 3× (without glycerol) and 7.65 mL water. Start polymerization by adding 20 μL TEMED and 200 μL APS.
10. Decant the overlying water from separating gel, rinse briefly the upper gel boundary by small volume of stacking gel solution and then pour the remaining stacking gel solution between glass plates using a pipette. Insert carefully a suitable comb in between the glass plates at an angle, in order to avoid trapping of air bubbles. Allow the gel to polymerize at room temperature.
11. For storage: remove the comb, dilute light gel buffer 3× with water, resulting in a 1× buffer and use this buffer immediately to rinse the sample wells. Fill the empty wells of stacking gel with the same 1× buffer and cover the assembly with aluminum foil to prevent drying. Store the gel for a maximum of 3 days at 4 °C.

3.3 BN Gel Electrophoresis

1. Assemble the electrophoresis device and fill the wells of stacking gel with the appropriate cathode buffer (for BN gel). Load the samples into the wells of the gel (*see* **Note 14**) adjusting equal loading volumes for all.
2. Load any unused (blank without sample) wells with a solution (buffer, detergent, sample dye) closely resembling that of neighboring wells (*see* **Note 15**).
3. Place appropriate cathode buffer in the upper chamber and anode buffer in the lower chamber (*see* **Note 16**).
4. Connect the electrophoresis device to power supply. Set the current to 15 mA and voltage to 100 V. Perform electrophoresis at 4 °C, in dark. Increase voltage to 500 V after the blue-colored sample running front completely enters the separating gel, taking care that the current does not exceed 15 mA.
5. Replace the dark blue cathode buffer with light blue cathode buffer (*see* **Note 16**) as the samples cover about 20% distance on separating gel. Resume electrophoresis at 500 V.
6. Stop the electrophoretic run as the blue running front starts leaving the gel. If the focus of study is on high molecular weight proteins, then the blue front should completely run out.
7. Dismantle the assembly, remove spacers and lift away one of the glass plates carefully so that the gel remains attached to other plate. Take care that the gel does not dry by spraying a little water on it. Cut the lanes of interest from BN gel for further denaturation and second-dimension electrophoresis. Perform all operations in dark/subdued light conditions.

3.4 In-Gel Denaturation

1. Transfer the cut BN gel lanes carefully to a thick plastic sheet in fume hood. Incubate the gel strips on plastic sheet with denaturation solution containing 2% SDS, 2% BME, and 66 mM $NaHCO_3$ at room temperature for 1 h. Overlay this solution such that the gel strips are completely submerged in it but not floating. Change the solution at interval of 20 min. Perform all operations in dark/subdued light conditions.
2. Wash the gel strips repeatedly with water (3–4 brief washes) to remove BME.

3.5 Second-Dimension Tricine-SDS Gel Assembly

1. After denaturation, place the gel strip from plastic sheet onto a clean, low fluorescence glass plate. Position it at distance of about 1.5 cm below the top, perpendicular to gel-running direction so that the end with high polyacrylamide concentration touches close to one of the spacers.
2. Mount the spacers at each side of the glass plate. Then, carefully place the second, clean, low fluorescent glass plate on top and fix with clamps. Fix the assembly vertical in the gel-casting unit.

3. Prepare the solutions for separating and sample gels as described in the Materials section:
4. Pour the 13% separating gel mixture with a glass pipette between the glass plates so that a gap of 2 cm between the separating gel and the BN gel strip is left. Carefully overlay the gel solution with water.
5. After the separating gel gets polymerized, decant the overlay water, rinse briefly the upper gel boundary with 5% denaturing (lower) stacking gel solution and then pour the 5% denaturing stacking gel solution, leaving a gap of 1 cm below the BN gel strip. Overly the denaturing stacking gel solution with water.
6. After polymerization of the denaturing stacking gel (lower), decant the overlay water. Pour the semi-native stacking gel (upper) solution until it touches the native gel strip (avoiding trapping of air bubbles) and fills the gaps between the strip and the spacers.
7. Add respective cathode and anode buffers for SDS Tricine gel to the upper and lower chambers of the gel unit. Connect the gel unit to power supply.

3.6 SDS-Tricine Gel Electrophoresis in Second Dimension

1. Perform the electrophoresis for second-dimension SDS-Tricine gels in dark, at 17 °C. Keep initial voltage to 30 V and current 60 mA.
2. As the proteins enter the separating gel, increase the voltage to 120–150 V taking care that the current does not exceed 30 mA. For overnight run, keep the voltage at 100 V and current 25 mA. Continue electrophoresis till the blue running front leaves the gel completely.
3. As the electrophoresis is completed, dismantle the assembly. Let the gel remain within low fluorescent glass plates. Wrap the glass plates containing gel with moist tissues and aluminum foil until scanning.

3.7 Gel Documentation

Scan the gel without removing from glass plates, using a variable wavelength scanner like Typhoon (GE Healthcare). Clean the surface of glass plates carefully with water and remove the spacers before scanning (*see* **Notes 17** and **18**).

3.8 Image Analysis and Data Interpretation

Perform image analysis using a software that is compatible with the gel documentation system used as well as suitable for differential quantitation and multiplex, multivariate analysis of samples, as anticipated by DIGE methodology (*see* **Notes 19** and **20**).

For interpretation of BN DIGE data in context to protein interactomes, please refer to **Notes 21** and **22** as important guidelines.

4 Notes

1. This protocol is designed for standard size gels (14 cm × 16 cm). The longer the migration distance is, the better the resolution of proteins! The same type of slab-gel electrophoresis apparatus can be used for first-dimension native and second-dimension denaturing gels.
2. The sample solubilization buffer mentioned here suits mitochondria as case example. One needs to select proper solubilization buffer as per the experimental system [16] and standardize the optimum solubilization conditions regarding duration, type of detergent and suitable detergent/protein ratio, which is a very critical step [17]. There is no limit for the nature of sample that can be checked with BN DIGE method. From crude cell/tissue extracts to fractionated and partially purified protein samples, anything can be tested [10]. However, for higher solubilization efficiency, more precision, and better data quality, it is advisable to standardize carefully the experimental set up and use fractionated samples when possible. It is also necessary to check for any possible interference of reagents used with fluorescence labeling. This information is provided by the manufacturers of fluorescent dyes for DIGE. The pH of solubilization buffer is often critical to preserve delicate protein assemblies. Hence, it is advisable to adjust the pH to 8.5 as required for DIGE, just prior to labeling. For mitochondrial system in present experimental set up, we confirmed that the supercomplexes of mitochondrial membrane respiratory chain proteins remain stable even at pH 8.5.
3. The most commonly employed detergents for BN/CN PAGE are dodecylmaltoside (DDM), Triton X-100, and digitonin. Among these, digitonin has been found to be mild enough to preserve even delicate protein–protein interactions [16], such as respiratory supercomplexes and not interfering with DIGE labeling.
4. Though use of CyDyes is proposed in the original protocol by Viswanathan et al. [4] and is successfully applied further in combination with BN PAGE [5, 19, 21], it is possible to substitute the CyDyes by other compatible, spectrally resolvable fluorescent dyes like G-dyes [19], depending on the availability and cost constraints. The strength of working solutions of fluorescent dyes ranges between 200 and 400 pmoles/μL. In given case example of mitochondrial proteins and in present experimental set up, we found optimum labeling efficiency with 400 pmoles/μL. It is not advisable to work with higher

concentrations of fluorescent dyes but rather to have more protein available for labeling.

5. It is recommended to use for safety reasons, the commercially available, ready-to-use solutions rather than working with the neurotoxic acrylamide powder. This also helps greatly to maintain high quality and reproducibility of the gels. Always wear gloves while handling these solutions in an extractor fume hood and use a pipetting aid.
6. For mitochondrial samples (mentioned here as case example), the buffers containing imidazole are suitable for first-dimension BN gel. However, Bis-Tris containing buffers give better results for chloroplast or cyanobacterial samples [9, 16, 21].
7. It is possible to reduce the strength of SDS and BME both, to 1% and accordingly adjust the incubation time of gel strips with the denaturation solution. The conditions mentioned here for the denaturation step were found optimum for given experimental set up. BME is known to interfere with fluorescence labeling, reducing the efficiency. Hence, one needs to standardize the procedure to achieve the desired efficiency of labeling as well as optimum denaturation of proteins for the following second-dimension gel electrophoresis. Use of $NaHCO_3$ is optional [5, 21]. We found it not interfering but rather beneficial, by conferring buffering capacity during the incubation step at room temperature, in presence of BME.
8. The given protocol (for the mentioned volumes of reaction mixtures, etc.) applies for standard size gels (14 cm × 16 cm × 1.5 mm) with 30 mL total volume for a separating gel and 10 mL total volume for a stacking gel.
9. The solubilization buffer as mentioned in material section contains 5% w/v detergent digitonin (suitable for mitochondrial samples). The volume of solubilization buffer added determines the desired detergent/protein ratio (8 g/g in this case). The final detergent concentration in the sample for loading is 1% (considering all dilutions). In given case example of mitochondria, 16 μL of solubilization buffer containing 5% digitonin was added to mitochondrial pellet for solubilization. Later, 32 μL of solubilization buffer pH 9.0 (without detergent) was added to adjust the pH of solubilizate to 8.5, as required for labeling. Achieving mild but effective sample solubilization to preserve delicate protein associations, while obtaining the desired protein concentration of even low abundant proteins for labeling, is a crucial initial step in BN DIGE experiment.
10. For the present experimental set up, we found optimum labeling with 400 pmoles of dye/50 μg of protein after solubilization.

11. Some proteins show preferential labeling with certain fluorescent dyes. To avoid any error as a consequence thereof, the dye swapping is essential.
12. In the given example, we mixed 50 μg protein of each sample, resulting in 150 μg of total protein load. One has to consider the nature of sample, efficiency of labeling, gel size, maximum sample volume, and protein amount as recommended for loading on the gel.
13. Addition of sample dye is crucial for solubilized membranes which contain interfering substances like lipids that cause anomalies by forming detergent micelles. Purified membrane proteins or water-soluble proteins can be loaded without adding sample dye [16].
14. For a 10-tooth comb (8.5 × 1.5 mm sample well), the sample volume should not exceed 220 μL.
15. This is crucial to avoid deformed electrophoretic patterns due to differences in ionic strength between sample-loaded and unused wells and to obtain uniformly straight runs of proteins in the sample lanes.
16. While working with crude protein extracts (in particular, the solubilizates from native membranes), contaminating species such as detergent-lipid micelles interfere with electrophoretic separation of proteins. Use of cathode buffer blue (dark) containing 0.02% (w/v) Coomassie blue G250 at the start of electrophoresis helps in pulling the lipid-detergent particles to the running front, thereby improving the resolution of proteins on gel. Purified protein assemblies, thus devoid of surplus detergent-lipid micelles are more fragile and should be separated in the presence of cathode buffer with decreased dye content of only 0.002% (w/v) [8]. Therefore, the dark blue cathode buffer used at beginning of electrophoresis is replaced by light blue cathode buffer as the samples traverse 20% of the distance on separating gel [16].
17. Prescan the gels at lower resolution (500 μm pixel area) to optimize the PMT (photomultiplier tube) value. Select the maximum possible value for PMT, at which the highest abundant protein does not show saturation. Perform final scan of gels at higher resolution (100 μm) using the optimized PMT value. Keep the resolution (pixel size) and PMT value constant for all gels under comparison.
18. If the labeling efficiency is low, it is not advisable to increase the sensitivity of detector (PMT value) as it will result in overall increase in background. It is also not recommended to increase the amount of fluorescent dye beyond a limit, hence the only way to enhance labeling signal is to increase the initial amount of protein for labeling.

19. Comparatively higher technical noise (variance) is an important factor that has to be considered while doing BN-DIGE. It is necessary to estimate the net variance (technical variance + biological variance) for a given system prior to experimental design. When the net variance is higher, the accuracy of differential quantification is less [6]. One can deal with this problem by implementing the following measures:
    (a) To include an appropriately large number of individual biological replicates or to pool all replicate samples, while introducing more experimental repetitions of the common pool.
    (b) Application of false discovery rate (FDR) as multiple testing correction method.
    (c) Use of software that applies the algorithm based on variance stabilizing transformations like the logarithmic function.
20. The resolution in first-dimension BN gel is relatively low resulting in ill-defined spot boundaries on the second-dimension SDS gel, especially for the low molecular mass soluble proteins (Fig. 1). The overall proteome resolved on a BN-SDS-PAGE shows great variation in spot size and shape [6]. This can potentially hamper the quantification by inducing errors in measurement of spot volumes. Hence, one has to be very careful during image analysis for selection of proper spot defining parameters. Visual inspection is always necessary to confirm the accuracy of spot boundaries (spot volume) and spot matching across the different gels (image warping).
21. In context to low resolution, when the detected spot on gel showing significant difference in abundance contains more than one protein species, as might frequently happen with BN DIGE system, it is necessary to cross check and validate the observation by implementing alternate approaches. The best suitable way to cross-check BN DIGE observations in controversial cases is to perform Western blotting with specific antibodies after BN/SDS PAGE of suitable replicate sample groups or Peptide Mass Fingerprinting (PMF) MALDI MS.
22. By employing BN DIGE methodology, one deals with quantitative aspects of protein interactomes. Thus, it is commonly observed that a given protein exists simultaneously in different pools (various assemblies and as individual), showing differential pattern for abundance change. One has to carefully analyze these data (change and no change in abundance) considering various interlinked aspects, such as differences in stability and activity, dynamic protein–protein interactions, structural and functional heterogeneity in overall proteome as well as the total pool of any given protein.

Acknowledgement

The authors would like to acknowledge with gratitude Dr. Manuela Kratochwil (former Ph.D. student of Prof. Dencher) for contributing the BN/SDS PAGE images (unpublished data, part of the doctoral thesis "Effects of ageing, calorie restriction and ageing-associated diseases on the mitochondrial proteome", 2015, by Manuela Kratochwil at the Technische Universität Darmstadt).

References

1. Hunzinger C, Wozny W, Schwall GP, Poznanovic´ S et al (2006) Comparative profiling of the mammalian mitochondrial proteome: multiple aconitase-2 isoforms including N-formylkynurenine modifications as part of a protein biomarker signature for reactive oxygen species. J Proteome Res 5:625–633
2. Groebe K, Krause F, Kunstmann B, Unterluggauer H et al (2007) Differential proteomic profiling of mitochondria from *Podospora anserina*, rat and human reveals distinct patterns of age-related oxidative changes. Exp Gerontol 42:887–898
3. Schwaßmann HJ, Rexroth S, Seelert H, Dencher NA (2007) Metabolism controls dimerization of the chloroplast F_0F_1 ATP synthase in *Chlamydomonas reinhardtii*. FEBS Lett 581:1391–1396
4. Viswanathan S, Ünlu M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358
5. Dani D, Shimokawa I, Komatsu T, Higami Y, Warnken U, Schokraie E, Schnölzer M, Krause F, Sugawa MD, Dencher NA (2010) Modulation of oxidative phosphorylation machinery signifies a prime mode of anti-ageing mechanism of calorie restriction in male rat liver mitochondria. Biogerontology 11:321–334
6. Dani D, Dencher NA (2008) Native DIGE: a new look at the mitochondrial membrane proteome. Biotechnol J 3:817–822
7. Schägger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 199:223–231
8. Neff D, Dencher NA (1999) Purification of multisubunit membrane protein complexes: isolation of chloroplast F_oF_1-ATP synthase, CF_o & CF_1 by blue native electrophresis. Biochem Biophys Res Commun 259:569–575
9. Rexroth S, Meyer zu Tittingdorf JMW, Krause F, Dencher NA, Seelert H (2003) Thylakoid membrane at altered metabolic state: challenging the forgotten realms of the proteome. Electrophoresis 24:2814–2823
10. Krause F (2006) Detection and analysis of protein-protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (membrane) protein complexes and supercomplexes. Electrophoresis 27:2759–2781
11. Wittig I, Karas M, Schägger H (2007) High resolution clear-native electrophoresis for in-gel functional assays and fluorescnce studies of membrane protein complexes. Mol Cell Proteom 6:1215–1225
12. Wumaier Z, Nübel E, Wittig I, Schägger H (2009) Two dimensional native electrophoresis for fluorescent and functional assays of mitochondrial complexes. Methods Enzymol 456:153–168
13. Krause F, Reifschneider NH, Vocke D, Seelert H, Rexroth S, Dencher NA (2004) "Respirasome"-like supercomplexes in green leaf mitochondria of spinach. J Biol Chem 279:48369–48375
14. Krause F, Scheckhuber CQ, Werner A, Rexroth S, Reifschneider NH, Dencher NA (2004) Supramolecular organization of cytochrome c oxidase- and alternative oxidase-dependent respiratory chains in the filamentous fungus *Podospora anserina*. J Biol Chem 279:26453–26461
15. Krause F, Reifschneider NH, Goto S, Dencher NA (2005) Active oligomeric ATP synthase in mammalian mitochondria. Biochem Biophys Res Commun 329:583–590
16. Krause F, Seelert H (2008) Detection and analysis of protein-protein interactions of organellar and prokaryotic proteomes by blue native and colorless native gel electrophoresis. Curr Protoc Protein Sci:18–19. Chapter 14, Unit 14.11
17. Frenzel M, Rommelspacher H, Sugawa MD, Dencher NA (2010) Ageing alters the supramolecular architecture of OxPhos complexes in rat brain cortex. Exp Gerontol 45:563–572

18. Poetsch A, Neff D, Seelert H, Schägger H, Dencher NA (2000) Dye removal, catalytic activity and 2D crystallization of chloroplast H^+-ATP synthase purified by blue native electrophoresis. Biochim Biophys Acta 1466:339–349
19. Schäfer E, Seelert H, Reifschneider NH, Krause F et al (2006) Architecture of active mammalian respiratory chain supercomplexes. J Biol Chem 281:15370–15375
20. Kuter K, Kratochwil M, Marx S-H, Hartwig S, Lehr S, Sugawa MD, Dencher NA (2016) Native DIGE proteomic analysis of mitochondria from substantia nigra and striatum during neuronal degeneration and its compensation in an animal model of early Parkinson's disease. Arch Physiol Biochem 1862:741–753
21. Peters K, Braun H-P (2012) Comparative analyses of protein complexes by blue native DIGE. Methods Mol Bio (Clifton, N.J.) 854:145–154

Chapter 6

Comparative Two-Dimensional Fluorescence Gel Electrophoresis

Doreen Ackermann and Simone König

Abstract

Two-dimensional comparative fluorescence gel electrophoresis (CoFGE) uses an internal standard to increase the reproducibility of coordinate assignment for protein spots visualized on 2D polyacrylamide gels. This is particularly important for samples, which need to be compared without the availability of replicates and thus cannot be studied using differential gel electrophoresis (DIGE). CoFGE corrects for gel-to-gel variability by co-running with the sample proteome a standardized marker grid of 80–100 nodes, which is formed by a set of purified proteins. Differentiation of reference and analyte is possible by the use of two fluorescent dyes. Variations in the y-dimension (molecular weight) are corrected by the marker grid. For the optional control of the x-dimension (p*I*), azo dyes can be used. Experiments are possible in both vertical and horizontal (h) electrophoresis devices, but hCoFGE is much easier to perform. For data analysis, commercial software capable of warping can be adapted.

Key words Comparative fluorescence gel electrophoresis, hCoFGE, 2D-PAGE, Protein coordinates, Protein grid

1 Introduction

Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) is one of the most important techniques in the biochemical laboratory. Due to its exceptional high resolution, it can provide maps of entire proteomes visualizing thousands of proteins as stained spots on a gel matrix. The separation technique is often used in preparation for protein identification. Proteome images in conjunction with the ID of the proteins provide an illustrative description of the analyte and thus call for archiving. However, the main crux of gel electrophoresis, gel-to-gel variability as a result of the limited control of acrylamide polymerization, is a major drawback. This problem was recognized for proteome expression studies, where changes of proteins after treatment are studied. These experiments must include at least three replicates of each sample type in order to guarantee statistical relevance of the data.

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_6,

For this situation, the DIGE technique was developed [1]. It is a well-thought-through system solution involving mass- and charge-matched fluorescent dyes to label control and treated samples, respectively, as well as an internal standard formed by pooling all samples in the experiment. Moreover, gel variation is minimized by stringent protocols and special hardware (6- or 12-gel electrophoresis chambers). DIGE has been commercialized as an analytical pipeline including dedicated software so that the user is provided with a list of regulated proteins at the end of the experiment.

However, often replicate samples are not available. In order to still ensure reproducibility, the CoFGE technology (Comparative Fluorescence Gel Electrophoresis; see original publications for further details [2–5]) was developed which has been shown to improve the error in the assignment of spot coordinates from more than 10% to below 1%. Thereby, an internal molecular weight (MW) standard of about 8–10 commercially available purified proteins covering the entire molecular weight (MW) range (typically 8–100 kDa) is co-run with the analyte. This multiplexing is possible by the use of two different fluorescent dyes (1—sample, 2—MW standard). In this way, the standard proteins experience the same gel variation as the analyte proteins. In order to have the standards available across the whole separation range, a series of marker wells (10–14) is added to the gel close to the trough for the p*I* strip (Fig. 1).

CoFGE can be performed in both vertical and horizontal electrophoresis devices; in fact, it was originally developed for the former

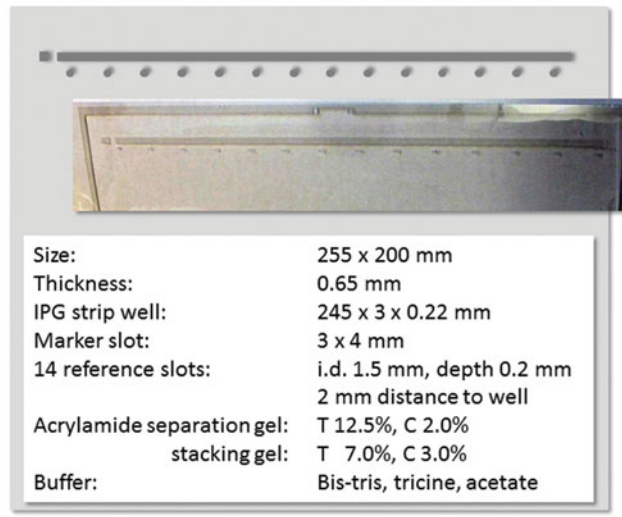

Fig. 1 Schematic of a Mercator gel for hCoFGE. CoFGE is enabled by the introduction of 14 round marker wells on the anodic gel side of the p*I*-strip (in the commercially available gel format for technical reasons; home-made gels had the wells on the cathodic side [4]). They hold 0.5 μL of protein solution (theoretical volume 0.35 μL). *Inset*: Photograph of top part of commercial Mercator gel

[2]. However, handling is considerably improved in the latter, and it is currently commercialized for this approach by the leading manufacturer in this area, SERVA Electrophoresis (Heidelberg, Germany). Thus, we focus on the protocol for hCoFGE below. Nevertheless, the major difference between vCoFGE and hCoFGE is the generation of the marker wells. While they are punched into the gel in hCoFGE (Fig. 1), in vCoFGE, they are generated by a pseudo 1D–PAGE set-up. To that end, a plastic comb with V-shaped teeth (initially cut from ordinary 1D–PAGE combs) is used in a stacking gel covering the p*I*-strip. For experimental details see original publication and its application by another laboratory [2, 6]. The control of the p*I* dimension is optional as commercial p*I*-strips are of high quality, and the improvement in spot coordinates is marginal. It is still required when strips from different suppliers are used. For that purpose, azo dyes were introduced [5].

This protocol describes a complete hCoFGE experiment using *Escherichia coli* lysate as test proteome. The result of this experiment is shown in Fig. 2b. Subsequent data analysis is software-dependent (Delta 2D 4.3, Decodon, Germany, in [2–5]). Any software capable of warping can be used following the workflow.

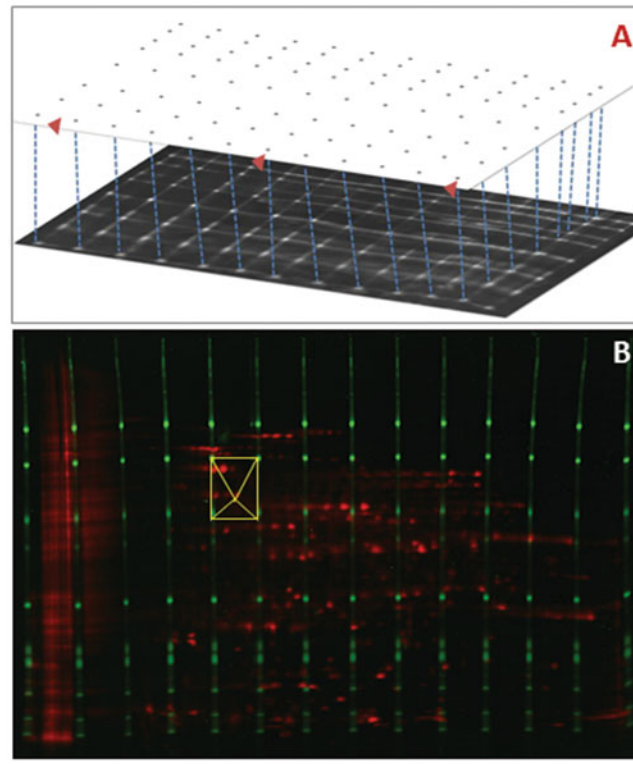

Fig. 2 (**a**) Matching of the ideal grid to a sample image. *Red triangles* mark additional reference points in case of optional p*I* control. (**b**) False-color image of the hCoFGE experiment described here. E. coli (*red*), marker grid (*green*). *Yellow lines* illustrate spot determination by triangulation (*see* **Note 6**).

2 Materials

Prepare all solutions using purified deionized water such as MilliQ water (Millipore) and analytical grade reagents. Observe waste disposal regulations.

2.1 Equipment

This protocol is based on the HPE-FlatTop Tower (Fig. 3, SERVA), but hPAGE devices from other vendors will work as well. Parameters need to be adjusted accordingly. The same is true for p*I*-strips (SERVA immobilized pH gradient (IPG) Blue Strip 4–7, 24 cm). CoFGE-ready-made gels are available from SERVA under the brand name Mercator (Fig. 1).

Further equipment:

1. Gel ScanFrame-Set, Paper Pools, IPG-Strip Equilibrator (SERVA).
2. Power supply (BioRad).
3. DryStrip Reswelling Tray, Ettan IPGphor II and Cup Loading Manifold, MultiTemp III, Typhoon 9400, ImageQuant software (GE Healthcare).
4. Ultrospec 2000 (Pharmacia Biotech).

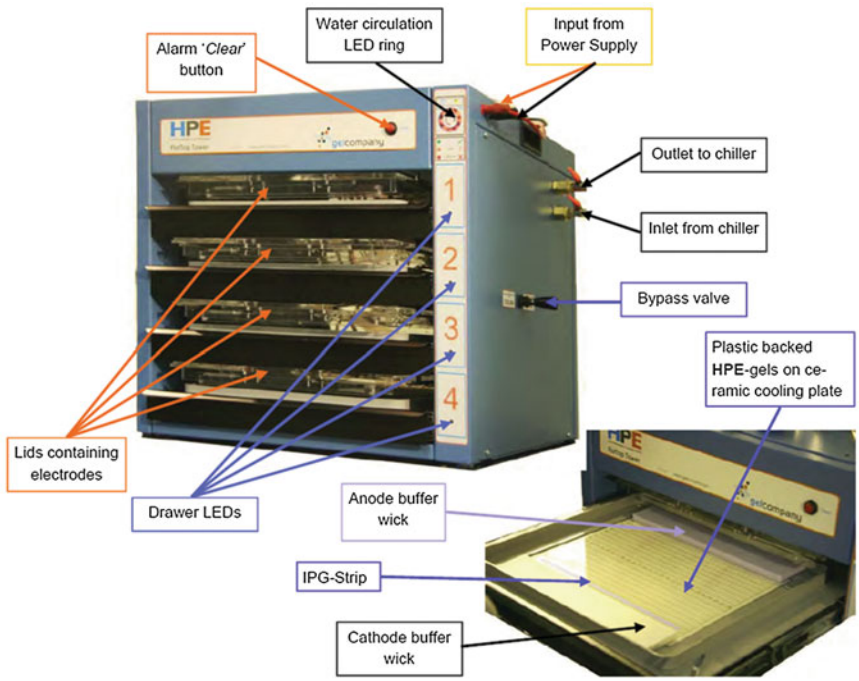

Fig. 3 HPE FlatTop Tower for four gels (SERVA, [7], reproduced with permission)

2.2 Chemicals and Solutions

2.2.1 Reference, Analyte, and Dyes

1. Grid mix: The reference mixture is formulated from stock solutions of commercial proteins (44.85 µL, 62.57 µg, pH 8.4; Table 1). The concentration of the individual proteins is a result of considerations, which might differ from lab to lab. Table 1 provides good starting values.
2. Analyte: *Escherichia coli* lyophilized cells (Sigma).
3. Dyes: For fluorescent labeling several products are on the market (e.g., CyDyes, GE [2]; G-Dyes, NH Diagnostics [2]; Sci-Dyes, SERVA). They all serve the purpose. Prepare as recommended by the manufacturer.
   - Sci 3 (1 µL, 400 pmol/µL).
   - G-Dye300 (1 µL, 400 pmol/µL).

2.2.2 Buffers

1. Lysis buffer I: 30 mM Tris, 8 M urea, 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; w/v).
2. Lysis buffer II: 7 M urea, 4% (w/v) CHAPS, 10 mM TCEP (tris (2-carboxyethyl) phosphine)).
3. Urea buffer I: 100 mM TrisBase, 8 M urea, pH 7.56.
4. Urea buffer II: 7 M urea, 0.5% CHAPS.

Table 1
Grid mix consisting of pure proteins available from Sigma-Aldrich. MWs are those given by the manufacturer, but attention needs to be paid to the form of the protein ultimately present on the gel. For myoglobin, e.g., this is the heme-free form, apomyoglobin (16.9 kDa). Proteins were dissolved in water and stored at −80 °C unless noted otherwise

Protein	Source	MW/kDa	c_{stock}/ pmol/µL	Amount of protein per 100 µL grid mix/µg	V_{stock}/ 100 µL grid mix/µL	Amount of protein per spot / ng
Ubiquitin	Bovine erythrocytes	8.56	100	10	11.7	24
α-Lactalbumin	Bovine milk	14.2	70.4[a]	10	10.0	24
Myoglobin	Horse heart	17.6	100	10	5.88	24
Trypsin inhibitor	Glycine max	~20	100	25	12.4	60
Trypsinogen	Bovine pancreas	23.98	100	15	6.26	36
GAPDH[b]	Rabbit muscle	~36	50	6	3.33	14.5
Ovalbumin[c]	Chicken egg	44.3	100	20	4.51	48
Albumin	Bovine	66.5	100	15	2.26	36
Phosphorylase B[b]	Rabbit muscle	97.0	10.3[d]	22.5	22.5	54

[a] Solubility in water according to the manufacturer 1 mg/mL/70.4 pmol/µL
[b] Dissolved in lysis buffer I
[c] Dissolved in HEPES buffer
[d] Solubility according to the manufacturer 10.3 pmol/µL in phosphate buffer (*see* **Note 1**)

5. SDS sample buffer: 7 M urea, 0.5% CHAPS, 4% (w/v) SDS.
6. Sample buffer: 8 M urea, 0.5% CHAPS.
7. IEF buffer: 8 M urea, 0.5% CHAPS, 2% (v/v) SERVAlyte 4–7, 2% (v/v) DTT.
8. Rehydration buffer: 8 M urea, 0.5% CHAPS, 1% (v/v) SERVAlyte 4–7, 0.2% (v/v) DTT, 0.004% (w/v) bromophenol blue.
9. Equilibration buffer I: 1.8 g urea, 50 mg DTT, 5 mL SERVA stock solution.
10. Equilibration buffer II: 1.8 g urea, 125 mg iodoacetamide, 5 mL SERVA stock solution.
11. HEPES buffer: 20 mM HEPES, 150 mM NaCl.

2.2.3 Solutions

1. Lysine solution: 1 µL, 2.5 M lysine (*see* **Note 2**).
2. Cleaning solvent: 1% formic acid, 5% acetonitrile, water.
3. Dithiothreitol (DTT) solution: 50 mM DTT, in 50 mM NH_4HCO_3, *see* **Note 3**.
4. Iodoacetamide solution: 50 mM in 50 mM NH_4HCO_3.

3 Methods

Work proceeds at room temperature unless otherwise noted.

3.1 Preparation of Grid Mix

3.1.1 Labeling

1. Incubate grid mix with Sci 3 for 30 min on ice.
2. Stop labeling with lysine solution for 10 min on ice. Aliquots can be temporarily stored at −80 °C until further use.

3.1.2 Reduction

1. Add 200 µL lysis buffer II to the labeled grid mix and place it into an ultrasonic bath for 15 min.
2. Rinse Amicon 3K filter units (*see* **Note 4**) by centrifuging with cleaning solvent (500 µL, 15 min, 14,000 × *g*, 20 °C).
3. Transfer labeled grid mix to the filter unit and centrifuge (75 min, 14,000 × *g*, 4 °C).
4. Rinse the filter unit with 100 µL urea buffer I by centrifuging (30 min, 14,000 × *g*, 4 °C).
5. For reduction add 100 µL DTT solution and vortex (45 min, 1000 rpm).
6. Centrifuge (30 min, 14,000 × *g*, 18 °C) and rinse with 100 µL urea buffer I (15 min, 14,000 × *g*, 18 °C).

3.1.3 Alkylation

1. Add 100 µL iodoacetamide solution to the filter unit and vortex in a BlackBox (25 min).

2. Stop the process by centrifuging (15 min, 14,000 × *g*, 4 °C) and rinsing with DTT solution (100 µL; vortex for 30 min; centrifuge for 90 min, 14,000 × *g*, 4 °C).

3.1.4 Purification

1. Wash the grid mix proteins on the filter with 100 µL urea buffer II (15 min, 14,000 × *g*, 4 °C) and twice with SDS sample buffer (100 µL; 30 min, 14,000 × *g*, 18 °C).
2. Re-dissolve the proteins in 90 µL SDS sample buffer and transfer them to an Eppendorf tube.
3. Store them at −32 °C until further use.
4. Prepare grid mix for use by diluting 6 µL of labeled and purified grid mix with 6 µL sample buffer.

3.2 Preparation of Analyte

1. Lyse *E. coli* (25 mg) in 1 mL lysis buffer I containing 1/10 tablet EDTA-free protease inhibitor cocktail Complete mini. Vortex (30 min) and centrifuge (12,000 × *g*, 4 °C).
2. Adjust the supernatant pH to 8.85.
3. Determine the protein concentration (10.9 mg/mL) at 590 nm using Bradford-based Cytoskeleton kit ADV01 or suitable alternatives.
4. Label *E. coli* lysate (50 µg, 4.6 µL) with G-Dye300 (30 min on ice).
5. Quench the reaction with lysine solution (10 min on ice).
6. For subsequent isoelectric focusing, add 6.6 µL IEF buffer. Vortex and centrifuge (1 min each).

3.3 Isoelectric Focusing

1. For passive rehydration of the IPG-strip without sample overnight (14 h), prepare a trough of the rehydration tray with 450 µL rehydration buffer.
2. Place the IPG-strip into the trough with the gel side down and overlay it with 3 mL mineral oil.
3. For cup-loading place the IPG strip gel side up into the manifold and pipet the sample (13.2 µL, total protein amount 50 µg) followed by mineral oil (10 µL) into a cup on the anodic side of the strip.
4. Perform focusing at 20 °C and 50 µA using the parameters shown in Table 2.

3.4 Second-Dimension Gel Electrophoresis

1. Equilibrate the IPG-strip for 15 min each in equilibration buffers I and II (6 mL each).
2. Place two electrode wicks separately into the wick tray and add 45 mL SERVA cathode and anode electrode buffer, respectively. Allow 15 min for buffer uptake.

Table 2
Isoelectric focusing program

Step	Voltage/V	Time/h	Slope
1	150	3	Step
2	300	3	Step
3	1000	6	Grad
4	8000	4	Grad
5	8000	3	Step
Total		19	
Holding	300	1	Step

Table 3
Gel electrophoresis program for one gel

Step	Voltage/V	Current/mA	Power/W	Time/min
1	100	7	1	30
2	200	13	3	30
3	300	20	5	10
4	1500	40	30	230
5	1500	50	40	40

3. Spread cooling fluid (3 mL) on the cooling plate of one drawer of the FlatTop Tower (*see* **Note 5**).
4. Load MW marker PageRuler Plus (5 μL; Prestained Protein Ladder, Thermo nb: 26,619 or suitable alternatives) into the marker well.
5. Place the IPG strip into the designated trough face-down with the anode (+) side toward the marker well.
6. Apply 0.5 μL labeled grid mix to each reference well immediately before the start of the second electrophoresis dimension.
7. Perform the second-dimension gel electrophoresis as shown in Table 3.
8. Remove the p*I*-strip after 75 min between **steps 3** and **4**.
9. Stop the run when the running front reaches the end of the gel.

3.5 Scanning

1. Scan gels immediately using the green laser for Sci 3 (532 nm, emission filter 580, band pass (BP) 30, photomultiplier tube

(PMT) 525) and the red laser for G-Dye300 (633 nm, emission filter 670, BP 30, PMT 490).

2. Set the resolution for the main scans to 100 μm per pixel.
3. Adapt the PMT response in such a way that the gel image shows the most intense protein spot slightly below saturation.
4. Visualize gel images using ImageQuant software.

3.6 Image Analysis

This paragraph describes the general workflow of spot coordinate correction by referencing to the marker grid. The individual steps are software-dependent.

1. Generate a reference grid (Fig. 2a) by running at least three, better more, gels of the same dimension under identical conditions for the grid mix (gels can optionally contain analyte). Use all grid images to detect the average location of the grid nodes. The x-coordinates of the nodes are determined by the well distances; the y-coordinates by the position of the marker protein spots. With these data, construct an ideal grid with drawing software (e.g., Powerpoint).
2. Match the individual marker grid for each gel to the ideal grid by assigning every experimental grid spot to the corresponding spot of the theoretical grid. This process determines the match vectors for the respective gel.
3. Apply the determined gel match vectors to the analyte proteome on this gel. Control the mapping of the match vectors manually.

4 Notes

1. The phosphate buffer recommended by the manufacturer may not be compatible with fluorescence labeling. Use lysis buffer I.
2. Proper quenching of the labeling reaction needs to be controlled. Use a higher concentration of lysine than recommended by the manufacturer to avoid mislabeling due to quenching failure [7].
3. Ammonium bicarbonate buffer is not stable. It will change its pH during the course of a day and thus needs to be prepared fresh each time [8].
4. Filter units contain polyethylene glycols (PEG) for preservation. They need to be carefully rinsed before use. Washing with 5% acetonitrile containing 0.1% formic acid at least three times is necessary to substantially reduce the PEG signal in mass spectrometry.

5. For the use of HPE FlatTopTower (Fig. 3), follow the detailed instructions of the manufacturer available in the manual [9].
6. Ubiquitin may form two spots when a DTT-containing buffer is used. They were not observed in Laemmli buffer (62.5 mM Tris, 50% glycerol, 2% (w/v) SDS, 0.01 (w/v) bromophenyl blue, 5% (v/v) mercaptoethanol). The reason for this was not determined as it did not have an effect on CoFGE experiments as long as the protocols were consistent.

Acknowledgement

This work was supported by the Transfer Prize 2013/2014 of the University of Münster. The authors thank Marina Gole, Alena Dreiling, and Diogo P. Duarte for technical assistance and acknowledge the collaboration with the licensee of the method, SERVA Electrophoresis GmbH, Heidelberg, Germany.

References

1. Ünlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
2. Ackermann D, Wang W, Streipert B, Geib B, Grün L, König S (2012) Comparative fluorescence two-dimensional gel electrophoresis using a gel strip sandwich assembly for the simultaneous on-gel generation of a reference protein spot grid. Electrophoresis 33:1406–1410
3. Ackermann D, Wang W, Grün L, König S, Patent application EP11167383.6, 25 May 2011
4. Hanneken M, König S (2014) Horizontal comparative fluorescence two-dimensional gel electrophoresis (hCoFGE) for improved spot coordinate detection. Electrophoresis 35:1118–1121
5. Hanneken M, Slais K, König S (2015) pI control in comparative fluorescence two-dimensional gel electrophoresis (CoFGE) using amphoteric azo dyes. Data Brief 3:221–228
6. Kotz S, Kullmann M, Crone B, Kalayda GV, Jaehde U, Metzger S (2015) Combination of 2D gel electrophoresis and a fluorescent carboxyfluorescein-diacetate-labeled cisplatin analogue allows the identification of intracellular cisplatin-protein adducts. Electrophoresis 36:2811–2819
7. Wang W, Ackermann D, Mehlich AM, König S (2010) False labelling due to quenching failure of N-hydroxysuccinimide-ester coupled dyes. Proteomics 10:1525–1529
8. Bayer M, König S (2016) Abundant cysteine side reactions in traditional buffers interfere with the analysis of posttranslational modifications and protein quantification – how to compromise. Rapid Commun Mass Spectrom 30:1823–1828
9. SERVA tower manual V4, 2016

Chapter 7

DIGE-Based Phosphoproteomic Analysis

Taras Stasyk and Lukas Alfons Huber

Abstract

Here, we describe the detailed step-by-step protocol for detection of phosphoproteins in two-dimensional difference gel electrophoresis (DIGE) gels. A standard DIGE protocol is combined with subsequent post-staining with phosphospecific fluorescent dye. The combination of these two methods complements DIGE-based proteome profiling by fluorescence detection of phosphoproteins in the same gel providing additional possibility for sensitive and accurate quantification of the differentially regulated phosphoproteins in biological samples.

Key words 2D-DIGE, Phosphoprotein, Phosphoproteomics

1 Introduction

Two approaches for detection of proteins employing fluorescent dyes, i.e. DIGE and Multiplexed Proteomics, greatly enhanced the utility of classical two-dimensional gel electrophoresis (2-DE) [1, 2]. For the DIGE method three protein samples are pre-stained by covalent labeling with different cyanine-based CyDyes, which then can be mixed and analyzed in a single gel [3]. The inclusion of a standard sample, which is a pool of each of the samples being compared, facilitates both spot matching and subsequent accurate quantification. Alternatively, for fluorescence detection of proteins separated with 2-DE, gels can be post-stained with fluorescent dyes, such as Deep Purple [4], SYPRO Ruby [5], or similar ruthenium-based dyes [6].

Protein phosphorylation among hundreds of post-translational modifications is in special focus since many years. A well-established post-staining method with fluorescent phosphosensor Pro-Q Diamond (Molecular Probes) is suitable for the detection of phosphoproteins directly in polyacrylamide gels and is also compatible with a total protein staining procedure with SYPRO Ruby [7].

We have developed several years ago a complementary approach to classical DIGE proteome profiling [8]. It combines

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_7,

Table 1
CyDye DIGE Fluor minimal dyes and Pro-Q Diamond phosphoprotein gel stain characteristics and scanning parameters, according to manufacturers

Fluor dye	Max. absorption (nm)	Max. fluorescence (nm)	Excitation/emission filter (Typhoon)	Excitation/emission filter (molecular Imager)
Cy2	451	509	488/520BP40	488/530BP30
Cy3	553	569	532/580BP30	532/605DF50
Cy5	645	664	633/670BP30	665/695DF55
Pro-Q Diamond	555	580	532/580BP30	532/605DF50

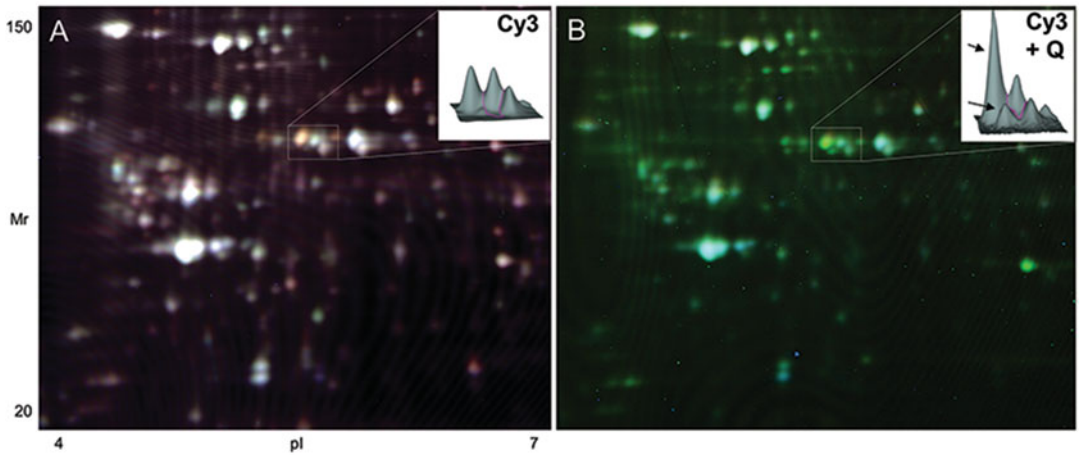

Fig. 1 DIGE of early endosomes purified from murine EpH4 cells in combination with Pro-Q Diamond phosphospecific fluorescent dye, merged images and 3D views of detailed sections of the gel. Early endosomes were purified by sucrose gradient centrifugation [14], 20 mg of protein was labeled with CyDye Fluorophores, mixed and separated by 2-DE [8]. Gel was scanned with a Typhoon 9410 scanner and visualized using a Image Quant 5.2 software. (**a**) Control (Cy2, *blue*) and cells treated with EGF for 5 min (Cy3, *green*) and for 40 min (Cy5, *red*). (**b**) The same gel post-stained with Pro-Q Diamont fluorescent dye and scanned as for the CyDyes. Phosphoproteins are visible as spots with increased *green* fluorescence

the standard DIGE protocol with subsequent post-staining of gels with Pro-Q Diamond phosphospecific stain. This was possible because Pro-Q Diamond and Cy3 have very similar spectra of fluorescence with excitation/emission maxima at 555/580 nm and 553/569 nm, respectively (*see* Table 1). In brief, after a standard DIGE experiment followed by imaging, gels were fixed and post-stained with Pro-Q Diamond. Proteins in gels, which were specifically stained with a phosphospecific dye appear as spots with increased Cy3-fluorescence, because of additive effect of original Cy3 signal and added fluorescence of Pro-Q Diamond sensor (Fig. 1). Such increased fluorescence can be normalized against

Cy2 (standard) and quantified as ratios (Cy3 + Q)/Cy2 to Cy3/Cy2 before and after post-staining for phosphoproteins. In general, spots with increased ratio (higher than 1) are detected as phosphorylated ones. Importantly, quantitative comparison between different samples comes from the DIGE application, whereas Pro-Q Diamond post-staining provides additional information on the phosphorylation state of differentially regulated protein spots. Of note, it was shown that fluorescence signal intensity of Pro-Q Diamond correlates well with the number of phosphorylated residues on a protein [7]. The here introduced protocol was applied previously by others and us for functional proteomics experiments [8–11].

2 Materials

2.1 Reagents

CyDye DIGE Fluor minimal dyes (Cy2, Cy3, Cy5) (GE Healthcare).

Pro-Q Diamond phosphoprotein gel stain (Molecular Probes).

RuBP (Ruthenium II tris (bathophenantroline disulfonate)) fluorescent protein staining solution.

2.2 Solutions

1. 2-DE Sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, buffered to pH 8.5 with HCl. Aliquots can be stored at −20 °C.
2. Equilibration buffer: 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue.
3. Fix solution: 50% methanol and 10% acetic acid. Prepare 500 mL fix solution per gel.
4. Destain solution: 20% acetonitrile, 50 mM sodium acetate, pH 4.0. To prepare 1 L of destain solution, combine and mix thoroughly: 50 mL of 1 M sodium acetate, pH 4.0, 750 mL of water, 200 mL of acetonitrile.
5. RuBP fluorescent protein staining solution: 20 mM RuBP stock, home-made according to [6].

2.3 Equipment

DeCyder Difference In-gel Analysis (DIA) software (GE Healthcare).

DeCyder Biological Variation Analysis (BVA) software (GE Healthcare).

Ettan Spot Picker (GE Healthcare).

ImageQuant (GE Healthcare).

IPGphor™ IEF System (GE Healthcare).

IPG strips (18 cm, pH 3-11, GE Healthcare).

PROTEAN electrophoretic system (Bio-Rad Laboratories).
Typhoon™ 9410 Imager (GE Healthcare).

3 Methods

3.1 Sample Preparation (TIMING 1 h)

1. Precipitate protein by chloroform-methanol (Wessel-Flügge method) [12], (*see* **Note 1**). Add 4 volumes of methanol followed by 1 volume of chloroform to protein sample to be analyzed by 2-D gel electrophoresis. Vortex and assure that there is only one phase.
2. Add 3 volumes of distilled water and vortex thoroughly.
3. Centrifuge 1 min at 16,000 × *g*, room temperature. Remove upper organic phase with drawn out Pasteur pipette without disturbing the interphase.
4. Add at least 3 volumes of methanol, vortex thoroughly and centrifuge at 16,000 × *g* for 2 min.
5. Carefully remove supernatant with drawn out Pasteur pipette without disturbing the pellet.
6. Air dry the pellet with care (overdrying makes the pellet very difficult to solubilize).
7. Solubilize protein samples in 2-D DIGE sample buffer. Determine protein concentration using Coomassie Plus Protein Assay.

3.2 Two-Dimensional Difference Gel Electrophoresis (TIMING 2 Days)

1. Label 40 μg of protein in 20 μL of sample buffer with 160 pmol of CyDye DIGE Fluor minimal dyes according to the manufacturer's instruction (Cy3, Cy5 for samples and Cy2 for internal control consisting of equal parts of all samples) (*see* **Notes 2** and **3**).
2. For isoelectric focusing (IEF) apply the samples by rehydration to IPG strips (18 cm, pH 3-11) overlaid with 1 mL liquid paraffin. The protocol for IEF, performed with a IPGphorTM Isoelectric focusing system, consisted of the following steps and parameters:
   (a) Active Rehydration with 50 μA/strip at 20 °C.
   (b) **Step 1** Step and hold: 30 V for 12 h.
   (c) **Step 2** Step and hold: 200 V for 1 h.
   (d) **Step 3** Gradient from 100 V to 500 V for 1 h.
   (e) **Step 4** Gradient from 500 V to 1000 V for 1 h.
   (f) **Step 5** Gradient from 1000 V to 8000 V for 2 h.
   (g) **Step 6** Step and hold 8000 V until total 42,000 Vh.

3. Upon completion of the first dimension, incubate strips in an Equilibration buffer, containing 1% DTT for 15 min and thereafter in the same buffer containing 2.5% iodoacetamide for 15 min. PAUSING POINT: the strips can be stored at −20 °C.
4. For the second dimension, transfer strips to the tops of 12.5% polyacrylamide gels and separate for the second dimension with the PROTEAN System for 12–14 h. Apply 7 mA per each gel, the voltage rose from app. 35 V up to 120 V during electrophoresis, which takes about 16 h.
5. After electrophoresis scan the gels using a TyphoonTM 9410 Imager at 100 dpi resolution or any other suitable and similar device. Optimize scan settings for a maximum signal of approximately 85,000 counts. Directly after scanning fix gels and store at 4 °C.
6. Image preparation and analysis. Crop images using ImageQuant 6.2 and perform image analysis using DeCyder 6.5 DIA software (Difference In-gel Analysis). Analyze at least four independent biological samples for each experimental setup and statistically analyze using DeCyder Biological Variation Analysis (BVA) software.

   PAUSING POINT: the gels can be stored for long time in cold room for subsequent phosphoprotein or total protein staining and spot picking for protein identification.

3.3 Detection of Phosphoproteins in DIGE-Gels

3.3.1 Staining Gels with Pro-Q Diamond Phosphoprotein Gel Stain (TIMING 1 Day)

1. Fix the gel. Immerse the gel in ~500 mL of fix solution and incubate at room temperature with gentle agitation for 60 min. Repeat the fixation overnight to ensure that all of the SDS is washed out of the gel.
2. Wash the gel. Incubate the gel in ~500 mL of water with gentle agitation for 20 min. Repeat this step for a total of three washes.
3. Scan the gel again for Cy2 (Standard) and Cy3 after washing in water, just prior gel staining with Pro-Q Diamond. Proceed with standard Pro-Q staining (*see* **Note 4**).
4. Stain the gel. Incubate the gel in the dark in 300 mL of Pro-Q Diamond phosphoprotein gel stain with gentle agitation for 90 min.
5. Destain the gel. Incubate the gel in 500 mL of destain solution with gentle agitation for 30 min at room temperature, protected from light. Repeat this procedure two more times (*see* **Note 5**).
6. Wash the gel. Wash twice with water at room temperature for 15 min per wash.

   PAUSING POINT: the gels can be stored for long time in cold room for subsequent total protein staining and spot picking for protein identification. Store gels in water in the presence of 0.02% NaN_3 in cold room sealed in plastic bags.

3.3.2 Imaging and Analysis (TIMING 4–8 h)

1. Scan gel again for Cy2 (Standard) and Cy3. Crop images using ImageQuant 6.2.
2. Detect spots using DIA program. Perform spot detection using DeCyder 6.5 DIA software.
3. BVA analysis. Download this file along with DIA file with detected spots in scanned 2D maps just before Pro-Q-Diamond staining (Subheading 3.3.1.3) to BVA and perform spot matching. Compare ratios of Cy3 before and after Pro-Q post-staining (normalized to Cy2 as a standard). Spots with increased ratio (higher than 1) are phosphorylated proteins. Consider 3–4 independent biological samples per group for statistical analysis (*see* **Note 6**).

3.3.3 Staining the Gel for Total Protein (Optional). Spot Picking for Protein Identification (TIMING 1 Day)

For spot picking post-stain DIGE-gel with Ruthenium-based fluorescent protein dye according to an improved staining protocol [13]. Commercial SYPRO Ruby protein gel stain can also be used.

1. Rinse the gel in water for 15 min.
2. Incubate the gel in 500 nM RuBP solution for 6 h.
3. Equilibrate the gel in water for 10 min and repeat once.
4. Destain the gel with 40% EtOH/10% acetic acid for 15 h.
5. Equilibrate the gel in water for 15 min and repeat once.
6. Scan at 457 nm excitation and 670 nm filter.

Excise protein spots from gels with Ettan Spot Picker and in-gel digest with trypsin for subsequent protein identification using mass spectrometry.

4 Notes

1. Always precipitate protein sample by the Wessel-Fluegge method [12]. Protein precipitation before two-dimensional gel electrophoresis is of particular importance when the gel is to be analyzed for phosphoprotein content. Lipid depletion minimizes background staining due to phospholipids and other cell constituents.
2. Reconstitute the dry CyDyes in anhydrous Dimethylformamide (DMF) ($\leq$ 0.005% H_2O, $\geq$99.8% pure, open for less than 3 months).
3. Label protein with CyDyes of double less than "standard" concentration (4 pmol per mg of protein). More intense signal from DIGE will make ratio (Cy3 + Q)/Cy2 to Cy3/Cy2 less prominent in phosphoprotein detection.
4. After washing in water, just prior gel staining with Pro-Q Diamond, scan gel again as for DIGE (for Cy2 and Cy3).

This DIA file (but not the original one!) will be used for comparison with the same scan after Pro-Q post-staining. The reason for this is that after fixation fluorescence of CyDyes can be a bit different; another advantage is that gel is scanned after washing with water (before and after Pro-Q Diamond) and is of the same size.

5. Extensive destaining can cause loss of signal. Do not re-use Pro-Q Diamond stain or dilute it, it might significantly compromise fluorescence intensity and linearity.
6. Because of normalization method in DIA spots, which are not stained/phosphorylated have decreased intensities, phosphoproteins appear as more intense spots. This threshold is sample-, staining- and labeling-dependent. Therefore, the here-described protocol has to be optimized for each protein sample.

References

1. Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4(12):3665–3685. doi:10.1002/pmic.200401031
2. Patton WF, Beechem JM (2002) Rainbow's end: the quest for multiplexed fluorescence quantitative analysis in proteomics. Curr Opin Chem Biol 6(1):63–69
3. Ünlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077. doi:10.1002/elps.1150181133
4. Mackintosh JA, Choi HY, Bae SH, Veal DA, Bell PJ, Ferrari BC, Van Dyk DD, Verrills NM, Paik YK, Karuso P (2003) A fluorescent natural product for ultra sensitive detection of proteins in one-dimensional and two-dimensional gel electrophoresis. Proteomics 3(12):2273–2288. doi:10.1002/pmic.200300578
5. Berggren KN, Schulenberg B, Lopez MF, Steinberg TH, Bogdanova A, Smejkal G, Wang A, Patton WF (2002) An improved formulation of SYPRO Ruby protein gel stain: comparison with the original formulation and with a ruthenium II tris (bathophenanthroline disulfonate) formulation. Proteomics 2(5):486–498. doi:10.1002/1615-9861(200205)2:5<486::AID-PROT486>3.0.CO;2-X
6. Rabilloud T, Strub JM, Luche S, van Dorsselaer A, Lunardi J (2001) A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. Proteomics 1(5):699–704. doi:10.1002/1615-9861(200104)1:5<699::AID-PROT699>3.0.CO;2-C
7. Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, Schulenberg B, Hendrickson J, Beechem JM, Haugland RP, Patton WF (2003) Global quantitative phosphoprotein analysis using multiplexed proteomics technology. Proteomics 3(7):1128–1144. doi:10.1002/pmic.200300434
8. Stasyk T, Morandell S, Bakry R, Feuerstein I, Huck CW, Stecher G, Bonn GK, Huber LA (2005) Quantitative detection of phosphoproteins by combination of two-dimensional difference gel electrophoresis and phosphospecific fluorescent staining. Electrophoresis 26(14):2850–2854. doi:10.1002/elps.200500026
9. Stasyk T, Schiefermeier N, Skvortsov S, Zwierzina H, Peränen J, Bonn GK, Huber LA (2007) Identification of endosomal epidermal growth factor receptor signaling targets by functional organelle proteomics. Mol Cell Proteomics 6(5):908–922. doi:10.1074/mcp.M600463-MCP200
10. Orsatti L, Forte E, Tomei L, Caterino M, Pessi A, Talamo F (2009) 2-D difference in gel electrophoresis combined with pro-Q Diamond staining: a successful approach for the identification of kinase/phosphatase targets. Electrophoresis 30(14):2469–2476. doi:10.1002/elps.200800780
11. Mattei B, Spinelli F, Pontiggia D, De Lorenzo G (2016) Comprehensive analysis of the membrane phosphoproteome regulated by oligogalacturonides in Arabidopsis thaliana. Front

Plant Sci 7:1107. doi:10.3389/fpls.2016.01107

12. Wessel D, Flügge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138(1):141–143
13. Lamanda A, Zahn A, Roder D, Langen H (2004) Improved ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-tobackground ratio and improved baseline resolution. Proteomics 4(3):599–608. doi:10.1002/pmic.200300587
14. de Araùjo ME, Huber LA, Stasyk T (2008) Isolation of endocitic organelles by density gradient centrifugation. Methods Mol Biol 424:317–331. doi:10.1007/978-1-60327-064-9_25

Chapter 8

DIGE Saturation Labeling for Scarce Amounts of Protein from Formalin-Fixed Paraffin-Embedded (FFPE) Tissue

Paul Dowling

Abstract

In this chapter, we describe the utility of difference gel electrophoresis (DIGE) as a proteomics platform for the global detection of expressed proteins in formalin-fixed paraffin-embedded (FFPE) tissues and its use for biomarker discovery/identification of proteins that may contribute to cancer development and progression. Formalin fixation and paraffin embedding of tissue is the standard processing methodology practiced in pathology laboratories worldwide, resulting in a highly stable form of tissue that is easily stored due to its inherent stability at room temperature. Consequently, FFPE tissues represent an attractive reservoir of clinical material for conducting retrospective protein biomarker analysis. A limitation for proteomics research in this type of clinical sample is the amount of viable protein that can be obtained from fixed tissues. Tissue biopsies are precious samples that can generally be acquired in very small amounts due to the invasive nature of the sample collection, mainly during surgery or biopsy. Subsequently, the amount of extracted protein can be, in many cases, very limited. The saturation DIGE technology has emerged as a useful method for protein analysis where only scarce amounts of protein are available. This approach can be adapted successfully to label low-level protein isolated from FFPE tissue.

Key words Formalin-fixed paraffin-embedded tissues, Saturation labeling

1 Introduction

The 2D difference gel electrophoresis (DIGE) technique, established on fluorophores covalently linked to amino acid side chain residues, has many advantages over traditional 2D gel non-labeling methods including high sensitivity, the linearity of the dyes utilized, and the standardization of inter-gel variability. Using an internal standard of pooled samples, which are run simultaneously with the analytical samples, provides an extra layer of quantification accuracy and statistical confidence.

In contrast to the minimal 2D DIGE technology where a significant amount of protein starting material is required (in

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_8,

most examples 50 μg is used), very small amounts of proteins are amenable to labeling using the saturation DIGE technology introduced by Kondo and co-workers [1]. Protein labeling in saturation DIGE is based on dyes coupled to a maleimide group that form a covalent bond with the thiol group of cysteine residues via a thioether linkage on all cysteine residues of a protein. This leads to an enhanced sensitivity as compared to the minimal DIGE strategy, when investigating samples in situations where protein amount is at a minimum. This strategy of labeling scarce protein amounts has been successfully employed by some research groups focusing on laser microdissected tissues [2–4].

Minimal labeling is associated with a predefined ratio of protein to CyDye DIGE Fluor resulting in only 2–5% of the total number of lysine residues being labeled [5]. However, saturation labeling involves adding CyDye DIGE Fluor in a controlled protein to dye ratio, facilitating conditions that allow for all available cysteine residues of a protein to be labeled [6, 7].

As with the minimal labeling approach, an internal standard should be incorporated within each gel as part of the DIGE protocol. In the case of saturation labeling, because a 2 dye system is used, the internal standard is labeled with one of the available CyDye DIGE Fluor saturation dye (for example, Cy3) and is run on every gel together with experimental samples labeled with the other CyDye DIGE Fluor saturation dye (for example, Cy5). The internal standard, labeled with one CyDye DIGE Fluor saturation dye, should comprise an aliquot from each biological sample within the experiment, ensuring that every spot on every gel is represented within the common internal standard [8].

Tissue-based proteomic studies are inherently attractive for relating protein biomarkers directly to disease and potentially identify new therapeutic targets. Whereas fresh and/or frozen tissue may represent attractive samples from which proteomic biomarker investigations may be conducted, they are often difficult to obtain in large numbers and/or amounts, with the extraction of sufficient amounts of protein challenging when the starting material is at a minimal level. Formalin-fixed and paraffin-embedded (FFPE) tissue collections, with attached clinical and outcome information, are invaluable resources for conducting retrospective protein biomarker investigations and performing translational studies of cancer and other diseases [9, 10]. FFPE material is a very precious resource and typical amount that are to be available for laboratory omics-based analysis tend to be small. Therefore, a strategy that allows labeling of scarce amounts of protein, such as the saturation difference in gel electrophoresis technology, is a very attractive approach prior to 2D gel-based analysis [11].

2 Materials

2.1 Equipment

1. Eppendorf Model 5417R centrifuge.
2. LP Vortex Mixer (ThermoFisher Scientific).
3. Digital Dry Baths/Block Heaters (ThermoFisher Scientific).

2.2 Reagents

1. CyDye DIGE Fluor Labeling Kit for Scarce Samples plus Preparative Gel Labeling containing: 100 nmol CyDye DIGE Fluor Cy3 saturation dye for analytical labeling; 100 nmol CyDye DIGE Fluor Cy5 saturation dye for analytical labeling; 400 nmol CyDye DIGE Fluor Cy3 saturation dye for preparative labeling (*see* **Note 1**).
2. 99.8% anhydrous dimethylformamide (DMF).
3. Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP).

2.3 Solutions

All solutions should be prepared with analytical grade chemicals and ultrapure water.

1. 1× sample buffer-7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS.
2. 2× sample buffer-7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) Pharmalyte™, 130 mM DTT (*see* **Note 2**).
3. DIGE Lysis buffer-30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS. Adjust to pH 8.0 with 1.0 M HCl. Aliquot and store at −15 °C to −30 °C. Stable for 3 months.
4. 2 mM TCEP solution-2.8 mg TCEP in 5 mL of HPLC-grade water (*see* **Note 3**).
5. Extraction buffer: 20 mM Tris-HCl buffer pH 9, containing 2% SDS.
6. 100% Ethanol.
7. 85% Ethanol.
8. 70% Ethanol.
9. 100% Xylene.

3 Methods

3.1 Deparaffinization of FFPE Slides

1. Up to two sections, each with a thickness of about 10 μm and area of up to about 100 mm^2, are combined in one preparation (*see* **Note 4**).
2. Immerse the slides in xylene for 10 min. Repeat this step once in fresh xylene solution for 10 min.
3. Immerse the slides in 100% ethanol for 5 min, followed by another immersion in 85% ethanol for 1 min, followed by another immersion in 70% ethanol for 1 min.

4. Immerse the slides in HPLC-grade water for 1 min. Remove excess water from the slide, taking care not to allow the sections to dry out.
5. Transfer the sections to a microcentrifuge tube using a clean sterile razor blade.

3.2 Protein Extraction

1. Add 50 μL of extraction buffer to the dewaxed FFPE tissues sections, with vortexing and vigorous pipetting for 30 s (*see* **Note 5**).
2. Incubate the dewaxed FFPE tissues sections with extraction buffer on a heating block at 100 °C for 20 min.
3. Centrifuge the sample for 60 s at 1000 × *g*, with vortexing for 5 s and incubate at 60 °C for 2 h.
4. Following this incubation, centrifuge the sample at 4 °C for 10 min at 16,000 × *g*. Transfer the supernatant (soluble fraction) to a new microcentrifuge tube.
5. Precipitate extracted proteins using the 2D clean-up kit, resuspend protein pellet in DIGE lysis buffer and quantify the protein yield using Bradford Reagent. Using this protocol, the expected protein yield is about 10–30 μg of protein.

3.3 Labeling Samples for Analytical Gels

1. Add a volume of protein lysate equivalent to 5 μg protein to a sterile microfuge tube. Make up the volume to 9 μL with cell lysis buffer.
2. Add 1 μL TCEP (2 nmol) to the protein lysate. Typically, 5 μg of protein lysate requires 2 nmol TCEP and 4 nmol dye for the labeling reaction (assuming an average cysteine content of 2%).
3. Mix vigorously by pipetting.
4. Spin down the sample in a microcentrifuge at 1000 × *g* for 30 s and incubate at 37 °C for 1 h, in the dark.
5. Add 2 μL dye (4 nmol) to the mixture (protein lysate plus TCEP).
6. Label a pooled protein extract with CyDye DIGE Fluor Cy3 saturation dye and label experimental protein extracts (e.g., control, test) with CyDye DIGE Fluor Cy5 saturation dye.
7. Mix vigorously by pipetting.
8. Spin down the sample in a microcentrifuge at 1000 × *g* for 30 s and incubate at 37 °C for 30 min, in the dark.
9. Prepare the 2× sample buffer by adding Pharmalytes (2% final) and DTT (130 mM final) to 1× sample buffer (7 M Urea, 2 M Thiourea, 4% CHAPS).
10. To stop the reaction, calculate the total volume of the labeling reaction and add an equal volume of 2× sample buffer (i.e., add 12 μL 2× sample buffer to 12 μL labeling reaction mixture).

11. Mix vigorously by pipetting.
12. Spin down the sample in a microcentrifuge.
13. Samples are ready for immediate use (*see* **Note 6**).

4 Notes

1. Product number: 25-8009-84.
2. Prepare fresh by adding DTT and Pharmalytes to 2× sample buffer and use immediately.
3. TCEP solution is unstable and should be used immediately.
4. Before deparaffinization, keep slides at room temperature for 1 h.
5. Use tubes that have a tight fitting cap and wrap the caps with parafilm.
6. Can be stored frozen for up to 1 month, at −70 °C, in the dark.

References

1. Kondo T, Seike M, Mori Y, Fujii K, Yamada T, Hirohashi S (2003) Application of sensitive fluorescent dyes in linkage of laser microdissection and two-dimensional gel electrophoresis as a cancer proteomic study tool. Proteomics 3(9):1758–1766. doi:10.1002/pmic.200300531
2. de la Cuesta F, Alvarez-Llamas G, Maroto AS, Donado A, Juarez-Tosina R, Rodriguez-Padial L, Pinto AG, Barderas MG, Vivanco F (2009) An optimum method designed for 2-D DIGE analysis of human arterial intima and media layers isolated by laser microdissection. Proteomics Clin Appl 3(10):1174–1184. doi:10.1002/prca.200900053
3. Sawhney S, Stubbs R, Hood K (2009) Reproducibility, sensitivity and compatibility of the ProteoExtract subcellular fractionation kit with saturation labeling of laser microdissected tissues. Proteomics 9(16):4087–4092. doi:10.1002/pmic.200800949
4. Arnold GJ, Fröhlich T (2012) 2D DIGE saturation labeling for minute sample amounts. Methods Mol Biol 854:89–112. doi:10.1007/978-1-61779-573-2_7
5. Villar M, Torina A, Nuñez Y, Zivkovic Z, Marina A, Alongi A, Scimeca S, La Barbera G, Caracappa S, Vázquez J, Fuente JL (2010) Application of highly sensitive saturation labeling to the analysis of differential protein expression in infected ticks from limited samples. Proteome Sci 8:43. doi:10.1186/1477-5956-8-43
6. McNamara LE, Kantawong FA, Dalby MJ, Riehle MO, Burchmore R (2011) Preventing and troubleshooting artefacts in saturation labelled fluorescence 2-D difference gel electrophoresis (saturation DiGE). Proteomics 11(24):4610–4621. doi:10.1002/pmic.201100135
7. Friedman DB, Lilley KS (2008) Optimizing the difference gel electrophoresis (DIGE) technology. Methods Mol Biol 428:93–124
8. Greengauz-Roberts O, Stöppler H, Nomura S, Yamaguchi H, Goldenring JR, Podolsky RH, Lee JR, Dynan WS (2005) Saturation labeling with cysteine-reactive cyanine fluorescent dyes provides increased sensitivity for protein expression profiling of laser-microdissected clinical specimens. Proteomics 5(7):1746–1757. doi:10.1002/pmic.200401068
9. Magdeldin S, Yamamoto T (2012) Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. Proteomics 12(7):1045–1058. doi:10.1002/pmic.201100550
10. Klopfleisch R, Weiss AT, Gruber AD (2011) Excavation of a buried treasure–DNA, mRNA, miRNA and protein analysis in formalin fixed, paraffin embedded tissues. Histol Histopathol 26(6):797–810
11. Giusti L, Lucacchini A (2013) Proteomic studies of formalin-fixed paraffin-embedded tissues. Expert Rev Proteomics 10(2):165–177. doi:10.1586/epr.13.3

Chapter 9

Comparative 3-Sample DIGE Analysis of Skeletal Muscles

Kay Ohlendieck

Abstract

The skeletal muscle proteome consists of a large number of diverse protein species with a broad and dynamic concentration range. Since mature skeletal muscles are characterized by a specific combination of contractile cells with differing physiological and biochemical properties, it is essential to determine specific differences in the protein composition of fast, slow, and hybrid fibers. Fluorescence two-dimensional gel electrophoresis (DIGE) is a powerful comparative tool to analyze fiber type-specific differences between fast and slow muscles. In this chapter, the application of the DIGE method for the comparative analysis of different subtypes of skeletal muscles is outlined in detail. A standardized proteomic workflow is described, involving sample preparation, protein extraction, differential fluorescence labeling using a 3-dye system, first-dimension isoelectric focusing, second-dimension slab gel electrophoresis, DIGE image analysis, protein digestion, and mass spectrometry.

Key words Difference gel electrophoresis, Mass spectrometry, Muscle types, Proteomics, Two-dimensional gel electrophoresis

1 Introduction

Two-dimensional gel electrophoresis has been extensively used for large-scale protein separation prior to mass spectrometric analysis [1], including the biochemical and proteomic characterization of skeletal muscle tissues [2]. In comparative proteomics, two-dimensional fluorescence difference gel electrophoresis (DIGE) [3] is a frequently employed protein separation method [4]. The technique was originally developed by Unlü et al. [5] and is an extremely robust and highly reproducible gel-based method of analytical protein biochemistry [6]. DIGE can be employed as a comparative approach with minimal or saturation protein labeling using pre-electrophoretic labeling with fluorescent 2-dye or 3-dye systems [7–9]. The availability of sophisticated 2D–DIGE software analysis tools is a great advantage for the routine quantitative analysis of multiple fluorescently labeled protein samples that have

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_9,

been separated on the same two-dimensional slab gel [10, 11]. The application of the DIGE technique greatly reduces analytical complications due to gel-to-gel variations [12], which has also been exploited in the field of skeletal muscle proteomics [13].

DIGE-based analyzes of skeletal muscle preparations with Cy2, Cy3, or Cy5 dyes are usually carried out with 50 µg protein aliquots per sample, and routinely detect a highly representative proportion of the muscle tissue proteome. This includes major protein species involved in the sarcomeric excitation–contraction–relaxation cycle, the glycolytic pathway, mitochondrial metabolism, the ion-handling apparatus, the cytoskeleton, the basal lamina, cellular signaling, and the stress response [14]. The extensive usage of the DIGE method in the field of skeletal muscle proteomics was instrumental for the differential analysis of muscle specification [15], muscle transformation [16], disuse-related muscular atrophy [17], hypoxia-associated changes in muscles [18], the effects of physical exercise [19, 20], and natural skeletal muscle aging [21–23], as well as the pathobiochemical evaluation of muscle diseases such as muscular dystrophy [24], myasthenia gravis [25], and collagen myopathy [26]. Changes in fiber type composition have been shown to occur in both physiologically challenged and pathologically insulted skeletal muscles [27]. Individual skeletal muscles are usually characterized by diverse fiber populations that consist of fast-glycolytic, slow-oxidative, intermediate fast-glycolytic/oxidative and mixed hybrid fibers [28]. Predominantly fast- versus slow-twitching muscles contain differing numbers of type I, I/IIa, IIa, IIa/IIx, IIx, IIx/IIb, and IIb fibers, which correlates relatively well with the histochemical distribution of slow versus fast isoforms of myosin heavy chains [29].

However, for the detailed evaluation of the molecular and cellular heterogeneity of subtypes of skeletal muscles and their physiological plasticity, more sophisticated biochemical approaches have to be utilized [30]. Here, we describe the application of the DIGE technique for the comparative analysis of different skeletal muscles. An optimized proteomic workflow, as shown in Fig. 1, is specifically outlined for the characterization of fast versus slow versus mixed skeletal muscles using a 3-dye DIGE approach. Importantly, this differential labeling method can be generally applied for the parallel analysis of 3 separate tissue specimens, such as normal, diseased, and therapeutically treated skeletal muscles [31]. The described 3-sample comparison is an optimized and standardized method that uses a 12-gel DIGE system with a minimum number of gels to achieve optimum results in relation to biological repeats, sample pairing, technical repeats, and reverse fluorescent dye labeling.

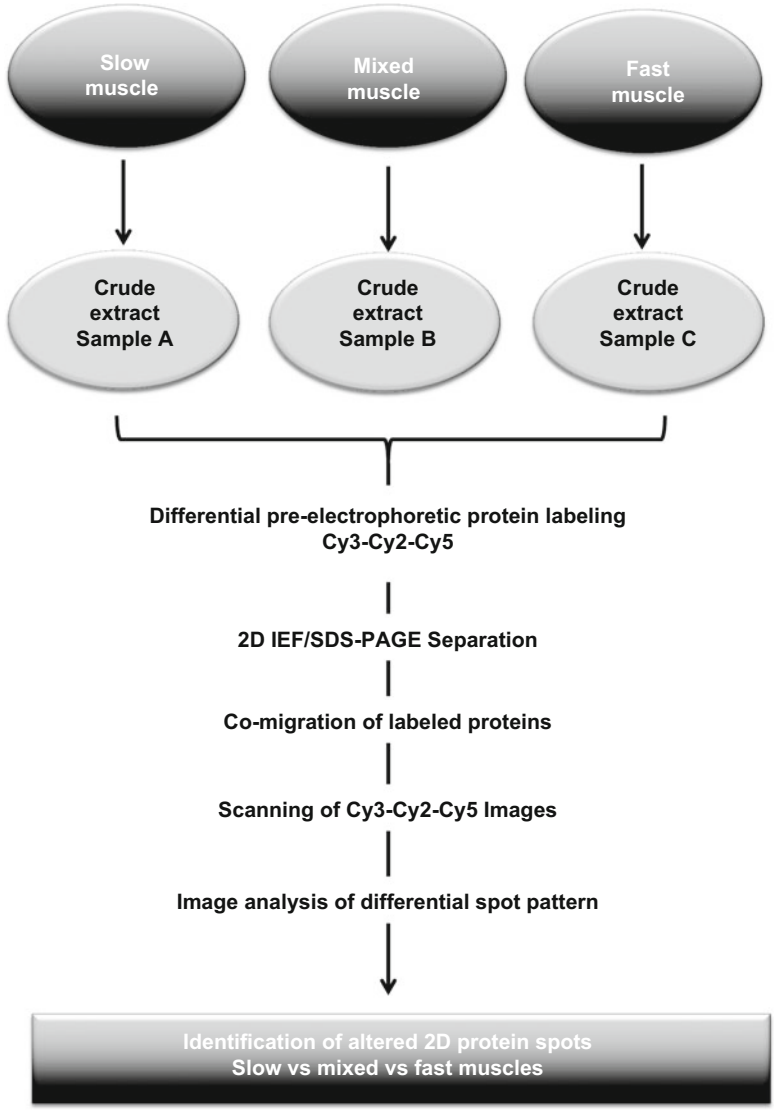

Fig. 1 Overview of the proteomic workflow for the profiling of different subtypes of skeletal muscles using fluorescence difference gel electrophoresis. Protein fractions are extracted from slow, fast, and mixed muscles and differentially labeled with Cy2, Cy3, and Cy5 dyes. Following separation by first-dimension isoelectric focusing (IEF) and second-dimension sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), gel images are scanned and protein spot patterns compared between differentially labeled protein fractions and pooled standards

2 Materials

2.1 Equipment

Unless otherwise stated, all equipment is from Amersham/GE Healthcare.

1. IPGphor IEF unit.
2. IPG DryStrip reswelling tray.

3. Manifold.
4. Sample loading cups.
5. Gel casting box.
6. Cassette racks.
7. Ettan DALT*twelve* multiple vertical slab gel unit.
8. Electrophoresis power supplies.
9. Glass plates suitable for fluorescence analysis.
10. Typhoon Trio variable mode imager.
11. ImageScanner UMax.
12. Vortex Genie-2 (Scientific Industries).
13. Stuart SSL4 shaker (Lennox Laboratory Supplies Ltd.).
14. Heto speedvac concentrator.
15. Eppendorf Model 5417R centrifuge.
16. Agilent Technologies 6340 Ion Trap LC mass spectrometer.
17. Agilent Technologies Nanoflow 1200 series system for peptide separation.

2.2 Reagents

All chemicals used should be of analytical grade. CyDye DIGE fluor minimal dyes should be stored at −20 °C in the dark.

1. CyDye DIGE fluor minimal dye Cy2 (for pooled standards).
2. CyDye DIGE fluor minimal dye Cy3 (for differential labeling).
3. CyDye DIGE fluor minimal dye Cy5 (for differential labeling).
4. Laemmli-type slab gel electrophoresis buffer system.
5. Immobilized linear pH gradient (IPG) strips.
6. pH 3–10 ampholytes.
7. IPG buffer.
8. Destreak agent.

2.3 Solutions

All solutions should be prepared with analytical grade chemicals and ultrapure water.

2.3.1 Preparation of Extracts from Different Subtypes of Skeletal Muscle

1. Lysis buffer: 1% pH 3–10 Ampholytes, 4% CHAPS, 9.5 M urea, 100 mM dithiothreitol. Ideally, the lysis buffer for the initial preparation of muscle tissue extracts should be supplemented with a commercially available protease inhibitor cocktail to avoid the degradation of sensitive skeletal muscle proteins (*see* **Note 1**). This solution can be dispensed into 1 mL Aliquots and stored at −20 °C.
2. DIGE lysis buffer: 9.5 M urea, 4% CHAPS, 30 mM Tris–HCl, pH 8.5 (*see* **Note 2**). This solution used during DIGE labeling can be dispensed into 1 mL aliquots and stored at −20 °C. This

solution should not be supplemented with reducing agents or protease inhibitors (*see* **Note 3**).

3. Reducing lysis buffer: 4% CHAPS, 9.5 M urea, 2% IPG buffer pH 3–10, 130 mM dithiothreitol. This reducing lysis buffer is for addition to samples after the dye labeling reaction and should ideally be supplemented with a commercially available protease inhibitor cocktail. This solution can be dispensed into 1 mL aliquot and stored at −20 °C.

2.3.2 Gel Electrophoresis

1. IPG rehydration buffer: Add 12 μL of Destreak and 0.002% Bromophenol Blue dye (*see* **Note 4**) to 1 mL lysis buffer.
2. SDS equilibrium buffer: 2% sodium dodecyl sulfate, 8 M urea, 30% glycerol, 50 mM Tris–HCl pH 8.8, and 0.002% Bromophenol Blue dye. Solution can be aliquoted and stored at −20 °C (*see* **Note 5**).
3. DTT equilibrium buffer: Add 100 mg dithiothreitol per 10 mL of SDS equilibrium buffer. Make this solution freshly prior to use in gel electrophoresis.
4. IA equilibrium buffer: Add 125 mg iodoacetamide per 10 mL of SDS equilibrium buffer. Make this solution freshly prior to use in gel electrophoresis.
5. 10× SDS buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS. Store this solution at room temperature.
6. Sealing solution: 1% agarose in 1× SDS buffer, and Bromophenol Blue dye. Heat solution until agarose has properly dissolved. Store the solution at room temperature (*see* **Note 6**).

2.3.3 Protein Visualization Using Coomassie Brilliant Blue

1. CBB Buffer A: 10% (w/v) ammonium sulfate, 2% (v/v) phosphoric acid.
2. CBB Buffer B: 5% (w/v) Commassie Brilliant Blue G-250 in water.
3. CBB staining solution: 2 mL Buffer B plus 80 mL Buffer A and 20 mL methanol.
4. Neutralization buffer: 0.1 M Tris at pH 6.5.
5. Wash solution: 25% (v/v) methanol.
6. Fixation solution: 20% (w/v) ammonium sulfate.

2.3.4 Reduction, Alkylation, and In-Gel Digestion

1. DTT solution: 10 mM DTT in 100 mM ammonium bicarbonate. Make this solution freshly prior to usage.
2. IA solution: 55 mM iodoacetamide in 100 mM ammonium bicarbonate. Make this solution freshly prior to usage.
3. Trypsin reconstitution solution: 50 mM acetic acid.
4. Trypsination buffer: 20 μg trypsin resuspended in 100 μL of reconstitution solution. Add 10 μL of this to 500 μL of 50 mM

ammonium bicarbonate. Make this solution freshly and use it immediately for trypsination of protein extracts.

5. Extraction solution: 1:2 (v/v) formic acid/acetonitrile. Make this solution freshly and use it immediately for peptide extraction.

3 Methods

3.1 Preparation of Tissue Extracts from Different Subtypes of Skeletal Muscle

1. Weigh four individual tissue samples from different subtypes of muscles that represent slow, fast, and mixed skeletal muscles (*see* **Note 7**). In the case of rodent muscle specimens, this could include the predominantly slow-twitching *soleus* muscle (samples A1–A4), the mixed *gastrocnemius* muscle (samples B1–B4) and the predominantly fast-twitching *tibialis anterior* muscle (samples C1–C4). This arrangement of samples A1–A4, B1–B4, and C1–C4 represents four biological repeats for each subtype of skeletal muscle under investigation. For technical repeats, each sample should be measured twice as outlined in the diagram of Fig. 2.
2. Place individual skeletal muscle tissue samples in liquid nitrogen and carefully grind them to a powder with mortar and pestle.
3. Add muscle powder to the appropriate buffer at a ratio of 1:10 (w/v). For DIGE analysis, transfer samples into DIGE lysis buffer (which contains no reducing agents and no protease inhibitors) and add equal volumes of 2× lysis buffer to protein extracts before placing them onto IEF strips. For the preparation of pick gels to be stained with Coomassie Brilliant Blue, add muscle powder to lysis buffer.
4. Solutions representing different subtypes of skeletal muscles should be well labeled and kept separate.
5. Briefly, vortex the solutions prepared for both DIGE analysis and pick gel preparation.
6. Incubate the suspensions on a rocker for 1 h at room temperature, with gentle vortexing every 10 min for 30 s.
7. Centrifuge at 20,000 × *g* for 20 min at 4 °C. Discard the pellet and uppermost fatty layer and save the protein-containing middle layer for subsequent analysis.
8. The protein concentration in muscle extracts should be determined by a reliable protein quantification method (*see* **Note 8**).
9. Dispense protein extracts into aliquots of 50 μL and store at −80 °C.

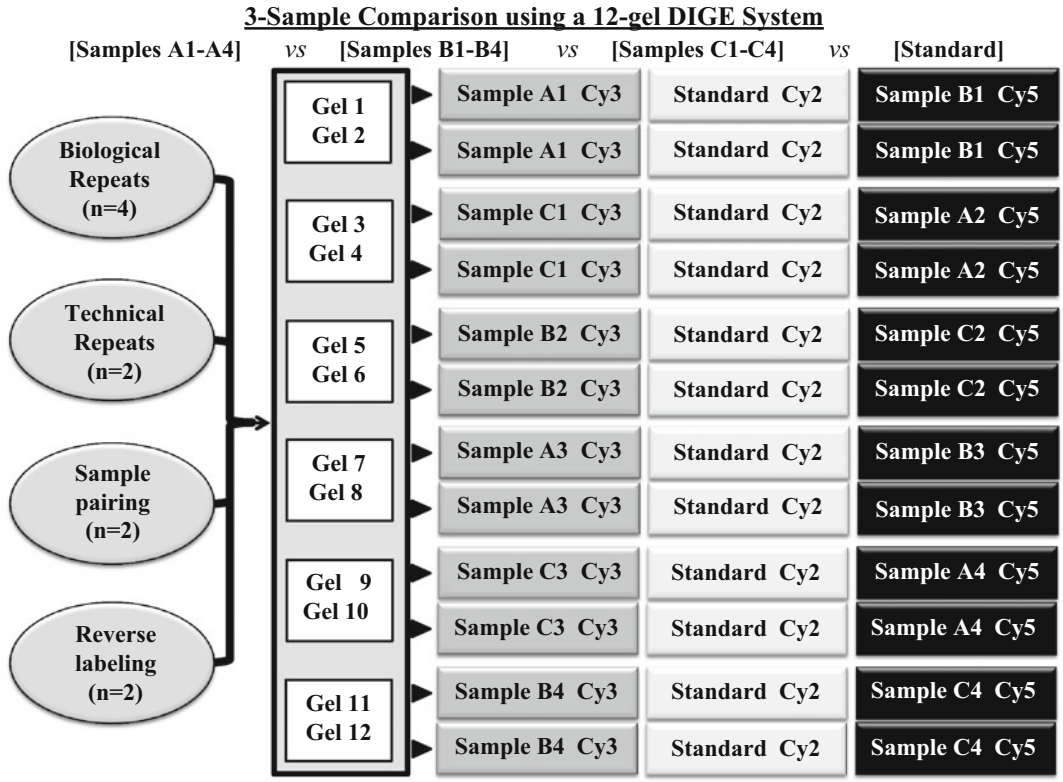

Fig. 2 Summarizing scheme of a 3-sample comparison using a 12-gel fluorescence difference gel electrophoresis (DIGE) 3-dye system. The outlined comparison is an optimized method that utilizes a minimum number of gels to achieve optimum results in relation to biological repeats, sample pairing, technical repeats and reverse fluorescent dye labeling

3.2 Differential 3-Sample Labeling of Muscle Proteins with Fluorescent CyDyes

1. Resuspend commercially available CyDye stock solutions in anhydrous dimethylformamide to give a final concentration of 1 mM dye. It is recommended that a fresh batch of dimethylformamide is used for the generation of a new stock solution of fluorescent dyes (*see* **Note 9**).
2. Briefly, vortex the vials containing individual CyDyes and centrifuge them at 12,000 × *g* for 10 s prior to the labeling reaction.
3. Dilute dyes 1:4 (v/v) with dimethylformamide to make a working solution of 200 pmol/μL. Working solutions can be stored in the dark at −20 °C.
4. Check whether the sample pH-value is at pH 8.5 prior to the labeling reaction.
5. As a general guide, add 1 μL of dye per 25 μg of skeletal muscle protein. For technical repeats, label twice 50 μg of each muscle extract sample with dye (*see* **Note 10**).

6. For the differential analysis of the predominantly slow-twitching *soleus* muscle, label 50 μg each of samples A1 and A3 using Cy3 dye, and 50 μg each of samples A2 and A4 using Cy5 dye.
7. For the differential analysis of the mixed *gastrocnemius* muscle, label 50 μg each of samples B2 and B4 using Cy3 dye, and 50 μg each of samples B1 and B3 using Cy5 dye.
8. For the differential analysis of the predominantly fast-twitching *tibialis anterior* muscle proteome, label 50 μg each of samples C1 and C3 using Cy3 dye, and 50 μg each of samples C2 and C4 using Cy5 dye.
9. For the internal standards, label 50 μg of pooled samples using the Cy2 dye. Overall 12 preparations of pooled standards are required for a 12-gel DIGE experiment with a 3-dye system.
10. *See* Fig. 2 for an overview of the differential dye labeling approach. The 3-sample comparison method described here is an optimized method that uses a 12-gel DIGE system with a minimum number of gels to achieve optimum results in relation to biological repeats ($n = 4$), sample pairing ($n = 2$), technical repeats ($n = 2$), and reverse fluorescent dye labeling.
11. Briefly, vortex the samples, centrifuge them at 12,000 × *g* for 10 s and then incubate the suspension on ice in the dark for 30 min.
12. Stop the labeling reaction by addition of 1 μL of 10 mM lysine per 25 μg of protein. Briefly, vortex the samples, centrifuge them at 12,000 × *g* for 10 s and then incubate them on ice in the dark for 10 min (*see* **Note 11**).

3.3 Rehydration of First-Dimension Gel Strips

1. Carefully fill the reservoir slots of the Ettan IPGphor DryStrip reswelling tray with 450 μL of rehydration buffer.
2. Remove plastic backing from 24 cm-long IPG strips of pH 3–10 by peeling from the (−) end and push the (+) end toward the top of strip holder.
3. Transfer first-dimension strips gel side down into rehydration buffer, ensuring that no bubbles remain trapped under the IPG strip.
4. Rehydration should be carried out for at least 12 h.

3.4 First-Dimension Isoelectric Focusing

1. Transfer first-dimension strips to the manifold gel side up on the Ettan Multiphor II system. Lift strips slowly by the (−) end and place the (+) end toward the (+) end marked on the IPGphor apparatus.
2. Cover first-dimension strips with cover fluid by the addition of 108 mL of drystrip cover fluid over the entire manifold.
3. Place wicks, wet with deionized water, onto ends of strips.

4. Place sample loading cups onto strips.
5. For analytical DIGE gels: Following differential DIGE labeling, add an equal volume of reducing lysis buffer. Pool the samples together to give 150 μg of total combined labeled extract per strip (50 μg Cy2-labeled protein fraction +50 μg Cy3-labeled protein fraction +50 μg Cy5-labeled protein fraction) as per experimental design (Fig. 2).
6. Position the electrodes in the correct orientation.
7. Carry out isoelectric focusing at 20 °C as follows: 4 h step at 80 V, 2 h step at 100 V, 1.5 h step at 500 V, 1.5 h step at 1000 V, 1 h step at 2000 V, 1 h step at 4000 V, 2 h step at 6000 V and a 2.5 h step at 8000 V.
8. After completion of the first-dimensional gel separation, IEF strips can be stored at −80 °C.
9. For preparative pick gels to be stained with Coomassie Brilliant Blue: Pipette 500 μg of the protein extract into cups. Subsequently, pipette protein into sample loading cups and carry out the same isoelectric focusing procedure as described above for DIGE gels.

3.5 Second-Dimensional Slab Gel Electrophoresis

1. Incubate first dimension strips in 10 mL DTT equilibrium buffer while rocking for 15 min.
2. Pour off solution and incubate while rocking in 10 mL IA equilibrium buffer for 15 min.
3. Pour off solution and wash strips briefly in 1× SDS running buffer.
4. With the help of SDS running buffer slide the first-dimension strips smoothly onto the second-dimension slab gels in order to reduce air bubbles forming between strip and slab gel.
5. Place strips with the (+) end to left, gel side facing out, onto a 12.5% resolving slab gel.
6. Press strips against the slab gel and then add warmed overlay sealing solution. This should eliminate air bubbles.
7. Place 12 separate gel sandwiches representing the differential DIGE-labeled preparations, as outlined in Fig. 2, in the Ettan DALT*twelve* tank.
8. Carry out the second-dimension gel electrophoresis step at 0.2 W/gel for 1 h, followed by 0.4 W/gel for 1 h and then 1.5 W/gel overnight until dye front runs off.
9. Following electrophoresis, carefully remove gels from plates and mark one corner of gel to track its orientation. Strips should remain with gels as each contains a unique number to allow tracking of samples. DIGE gels should be stored in

darkness to protect the fluorescence signal of individually labeled skeletal muscle proteins.

10. Preparative pick gels should ideally be run at the same time as analytical DIGE gels as to eliminate any potential technical discrepancies arising from the second-dimension separation step.

3.6 Coomassie Brilliant Blue Staining of Pick Gels

1. For the picking of protein spots of interest, a suitable slab gel has to be stained to visualize individually separated proteins. For the proper visualization of protein spots, a total protein amount of 500 μg should be loaded per pick gel (*see* **Note 12**).
2. Following gel electrophoretic separation, the slab gel that represents the pick gel is washed in distilled water.
3. Transfer the slab gels carefully to a clean dish containing Coomassie Brilliant Blue staining solution and incubated for 6 h or overnight with gentle agitation.
4. Following staining, transfer pick gels to neutralization buffer for 3 min.
5. Incubate the gels in wash solution for 1 min.
6. Following washing, slab gels are transferred to the fixation solution (*see* **Note 13**).
7. Scan Commassie Brilliant Blue-stained preparative slab gels using a suitable instrument, such as the ImageScanner UMax.
8. Add the information from the gel images to the Progenesis software program to serve as a pick gel and align them with the DIGE-labeled gel images.

3.7 Image Analysis of Protein Spot Patterns

1. Scan CyDye-labeled gels using a suitable variable mode imager, such as the Amersham Biosciences/GE Healthcare Typhoon Trio apparatus.
2. For image acquisition, scan Cy2, Cy3, and Cy5-labeled muscle proteins at wavelengths of 488 nm, 532 nm, and 633 nm, respectively. Photomultiplier tube PMT values should be optimized so that the volume of the most abundant protein spot is between 80,000 and 99,000 when scanned at a resolution of 100 μm (*see* **Note 14**).
3. Evaluate the images representing samples from fast, slow, and mixed skeletal muscles using 2D gel analysis software, such as Progenesis SameSpots analysis software (NonLinear Dynamics, Newcastle upon Tyne, UK).
4. Normalize the differential gel images against their corresponding pooled image. All two-dimensional gels are aligned to the reference image.

5. Following detection and establishments of individual spot patterns, gels are placed into 3 main groups (fast muscle versus slow muscle versus mixed muscle) and are then analyzed to determine significant changes in the abundance of distinct 2D protein spots.
6. Calculate paired Student's *t*-test values for each protein spot across all slab gels. An ANOVA score of 0.5 is required for spots to be included in the subsequent detailed evaluation of changes in muscle protein expression patterns.
7. Then principal component analysis (PCA) should be verified. Changes displaying power of <0.8 should be removed from the analysis.
8. All remaining changed protein spots that exhibit a fold-change of 1.5 or greater and meet the significance criteria should be visually checked on the aligned gels to ensure feasibility.
9. Scan Coomassie-stained preparative gels using a suitable instrument, such as the ImageScanner UMax from Amersham Biosciences/GE Healthcare. Images are added to the Progenesis software program as a pick gel and aligned to analytical DIGE-based gels.

3.8 Excision of Protein Spots from Two-Dimensional Gels

Protein spots of interest can be removed and transferred manually from two-dimensional gels or alternatively can be extracted by an automated spot-picking workstation (*see* **Note 15**). For manual spot picking, follow the below steps:

1. Cut off the ends of sterile pipette tips.
2. Place the tip of a sterile pipette over the individual protein spot.
3. Press firmly down through the gel matrix.
4. Carefully take up the plug into the pipette tip.
5. For transfer, the plug should be aspirated into a fresh plastic tube.

3.9 Reduction and Alkylation of Preparative Protein Spots

1. After the addition of 500 μL of neat acetonitrile, incubate gel plugs for 10 min while shaking. Briefly, spin down the suspension and remove liquid.
2. Add 30 μL of DTT solution and incubate gel plugs for 30 min at 56 °C while shaking.
3. Chill tubes to room temperature and add 500 μL of acetonitrile and incubate gel plugs for 10 min at room temperature. Remove all liquid.
4. Add 30 μL of IA solution and incubate gel plugs for 20 min at room temperature.
5. Shrink gel plugs with acetonitrile and remove all liquid (*see* **Note 16**).

3.10 Enzymatic Digestion of Muscle Proteins for Mass Spectrometric Analysis

1. In-gel digestion is initiated by the addition of 50 µL of trypsin buffer.
2. Incubate gel plugs with trypsin for 30 min at 4 °C (*see* **Note 17**).
3. Add more trypsin buffer to fully cover gel plugs and incubate muscle proteins for a further 90 min at 4 °C (*see* **Note 18**).
4. Incubate the suspension overnight at 37 °C (*see* **Note 19**).
5. Add 100 µL of extraction buffer and incubate proteins for 15 min at 37 °C with shaking.
6. Using sterile gel-loading tips, transfer all supernatant fractions into individual 0.2 mL plastic tubes.
7. Dry down peptides in a standard speedvac concentrator (*see* **Note 20**).

3.11 Mass Spectrometric Identification of Individual Protein Species

Depending on the type of mass spectrometer available, procedures may vary, such as apparatuses using linear ion traps, quadrupole, orbitrap, Fourier transform ion cyclotron resonance, or time-of-flight methodology [32–37]. Individual classes of mass spectrometers differ considerably in their performance in relation to mass accuracy, resolving power, sensitivity and dynamic range, as well as throughput capacity and available fragmentation modes [36]. Below steps describe the routine usage of an Agilent Technologies 6340 Ion Trap LC mass spectrometer:

1. For the identification of a protein spot of interest, reconstitute the generated peptides in 12 µL of 0.1% formic acid.
2. Vortex samples briefly.
3. Sonicate samples for 5 min.
4. To remove any gel particles, centrifuge samples for 20 min in cellulose spin filter tubes at 14,000 × *g*.
5. Using fresh tips for each sample, pipette samples to individually labeled LC-MS vials.
6. To identify muscle-associated proteins of interest, analyze peptide mixtures on an ion trap LC mass spectrometer by injecting 5 µL of sample.
7. Although conditions have to be usually optimized with specific mass spectrometers, using a 10 min gradient of 5–100% acetonitrile/0.1% formic acid and a post run of 1 min through a Zorbax 300SB C18 µm column gives reliable results with skeletal muscle proteins.
8. Separate peptides with a nanoflow Agilent 1200 series system, equipped with a Zorbax 300SB C18 5 µm, 4 mm 40 nl precolumn and a Zorbax 300SB C18 5 µm, 43 mm × 75 µm

analytical reversed phase column using HPLC-Chip technology.

9. Mobile phases should be (A): 0.1% formic acid, and (B): 90% acetonitrile and 0.1% formic acid.
10. Load samples into the enrichment at a capillary flow rate set to 4 μL/min with a mix of A and B at a ratio 19:1.
11. Elute tryptic peptides with a linear gradient of 5–70% solvent B over 6 min, 70–100% for 1 min and 100% for 1 min with a constant nano pump flow of 0.60 mL/min.
12. A 1 min post-time of Solvent A should be used to remove sample carryover.
13. Set the capillary voltage to 2000 V.
14. The flow rate and the temperature of the drying gas should be 4 L/min and 300 °C, respectively.
15. In order to identify distinct protein species, utilize Internet-based database search engines, such as Mascot MS/MS Ion search from Matrix Science (London, UK; NCBI database).

4 Notes

1. Suitable combinations of protease inhibitors for specific applications are usually determined by trial and error experiments. For the biochemical and proteomic analysis of mammalian skeletal muscle samples, a suitable protease inhibitor cocktail for the preparation of crude skeletal muscle extracts is represented by the addition of 1 tablet of "Complete Mini" (Roche Diagnostics, Mannheim, Germany) per 10 mL of lysis buffer.
2. It is critical that the pH value of the DIGE lysis buffer is verified as being pH 8.5 and adjusted, if necessary, using 30 mM Tris–HCl.
3. The DIGE lysis buffer should not contain any chemicals that may interfere with the CyDye labeling reaction.
4. Besides using low-fluorescence glass plates during the DIGE analysis, it is also recommended to completely run off the Bromophenol Blue dye front in DIGE gel systems or omit this dye in order to prevent the potential interference with fluorescent signals.
5. The SDS equilibrium buffer is a stock solution. Dithiothreitol or iodoacetamide must be added prior to use.
6. Agarose sealing solution should be melted prior to use.
7. For studies with mouse skeletal muscles, approximately 50 mg wet weight of each individual subtype of tissue should be obtained from adult animals for comparative DIGE-based

studies. In the case of very small muscles, tissue specimens from several animals have to be pooled for the initial preparative steps.

8. The accurate determination of the protein concentration in samples is crucial prior to the comparative DIGE analysis using high-resolution two-dimensional gel electrophoresis. Suitable protein quantification assays are commercially available, such as the 2-D Quant Kit from Amersham Biosciences/GE Healthcare (Little Chalfont, Bucks., UK).
9. Dye stock solutions can be stored in the dark at −20 °C.
10. For the comparative analysis of three different biological samples using a 12-gel DIGE experiment with a 3-dye system, overall 36 fluorescently labeled preparations are required. This includes 12 internal standards consisting of 50 μg of pooled samples labeled with Cy2 dye, as well as 24 samples that represent 8 repeats of 50 μg each of 3 different specimens with a differential label using Cy3 and Cy5 dyes.
11. CyDye-labeled samples can be used immediately for electrophoretic separation or stored in the dark at −80 °C.
12. The loading of a total amount of 150 μg protein per DIGE gel is suitable for the differential analysis of fluorescently labeled proteins (50 μg Cy2-labeled protein +50 μg Cy3-labeled protein +50 μg Cy5-labeled protein), but this amount of protein is usually not sufficient for the routine analysis of a typical Coomassie-stained pick gels. In our experience, 500 μg of total muscle protein gives satisfactory results for the positioning and manual extraction of protein spots following staining with Coomassie Brilliant Blue.
13. Washing slab gels with distilled water removes acetic acid and this greatly reduces the formation of ripples at the edge of slab gels and also eliminates air bubbles between the gel and flatbed scanner.
14. The optimization of PMT values guarantees that no individual protein spot will be saturated on the slab gel, therefore allowing accurate and quantifiable analysis.
15. As an alternative to the manual removal and transfer of gel plugs containing protein spots of interest, an automated spot-picking workstation (such as the highly reliable Ettan spot picker from Amersham Biosciences/GE Healthcare) can be employed. The spot-picking map of excised protein spots can be exported from the Progenesis software program to the automated device. Proteins can be conveniently excised into 96-well plastic plates for subsequent analysis.
16. Gel plug samples are now ready for in-gel digestion to generate distinct peptide populations. Alternatively, gel plugs can be stored at −20 °C.

17. Alternatively, proteolytic digestion can be achieved by using a combination of two proteolytic enzymes, Lys-C and trypsin. Lys-C/trypsin-mixtures are commercially available from several sources, such as Promega. The initial digestion should be carried out with sequencing-grade Lys-C at a ratio of 1:100 (protease:protein) for 4 h at 37 °C, followed by dilution with four times the initial sample volume in 50 mM ammonium bicarbonate. Samples should subsequently be digested with sequencing-grade trypsin at a ratio of 1:25 (protease:protein) overnight at 37 °C. Halt the digestion process by acidification with 2% trifluoroacetic acid in 20% acetonitrile.
18. Incubating with trypsin buffer at 4 °C allows the slow and efficient diffusion of the protease into gel plugs.
19. Following trypsination, peptide mixtures can be stored at −20 °C.
20. Dried-down peptide extracts can be stored at −20 °C for future analysis.

Acknowledgements

Research in the author's laboratory has been supported by project grants from the Irish Higher Education Authority, the Irish Health Research Board, and Muscular Dystrophy Ireland.

References

1. Oliveira BM, Coorssen JR, Martins-de-Souza D (2014) 2DE: the phoenix of proteomics. J Proteomics 104:140–150
2. Murphy S, Dowling P, Ohlendieck K (2016) Comparative skeletal muscle proteomics using two-dimensional gel electrophoresis. Proteomes 4:27
3. Minden JS, Dowd SR, Meyer HE et al (2009) Difference gel electrophoresis. Electrophoresis 30:S156–S161
4. Timms JF, Cramer R (2008) Difference gel electrophoresis. Proteomics 8:4886–4897
5. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
6. Arentz G, Weiland F, Oehler MK et al (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9:277–288
7. Alban A, David SO, Bjorkesten L et al (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3:36–44
8. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5:3105–3115
9. Viswanathan S, Unlü M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358
10. Tonge R, Shaw J, Middleton B et al (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396
11. Marouga R, David S, Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382:669–678
12. Karp NA, Kreil DP, Lilley KS (2004) Determining a significant change in protein expression with DeCyder during a pair-wise comparison using two-dimensional difference gel electrophoresis. Proteomics 4:1421–1432

13. Malm C, Hadrevi J, Bergström SA et al (2008) Evaluation of 2-D DIGE for skeletal muscle: protocol and repeatability. Scand J Clin Lab Invest 68:793–800
14. Carberry S, Zweyer M, Swandulla D et al (2013) Application of fluorescence two-dimensional difference in-gel electrophoresis as a proteomic biomarker discovery tool in muscular dystrophy research. Biology (Basel) 2:1438–1464
15. Hadrévi J, Hellström F, Kieselbach T et al (2011) Protein differences between human trapezius and vastus lateralis muscles determined with a proteomic approach. BMC Musculoskelet Disord 12:181
16. Donoghue P, Doran P, Wynne K et al (2007) Proteomic profiling of chronic low-frequency stimulated fast muscle. Proteomics 7:3417–3430
17. Moriggi M, Cassano P, Vasso M et al (2008) A DIGE approach for the assessment of rat soleus muscle changes during unloading: effect of acetyl-L-carnitine supplementation. Proteomics 8:3588–3604
18. De Palma S, Ripamonti M, Vigano A et al (2007) Metabolic modulation induced by chronic hypoxia in rats using a comparative proteomic analysis of skeletal muscle tissue. J Proteome Res 6:1974–1984
19. Egan B, Dowling P, O'Connor PL et al (2011) 2-D DIGE analysis of the mitochondrial proteome from human skeletal muscle reveals time course-dependent remodelling in response to 14 consecutive days of endurance exercise training. Proteomics 11:1413–1428
20. Burniston JG, Kenyani J, Gray D et al (2014) Conditional independence mapping of DIGE data reveals PDIA3 protein species as key nodes associated with muscle aerobic capacity. J Proteome 106:230–245
21. Doran P, O'Connell K, Gannon J et al (2008) Opposite pathobiochemical fate of pyruvate kinase and adenylate kinase in aged rat skeletal muscle as revealed by proteomic DIGE analysis. Proteomics 8:364–377
22. Staunton L, Zweyer M, Swandulla D et al (2012) Mass spectrometry-based proteomic analysis of middle-aged vs. aged vastus lateralis reveals increased levels of carbonic anhydrase isoform 3 in senescent human skeletal muscle. Int J Mol Med 30:723–733
23. Capitanio D, Vasso M, De Palma S et al (2016) Specific protein changes contribute to the differential muscle mass loss during ageing. Proteomics 16:645–656
24. Doran P, Martin G, Dowling P et al (2006) Proteome analysis of the dystrophin-deficient MDX diaphragm reveals a drastic increase in the heat shock protein cvHSP. Proteomics 6:4610–4621
25. Gomez AM, Vanheel A, Losen M et al (2013) Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis. J Neuroimmunol 261:141–145
26. De Palma S, Capitanio D, Vasso M et al (2014) Muscle proteomics reveals novel insights into the pathophysiological mechanisms of collagen VI myopathies. J Proteome Res 13:5022–5030
27. Dowling P, Murphy S, Ohlendieck K (2016) Proteomic profiling of muscle fibre type shifting in neuromuscular diseases. Expert Rev Proteomics 13:783–799
28. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiol Rev 91:1447–1531
29. Pette D, Staron RS (2000) Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech 50:500–509
30. Holland A, Ohlendieck K (2013) Proteomic profiling of the contractile apparatus from skeletal muscle. Expert Rev Proteomics 10:239–257
31. Doran P, Wilton SD, Fletcher S et al (2009) Proteomic profiling of antisense-induced exon skipping reveals reversal of pathobiochemical abnormalities in dystrophic mdx diaphragm. Proteomics 9:671–685
32. Yates JR, Ruse CI, Nakorchevsky A (2009) Proteomics by mass spectrometry: approaches, advances, and applications. Annu Rev Biomed Eng 11:49–79
33. Cox J, Mann M (2011) Quantitative, high-resolution proteomics for data-driven systems biology. Annu Rev Biochem 80:273–299
34. Angel TE, Aryal UK, Hengel SM et al (2012) Mass spectrometry-based proteomics: existing capabilities and future directions. Chem Soc Rev 41:3912–3928
35. Altelaar AF, Heck AJ (2012) Trends in ultrasensitive proteomics. Curr Opin Chem Biol 16:206–213
36. Zhang Z, Wu S, Stenoien DL et al (2014) High-throughput proteomics. Annu Rev Anal Chem 7:427–454
37. Faini M, Stengel F, Aebersold R et al (2016) The evolving contribution of mass spectrometry to integrative structural biology. J Am Soc Mass Spectrom 27:966–974

Chapter 10

DIGE Analysis of ProteoMiner™ Fractionated Serum/Plasma Samples

Sandra Murphy and Paul Dowling

Abstract

The discovery of clinically relevant biomarkers using gel-based proteomics has proven extremely challenging, principally because of the large dynamic range of protein abundances in biofluids such as blood and the fact that only a small number of proteins constitute the vast majority of total blood protein mass. Various separation, depletion, enrichment, and quantitative developments coupled with improvements in gel-based protein quantification technologies, specifically difference gel electrophoresis (DIGE), have contributed to significant improvements in the detection and identification of lower abundance proteins. One of these enrichment technologies, Proteominer, will be the focus of this chapter. The Proteominer technology a utilizes hexapeptide bead library with huge diversity to bind and enrich low-abundance proteins but at the same time suppressing the concentration of high-abundance proteins in subsequent analysis.

Key words Hexapeptide, Plasma, ProteoMiner™, Serum

1 Introduction

An approach for the detection of low abundant proteins has been developed that utilizes combinatorial peptide ligand libraries (CPLL) to capture and analyze this "low-abundance proteome" in association with gel-based quantification methods. The idea of libraries containing millions of peptides, produced on the basis of a "one-bead, one-peptide" approach, was discussed in a seminal paper by Lam and co-workers [1]. This technology is presently commercially available under the trade name of Proteominer™ (Bio-Rad Laboratories, Hercules, CA, USA) and has been widely used for analysis of complex biological samples such as serum or plasma [2–4].

Proteominer technology has its foundations on the interaction of complex protein samples with a large, highly diverse library of hexapeptides bound to chromatic support beads [5, 6]. Each bead carries a large number (billions) of copies of the same peptide bait, therefore the beads are thus different from each other, with all

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_10,

possible combinations of hexapeptides present. In theory, each unique hexapeptide binds to a unique protein sequence. When a complex biological sample, serum, or plasma, for example, is applied to the beads, high-abundance proteins like albumin saturate their high-affinity ligands quickly, and excess unbound protein is removed during the washing steps [7, 8]. In contrast, low-abundance proteins are concentrated on their specific affinity ligands, as they never saturate their unique binding sites. In addition, the sequential elution kit employs multiple elution reagents to sequentially elute proteins based on their different properties and separate biological samples into four distinct fractions [9]. This further allows researchers to both compress the dynamic range of proteins and also extend protein detection and quantification by analyzing each of the four fraction separately. When analyzed in downstream applications, the number of proteins detected is dramatically increased. Proteominer technology can also be employed to reduce the dynamic range of other biofluids, including cerebrospinal fluid [10], together with the more traditional samples such as serum/plasma. Proteominer can be used for differential expression analysis and is compatible with current downstream protein analysis techniques. Proteominer technology enriches and concentrates low-abundance proteins that cannot be detected through traditional gel-based methods when an investigation of raw unfractionated biofluids is used. Thus, it can significantly increase the sensitivity of DIGE-based proteomics analyses and provides a tool for digging deeper into the proteome [11, 12].

2 Materials

2.1 Equipment

1. Eppendorf Model 5417R centrifuge.
2. LP Vortex Mixer (ThermoFisher Scientific).
3. Rotating Mixer (BenchMark).

2.2 Reagents

1. Proteominer Sequential Elution Large-Capacity Kit (Catalog #163-3011).
2. 2D clean-up kit (GE Healthcare).
3. Bradford assay (Bio-Rad, 500-0205).
4. Bovine serum albumin (BSA) solution (Sigma, A9543).

2.3 Solutions

1. Elution Reagent 1. 5 mL 1 M sodium chloride, 20 mM HEPES, pH 7.4.
2. Elution Reagent 2. 5 mL 200 mM glycine, pH 2.4.
3. Elution Reagent 3. 5 mL 60% ethylene glycol in water.

4. Elution Reagent 4. 5 mL 33.3% 2-propanol, 16.7% acetonitrile, 0.1% trifluoroacetic acid.
5. Plasma Preparation Buffer. 1.5 mL 1 M sodium citrate, 20 mM HEPES, pH 7.4.
6. Wash Buffer. 50 mL PBS buffer (150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4).

3 Methods

3.1 Column Preparation—Proteominer Large-Capacity

1. This protocol has been optimized for plasma and serum samples with protein concentrations of >50 mg/mL (requires total protein load >50 mg).
2. Remove the top cap of the spin column and snap off the bottom cap prior to use. The bottom cap will be used as a plug for the remainder of the protocol (*see* **Note 1**).
3. Place the spin column in a collection tube and centrifuge at 1000 × *g* for 60 s to remove the storage solution. Discard the storage solution.
4. Replace the bottom cap (from **step 1**) and add 600 μL of wash buffer to the beads. Rotate column end-over-end for 5 min (*see* **Note 2**).
5. Remove the top cap and then the bottom cap of the spin column. Place the spin column in the collection tube and replace the top cap. Centrifuge at 1000 × *g* for 60 s. Discard the solution.
6. Wash the beads a second time by repeating **steps 4** and **5**.
7. Replace the bottom cap on a spin column. The column now contains washed and prepared beads (100 μL) ready for sample binding.

3.2 Sample Binding (Serum/Plasma)

1. Centrifuge the serum/plasma sample at 16,000 × *g* for 10 min at 4 °C to remove any debris from the sample that might compromise the binding of proteins to the beads.
2. Add 1 mL of serum/plasma to the column. Replace the top cap and tap the column to mix. Rotate column on a platform or rotational shaker for 2 h at room temperature (*see* **Note 3**).

3.3 Sample Wash

1. Remove bottom cap, place the column in a collection tube and centrifuge at 1000 × *g* for 60 s. Discard collected material.
2. Replace the bottom cap and add 600 μL of wash buffer to the column. Replace top cap and rotate from end-over-end for 5 min.
3. Remove bottom cap, place the column in a collection tube and centrifuge at 1000 × *g* for 60 s. Discard collected material.
4. Repeat **steps 2** and **3** two more times.

3.4 Elution

1. Carefully add 200 μL wash buffer on all sides of the column to ensure none of the beads are stuck to the sides of the column.
2. Centrifuge at 1000 × *g* for 60 s. Discard collected material.
3. Attach bottom cap to the column. Add 100 μL of elution reagent 1 to the spin column. Incubate at room temperature for 10 min with periodic vortexing during this period.
4. Remove bottom cap and place column in a collection tube (marked "elution 1 proteins") and centrifuge at 1000 × *g* for 60 s to collect the elution (*see* **Note 4**).
5. Repeat **steps 3** and **4** two more times and collect both elutions in the collection tube (elution 1 proteins).
6. Attach bottom cap to the column and add 100 μL of elution reagent 2 to the spin column. Incubate at room temperature for 10 min with periodic vortexing during this period.
7. Remove bottom cap and place column in a collection tube (marked "elution 2 proteins") and centrifuge at 1000 × *g* for 60 s to collect the elution (*see* **Note 4**).
8. Repeat **steps 6** and 7 two more times and collect both elutions in the collection tube (elution 2 proteins) (*see* **Note 5**).
9. Attach bottom cap to the column. Add 100 μL of elution reagent 3 to the spin column. Incubate at room temperature for 10 min with periodic vortexing during this period.
10. Remove bottom cap and place column in a collection tube (marked "elution 3 proteins") and centrifuge at 1000 × *g* for 60 s to collect the elution (*see* **Note 4**).
11. Repeat **steps 9** and **10** two more times and collect both elutions in the collection tube (elution 3 proteins).
12. Attach bottom cap to the column. Add 100 μL of elution reagent 4 to the spin column. Incubate at room temperature for 5 min with periodic vortexing during this period.
13. Remove bottom cap and place column in a collection tube (marked "elution 4 proteins") and centrifuge at 1000 × *g* for 60 s to collect the elution (*see* **Note 4**).
14. Repeat **steps 12** and **13** two more times and collect both elutions in the collection tube (elution 4 proteins).
15. Store elutions at −20 °C or proceed with downstream analysis.

3.5 Sample Precipitation Using 2D Clean-Up Kit

1. All steps should be performed on ice (4 °C) at all times unless otherwise stated according to the manufacturer's manual.
2. Pre-chill the wash buffer prior to use for at least 1 h at −20 °C.
3. Transfer 100 μL from each of the Proteominer sequentially fractionated samples into microcentrifuge tubes.
4. For each sample, add 3 volumes of precipitant to 1 volume of sample. Vortex and incubate on ice for 15 min.

5. Add 3 volumes of co-precipitant to the mixture and vortex briefly to mix.
6. Centrifuge at 20,000 × *g* for 5 min with the hinge side of the microcentrifuge tubes to the outside.
7. Remove the tube immediately and discard the supernatant using a pipette. Avoid disturbing the pellet.
8. Reposition the microcentrifuge tubes as before in the centrifuge as before and centrifuge at 20,000 × *g* for 60 s to bring down the remaining liquid to the bottom of the tube. Remove all the liquid from the tube.
9. Without disturbing the pellet, add co-precipitant onto the pellet. The volume of co-precipitant used is 3–4 times the volume of the pellet. Leave the tube on ice for 5 min.
10. Reposition the microcentrifuge tubes as before in the centrifuge and centrifuge at 20,000 × *g* for 5 min.
11. Using a pipette, remove and discard the wash solution without disturbing the pellet.
12. Add a sufficient volume of deionized water to cover the pellet. Vortex to mix and ensure the pellet dislodges from the side of the microcentrifuge tube.
13. Add 1 mL of the pre-chilled wash buffer and 5 μL of the wash additive. Vortex vigorously for 10 s.
14. Incubate the tubes at −20 °C for 2 h. Vortex for 30 s once every 15 min during this 2 h period.
15. Reposition the microcentrifuge tubes as before in the centrifuge and centrifuge at 20,000 × *g* for 5 min.
16. Remove and discard the supernatant. A white pellet should now be visible. Allow the pellet to air-dry briefly for 2–3 min (*see* **Note 6**).
17. Solubilize protein pellet in DIGE lysis buffer with sonication.
18. Protein concentrations can be determined using the Bradford protein assay kit (Bio-Rad, 500-0205) based on the manufacturer's instructions. Perform the assay in triplicate measure the absorbance at 595 nm and determine the protein concentration from a standard curve.
19. Proceed to DIGE labeling.

4 Notes

1. Take caution to ensure the bottom cap is tightly attached.
2. Replace the top cap and tap the spin column gently on a bench to mix the beads with the wash buffer.

3. Add 900 μL plasma (non-heparinized) and 100 μL plasma preparation buffer to spin column.
4. This elution contains your eluted proteins. Do not discard.
5. If using plasma samples, a white precipitate will form when adding elution reagent 2. Before using eluent in downstream applications, spin and use supernatant
6. Over-drying the pellet can result in difficulty in resuspension of the pellet.

References

1. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ (1991) A new type of synthetic peptide library for identifying ligand-binding activity. Nature 354 (6348):82–84. doi:10.1038/354082a0
2. Li L (2015) Dynamic range compression with ProteoMiner™: principles and examples. Methods Mol Biol 1295:99–107. doi:10.1007/978-1-4939-2550-6_9
3. Milan E, Lazzari C, Anand S, Floriani I, Torri V, Sorlini C, Gregorc V, Bachi A (2012) SAA1 is over-expressed in plasma of non small cell lung cancer patients with poor outcome after treatment with epidermal growth factor receptor tyrosine-kinase inhibitors. J Proteomics 76 Spec No:91–101. doi:10.1016/j.jprot.2012.06.022
4. Fernández C, Santos HM, Ruíz-Romero C, Blanco FJ, Capelo-Martínez JL (2011) A comparison of depletion versus equalization for reducing high-abundance proteins in human serum. Electrophoresis 32(21):2966–2974. doi:10.1002/elps.201100183
5. Boschetti E, Giorgio Righetti P (2008) Hexapeptide combinatorial ligand libraries: the march for the detection of the low-abundance proteome continues. BioTechniques 44 (5):663–665. doi:10.2144/000112762
6. Righetti PG, Boschetti E (2008) The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. Mass Spectrom Rev 27(6):596–608. doi:10.1002/mas.20178
7. Hartwig S, Czibere A, Kotzka J, Passlack W, Haas R, Eckel J, Lehr S (2009) Combinatorial hexapeptide ligand libraries (ProteoMiner): an innovative fractionation tool for differential quantitative clinical proteomics. Arch Physiol Biochem 115(3):155–160. doi:10.1080/13813450903154224
8. Bandow JE (2010) Comparison of protein enrichment strategies for proteome analysis of plasma. Proteomics 10(7):1416–1425. doi:10.1002/pmic.200900431
9. Matysiak J, Hajduk J, Mayer F, Hebeler R, Kokot ZJ (2016) Hyphenated LC-MALDI-ToF/ToF and LC-ESI-QToF approach in proteomic characterization of honeybee venom. J Pharm Biomed Anal 121:69–76. doi:10.1016/j.jpba.2016.01.009
10. Mouton-Barbosa E, Roux-Dalvai F, Bouyssié D, Berger F, Schmidt E, Righetti PG, Guerrier L, Boschetti E, Burlet-Schiltz O, Monsarrat B, Gonzalez de Peredo A (2010) In-depth exploration of cerebrospinal fluid by combining peptide ligand library treatment and label-free protein quantification. Mol Cell Proteomics 9 (5):1006–1021. doi:10.1074/mcp.M900513-MCP200
11. Sihlbom C, Kanmert I, Bahr H, Davidsson P (2008) Evaluation of the combination of bead technology with SELDI-TOF-MS and 2-D DIGE for detection of plasma proteins. J Proteome Res 7(9):4191–4198. doi:10.1021/pr800340c
12. Fekkar A, Pionneau C, Brossas JY, Marinach-Patrice C, Snounou G, Brock M, Ibrahim-Granet O, Mazier D (2012) DIGE enables the detection of a putative serum biomarker of fungal origin in a mouse model of invasive aspergillosis. J Proteome 75(9):2536–2549. doi:10.1016/j.jprot.2012.01.040

Part IV

Comparative DIGE Tissue Proteomics

Chapter 11

DIGE Analysis of Human Tissues

Cecilia Gelfi and Daniele Capitanio

Abstract

Two-dimensional difference gel electrophoresis (2-D DIGE) is an advanced and elegant gel electrophoretic analytical tool for comparative protein assessment. It is based on two-dimensional gel electrophoresis (2-DE) separation of fluorescently labeled protein extracts. The tagging procedures are designed to not interfere with the chemical properties of proteins with respect to their p*I* and electrophoretic mobility, once a proper labeling protocol is followed. The two-dye or three-dye systems can be adopted and their choice depends on specific applications. Furthermore, the use of an internal pooled standard makes 2-D DIGE a highly accurate quantitative method enabling multiple protein samples to be separated on the same two-dimensional gel. The image matching and cross-gel statistical analysis generates robust quantitative results making data validation by independent technologies successful.

Key words Two-dimensional electrophoresis, 2-D DIGE, CyDye DIGE fluors, Differential analysis

1 Introduction

An essential need in biomedical research is the understanding of the molecular, cellular and environmental events that drive cellular behavior in diseases leading to the identification of therapeutic interventions. At variance from the genome that remains nearly constant over time, the proteome changes dynamically according to cell type and in response to various stimuli. Furthermore, the number of different proteins grows dramatically if one considers alternative splicing and post-translational modifications (PTMs). For this reason, proteomic exploration, by allowing the large-scale characterization of proteins in a cell, tissue or organism, gave us insights into the perturbations of signaling networks that mediate cellular activity and facilitated the characterization of putative markers for disease onset and progression, and discovery of new drug targets [1, 2]. Furthermore, continuous technological and methodological developments have extended the application of proteomic studies to clinical samples, which are often limited in sample amount. Thus, quantitative proteomics has now a great potential to

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_11,

discover, validate, and accurately quantify biomarkers in body fluids [3] and tissues [4], taking into consideration also protein PTMs [5].

The 2-D DIGE method was first described in 1997 [6] as a modification of 2-DE [7–9]. It has been developed with the aim to overcome the lower gel reproducibility and to increase the sensitivity of 2-DE methodology by labeling samples with fluorescent dyes allowing the separation of multiple samples in the same gel. Furthermore, after gel excision of spots of interest, it permits their identification by mass spectrometry (MS), including specific proteoforms and unknown post-translational modifications (PTMs) [10–13]. Protein samples are labeled with up to three spectrally distinct, charge- and mass-matched fluorescent dyes (CyDye DIGE fluors) [6, 14, 15], i.e., Cy2, Cy3, and Cy5. CyDyes DIGE fluors have an N-hydroxysuccinimide ester reactive group and are designed to covalently bind the epsilon amino group of lysine via an amide bond (Fig. 1).

Furthermore, the amount of dye added to samples limits the reaction, hence this method is referred to as "minimal labeling." This procedure ensures that only 1% to 2% of the total number of lysine residues are fluorescently labeled. The minimal labeling procedure is based on the fact that the same protein, labeled with any of the CyDye DIGE fluors, migrates to the same position on a 2-D gel [16] eliminating the intra-gel variation. After labeling, protein extracts from different samples are mixed and separated on the same 2-D gel. Samples are then visualized using an imager equipped with appropriate laser wavelengths for detecting CyDyes and digital images from each sample are analyzed by a dedicated software. The greatest advance introduced by 2-D DIGE for semi-quantitative studies is the use of a labeled internal standard made by coupling all samples involved in the study tagged with one of the CyDye DIGE fluors (usually Cy2 in a three-dye experiment or Cy3 in a two-dye experiment) (Fig. 2) [17, 18].

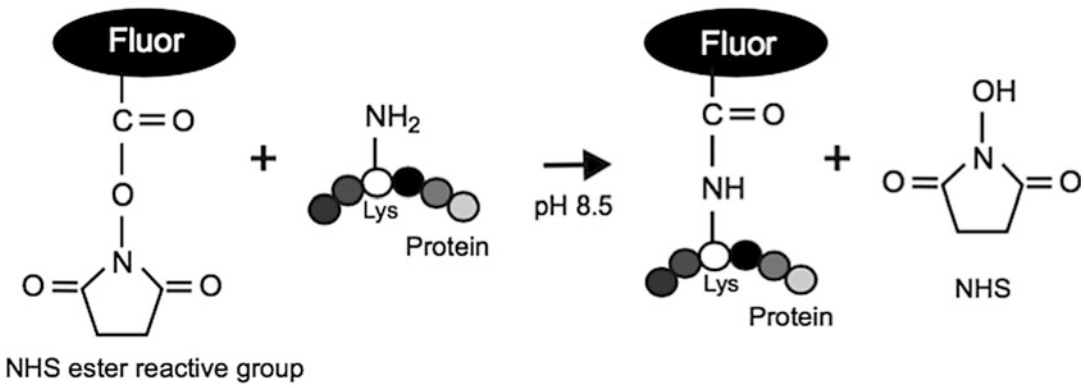

Fig. 1 *N*-hydroxysuccinimide (NHS) ester labeling of proteins

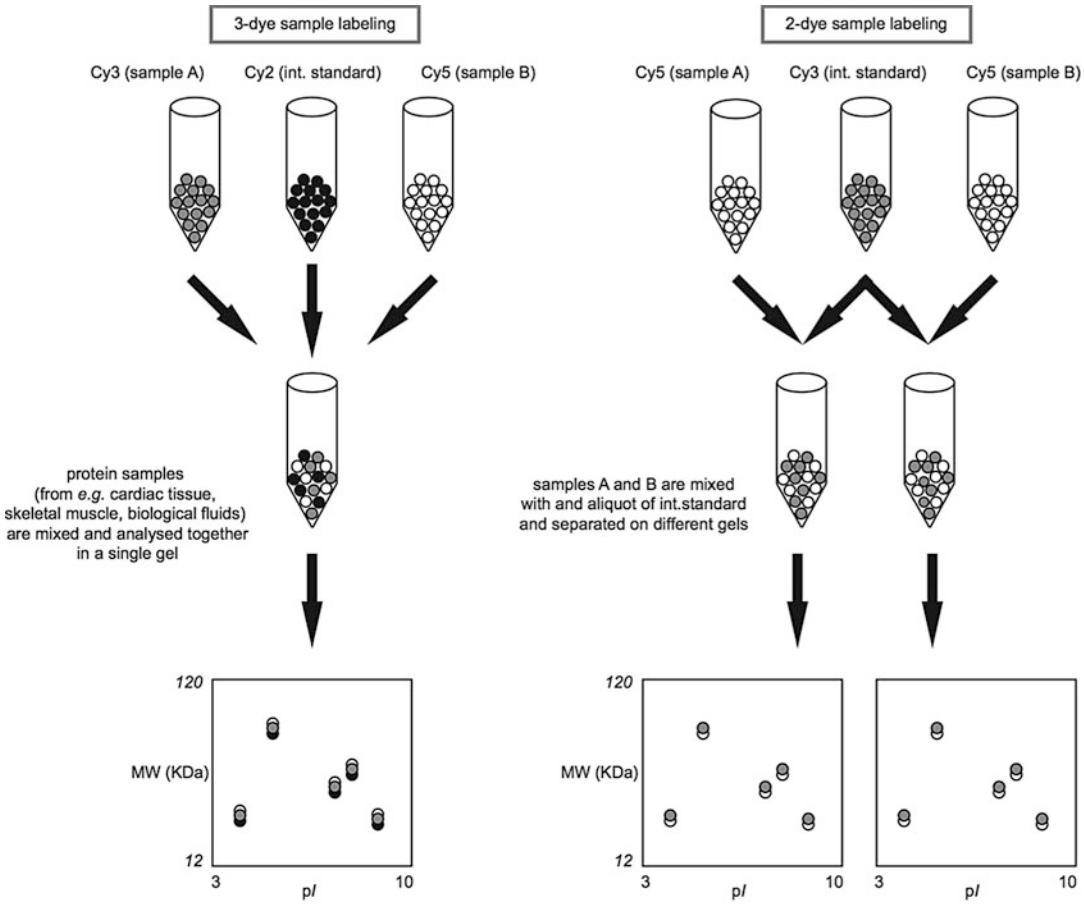

Fig. 2 Scheme of a DIGE labeling protocol, using three or two fluors

The use of internal standard ensures that every protein from all samples is present in it, thus linking each sample in a gel to a common internal standard. In addition, by reducing the experimental variation it allows an accurate quantification of the biological variation obtained from the statistical analysis of spot abundance between gels [19]. Furthermore, fluors are characterized by a quite good sensitivity with a detection capability of 125 pg of a single protein and a linearity of dye intensity over five orders of magnitude. In this chapter, the practical procedures to perform a standardized and reproducible 2-D DIGE experiment starting from tissue protein extracts are described step by step and troubleshooting and solutions are provided.

It is well known that this technology has various technical limitations, particularly when applied to crude extracts: it underrates low abundant elements, integral membrane proteins and proteins with high molecular mass [20]. In addition, proteins characterized by extreme p*I*s require the use of multiple narrow-range p*I* gels in the first dimension [21] which cannot be routinely

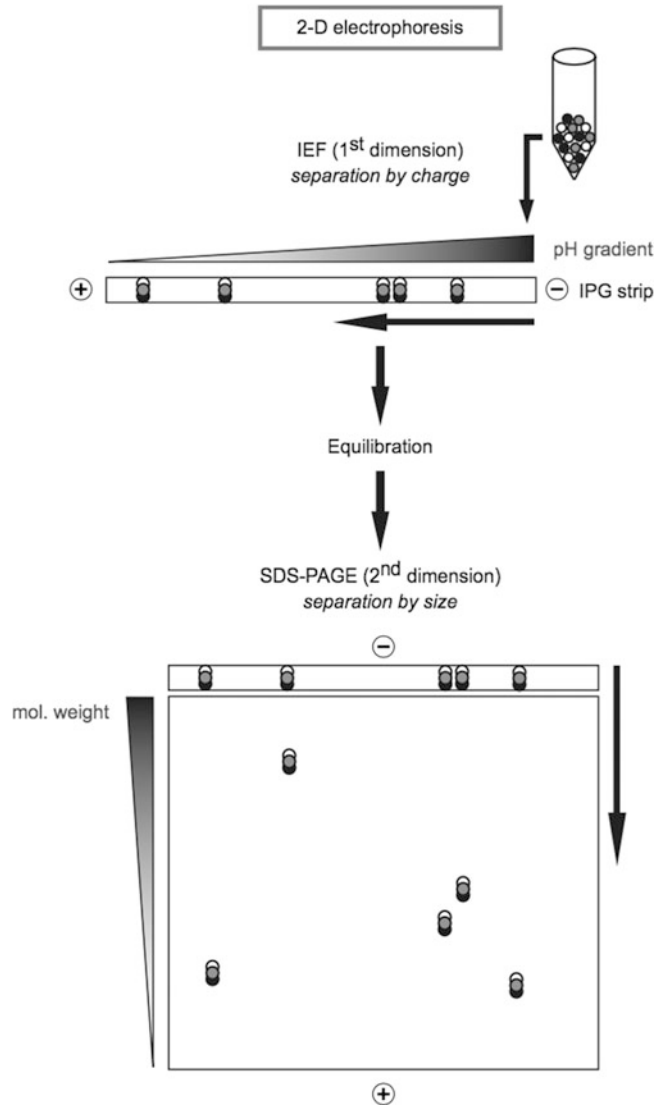

Fig. 3 2-D electrophoresis workflow

adopted in differential studies since it can be sample and time-consuming. However, there are also many advantages in using 2-D DIGE for the study of tissues; this technology has the highest separation power for the most part of intact proteins and it allow proteoforms and unknown PTMs identification and quantification. Actually, separation is based on two unique characteristics of an intact protein: the p*I*, which depends on protein sequence, and the apparent molecular mass (Fig. 3). The combination of isoelectric focusing (IEF) on immobilized pH gradient (IPG), a technology characterized by an unraveling resolving power, since it allows the separation of proteins with difference of one thousandth unit of p*I*

[22] in the first dimension, and SDS gel-based separation in the second, decreases the protein dynamic range and increases protein detectability.

However, the latter is hampered by gel extraction which lowers the sensitivity of this methodology due to MS background noise caused by not completely polymerized acrylamide derivatives [23] extracted with peptides during the proteolytic spots digestion step for MS identification. However, for specific purposes, 2-D DIGE is a technique that enables one to directly compare a set of two protein complements and provides robust quantitative results (Fig. 4).

On the other hand it should also be considered that this approach, despite its good performances, is quite expensive, time-consuming and requires specific skills and dedicated instrumentation which are not always present in proteomic laboratories.

2 Materials and Instruments

All solutions utilize ultrapure water (18 MΩcm at 20 °C) and analytical grade reagents. Solutions are filtered prior to use.

2.1 Sample Preparation and Quantitation

1. Tissue homogenization for sample disruption (*see* **Note 1**).
2. Lysis Buffer: 30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), pH 8.5. For a final volume of 100 mL dissolve 364 mg of Tris, 42 g of urea, 15.2 g of thiourea and 4 g of CHAPS in about 50 mL of water. Adjust the pH with dilute HCl and make up to 100 mL with water. Prepare aliquots of 1 mL and store at −20 °C. Prior to use add 1 μL of 10 mM phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 2**).
3. Sonicator (*see* **Note 3**).
4. Precipitation method to purify protein sample to remove non-protein impurities (*see* **Note 4**).
5. pH indicator strips (pH 4.5–10.0) to check the pH of the protein extract and 50 mM sodium hydroxide to adjust the pH.
6. Quantitation method to determine sample concentration (*see* **Note 5**).

2.2 Protein Labeling

1. CyDye™ DIGE fluor Cy2, Cy3, and Cy5 minimal dye (GE Healthcare) (*see* **Note 6**).
2. 99.8% DMF (anhydrous dimethylformamide) for CyDye solubilization. Solubilized dyes must be used within 3 months of opening (*see* **Note 7**).
3. 10 mM lysine solution. The solution can be stored at 4 °C for several months.

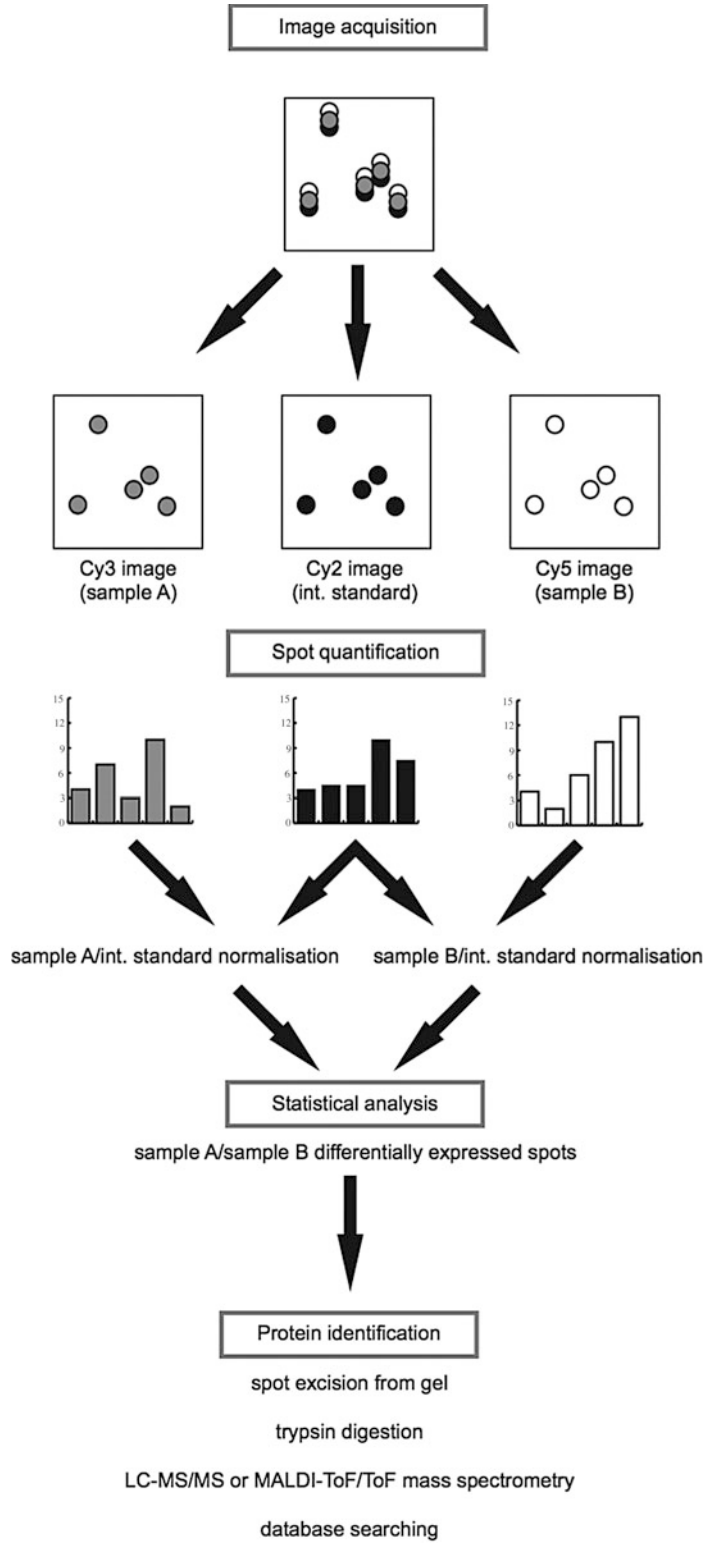

Fig. 4 Image acquisition and data processing scheme

2.3 Gel Electrophoresis

2.3.1 Isoelectric Focusing (IEF)

1. 2× sample buffer: 8 M urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) carrier ampholytes (IPG buffer) pH 3–10 for IEF. For a final volume of 100 mL dissolve 48.05 g of urea, 2 g of DTT, 4 g of CHAPS in 50 mL of water, add 1 mL of IPG buffer pH 3–10. Add water to a volume of 100 mL. Aliquots can be stored at −20 °C.
2. Immobiline™ DryStrip IPG gel strips (GE Healthcare). Precast Immobiline DryStrip are available with different lengths (7, 11, 13, 18, and 24 cm) and different pH gradients (3.5–4.5, 4.5–5.5, 4–7 linear, 6–11 linear, 3–10 non linear and linear, 3–11 NL and L, etc.).
3. Rehydration stock solution: 7 M urea, 2 M thiourea, 2% (w/v) CHAPS. For a final volume of 100 mL, weigh out 42.04 g of urea, 15.22 g of thiourea and 2 g of CHAPS and prepare a solution as in previous steps. Store at −20 °C. Prior to use add 65 mM DTT and 0.5% (v/v) IPG buffer with a pH range corresponding to IPG strip pH gradient.
4. Bromophenol blue as tracking dye.
5. IPG cover fluid (GE Healthcare).
6. IPGphor isoelectric focusing units (GE Healthcare) or similar for first-dimension isoelectric focusing of proteins.
7. IPGphor strip holder (GE Healthcare) having the same length of the IPG strips or, alternatively, the IPGphor Manifold (GE Healthcare) that allows protein separation on 7–24 cm long Immobiline DryStrips.

2.3.2 IPG Strip Equilibration

1. Equilibration buffer: 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 375 mM Tris–HCl pH 8.8. For a final volume of 100 mL dissolve 36.036 g of urea, 2 g of SDS, add 20 mL of glycerol and 20 mL of Tris–HCl pH 8.8 and prepare a solution as in previous steps. Store at −20 °C. Prior to use, separate the equilibration buffer into 2 aliquots and add 65 mM DTT (100 mg per 10 mL equilibration buffer) and 135 mM iodoacetamide (250 mg per 10 mL equilibration buffer), respectively.

2.3.3 SDS-PAGE

1. Resolving gel buffer 5×: 1.875 M Tris–HCl, pH 8.8. Add about 200 mL of water to a graduated 1-L cylinder or a glass beaker. Weigh out 227.1 g of Tris and add water to a volume of 900 mL. Mix and adjust the pH with concentrated HCl. Make up to 1 L with water and store at 4 °C in a glass bottle.
2. 40% acrylamide/Bis solution (39:1 acrylamide/Bis): To prepare 1 L of stock solution weigh out 390 g of acrylamide monomer and 10 g of Bis (cross-linker) and dissolve in a graduated cylinder with about 400 mL of water. Mix and

make up to 1 L with water. Store at 4 °C in a glass bottle (*see* **Note 8**).

3. 10% ammonium persulfate (APS): Prepare aliquots of 1 mL and store at −20 °C.
4. *N,N,N,N′*-tetramethyl-ethylenediamine (TEMED). Store at 4 °C.
5. SDS-PAGE running buffer (10×): 250 mM Tris, 1.92 M glycine and 1% SDS. For a final volume of 1 L, weigh out 30.2 g of Tris and 144.2 g of glycine and dissolve in about 600 mL of water. Mix, filter the solution, add 10 g of SDS and make up to 1 L with water. Store at 4 °C in a glass bottle.
6. 30% (v/v) isopropanol. Store at room temperature in a dark glass bottle.
7. 0.5% agarose sealing solution: For a final volume of 100 mL, weigh out 0.5 g of agarose, add 100 mL of 1× SDS-PAGE running buffer and melt in a heating block or boiling water (*see* **Note 9**).
8. Low-fluorescence glass plates (GE Healthcare).
9. Gel casting cassettes.
10. Electrophoretic unit.

2.4 Image Acquisition and Analysis

1. Typhoon variable mode imager (GE Healthcare) or similar.
2. Any one of the commercially available DIGE image analysis software packages (e.g., DeCyder from GE Healthcare, Progenesis samespots from Nonlinear Dynamics, Melanie from Bio-Rad) developed for spot detection, matching and differential protein expression analysis.

   The most important steps involved in image analysis are:

   - Spot detection.
   - Background subtraction.
   - In-gel normalization.
   - Artifacts removal.
   - Gel matching.
   - Statistical analysis.

3 Methods

3.1 Protein Extraction

1. To perform a 2-D DIGE experiment involving multiple samples, we suggest to determine the protein extraction yield prior to defining the experimental plan. In our experience, in the case of muscle and heart tissues, at least 10–15 mg are required

since the protein yield is around 1 mg/10 mg, but other tissues (e.g., aorta or tendon) provide lower protein amount.

2. Grind the frozen tissue in a dry ice cooled mortar, then apply the sample homogenization method of choice (*see* **Note 1**).
3. After sample disruption, add lysis buffer and sonicate the sample on ice (20s–bursts, with 60s–rest between bursts) until the particulate is completely dissolved, or until a plateau of particulate solubilization is reached.
4. Centrifuge the sample at 12,000 × *g* for 5 min at 4 °C to remove any insoluble material.
5. Precipitate proteins in order to separate them from non-protein impurities (salts, nucleic acids, lipids, etc.).
6. Resuspend pellets with lysis buffer for DIGE.
7. Check the pH of the sample on ice. The sample pH must be pH 8.0–9.0. Adjust the pH using dilute NaOH, if required.
8. Accurately quantify protein samples.

3.2 Protein Labeling

1. Leave CyDye to warm for 5 min at room temperature before use.
2. After 5 min, add the specified volume of DMF to each vial of CyDye to prepare a CyDye stock solution (i.e., 5 μL of DMF for the 5 nmol pack size). The stock solution is stable for 3 months at −20 °C.
3. Vortex vigorously for 30 s.
4. Centrifuge the vial for 30 s at 12,000 × *g* in a microcentrifuge.
5. Prepare the CyDye working solution adding 1 volume of CyDye stock solution to 1.5 volumes of high-grade DMF to make 400 μM CyDye solution. For example, take 2 μL CyDye stock solution and add 3 μL DMF to give 400 pmol of CyDye in 1 μL. The CyDye fluor working solution is stable for 1 week at −20 °C.
6. Label 50 μg of each sample with 400 pmol of dye by adding 1 μL working solution.
7. Carry out the labeling reaction by incubating the sample on ice for 30 min in the dark.
8. Add 1 μL (for 50 μg of sample labeled) of 10 mM lysine solution to stop the reaction. Mix by pipetting and spin briefly in a microcentrifuge.
9. Leave for 10 min on ice in the dark.
10. After labeling, samples can be stored at −70 °C for at least 3 months.

3.3 Loading Samples onto IPG Strips

1. Add to the CyDye-labeled samples an equal volume of 2× sample buffer and leave on ice for 10 min.
2. Combine the differentially labeled samples (two or three, *see* Fig. 2) into a microfuge tube and mix. One of these samples should be the internal standard (*see* **Note 10**).
3. Add to the samples the appropriate volume of rehydration solution (*see* **Note 11**).
4. Pipette the appropriate volume of rehydration solution into each holder removing any larger bubbles (*see* **Note 12**).
5. Remove the protective cover from the IPG strip and position the strip with the gel upside down. Be careful not to trap bubbles under the IPG strip.
6. Apply the appropriate volume of IPG cover fluid on the strip (*see* **Note 13**) to minimize evaporation and urea crystallization.
7. Allow the IPG strip to rehydrate for a minimum of 10 h (*see* **Note 14**).

3.4 First Dimension (IEF)

1. Run the appropriate IEF protocol (*see* **Note 15**).
2. If the IPG strips are not run immediately on the second dimension, they can be stored at −80 °C in a sealed container. Do not equilibrate strips prior to storage.

3.5 IPG Strip Equilibration

1. Separate the equilibration buffer into two aliquots and add 65 mM DTT (100 mg per 10 mL equilibration buffer) and 135 mM iodoacetamide (250 mg per 10 mL equilibration buffer) just prior to use.
2. Equilibrate strips for 15 min in the first solution (DTT) followed by equilibration in the second solution (iodoacetamide) for 8 min.
3. After equilibration, place the strips on filter paper moistened with deionized water.

3.6 Second Dimension (SDS-PAGE)

1. Assemble the gel cassette.
2. Prepare a sufficient volume of gel solution without adding 10% (w/v) APS and TEMED. A commonly used second dimension gel for 2-DE is a homogeneous gel containing 12% total acrylamide (*see* **Note 16**).
3. Degas the gel solution for 10–15 min.
4. Add 10% APS solution and TEMED to the gel solution in order to make a 0.5% (v/v) APS/0.03% TEMED gel solution.
5. Pour the gel solution into the gel cassette and pipette 1–1.5 mL of 30% (v/v) isopropanol solution on top.
6. Allow gels to polymerize for at least 1–2 h.

7. Disassemble the gel cassette.
8. Prepare the appropriate volume of the 1× running buffer by diluting the 10X SDS-PAGE running buffer.
9. Melt the agarose sealing solution and, for each Immobiline DryStrip, slowly pipette the solution up to the top of the glass plate (*see* **Note 17**).
10. Carefully place the Immobiline DryStrip between the two glass plates holding one end of the strip with forceps. Using a thin plastic spacer, push the strip until it comes in contact with the surface of the gel avoiding the trapping of air bubbles (*see* **Note 18**).
11. Insert the glass plates into the electrophoresis apparatus and start the run (*see* **Note 19**).
12. At the end of the run the gels are ready to be scanned.

3.7 Image Acquisition

1. After electrophoresis, take the images of the gel at the appropriate wavelength using a laser-based imager such as a Typhoon variable mode scanner (GE Healthcare) [24] (*see* **Note 20**) or alternatively images can be taken using a scanning CCD camera [25–27].
2. Rinse the gel sandwich with distilled water and wipe it dry with lint-free towels.
3. Place the gel sandwich on the scanner platen and take the images according to the manufacturer's instructions.

3.8 Image Analysis

Here, we describe the principal steps of image analysis performed using the DeCyder software [14, 17, 24, 28, 29].

1. Upload gel images into the DeCyder database using the Image Loader module. Within this module it is possible to create a project file and to edit (e.g., cropping, printing, flipping, and rotating) gel images.
2. For each gel, create a workspace in the DIA (Differential In-gel Analysis) module.
3. Perform protein spot detection and quantification on images derived from the same gel by selecting the *Process Gels* function. A set of images is merged together thereby incorporating all spot features in a single image (*see* **Note 21**).
4. Use the *Exclude Filter* function in order to eliminate non-proteinaceous spots (based on their physical characteristics). Numerical data for individual spots are calculated (volume, area, peak height, and slope). The filter excludes spots on the basis of these values.

5. Background subtraction is performed automatically. Spot volumes are always expressed with the background subtracted (*see* **Note 22**).
6. Save DIA workspaces. These files contain all the data associated with the loaded spot maps.
7. Open the BVA (Biological Variation Analysis) module and create a BVA workspace. BVA matches multiple images from different gels to provide statistical data on differential protein expression levels between multiple groups.
8. Import all the DIA workspaces composing your project.
9. Select a Master Gel among the uploaded images. All gel images will be matched to the Master Gel before data analysis (*see* **Note 23**).
10. Perform gel matching by selecting the *Process: Match* function and then checking the *Match All* box (*see* **Note 24**).
11. Assign the experimental group to gel images (e.g., control and treated groups, different time points, conditions, etc.) in the *Experimental Design View* of the Spot Map Mode, this facilitate the inter-group statistical analyses. To add a group, click on the *Add* button, choose a name and a description, select a color and confirm the choice. Drag and drop gel images to the appropriate group folder.
12. Save the BVA workspace and open the EDA (Extended Data Analysis) module.
13. Import the BVA workspace in EDA to perform both univariate and multivariate statistical analyses of protein expression.
14. Create a Base Set in the *Setup* page by removing unassigned gel images and spots with too many missing values, as they can affect the results.
15. Perform *Differential Expression Analysis* by accessing the *Calculation* page. This function permits to investigate differential expression between two or more experimental groups. There are different sub-analyses related to the experimental setup. Student's t-test is used to evaluate the hypothesis of a difference between two groups. If more than two groups are included into the experimental design ANOVA must be used (*see* **Note 25**). Visualize results in the *Results* page.
16. From the *Calculation* page, select *Principal Component Analysis (PCA)*. This analysis is essentially a method for reducing the dimension of the variables in a multidimensional space getting a simpler view of the proteins and the spot maps in the data set. It is thus possible to detect outliers in the data set and to identify spot maps that have similar expression profiles.
17. Select *Pattern Analysis*. This process consists of algorithms that can help to find the subsets of the data that show similar

expression patterns. There are different types of pattern algorithms, one of the most important is *Hierarchical Clustering*, a method that combines or splits data pairwise and generates a tree-like structure called dendrogram. Protein and spot maps with similar expression profiles are grouped together.

18. Save EDA workspace to complete the analysis.

3.9 Troubleshooting Guide

In Table 1, the main troubles that can occur when running a 2-D DIGE experiment and how they can be bypassed are described.

Table 1
Troubleshooting table

Problem	Possible reason	Solution
No spots or reduced spot number	Sample load is insufficient	Re-check sample concentration.
	The sample does not migrate completely into the IPG strip	Adjust the amounts of detergent, reducing agent, and ampholytes in the rehydration buffer to favor sample solubilization. Check that the IPG strip is correctly placed on the isoelectric focusing unit.
	Low pH prior to labeling	Check pH, it should be 8.5 immediately prior to labeling. If necessary, increase pH using higher pH lysis buffer containing 30 mM Tris (pH 9.0–10.0) or use 50 mM NaOH.
	Presence of molecules that compete for dye	Thiol agents (e.g., DTT) and primary amines (e.g., ampholytes) present in sample during labeling compete for CyDye DIGE fluor minimal dye. Dilute protein lysate with thiol-/amine-free lysis buffer. Clean sample by precipitation, or increase the amount of dye in the labeling reaction.
Horizontal streaking on gels	Proteins remain at the origin of the 1st dimension	Modify sample preparation protocols. Adjust concentrations of urea, detergents, ampholytes, and reducing agent.
	Protein overload	Decrease the sample amount. Extend focusing time by prolonging initial low-voltage steps.
	Nucleic acids are bound to proteins	Treat samples with an endonuclease to reduce viscosity and increase protein uptake into the IPG strip.
	Over or Under-focusing	Reduce or prolong focusing time.
	Ionic impurities in the sample	Desalt/dilute the sample so that salts are less than 10 mM. Protein precipitation may be useful. If the sample cannot be modified, reduce the effect of ionic impurities by limiting the IEF voltage to 100–150 V for 2 h. This allows ions to move to the ends of the IPG strip. Then resume a normal voltage step program.
	Ionic detergent in the sample.	If SDS is used in sample preparation, its concentration into the reydration solution must not exceed 0.25%. Ensure that the nonionic detergent present must be at least eight times higher than the concentration of any ionic detergent to remove SDS from proteins.

(continued)

Table 1
(continued)

Problem	Possible reason	Solution
Vertical streaking on gel	Pinpoint streaks	Presence of particulate material in gel. Use purified water for all buffers. Wash glass plates.
		Excessive DTT. Reduce concentration of DTT to 50 mM or less.
	Broad streaks connected to spots	Possible protein aggregation due to incomplete reduction/alkylation. Use more reducing agent in buffer. More SDS for equilibration (0.1% w/v) or increasing equilibration time may also help.
Vertical gaps in gel	Air bubbles	Ensure there are no air bubbles between the agarose and polyacrylamide gel when applying the IPG strip to the gel.
	High salt concentration	Desalt/dilute the samples before sample preparation for 2-DE. Protein precipitation may be useful.
	IPG strip not fully rehydrated	Ensure that the IPG strips are rehydrated with sufficient volume of rehydration solution. Check that the rehydration solution is evenly spread along the entire length of the IPG strip.
Individual spots seen as multiple spots	Protein carbamylation	Do not heat the sample once dissolved in rehydration sample buffer. Urea containing solutions should not be heated above 37 °C.
	Spots vertically twinned	Incorrect IPG strip placement on the 2nd dimension gel. IPG strips need to be placed with their plastic backing against the glass plate.
Distortion of spots	Top surface of second dimension is not flat	Immediately overlay gel with water-saturated isopropanol after casting.
	Incomplete/too rapid polymerization	Degas solutions before casting. Increase or decrease the concentration of TEMED/APS to accelerate or slow down polymerization.

4 Notes

1. The tissue should be disrupted and kept at low temperature to minimize heat generation and proteolysis. There are different disruption methods, both mechanical and chemical [30, 31]. Gentle lysis methods (osmotic lysis [32], freeze–thaw lysis [30, 33, 34], enzymatic lysis [35, 36]) are generally adopted when the sample of interest is easily soluble. Vigorous lysis methods, like sonication [37, 38], grinding [39–41], mechanical homogenization [33, 42], glass bead homogenization [43], are adopted to break solid tissue or cells with tough cell walls.
2. Appropriate sample preparation is a key element for 2D-DIGE results. The optimal procedure for each sample type is usually empirically established. Ideally, the process results in a

complete solubilization, disaggregation, denaturation, and reduction of proteins. Since CyDye DIGE fluors minimal dyes label primary amines, it should be reminded that DTT and ampholytes, being exogenous sources of amines, must be avoided prior labeling.

3. Sonicate samples in short bursts to counteract heating. Cool on ice between bursts.

   Sonication is complete when the solution appears significantly less cloudy than the starting solution.
4. Protein precipitation is utilized to selectively separate proteins of interest from contaminating substances. Current methods include TCA precipitation, acetone precipitation, TCA combined with acetone precipitation, etc., and have several disadvantages like incomplete precipitation, difficult protein resuspension, introduction of ions that can interfere with IEF. Kits specifically designed for protein precipitation for 2-DE are commercially available (e.g., 2-D Clean-Up kit from GE Healthcare).
5. Electrophoretic analysis of protein samples requires accurate quantification of the sample to be analyzed. Use a standard protein quantitation method or, alternatively, use a kit designed specifically for the accurate determination of protein concentration in samples to be analyzed by 2-DE (e.g., PlusOne™ 2-D Quant kit from GE Healthcare). The recommended concentration of the protein lysate required for minimal labeling is between 5 and 10 mg/mL.
6. Store CyDyes in the dark at −20 °C.
7. The quality of the DMF is critical to ensure a successful protein labeling. Anhydrous DMF must be used to overcome water contamination thus new, never opened, bottles are suggested since amines, produced by old DMF, react with the CyDyes reducing the concentration of fluors available for protein labeling.
8. Acrylamide is a neurotoxin; it is important to wear gloves and use appropriate handling precautions.
9. Prepare the agarose sealing solution during equilibration.
10. Two kinds of experimental design can be performed: the two- or three-color experiment. In the two-color experiment, samples are labeled with the same fluor (usually Cy5) and the internal standard with Cy3 (the internal standard is created by pooling an aliquot of all samples and it is labeled with one of the CyDye fluors). In the second case, all samples are labeled with two fluors (Cy3 and Cy5), enabling dye swapping to control any dye-specific effects that might result from preferential labeling or different fluorescence characteristics of the

gel at the different excitation wavelengths. Here the internal standard is labeled with Cy2. The major difference between the two experimental designs is represented by the number of gels which is higher in a two-color experiment.

11. Just prior to use add to the rehydration stock solution the appropriate amount of IPG Buffer (0.5% v/v), 65 mM DTT, a trace of Bromophenol blue and the samples. The volume of the rehydration solution depends on the IPG strips' length (i.e., for 24-cm IPG strips the rehydration solution volume required per strip is 450 μL). Make sure the IPG buffer matches the pH gradient of the strip used for IEF.
12. Select the strip holders corresponding to the IPG strip length. Wash each holder with detergent to remove residual protein and rinse with distilled water. Before use the holders must be carefully dried.
13. The IPG cover fluid volume, to be applied on the strip, depends on the length of the holder used. Add the oil until the entire IPG strip is covered.
14. It is possible to rehydrate strips in absence of protein samples. In this case protein samples will be loaded after rehydration, using a cup loading technique.
15. A typical IEF protocol generally proceeds through a series of voltage steps that begins at a relatively low value. Voltage is gradually increased to the final desired focusing voltage, which is held for up to several hours. A low initial voltage minimizes sample aggregation. It is important to remember that focusing parameters for different pH gradients and different protein loading need to be optimized. A typical protocol for a 24 cm, pH 3–10 IPG strip consist in 12 h rehydration followed by a 200 V step for 2 h, 500 V for 2 h, 1000 V for 2 h, 2000 V for 1½ h, 3000 V for 1½ h, a gradient 3000–8000 V for 5 h and then maintaining 8000 V for 2 h.
16. Single percentage gels offer better resolution for a particular MW window. When a gradient gel is used the separation interval is wider and spots are sharper because the decreasing pore size functions to minimize diffusion. Remember that stacking gels are not necessary for 2D gels.
17. Take care not to introduce bubbles and do not allow the agarose to cool or solidify before placing the strip.
18. By convention, the acidic end of the strip is placed on the left.
19. Recommended running condition:
    - 16 h overnight run, 15 °C (2 W per gel).
    - 8 h duration, 15 °C (4 W per gel).
    - 4 h duration, 15 °C (8 W per gel).

Table 2
Excitation and emission characteristics for the three common DIGE fluors

Fluorescent dye	Laser excitation source (nm)	Emission filter (nm)
DIGE fluor Cy3 minimal dye	Green (532)	580 BP 30
DIGE fluor Cy5 minimal dye	Red (633)	670 BP 30
DIGE fluor Cy2 minimal dye	Blue2 (488)	520 BP 40

The run is finished when the bromophenol blue dye front reaches the bottom of the gel.

20. The excitation and emission wavelength used for all three CyDyes are listed in Table 2.
21. An estimation of the number of spots present on the images must be entered—it is recommended that this value is overestimated to compensate for the detection of non-proteinaceous spots on the image.
22. Background is subtracted by excluding the lowest 10th percentile pixel value on the spot boundary from all other pixel values within the spot boundary. The spot volume is the summation of these corrected values.
23. When creating a BVA workspace, the image containing the largest number of spots will be automatically set as the Master Gel. The Master Gel function can be assigned to a different image by selecting the new image and by checking the *Master (M)* box in the *Spot Map Functions* area.
24. To optimize gel matching, landmarks can be set in gel regions where matching has been unsuccessful by selecting a clearly defined spot in all the internal standard images and by clicking the *Add Match* (when all selected spots are unmatched) or the *Break + Add* (when some spots are wrongly matched) buttons. When landmarking is complete, rematch the gels.
25. The simplest form of ANOVA is known as one-way ANOVA and it is used to test for differences in standardized abundance among experimental groups, the test is accompanied by *Tukey's* test (post hoc test) to get an indication of which group is different. The two-way ANOVA is used to analyze two conditions in an experimental design in which two independent factors are taken into consideration. It calculates the significance of the difference between groups with the same condition 2 and different condition 1 (indicated as two-way ANOVA Condition 1) and the other way around (two-way ANOVA Condition 2). The two-way ANOVA analysis also calculates a significance value of the mutual effect of the two factors (two-way ANOVA interaction). Significantly changed proteins having a p-value <0.01 are typically considered.

Acknowledgements

This work was supported by the Telethon foundation (grant N. GGP08107D to C.G.), EU community (grant BIO-NMD N. 241665 to C.G.) and Italian Ministry of University and Scientific Research (grant FIRB RBRN07BMCT to C.G. and PRIN 2015FBNB5Y).

References

1. Lee JM, Kohn EC (2010) Proteomics as a guiding tool for more effective personalized therapy. Ann Oncol 21(Suppl 7):vii205–vii210. doi:10.1093/annonc/mdq375
2. Pal R, Alves G, Larsen JP, Moller SG (2014) New insight into neurodegeneration: the role of proteomics. Mol Neurobiol 49 (3):1181–1199. doi:10.1007/s12035-013-8590-8
3. Huang Z, Ma L, Huang C, Li Q, Nice EC (2016) Proteomic profiling of human plasma for cancer biomarker discovery. Proteomics 17 (6). doi:10.1002/pmic.201600240
4. Kim EY, Kim WK, Oh KJ, Han BS, Lee SC, Bae KH (2015) Recent advances in proteomic studies of adipose tissues and adipocytes. Int J Mol Sci 16(3):4581–4599. doi:10.3390/ijms16034581
5. Pagel O, Loroch S, Sickmann A, Zahedi RP (2015) Current strategies and findings in clinically relevant post-translational modification-specific proteomics. Expert Rev Proteomics 12(3):235–253. doi:10.1586/14789450.2015.1042867
6. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077. doi:10.1002/elps.1150181133
7. Garrels JI (1979) Two dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J Biol Chem 254(16):7961–7977
8. Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26(3):231–243
9. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250(10):4007–4021
10. Blundon MA, Schlesinger DR, Parthasarathy A, Smith SL, Kolev HM, Vinson DA, Kunttas-Tatli E, McCartney BM, Minden JS (2016) Proteomic analysis reveals APC-dependent post-translational modifications and identifies a novel regulator of beta-catenin. Development 143(14):2629–2640. doi:10.1242/dev.130567
11. Burnham-Marusich AR, Plechaty AM, Berninsone PM (2014) Size-matched alkyne-conjugated cyanine fluorophores to identify differences in protein glycosylation. Electrophoresis 35(18):2621–2625. doi:10.1002/elps.201400241
12. Qu Z, Meng F, Zhou H, Li J, Wang Q, Wei F, Cheng J, Greenlief CM, Lubahn DB, Sun GY, Liu S, Gu Z (2014) NitroDIGE analysis reveals inhibition of protein S-nitrosylation by epigallocatechin gallates in lipopolysaccharide-stimulated microglial cells. J Neuroinflammation 11:17. doi:10.1186/1742-2094-11-17
13. Albrethsen J, Miller LM, Novikoff PM, Angeletti RH (2011) Gel-based proteomics of liver cancer progression in rat. Biochim Biophys Acta 1814(10):1367–1376. doi:10.1016/j.bbapap.2011.05.018
14. Gharbi S, Gaffney P, Yang A, Zvelebil MJ, Cramer R, Waterfield MD, Timms JF (2002) Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. Mol Cell Proteomics 1(2):91–98
15. Zhou G, Li H, DeCamp D, Chen S, Shu H, Gong Y, Flaig M, Gillespie JW, Hu N, Taylor PR, Emmert-Buck MR, Liotta LA, Petricoin EF 3rd, Zhao Y (2002) 2D differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. Mol Cell Proteomics 1(2):117–124
16. Swatton JE, Prabakaran S, Karp NA, Lilley KS, Bahn S (2004) Protein profiling of human postmortem brain using 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Mol Psychiatry 9(2):128–143. doi:10.1038/sj.mp.4001475. 4001475 [pii]
17. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional

difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3 (1):36–44. doi:10.1002/pmic.200390006

18. Knowles MR, Cervino S, Skynner HA, Hunt SP, de Felipe C, Salim K, Meneses-Lorente G, McAllister G, Guest PC (2003) Multiplex proteomic analysis by two-dimensional differential in-gel electrophoresis. Proteomics 3 (7):1162–1171. doi:10.1002/pmic.200300437
19. Marouga R, David S, Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382(3):669–678. doi:10.1007/s00216-005-3126-3
20. Rabilloud T, Chevallet M, Luche S, Lelong C (2010) Two-dimensional gel electrophoresis in proteomics: past, present and future. J Proteomics 73(11):2064–2077. doi:10.1016/j.jprot.2010.05.016
21. Gorg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4 (12):3665–3685. doi:10.1002/pmic.200401031
22. Righetti PG, Gelfi C, Chiari M (1996) Isoelectric focusing in immobilized pH gradients. Methods Enzymol 270:235–255
23. Righetti PG, Gelfi C (1997) Electrophoresis gel media: the state of the art. J Chromatogr B Biomed Sci Appl 699(1–2):63–75
24. Yan JX, Devenish AT, Wait R, Stone T, Lewis S, Fowler S (2002) Fluorescence two-dimensional difference gel electrophoresis and mass spectrometry based proteomic analysis of *Escherichia coli*. Proteomics 2(12):1682–1698. doi:10.1002/1615-9861(200212)2:12<1682::AID-PROT1682>3.0.CO;2-Y
25. Dominguez-Santos R, Kosalkova K, Garcia-Estrada C, Barreiro C, Ibanez A, Morales A, Martin JF (2017) Casein phosphopeptides and CaCl2 increase penicillin production and cause an increment in microbody/peroxisome proteins in *Penicillium chrysogenum*. J Proteomics 156:52–62. doi:10.1016/j.jprot.2016.12.021
26. Dautel F, Kalkhof S, Trump S, Michaelson J, Beyer A, Lehmann I, von Bergen M (2011) DIGE-based protein expression analysis of B [a]P-exposed hepatoma cells reveals a complex stress response including alterations in oxidative stress, cell cycle control, and cytoskeleton motility at toxic and subacute concentrations. J Proteome Res 10(2):379–393. doi:10.1021/pr100723d
27. Lim LC, Looi ML, Zakaria SZ, Sagap I, Rose IM, Chin SF, Jamal R (2016) Identification of differentially expressed proteins in the serum of colorectal cancer patients using 2D-DIGE proteomics analysis. Pathol Oncol Res 22 (1):169–177. doi:10.1007/s12253-015-9991-y
28. Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, Caprioli RM (2004) Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 4(3):793–811. doi:10.1002/pmic.200300635
29. Gade D, Thiermann J, Markowsky D, Rabus R (2003) Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp. strain 1. J Mol Microbiol Biotechnol 5(4):240–251. doi:10.1159/000071076. MMB2003005004240 [pii]
30. Bollag D, Edelstein SJ (1991) Protein extraction. In: Protein methods. Wiley-Liss, New York
31. Scopes R (1987) Making an extract. In: Protein purification: principles and practice, 2nd edn. Springer Verlag, New York
32. Pennington SR, Wilkins MR, Hochstrasser DF, Dunn MJ (1997) Proteome analysis: from protein characterization to biological function. Trends Cell Biol 7(4):168–173. doi:10.1016/S0962-8924(97)01033-7. S0962-8924(97)01033-7 [pii]
33. Lenstra JA, Bloemendal H (1983) Topography of the total protein population from cultured cells upon fractionation by chemical extractions. Eur J Biochem 135(3):413–423
34. Toda T, Ishijima Y, Matsushita H, Yoshida M, Kimura N (1994) Detection of thymopoietin-responsive proteins in nude mouse spleen cells by two-dimensional polyacrylamide gel electrophoresis and image processing. Electrophoresis 15(7):984–987
35. Cull M, McHenry CS (1990) Preparation of extracts from prokaryotes. Methods Enzymol 182:147–153
36. Jazwinski SM (1990) Preparation of extracts from yeast. Methods Enzymol 182:154–174
37. Kawaguchi S, Kuramitsu S (1995) Separation of heat-stable proteins from Thermus thermophilus HB8 by two-dimensional electrophoresis. Electrophoresis 16(6):1060–1066
38. Teixeira-Gomes AP, Cloeckaert A, Bezard G, Dubray G, Zygmunt MS (1997) Mapping and identification of *Brucella melitensis* proteins by two-dimensional electrophoresis and microsequencing. Electrophoresis 18(1):156–162. doi:10.1002/elps.1150180128
39. Gorg A, Boguth G, Obermaier C, Posch A, Weiss W (1995) Two-dimensional polyacrylamide gel electrophoresis with immobilized

pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. Electrophoresis 16(7):1079–1086

40. Gorg A, Postel W, Domscheit A, Gunther S (1988) Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (Hordeum vulgare): method, reproducibility and genetic aspects. Electrophoresis 9 (11):681–692. doi:10.1002/elps.1150091103
41. Gorg A, Postel W, Gunther S (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9(9):531–546. doi:10.1002/elps.1150090913
42. Dignam JD (1990) Preparation of extracts from higher eukaryotes. Methods Enzymol 182:194–203
43. Blomberg A, Blomberg L, Norbeck J, Fey SJ, Larsen PM, Larsen M, Roepstorff P, Degand H, Boutry M, Posch A et al (1995) Interlaboratory reproducibility of yeast protein patterns analyzed by immobilized pH gradient two-dimensional gel electrophoresis. Electrophoresis 16(10):1935–1945

Chapter 12

DIGE Analysis of Animal Tissues

Alessio Di Luca, Ruth Hamill, Anne Maria Mullen, and Giuliano Elia

Abstract

Two-dimensional difference gel electrophoresis (2D-DIGE) is an acrylamide gel electrophoresis-based technique for protein separation and quantification in complex mixtures. The technique addresses some of the drawbacks of conventional 2D polyacrylamide gel electrophoresis (2D-PAGE), offering improved sensitivity, more limited experimental variation and accurate within-gel matching. DIGE is based on direct labeling of proteins with isobaric fluorescent dyes (known as CyDyes: Cy2, Cy3, and Cy5) prior to isoelectric focusing (IEF). Here, up to two samples and a reference pool (internal standard) can be mixed and loaded onto IEF for first dimension prior to SDS-PAGE separation in the second dimension. After the electrophoretic run, the gel is imaged at the specific excitation wavelength for each dye, in sequence, and gel scans are recorded separately. For each individual protein spot, intensities recorded at the different wavelengths are integrated and the ratio between volumes normalized to that of the internal standard. This provides an immediate appreciation of protein amount variations under the different conditions tested. In addition, proteins of interest can still be excised and identified with conventional mass spectrometry techniques and further analyzed by other biochemical methods. In this chapter, we describe the application of this methodology to separation and quantitation of proteins mixtures from porcine muscle exudate, collected following centrifugation of muscle specimens (centrifugal drip) for the characterization of quality parameters of importance in the meat industry.

Key words 2D-DIGE, CyDye DIGE fluor, Internal standard, Isoelectric focusing, SDS-PAGE, Image analysis, Machine learning algorithm, Mass spectrometry, Porcine muscle exudate, Centrifugal drip

1 Introduction

In 1975, O'Farrell [1] developed a technique for the separation of complex protein mixtures by high-resolution two-dimensional electrophoresis (2D-PAGE). In this technique, proteins are first separated according to their isoelectric point in an isoelectric focusing (IEF) experiment in presence of urea, detergents, and 1,4-dithiothreitol (DTT). Then, proteins are further separated according to their molecular mass in a second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and can be quantified by staining with either silver or Coomassie

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_12,

Brilliant Blue [1]. Depending on the pH gradient and dimension of the gels used, 2D-PAGE can resolve more than 5000 proteins simultaneously. Furthermore, this technique provides information about the isoform composition of intact proteins, which reflects changes in post-translational modifications (such as phosphorylation, glycosylation, or limited proteolysis). Moreover, 2D-PAGE also permits the isolation of proteins for further structural analyses by mass spectrometric techniques (e.g., MALDI-TOF MS, ion trapping instruments). Despite these benefits, a major problem of 2D-PAGE has been the limited spatial reproducibility between 2D gels, due to lack of homogeneity in thermal and electric fields, limited dynamic range and gel to gel variability [1, 2]. 2D-difference gel electrophoresis (2D-DIGE) technology circumvents many of these issues [3–5]. Here, one or two samples and a reference pool (internal standard) are labeled using three different isobaric, fluorescent cyanine dyes (CyDyes) before IEF and separation on a single 2D gel. These CyDyes have similar chemical and electrophoretic properties but differ in spectral properties. After excitation and imaging at both wavelengths, the images are overlaid and "subtracted" (normalized), whereby only differences between the samples are visualized. Due to the simultaneous migration of both samples on the same gel, experimental variations in spot positions and protein abundance are eliminated, thus facilitating image analysis. One of these three cyanine dyes is used to label an internal standard, which is run together with all samples on all gels belonging to the same series of experiments. This internal standard is normally obtained by pooling an equimolar amount of proteins extracted from all the samples in the experiment, which are labeled with one of the dyes [2, 3]. To complete the identification of the proteins differentially expressed between the various samples, gel images are captured in a digital format. Many procedures and different instrumentation are available for the acquisition of 2D gel images, including modified scanners, laser densitometers, charge-coupled device (CCD) cameras, and fluorescent and phosphor imagers. The saved images are then subjected to computer-assisted image analysis to reduce the background, match the spots identified between the different samples, and to identify any changes between conditions of interest [2]. Moreover, another advantage of 2D-DIGE technology compared to 2D-PAGE is that it can generate sufficient data to apply discriminatory methods such as machine learning algorithm [6].

Here, we show that using 2D-DIGE it is possible to separate proteins mixtures from porcine muscle exudate, collected following centrifugation (centrifugal drip) [7–9]. A typical example of a 2D-DIGE gel of this type of samples is shown in Fig. 1. Two fluorophores were used, Cy3 dye fluor to label the internal standard and Cy5 dye fluor to label the samples using the 'minimal labeling' technique, in which only a small percentage of the lysine residues

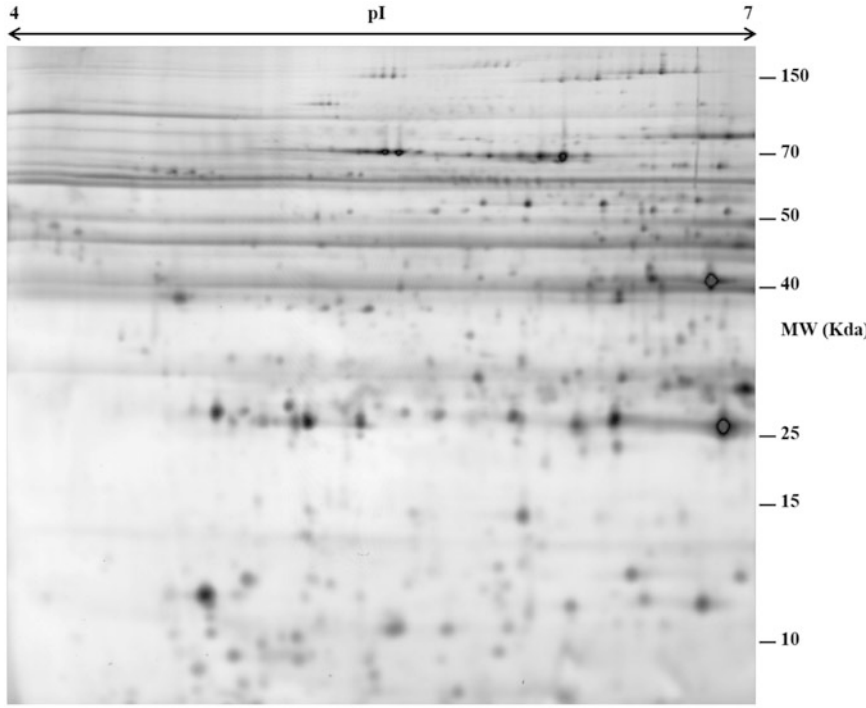

Fig. 1 A typical example of a 2D-DIGE gel of a porcine muscle exudate (centrifugal drip) sample. The image shown was obtained scanning the gel at 100 μm resolution in a Typhoon scanner 9200 (*see* Subheading 3) at the wavelength of 633 nm (*red laser*), specific for the CyDye5. The gradient of p*I* tested is shown above the gel image and the MW marker positions are indicated on the right-hand side

present in the proteins are labeled [10]. 2D-DIGE revealed a total of 376 distinct protein spots in the porcine exudate proteome. Analysis of protein concentration changes in animals of divergent phenotype led to identification of putative markers of meat quality (a hierarchical clustering analysis of these results is shown, as an example, in Fig. 2), important for pork meat industry [6–9].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18.2 MΩ at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated). Diligently follow all waste disposal regulations when disposing waste materials. Moreover, all equipment and materials that come into contact with gel samples must be clean and free of keratin contamination. Minimize handling and manipulations of samples.

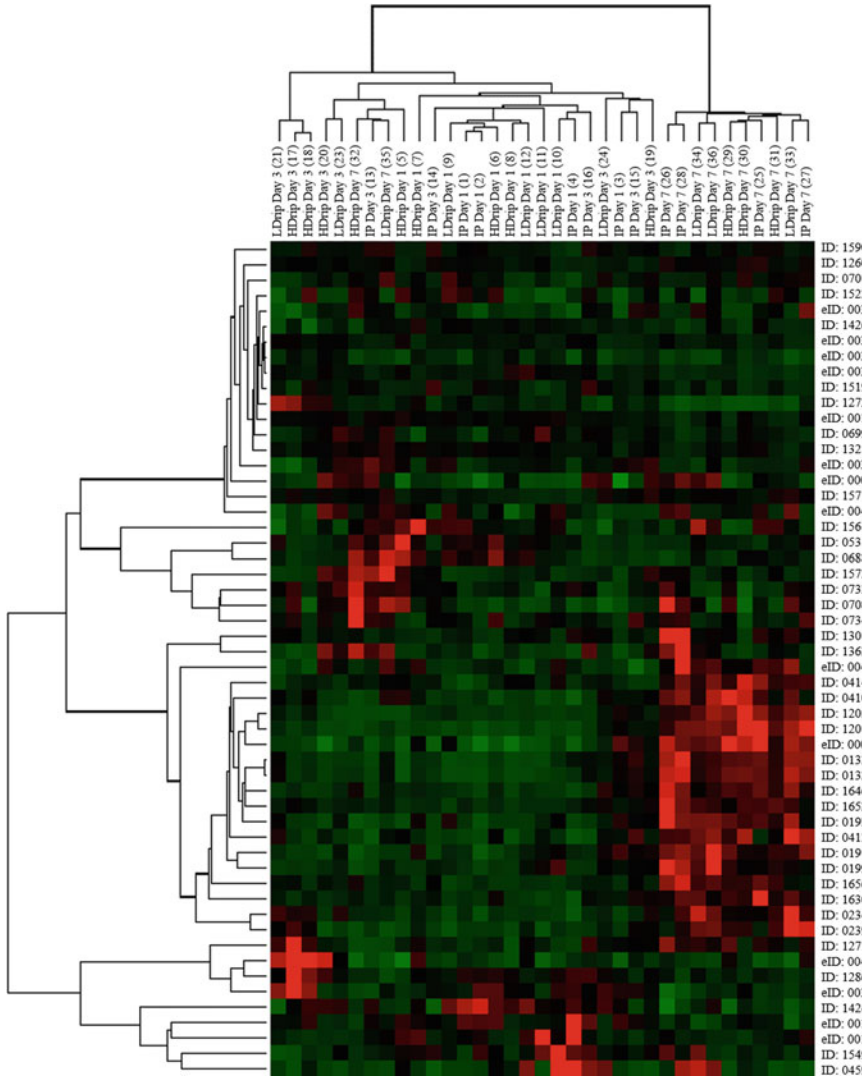

Fig. 2 Clustergram of 53 protein biomarkers identified by 2D-DIGE, showing different abundance pattern of proteins in porcine muscle exudate across days post mortem. HDrip, IP and LDrip indicate animal phenotypes divergent for water holding capacity, a quality parameter important for pork meat industry. A subset of these 53 biomarkers has been shown to discriminate between high- and low-drip samples with high accuracy [6]

2.1 Buffer Recipes for First Dimension Isoelectric Focusing (IEF)

1. DIGE lysis buffer: 9.5 M Urea, 2% CHAPS, pH 8.5 at 4 °C (*see* **Note 1**). Dissolve 28.53 g Urea in approximately 28 ml of water (*see* **Note 2**). Make up to 48 ml with water. Add 0.5 g Amberlite, stir for 10 min and filter under vacuum with Whatman filtration apparatus using 0.25 μm filters. Add 1 g CHAPS and 1 ml 1 M Tris, pH 8.0 to 48 ml of this solution. Mix and adjust pH to 8.5 at 4 °C with HCl if required (*see* **Note 3**). Dispense into microfuge tubes in 1 ml aliquots, label clearly and store in freezer at −20 °C (*see* **Note 4**).

2. 2× samples buffer: 9.5 M Urea, 2% CHAPS, 2% DTT, 1.6% Pharmalyte (*see* **Note 1**). Dissolve 28.53 g Urea in approximately 28 ml of water (*see* **Note 2**). Make up to 48 ml with water. Add 0.5 g Amberlite, stir for 10 min and filter under vacuum with Whatman filtration apparatus using 0.25 μm filters. Add 1 g CHAPS, 1 g DTT, 0.8 ml Pharmalyte pH 3–10 (*see* **Note 5**) to 48 ml of this solution. Make up to 50 ml if necessary and mix. Dispense into microfuge tubes in 1 ml aliquots, label clearly and store in freezer at −20 °C (*see* **Note 4**).
3. Rehydration buffer: 8 M Urea, 0.5% CHAPS, 0.2% DTT, 0.2% Pharmalyte. Dissolve 19.3 g Urea in 25.6 ml of water. Add 0.5 g Amberlite, stir for 10 min and filter under vacuum with Whatman filtration apparatus using 0.25 μm filters. Weigh and add 0.06 g DTT and 0.15 g CHAPS. Add 150 μl Pharmalyte, pH 3–10 and few grains of Bromophenol Blue (*see* **Note 5**). Make up to 40 ml with water, mix and dispense into microfuge tubes in 1 ml aliquots, label and store in freezer at −20 °C (*see* **Note 4**).
4. 1.5 M Tris–HCl solution, pH 8.8. Dissolve 18.171 g Tris in 30 ml of water. Adjust the pH to 8.8 (at 20 °C) with HCl (*see* **Note 6**). Make up to a final volume to 100 ml with water.
5. Equilibration buffer: 6 M Urea, 50 mM TrisCl pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS. Weigh 180 g Urea and combine with 17 ml 1.5 M Tris–HCl solution (pH 8.8) and 176 ml of 85% Glycerol. Make up to 500 ml with water and dissolve completely. Finally, add 10 g SDS and dissolve (*see* **Note 7**). Dispense into Universal Containers in 10 ml and/or 20 ml aliquots and store at −20 °C (*see* **Note 4**).
6. Reducing equilibration buffer: 6 M Urea, 50 mM TrisCl pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS, 1% (w/v) DDT. Gently defrost 20 ml aliquot of equilibration buffer and add 0.2 g DDT (*see* **Note 8**). Mix well (*see* **Note 4**).
7. Alkylation equilibration buffer: 6 M Urea, 50 mM TrisCl pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide (IAA). Gently defrost 20 ml aliquot of equilibration buffer and add 0.5 g IAA (*see* **Note 8**). Mix well (*see* **Note 4**).

2.2 Buffer Recipes for Second Dimension SDS-PAGE

1. Tris-Glycine running buffer (*see* **Notes 9** and **10**): 25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS. 1×, Weigh 6.06 g Tris Base and 28.82 g Glycine and transfer to a beaker. Add in the beaker 1.5 l of water and mix well. Add 2.0 g SDS as last and dissolve (*see* **Note 7**). Transfer to graduated cylinder and bring final volume up to 2 liters, dissolve completely and store in a Duran bottle. 10×, Weigh 60.57 g Tris Base and 288.82 g Glycine

and transfer to a beaker. Add in the beaker 1.5 l of water and mix well. Add 20.0 g SDS as last and dissolve (*see* **Note 7**). Transfer to graduated cylinder and bring final volume up to 2 l, dissolve completely and store in a Duran bottle.

2. Agarose sealing solution: Weigh 1.0 g of Agarose and dissolve in 100 ml of 1× Tris-Glycine running buffer by heating gently in a microwave. Add a few grains of Bromophenol Blue to color. Store in Duran bottle and microwave when required (*see* **Note 11**).
3. Water saturated butanol: Mix 100 ml Butanol and 100 ml of water. Shake and wait until the Butanol and water are separated and then use the top layer.

2.3 Specialist Materials and Equipment for the First and Second Dimension

1. Amersham CyDye DIGE Fluors minimal dyes (Cy3 and Cy5 dye fluors) (e.g., Ettan DIGE, GE Healthcare, UK; other suppliers are available).
2. 99.8% anhydrous dimethylformamide (DFM) (*see* **Note 12**).
3. 10 mM Lysine (to stop the reaction).
4. Immobiline DryStrip gels (IPG strips), pH 4–7 24 cm (*see* **Note 13**).
5. Rehydration tray.
6. Immobiline DryStrip Cover Fluid.
7. Ettan IPG Phor3 Isoelectric Focusing System (e.g., GE Healthcare, UK; other suppliers are available).
8. Protogel (e.g., National Diagnostics, USA; other suppliers are available).
9. Protogel buffer (e.g., National Diagnostics, USA; other suppliers are available).
10. Ammonium persulfate.
11. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).
12. DALT six gels caster (e.g., GE Healthcare, UK; other suppliers are available).
13. GealSeal.
14. PROTEAN Plus Dodeca Cell (e.g., Bio-Rad Hercules, CA, USA; other suppliers are available) and MultiTemp cooling unit (e.g., Amersham Biosciences, Uppsala, Sweden; other suppliers are available).
15. Low-fluorescence glass plate, 3 mm thick.
16. Electrophoresis Power Supplies.
17. Typhoon scanner 9200 (e.g., GE Healthcare, UK; other suppliers are available).
18. Progenesis SameSpots (e.g., Nonlinear Dynamics, Waters, UK; other suppliers are available).

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Please read protocols carefully in advance and make sure all materials and equipment are available before starting the experiment. Moreover, all equipment and materials that come into contact with gel samples must be clean and free of keratin contamination. Minimize handling and manipulations of samples.

3.1 CyDye Reconstitution and Protein Sample Labeling

1. Normalize the samples to a protein concentration of 10 mg/ml with DIGE lysis Buffer (*see* **Notes 14** and **15**).
2. Reconstitute each CyDye (Cy3 and Cy5 dye fluors; on reconstitution in DMF the CyDye will give a deep color; Cy2-yellow, Cy3-red, Cy5-blue) in 99.8% anhydrous DMF to a stock solution of 1 mM according to Amersham instruction. Firstly, take a small volume of DMF from its original container and dispense into a fresh microfuge tube (*see* **Note 12**). Remove the CyDye from the −15 °C to −30 °C freezer and leave to warm for 5 min at room temperature. After 5 min add the specified volume of DMF to each new vial of CyDye to reach a CyDye stock solution of 1 mM (*see* **Note 16**). Replace the cap on the dye microfuge tube and vortex vigorously for 30 s, then centrifuge the microfuge tube for 30 s at 12,000 × *g* in a microcentrifuge prior to use (*see* **Note 17**).
3. From the CyDye stock solution, prepare the CyDye working solution (400 pmol/μl) by dilution with DMF (Amersham instruction). Briefly spin down CyDye stock solution prepared in **step 2**, in a microcentrifuge. Add one volume of CyDye stock solution to 1.5 volumes of high-grade DMF, to make 400 μM CyDye solution. For example, mix 2 μl CyDye stock solution with 3 μl DMF to give 400 pmol CyDye in 1 μl (*see* **Note 18**).
4. Before starting the minimal labeling of protein samples [10], randomize and gently (on ice) defrost both, samples and pool sample. Add 1 μl of diluted CyDye to the microfuge tube containing the protein sample (i.e., 50 μg of protein is labeled with 400 pmol of dye for the labeling reaction). Bulk labeling reactions for the pool sample can also be done using more protein and dye. In our study [8], Cy5 was used for the preparation of the CyDye working solution for the individual samples, whereas Cy3 was used for preparation of the CyDye working solution for the pooled sample (internal standard). Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 min in the dark and then add 1 μl of 10 mM lysine to stop the reaction. Mix by pipetting and spin briefly in a microcentrifuge. Leave for 10 min on ice in the dark. Samples can now be used or stored for up to 3 months at −70 °C in the dark.

3.2 IPG Strip In-Gel Rehydration

1. Before processing the samples that have been CyDye labeled in the first dimension, add an equal volume of 2× sample buffer (e.g., one protein sample labeled with one CyDye is 20 μl, add an equal volume of 2× sample buffer, 20 μl + 20 μl = 40 μl) and then leave on ice for 10 min in the dark. A similar procedure should be used for the pooled sample, and then leave on ice for 10 min in the dark. Add the sample and the pool sample together (40 μl × 2 = 80 μl).
2. Thaw the required volume of rehydration buffer. The total volume required for a 24 cm strip is 450 μl (450 μl−80 μl = 370 μl of rehydration buffer) (*see* **Note 19**). Ensure that the rehydration tray is clean and dry and is level before starting the passive in-gel rehydration.
3. Carefully pipette each sample (prepared above), along the length of an individual groove in the tray (*see* **Note 20**).
4. Remove the protective cover from the IPG strip, this exposes the gel (which is sticky). Carefully place the strip with the gel side facing down onto the sample/rehydration solution (*see* **Note 21**).
5. Overlay each strip with approximately 2.0 ml IPG Cover Fluid to prevent evaporation and urea crystallization.
6. Slide the lid onto the rehydration tray and allow strips to rehydrate for a minimum of 10 h to overnight.

3.3 First Dimension Isoelectric Focusing (IEF) Using IPGphor III with Ceramic Manifold

1. Ensure that IPGphor bed is clean and dry before placing the ceramic manifold onto the unit. Check that the ceramic manifold is level by placing the small circular spirit level on the center of the manifold tray (*see* **Note 22**).
2. Pour Immobiline DryStrip Cover fluid into all channels where the strips are to be run. Additionally, pour cover fluid into the adjacent unused channel at each side of the strips.
3. Place the strips under the cover fluid face up in the tray, with the anodic (+, pointed) end of the IPG strip resting on the dot etched into the bottom of the channel (*see* **Note 23**). Centre the strip in the manifold channel.
4. Separate the appropriate number of paper electrode wicks (two per IPG strip). Wet each wick thoroughly with water and allow excess to drain off. Place one wick at each end of the strip in such a way that the wick overlaps the end of the gel on the strip.
5. Place the electrode assembly on top of all wicks, then swivel the cams into the closed position taking care not to move the electrode.
6. Check that all strips are covered in cover fluid to prevent evaporation and urea crystallization.

7. Close the IPGphor lid. Switch the IPGphor on (the switch is at the back of the unit). Program the IPGphor either from the front of the unit or from the computer.
8. The focusing program used in our study was performed using Ettan IPG Phor3 under the following conditions: S1. 3500 V at 75,000 V h; S2. gradient 8000 V for 10 min; S3. 8000 V for 1 h, S4. holding step at 100 V (*see* **Note 24**). Begin IEF.
9. As isoelectric focusing proceeds, the dye migrates toward the anode (*see* **Note 25**). At the end of the focusing program switch the IPGphor off.
10. When the run is complete if the second dimension cannot be performed directly after IEF, the IPG strips should be immediately stored in gel bags and in a metal cassette, at −80 °C until required.
11. After each use the ceramic manifold must be carefully cleaned with strip cleaning fluid. Rinse thoroughly with distilled water and then deionized water, allow to air dry (*see* **Note 26**).

3.4 Casting Homogenous Gels in DALT Six Gel

1. Before preparing the glass cassettes make sure that all are clean and dry. Prepare the low-fluorescence glass plate and tape along the sides (*see* **Note 27**).
2. Set up the DALT six gel caster in a tray and remove the faceplate.
3. Fill the caster by first placing a separator sheet against the back wall and then alternating gel cassettes with separator sheets. The short plate should be closest to the pouring channel. Finish with a separator sheet and fill any remaining space with blank cassette and thicker spacer sheets.
4. Smear a light coating of GelSeal evenly onto the gasket in the faceplate.
5. Slide the faceplate onto the caster with the bottom (v-shaped) slots resting on their respective screws. Screw these and the remaining two screws into holes at the bottom of the faceplate and tighten all screws evenly.
6. Clamp the faceplate with the six clamps provided.
7. Prepare the monomer solution (in our study 12% SDS-PAGE gel) in a beaker, adding for a solution of about 600 ml, 240 ml of protogel, 156 ml of protogel buffer, 201 ml of water, adding lastly 2.4 ml 10% APS and 240 μl TEMED. Once these have been added polymerization will begin (*see* **Note 27**).
8. Pour the acrylamide solution evenly into the channel at the back of the caster until the level of solution is about 0.5 cm below the level of the shorter glass plate.

9. Layer approximately 1.5 ml of water saturated butanol onto the top of each gel. This can be washed off with water after 1 h.
10. Gels are left to polymerize overnight.
11. On the morning when the gels are to be run dismantle the DALT six gel caster and examine the gels. The gels should be clear with no distortions swirls or air bubbles.

3.5 Equilibration of IPG Gel Strips and Second Dimension SDS-PAGE Gel Loading

1. Prepare the reducing equilibration buffer (*see* **Notes 28** and **29**) by adding DTT to stock equilibration buffer for a final concentration of 1% (w/v) DTT (*see* **Note 8**).
2. Carefully place IPG strips into equilibration tubes with reducing equilibration buffer, one strip per tube (if used). Leave for 15 min minimum, on rocking platform to equilibrate.
3. Prepare the alkylation equilibration buffer by adding IAA to the stock equilibration buffer to a final concentration of 2.5% (w/v) IAA (*see* **Note 8**).
4. Pour off the first equilibration buffer from each tube and replace with alkylation equilibration buffer. Leave strips to equilibrate for 15 min on rocking platform.
5. In the meantime, prepare the agarose sealing solution by melting using a medium setting in the microwave (*see* **Note 11**). Each 1.5 mm gel will require approximately 2 ml of Agarose.
6. Before applying IPG strips to SDS gels, rinse the IPG strip with 1× Tris-Glycine running buffer, and place across the top of the SDS gel between the glass plates with the plastic side of the strip facing the back. The positive end of the strip (+) should be positioned to the left or hinged side of the gel plate (*see* **Note 30**).
7. Pipette the molten (cooled) agarose on top of the IPG strip, making sure that no air bubbles are trapped between the strip and gel. Once the Agarose has set, the plate is ready to run in a separation unit (*see* **Note 31**).

3.6 Running Gels in BioRad Separation Unit

1. Prepare sufficient 1× Tris-Glycine running buffer for the second dimension using PROTEAN Plus Dodeca Cell. This tank can require up to 23 l depending on the number of gels run (*see* **Note 10**).
2. Switch the MultiTemp cooling unit on and set to 15 °C (*see* **Note 32**).
3. Make sure that the Separation Unit (the tank) is level before filling with buffer. Also check that the outlet/waste tap at the rear of the unit is closed.
4. Only half fill the tank with buffer (the rest can be added when all plates and blanks are in place, otherwise you may end up overfilling the tank) and allow the 1× Tris-Glycine running

buffer to equilibrate in the tank for a least 1 h before loading gels.

5. Lubricate all gels and blank cassettes with 1× Tris-Glycine running buffer before inserting into gaskets.
6. Check that gels are in the correct orientation. The hinge side should be at the bottom and the IPG strip to the right side of tank.
7. Make sure that the IPG strips are to the right side of the gaskets (rubber slots into which the glass cassette is placed).
8. Once the gels are stacked, overlay them with 1× Tris-Glycine running buffer which should come up just to the start of the glass spacer (*see* **Note 33**).
9. Place the lid firmly onto the unit and switch the pump on at the top setting (*see* **Note 34**). Cover the tank with a dark blanket to keep gels in the dark.
10. Insert the leads from the lid of the separation tank into the electrophoresis power supply and set the run conditions of the gels as follows: S1. 0.2 W per gel for 1 h; S2. 1 W per gel overnight (*see* **Note 35**); S3. if necessary, the power can be increased the following morning up to 5–7 W per gel. The run is completed when the dye front (which moves in a vertical line from right to left) reaches the end of the gel (*see* **Note 36**).

3.7 Image Analysis

1. Scan directly the gels in the low-fluorescence glass plates after the second dimension to ensure that all gels have the same dimensions, this will simplify spot matching of different gels. The exterior surface of the glass plates must be carefully cleaned with water and dried with a lint-free laboratory wipe before the gel cassette is positioned on the scanner.
2. Each fluorescent dye should be consecutively excited to avoid fluorescence crosstalk and scanned at a final resolution of 100 pixels with a proper filter (*see* **Note 37**).
3. After the scan, the software provides an initial image overlay of the scanned gel, thus giving a quick overview of differences between the labeled extracts. At least three gels of three individual protein extracts per experimental condition should be run, normalized and compared before qualitative and quantitative changes can be considered significant.

4 Notes

1. The standard DIGE lysis buffer must not contain any components, which will compete with the protein for the CyDyes during sample labeling (DTT, protease inhibitors or

ampholytes). The DTT and ampholytes are introduced after the labeling by the addition of the "2× samples buffer" to the labeled sample.

2. This reaction is endothermic and the urea will only dissolve when the solution has reached room temperature as it is close to its maximum solubility (108 g in 100 ml water at 20 °C).
3. The pH of the labeling reaction is also crucial and so the DIGE lysis buffer is adjusted to pH 8.5 at 4 °C (the temperature at which the labeling reaction takes place).
4. Never heat urea solution above 37 °C to avoid protein carbamylation. Do not freeze any thawed buffers containing urea. Make up aliquots, freeze and thaw aliquots as required (discard any remaining thawed material).
5. A broad pH range of Pharmalyte can be used for both the 3–10 range and 4–7 immobilized pH gradient (IPG) strips. However, if a short pH range strips (e.g., 3.5–4.5 or 5–6) are used, the pH range of Pharmalyte should match that of IPG strips.
6. The addition of HCl to the Tris solution will cause a rise in temperature and as dpH/dt for Tris is −0.028 unit x deg-1 any temperature difference must be taken into account.
7. Always weigh and then add SDS in the solution in the fume hood wearing a mask, it may be necessary to turn off the fume hood while weighing the SDS however as soon as SDS is in solution the fume hood should be switched on to remove the SDS.
8. 10 ml of equilibration buffers (reducing or alkylation) is required for each sample/equilibration if tube/IGP strips are used. It is also possible to use 10 ml disposable pipettes for equilibration by snapping off the top and using parafilm to seal both ends (5 ml of the buffer is required) or a rehydration tray (3 ml of the buffer is required) can be used. Prepare these two solutions (adding DDT or IAA to the defrost equilibration buffers) just in advance of use.
9. It is important not to adjust the pH of the Tris-Glycine running buffer. The presence of chloride ions in the running buffer from HCl (as a result of adjusting the solution's pH) will slow down the migration of sample proteins. Proteins are coated in SDS which imparts a negative charge and thus they migrate toward the bottom of the gel. The presence of a negative ion which is smaller and much more efficient at migration than the larger proteins will therefore impede the progress of proteins through the gel matrix.
10. 1× is the dilution of Tris-Glycine running buffer used for running the gels, however as a large volume (up to 23 l depending on number of gels) is required for running the

gels, a less diluted solution (10×) can be prepared which will be diluted with water in the tank (e.g., PROTEAN Plus Dodeca Cell; Bio-Rad Hercules, CA, USA; other suppliers are available).

11. It is important to keep the lid loose and to gently boil only: caution hot material!
12. The DMF should be fresh (less than 3 months old after first opening), because it degrades to amine compounds, which interfere with the labeling reaction.
13. Different pH ranges (e.g., 3–10, 4–5) and strip lengths (e.g., 7, 18 cm) can be used according to the complexity of the specimens and the strategies used for the analysis. A good first-dimension separation ensures that spots in the second dimension are well separated, even with high protein loads.
14. A simple sample preparation is pivotal in 2D-PAGE protocols to ensure a greater reproducibility. The fundamental steps in sample preparation are (1) cell disruption, (2) inactivation or removal of interfering substances, and (3) subsequent solubilization of the proteins [2]. The specimen used in our studies is an exudate collected post mortem from muscle tissue following centrifugation (centrifugal drip [9]). The concentration of the centrifugal drip samples was determined in triplicate according to a modified Bradford assay protocol using a bovine serum albumin (BSA) standard [11].

    The protein concentration should be normalized with DIGE lysis Buffer to a protein concentration between $\geq$1 mg/ml and $\leq$20 mg/ml. Best results are obtained with final protein concentrations between 5 and 10 mg/ml according to Amersham instruction. Check randomly the pH (with litmus paper) of some samples. Indeed, it is essential that the pH of the protein solution used with a CyDye DIGE Fluor is between pH 8.0 and 9.0. A protein pH solution below 8.0 result in little or no protein labeling. Moreover, the protein solution should not contain any added primary amine compounds before labeling. Indeed, primary amines, such as ampholytes, will compete with the proteins for CyDye, resulting in fewer CyDye-labeled proteins, which might affect the data after scanning and spot detection.
15. After all samples have been normalized to a concentration of 10 mg/ml with Standard DIGE Lysis Buffer, it is recommended to prepare aliquots of 50 μg of proteins, which is the amount of proteins that will be labeled with CyDye (a duplicate of each sample can be prepared in the eventuality of any problems). It is recommended to randomize the samples preparation to reduce batch effect. It is also suggested to prepare a pool generated from equal amounts of all the samples that have

been normalized at the concentration of 10 mg/ml with Standard DIGE Lysis Buffer in one Eppendorf that will be used as internal standard with a bulk labeling reaction. It is important to prepare an amount of pool sample that should be more than enough to load in each gel within an experiment. A duplicate of the pool samples can be prepared in the eventuality of any problems. Aliquots and pool samples should be stored in the freezer at −80 °C.

16. Displacement of CyDye during manufacture or shipment of the fluors can be recovered to the bottom of the tube by pipetting the DMF down the side of the tube, vortexing vigorously, and centrifuging.
17. Unused CyDye stock solution should be returned to the freezer (−15 °C to −30 °C) as soon as possible and stored in the dark. The reconstituted CyDye stock solution dyes are stable and usable until the expiry date detailed on the tube or for 2 months, whichever is sooner.
18. Add the DMF first to the microfuge tube, followed by CyDye. 1 μl of the diluted dye now contains 400 pmol, whereas in each tube of CyDye there will be a 1 mM CyDye stock solution.

    Amersham instruction recommended that 50 μg of protein is labeled with 400 pmol of CyDye, however, between 100 pmol and 1000 pmol per 50 μg of protein can be used. If labeling more than 50 μg of protein, then the same fluor to protein ratio must be used for all samples on the same gel.

    The CyDye working solution is only stable for 1 week at −15 °C to −30 °C.
19. The total volume of rehydration buffer used varies with the length of IPG strip, for example 340 μl is loaded onto an 18 cm strip. For other volumes check the booklet which accompanies each pack of strips to see what the correct loading volume should be.
20. Try to avoid air bubbles, however if they occur use a needle to burst them before applying the IPG strip.
21. Dry IPG strip is easily moistened. Take dry IPG strip from freezer just prior use. Do not allow IPG strip set at room temperature more than 10 min. Moreover, try to avoid trapping air bubbles under the strip as this will result in uneven rehydration.
22. The Ettan IPGphor is capable of producing thousands of volts. Before operating familiarize yourself with the operating instructions and warnings of this unit (cf. "Ettan IPGphor Cup Loading Manifold," Amersham Biosciences user manual).
23. The end of the gel and not the end of the plastic should be aligned to this mark.

24. This method of focusing is conducted at high voltages and low currents due to low ionic strength within the strips. During IEF, the current decreases while the voltage increases. A low initial voltage minimizes sample aggregation and allows the parallel separation of samples with differing salt concentrations. The program used is dependent on the size of strip being focused and the quantity of protein on the strip. If the program does not produce optimal focusing for a specific sample, it may need to be adjusted [2].
25. The dye front leaves the IPG strip well before focusing is complete, so clearing of the dye is no indication that the sample is focused.
26. Do not use abrasive cleaning agents or pads. Moreover, the electrode assemblies should be wiped clean with paper.
27. Warning! Acrylamide is a neurotoxin, always wear protective gloves when handling. It is also possible to use a precasted polyacrylamide DIGE gels in a low fluorescent glass cassette rather than prepare home-casted polyacrylamide gels to increase the quality, reproducibility, and safety.
28. Before the second-dimension separation, it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with SDS [2].
29. Before equilibrating IPG strips check that your gels are of usable quality.
30. If the glass plates are slightly wet with 1× Tris-Glycine running buffer, the IPG strip will slide into place more easily. However, it is important to drain off surplus buffer before sealing with agarose otherwise the agarose will not set properly.
31. Embedding in agarose is not absolutely necessary, but it ensures much better contact between the IPG gel strip and the top of the SDS gel.
32. Check that there is sufficient water in this unit.
33. This ensures that all of the gel is under buffer, but be careful to not overfill the tank.
34. The power switch for the pump is at the back, once it is switched on it should be possible to see buffer mixing in the separation tank.
35. This can be programmed into an electrophoresis power supplies.
36. The gels so obtained should be scanned and analyzed as soon as possible. Indeed, it is possible to store the gels at dark and 4 °C for 1 week (sealing the gels with films to avoid dehydration), but it is recommended to scan and analyze the gels within 1 day, trying to use the same parameters between gels. To do so,

it is also important to not run too many gels in the same day, six gels is the optimal number.

37. In our study the DIGE gels were scanned at 100 μm resolution using a Typhoon scanner 9200 (GEHealthcare) at two different wavelengths (CyDye3, green laser 532 nm and CyDye5, red laser 633 nm) [8].

References

1. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250(10):4007–4021
2. Gorg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4(12):3665–3685. doi:10.1002/pmic.200401031
3. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3 (1):36–44. doi:10.1002/pmic.200390006
4. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1(3):377–396. doi:10.1002/1615-9861(200103)1:3<377::AID-PROT377>3.0.CO;2-6
5. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077. doi:10.1002/elps.1150181133
6. Di Luca A, Hamill RM, Mullen AM, Slavov N, Elia G (2016) Comparative proteomic profiling of divergent phenotypes for water holding capacity across the post mortem ageing period in porcine muscle exudate. PLoS One 11(3):e0150605. doi:10.1371/journal.pone.0150605
7. Di Luca A, Elia G, Hamill R, Mullen AM (2013) 2D DIGE proteomic analysis of early post mortem muscle exudate highlights the importance of the stress response for improved water-holding capacity of fresh pork meat. Proteomics 13(9):1528–1544. doi:10.1002/pmic.201200145
8. Di Luca A, Elia G, Mullen AM, Hamill RM (2013) Monitoring post mortem changes in porcine muscle through 2-D DIGE proteome analysis of Longissimus muscle exudate. Proteome Sci 11(1):9. doi:10.1186/1477-5956-11-9
9. Di Luca A, Mullen AM, Elia G, Davey G, Hamill RM (2011) Centrifugal drip is an accessible source for protein indicators of pork ageing and water-holding capacity. Meat Sci 88 (2):261–270. doi:10.1016/j.meatsci.2010.12.033
10. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5(12):3105–3115. doi:10.1002/pmic.200500083
11. Ramagli LS, Rodriguez LV (1985) Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 6(11):559–563. doi:10.1002/elps.1150061109

Chapter 13

Rapid 2D DIGE Proteomic Analysis of Mouse Liver

Shotaro Kamata and Isao Ishii

Abstract

Several years have passed since LC-MS(/MS) became the mainstream for proteomic analysis; however, conventional 2D DIGE (two-dimensional difference gel electrophoresis) continues to be an important technology that enables rapid and direct visualization of hundreds to thousands of proteins and their quantitative analyses. We can get global proteomic views using 2D DIGE within 3 days, and then identify proteins with differential expression levels using MALDI-TOF/MS and MASCOT search engine within a week. Here, we describe our routine 2D DIGE proteomic analysis of the liver isolated from mice in pathological conditions.

Key words 2D DIGE, Isoelectric focusing, Proteomics, MALDI-TOF/MS

1 Introduction

Proteomic analysis that examines large numbers of proteins for their associations with physiological and pathological conditions may contribute to the understanding of molecular mechanisms underlying them. Among several proteomic technologies so far developed, 2D DIGE (two-dimensional difference gel electrophoresis) provides rapid global views of protein expression and important information about changes in p*I*s (isoelectric points) and molecular weights caused by molecular events including posttranslational modification (e.g., phosphorylation), alternative splicing, and degradation. Although conventional 2D DIGE had encountered technical difficulties in consistent fluorescent labeling of proteins and 2D electrophoresis, and thus high levels of gel-to-gel variations and low reproducibility, the introduction of cyanine-based fluorescent labeling, a variety of premade dried strips for isoelectric focusing (IEF), and a pooled internal standard to enable spot matching between the gels using PC software has overcame these limitations [1].

GE Healthcare has been the major supplier of 2D DIGE system reagents and we routinely use their CyDye DIGE Fluor (Cy2, Cy3,

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_13,

Table 1
Characteristics of CyDye Fluors

Fluor	Fluorescence color	Max. absorption wavelength (nm)	Max. fluorescence wavelength (nm)	Molar extinction co-efficient	MW added to protein (Da)
Cy2	Green	491	509	135,000	434
Cy3	Orange	553	569	133,000	466
Cy5	Red	645	664	217,000	464

or Cy5; Table 1) minimal dyes and Immobiline DryStrips. CyDye DIGE Fluors have an NHS ester reactive group that covalently attaches to the lysine ε-amino residue of proteins via an amide linkage. The quantity of dye added to the sample is limiting in the labeling reaction (200 pmol per 25 μg protein), which ensures that the dyes label ~1–2% of the lysine residues and then only a single lysine per protein (irrespective of its molecular size). The three dyes have matched sizes (MW 434–466) and charges (+1) such that the same protein labeled with each CyDye will overlay in 2D electrophoresis gels. Moreover, the lysine carries a +1 charge around neutral/acidic pH and CyDye also carry an intrinsic +1 charge. Therefore, the p*I*s of proteins do not significantly alter by CyDye labeling. GE Healthcare also provides various types of Immobiline DryStrips (various lengths and pH ranges), which enables easy and reproducible separations of targeted proteins by IEF. Here, we present our routine 2D DIGE proteomic approaches to investigate mouse (hepatic) proteins [2, 3] that may be involved in fatty liver formation caused by a methionine/choline-deficient (MCD) diet [4].

2 Materials

Prepare all solutions using ultrapure water and reagents of analytical or molecular biology grade.

2.1 Equipment

1. Micro Smasher MS-100R homogenizing system (TOMY, Tokyo, Japan).
2. Zirconia beads (5-mm diameter, TOMY).
3. 2-mL tube specific for bead homogenization (TOMY).
4. Immobiline DryStrips (pH 3–10 NL, 18 cm, GE Healthcare).
5. CoolPhoreStar IPG-IEF Type-PX system (Anatech, Tokyo, Japan).
6. Low-melting-point agarose (Agarose, low gelling temperature, Sigma-Aldrich).

7. 12.5% SDS-PAGE gel precasted with low-fluorescent glass plates (14 cm × 14 cm, DRC, Tokyo, Japan).
8. ERICA-*S* high-speed electrophoresis system (DRC).
9. Typhoon Trio image scanner (GE Healthcare).
10. DeCyder 2D (Ver. 6.5) Differential Analysis software (GE Healthcare).
11. Silver Stain MS kit (Wako, Tokyo, Japan).
12. μFocus MALDI plate (900 μm, 384 circles, Hudson Surface Technology [Old Tappan, NJ, USA]).
13. AXIMA-CFR plus MALDI-TOF/MS (Shimadzu, Kyoto, Japan).
14. Peptide calibration standards (~1000–3200 Da, Bruker Daltonics).
15. MASCOT Search engine (Matrix Science; http://www.matrixscience.com).

2.2 Buffers and Solutions

2.2.1 Liver Homogenate Preparation and CyDye Labeling

1. Urea buffer: 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS). Weigh 8.40 g urea, 3.04 g thiourea, and 800 mg CHAPS, and transfer to the 50-mL plastic tube. Add water to a volume of 20 mL (*see* **Note 1**).
2. Lysis buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4). Mix 10 mL of Urea buffer, 50 μL of 200 mM PMSF (in methanol), and 10 μL of 1 M Na_3VO_4.
3. Tris-lysis buffer, pH 8.5: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM Tris–HCl, 1 mM PMSF, 1 mM Na_3VO_4. Mix 2 mL of Urea buffer, 80 μL of 1 M Tris–HCl, pH 8.8, 10 μL of 200 mM PMSF, and 2.0 μL of 1 M Na_3VO_4 and adjust pH to 8.5 with concentrated HCl.
4. DTT-lysis buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 130 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM Na_3VO_4, 2% IPG buffer (pH 3–10 NL [non-liner], GE Healthcare). Weigh 20 mg DTT and transfer to the 1.5 mL plastic tube. Add 1 mL of Urea buffer, 5 μL of 200 mM PMSF, 1.0 μL of 1 M Na_3VO_4, and 20 μL of IPG buffer.
5. Bio-Rad Protein Assay (Bradford dye-binding method, *see* **Note 2**).
6. CyDye reagents (500 μM): Dissolve 5 nmol of CyDye DIGE Fluor Cy2, Cy3, or Cy5 minimal dyes (GE Healthcare) in 10 μL of *N*, *N*-dimethylformamide (superdehydrated, Wako).
7. Stop solution: 10 mM L-Lysine

2.2.2 Two-Dimensional Electrophoresis

1. Swelling reagent: 7 M urea, 2 M thiourea, 2% [v/v] Triton X-100, 13 mM DTT, 2.5 mM acetic acid, 1% IPG buffer, 0.0005% bromophenol blue (BPB). Weigh 8.40 g urea, 3.04 g thiourea, and 40.0 mg DTT, and transfer to the 50-mL plastic tube. Add 2 mL of 20% (v/v) Triton X-100, 500 μL of 100 mM acetic acid, 200 μL of IPG buffer, 100 μL of 0.1% BPB, and water to a volume of 20 mL.
2. Equilibration buffer: 50 mM Tris–HCl, pH 6.8, 6 M urea, 2% sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 0.0005% BPB. Weigh 7.26 g urea and transfer to the 50-mL plastic tube. Add 4 mL of 10% SDS, 1 mL of 1 M Tris–HCl, pH 6.8, 6 mL of glycerol, 100 μL of 0.1% BPB, and water to a volume of 20 mL.
3. Reducing buffer: 50 mM Tris–HCl, pH 6.8, 6 M urea, 2% SDS, 30% [v/v] glycerol, 65 mM DTT, 0.0005% BPB. Dissolve 100 mg of DTT in 10 mL of Equilibration buffer.
4. Alkylating buffer: 50 mM Tris–HCl, pH 6.8, 6 M urea, 2% SDS, 30% [v/v] glycerol, 4.5% iodoacetamide, 0.0005% BPB. Dissolve 450 mg of iodoacetamide in 10 mL of Equilibration buffer.
5. Tris/glycine/SDS electrophoresis buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS. Mix 30.3 g of Tris, 144 g of glycine, 10 g of SDS, and water to a volume of 1 L to make 10× stock solution. Dilute the stock solution 10 times with water before use.

2.2.3 MALDI-TOF/MS Analysis

1. Ammonium bicarbonate solution: 100 mM ammonium bicarbonate (NH_4HCO_3).
2. Acetonitrile: LC-MS grade acetonitrile.
3. Matrix solution: 10 mM α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1% trifluoroacetic acid (TFA)/50% acetonitrile.
4. Trypsin solution: 10 ng/μL of Trypsin Gold, Mass Spectrometry Grade (Promega) and 0.01% ProteaseMAX Surfactant, Trypsin Enhancer (Promega) in 25 mM NH_4HCO_3.

3 Methods

Carry out all procedures at room temperature unless otherwise noted.

3.1 Specimen Preparation

1. For mouse liver sampling, anesthetize mice with isoflurane inhalation.
2. Make incisions in their abdomens to expose the liver.
3. Isolate liver aliquots (~150 mg).
4. Snap freeze tissue specimens with liquid nitrogen and store at –80 °C until use.

3.2 DryStrip Swelling (Day 1)

1. Make Swelling reagent (5 mL per strip).
2. Immerse an Immobiline DryStrip (pH 3–10 NL, 18 cm) in 5 mL of Swelling reagent using a sealed 1 cm dia. × 20 cm cylinder-type plastic tube. Make sure that the gel side (not the retainer side) is up and no air bubbles on it.
3. Incubate at 20 °C for 8–24 hours for gel swelling using CoolPhoreStar IPG-IEF Type-PX system (Anatech) or any cool incubators.

3.3 Sample (Liver Homogenate) Preparation and CyDye Labeling (Day 2)

1. Prepare Urea buffer and then Lysis buffer.
2. Weigh frozen liver aliquots, transfer 50–120 mg to 2-mL tubes (specific for bead homogenization), and then, add single Zirconia bead and ice-cold Lysis buffer (1 mL per 100 mg liver). Keep on ice during these procedures.
3. Homogenize using Micro Smasher MS-100R homogenizing system (4100 rpm, 30 s × 3 times [30 s intervals], 4 °C).
4. Centrifuge (16,000 × *g*, 5 min, 4 °C) and transfer the supernatant (by removing the pellet and floating oil) to new 1.5-mL plastic tube.
5. Centrifuge (20,000 × *g*, 25 min, 4 °C) and transfer the supernatant to new 1.5-mL plastic tube. During this centrifugation, prepare Tris-lysis buffer.
6. Measure protein concentrations using Bio-Rad Protein Assay and bovine serum albumin (BSA) for protein standard (*see* **Note 2**).
7. Dilute the supernatant to 10 mg/mL and add 1/3 volume of Tris-lysis buffer, pH 8.5.
8. Add 200 pmol (0.4 μL) of CyDye DIGE Fluor Cy2, Cy3 or Cy5 to 3.33 μL (25 μg) of diluted samples (*see* **Note 3**) (Fig. 1).
9. Mix well, spin down, and stand on ice for 30 min in the dark.
10. Add 0.5 μL of 10 mM L-Lysine as Stop solution, mix well, spin down, and stand on ice for 10 min in the dark. During the incubation, prepare DTT-lysis buffer.
11. Mix all three samples (Cy2, Cy3, and Cy5-labeled) and add an equal volume (12.69 μL) of DTT-lysis buffer. Mix well, spin down, and stand on ice for 10 min in the dark.

3.4 Isoelectric Focusing (IEF) (Day 2)

1. Set the swelled DryStrip on CoolPhoreStar IPG-IEF Type-PX system.
2. Apply the mixed CyDye-labeled sample on the anode end of the DryStrip.
3. Apply 200 mL of silicon oil to protect the strip from drying (*see* **Note 4**).

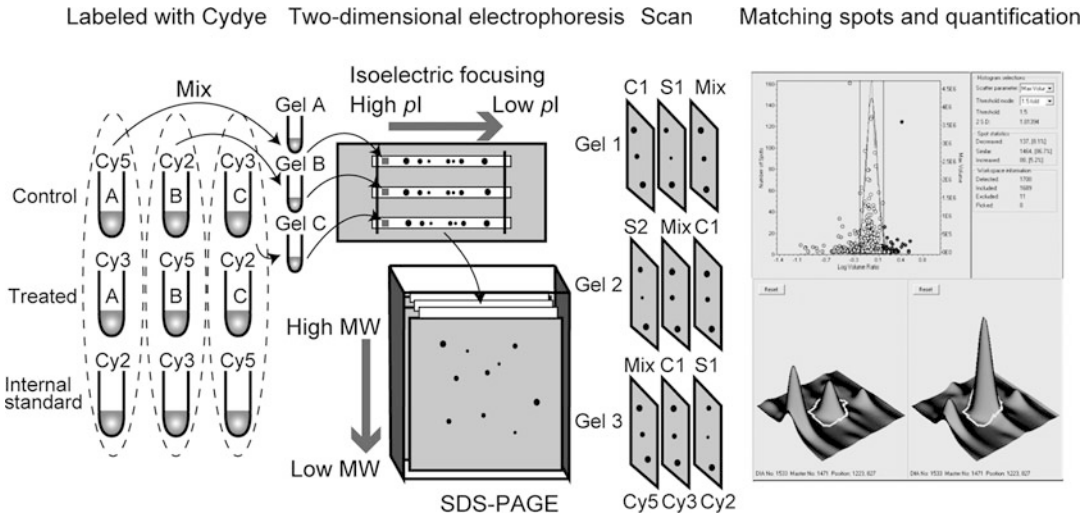

Fig. 1 Typical procedures for 2D DIGE analysis. Three samples (control, +stimuli, and all-(sample) mix in this case) are labeled with CyDye 2, 3, or 5, subjected to isoelectric focusing and then SDS-PAGE. The gels are scanned for each fluorescence through low-fluorescence glass plates using a fluorescence imager. All-mix samples are used for calibration of each spot using the DeCyder software (*see* **Note 3**). Using this procedure, we can quantify fluorescence (a reflection of protein amounts) for all major protein spots across the 2D gels

4. Start IEF in the following condition: 500 constant volts for 2 h, linear gradient from 500 to 3500 V for 6 h, and finally 3500 constant volts for 10 h at 20 °C.

3.5 SDS-PAGE and Fluorescence Detection (Day 3)

1. At ~1 h prior to the end of IEF, start the preparations of Equilibration buffer, Reducing buffer, and Alkylation buffer.
2. Stop IEF and remove even a trace amount of silicon oil from the strip.
3. Rinse the strip with water, and incubate in Reducing buffer (2.5 mL per strip) with shaking using a packing plastic tray of the Immobiline DryStrip (to save buffer volumes) for 30 min.
4. Incubate in Alkylation buffer (2.5 mL per strip) with shaking using the packing plastic tray for 15 min. During this incubation, prepare 0.5% low-melting-point agarose in Tris/Glycine/SDS electrophoresis buffer (~500 μL per sample) with heat (microwave) and SDS-PAGE system.
5. Place the strip (after the IEF; both strip ends can be cut off if no proteins of interest are in it) on the top of 12.5% SDS-PAGE gel precasted with low-fluorescent glass plates (14 cm × 14 cm) and seal with 450 μL of heated (dissolved) 0.5% agarose. Take care not to make any bubbles.
6. Electrophoresis (200 constant volts, 4 h with ERICA-*S* high-speed electrophoresis system).

7. Scan fluorescence of the gel (in the glass plates) with Typhoon Trio image scanner. The use of low-fluorescent glass plates enables fluorescence detection without detachment of the gel from the plates.
8. Analyze the scan data with DeCyder 2D (Ver. 6.5) software.
9. If needed, start swelling of another DryStrip.

3.6 Sample Preparation and Additional 2DE for Silver Staining (Day 4–5)

1. Mix 100–200 μg of liver homogenate (from Subheading 3.3, **step 6**) and an equal volume of DTT-lysis buffer.
2. Run 2DE with the same procedures.
3. After electrophoresis, stain the gel with Silver Stain MS kit (silver staining)
4. Store the gel at 4 °C until gel excision.

3.7 MALDI-TOF/MS Analysis (Day 6)

1. Excise single targeted brown/black-stained spot from the gel using a micro-spatula and transfer to a 1.5-mL plastic tube.
2. Add 100 μL of a yellowish mixture of De-staining solutions A and B (in the Silver Stain MS kit) and shake using a test tube shaker for 15 min.
3. Spin down briefly using a desktop mini-centrifuge (2000 × *g*), aspirate the buffer, and repeat such wash with another 100 μL mixture of De-staining solutions A and B. During this procedure, the color of the gel piece changes from brown to yellow (*see* **Note 5**).
4. Add 200 μL of 50 mM ammonium bicarbonate (NH_4HCO_3): acetonitrile (1:1) to the gel, shake for 5 min using a tube shaker, spin down, and aspirate the buffer.
5. Repeat such NH_4HCO_3/acetonitrile wash additional three times (5 min × 3). During these procedures, the color of the gel piece changes from yellow to colorless.
6. Add 200 μL of acetonitrile to the gel piece and incubate for 5 min. During the incubation, the color of gel piece changes from colorless to white [Dehydration step].
7. Spin down and rinse the gel piece with 200 μL of 100 mM NH_4HCO_3 (5 min incubation; the color changes from white to colorless).
8. Spin down and rinse the gel piece with 200 μL of acetonitrile (5 min incubation; the color changes from colorless to white).
9. Spin down and rinse the gel piece with 200 μL of 100 mM NH_4HCO_3 (10 min incubation; the color changes from white to colorless).
10. Spin down and rinse the gel piece with 200 μL of 50 mM NH_4HCO_3:50% acetonitrile (1:1) (10 min incubation).

11. Spin down and rinse the gel piece with 200 μL of acetonitrile (10 min incubation; the color changes from colorless to white).
12. Dry the pellet using a rotary vacuum concentrator for 10 min.
13. Add 10 μL of trypsin solution (Promega) and incubate at 50 °C for 1 h.
14. Add 3 μL of 2% TFA.
15. Place 0.5 μL of Matrix solution, 1.0 μL of the trypsin digest, and then another 0.5 μL of Matrix solution on a μFocus MALDI plate (air dry).
16. Obtain positive ion mass spectrum (700–4000 m/z range) using MALDI-TOF/MS and peptide calibration standards with a reflectron mode.

3.8 Protein Database Search (Day 7)

Proteins can be identified by matching the peptide mass finger prints with the Swiss-Prot protein database using the MASCOT Search engine (Matrix Science, http://www.matrixscience.com). We routinely use the following parameters: taxonomy, *Mus musculus*; enzyme, trypsin; and allowing 1 missed cleavage. We select carbamidomethylation as a fixed modification and allow the oxidation of methionine as a variable. The peptide mass tolerance is set at 0.5 Da and the significance threshold is set at $p<0.05$ probability based values on Mowse Scores ($\geq$55). An exemplary one-week schedule for 2D DIGE proteomic analysis is given in Table 2.

Table 2
Exemplary one-week schedule for 2D DIGE proteomic analysis

2D DIGE			Protein identification	
Day 1	DryStrip swelling start	8~24 h		
Day 2	Sample preparation CyDye labeling DryStrip swelling end Isoelectric focusing start	2 h 1 h 18 h		
Day 3	Isoelectric focusing end SDS-PAGE (14 cm × 14 cm) Fluorescence detection	 4 h 1 h	DryStrip swelling start	8~24 h
Day 4	DeCyder analysis	1 gel/h	Sample preparation DryStrip swelling end Isoelectric focusing start	1 h 18 h
Day 5			Isoelectric focusing end SDS-PAGE (14 cm × 14 cm)	 4 h
Day 6			Silver stain Trypsin digestion MALDI-TOF/MS analysis	1 h 4 h 10 spots/h
Day 7			Protein database search	50 data/h

4 Example Analysis

Fatty liver (hepatic steatosis) is a reversible condition wherein large vacuoles of triacylglycerol accumulate in hepatic cells. Non-alcoholic fatty liver disease (NAFLD) has been rapidly increasing in parallel to the increased prevalence of obesity and type II diabetes, which could progress to more severe clinical conditions such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [5]. However, initial molecular events underlying the onset of hepatic steatosis and critical factors that affect the progression (to NASH) are still uncertain. Several groups pursue this by proteomic approaches.

In our study, C57BL/6J mice (8-week-old males, Japan SLC, Shizuoka, Japan) were fed *ad libitum* with a MCD diet (MCDD, Oriental Yeast, Tokyo, Japan) or its control diet (MCD diet supplemented with 0.51% methionine and 20 ppm choline bitartrate) for 5 weeks. Methionine and choline are important precursors of phosphatidylcholine that comprises the outer coat of very low-density lipoprotein (VLDL); therefore, their deficiency could cause defective hepatic VLDL secretion and thus hepatic triacylglycerol accumulation [6]. Five-week-feeding with MCD diet caused a progressive weight loss (Fig. 2a) and fatty liver formation (Fig. 2b) as previously reported [6]. For total 8 samples, we used four SDS-PAGE gels; each gel contained (1) MCD diet sample, (2) control sample, and then (3) eight sample mix (for calibration), which were labeled with Cy2, Cy3, or Cy5 (*see* **Note 3**).

Our 2D DIGE and its qualitative analysis using the DeCyder software identified that several hepatic proteins were up- or down-regulated in fatty liver of MCD diet-fed mice (Fig. 2c, d). These include sulfotransferase 1 family member D1 (Sult1d1; spot 1), serotransferrin (Trf, spot 2), and serum albumin (Alb, spot 3) as up-regulated proteins, and major urinary protein 2 (Mup2, spot 9), carboxylesterase 3B (Ces3b; spot 10), and ATP-citrate synthase (Acly; spot 11) as down-regulated proteins (Table 3). Expression of aldehyde dehydrogenase X, mitochondrial (Aldh1b1) was up-regulated and those of selenium-binding protein 2 (Selenbp2), glutathione S-transferase P 1 (Gstp1), formimidoyltransferase-cyclodeaminase (Ftcd), and 3-hydroxyanthranilate 3,4-dioxygenase (Haao) were down-regulated, which were generally consistent with previous results by Lee et al. [7]. The other proteomic changes are novel observations. All these experimental results were obtained within 1 week.

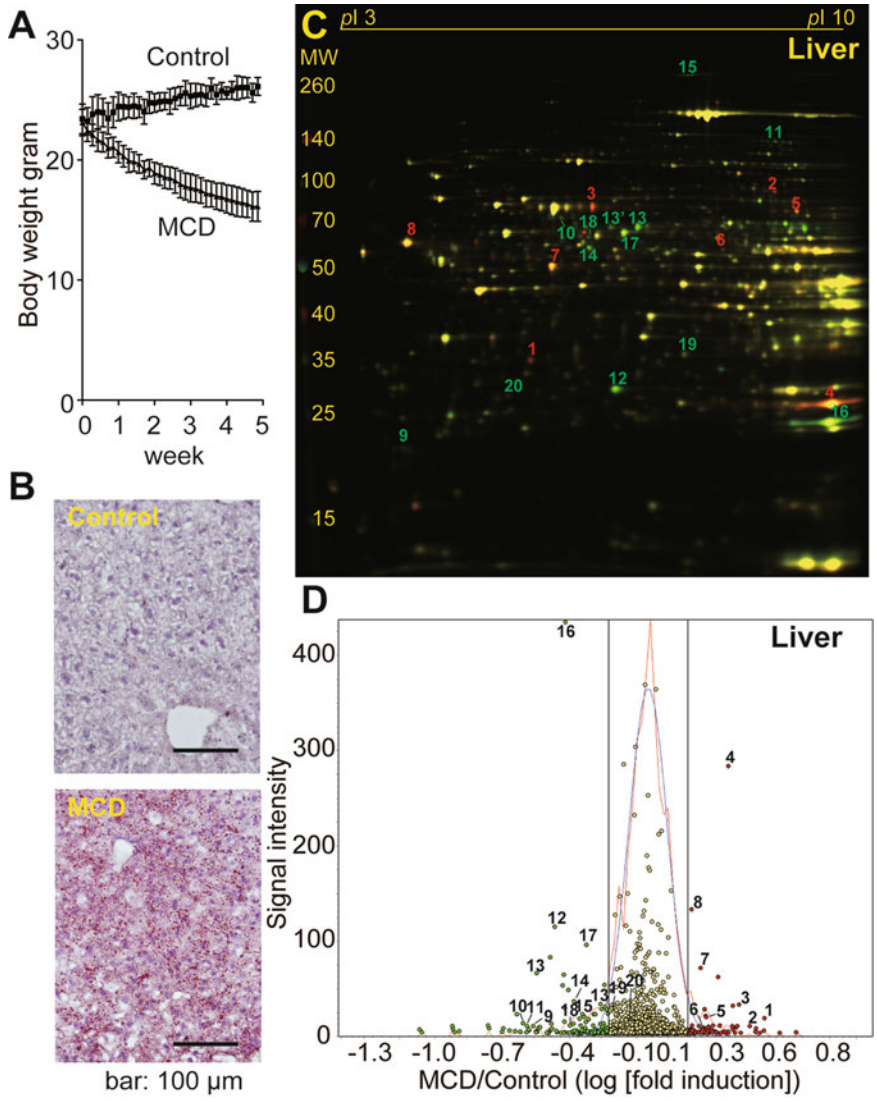

Fig. 2 2D DIGE proteomic analysis of fatty liver. Mice were fed MCD diet to induce fatty liver formation in mice. (**a**) Body weight changes after feeding with the MCD diet and its control diet. Data are mean ± SD ($n = 4$ each). (**b**) Oil *red* O-stained hepatic sections from mice fed MCD diet and its control diet for 5 weeks. (**c**) The representative florescent image in which up-regulated proteins are labeled in *red* and down-regulated proteins are in green. Approximate p*I* values and molecular weights (MW; kDa) are indicated. (**d**) Quantitative profiling of the above image by the DeCyder software. The *x*-axis represents log[(MCD/Control) fold induction] and *y*-axis represents spot signal intensity. The *red line* represents spot number distribution and blue line its Gaussian approximation. The *two black straight lines* represent 1.5 and –1.5 fold-change

Table 3
Examples for differentially expressed hepatic proteins in mice fed MCD diet. The identified 20 differentially expressed (significantly up- or down-regulated; $p<0.05$) hepatic proteins are listed. Their spot ID (identical to Fig. 2c, d), *p* value, protein name, Mowse score, sequence coverage, peptide matches, estimated molecular weights (Da), and estimated p*I*s are shown

Spot ID	Fold change	*p*-Value	Protein name	Mowse score	Sequence coverage (%)	Peptide matches	MW	p*I*
Up-regulated proteins								
1	2.86	0.003	Sulfotransferase 1 family member D1 (Sult1d1)	82	47	15/74	35,232	5.55
2	2.24	0.044	Serotransferrin (Trf)	158	45	33/95	78,878	6.94
3	2.17	0.030	Serum albumin (Alb)	170	42	20/40	70,700	5.75
4	2.14	0.030	Glutathione S-transferase Mu 1 (Gstm1)	148	79	26/88	26,069	7.71
5	1.85	0.010	Transketolase (Tkt)	207	66	24/65	68,272	7.23
6	1.74	0.001	Aldehyde dehydrogenase X, mitochondrial (Aldh1b1)	192	55	26/85	58,087	6.59
7	1.70	0.046	S-adenosylmethionine synthase isoform type-1 (Mat1a)	76	24	9/32	44,051	5.51
8	1.45	0.008	Protein disulfide-isomerase (P4hb)	212	63	32/96	57,422	4.77
Down-regulated proteins								
9	–9.00	0.004	Major urinary protein 2 (Mup2)	152	77	17/36	20,935	5.04
10	–3.47	0.002	Carboxylesterase 3B (Ces3b)	57	31	14/100	63,365	5.65
11	–3.44	0.004	ATP-citrate synthase (Acly)	70	28	27/87	120,564	7.13

(continued)

Table 3
(continued)

Spot ID	Fold change	*p*-Value	Protein name	Mowse score	Sequence coverage (%)	Peptide matches	MW	p*I*
12	–2.95	0.010	Indolethylamine N-methyltransferase (Inmt)	108	48	13/46	30,068	6.00
13	–2.81	0.007	Carboxylesterase 3A (Ces3a)	94	23	14/39	63,677	5.78
14	–2.54	0.026	Selenium-binding protein 2 (Selenbp2)	68	18	10/31	53,147	5.78
15	–2.46	0.008	Fatty acid synthase (Fasn)	227	36	85/131	274,994	6.13
16	–2.30	0.025	Glutathione S-transferase P 1 (Gstp1)	73	44	10/42	23,765	7.68
17	–1.95	0.010	Bifunctional epoxide hydrolase 2 (Ephx2)	86	32	19/58	63,045	5.85
18	–1.87	0.019	Formimidoyltransferase-cyclodeaminase (Ftcd)	78	41	17/73	59,529	5.79
13′	–1.67	0.009	Carboxylesterase 3A (Ces3a)	61	16	8/21	63,677	5.78
19	–1.55	0.015	3-hydroxyanthranilate 3,4-dioxygenase (Haao)	105	33	10/18	32,955	6.09
20	–1.37	0.036	Guanidinoacetate N-methyltransferase (Gamt)	139	69	11/38	26,604	5.43

5 Notes

1. It will take some time to dissolve urea and thiourea (endothermic reactions); however, do not heat to avoid their degradation. Urea buffer can be stored at –80 °C.
2. For measurements of protein contents, use the Bradford dye-binding method (e.g., Bio-Rad Protein Assay) but not the bicinchoninic acid (BCA) method (e.g., Pierce BCA Protein Assay Kit). Even with the Bradford dye-binding method, protein standard samples should be measured in the presence of same volume of Lysis buffer, which may influence the calibration.
3. There are very subtle differences between CyDye DIGE Fluors (Cy2, Cy3, and Cy5) for their preferences to label specific tissue proteins. For this reason, we usually try to use different CyDye Fluors for labeling each sample pool. For example, we use Cy2 for control sample No.1, Cy3 for stimulated sample No.1, and Cy5 for all-mix sample in Gel 1 while Cy3 for control sample No.2, Cy5 for stimulated sample No.2, and Cy2 for all-mix sample in Gel 2, and Cy5 for control sample No.3, Cy2 for stimulated sample No.3, and Cy3 for all-mix sample in Gel 3. Such efforts may contribute to distinguish protein expressional changes from differential labeling by different CyDyes.
4. Silicon oil is reusable. After every use, silicon oil should be stored with beads-type silica gel and filtrated through a paper filter just before reuse.
5. Make sure not to lose small gel pieces during the following wash and drying processes.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Numbers 15J03367 (to S.K.), 16H05107 (to I.I.), and 16K15194 (to I.I.).

References

1. Arentz G, Weiland F, Oehler MK, Hoffmann P (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9(3-4):277–288. doi:10.1002/prca.201400119
2. Hagiya Y, Kamata S, Mitsuoka S, Okada N, Yoshida S, Yamamoto J, Ohkubo R, Abiko Y, Yamada H, Akahoshi N, Kasahara T, Kumagai Y, Ishii I (2015) Hemizygosity of transsulfuration genes confers increased vulnerability against acetaminophen-induced hepatotoxicity in mice. Toxicol Appl Pharmacol 282(2):195–206. doi:10.1016/j.taap.2014.11.015
3. Kamata S, Akahoshi N, Ishii I (2015) 2D DIGE proteomic analysis highlights delayed postnatal repression of alpha-fetoprotein expression in homocystinuria model mice. FEBS Open Bio 5:535–541. doi:10.1016/j.fob.2015.06.008

4. Anstee QM, Goldin RD (2006) Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 87(1):1–16. doi:10.1111/j.0959-9673.2006.00465.x
5. Bedossa P (2017) Pathology of non-alcoholic fatty liver disease. Liver Int 37(Suppl 1):85–89. doi:10.1111/liv.13301
6. Rizki G, Arnaboldi L, Gabrielli B, Yan J, Lee GS, Ng RK, Turner SM, Badger TM, Pitas RE, Maher JJ (2006) Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1. J Lipid Res 47 (10):2280–2290. doi:10.1194/jlr.M600198-JLR200
7. Lee SJ, Kang JH, Iqbal W, Kwon OS (2015) Proteomic analysis of mice fed methionine and choline deficient diet reveals marker proteins associated with steatohepatitis. PLoS One 10(4): e0120577. doi:10.1371/journal.pone.0120577

Chapter 14

Proteomic Analysis of Lung Tissue by DIGE

Jarlath E. Nally and Simone Schuller

Abstract

Lungs perform an essential physiological function, mediated by a complex series of events that involve the coordination of multiple cell types to support not only gaseous exchange, but homeostasis and protection from infection. Guinea pigs are an important animal disease model for a number of infectious and noninfectious pulmonary conditions and the availability of a complete genome facilitates comprehensive analysis of tissues using the tools of proteomics. Here, we describe the application of 2-D Difference Gel Electrophoresis (DIGE) to compare, quantify, and identify differential protein expression of proteins in lung tissue from guinea pigs with leptospiral pulmonary hemorrhage syndrome (LPHS) compared to noninfected controls. 2-D DIGE is a powerful technique that provides novel insights into the dynamics of the complex lung proteome during health and disease.

Key words DIGE, SDS-PAGE, Proteomics, Lung, Pulmonary, CyDye, Guinea pigs

1 Introduction

The lungs, one of the largest organs of the human body, facilitate respiration. This physiological function is regulated by a complex series of events that involve the coordination of multiple cell types, including pneumocytes, bronchial epithelium, alveolar macrophages, endothelial cells, and interstitial cells, that not only supports gaseous exchange, but homeostasis and protection from infection. Transcriptomic analysis of lung tissue indicates that 73% of all human genes are expressed in the lung, of which 183 are expressed at elevated levels compared to other tissue types [1–3]. A comprehensive analysis of the complete proteome of the human lung is being performed (http://www.proteinatlas.org/humanproteome/lung) [4]. Proteomic analysis of lung tissue is being used to provide novel insights into the mechanisms of many disease processes including infectious [5, 6] and noninfectious conditions [7–11].

Guinea pigs represent an important disease model for a number of infectious and noninfectious pulmonary conditions such as

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_14,

LPHS [12], tuberculosis [13], Legionnaires disease [14], allergic asthma [15], chronic bronchitis [16], and preterm respiratory distress syndrome [17]. Guinea pigs share a number of similarities with humans with regard to hormonal and immunologic responses [18], pulmonary physiology [19], and the corticosteroid response [20]. The immunological genes of guinea pigs are more similar to humans than that of the mouse [21]. This species therefore represents a particularly important model for the human immune system. While the Broad Institute originally sequenced the guinea pig genome to 2× coverage as part of the Mammalian Genome Project to annotate the human genome, the guinea pig genome has now been published to full (7×) coverage (https://www.broadinstitute.org/guinea-pig/guinea-pig-genome-project). In addition, low sequence coverage from two outbred guinea pig strains, one additional inbred strain, and a Peruvian guinea pig as part of a SNP discovery project are currently being added. These findings are freely accessible to researchers and have opened up important new avenues of research investigations using genomic, transcriptomic, and proteomic techniques in this species.

Both gel-free and gel-based proteomics techniques have been successfully applied to examine the dynamics of the proteome of lung tissue and have demonstrated the power of both the approaches. Gel free "shot gun" techniques are considered fast and reliable, but gel-based techniques, and in particular 2-D differential gel electrophoresis (DIGE), while being more labor intensive, provides a visual control and analysis of the sample, allowing for the targeted identification of protein spots of interest and the differentiation of selected protein isoforms and their respective posttranslation modifications [22].

Gel-based proteomics relies principally on sodium dodecyl sulfate polyacrylamide-gel electrophoresis (GE) to separate complex protein samples using both one-dimensional (1D) and two-dimensional (2D) separation techniques. 1D GE allows for the separation of proteins according to molecular mass. In contrast, 2D GE is based on the separation of proteins according to both isoelectric point (Isoelectric focusing) and molecular mass (GE) which results in highly resolved protein spots.

While 2D GE provides a good overview of the molecular mass and isoelectric point of the majority of proteins in a complex sample, gel-to-gel variability can limit the ability to directly compare protein abundance between samples. This limitation has been overcome with the development of 2D DIGE, a technique that uses fluorescent labeling of protein samples prior to protein separation in the same gel [5, 23]. While different dye sets are commercially available, this article will focus on the use of the cyanine-based dyes (CyDyes): Cy2, Cy3, and Cy5. Protocols have been developed such that a restricted number (~5%) of lysine residues on each protein are labeled with CyDyes [24, 25]. This fluorescent minimal labeling

approach [26] allows for the separation of proteins from two different samples, labeled with two different CyDyes, to be separated on the same gel, and thus minimizes gel-to-gel variability. It also allows for the inclusion of an internal standard to normalize spot intensities across multiple gels. Further, fluorescent dyes increase the sensitivity of GE, allowing for the detection of up to 0.2 fmol of protein over a broad linear range (ca 20,000-fold concentration range) for quantitation, permitting the precise measurement of a wide range of protein spot intensities [22].

The principle of 2D DIGE is simple: two protein samples, labeled with different fluorescent CyDyes, typically Cy3 and Cy5, are separated on a single gel which also contains an internal standard. The internal standard comprises an equal amount of proteins from all samples included in the study labeled with a third CyDye, typically Cy2 [27]. Proteins with similar characteristics labeled with different fluorescent dyes co-migrate to the same position on the gel. Protein spots can then be imaged using a fluorescence scanner, equipped with filters to pick up the frequencies of the individual fluorescent dyes, thus generating three images representative of samples labeled with Cy3 and Cy 5, as well as the internal standard labeled with Cy2. The same internal standard can be used to normalize the relative spot intensities of protein spots across multiple gels and thus compare the relative abundance of specific proteins in each sample of interest [28]. Differentially expressed protein spots can then be identified and quantified by software analysis, and selected for further analysis, e.g., identification by mass spectrometry.

Here, we describe the application of 2D DIGE as used to compare the proteome of lung tissue from guinea pigs with leptospiral pulmonary hemorrhage syndrome (LPHS) to that of noninfected controls [5, 29]. All proteins identified in this experiment were used to generate a 2-D guinea pig lung proteome map (http://proteomics-portal.ucd.ie/). This provides a reference gel map, facilitating future gel-based proteomic studies on the lungs of guinea pigs.

2 Materials

2.1 Reagents and Consumables

Use analytical grade reagents whenever possible.

1. Amidosulfobetaine-14 (ASB-14).
2. Acrylamide stock, available from various commercial sources (*see* **Note 1**).
3. Bind-silane (GE Healthcare).
4. CyDyes (Cy2, Cy3, Cy5); available from various commercial sources.

5. Dimethylformamide (DMF, anhydrous).
6. Electrode pads (precut).
7. Fluorescent stain (SyproRuby®; Deep Purple™).
8. Immobilised pH gradient (IPG) strips (pH 3–10 NL, pH 4–7; 7 cm and 24 cm Immobiline DryStrips).
9. IPG strip cover fluid (GE Healthcare).
10. IPG buffer corresponding to the pH range of the IEF strips.
11. Lint free tissue wipes.
12. Low protein retention tubes.
13. pH indicator paper with a narrow pH range (e.g., 7.5–9.5).

2.2 Equipment

1. Sonicator.
2. DryStrip rehydration tray (GE Healthcare).
3. IPGphor IEF apparatus (GE Healthcare).
4. Low fluorescent glass plates 24 cm (GE Healthcare).
5. Stand for casting large gels including blank cassettes and separator sheets (GE Healthcare).
6. DaltSix electrophoresis and cooling unit (GE Healthcare).
7. Fluorescent gel imager (e.g., Typhoon 9400 Variable Mode Imager).
8. Image analysis software (Progenesis® same spots, DCyder, ImageQuant by GE Healthcare, ImageJ).

2.3 Preparation of Reagents

All the solutions are prepared with ultrapure/double-distilled water unless otherwise stated.

1. *1% Bromophenol blue stock solution*: Mix 100 mg Bromophenol Blue and 60 mg Tris base. Bring up to 10 ml final volume.
2. *Solubilization Buffer*: 7 M Urea, 2 M Thiourea, and 1% ASB-14; Mix 42.042 g of Urea, 15.22 g of Thiourea, and 1 g of ASB-14. Bring up to 100 ml with double distilled water. Make 1 ml aliquots and freeze at −20 °C.
3. *Dye solutions:* Cy2, Cy3, and Cy5 dyes are commercially sourced and should be stored at −20 °C until use. After centrifugation to ensure reagents are at the bottom of the tubes, dyes are reconstituted by adding fresh dimethylformamide (DFM) to obtain 1 mm stock solutions. The stock solutions are then further diluted with DFM to 400 pmol/μl working solutions.
4. *Quenching solution*: Prepare 1 μl of 10 mM lysine per μl of CyDye added.
5. *Rehydration buffer*: Solubilization buffer containing 30 mM DTT, 0.5% IPG buffer (0.5%), and 1 μl of Bromophenol blue stock solution. Use IPG buffer corresponding to the pH range

of the IPG strip. Final volumes are dependent on strip length (125 µl for 7 cm and 450 µl for 24 cm strips).

6. *12% Polyacrylamide gels (makes six 24 cm gels):* Mix 253 ml of double distilled water, 187.5 ml of 40% Acrylamide, 6 ml of 10% SDS, and 150 ml of Tris Buffer pH 8.8. Degas for 2 h using a vacuum pump, while mixing with a stir bar, then add 166 µl TEMED and 3.3 ml of 10% APS just before pouring the gel. Stock solutions of 10% APS can be prepared ahead of time and stored in aliquots at −20 °C.
7. *Equilibration buffer:* 6 M urea, 75 mm Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, and 0.002% bromophenol blue. For 200 ml buffer, mix 72.1 g Urea, 6.7 ml of Tris–HCl ph 8.8, 69 ml of Glycerol, 4 g SDS, 400 µl Bromophenol blue stock solution and add double distilled water to 200 ml. Freeze 20 ml aliquots at -20 °C. Add DTT or Iodoacetamide, to a final concentration of 1% or 2.5% respectively, just before use. Calculate 20 ml of Equilibration buffer and 0.2 g of DTT or 0.5 g of Iodoacetamide per strip.
8. *10× SDS Electrophoresis Buffer:* Mix 60.5 g Tris base, 288.2 g Glycine, and 20 g SDS. Bring up to 2 l with double distilled water. To obtain 1× SDS running buffer dilute 1:9 with double distilled water. To obtain 2× SDS running buffer dilute 1:5 with double distilled water. You need about 4 l of 1× SDS running buffer and 1 l of 2× SDS running buffer to complete this experiment.
9. *Agarose Sealing solution*: Mix 0.5 g Agarose, 200 µl of 1% Bromophenol stock solution. Bring up to 100 ml with 1× Electrophoresis buffer.
10. *Sypro Fixation buffer*: Mix 500 ml methanol and 70 ml acetic acid. Bring up to 1 l with double distilled water.
11. *Sypro Wash buffer*: Mix 100 ml methanol and 70 ml acetic acid. Bring up to 1 l with double distilled water.

3 Methods

3.1 Experimental Design

When planning a 2D DIGE experiment, several factors including the number of biological replicates, sample randomization, and general time management should be considered [28].

1. *Number of biological replicates:* The number of samples to be included in the experiment should be decided based on the expected biological variation across samples (e.g., lung tissues from a standardized laboratory animal model versus patient materials). The heterogeneity of samples can be estimated by performing preparatory 1D and/or 2D GE and comparing

Table 1
Experimental design. Comparison of protein abundance between six biological replicates of disease (A1–A6) and control groups (B1–B6). Samples are labeled with either Cy3 or Cy5 using a "dye swap." The pooled internal standard is labeled with Cy2. Samples are randomized to one of six gels

Gel	Cy2 Pooled internal standard	Cy3 50 μg in 400 pmol of Cy3	Cy5 50 μg in 400 pmol of Cy5
1	50 μg (4.17 μg each of samples A1–6 and B1–6)	B2	A5
2	50 μg (4.17 μg each of samples A1–6 and B1–6)	A1	B3
3	50 μg (4.17 μg each of samples A1–6 and B1–6)	A6	B1
4	50 μg (4.17 μg each of samples A1–6 and B1–6)	A3	B5
5	50 μg (4.17 μg each of samples A1–6 and B1–6)	B4	A2
6	50 μg (4.17 μg each of samples A1–6 and B1–6)	B6	A4

protein band/spot patterns. A minimum of three samples per group should be included in an experiment to allow for a meaningful statistical analysis. To limit run-to-run variability, all gels to be included in the analysis should be run in parallel either in one or more 6 or 12 gel appliances.

2. *Sample randomization*. In order to limit sample labeling bias, samples should be randomized so that equal numbers of both groups are labeled with Cy3 and Cy5 (dye swap). Cy2 is used to label the internal standard. Tonge et al. (2001) compared the variability of different CyDye combinations and found that a comparison of Cy3 and Cy5 labeled samples was less variable than other dye combinations, likely due to the fact that Cy2 is a slightly weaker fluorescent agent and therefore associated with a less favorable signal-to-noise ratio [25]. Karp and Lilley (2005) confirmed this observation. Labeled samples are then randomized to the gels in order to remove bias due to systematic errors caused by experimental artifact. An example for sample randomization on 6 gels is shown in Table 1.
3. *Time management*. The full experiment will take 3 days to complete. The workflow can be interrupted at various stages as indicated; however, careful planning is necessary to ensure vital equipment and facilities are available when they are needed.

3.2 Sample Preparation

1. *Choice of sample*. Careful consideration should be given to the choice of sample. Lung tissue is complex, containing a multitude of different structures and cell types, as well as blood and potentially oedema fluid. Because of the high sensitivity of DIGE to detect differences in protein abundance, structures

of interest should be chosen carefully (e.g., non-hemorrhagic, non-oedematous areas; bronchi vs parenchyma) to avoid introduction of artifactual differences in protein abundance. Laser microdissection is a useful technique to capture specific areas of interest [30].

2. *Protein solubilization*. Several methods can be used for sample preparation including grinding of frozen tissue, glass bead solubilization, and sonication. When using sonication, a good approach is to place 0.2 g of flash frozen lung tissues (flash frozen at the time of collection) in a 1.5 ml low protein retention Eppendorf tube containing 1 ml of solubilization buffer. The sample is sonicated in short bursts at maximum capacity, while being kept on ice to avoid overheating. The samples are then incubated overnight at room temperature. Samples are then centrifuged at 12,000 × *g* for 5 min and the supernatant and pellet were separated, aliquoted, and frozen until further analysis. Whichever method is used, efficient solubilization of the majority of proteins should be examined by comparing protein band patterns of supernatant and pellet on a one dimensional gel (Fig. 1).
3. *Protein quantification*. To ensure correct labeling and equal loading, protein concentrations of the samples are quantified using an assay compatible with reducing and/or detergent agents, e.g., RC/DC protein assay kit; Bio-Rad.
4. *Determination of spot resolution*. As part of the preparatory work, the degree of protein spot resolution and spread over pH ranges for your samples should be determined via 2-D gel electrophoresis (Fig. 2). For this purpose, 25 μg of proteins can be loaded onto 7 cm IPG strips starting with a pH range of 3–10. After second dimension separation, the gels are inspected for spots at the extremes of this range and for overlapping of spots. The ideal pH range for your sample is the one that includes the majority of spots while providing enough resolution to minimize overlapping of individual protein spots (Fig. 2). Often a pH range of 4–7 adequately fulfils these requirements. Alternatively, 2D DIGE analysis can be performed on several pH ranges or the use of IPG strips with a nonlinear gradient, which provide higher resolution in the middle of the gel and lower resolution at the higher and lower end of the pH spectrum of the strip. When comparing lung tissue from guinea pigs with LPHS compared to noninfected controls by DIGE, over 1500 protein spots were aligned across all biological replicates; only 5 proteins spots were detectable as differentially expressed over a pH range of 3–10 compared to 130 proteins spots that were characterized as differentially expressed over a pH range of 4–7 due to improved protein resolution (data not shown) [5].

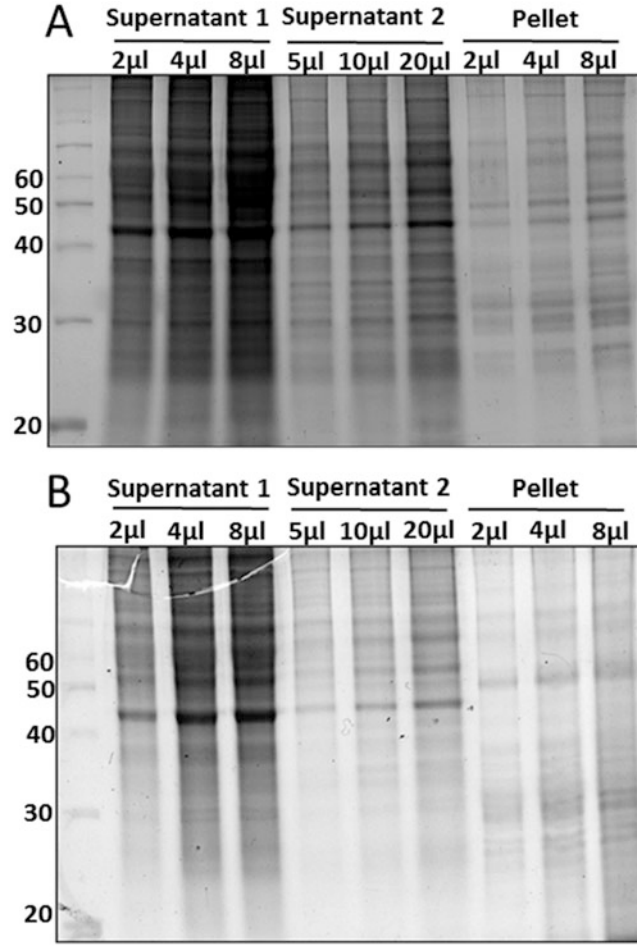

Fig. 1 Optimization of sample preparation. Comparison of protein fractions from lung tissue of noninfected (**a**) and infected (**b**) guinea pigs after one or two sonication steps in solubilization buffer. Comparison of protein profiles between pellets and supernatants by 1D–SDS PAGE suggests that the majority of proteins are solubilized in supernatant 1. There were no significant differences in protein band patterns between solubilized proteins present in the supernatant after one (supernatant 1) or two (supernatant 2) sonication steps. Based on these results one sonication step was judged sufficient for solubilization of the majority of proteins from lung tissue for 2-D DIGE. Gels were stained with SyproRuby stain. Molecular mass markers are indicated

5. *Check sample quality.* Running of preparatory gels also provides the opportunity to screen for obvious differences in spot patterns between groups or technical problems with the sample, including streaking or lack of high molecular weight proteins indicative of protein sample degradation.

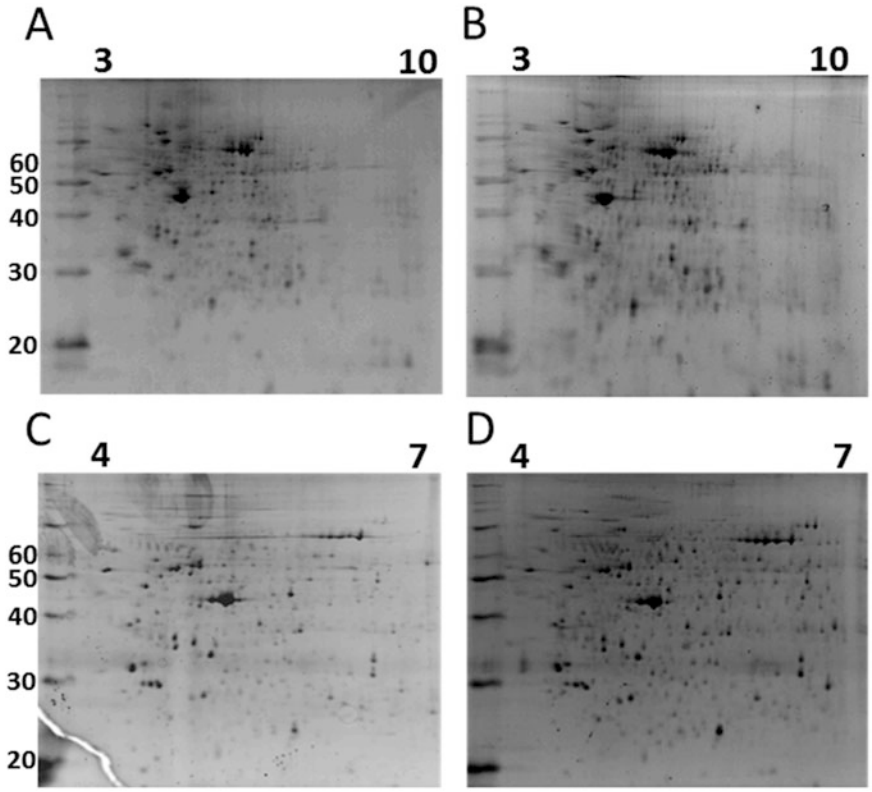

Fig. 2 Determination of protein spot resolution and pH range of proteins from lung tissue of infected and noninfected guinea pigs via 2-D gel electrophoresis. Lung tissue from noninfected (**a**, **c**) and infected guinea pigs (**b**, **d**) were separated over pH 3–10 (**a**, **b**) or pH 4–7 (**c**, **d**). Images show good protein spot resolution. A number of protein spots were present outside the pH 4–7 range. Limited differences in protein spot patterns between infected and noninfected lung tissues are detected. Twenty-five μg of protein were loaded onto each gel. Gels were stained with SyproRuby stain. Molecular mass markers are indicated

3.3 Labeling Procedure

Powder-free gloves should be worn for all the procedures to avoid keratin contamination, which will interfere with mass spectrometry.

1. Samples are defrosted.
2. To optimize fluorescent labeling, each sample pH is adjusted to pH 8.5 with 50 mM NaOH (optimal labeling is between pH 8.0 and 9.0) (*see* **Note 2**).
3. The optimal protein concentration for labeling is 0.5–10 μg/μl. To avoid pipetting errors, it is helpful to adjust all samples to the same protein concentration before going into the experiment. This considerably simplifies the task of combining samples for the internal standard and final sample mixes.
4. The internal standard is prepared by pooling equal amounts of sample from all samples included in the experiment (*see* **Note 3**).

5. For each gel, 50 μg of protein from samples are labeled with 400 pmol of Cy3 or Cy5 (*see* **Note 4**). For each gel, 50 μg of protein of pooled internal standard is labeled with Cy2. The samples are left to incubate on ice for 30 min (*see* **Note 5**). The labeling is then quenched by the addition of 1 μl of 10 mM lysine per μl of CyDye added and samples are incubated for 10 min at room temperature. During the entire experiment, labeled samples are protected from light in order to limit degradation of the CyDye labels. After labeling the samples are combined as per experimental design.

3.4 Gel Preparation

As much of the quality of the final images relies on the quality of the gels, specific care should be given to producing high quality gels for DIGE. To ensure all the gels in the experiment have the same chemical and physical properties, a multi-gel caster allowing for the simultaneous pouring of all gels is necessary.

1. Low fluorescent plates are carefully cleaned (*see* **Note 6**) and front and back plates assembled.
2. The caster is prepared and filled, starting with a separator sheet and then by alternating plates and separator sheets until full.
3. Finish with a separator sheet (*see* **Note 7**).
4. The caster is then positioned upright on an absolutely level surface to ensure horizontal gel surfaces.
5. The gel matrix is then poured into the caster via a funnel avoiding the introduction of air bubbles (*see* **Note 8**).
6. The surface of the gels is then generously sprayed with 0.1% SDS solution. The gels should be given several hours for polymerization. They can be stored at 4 °C in 2D running buffer for up to 4 days.

3.5 Rehydration of IPG Strips

1. Prepare rehydration solution. Pipette the required amount per strip in separate labeled tubes. Then add required amount of sample and solubilization buffer to add up to the desired volume (*see* **Note 9**). Again, this is simplified if all samples have been standardized to the same protein content. Carefully mix by pipetting up and down. Briefly spin down samples to remove all bubbles.
2. Remove IPG Strips from freezer.
3. Prepare rehydration tray. Make sure it stands level.
4. Carefully pipette final rehydration solution into wells. There should be no bubbles. Burst bubbles with a needle before placing the IPG strip with the gel side facing down onto the rehydration solution. Be careful to record strip numbers associated with each sample. Carefully cover strips with cover fluid

to avoid desiccation. Cover the tray with lid, then put light excluding cover over the tray. Allow IPG strips to rehydrate for 10–20 h at room temperature.

3.6 2D SDS-PAGE

1. *Isoelectric focusing*. Isoelectric focusing can be performed using an Ettan IPGphor IEF System (GE Healthcare). The strip holder is positioned in the machine and all channels evenly covered with 108 ml of Immobiline DryStrip cover fluid. IPG Strips are placed, face up in the tray with the anodic (+) end of the strip resting on the appropriate mark on the tray. Precut electrode pads are wet with 150 μl of deionized water and blotted until they are almost dry. The pads are placed on the ends of the IPG strips. The electrode assembly is placed over the top of all pads and locked (*see* **Note 10**) and the cover of the Ettan IPGphor is closed. Ensure that samples are run in the dark. Perform Isoelectric focusing according to the length and pH range of selected IPG strips. For 24 cm pH 4–7 strips, 3500 V for 75,000 VH (step 1), a gradient to 8000 V for 10 min (step 2), followed by 8000 V for 1 h (step 3), and 100 V for 5 h (holding step) work well. After isoelectric focusing strips can be frozen at −80 °C for 2–4 days.
2. *Strip equilibration*. Strips are transferred into equilibration buffer with added 1% DTT and incubated on a shaker for 10 min. After a brief rinse with water, the strips are transferred into the second equilibration buffer containing 2.5% iodoacetamide for 10 min. Placing the IPG strip, with the gel side facing inward, in a Petri dish works well for this procedure.
3. *SDS-PAGE*. The strips are rinsed with electrophoresis running buffer and overlaid on 12% acrylamide gels, which are prepared upright in a stand (*see* **Note 11**). Agarose gel with bromophenol blue (tracking dye) is used to seal the strips (*see* **Note 12**). The gels are run using a DaltSix electrophoresis unit (GE Healthcare). Prepare the tank by inserting the anode assembly unit. Fill the unit with 1× SDS Electrophoresis buffer and turn the pump on. Turn on the cooler system (15 °C). Insert the prepared gels into the unit. Fill the unused spaces with blank cassettes. Add more 1× SDS Electrophoresis buffer until the buffer is at or just below the "LBC (lower buffer chamber) start fill" line. Slide on the UBC (upper buffer chamber). Fill the upper chamber with 2× SDS Electrophoresis buffer to between the fill lines (approximately 0.8 l). Fill the LBC with 1× SDS Electrophoresis buffer to the same level as the upper chamber (approximately 4 l in total). Put the lid on the unit. Start the run (*see* **Note 13**). At the end of the run, when the dye front has just migrated off the end of the gel, switch off the power pack, disconnect and remove gels.

3.7 Image Acquisition and Analysis

1. *Scanning.* Gels can be scanned inside the glass plates. Plates should be thoroughly cleaned before scanning because high fluorescent specs might artificially increase the upper end of the dynamic range thus preventing the detection of low intensity spots. A Typhoon™ variable mode imager (GE Healthcare) is commonly used for gel scanning. This scanner has band pass filters to image each of the three CyDyes (520 nm for Cy2, 580 nm for Cy3, and 670 nm for Cy5). The gels are prescanned at a pixel size of 500 μm and the photomultiplier tube (PMT) voltage adjusted in order to ensure that the most intense spot on the gel is at the upper end of the dynamic range, thus ensuring that the full dynamic range of the detector is used. For the final scans, gels are scanned at a pixel size of 100 μm.
2. *Principle component analysis (PCA)* is a multivariate statistical analysis technique, which allows for easy detection of outlying gels and visualization of clustering of results of the biological replicates according to their group. PCA is performed by comparing spot with significant differences in normalized spot volumes between the groups present on all gels. It provides a global perspective over the experimental variation, thus giving an idea whether the variation seen is due to biologic variation according to the grouping, or technical/random biological noise in the system. An example for good clustering of biological replicates into groups is shown in Fig. 3.
3. *Spot analysis.* Differences in spot volumes can be analyzed using dedicated software packages such as SameSpots (TotalLab) or DeCyder (GE Healthcare), PDQuest (Bio-Rad), Delta2D (Decodon). Freeware programmes such as ImageJ or QuickTime can also be used for image visualization and annotation.

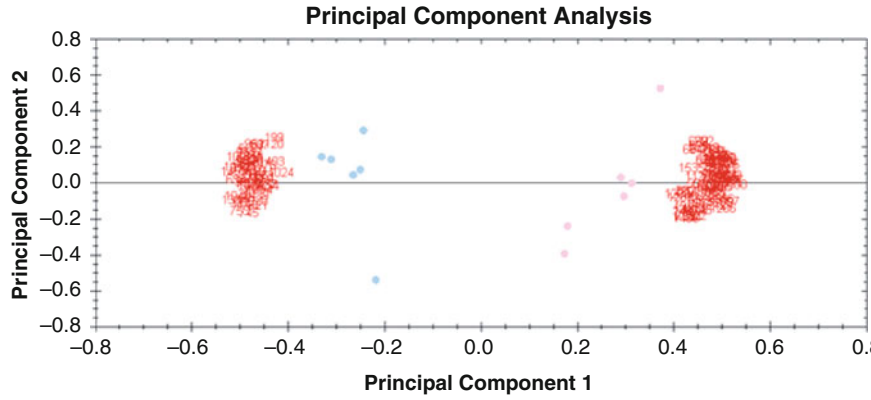

Fig. 3 Principle component analysis biplot of 2-D DIGE pH 4–7. Principle component analysis (PCA) allows for easy detection of outlying results and visualization of clustering of results of the six biological replicates according to their group [5]. The biplot shows excellent clustering of expression levels of significantly differently expressed spots on the six gels according to the relevant group (LPHS *pink*; control *blue*)

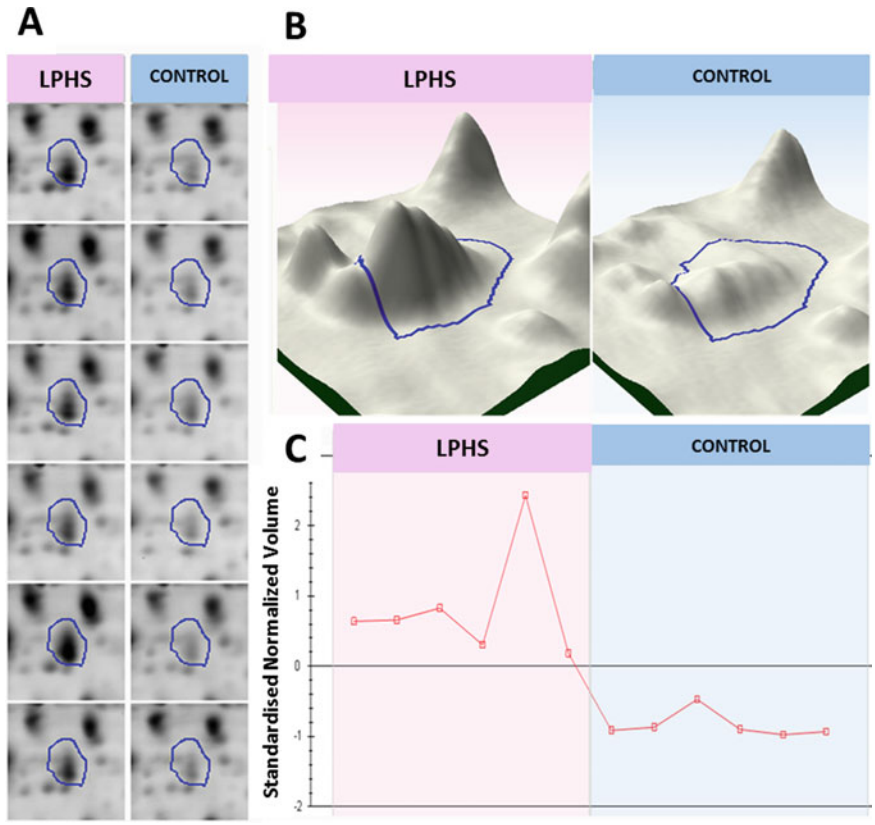

Fig. 4 Three different ways to illustrate differences in spot volumes using spot 695 from the pH 4–7 experiment [5]. (**A**) 2-D image of protein spot 695 from 12 biological replicates. (**B**) 3-D image of mean spot volumes of spot 695 in LPHS and control samples; (**C**) Graphic illustration of spot volumes for spot 695 in LPHS and control groups. The spot intensities (**A**) and volumes (**B**, **C**) of protein spot 695 are significantly higher in LPHS lung tissue compared to controls. This protein was identified by mass spectrometry as alpha-1-antiproteinase S precursor, an acute phase protein [5]

Gel images are inspected and imported into the selected programme. Prior to analysis, images are first cropped (making sure high signal areas at the borders of the gels and spot picking reference tags are removed), then the samples on each gel assigned to the respective experimental groups (*see* **Note 14**). Spots are then aligned by the programme and the spot volumes of the individual samples normalized against the internal standard. Spot volumes of protein spots aligned across all gels are then compared between groups. Statistical criteria for significant differences in spot volumes typically are set at $p < 0.05$, power > 0.8, and $q < 0.01$ (false discovery rate). While the software will do most of this work automatically, visual inspection of single protein spots of interest is advised before assembling the final list of protein spots for picking. Figure 4 illustrates the analysis of a protein spot with significant

differences in abundance in lung tissue from experimentally infected guinea pigs (LPHS) compared to the noninfected control group [5].

3.8 Generation of a Master Gel and Spot Picking

For protein identification, protein spots can be picked from the gels included in the 2D DIGE analysis, or alternatively from separately run master gels. The advantage of using master gels is that greater amounts of protein can be loaded. Typically, 500–1000 μg of protein are loaded per master gel.

1. *Preparation of master gels for spot picking.* Before pouring the gel for second dimension separation, the front glass plate should be treated with a solution containing 80% ethanol, 2% acetic acid, and 0.1% Bind-silane (GE Healthcare) in order to immobilize the gel on the front plate during staining and spot picking. Once dry, a reference marker is attached on the midpoint of the left and right side margins. These markers served as coordinates and reference points when using an automated spot picker.
2. *Protein separation.* Proteins are separated using the same protocol as described for DIGE.
3. *Fixation and staining.* After second dimension protein separation, the two glass plates are opened and the gel that is immobilized to the front plate, is first placed in fixation solution (50% methanol and 7% acetic acid) for 1 h and then stained with SyproRuby stain (Invitrogen) overnight. After staining, the gel is destained using 10% methanol and 7% acetic acid for 1 h to reduce background. If the gel is to be scanned again using the Typhoon™ variable mode imager, the glass plates are reassembled, and scanned with the front plate facing up, as previously described. A spot picking list with the coordinates of all selected spots can be produced using SameSpots (TotalLab). Because gels are scanned with the front plate on the top of the gel, and automated spot pickers typically work with the front plate under the gel, the X coordinate has to be corrected to account for the inversed image using the formula Xcorrected = Xmax-x (X being the corrected coordinate, Xmax being the width of the image in pixels, and x being the previous coordinate). This step is obviously not necessary if a combined scanner-spot picker robot is used.

4 Notes

1. Acrylamide is a neurotoxin. It is important to wear appropriate personal protective equipment and use appropriate handling precautions.

2. To test sample pH, pipette one droplet of sample and standard on pH indicator paper. If the pH is below 8 the CyDye will not bind, and if it is above 9, multiple dye molecules can bind to the protein, or to different amino acids, which will be negatively charged at high pH.
3. Make sure you have enough standard for all gels included in the experiment, plus extra for potential repeats and pipetting losses.
4. The protein to dye ratio recommended by the manufacturer is 400 pmol dye for 50 μg protein. It is possible to work with 25 μg protein and 200 pmol/dye without loss of sensitivity in case of very small samples or to reduce costs for the CyDye.
5. To synchronize the labeling time, the dye can be applied to the inner wall of the sample tube. The tubes are then all spun together, vortexed and spun again in a microcentrifuge to mix the dye well with the sample.
6. As gels are scanned between the glass plates, these have to be absolutely free of stain or dust. First wash the plates with water, and use ethanol and lint-free tissue to carefully clean the plates before casting the gels.
7. To avoid leakage, remove the gray foam seal from the groove in the faceplate of the caster and lubricate with Vaseline to help ensure a liquid tight seal and then put back in place. Be careful to also obtain a good seal between the bottom of the plates and the casting stand to avoid leakage of the gel matrix.
8. Gentle tapping of the caster stand from both sides with styrofoam blocks allows for trapped air to be released to the surface after pouring.
9. Some authors advise to not include the protein samples in the rehydration solution. Cup loading is used instead.
10. When using 24 cm strips, the top and bottom electrode units have to be placed with the electrodes facing away from each other in order to accommodate the length of the strips.
11. By convention the strips are placed with the acidic end to the left of the gel. Position the strips with the plastic backing against the inside of the back plate. The gel surface of the strip should not be touching the front plate. Then gently push the strip down until the entire lower edge of the strip is in contact with the top surface of the gel.
12. Allow for enough time for the agarose gel to cool down and solidify before moving the gels.
13. You can set up the power pack to 4 W/ gel for 1 h followed by 17 W/ gel until the bromophenol blue gets out of the gels (approx. 4:30 min). However, it works well to run the gels at

0.5 W/gel for 1 h and then at 2 W/gel overnight. The power is increased the following morning to 17 W/gel until the tracking dye reaches the bottom edge of the gel. This allows for enough time to perform the scanning of the gels in one go on day 3 of the experiment.

14. After scanning of the gels, crop areas with spacers, IPG strips, and other areas, which might show autofluorescence and therefore interfere with the analysis.

Acknowledgments

USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References

1. Yu NY-L, Hallström BM, Fagerberg L, Ponten F, Kawaji H, Carninci P, Forrest AR, Hayashizaki Y, Uhlén M, Daub CO (2015) Complementing tissue characterization by integrating transcriptome profiling from the Human Protein Atlas and from the FANTOM5 consortium. Nucleic Acids Res :gkv608
2. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K (2014) Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics 13(2):397–406
3. Lindskog C, Fagerberg L, Hallström B, Edlund K, Hellwig B, Rahnenführer J, Kampf C, Uhlén M, Pontén F, Micke P (2014) The lung-specific proteome defined by integration of transcriptomics and antibody-based profiling. FASEB J 28(12):5184–5196
4. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A (2015) Tissue-based map of the human proteome. Science 347(6220):1260419
5. Schuller S, Sergeant K, Renaut J, Callanan JJ, Scaife C, Nally JE (2015) Comparative proteomic analysis of lung tissue from guinea pigs with leptospiral pulmonary haemorrhage syndrome (LPHS) reveals a decrease in abundance of host proteins involved in cytoskeletal and cellular organization. J Proteome 122:55–72. doi:10.1016/j.jprot.2015.03.021
6. Zhao D, Liang L, Li Y, Liu L, Guan Y, Jiang Y, Chen H (2012) Proteomic analysis of the lungs of mice infected with different pathotypes of H5N1 avian influenza viruses. Proteomics 12(12):1970–1982. doi:10.1002/pmic.201100619
7. Chang DW, Hayashi S, Gharib SA, Vaisar T, King ST, Tsuchiya M, Ruzinski JT, Park DR, Matute-Bello G, Wurfel MM, Bumgarner R, Heinecke JW, Martin TR (2008) Proteomic and computational analysis of bronchoalveolar proteins during the course of the acute respiratory distress syndrome. Am J Respir Crit Care Med 178(7):701–709. doi:10.1164/rccm.200712-1895OC
8. Konsavage WM, Umstead TM, Wu Y, Phelps DS, Shenberger JS (2013) Hyperoxia-induced alterations in the pulmonary proteome of juvenile rats. Exp Lung Res 39(2):107–117. doi:10.3109/01902148.2013.763871
9. Korfei M, von der Beck D, Henneke I, Markart P, Ruppert C, Mahavadi P, Ghanim B, Klepetko W, Fink L, Meiners S, Kramer OH, Seeger W, Vancheri C, Guenther A (2013) Comparative proteome analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP) and organ donors. J Proteome 85:109–128. doi:10.1016/j.jprot.2013.04.033
10. Liu YF, Chen YH, Li MY, Zhang PF, Peng F, Li GQ, Xiao ZQ, Chen ZC (2012) Quantitative proteomic analysis identifying three

annexins as lymph node metastasis-related proteins in lung adenocarcinoma. Med Oncol 29 (1):174–184. doi:10.1007/s12032-010-9761-3

11. Hu J, Gao Z, Wang X, Gu M, Liang Y, Liu X, Hu S, Liu H, Liu W, Chen S, Peng D, Liu X (2016) iTRAQ-based quantitative proteomics reveals important host factors involved in the high pathogenicity of the H5N1 avian influenza virus in mice. Med Microbiol Immunol. doi:10.1007/s00430-016-0489-3
12. Nally JE, Chantranuwat C, Wu X-Y, Fishbein MC, Pereira MM, da Silva JJP, Blanco DR, Lovett MA (2004) Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. Am J Pathol 164(3):1115–1127
13. McMurray D, Collins F, Dannenberg A Jr, Smith D (1996) Pathogenesis of experimental tuberculosis in animal models, Tuberculosis. Springer, New York, pp 157–179
14. Breiman RF, Horwitz MA (1987) Guinea pigs sublethally infected with aerosolized *Legionella pneumophila* develop humoral and cell-mediated immune responses and are protected against lethal aerosol challenge: a model for studying host defense against lung infections caused by intracellular pathogens. J Exp Med 165(3):799–811
15. Ricciardolo FL, Nijkamp F, Rose VD, Folkerts G (2008) The guinea pig as an animal model for asthma. Curr Drug Targets 9(6):452–465
16. Luo Y-L, Zhang C-C, Li P-B, Nie Y-C, Wu H, Shen J-G, Su W-W (2012) Naringin attenuates enhanced cough, airway hyperresponsiveness and airway inflammation in a guinea pig model of chronic bronchitis induced by cigarette smoke. Int Immunopharmacol 13 (3):301–307
17. Koshy S, Beard LL, Kuzenko SR, Li T, Folkesson HG (2011) Lung fluid absorption is induced in preterm guinea pigs ventilated with low tidal volumes. Exp Lung Res 37 (1):44–56
18. Dascher CC, Hiromatsu K, Naylor JW, Brauer PP, Brown KA, Storey JR, Behar SM, Kawasaki ES, Porcelli SA, Brenner MB (1999) Conservation of a CD1 multigene family in the guinea pig. J Immunol 163(10):5478–5488
19. Meurs H, Santing RE, Remie R, Van Der Mark TW, Westerhof FJ, Zuidhof AB, Bos IST, Zaagsma J (2006) A guinea pig model of acute and chronic asthma using permanently instrumented and unrestrained animals. Nat Protoc 1(2):840–847
20. Gaumer H, Salvaggio J, Weston W, Claman H (1974) Cortisol inhibition of immunologic activity in guinea pig alveolar cells. Int Arch Allergy Immunol 47(6):797–809
21. Padilla-Carlin DJ, McMurray DN, Hickey AJ (2008) The guinea pig as a model of infectious diseases. Comp Med 58(4):324–340
22. Friedman DB, Lilley KS (2008) Optimizing the difference gel electrophoresis (DIGE) technology. Methods Mol Biol 428:93–124
23. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077. doi:10.1002/elps.1150181133
24. Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, Tonge R (2003) Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. Proteomics 3(7):1181–1195. doi:10.1002/pmic.200300439
25. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1(3):377–396. doi:10.1002/1615-9861(200103)1:3<377::aid-prot377>3.0.co;2-6
26. Minden JS (2012) Two-dimensional difference gel electrophoresis. Methods Mol Biol 869:287–304. doi:10.1007/978-1-61779-821-4_24
27. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3 (1):36–44. doi:10.1002/pmic.200390006
28. Dautel F, Kalkhof S, Trump S, Lehmann I, Beyer A, von Bergen M (2011) Large-scale 2-D DIGE studies-guidelines to overcome pitfalls and challenges along the experimental procedure. J Integr OMICS 1(9)
29. Schuller S, Sergeant K, Renaut J, Callanan JJ, Scaife C, Nally JE (2015) A 2-D guinea pig lung proteome map. Data Brief 4:140–145
30. Kondo T, Seike M, Mori Y, Fujii K, Yamada T, Hirohashi S (2003) Application of sensitive fluorescent dyes in linkage of laser microdissection and two-dimensional gel electrophoresis as a cancer proteomic study tool. Proteomics 3(9):1758–1766. doi:10.1002/pmic.200300531

Chapter 15

Comparative Testis Tissue Proteomics Using 2-Dye Versus 3-Dye DIGE Analysis

Ashling Holland

Abstract

Comparative tissue proteomics aims to analyze alterations of the proteome in response to a stimulus. Two-dimensional difference gel electrophoresis (2D-DIGE) is a modified and advanced form of 2D gel electrophoresis. DIGE is a powerful biochemical method that compares two or three protein samples on the same analytical gel, and can be used to establish differentially expressed protein levels between healthy normal and diseased pathological tissue sample groups. Minimal DIGE labeling can be used via a 2-dye system with Cy3 and Cy5 or a 3-dye system with Cy2, Cy3, and Cy5 to fluorescently label samples with CyDye flours pre-electrophoresis. DIGE circumvents gel-to-gel variability by multiplexing samples to a single gel and through the use of a pooled internal standard for normalization. This form of quantitative high-resolution proteomics facilitates the comparative analysis and evaluation of tissue protein compositions. Comparing tissue groups under different conditions is crucially important for advancing the biomedical field by characterization of cellular processes, understanding pathophysiological development and tissue biomarker discovery. This chapter discusses 2D-DIGE as a comparative tissue proteomic technique and describes in detail the experimental steps required for comparative proteomic analysis employing both options of 2-dye and 3-dye DIGE minimal labeling.

Key words 2-Dye labeling, 3-Dye labeling, CyDyes, Difference gel electrophoresis, Isoelectric focusing, Mass spectrometry, Protein digestion, Protein identification, Protein separation, Tissue proteomics, Two-dimensional gel electrophoresis

1 Introduction

Proteins are biomolecules that facilitate all cellular processes and are extremely functionally diverse [1]. Proteomes contain vast amounts of biological information, which is not accessible by genomics or transcriptomics, and can be characterized by altered protein abundances, cell and time-dependent expression profile patterns, and posttranslational modifications [2]. Interrogation of biological systems, including tissue analysis, is most relevant at the protein level. Tissue proteomics is an ever-growing field as tissue pathogenesis is complex due to the many pathological factors that can affect and influence it, such as genetic abnormalities, acquired autoimmune

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_15,

defects, or traumatic injury [3, 4]. Tissue proteomics is a powerful analytical tool that can analyze global protein populations from both healthy and pathological tissue samples [5, 6]. Biochemical insights from proteomic methodologies are of considerable interest in the field of testis and spermatozoa biology and are imperative for enhancing the discovery of testis specific protein biomarkers. Tissue biopsies can be complex in composition, as seen in testis research, and may comprise of proteins from the epididymis, vascular tissue, connective tissue, or adipose tissue [5, 7].

Proteomic analysis of tissue samples can be carried out for qualitative purposes to identify proteins in a sample or for quantitative analysis to compare the relative abundances of samples to investigate altered protein expression profiles [8]. Two-dimensional gel electrophoresis (2D-GE) coupled with liquid chromatography mass spectrometry (LC-MS) is a routinely used and highly reproducible bioanalytical method that can quantify and subsequently identify hundreds to thousands of proteins for the systematic assessment of crude tissues, subcellular tissue fractions, or isolated protein complexes [9, 10]. In 1975, O'Farrell revolutionized modern biochemistry by introducing a new protein separation technique called two-dimensional gel electrophoresis (2D-GE). 2D-GE is a principal method of high-resolution protein separation, separating proteins in the first dimension by isoelectric point (p*I*) and molecular mass in the second dimension [11]. Proteins are separated in the first dimension with isoelectric focusing (IEF), where proteins are separated along a pH gradient based on their p*I* until the overall net charge is equal to zero [12]. Proteins are subsequently separated in the second dimension, based on their molecular mass, in an acrylamide gel matrix by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE). Proteins have unique electrophoretic mobility patterns, with smaller proteins migrating through the gel faster than the larger proteins. Coupling these two independent biochemical protein separation techniques allows for a higher resolution of protein separation than with other techniques [11].

In 1997, Minden and colleagues [13] modified 2D–GE so that only a single gel was required to reproducibly detect altered abundances between two protein samples, by fluorescently tagging samples with different cyanine dyes, CyDyes. This advanced technique was coined as DIGE, difference gel electrophoresis. Fluorescence DIGE is the most sensitive and reproducible form of 2D-GE as it allows multiple samples to be quantified in a single gel, thus eliminating gel-to-gel variability [14]. DIGE enables the direct comparison of two different protein populations on the same high-resolution 2D gel system and thus can provide a plethora of information about the proteome under investigation [15]. DIGE allows

tissue sample proteomes to be pre-electrophoretically labeled with sensitive fluorescent CyDyes in a 2-dye or a 3-dye system [13]. Using 2D-DIGE for quantitative proteomic investigations is a superior form of 2D-GE as it has the capability of identifying a range of protein species from structural proteins associated with the cytoskeleton, as well as metabolic proteins and enzymes, extracellular matrix proteins, cytokines, transporters, signaling proteins, ion-handling proteins, and molecular chaperones [8]. DIGE coupled with LC-MS/MS is a highly sensitive, robust, and powerful method for modern comparative tissue proteomics.

DIGE can be applied in two forms, with minimal or saturation labeling. Saturation labeling involves the complete labeling of protein thiol groups with CyDyes, which react via maleimide with free SH groups [16]. For the purpose of this chapter, I will be focusing solely on minimal labeling. DIGE minimal labeling is a highly sensitive method that is capable of detecting and quantifying small changes in protein abundance as it has a detection limit as low as 0.5 fmol protein and a detection range of more than 10,000-fold concentration range [13, 17]. Minimal CyDye labelling is a nucleophilic substitution reaction of the CyDye covalently attached N-hyroxysuccinimidyl (NHS) ester with amine groups of lysine amino acids [16]. The three CyDyes are size and charge matched allowing fluorescently labeled proteins to be multiplexed and run on the same analytical gel [13]. CyDyes label a "minimal" proportion, approximately 2–3%, of lysine resides of proteins in the sample and therefore do not impinge on the downstream LC-MS identification of proteins [18, 19].

DIGE minimal labeling can be applied as a two-dye multiplex (one sample and a pooled internal standard) or as a three-dye multiplex approach (two samples and a pooled internal standard) as illustrated in Fig. 1. Figure 2 outlines the application of a 2-dye and 3-dye DIGE approach for studying total protein extracts from normal versus affected testis tissue using a similar style as seen in recent work by Jockusch et al. [5] and Holland and Ohlendieck [4]. Both minimal labeling options have their benefits and drawbacks, and are user and experimental specific. The 3-dye approach with Cy2, Cy3, and Cy5 provides less gel-to-gel variability and thereby increases sample reproducibility, as two samples can be coresolved on the same gel with an internal standard. The 3-dye method is usually preferred when samples being compared potentially have significant genetic variation, for example in humans and in some animal species [17]. For this method Cy2 is used to label the pooled internal sample for normalization, Cy3 and Cy5 are used for sample labeling [20]. However, labeling with 3-dyes can result in a lower signal-to-noise ratio [20]. Karp et al. [21] suggested that when employing the 3-dye method there is a potential

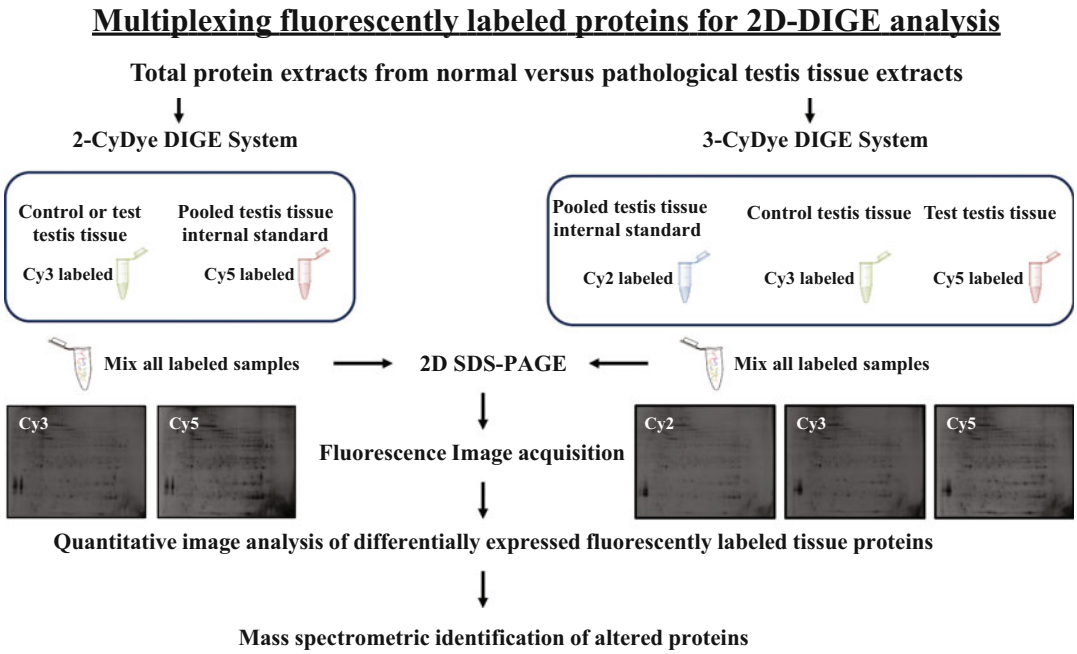

Fig. 1 2D–DIGE workflow for a 2-dye and 3-dye multiplex system. Shown is an overview of the principal approach used in both a 2-dye and 3-dye DIGE experiment for studying the global protein alterations from complex testis tissue proteomes. Protein is extracted from normal and pathogenic testis tissue samples, fluorescently labeled with CyDyes for a 2-dye or 3-dye multiplex approach, proteins are separated via 2D-GE based on their isoelectric point and molecular mass, 2D-DIGE images are acquired and quantitatively compared, and proteins with altered expression profiles are extracted and identified by mass spectrometry. Abbreviations used: *2D-GE* two-dimensional gel electrophoresis, *DIGE* difference gel electrophoresis, *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis

that the *p*-values skew toward 1 potentially suggesting that standardized volumes are not truly independent. This can have downstream processing drawbacks at the quantification stages, where the *q*-value can no longer be used to control the false-discovery rate. The 2-dye labeling approach employing Cy3 and Cy5 can and is routinely employed as an alternative technique. The 2-dye method is more reproducible than the 3-dye approach. This method uses Cy3 and Cy5 to label the pooled internal standard and the sample, respectively [20]. Reciprocal labeling, where the order of sample labeling is reversed, can be carried out to distinguish any potential sample-dependent differences and, the less common, dye-dependent differences [18]. While this approach is more costly, the quantitative data should be stronger [14].

While many researchers are moving toward label-free mass spectrometry for proteome wide quantification, DIGE remains a competitive, important, and relevant method in the field of proteomics [6, 10, 22]. Depending on sample complexity, DIGE permits the direct visualization of hundreds to thousands of protein species

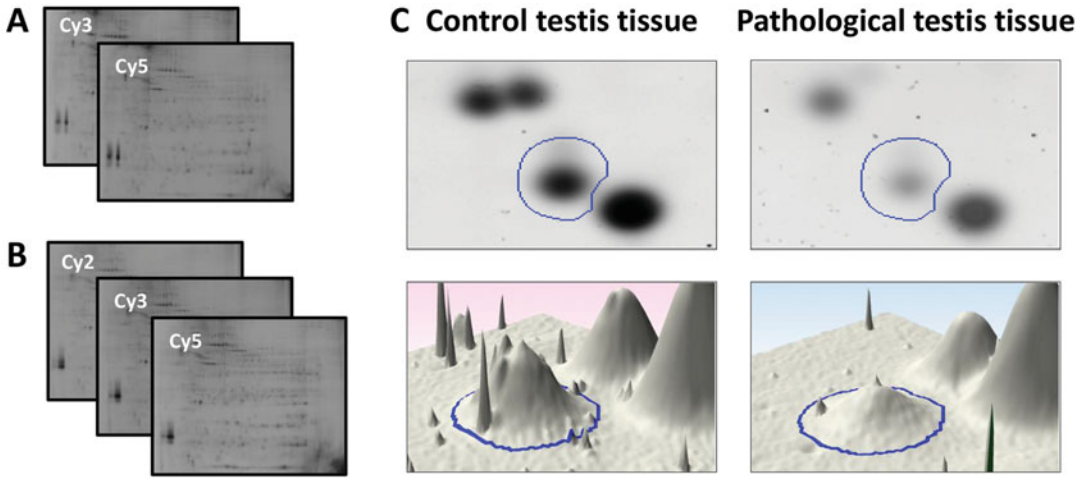

Fig. 2 2D-DIGE quantitative analysis of testis tissue proteins. Gel overlays of (**a**) a 2-dye DIGE system, and (**b**) a 3-dye DIGE system can be normalized to the internal pooled standard and subsequently quantitatively compared and analyzed to identify proteins with different abundance levels. (**c**) Shown are two statistically significant ($p \leq 0.05$) protein spots with major differences in abundances in the control testis tissue compared to the pathological testis tissue, as analyzed using Progenesis SameSpots (NonLinear Dynamics) software. The *top image* shows the difference in protein abundances at the gel level and the bottom image is a 3D visual representation of the difference in volume ratios in the control versus diseased testis tissue

separated by p*I* and molecular mass and the relative quantification of protein abundance [23]. Proteins that are differentially expressed can be excised from the gel slab, processed and subsequently identified by mass spectrometry. This comparative technique has the capabilities of identifying altered protein abundances and relaying crucial insight on individual proteins and their potential biological role [5, 18, 19]. While the field of proteomics is constantly evolving due to continuous advancements in mass spectrometry, allowing the identification and quantification of proteins and looking at protein-associated modifications, protein-protein interactions and localization, gel electrophoresis and more specifically 2D-DIGE is still relevant [6]. DIGE represents a key tool for tissue protein biomedical research and extensive use of this method can be instrumental in establishing the dynamic tissue proteome signature in pathological conditions and provide crucial insight for biomarker discovery and understanding the underlying pathophysiological mechanisms [7, 8].

This chapter introduces 2D-DIGE as a comparative tissue proteomic electrophoretic technique, compares 2-dye and 3-dye minimal DIGE labeling, delivers a complete experimental protocol with technical assistance, and provides crucial technical notes and troubleshooting insights that should be considered when carrying out 2D-DIGE minimal labeling experiments.

2 Materials

2.1 Equipment

Equipment for protein extraction from tissue samples, protein labelling, gel electrophoresis, gel imaging, quantitative gel analysis, and mass spectrometry was from the following suppliers:

1. pH test paper 7.5-9.5 (GE Healthcare) (*see* **Note 1**).
2. Hand-held IKA T10 Basic Homogeniser (IKA Labortechnik).
3. Immobilized pH gradient (IPG) strips-Immobiline DryStrips (240 × 3 × 0.5 mm, linear 4-7/6-9/3-10 pH; GE Healthcare); alternative sources are Bio-Rad, Sigma-Aldrich, and Isogen Lifesciences (*see* **Note 2**).
4. IPG DryStrip reswelling tray (GE Healthcare).
5. First dimensional electrophoresis unit-Ettan™ IPGphor™ apparatus (GE HealthCare); alternative equipment is PROTEAN IEF System (Bio-Rad), Multiphor II Horizontal Electrophoresis Unit (PerkinElmer) or UniPhor Horizontal Electrophoresis Unit (Sigma-Aldrich).
6. Manifold (GE Healthcare).
7. 2D-gel casting unit (GE Healthcare).
8. Low fluorescent 2D glass plates (GE Healthcare).
9. Cassette racks (GE Healthcare).
10. Second-dimensional electrophoresis unit-Ettan DALT*twelve* multiple vertical slab gel electrophoretic system (GE Healthcare); alternative equipment is PROTEAN Plus Dodeca™ Cell (Bio-Rad).
11. Laser scanning imaging device-Typhoon 9400™ Trio variable mode image scanner (GE Healthcare); alternative equipment is FLA 5100 Imaging System (FUJIFILM) or Ettan DIGE imager (GE HealthCare).
12. Analysis software-Progenesis SameSpots (Nonlinear Dynamics)-the most automated of the softwares available for DIGE analysis-alternative software is DeCyder™ (GE HealthCare); or Delta2D (DECODON).
13. Thermomixer (Eppendorf).
14. Model 5417R Centrifuge (Eppendorf).
15. Heto speedvac concentrator (GE Healthcare).
16. LC-MS/MS system for protein identification-nanoflow agilent 1200 series system, equipped with a Zorbax 300SB C18μm, 4 mm 40 nL pre-column for the separation of peptides (Agilent Technologies) coupled to an Agilent 6340 Ion Trap LC mass spectrometer. Other mass spectrometers are also capable of analysis.

2.2 General Reagent Solutions

To achieve successful experiments use high-quality electrophoresis/proteomic grade chemicals. All buffers and solutions should be made up in high quality water (mass spectrometry grade or of 18 MΩ or less) in order to keep contamination of protein samples to a minimum. Specific reagent solutions for protein extraction from tissue samples, gel electrophoresis, protein labeling, gel imaging, and mass spectrometry were from the following suppliers:

1. CyDye DIGE flours for minimal labeling (Cy2, Cy3 and Cy5) (Sigma).
2. Destreak agent (GE Healthcare).
3. DryStrip cover fluid (GE Healthcare).
4. IPG buffer (GE Healthcare).
5. IPG strips (GE Healthcare).
6. LC-MS Chromasolv water (Fluka).
7. Protease inhibitors (Roche).
8. Quick Start™ Bradford Protein Assay Kit (Bio-Rad).
9. ReadyPrep 2-D Cleanup Kit (Bio-Rad).
10. Sequence grade modified trypsin-lyophilized (Promega).

2.2.1 Preparation of Crude Tissue Sample Extracts

1. Tissue lysis buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM Dithiothreitol (DTT), 2% (w/v) IPG ampholytes pH 3–10, 10 mM Na-HEPES (pH 8.0), DNase, protease and phosphatase inhibitors (*see* **Note 3**).
2. ReadyPrep 2-D Cleanup Kit (Bio-Rad); alternative sources are 2D clean-up kit (GE HealthCare) and 2-D Sample Prep Kits (Pierce Biotechnology).
3. Quick Start™ Bradford Protein Assay Kit (Bio-Rad) or 2D-Quant kit (GE HealthCare).

2.2.2 CyDye Labeling (Minimal Dye Approach)

1. DIGE compatible lysis buffer: 9.5 M urea, 2 M thiourea, 4% CHAPS, 2% (w/v) IPG ampholytes pH 3-10, 10 mM DTT, 30 mM Tris-HCl, pH 8.5 (*see* **Note 4**).
2. Quenching solution: 10 mM lysine.
3. 2× sample buffer: 8 M urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) ampholytes pH 3-10.
4. 50 mM NaOH.

2.2.3 2D SDS-PAGE

1. 12.5% acrylamide gel: acrylamide (10%), SDS (0.1%), ammonium persulfate, TEMED, 0.37 M Tris–HCl, pH 8.8 (*see* **Note 5**).
2. IPG-strip rehydration buffer: 12 μL Destreak reagent and 0.002% (w/v) bromophenol blue per 1 mL of DIGE compatible lysis buffer (*see* **Note 6**).

3. Equilibration buffer stock: 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 50 mM Tris–HCl, pH 8.8 (*see* **Note 6**).
4. Equilibration buffer I (reduction buffer): 1% (w/v) DTT in equilibration stock (*see* **Note 7**).
5. Equilibration buffer II (alkylation buffer): 2.5% (w/v) Iodoacetamide (IAA) in equilibration stock (*see* **Note 7**).
6. Bind-silane: add 4 mL bind-silane to acetic acid and dH_2O mixture (220 μL acetic acid, 1 L dH_2O) (*see* **Note 8**).
7. 10× SDS running buffer: 25 mM Tris–base, 190 mM glycine, 1% (w/v) SDS. Dilute to a 1× solution before use.
8. Agarose sealing solution: 1% (w/v) agarose, 0.1% (w/v) SDS, 0.002% (w/v) bromophenol blue and 125 mM Tris (pH 6.8). Heat solution in microwave until agarose has fully dissolved (*see* **Note 9**).

2.2.4 Protein Visualization Using Coomassie Brilliant Blue

1. Buffer A: 10% (w/v) ammonium sulfate, 2% (v/v) phosphoric acid.
2. Buffer B: 5% (w/v) Coomassie Brilliant Blue G-250 in H_2O.
3. Coomassie staining solution: add 2 mL Buffer B to 80 mL Buffer A and bring to a final volume of 100 mL with methanol.
4. Neutralization buffer: 0.1 M Tris, pH 6.5.
5. Wash buffer: 25% (v/v) methanol.
6. Fixation buffer: 20% (w/v) ammonium sulfate.

2.2.5 In-Gel Digestion of Protein for Mass Spectrometric Identification

1. DTT solution: 10 mM DTT in 100 mM ammonium bicarbonate.
2. IAA solution: 55 mM iodoacetamide in 100 mM ammonium bicarbonate.
3. Trypsination buffer: 20 μg sequencing grade-modified trypsin in 100 μL resuspension buffer (*see* **Note 10**). For in-gel digestion, dissolve 10 μL aliquots into 500 μL 50 mM ammonium bicarbonate.
4. Extraction buffer: 1:2 (v/v) formic acid: acetonitrile.
5. Formic acid/acetonitrile (1:2 (v/v)).

3 Methods

3.1 Preparation of Crude Tissue Protein Extracts (See Note 11)

1. Weigh testis tissue samples from both control and test pathological groups and place ~100 mg tissue sample in an appropriate size tube for homogenization and add 1 mL lysis buffer to give a tissue to lysis buffer ratio of 1:10 (*see* **Note 12**).

2. Lyse tissue samples by (a) using a hand held homogenizer, (b) a sonication probe, or (c) manual grinding in the presence of liquid nitrogen with a pestle and mortar.
3. Incubate the tissue lysates at 4°C with agitation for 2 h.
4. Centrifuge lysates at top speed in a 1.5 mL microcentrifuge for 20 min at 4°C. Retain the protein containing middle layer for proteomic evaluation and leave behind the top lipid layer and the pelleted debris at the bottom.
5. Precipitate protein from tissue lysate using acetone or a 2D cleanup kit according to the manufacturer's recommendations overnight at –20°C. The following day, pellet the sample by centrifugation at top speed for 5 min at 4°C. Air-dry the protein pellet for 2 min and resuspend pellet in an appropriate DIGE compatible lysis buffer (*see* **Note 13**).
6. Determine the protein concentration using a Bradford assay or 2D quant kit. Standards and samples should be made up in lysis buffer as urea, CHAPS and DTT affect the Bradford assay. Using a 96-well plate add 5 µL sample/standard to 250 µL Bradford reagent, mix and measure the OD at 595 nm. The blank should be made up with lysis buffer (*see* **Note 14**).

3.2 Protein Labeling with Fluorescent CyDyes

1. Allow CyDye stock solutions to equilibrate to room temperature for 15 min before beginning.
2. Resuspend CyDyes in anhydrous Dimethyl formamide (DMF) to give a stock CyDye concentration of 1 mM (*see* **Note 15**). Briefly vortex and centrifuge CyDyes at 12000 × *g* for 30 s. Stock solutions can be used immediately or stored in the dark at −80°C for up to 2 months.
3. Dilute stock CyDyes 1:5 (v/v) with DMF to make a working solution of 200 ρmol. CyDye working solutions are only stable for 1 week at −20°C.
4. Prior to protein labeling it is essential to check the pH of the sample and ensure it is at pH 8.5 for accurate labeling.
5. Add 1 µL CyDye (at 200 ρmol) per 25 µg tissue protein. If you are using a 2-dye system label 50 µg per sample with 2 µL Cy3 and label 50 µg pooled internal standard with 2 µL Cy5. If you are using a 3-dye system label 50 µg per control sample with 2 µL Cy3, label 50 µg per test sample with 2 µL Cy5, and label 50 µg pooled internal standard with 2 µL Cy2.
6. Briefly vortex and centrifuge samples and incubate protein/CyDye mixtures on ice for 30 min in the dark.
7. Quench the labeling reaction with the addition of 1 µL 10 mM lysine per 25 µg protein. Briefly vortex and centrifuge samples and incubate protein/CyDye/lysine mixtures on ice for 10 min

in the dark. Samples are now CyDye labeled and can be immediately used for 2D-electrophoretic separation or stored at -80°C for 3 months in the dark.

3.3 First Dimension Protein Separation-Iso-Electric Focusing (IEF)

1. Following CyDye labeling of tissue proteins add an equal volume of 2× sample buffer to labeled samples and leave on ice for 10 min.
2. Pool protein samples that will be separated on the same first and second dimensional gel. For a 2-dye multiplex add 25 μg Cy3 labeled sample plus 25 μg Cy5 labeled pooled internal standard (i.e., 50 μg protein per IPG strip). For a 3-dye multiplex add 25 μg Cy3 labeled control sample, 25 μg Cy5 labeled test sample plus 25 μg Cy2 labeled pooled internal standard (i.e. 75 μg protein per IPG strip).
3. IEF strip rehydration. For the separation of tissue protein lysates in the first dimension using 24 cm pH 3-10 NL IPG Immobiline DryStrips will give superior separation and resolution of proteins. Add CyDye labeled proteins with appropriate volume of DIGE compatible rehydration buffer for strip length (*see* Table 1).
4. Rehydrate IPG strips in a DryStrip rehydration tray with sample at room temperature overnight to allow passive diffusion of proteins into the gel strip. Add ~1 mL PlusOne Drystrip Cover fluid to each well to prevent strips from drying out.
5. Isoelectric focusing (IEF)-separation of proteins in the first dimension. Load rehydrated strips onto the manifold in IPGphor gel side up.
6. Put paper wicks at each end of the strips and wet wicks with 150 μL 100 mM DTT.
7. Apply voltage clamps onto either end of the strip.
8. Cover manifold with 108 mL PlusOne Drystrip Cover Fluid.

Table 1
Volume of rehydration buffer required based on strip length for isoelectric focusing

Strip length (cm)	Total volume (μL)
7	125
11	200
13	250
18	350
24	450

Table 2
IEF running protocols

Method	Volts	Duration (min)
Step-and-hold	100	120
Step-and-hold	500	90
Step-and-hold	1000	60
Step-and-hold	2000	60
Step-and-hold	4000	60
Step-and-hold	6000	120
Step-and-hold	8000	240
Step-and-hold	500	180
Step-and-hold	8000	240

The IEF running conditions outlined in Table 2 can be used for 18 and 24 cm strips and are appropriate for protein separation based on p*I* for all pH ranges. The proteins will be separated for a total of 70,000 Vh

9. Set IPGphor to focus strips as outlined in Table 2.
10. Equilibration of IEF strip. After IEF protein separation in the first dimension strips need to be equilibrated prior to SDS-PAGE protein separation in the second dimension. Reduce focused strips by incubating strips in equilibration buffer A for 10 min with gentle agitation.
11. Alkylate strips by incubating strips in equilibration buffer B for 10 min with gentle agitation in the dark.

3.4 Second Dimension Protein Separation-SDS-PAGE

1. Clean low fluorescent glass plates with 70% EtOH and lint-free tissue.
2. Assemble the gel cassette (24 × 18 cm) with 1.5 mm thick spacers.
3. Pour gels (10–1.5% polyacrylamide) and avoid air-bubbles.
4. Overlay the top of the gels with 750 μL isopropanol to get an even gel surface. Allow gels to polymerize for ≥ 8 h (*see* **Note 16**). Once polymerized drain off the isopropanol and rinse well with water to remove.
5. If gels are polymerizing overnight layer tops of gels with 0.2% SDS and cover to prevent gels from drying out.
6. Briefly wash equilibrated strips in 1× SDS running buffer.
7. Load equilibrated strips onto polyacrylamide gel for protein separation in the second dimension.

Table 3
DIGE image acquisition mode settings

CyDye	Excitation filter (nm)	Emission filter (nm)
Cy2	488 (blue)	520 BP
Cy3	532 (green)	580 BP
Cy5	633 (red)	670 BP

8. Seal IPG strip in place using a 1% agarose sealing solution.
9. Run 2D-DIGE gels in an ETTAN-DALT, at 0.5 W/gel for the first 60 min and then increase power to 15 W/gel until the solvent reaches the bottom of the gel but before it runs off.

3.5 DIGE Gel Image Acquisition and Quantitative Analysis

1. Visualize CyDye labeled proteins using a Typhoon Trio 9400 variable mode image scanner (GE Healthcare). Image acquisition for DIGE gels requires setting the correct laser for excitation and emission filter wavelength to each CyDye as outlined in Table 3.
2. Perform all prescans at 500 μm low resolution to optimize the photomultiplier tube (PMT) value for each laser and then perform optimized scans for final image acquisition at 100 μm high resolution and save raw files in 16-bit .tif format. The PMT value for each gel is variable and should be adjusted for each image (400–700 V) to give a maximum pixel volume between 85,000 and 95,000 (*see* **Note 17**).
3. Analyze the DIGE images using Progenesis SameSpots (Non-Linear Dynamics) software, or appropriate alternative software, to detect apparent differences in protein abundances between groups.
4. Align gels to adjust for any gel-to-gel variation and if needed crop sections of the gels that are redundant areas for analysis (IPG strip and the solvent front).
5. Select the appropriate gel for the pooled internal standard to facilitate spot matching and protein normalization and set up experimental design.
6. Carry out statistical analysis to generate a list of significant protein spots. Tag significant proteins ($p \leq 0.05$) with a fold change of ≥ 1.5 and a power score ≥ 0.8 and export spot list.
7. Use this list of statistically significant differentially expressed protein spots for further analysis, such as identifying proteins by mass spectrometry.

3.6 Spot Picking and Gel Destaining

1. Spot picking can be done manually or alternatively with an automated robotic spot picker. In both cases use Coomassie stained spot picking gel that was run in parallel with the DIGE experimental gels or use experimental DIGE gels that were subsequently Coomassie stained (*see* **Note 18**).
2. For manual excision use a sterile pipette tip with the end removed to excise spot and put gel plug in a labeled 1.5 mL tube or 96-well plate (*see* **Notes 19** and **20**). If you are trying to identify low-abundance proteins, it is good practice to enrich for the proteins of interest or pool protein from multiple DIGE gels (*see* **Note 21**).
3. Destain Coomassie stained gel plugs with 100 μL 100 mM ammonium bicarbonate/acetonitrile solution (1:1) to each gel plug, and incubate for 30 min at room temperature with occasional vortexing.
4. Add 500 μL neat acetonitrile and incubate for 10 min at room temperature to dehydrate and shrink gel plug.
5. Remove solution, leaving behind the gel plug (*see* **Note 21**).

3.7 In-Gel Protein Digestion and Peptide Extraction

1. Resuspend 20 μg trypsin in 100 μL reconstitution buffer (*see* **Note 22**). For a working trypsin concentration add 10 μL reconstituted trypsin to 500 μL 50 mM ammonium bicarbonate.
2. Add 50 μL trypsin to gel plugs and incubate for 30 min at 4°C to allow for slow trypsin absorption into gel matrix.
3. If required add more trypsin/buffer to ensure gel plug is submerged in the solution and incubate for another 90 min at 4°C.
4. Add 20 μL 50 mM ammonium bicarbonate to cover gel plug and incubate at 37°C overnight in a thermomixer at 250 rpm.
5. Add 100 μL extraction buffer to each sample and incubate for 15 min at 37°C in thermomixer as before.
6. Remove the peptide containing supernatant and transfer to a new sterile microcentrifuge tube.
7. Dry down peptides in a vacuum centrifuge (*see* **Note 21**).

3.8 Liquid Chromatography–Mass Spectrometry

1. Resuspend dried peptides in 15 μL 0.1% formic acid.
2. To ensure complete resuspension of peptides vortex and sonicate samples for 10 min (*see* **Note 23**).
3. Centrifuge samples at 14000 × *g* for 20 min at 4°C and transfer top 12 μL to a LC-MS vial (*see* **Note 24**).
4. Separating peptides on a nanoflow Agilent 1200 series system coupled with a Model 6340 Ion Trap LC-MS apparatus

(Agilent Technologies) can allow analysis of peptide mixtures and identify tissue-associated protein digests (*see* **Note 25**).

5. Inject 5 μL peptide mixture into the nanoflow LC system and load analytical samples into the enrichment capillary at a flow rate set to 2 μL/min. The mobile phase of solvent A: 0.1% formic acid and solvent B: 90% acetonitrile/0.1% formic acid were set at a ratio of 19:1 for solvent A to solvent B (*see* **Note 25**).
6. Separate peptide samples over a 10 min gradient of 5–100% acetonitrile/0.1% formic acid and an equilibrating post run of 5 min through a Zorbax 300SB C18 5 μm column, 43 mm × 75 μm analytical reverse phase column employing HPLC-Chip technology.
7. Elute tryptic peptides with a continuous linear gradient of 5–70% solvent B for 6 min, 70–100% solvent B for 1 min and 100% solvent B for 1 min, with a constant nano-pump flow rate of 0.6 mL/min. Run a 2 min post-time of solvent A to minimize sample carryover.
8. Capillary voltage should be set at 1700–2000 V, flow rate at 4 μL/min, and drying gas temperature at 300°C.
9. Database searches can be conducted with MASCOT MS/MS Ion Search (Matrix Science, London, UK) for protein identification. For confident protein identifications search parameters for filtering data with MASCOT should be set to (a) two missed cleavages by trypsin, (b) carboxymethylation of cysteines as a fixed modification, (c) oxidation of methionine as a variable modification, (d) minimum of two unique peptides, and (e) a MASCOT score $\geq$ 49. It is crucial to cross reference the p*I* values and molecular masses of the identified proteins to their corresponding position on the DIGE gel.

4 Notes

1. Narrow pH range test papers are more accurate than broad range test papers.
2. IEF strips are available in different pH ranges and sizes; pH 3–10 NL strips are a good starting point for whole-cell tissue extracts. Nonlinear (NL) indicates that the strip has a pH gradient, with increased resolution between pH 5 and 7. In practice, using 24 cm pH 3–10 NL strips improves the resolution of proteins in the region where most proteins lie. Alternatively, narrower pH range strips (1 pH unit or pH 4–7) are available for closer study of proteins within a region of interest.
3. As soon as lysis occurs, proteolysis, de-phosphorylation, and denaturation begin. It is important to prepare samples on ice

(4 °C) to reduce this and to supplement all samples with appropriate inhibitors (e.g., PMSF, Na_3VO_4, or a protease inhibitor cocktail).

4. Use the appropriate IPG buffer that corresponds to the pH range of the IEF strips being used in the experiment. The pH of the lysis buffer is critical for the CyDye labeling reaction. Store lysis buffer in 1 mL aliquots at −80 °C.
5. Alternatively, this step can be bypassed by purchasing premade 2D gels that do not contain any fluorescent contaminants. Spot resolution and pattern reproducibility can be by the use of precast IPG strips and precast gels for first and second dimension protein separation, respectively.
6. When carrying out DIGE, the bromophenol blue tracking dye may interfere with fluorescent signals and should be omitted but can be used for Coomassie staining.
7. Use equilibration buffer stock to prepare equilibration buffers I and II as needed using 10 mL per IEF strip. Stocks can be stored at −20°C for 3–4 months. Equilibration buffer I and II should be made up fresh on the day of use.
8. To assist accurate spot picking, gels can be immobilized to prevent swelling or shrinking during the staining process. Treat one of the low fluorescent glass plates (one pair of plates used per gel) with bind-silane to achieve this. Alternatively, low fluorescent plastic-backed plates can be used.
9. Agarose sealing solution must be melted before use. Allow solution to cool to $\leq$ 60 °C before use.
10. Make 10 μL aliquots of trypsinization buffer and store at −20 °C for up to 2 weeks.
11. Wear gloves at all times to avoid keratin contamination, which is a key obstacle in further analysis by MS.
12. During sample preparation it is crucial to keep all samples on ice to prevent samples overheating and causing protein modification and protein loss.
13. Ensure that all the solutions containing urea are prepared freshly. To prevent the formation of cyanate which causes protein carbamylation and subsequent formation of charge trains on the 2D gel, do not heat urea containing solutions above room temperature (18–21 °C).
14. Samples being assayed by the Bradford method should be read within 1 h of adding Bradford reagent and where possible kept covered to keep light out.
15. Reconstitute CyDye in quality-grade (> 99.5% pure) anhydrous DMF. Low-quality DMF can result in poor labeling efficiency and shortened shelf life for CyDyes. Store CyDye

solutions at −80 °C and allow them to equilibrate to ice temperature before opening the tube.

16. If gels are to be used for spot picking for MS identification gels should be allowed to polymerize for ≥ 8 h. Unpolymerized acrylamide can induce side-chain and amino terminal protein modifications that can lead to downstream MS identification problems.
17. The maximum pixel intensities of all three CyDye images need to be similar. If the most intense protein spot is saturated (≥ 100,000), the gels need to be rescanned using a lower PMT to ensure all the protein spots fall within the linear dynamic range. It is imperative that the pixel volume is in the correct range (85,000–95,000) to obtain meaningful quantitative comparison between the gel images.
18. Alternative gel staining such as silver staining can be employed. Silver staining is highly sensitive with a protein detection limit of less than 1 ng, however the protocol requires modifications for mass spectrometry compatibility. As a consequence, protein digestion and peptide extraction may be compromised and could potentially lead to poor mass spectrometric protein identification.
19. While manually excising protein spots for identification carry out all steps under sterile conditions in a laminar flow hood to minimize contamination.
20. Print out master gel image from quantitative analysis and use as a visual tool to ensure you are picking the protein spots of interest. It is a good idea to use reference spots to confirm you are picking the correct spot of interest.
21. Potential pause point-samples can be stored at −80 °C for several weeks.
22. Make up trypsin shortly before use. Sequencing grade-modified trypsin reduces auto-digestion, which may otherwise result in additional peptide fragments in a sample, which could interfere with downstream database searching of fragmented peptide masses.
23. Use a sonication bath to ensure protein is fully resuspended. If the pellet is over-dried it will not dissolve in sample buffer.
24. If needed samples can be centrifuged in cellulose spin filter tubes to remove any remaining gel particles.
25. Tissue peptide samples can be analyzed on different mass spectrometers and methods should be tailored for specific equipment.

5 Troubleshooting

- It can be beneficial to run a trial test gel prior to running a full DIGE experiment to make sure your experiment is a success.
- A common reason for poor 2D-DIGE results is in sample preparation- protein concentration and protein labeling. The presence of Tris (> 50 mM) or due to the pH of the protein lysate (< pH 8.0) can result in incomplete labeling and causing a weak fluorescent signal.
- Poor separation of proteins in the first dimension could be due to the presence of interfering contaminants (e.g. nucleic acids and salts), this can be overcome by the use of 2D clean-up kits.
- Poor separation of proteins in the second dimension could be due to carbamylation of proteins, if urea is present in the sample to not heat above 30 °C. If there is vertical streaking on the gel it is generally an indication that an inappropriate concentration of SDS was used.
- If protein identification problems are mainly due to low starting material or due to the parameters selected for the database search (try setting less stringent conditions for database searching: allow for 1–2 missed trypsin cleavages, reduce the mass error for MS to 100–200 ppm and MS/MS to 0.5 Da).

Acknowledgments

Research in the author's laboratory has been supported by project grants from the Irish Higher Education Authority (BioAT) and Muscular Dystrophy Ireland.

References

1. Meissner F, Mann M (2014) Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology. Nat Immunol 15(2):112–117. doi:10.1038/ni.2781
2. Wilhelm M, Schlegl J, Hahne H et al (2014) Mass-spectrometry-based draft of the human proteome. Nature 509(7502):582–587. doi:10.1038/nature13319
3. Dubowitz V, Sewry CA, Oldfors A (2013) Muscle biopsy: a practical approach, 4th edn. Saunders Elsevier, Philadelphia
4. Holland A, Ohlendieck K (2015) Comparative profiling of sperm proteome. Proteomics 15 (4):632–648. doi:10.1002/pmic.201400032
5. Jockusch H, Holland A, Staunton L, Schmitt-John T, Heimann P, Dowling P, Ohlendieck K (2014) Pathoproteomics of testicular tissue deficient in the GARP component VPS54: the wobbler mouse model of globozoospermia. Proteomics 14(7–8):839–852. doi:10.1002/pmic.201300189
6. Holland A, Ohlendieck K (2014) Proteomic identification of muscle-associated biomarkers of amyotrophic lateral sclerosis using the wobbler mouse model of primary motor neuropathy. J Integr OMICS 4(2):57–68. doi:10.5584/jiomics.v4i2.171
7. Holland A, Ohlendieck K (2014) Comparative proteomics for studying muscular dystrophy:

intrinsic biological and analytical issues associated with the systemic utilization of tissue specimens. J Proteomics Bioinform S10:002. doi:10.4172/jbs.S10-002

8. Dowling P, Holland A, Ohlendieck K (2014) Mass spectrometry-based identification of muscle-associated and muscle derived proteomic biomarkers of dystrophinopathies. J Neuromuscul Dis 1(1):15–40. doi:10.3233/JND-140011
9. Rabilloud T, Lelong C (2011) Two-dimensional gel electrophoresis in proteomics: a tutorial. J Proteome 74(10):1829–18241. doi:10.1016/j.jprot.2011.05.040
10. Murphy S, Dowling P, Ohlendieck K (2016) Comparative skeletal muscle proteomics using two-dimensional gel electrophoresis. Proteomes 4(3):27. doi:10.3390/proteomes4030027
11. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250(10):4007–4021
12. Rible H (1973) Historical and theoretical aspects of isoelectric focusing. Ann N Y Acad Sci 209:11–22
13. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077. doi:10.1002/elps.1150181133
14. Arentz G, Weiland F, Oehler MK, Hoffmann P (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9(3–4):277–288. doi:10.1002/prca.201400119
15. Minden JS (2012) DIGE: past and future. Methods Mol Biol 854:3–8. doi:10.1007/978-1-61779-573-2_1
16. Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, Tongue R (2003) Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. Proteomics 3(7):1181–1195. doi:10.1002/pmic.200300439
17. Tonge R, Shaw J, Middleton B, Rowlingson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1((3)):377–396. doi:10.1002/1615–9861(200103)1:3,377::AID-PROT377>3.0.CO;2–6
18. Viswanathan S, Unlu M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protoc 1(3):1351–1358. doi:10.1038/nprot.2006.234
19. Minden J (2007) Comparative proteomics and difference gel electrophoresis. BioTechniques 43(6):739–745
20. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5(12):3105–3115. doi:10.1002/pmic.200500083
21. Karp NA, McCormick PS, Russell MR, Lilley KS (2007) Experimental and statistical consideration to avoid false conclusions in proteomics studies using differential in-gel electrophoresis. Mol Cell Proteomics 6(8):1354–1364. doi:10.1074/mcp.M600274-MCP200
22. Holland A, Schmitt-John T, Dowling P, Meleady P, Henry M, Clynes M, Ohlendieck K (2014) Intricate effects of primary motor neuropathy on contractile proteins and metabolic muscle enzymes as revealed by label-free mass spectrometry. Biosci Rep 34(4):331–343. doi:10.1042/BSR20140029
23. Beckett P (2012) The basics of 2D DIGE. Difference gel electrophoresis (DIGE): methods and protocols. Methods Mol Biol 854:9–18. doi:10.1007/978-1-61779-573-2_2

Chapter 16

DIGE Analysis of Fish Tissues

Joanna Nynca, Mariola A. Dietrich, and Andrzej Ciereszko

Abstract

Two-dimensional difference gel electrophoresis (2D-DIGE) appears to be especially useful in quantitative approaches, allowing the co-separation of proteins of control samples from proteins of treatment/disease samples on the same gel, eliminating gel-to-gel variability. The principle of 2D-DIGE is to label proteins prior to isoelectric focusing and use three spectrally resolvable fluorescent dyes, allowing the independent labeling of control and experimental samples. This procedure makes it possible to reduce the number of gels in an experiment, allowing the accurate and reproducible quantification of multiple samples. 2D-DIGE has been found to be an excellent methodical tool in several areas of fish research, including environmental pollution and toxicology, the mechanisms of development and disorders, reproduction, nutrition, evolution, and ecology.

Key words 2D-DIGE, Minimal labeling, CyDye, Fish, Tissue

1 Introduction

In comparative proteomic studies, it is critical to identify and quantify proteins and their proteoforms. This objective is especially challenging in gel-based methods due to a need to perform several replicate runs to counteract high variability in running gels. Two-dimensional difference gel electrophoresis (2D-DIGE) appears to be especially useful in quantitative approaches, allowing the co-separation of proteins of control samples from proteins of treatment/disease samples on the same gel, eliminating gel-to-gel variability [1]. Contrary to conventional 2-DE, the principle of 2D-DIGE is to label proteins prior to isoelectric focusing. Three spectrally resolvable fluorescent dyes (CyDyes™; Cy5, Cy3, and Cy2) are currently used in most studies, allowing the independent labeling of control and experimental samples. The third dye Cy2 is used to label an internal standard (pool created from a mixture of all samples in the experiment), allowing for precise quantitative analysis [2, 3]. After electrophoresis, each fluorescence signal is scanned independently and, after overlaying changes in protein expression,

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_16,

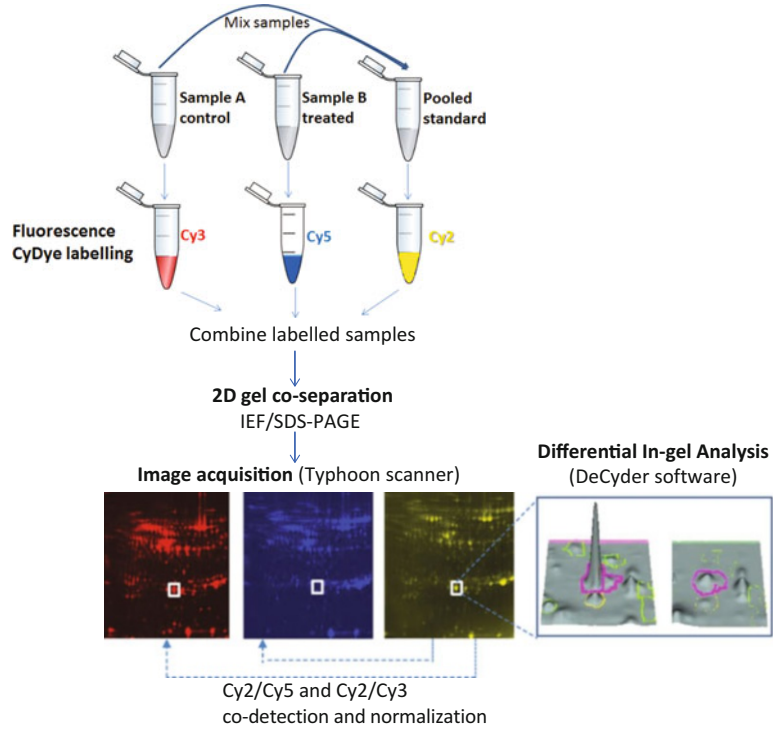

Fig. 1 Workflow for a minimal CyDye labeling experiment

both down-regulated and up-regulated proteins can be visualized (Fig. 1). The use of software tools specifically designed for the evaluation of CyDye™ stained gels enables spot detection, in-gel normalization, and the calculation of protein spot ratios for quantification. The introduction of 2D-DIGE had a profound impact on proteomic research; it minimizes the number of gels in an experiment, allowing the accurate and reproducible quantification of multiple samples.

There are several areas of fish research where 2D-DIGE have been found to be an excellent methodical tool. 2D-DIGE has been indicated as a biomarker discovery tool in aquatic toxicology [1]. Indeed, the most prolific research so far was performed in the studies of environmental pollution and toxicology (Table 1). These studies significantly contributed to ecotoxicology and help obtain advanced mechanistic understanding of the impact of aquatic pollutants in organisms. These pollutants include metals (mercury, cadmium, copper), perfluorochemicals, brominated flame retardant congeners, hormonal substances, pharmaceuticals, and disinfectants. Another important area of research is related to the use of fish as vertebrate models for studying the mechanisms of development and disorders. For toxicological and model studies, research is mainly performed with the use of model species, such as

Table 1
2D-DIGE studies of fish tissues

Area of research	Main subject	Species	References
Genetics	Population proteomics in cardiac protein expression	*Fundulus*	[4]
	Population proteomics of the European hake	European hake	[5]
Environmental pollution/toxicology	Integrated Biomarker Proteomic index to assess the effects of freshwater pollutants	European eel	[6]
	The mechanism of neurotoxical action of inorganic mercury	Medaka	[7]
	Chronic mercury hepatotoxicity	Medaka	[8]
	Cadmium involvement in heat acclimation process	European bullhead	[9]
	Proteomic response to sublethal cadmium exposure	European bullhead	[10]
	Comparative proteomics of Copper exposure and toxicity	Rainbow trout, common carp, gibel carp	[11]
	Proteomic of hepatic tissue and cyanobacterial blooms	Common carp	[12]
	Protein expression signatures after in vivo exposure to perfluorooctane sulfonate	European eel ‘	[13]
	Proteomic responses after perfluorooctane sulfonate exposure	European eel	[14]
	Ecotoxicoproteomics of perfluorooctane sulfonate action	European bullhead	[15]
	Proteomic analysis after exposure to perfluorononanoic acid	Zebrafish	[16]
	Toxicity of PBDE congeners BDE47, BDE153, and BDE154	Atlantic salmon	[17]
	Tetrabromobisphenol-A (TBBPA) toxicity	Zebra fish	[18]
	Ivermectin toxicity	Gilthead sea bream	[19]
	Malachite green toxicity	Asian catfish	[20]
	Long-term effects of malachite green	Asian catfish	[21]
	Cyclosporine A toxicity	Zebrafish	[22]
	Estrogenic and metabolic effects of 17 alpha-ethinylestradiol	Zebrafish	[23]
Breeding/husbandry	Domestication and immune response	Eurasian perch	[24]
	Hypoxia in juvenile Eurasian perch under domestication	Eurasian perch	[25]
	Proteomic evaluation of potentiated sulfa treatment	Gilthead sea bream	[26]

(continued)

Table 1
(continued)

Area of research	Main subject	Species	References
Fish models	Zebra fish as a model for regenerative potential of mammalian heart	Zebrafish	[27]
	Hypoxia-induced changes in skeletal muscle proteome	Zebrafish	[28]
	Zebra fish as an alternate model animal in sleep research	Zebrafish	[29]
	Phenanthrene concentrations and embryo proteome	Zebrafish	[30]
	Xiphophorus Gordon-Kosswig melanoma model	Platyfish X. maculams Jp 163 A, and the swordtail X. helleri (Sarabia)	[31]
	Animal model for human melanoma	Genus *Xiphophorus*	[32]
	Model fish species exposed to individual pesticides and a binary mixture	Fathead minnows	[33]
	Myopia-related marker proteins	Tilapia	[34]
	Hypoxia-related proteins in medaka brain tissue	Medaka	[35]
	Chronic ethanol administration and brain proteins	Zebrafish	[36]
Nutrition and food technology	Metabolic processes involved in the winter thermal stress	Gilthead seabream	[37]
	Performance and physiological effects of three different commercial feeds	Gilthead seabream	[38]
	Slaughtering techniques and postmortem integrity of fish filet proteins	European sea bass	[39]
	Postmortem storage temperature and muscle protein degradation	Sea bass	[40]
	Protein expression profile in relation to increasing levels of phospholipids	Pikeperch	[41]
Reproduction/development	Reproductive performance and oocyte proteomic profile	Eurasian perch	[42]
	Transcriptional and proteomic profiling of spermatogenesis	Flatfish	[43]
	Testis proteome in wild-caught and hormone-treated F1 fish	Senegalese sole	[44]
	Proteome during embryonic development	Zebrafish	[45]
	Activation of sperm motility	Common carp	[46]
	Cryopreservation-related changes in sperm proteome	Common carp	[47]
	Cryopreservation-related changes in sperm proteome	Rainbow trout	[48]
	Seminal plasma proteome in relation to blood plasma proteome	Common carp	[49]

zebrafish and medaka. Studies related to fish reproduction and nutrition are performed with the use of important aquaculture species, such as freshwater carp, rainbow trout, pikeperch and Eurasian perch, and saltwater species such as Senegalese sole, flatfish, and sea bass. Results of reproductive studies significantly contributed to the better understanding of the function of the fish male reproductive system, the development of oocytes, and embryo development [50]. Proteomic research on fish nutrition furthered advances in diet formulation and food technology. The acquired knowledge is a prerequisite for better control of fish reproduction, the improvement of hatchery procedures, and consequently raising of fish production in captivity. Proteomic studies with the use of 2D-DIGE have also been found to be a promising emerging tool in population genetics, allowing better understanding of the mechanisms important for evolution and ecology.

Here we described the methodology of DIGE applied in fish research, focusing specifically on sample preparation and sample labeling using minimal labeling strategy.

2 Materials

2.1 Technical Equipment (See Note 1)

1. Pippetes.
2. Homogenizer.
3. Sonicator.
4. Refrigerated centrifuge.
5. Spectrophotometer or a microtiter-plate reader set up to 595 nm.
6. Microplates or cuvettes.
7. Reswelling tray (GE Healthcare).
8. IPG box (GE Healthcare).
9. Isoelectric focusing system (IPGphore IEF system; GE Healthcare) (*see* **Note 1**).
10. Ceramic IPGphore manifold with electrodes.
11. Immobiline DryStrips (*see* **Note 2**).
12. Paper electrode wicks.
13. Electrophoresis unit (Ettan DALT six apparatus).
14. Power supply.
15. Fluorescent scanner equipped with appropriate excitation wavelength (488, 532, 633 nm) and emission filters (Typhoon 9400 scanner, GE Healthcare).
16. DeCyder 2D Differential analysis Software (GE Healthcare) or similar software.

2.2 Buffers and Solutions

2.2.1 Sample Preparation (See Note 3)

1. DIGE lysis buffer: 30 mM (w/v) Tris, 7 M (w/v) urea, 2 M (w/v) thiourea, 4% (w/v) CHAPS, pH 8.5 (*see* **Note 4**).
2. Protease inhibitors: 200 mM stock solution of phenylmethanesulfonyl fluoride (PMFS) in isopropanol (store at room temperature); 1 mg/mL leupeptin in water (store frozen in aliquots), 1 mg/mL aprotinin in water (store frozen in aliquots), 1 mg/mL pepstatin in methanol (store frozen in aliquots). Commercially available protease inhibitor cocktails can be used instead.
3. Precipitation solutions: 2D Clean-up-kit (GE Healthcare); Acetone stored at −20 °C, trichloroacetic acid (TCA).
4. IPG buffer (*see* **Note 5**).

2.2.2 Protein Quantitation

1. Coomassie (Bradford) Protein Assay Kit (ThermoScientific).
2. Bovine serum albumin (BSA) standard at a concentration of 2 mg/mL.

2.2.3 CyDye™ Minimal Labeling

1. CyDye™ minimal dyes (Cy ™2, Cy ™3, Cy ™5).
2. DIGE lysis buffer: 30 mM (w/v) Tris, 7 M (w/v) urea, 2 M (w/v) thiourea, 4% (w/v) CHAPS, pH 8.0 (*see* **Note 6**).
3. 99.8%, anhydrous dimethylformamide (DMF) (*see* **Note 7**).
4. DIGE "stop" solution: 10 mM L-lysine.

2.2.4 IEF

1. 2× DIGE lysis buffer: 7 M (w/v) urea, 2 M (w/v) thiourea, 4% (w/v) CHAPS, 2% DTT, 2% IPG buffer (*see* **Note 5**).
2. Mineral oil protects IPG strips from drying during IEF (Cover fluid; GE Healthcare).
3. Equilibration buffer: 6 M (w/v) urea, 2% (w/v) SDS, 30% (w/v) glycerol, 50 mM Tris–HCl, pH 8.8. This buffer is either supplemented with 50 mM DTT or 100 mM iodoacetamide (IAA).

2.2.5 SDS-PAGE

1. Ready-to-use precast 12.5% polyacrylamide gels in low fluorescent glass cassette (*see* **Note 8**).
2. DIGE Buffer Kit containing Cathode and Anode buffers. Prepare buffers according to the manufacturer's protocol.
3. Agarose solution (0.4% (w/v) agarose in cathode buffer (125 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) bromophenol blue).

3 Methods

Sample preparation represents the first step in the experiment and therefore is a very crucial step for successful 2D DIGE electrophoresis-based proteomic analysis (*see* **Note 9**). Although a

universal procedure for sample preparation is highly desirable, there is no single method that can be applied to all types of fish tissues. A general protocol described below for the preparation of fish sample includes fundamental steps such as (1) cell disruption (homogenization with lysis buffer), (2) removing of interfering components (sample clean-up), and (3) protein solubilization. Different preparation methods of various fish tissues are summarized in details in Table 2. This protocol describes minimal labeling.

3.1 Sample Preparation

The sample is prepared as for classical 2D gel electrophoresis, except that primary amines, carrier ampholytes, and thiols are omitted from the buffers.

1. Freeze fish tissue in liquid nitrogen and store at −80 °C.
2. For homogenization use 100 mg of the tissue.
3. Place the cells or tissue sample in a 1.5-mL centrifuge tube.
4. Homogenize the tissue a few passes or turns of the pestle on liquid nitrogen.
5. Sonicate in lysis buffer (with proteinase inhibitor cocktail) three times × 10 s pulses on ice (depending on sample size).
6. To remove unbroken cells and insoluble debris centrifuge (12,000 × *g* for 5–15 min, 4 °C) the lysate.
7. Collect the supernatant to 1.5 mL eppendorf tubes.
8. Precipitate the protein using acetone. To collected supernatant (equivalent to 50 μg of total protein) add four volumes of ice-cold acetone and incubate at −20 °C for 120 min. Pellet the proteins from this 80% acetone precipitation step by centrifugation for 10 min in a microfuge at maximum speed.
9. Allow the pellet to air dry but do not over-dry.
10. Add lysis buffer to the pellet and gently disperse it using a pipet fitted with a pipet tip. Sonicate the sample for 10 min in a sonicating water bath and incubate on a vortex shaker at 20 °C for 60 min.
11. Alternatively, precipitate the protein using Clean-up Kit according to the manufacturer's instructions (GE Healthcare).

3.2 Protein Quantitation

1. Assess the protein concentration according to the Thermo-Scientifc user guide.
2. A standard curve should be made with BSA dissolved in lysis buffer at a concentration range of 0.5–2.5 mg/mL. The blank, the standards, and samples should be made with lysis buffer (*see* **Note 10**).
3. Place standard human albumin or real samples in triplicate in polystyrene microtiter plates, and add assay reagents.

Table 2
Methods of fish cell and tissue preparation for 2D-DIGE

Method of cell or tissue disruption	Fish cell or tissue	Species	References
Homogenization, extraction with TriReagent/Trizol reagent, optionally washing with 0.3 M guanidinium chloride in ethanol, centrifugation (13,000 × *g*; 10 min; RT), precipitation with ice-cold acetone (−20 °C, overnight)	Brain Liver Gill	Medaka (*Oryzias latipes*) Zebrafish (*Danio rerio*) Common carp (*Cyprinus carpio*) Gibel carp (*Carrassius auratus gibelio*) Rainbow trout (*Oncohynchus mykiss)*	[35] [18, 23] [11]
Homogenization in homogenization buffer (10 mM Tris–HCl; 250 mM sucrose; 1 mM EDTA; pH 7), centrifugation (1000 × *g*; 8 min; 4 °C) followed by second centrifugation of supernatant (7700 × *g*; 10 min; 4 °C)	Gill	European bullhead (*Cottus gobio*)	[9]
Protein extraction in ProteoPrep Chaotropic Extraction Reagent (Sigma), homogenization, sonication, centrifugation (10,000 × g; 10 min; 4 °C)	Fin	Platyfish (*Xiphophorus maculatus)*	[31]
Homogenization in TNE buffer (50 mM Tris–HCl pH 7.6; 150 mM NaCl; 2 mM EDTA pH 8.0; 1 mM Na_3VO_4; 1 μL leupeptin, 2 μL pepstatin; 0.1 μL aprotinin; 1% NP-40; 1 mM PMSF; 2 mM DTT;), sonication (10 min, 4 °C), precipitation using 2-D Clean-Up kit (GE Healthcare)	Testis	Senegalese sole (*Solea senegalensis*)	[43, 44]
Sonication in 94 mM NaCl, 27 mM KCl, 50 mM glycine, 15 mM Tris–HCl, pH 7.5, 0.1% Triton) on ice six times for 5 s at 30% amplitude, incubation on ice for 1 h and centrifuged for 10 min at 14,000 × *g* at 4 °C., Clean-up kit Precipitation with Clean-up kit, in ice-cold acetone or in TCA/acetone, centrifugation (10,000 × *g*; 10 min; 4 °C)	Sperm PBMC Hepatocytes Brain	Common carp (*Cyprinus carpio*) European eel (*Anguilla anguilla*) Marine medaka (*Oryzias melastigma*) Medaka (*Oryzias latipes*)	[50] [6, 14] [8] [7]

Dilution in lysis buffer (7 M Urea, 2 M Thiourea, 2% w/v CHAPS, 10–20 mM Tris), optionally sonication, centrifugation	Seminal plasma	Common carp (*Cyprinus carpio*)	[49]
		Rainbow trout (*Oncorhynchus mykiss*)	[48]
	Blood plasma	Common carp (*Cyprinus carpio*)	[49]
	Serum	Eurasian perch (*Perca fluviatilis*)	[24, 25]
		Sea bream (*Sparus aurata*)	[38]
	PBMC	Asian catfish *(Pangasianodon hypophthalmus)*	[21]
Homogenization in ND-RIPA buffer (50 mM Tris; 1% CHAPS; 150 mM NaCl; 1% Triton X-100; 1% Nonidet P-40; 2 mM NaF; 2 mM Na_3VO_4; protease inhibitor cocktail; pH 7.5) on ice, sonication, precipitation in cold pure acetone (1:4; −30 °C, 2 h) and centrifugation (10,000 × *g*; 5 min; 4 °C)	PBMC	Asian catfish *(Pangasianodon hypophthalmus)*	[20]
	Oocyte	Eurasian perch (*Perca fluviatilis*)	[42]
	Gill	European bullhead (*Cottus gobio*)	[9]
Homogenization or sonication in lysis buffer (30 mM Tris; 7 M urea; 2 M thiourea; 4% CHAPS) on ice, centrifugation (14,000 × *g*; 5 min, 4 °C), optionally precipitation with Clean-up kit, or ice-cold acetone or in TCA/acetone	Brain	Fathead minnow *(Pimephales promelas)*	[33]
		Zebrafish (*Danio rerio*)	[29, 36]
	Heart	Zebrafish (*Danio rerio*)	[27]
		Fundulus heteroclitus, F. grandis	[4]
	Liver	Zebrafish (*Danio rerio*)	[16]
		Sea bream (*Sparus aurata*)	[19, 26, 37, 38]
		Pikeperch (*Sander lucioperca*)	[41]
		Common carp (*Cyprinus carpio*)	[12]
		Atlantic salmon (*Salmo salar*)	[17]
	Muscle	European sea bass (*Dicentrarchus labrax*)	[39, 40]
		Zebrafish (*Danio rerio*)	[28]
	Embryo	Zebrafish (*Danio rerio*)	[22, 30, 45]

PBMC peripheral blood mononuclear cells

4. Measure the optical densities (595 nm) with a microtiter-plate reader.
5. Interpolate the protein concentrations in the samples from the regression analysis of the standard curve.

The recommended protein concentration is between 5 and 10 mg/mL (*see* **Note 11**).

3.3 CyDye™ Minimal Labeling

Prior to labeling, solubilize sample in DIGE lysis buffer. The optimal pH range for minimal labeling is between pH 8.0 and 9.0 (*see* **Note 12**).

3.3.1 Preparation of CyDye DIGE Fluor Minimal Dyes

1. Reconstitute CyDye in anhydrous DMF to a concentration of 1 nmol/μL (stock solution) by adding 5 μL to each vial of CyDye according to the manufacturer's protocol.
2. Vortex and spin down the dye stock solution in a microfuge.
3. To create 400 pmol/μL of working dye solution dilute 1 volume of the stock CyDye in 1.5 volume of DMF (*see* **Note 13**).

The amount of CyDye used in the labeling reaction will have to be determined individually for the experiment.

3.3.2 Protein Labeling with the CyDye DIGE Fluor Minimal Dyes

Prepare an experimental design whereby the biological replicates within an experiment group are labeled equally with either Cy3 and Cy5 (i.e., for an experiment with six biological replicates per group, three should be labeled with Cy3 and three with Cy5) (*see* **Note 14**, Table 3).

1. For sample labeling, add 1 μL (400 pmol) Cy3 or Cy5 dyes to 50 μg of each sample within an experimental group (control, treated) and mix by vortexing (*see* Table 3).

Table 3
Experimental set up for CyDye™ labeling of six control samples and six treated samples with the incorporation of a pooled internal standard

	Cy2	Cy3	Cy5
Gel 1	50 μg Pooled Std.	50 μg Control 1	50 μg Treated 3
Gel 2	50 μg Pooled Std.	50 μg Treated 1	50 μg Control 4
Gel 3	50 μg Pooled Std.	50 μg Control 2	50 μg Treated 4
Gel 4	50 μg Pooled Std.	50 μg Treated 2	50 μg Control 5
Gel 5	50 μg Pooled Std.	50 μg Control 3	50 μg Treated 6
Gel 6	50 μg Pooled Std.	50 μg Treated 5	50 μg Control 6

Total Internal standard 300 μg (from a pool of 12 × 25 μg, each from control sample 1–6 and treated sample 1–6)

2. Centrifuge briefly in a microcentrifuge to collect the solution at the bottom of the tube. Leave the tube on ice and in the dark for 30 min.
3. Add 1 μL of 10 mM L-lysine to stop the reaction. Mix and spin the tube briefly in a microcentrifuge. Leave the tube on ice and in the dark for 10 min.
4. To create internal standard take an aliquot of 25 μg from each sample and mix them together in one vial (*see* **Note 15**).
5. Add a volume of pooled internal standard equivalent to $n \times 50$ μg protein to a microcentrifuge tube (n is the number of gels in the experiment).
6. Add n μL of diluted Cy2 to the microcentrifuge tube containing the pooled standard.
7. Mix and centrifuge briefly. Leave on ice for 30 min in the dark.
8. Add n μL of 10 mM L-lysine to stop the reaction. Mix and spin briefly in a centrifuge. Leave for 10 min in the dark.

Labeling is now finished. The labeled samples can be processed immediately or stored for up to 3 months at −80 °C in the dark.

3.4 Preparation for Loading the Samples onto IPG Strips

1. Mix appropriate 50 μg of Cy3-labeled sample and 50 μg of Cy5-labeled sample with Cy2-labeled pooled internal standards to give a total of 150 μg protein load/gel according to the experimental design (*see* Table 3).
2. Add an equal volume of 2 × lysis buffer to each pooled sample and leave on ice for 10 min.
3. Adjust the volume of the sample with a 1:1 mix of DIGE "lysis" buffer and 2 × lysis buffer, with a trace amount of bromophenol blue, to a total of 450 μL.

This protocol use 24 cm strip and rehydration sample loading for example.

3.5 IEF

1. Distribute the sample solution mixture evenly in the reswelling tray.
2. Remove protective foil from the IPG gel strips and rehydrate the IPG strips (remember gel-side down) for at least 8 h at RT in the dark.
3. Place the rehydrated IPG gel strips gel-side up with the basic end toward cathode in the manifold on the IPGphor unit.
4. Apply electrode paper wicks, soaked with water to each end of the strip.
5. Position the electrodes.
6. Cover the entire surface of the gels with Cover Fluid.

7. Perform IEF according to the manufacturer's instructions. The optimal gradient depends on the sample and length and pH range of IPG strip.
8. After the IEF run, the strips can be used for the second dimension immediately or can be stored at −80 °C.

3.6 SDS-PAGE

1. Equilibrate the strips in room temperature in equilibration buffer firstly supplemented with 50 mM DTT (15 min), secondly with 100 mM IAA (15 min).
2. Rinse the strips in running buffer (cathodic).
3. Place IPG strip on top of the second-dimension gel with the plastic touching the back glass and the acidic end of the strip toward the left. Gently push the IPG strip down until it contacts the stacking gel.
4. Cover the IPG strip with melted agarose until it just covers the IPG strip. Make sure there are no bubbles.
5. Place the gel in the electrophoresis unit, and fill the upper and lower chambers with tank buffer.
6. Electrophorese at a constant current with a maximum voltage set at 500. The current at 10–25 mA per gel is set (e.g., 25 mA per gel requires ~10 h to complete and 15 mA per gel takes ~16 h).

3.7 Scanning the Gels

1. After electrophoresis, leave the gels between the glass plates and acquire the images using the Typhoon 9400 scanner (*see* **Note 3**). Therefore, choose excitation wavelengths and emission filters specific for each of the CyDyes according to the Typhoon user guide.
2. Before scanning clean the glass plates thoroughly to avoid introducing any fluorescent particles (dust, lint, etc.).
3. Prescan the gel images using low-resolution setting (200 μm) so that the final image capture setting can be optimized to avoid saturation.
4. The final image is scanned at 100 μm resolution.

An example gel is presented in Fig. 2.

3.8 Image Analysis

1. Perform image analysis using appropriate software (DeCyder GE Healthcare). The outcome of image analysis is a list of difference-protein spots that indicate significant differences between the two groups (control and treated) being compared.

These protein spots can then be excised for further analysis, such as by mass spectrometry to identify the protein.

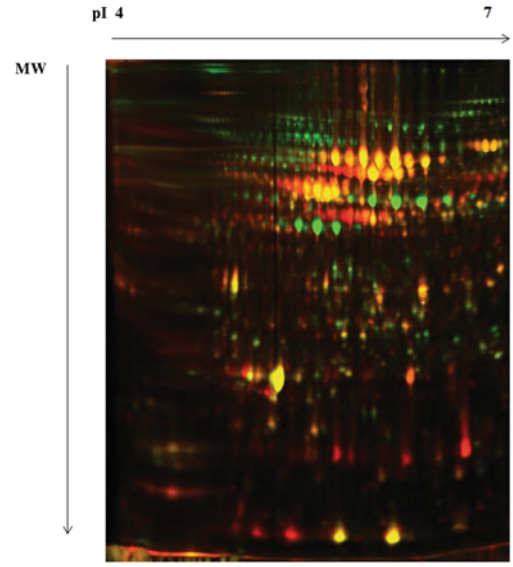

Fig. 2 An overlay fluorescence image of rainbow trout seminal and blood plasma labeled with three different dyes (internal standard with Cy2, blood plasma with Cy3, seminal plasma with Cy5). Internal standard and sample of seminal and blood plasma were co-separated, 50 μg protein of each sample and in total of 150 μg on 18 cm IPG dry strip pH 4–7 during IEF followed by the separation by SDS-PAGE

4 Notes

1. The experiment is performed using equipment from GE Healthcare (Sweden), however suitable equipment is also available from BioRad.
2. The optimal pH range and length of the IPG strips for IEF depends on the sample and should be analyzed to optimize the resolution. Choose shorter strips for fast screening or when the most abundant proteins are of interest. Use longer strips for maximal resolution and loading capacity. Use a pH interval of 3–10 for an overview of total protein distribution. For increased resolution between pH 5 and 7, use 3–10 NL (Non Linear) to distribute the proteins more evenly over the gel.
3. Use high-purity water and chemicals for all buffers. Do not change chemicals during an experiment to avoid variability of the results. Several reagents used for the sample preparation are toxic. For safety reasons use protective gloves and glasses. Wear gloves at all times to avoid keratin contamination, which is a key obstacle to further analysis by mass spectrometry.

4. Never heat urea solutions above room temperature (30 °C) as this will cause the formation of cyanate, which carbamylates protein and produces charge trains in 2D gels.
5. Use the appropriate IPG buffer that corresponds to the pH range of the IEF strips in the experiment. IPG buffers are ampholyte-containing buffer concentrates, each IPG buffer type produces more uniform conductivity along the Immobiline DryStrip during focusing, resulting in wider latitude in run times. IPG buffers also eliminate high background staining.
6. For efficient labeling the protein samples should have optimally pH 8.5; it must be above pH 8.0. Proteins have some inherent buffering capacity and may have decreased the pH value of the sample solution below pH 8. Samples which have been cleaned up with TCA acetone or the Ettan™ 2-D clean up kit can be acidic. Beware: Sometimes, when samples have been transported in dry ice and the tubes have not been sealed well enough, CO_2 has diffused into the samples and caused a strong drop of the pH value. In this case, it might be necessary to adjust the pH value with 250 mM NaOH.
7. DMF should be less than 3 months old from the day of opening. The quality of the DMF is critical to ensure that the protein labeling is successful. The DMF must be anhydrous and every effort should be used to ensure it is not contaminated with water. DMF after opening, over a period of time, will degrade with amine compounds being produced. Amines will react with the NHS ester CyDye reducing the concentration of dye available for protein labeling.
8. Reproducibility of spot patterns can be facilitated by the use of precast gels for the second dimension.
9. The sample preparation protocol should be kept as simple as possible to avoid protein loss and to ensure reproducibility of the results. Handling of sample should be carried out on ice to minimize proteolytic degradation. The addition of protease inhibitors is also recommended.
10. As several constituents of the lysis buffer (urea, CHAPS, and DTT) may affect protein concentration measurements, the standards and samples should all be made up in lysis buffer. If the sample is too concentrated, dilute with lysis buffer.
11. Independently from the experiment size, at least 75 μg of each sample is required: 50 μg for sample labeling, 25 μg for the creation of internal standard.
12. Test sample pH by spotting 0.1–0.5 μL of the sample on a standard pH indicator strip.
13. It is recommended to use 400 pmol of dye for labeling 50 μg of protein.

14. To avoid a bias in the results due to specific fluorescent properties of the dyes, swap the dyes in such a way that for each condition replicates exist that are labeled with either Cy3 or Cy5 (Table 3).
15. Label slightly more protein for the Cy2 internal standard than according to the independent number of gels (e.g., 10% more) to allow for variation in pipetting. This is to ensure that there is sufficient Cy2-labeled protein for all the gels.

Acknowledgements

This work was supported by a Projects: 2011/01/D/NZ9/00619; 2011/01/D/NZ9/00628; 2015/17/B/NZ9/01542 and 2016/21/B/NZ9/03620 from the National Science Centre, as well as funds appropriated to the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences.

References

1. Martyniuk CJ, Alvarez S, Denslow ND (2012) DIGE and iTRAQ as biomarker discovery tools in aquatic toxicology. Ecotoxicol Environ Saf 76:3–10
2. Beckett P (2012) The basis in 2D DIGE. In: Cramer A, Westermeier R (eds) Difference gel electrophoresis (DIGE); methods and protocols, Methods in molecular biology, vol 854. Humana, Clifton, NJ, pp 9–19
3. Arentz G, Weiland F, Oehler MK, Hoffmann P (2015) State of the art of 2D DIGE. Proteomics Clin Appl 9:277–288
4. Rees BB, Andacht T, Skripnikova E, Crawford DL (2011) Population proteomics: quantitative variation within and among populations in cardiac protein expression. Mol Biol Evol 28:1271–1279
5. Gonzalez EG, Krey G, Espineira M, Diez A, Puyet A, Bautista JM (2010) Population proteomics of the European hake (*Merluccius merluccius*). J Proteome Res 9:6392–6404
6. Roland K, Kestemont P, Dieu M, Raes M, Silvestre F (2016) Using a novel "integrated biomarker proteomic" index to assess the effects of freshwater pollutants in European eel peripheral blood mononuclear cells. J Proteomics 137:83–96
7. Wang YY, Wang DZ, Lin L, Wang MH (2015) Quantitative proteomic analysis reveals proteins involved in the neurotoxicity of marine medaka *Oryzias melastigma* chronically exposed to inorganic mercury. Chemosphere 119:1126–1133
8. Wang MH, Wang YY, Zhang L, Wang J, Hong HS, Wang DZ (2013) Quantitative proteomic analysis reveals the mode-of-action for chronic mercury hepatotoxicity to marine medaka (*Oryzias melastigma*). Aquat Toxicol 130:123–131
9. Dorts J, Kestemont P, Thezenas ML, Raes M, Silvestre F (2014) Effects of cadmium exposure on the gill proteome of *Cottus gobio*: modulatory effects of prior thermal acclimation. Aquatic Toxicol 154:87–96
10. Dorts J, Kestemont P, Dieu M, Raes M, Silvestre F (2011) Proteomic response to sublethal cadmium exposure in a sentinel fish species, *Cottus gobio*. J Proteome Res 10:470–478
11. Eyckmans M, Benoot D, Van Raemdonck GAA, Zegels G, Van Ostade XWM, Witters E, Blust R, De Boeck G (2012) Comparative proteomics of copper exposure and toxicity in rainbow trout, common carp and gibel carp. Comp Biochem Physiol D Genomics Proteomics 7:220–232
12. Jiang JL, Wang XR, Shan ZJ, Yang LY, Zhou JY, Bu YQ (2014) Proteomic analysis of hepatic tissue of *Cyprinus carpio* L. exposed to cyanobacterial blooms in Lake Taihu, China. PLoS One 9:e88211. doi:10.1371/journal.pone.0088211
13. Roland K, Kestemont P, Loos R, Tavazzi S, Paracchini B, Belpaire C, Dieu M, Raes M, Silvestre F (2014) Looking for protein expression signatures in European eel peripheral blood mononuclear cells after in vivo exposure

to perfluorooctane sulfonate and a real world field study. Sci Total Environ 468:958–967

14. Roland K, Kestemont P, Henuset L, Pierrard MA, Raes M, Dieu M, Silvestre F (2013) Proteomic responses of peripheral blood mononuclear cells in the European eel (*Anguilla anguilla*) after perfluorooctane sulfonate exposure. Aquat Toxicol 128-129:43–52
15. Dorts J, Kestemont P, Marchand PA, D'Hollander W, Thezenas ML, Raes M, Silvestre F (2011) Ecotoxicoproteomics in gills of the sentinel fish species, *Cottus gobio*, exposed to perfluorooctane sulfonate (PFOS). Aquat Toxicol 103:1–8
16. Zhang W, Liu Y, Zhang HX, Dai JY (2012) Proteomic analysis of male zebrafish livers chronically exposed to perfluorononanoic acid. Environ Int 42:20–30
17. Softeland L, Petersen K, Stavrum AK, Wu T, Olsvik PA (2011) Hepatic in vitro toxicity assessment of PBDE congeners BDE47, BDE153 and BDE154 in Atlantic salmon (*Salmo salar* L.) Aquat Toxicol 105:246–263
18. De Wit M, Keil D, Remmerie N, van der Ven K, van den Brandhof EJ, Knapen D, Witters E, De Coen W (2008) Molecular targets of TBBPA in zebrafish analysed through integration of genomic and proteomic approaches. Chemosphere 74:96–105
19. Varo I, Rigos G, Navarro JC, del Ramo J, Calduch-Giner J, Hernandez A, Pertusa J, Torreblanca A (2010) Effect of ivermectin on the liver of gilthead sea bream *Sparus aurata*: a proteomic approach. Chemosphere 80:570–577
20. Pierrard MA, Kestemont P, Delaive E, Dieu M, Raes M, Silvestre F (2012) Malachite green toxicity assessed on Asian catfish primary cultures of peripheral blood mononuclear cells by a proteomic analysis. Aquat Toxicol 114:142–152
21. Pierrard MA, Kestemont P, Phuong NT, Tran MP, Delaive E, Thezenas ML, Dieu M, Raes M, Silvestre F (2012) Proteomic analysis of blood cells in fish exposed to chemotherapeutics: evidence for long term effects. J Proteomics 75:2454–2467
22. Ponnudurai RP, Basak T, Ahmad S, Bhardwaj G, Chauhan RK, Singh RA, Lalwani MK, Sivasubbu S, Sengupta S (2012) Proteomic analysis of zebrafish (*Danio rerio*) embryos exposed to cyclosporine A. J Proteomics 75:1004–1017
23. De Wit M, Keil D, van der Ven K, Vandamme S, Witters E, De Coen W (2010) An integrated transcriptomic and proteomic approach characterizing estrogenic and metabolic effects of 17 alpha-ethinylestradiol in zebrafish (*Danio rerio*). Gen Comp Endocrinol 167:190–201
24. Douxfils J, Lambert S, Mathieu C, Milla S, Mandiki SNM, Henrotte E, Wang N, Dieu M, Raes M, Rougeot C, Kestemont P (2014) Influence of domestication process on immune response to repeated emersion stressors in Eurasian perch (*Perca fluviatilis*, L.) Comp Biochem Physiol A Mol Integr Physiol 173:52–60
25. Douxfils J, Deprez M, Mandiki SNM, Milla S, Henrotte E, Mathieu C, Silvestre F, Vandecan M, Rougeot C, Melard C, Dieu M, Raes M, Kestemont P (2012) Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication–-clues to physiological acclimation and humoral immune modulations. Fish Shellfish Immunol 33:1112–1122
26. Varo I, Navarro JC, Rigos G, Del Ramo J, Calduch-Giner JA, Hernandez A, Pertusa J, Torreblanca A (2013) Proteomic evaluation of potentiated sulfa treatment on gilthead sea bream (*Sparus aurata* L.) liver. Aquaculture 376:36–44
27. Gomes RSM, Skroblin P, Munster AB, Tomlins H, Langley SR, Zampetaki A, Yin XK, Wardle FC, Mayr M (2016) "Young at heart": regenerative potential linked to immature cardiac phenotypes. J Mol Cell Cardiol 92:105–108
28. Chen K, Cole RB, Rees BB (2013) Hypoxia-induced changes in the zebrafish (*Danio rerio*) skeletal muscle proteome. J Proteomics 78:477–485
29. Purushothaman S, Saxena S, Meghah V, Lakshmi MGM, Singh SK, Swamy CVB, Idris MM (2015) Proteomic and gene expression analysis of zebrafish brain undergoing continuous light/dark stress. J Sleep Res 24:458–465
30. Gundel U, Kalkhof S, Zitzkat D, von Bergen M, Altenburger R, Kuster E (2012) Concentration-response concept in ecotoxicoproteomics: effects of different phenanthrene concentrations to the zebrafish (*Danio rerio*) embryo proteome. Ecotoxicol Environ Saf 76:11–22
31. Perez AN, Oehlers L, Heater SJ, Booth RE, Walter RB, David WM (2012) Proteomic analyses of the Xiphophorus Gordon-Kosswig melanoma model. Comp Biochem Physiol C Toxicol Pharmacol 155:81–88
32. Lokaj K, Meierjohann S, Schuetz C, Teutschbein J, Schartl M, Sickmann A (2009) Quantitative differential proteome analysis in an animal model for human melanoma. J Proteome Res 8:1818–1827
33. Biales AD, Bencic DC, Flick RL, Blocksom KA, Lazorchak JM, Lattier DL (2011) Proteomic

analysis of a model fish species exposed to individual pesticides and a binary mixture. Aquat Toxicol 101:196–206

34. Jostrup R, Shen W, Burrows JTA, Sivak JG, McConkey BJ, Singer TD (2009) Identification of myopia-related marker proteins in tilapia retinal, RPE, and choroidal tissue following induced form deprivation. Curr Eye Res 34:966–975
35. Oehlers LP, Perez AN, Walter RB (2007) Detection of hypoxia-related proteins in medaka (*Oryzias latipes*) brain tissue by difference gel electrophoresis and de novo sequencing of 4-sulfophenyl isothiocyanate-derivatized peptides by matrix-assisted laser desorption/ionization time-of-flight mass, spectrometry. Comp Biochem Physiol C Toxicol Pharmacol 145:120–133
36. Damodaran S, Dlugos CA, Wood TD, Rabin RA (2006) Effects of chronic ethanol administration on brain protein levels: a proteomic investigation using 2-D DIGE system. Eur J Pharmacol 547:75–82
37. Richard N, Silva TS, Wulff T, Schrama D, Dias JP, Rodrigues PML, Conceicao LEC (2016) Nutritional mitigation of winter thermal stress in gilthead seabream: associated metabolic pathways and potential indicators of nutritional state. J Proteomics 142:1–14
38. Ghisaura S, Anedda R, Pagnozzi D, Biosa G, Spada S, Bonaglini E, Cappuccinelli R, Roggio T, Uzzau S, Addis MF (2014) Impact of three commercial feed formulations on farmed gilthead sea bream (*Sparus aurata*, L.) metabolism as inferred from liver and blood serum proteomics. Proteome Sci 12:44. doi:10.1186/s12953-014-0044-3
39. Addis MF, Pisanu S, Preziosa E, Bernardini G, Pagnozzi D, Roggio T, Uzzau S, Saroglia M, Terova G (2011) 2D DIGE/MS to investigate the impact of slaughtering techniques on postmortem integrity of fish filet proteins. J Proteomics 75:3654–3664
40. Terova G, Addis MF, Preziosa E, Pisanu S, Pagnozzi D, Biosa G, Gornati R, Bernardini G, Roggio T, Saroglia M (2011) Effects of postmortem storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: analysis by 2-D DIGE and MS. Proteomics 11:2901–2910
41. Hamza N, Silvestre F, Mhetli M, Ben Khemis I, Dieu M, Raes M, Cahu C, Kestemont P (2010) Differential protein expression profile in the liver of pikeperch (*Sander lucioperca*) larvae fed with increasing levels of phospholipids. Comp Biochem Physiol D Genomics Proteomics 5:130–137
42. Castets MD, Schaerlinger B, Silvestre F, Gardeur JN, Dieu M, Corbier C, Kestemont P, Fontaine P (2012) Combined analysis of *Perca fluviatilis* reproductive performance and oocyte proteomic profile. Theriogenology 78:432–442
43. Forne I, Castellana B, Marin-Juez R, Cerda J, Abian J, Planas JV (2011) Transcriptional and proteomic profiling of flatfish (*Solea senegalensis*) spermatogenesis. Proteomics 11:2195–2211
44. Forne I, Agulleiro MJ, Asensio E, Abian J, Cerda J (2009) 2-D DIGE analysis of Senegalese sole (*Solea senegalensis*) testis proteome in wild-caught and hormone-treated F1 fish. Proteomics 9:2171–2181
45. Lucitt MB, Price TS, Pizarro A, Wu W, Yocum AK, Seiler C, Pack MA, Blair IA, FitzGerald GA, Grosser T (2008) Analysis of the zebrafish proteome during embryonic development. Mol Cell Proteomics 7:981–994
46. Dietrich MA, Dietrich GJ, Mostek A, Ciereszko A (2016) Motility of carp spermatozoa is associated with profound changes in the sperm proteome. J Proteomics 138:124–135
47. Dietrich MA, Arnold GJ, Fröhlich T, Otte KA, Dietrich GJ, Ciereszko A (2015) Proteomic analysis of extracellular medium of cryopreserved carp (*Cyprinus carpio* L.) semen. Comp Biochem Physiol D Genomics Proteomics 15:49–57
48. Nynca J, Arnold GJ, Fröhlich T, Ciereszko A (2015) Cryopreservation-induced alterations in protein composition of rainbow trout semen. Proteomics 15:2643–2654
49. Dietrich MA, Arnold GJ, Nynca J, Fröhlich T, Otte KA, Ciereszko A (2014) Characterization of carp seminal plasma proteome in relation to blood plasma. J Proteomics 98:218–232
50. Ciereszko A, Dietrich MA, Nynca J (2017) Fish semen proteomics–new opportunities in fish reproductive research. Aquaculture 472:81–92

Part V

Auxillary DIGE Methods

Chapter 17

Protein Digestion for DIGE Analysis

Sandra Murphy and Kay Ohlendieck

Abstract

In-gel digestion of protein spots derived from two-dimensional gels and their subsequent identification by mass spectrometry is involved in a multitude of mass spectrometry-driven proteomic experiments, including fluorescence difference gel electrophoresis (DIGE). This type of proteomic methodology has been involved in the establishment of comparative proteome maps and in the identification of differentially expressed proteins and protein isoforms in health and disease. Most in-gel digestion protocols follow a number of common steps including excision of the protein spots of interest, de-staining, reduction and alkylation (for silver-stained gels), dehydration and overnight digestion with the proteolytic enzyme of choice. While trypsin has been a mainstay of peptide digestion for many years, it does have its shortcomings, particularly related to incomplete peptide digestion, and this has led to a rise in popularity for other proteolytic enzymes either used alone or in combination. This chapter discusses the alternative enzymes available and describes the process of in-gel digestion using the enzyme trypsin.

Key words Trypsin, Alternative proteolytic enzymes, Protein digestion, Two-dimensional gel electrophoresis, Mass spectrometry

1 Introduction

First described in 1975 [1, 2], two-dimensional gel electrophoresis (2D-GE) has become a core technology in the field of proteomics. The advent of 2D difference gel electrophoresis (2D-DIGE) [3] further improved the field, enabling higher sensitivity and reproducibility to be achieved [4]. While a plethora of protocols are available and optimization is required for individual experiments, the following steps are generally common to all methodologies. In 2D-DIGE, proteins are labeled with one of three mass and charge-matched fluorescent dyes known as CyDyes (Cy2, Cy3, and Cy5) [5], enabling samples to be pooled together and run on the same gel reducing issues of gel-to-gel variation [6]. Proteins are separated first by their isoelectric point in isoelectric focusing and subsequently by their molecular weight in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [7]. The resulting 2D gels are scanned and image analysis, involving spot detection

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_17,

and spot matching, is performed [8]. Protein spots of interest are isolated from a preparative 2D gel, either manually or using a robotic spot picker and the subsequent gel pieces are de-stained, dehydrated with acetonitrile and then rehydrated in a protease enzyme solution [9]. Digestion is usually conducted overnight and the resulting peptides are extracted and analyzed by mass spectrometry [10].

Protein digestion represents one of the critical steps in a proteomic workflow in both gel-based ("in-gel" protein digestion) and gel-free methodologies ("in-solution" protein digestion) [11]. The extracted peptides are analyzed by mass spectrometry and in order to identify the proteins the experimentally obtained peptide masses are compared to theoretical peptide masses of proteins in a database [12]. Peptide mass fingerprinting works particularly well with proteins obtained from high-resolution 2D gel electrophoresis [13]. Thus in this type of bottom-up or shotgun proteomics approach, in which peptides are used to identify their corresponding proteins, the choice of an optimal proteolytic enzyme is paramount.

Trypsin is the most frequently used conventional enzyme for protein digestion given its high specificity and ability to produce peptide fragments within the preferred mass range for fragmentation by tandem mass spectrometry [14]. Despite its popularity trypsin does have some shortcomings, particularly related to incomplete digestion. Some of these mis-cleavages are due to the inability of trypsin to cleave C-terminal to arginine or lysine in situations where they are directly followed by proline [15]. Trypsin is also less effective for the digestion of tightly folded proteins and membrane proteins, which can show resistance to trypsin and few available trypsin cleavage sites respectively [16]. For this reason, alternative proteases to improve qualitative proteome coverage have been proposed [17, 18]. LysC is one of the more frequently used enzymes of these alternative proteases, and is often used in conjunction with trypsin to reduce the number of mis-cleaved peptides and to improve digestion of tightly folded proteins [19]. A double digestion with LysC and trypsin can be achieved sequentially with samples digested first by LysC and then by trypsin overnight [20, 21]. Alternatively, LysC/trypsin mixes are available from a number of vendors.

AspN can also be used in conjunction with other proteases to improve proteome coverage [22]. Choudhary and colleagues [23] have shown that sequence coverage of the recombinant protein tissue plasminogen activator increased from 65% when digested with trypsin alone to 93.9% when digested with a combination of trypsin, LysC, and AspN. While chymotrypsin and trypsin tend to produce similar numbers and lengths of peptides [24], chymotrypsin helps in the identification of hydrophobic proteins mainly due to its ability to cleave peptides at the carboxyl side of hydrophobic amino acids [25, 26]. The serine protease GluC may also improve

proteome coverage and enable the study of post-translational modifications, particularly when used in conjunction with other enzymes. Biringer et al. [24] demonstrated that combining trypsin and GluC-derived peptides resulted in improved coverage of post-translational modification sites such as phosphorylation sites. GluC, AspN, and chymotrypsin are useful for identifying post-translational modifications on histone proteins as they generate peptides which are long enough to enable the identification of PTMs occurring on individual histones [27]. Such in-depth analysis is not possible with trypsin given the shorter length of trypsin-derived peptides.

Although not widely used in the field of proteomics, LysN used in combination with trypsin has been shown to increase protein coverage (although only a modest increase in the number of proteins identified was reported, primarily due to a high degree of overlap between the two enzymes) [28]. LysN-generated peptides are particularly amenable to electron transfer dissociation (ETD), producing peptide fragments dominated by *c*-ions. This generates simple sequence ladders of peptides which facilitates de novo sequencing and the analysis of post-translational modifications [29]. ArgC digestion alone is inferior to that of trypsin in terms of the number of peptides and proteins identified. However, when combined with trypsin it improves the average sequence coverage per protein and thus represents an attractive option for augmenting proteome coverage [30].

Some of the key characteristic of these alternative enzymes, including their specificity, suggested dilutions and applications are summarized in Table 1. Thus while advances in the sensitivity and speed of mass spectrometers is suspected to pave the way for increased coverage of the proteome, the use of alternative proteases and combinations of multiple proteases may also aid in this endeavor. In this chapter, we describe a protein digestion protocol for in-gel digestion using trypsin as the protease. This method may be modified to incorporate other enzymes as determined by the final user.

2 Materials

2.1 Equipment

Equipment for the in-gel digestion of protein spots of interest (as identified from DIGE analysis):

1. Thermomixer comfort (Eppendorf).
2. Model 5417R centrifuge (Eppendorf).
3. Heto speedvac concentrator (Medical Supply Company).
4. Agilent 6340 Ion Trap LC mass spectrometer using electrospray ionization (Agilent Technologies).

Table 1
Proteolytic enzymes for protein digestion in a proteomics workflow

Enzyme	Recommended ratio enzyme/protein	Specificity	Applications
Trypsin	1:25	R. K	Trypsin is the most commonly used proteolytic enzyme. It is efficient, specific, and produces peptides amenable to CID fragmentation.
LysC	1:100	K	Typically used in conjunction with trypsin as it can cleave lysines which are followed by proline residues. Therefore, it compensates for trypsin mis-cleavages Also used in phospho-peptide enrichment protocols
Chymotrypsin	1:60	Y, W, F	Useful for digestion of peptides arising from hydrophobic proteins. This protease is particularly useful for studying the transmembrane regions of membrane proteins
GluC	1:20–1:200	D, Z	Useful for the study of PTMs. Can be combined with other proteases to increase proteome coverage
ArgC	1:20–1:200	R, K	Useful for the study of PTMs, especially histone PTMs. Can be combined with other proteases to increase proteome coverage
AspN	1:20–1:200	D, C	Useful for the study of PTMs
LysN	1:20–1:200	K	Useful for epigenetic studies since it is capable of cleaving methylated lysines

The table shows the recommended ratio and specificity of seven commercially available proteases and gives notes on their potential applications

5. Nanoflow Agilent 1200 series system, equipped with a Zorbax 300SB C18 μm, 4 mm 40 nl pre-column (Agilent Technologies).

2.2 Reagent Solutions

All reagents should be prepared with ultrapure water to limit contamination of samples. The following reagents are required:

2.2.1 Destaining of Preparative Slab Gels

1. Destain for Coomassie gels: 1:1 (v/v) 100 mM ammonium bicarbonate/acetonitrile.
2. Destain for silver-stained gels: 1:1 (v/v) 30 mM potassium ferricyanide/100 mM sodium thiosulfate.

2.2.2 Reduction and Alkylation of Silver-Stained Gel Plugs

1. Reducing buffer: 10 mM DTT (dithiothreitol) in 100 mM ammonium bicarbonate.
2. Alkylating buffer: 55 mM IAA (iodoacetamide) in 100 mM ammonium bicarbonate.

2.2.3 Trypsin Digestion and Peptide Extraction

1. Trypsin reconstitution buffer: 50 mM acetic acid.
2. Trypsination buffer: 20 μg sequencing-grade trypsin resuspended in 100 μl of reconstitution buffer. Add 10 μl of this to 500 μl of 50 mM ammonium bicarbonate.
3. Extraction buffer: 1:2 (v/v) 5% formic acid/acetonitrile. Make this solution just before use.

3 Methods

3.1 Destaining for Coomassie and Silver-Stained Gels

1. Preparative slab gels for spot picking can be stained with either Coomassie or silver stain. The destaining protocol will vary depending on the type of staining used (*see* **Note 1**).
2. Rinse preparative slab gel in ultrapure water for 2–3 h in a laminar flow hood (*see* **Note 2**).
3. Excise protein spots of interest manually using a sterile pipette tip with the end cut off. Place the tip (cut side down) firmly on top of the protein spot of interest and take up the gel piece (*see* **Note 3**). Transfer to a 1.5 ml microcentrifuge tube. Label each tube with its corresponding spot number.
4. For Coomassie stained gels, add 100 μl of 100 mM ammonium bicarbonate/acetonitrile solution (1:1) to each gel piece. Incubate for 30 min (ensure gel plugs are completely destained) at room temperature with occasional vortexing. Add 500 μl of neat acetonitrile and incubate for 10 min at room temperature with shaking (*see* **Note 4**).
5. Remove solution. Gel plugs are ready for in-gel digestion. Alternatively, they may be stored at −20 °C for a couple of weeks.
6. For silver-stained gels, add 100 μl of a 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1) solution to each gel plug. Once the brown color disappears, wash the plugs with ultrapure water 3–4 times (or until the plugs are completely clear) (*see* **Note 5**).

3.2 Reduction and Alkylation

1. This is required for silver-stained gels only. Add 500 μl of neat acetonitrile and incubate for 10 min. Centrifuge briefly and remove all liquid.
2. Add 50 μl of 10 mM DTT in 100 mM ammonium bicarbonate and incubate for 30 min at 56 °C.
3. Cool tubes to room temperature (22 °C) and add 500 μl of neat acetonitrile and incubate for 10 min at room temperature. Aspirate the liquid.

4. Add 50 µl of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and incubate for 20 min at room temperature in the dark.
5. Add 500 µl of neat acetonitrile and incubate for 1 0 min at room temperature with shaking.
6. Aspirate all the liquid.
7. Gel plugs are now ready for in-gel digestion (*see* **Note 6**).

3.3 In-Gel Digestion

1. Resuspend 20 µg trypsin in 100 µl of reconstitution buffer (*see* **Note 7**). Add 10 µl aliquots to 500 µl 50 mM ammonium bicarbonate and add 50 µl of this mixture to gel plugs. Incubate for 30 min at 4 °C.
2. Add more buffer to fully cover gel plugs and incubate for a further 90 min at 4 °C (*see* **Note 8**).
3. Add 10–20 µl 100 mM ammonium bicarbonate (*see* **Note 9**) to fully cover gel plugs (*see* **Note 10**) and incubate overnight at 37 °C.

3.4 Peptide Extraction

1. Add 100 µl extraction buffer (*see* **Note 11**) to each gel plug and incubate for 15 min at 37 °C with shaking (*see* **Note 12**).
2. Remove the peptides-containing supernatant and transfer to a fresh microcentrifuge tube.
3. Dry down in a vacuum centrifuge.
4. Can proceed to LC-MS or can store dried peptides at −20 °C for a few months prior to usage.

3.5 LC-MS Analysis

1. Resuspend dried peptides in 15 µl 0.1% formic acid.
2. Vortex well and sonicate for 5 min to ensure full resuspension of peptides.
3. Centrifuge at 14,000 × *g* for 20 min and transfer supernatant to labeled LC-MS vials.
4. Analyze peptide mixtures on an ion-trap LC mass spectrometer.
5. Specific conditions will need to be optimized for individual mass spectrometers. However, using a 15 min gradient of 10–90% solvent B (50% acetonitrile and 0.1% formic acid) and a 1–5 min post run time of solvent A (0.1% formic acid) through a Zorbax 300SB C18µm column works well for most proteins.
6. For example, separate peptides with a nanoflow Agilent 1200 series system, equipped with a Zorbax 300SB C18 5 µm, 4 mm 40 nl pre-column and a Zorbax 300SB C18 5 µm, 43 mm × 75 mm analytical reversed phase column using HPLC-Chip technology.

7. Load 5 μl of sample into the enrichment at a capillary flow rate set to 2 μl/min with a mix of solvents A and B at a ratio of 19:1.
8. Set the capillary voltage to 1700 V, the flow rate to 4 μl/min and the drying gas temperature to 300 °C.
9. For protein identification, database searches may be conducted with MASCOT MS/MS Ion search (Matrix Science, London, UK) (*see* **Note 13**).

4 Notes

1. Coomassie stains are easily removed from gel plugs prior to in-gel digestion, but are less sensitive than other stains with a detection limit of 100 ng. Silver staining is highly sensitive, with a detection limit of less than 1 ng. However, silver staining is not compatible with mass spectrometry and therefore requires a more advanced destaining protocol. This may hinder protein digestion and peptide extraction and thus lead to poor mass spectrometric identification.
2. At all stages it is vital to limit, as much as possible, keratin contamination. Due to their proteinaceous nature, keratin contaminants will undergo enzymatic digestion and thus will be present in the final peptide mixture. This is a problem particularly for the detection of low abundance peptides by mass spectrometry as such peptides can be masked by keratin (if keratin is present at higher levels than the proteins of interest). This is especially true for mass spectrometers run in data-dependent mode. In order to limit keratin contamination, wear a lab coat and gloves, work in a laminar flow hood as much as possible, keep all pipette tip boxes, reagent bottles, and sample vials closed/covered when not in use and employ only HPLC grade reagents.
3. For manual spot picking print off an image of the 2D gel (2D-DIGE gel image), ensuring that the image fills an A4 page so that it will be the same size as a 2D gel. Circle the protein spots of interest on the paper and number them. Label microcentrifuge tubes with the spot numbers. Place the gel on a thick plastic bag, leave one side sealed and three sides open for ease of access. Place the image of the gel underneath the actual gel and align so that the circled protein spots on the paper align to the spots on the gel. Cut out spots of interest and place in its corresponding numbered tube.
4. Acetonitrile dehydrates and shrinks the gel plugs.
5. Can wash gel plugs with 100 μl of 200 mM ammonium bicarbonate for 20 min at room temperature with shaking if gel plugs are not destaining with water.

6. Gel plugs can be processed immediately or stored at −20 °C for a few weeks.
7. Make shortly before use. Ensure to use sequencing-grade trypsin, which has been modified to reduce autolysis which may otherwise result in additional peptide fragments in a sample which could interfere with database searching of fragment masses.
8. Incubating at 4 °C enables the slow and efficient diffusion of trypsin into gel plugs.
9. While our lab uses a 100 mM ammonium bicarbonate buffer, other groups use 25 mM ammonium bicarbonate to reduce any possible salt interference with ionization in mass spectrometry [31].
10. It is important to keep gel plugs immersed throughout the digestion procedure.
11. Volumes may be adjusted depending on the volume of the gel matrix. There should be an approximate ratio of 1:2 between the volume of the digest and that of the extraction buffer.
12. Other extraction protocols are available. These include extraction with 5% TFA/50% acetonitrile with vortexing and sonication [32], 66% acetonitrile/0.1% TFA with sonication [31] and 100% acetonitrile for 10 min at 25 °C (repeated three times).
13. Recommended parameters for filtering data obtained from searches with MASCOT include (a) a maximum of two missed cleavages by trypsin, (b) carboxymethylated cysteines as a fixed modification, (c) methionine oxidation as a variable modification, (d) a minimum of two unique peptides, and (e) a MASCOT score greater than 49. All of the p*I* values and molecular masses of the identified proteins should be compared to their corresponding position on two-dimensional preparative slab gels.

Acknowledgements

Research in the author's laboratory has been supported by project grants from the Irish Higher Education Authority, the Irish Health Research Board and Muscular Dystrophy Ireland, as well as a Hume Scholarship from Maynooth University.

References

1. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021
2. Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for

induced point mutations in mammals. Humangenetik 26:231–243
3. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
4. Marouga R, David S, Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382:669–678
5. Wu WW, Wang G, Baek SJ, Shen RF (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. J Proteome Res 5:651–658
6. Tonge R, Shaw J, Middleton B et al (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1:377–396
7. Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685
8. Dowsey AW, Morris JS, Gutstein HB, Yang GZ (2010) Informatics and statistics for analyzing 2-d gel electrophoresis image. Methods Mol Biol 604:239–255
9. Shevchenko A, Tomas H, Havlis J et al (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860
10. Tannu NS, Hemby SE (2006) Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. Nat Protoc 1:1732–1742
11. Choksawangkarn W, Edwards N, Wang Y et al (2012) Comparative study of workflows optimized for in-gel, in-solution, and on-filter proteolysis in the analysis of plasma membrane proteins. J Proteome Res 11:3030–3034
12. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422:198–207
13. Thiede B, Höhenwarter W, Krah A et al (2005) Peptide mass fingerprinting. Methods 35:237–247
14. Olsen JV, Ong SE, Mann M (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics 3:608–614
15. Siepen JA, Keevil EJ, Knight D, Hubbard SJ (2007) Prediction of missed cleavage sites in tryptic peptides aids protein identification in proteomics. J Proteome Res 6:399–408
16. Griffin NM, Schnitzer JE (2011) Overcoming key technological challenges in using mass spectrometry for mapping cell surfaces in tissues. Mol Cell Proteomics 10:R110.000935
17. Giansanti P, Tsiatsiani L, Low TY, Heck AJ (2016) Six alternative proteases for mass spectrometry-based proteomics beyond trypsin. Nat Protoc 11:993–1006
18. Swaney DL, Wenger CD, Coon JJ (2010) Value of using multiple proteases for large-scale mass spectrometry-based proteomics. J Proteome Res 9:1323–1329
19. Glatter T, Ludwig C, Ahrné E et al (2012) Large-scale quantitative assessment of different in-solution protein digestion protocols reveals superior cleavage efficiency of tandem Lys-C/trypsin proteolysis over trypsin digestion. J Proteome Res 11:5145–5156
20. Dowling P, Hayes C, Ting KR et al (2014) Identification of proteins found to be significantly altered when comparing the serum proteome from multiple myeloma patients with varying degrees of bone disease. BMC Genomics 15:904
21. Holland A, Henry M, Meleady P et al (2015) Comparative label-free mass spectrometric analysis of mildly versus severely affected mdx mouse skeletal muscles identifies annexin, lamin, and vimentin as universal dystrophic markers. Molecules 20:11317–11344
22. Guo X, Trudgian DC, Lemoff A et al (2014) Confetti: a multiprotease map of the HeLa proteome for comprehensive proteomics. Mol Cell Proteomics 13:1573–1584
23. Choudhary G, Wu SL, Shieh P, Hancock WS (2003) Multiple enzymatic digestion for enhanced sequence coverage of proteins in complex proteomic mixtures using capillary LC with ion trap MS/MS. J Proteome Res 2:59–67
24. Biringer RG, Amato H, Harrington MG et al (2006) Enhanced sequence coverage of proteins in human cerebrospinal fluid using multiple enzymatic digestion and linear ion trap LC-MS/MS. Brief Funct Genomic Proteomic 5:144–153
25. Temporini C, Calleri E, Cabrera K et al (2009) On-line multi-enzymatic approach for improved sequence coverage in protein analysis. J Sep Sci 32:1120–1128
26. Vermachova M, Purkrtova Z, Santrucek J et al (2011) New protein isoforms identified within Arabidopsis thaliana seed oil bodies combining chymotrypsin/trypsin digestion and peptide fragmentation analysis. Proteomics 11:3430–3434
27. Tweedie-Cullen RY, Brunner AM, Grossmann J et al (2012) Identification of combinatorial patterns of post-translational modifications on

individual histones in the mouse brain. PLoS One 7:e36980

28. Hohmann L, Sherwood C, Eastham A et al (2009) Proteomic analyses using Grifola frondosa metalloendoprotease Lys-N. J Proteome Res 8:1415–1422
29. Taouatas N, Drugan MM, Heck AJ, Mohammed S (2008) Straightforward ladder sequencing of peptides using a Lys-N metalloendopeptidase. Nat Methods 5:405–407
30. Wiśniewski JR, Mann M (2012) Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. Anal Chem 84:2631–2637
31. Katayama H, Nagasu T, Oda Y (2001) Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 15:1416–1421
32. Jiménez CR, Huang L, Qiu Y, Burlingame AL (2001) In-gel digestion of proteins for MALDI-MS fingerprint mapping. Curr Protoc Protein Sci, Chapter 16:Unit 16.4

Chapter 18

Subcellular Fractionation for DIGE-Based Proteomics

Sandra Murphy

Abstract

Mass spectrometry-based protein methodologies have revolutionized the field of analytical biochemistry and enable the identification of hundreds to thousands of proteins in biological fluids, cell lines, and tissue. This methodology requires the initial separation of a protein constellation and this has been successfully achieved using gel-based techniques, particularly that of two-dimensional difference gel electrophoresis (2D-DIGE). However, given the complexity of the proteome, fractionation techniques may be required to optimize the detection of low-abundance proteins, which are often under-represented, but which may represent important players in health and disease. Such subcellular fractionation protocols typically utilize density-gradient centrifugation and have enabled the enrichment of crude microsomes, the cytosol, the plasmalemma, the nuclei, and the mitochondria. In this chapter, we describe the experimental steps involved in the enrichment of crude microsomes from skeletal muscle using differential centrifugation and subsequent verification of enrichment by gel electrophoresis and immunoblotting, prior to comparative DIGE analysis.

Key words Muscle proteomics, Ultracentrifugation, Subcellular fractionation, Two-dimensional gel electrophoresis, Microsomal enrichment strategy

1 Introduction

Since the elucidation of the human genome in 2001 [1], scientific research has entered the "post-genomic era" in which proteomics has come to the fore. Despite only containing approximately twice the number of genes found in a simple roundworm [2] or a fruit fly [3], the human species is virtually unrivaled in its complexity, suggesting that proteins and not genes are responsible for the intricacy of an organism. This has thus led to the emergence of proteomics as a core technology for the enhanced understanding of an organism.

Mass spectrometry-based proteomics has developed as the methodology of choice for the large-scale profiling of proteins in health and disease [4–6]. The high resolution and reproducibility of modern mass spectrometers enables the routine identification and quantitation of hundreds and even thousands of proteins [7],

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_18,

rendering such technology the "gold standard" for the large-scale analysis of a proteome. Two-dimensional gel electrophoresis was first described in 1975 as a means of separating proteins from complex biological materials using orthogonal methods. First proteins are separated by their isoelectric point in isoelectric focusing and second by their molecular mass by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [8, 9]. However as issues pertaining to gel-to-gel variation limited the reproducibility of such studies, two-dimensional difference gel electrophoresis (2D-DIGE) became the method of choice. 2D-DIGE involves the fluorescence labeling of paired samples with two or three different dyes enabling them to be run on the same gel, thus circumventing the need to run multiple 2D gels [10, 11]. Software, such as Progenesis SameSpots and DeCyder 2D, facilitates the detection of differential protein expression and such proteins can then be excised from the gel and identified by mass spectrometry [10]. Comparative proteomic studies utilizing 2D-DIGE have enabled the identification of proteins involved in gestational diabetes [12], sarcopenia of old age [13], cancer [14, 15], and neuromuscular disease [16] along with a plethora of other conditions.

One of the major difficulties with comparative proteomics is the high dynamic range and complexity of the proteome. This is particularly evident in bodily fluids such as human plasma where excesses of 10 orders of magnitude exist between the concentrations of the most highly abundant proteins such as albumin and the proteins of lowest abundance such as enzymes and interleukins [17–20]. This concentration range far exceeds the dynamic range of proteomic analytical tools, thus rendering the detection of such low-copy number proteins technically challenging. Similar analytical difficulties also exist with the mass spectrometric analysis of tissue in general and muscle in particular. Individual skeletal muscles represent a heterogeneous assembly of fast-glycolytic, fast-oxidative-glycolytic, slow-oxidative and hybrid fiber types, each with considerably different protein constellations [21]. Complexity also exists on the molecular level with skeletal muscle expressing a particularly large number of different protein isoforms [22]. While this huge variety of proteoforms is essential for muscle plasticity, it represents an issue for both the gel electrophoresis and mass spectrometry aspects of a proteomic workflow. Low abundance proteins are typically underrepresented on 2D gels due to masking effects from other higher abundance proteins with similar p*I* and molecular mass values [23], while on the level of mass spectrometry highly abundant peptides lead to ion suppression of low abundance peptides limiting their isolation, fragmentation, and detection [6, 24].

These difficulties have forced researchers to use a combination of proteomic methodologies, encompassing whole tissue proteomics and organelle proteomics, to give optimal coverage of the proteome [25]. Similar to immunodepletion for serum samples

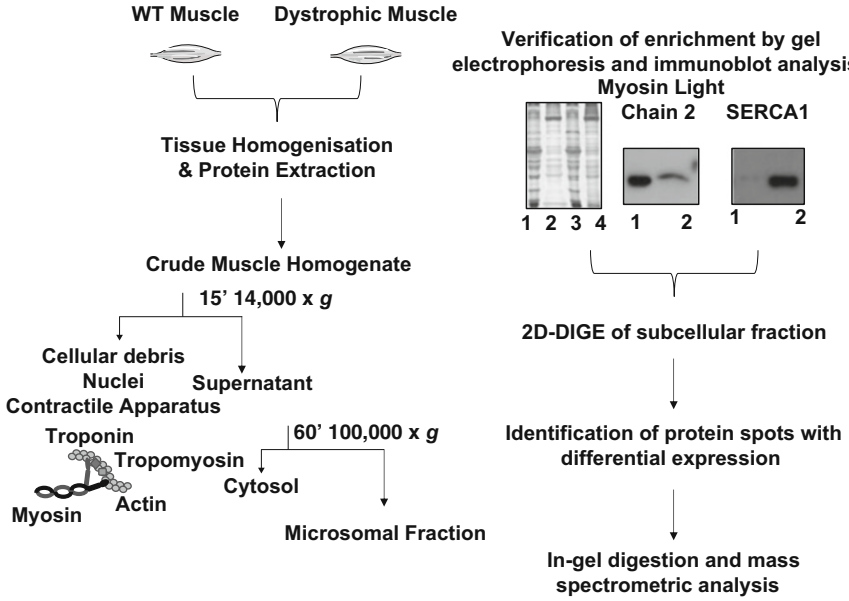

Fig. 1 Overview of the subcellular fractionation protocol. Illustrated on the left is a flowchart showing the subcellular fractionation protocol for the enrichment of crude microsomes from wild-type (WT) versus dystrophic skeletal muscle homogenates. Shown on the right is the verification analysis using gel electrophoresis and immunoblotting, where *lanes 1–4* refer to WT total extracts, WT crude microsomes, dystrophic *mdx-4cv* total extracts and dystrophic *mdx-4cv* crude microsomes, respectively. Comparative 2D-DIGE and mass spectrometric analysis are used to enable the identification of differentially expressed low abundance proteins in subcellular fractions

[26], subcellular fractionation for tissue offers a means of reducing sample complexity thus enabling the mass spectrometric detection of low abundance proteins. These types of sub-proteomic studies have largely concentrated on mitochondria given their important roles in health and disease. Mitochondrial dysfunction and oxidative stress are associated with numerous disorders; in particular cardiac abnormalities, skeletal muscle disorders, and age-related neurodegenerative conditions [27–29]. However, a variety of other cellular fractions can also be enriched by using subcellular fractionation, typically microsomes, sarcolemma, cytosol, and the contractile apparatus [16, 30, 31]. Here, we describe in detail the subcellular fractionation of skeletal muscle to give crude microsomes which can be subsequently analyzed by 2D-DIGE, as illustrated in Fig. 1.

2 Materials

2.1 Equipment

Equipment for the preparation of crude skeletal muscle homogenates and crude microsomes, as well as gel electrophoresis and immunoblotting:

1. Hand-held IKA T10 Basic Homogeniser (IKA Labortechnik).
2. Thermomixer comfort (Eppendorf).
3. Model 5417R centrifuge (Eppendorf).
4. Optiseal 4.9 ml polyallomer centrifuge tubes (Beckman Coulter).
5. Optima L-100 XP ultracentrifuge (Beckman Coulter).
6. NVT 90 rotor (Beckman Coulter).
7. Aluminum spacer part number 362198 (Beckman Coulter).
8. Mini-gel PROTEAN Tetra Cell system (Bio-Rad).
9. Transblot Cell for electrophoretic transfer of proteins to Immobilon NC-pure nitrocellulose membranes (Bio-Rad).
10. Exposure cassettes (Kodak).

2.2 Reagent Solutions

All reagents were prepared with ultrapure water to limit contamination of samples. Commercially available reagents, materials and solutions for sample preparation, gel electrophoresis and immunoblotting include:

1. Ultrapure Protogel acrylamide stock solution.
2. Protogel resolving buffer 4×.
3. Laemmli-type reducing buffer.
4. Protein molecular mass standard.
5. Whatman nitrocellulose transfer membrane.
6. BM Chemiluminescence western blotting kit.
7. X-ray film.
8. Protease inhibitor cocktail.
9. Phosphate-buffered saline tablets.
10. Primary antibodies; ab2818 to SERCA1 and ab92721 to MLC2 (Abcam).
11. Secondary peroxidase-conjugated antibody.

2.2.1 Preparation of Crude Homogenates and Resuspension of Microsomal Pellets

1. Homogenization buffer: 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5 mM EDTA, pH 7.0. This buffer should be supplemented with protease inhibitors, such as one tablet of a commercially available protease inhibitor cocktail per 10 ml buffer. Dispense into 1 ml aliquots and store at −20 °C.
2. DIGE buffer: 9.5 M urea, 2 M thiourea, 65 mM CHAPS, 100 mM DTT, supplemented with a protease inhibitor cocktail. Microsomal pellets can be resuspended in DIGE lysis buffer for subsequent DIGE analysis or homogenization buffer for immunoblot analysis.

2.2.2 Electrophoretic Transfer and Immunoblotting

1. 10× SDS running buffer: 25 mM Tris, 1.92 M glycine, 1% (w/v) SDS. Dilute to 1× before use and use as gel electrophoresis buffer if using hand-cast gels.
2. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
3. Ponceau staining solution: 0.1% (w/v) Ponceau S dye in 5% (v/v) acetic acid.
4. Phosphate-buffered saline solution: 1 PBS tablet per 100 ml water (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4).
5. Blocking buffer:5% (w/v) fat-free milk solution in PBS.
6. Primary antibody: Appropriately diluted primary antibody in blocking buffer.
7. Secondary antibody: Appropriately diluted secondary antibody in blocking buffer.

3 Methods

3.1 Preparation of Crude Skeletal Muscle Homogenates

All the steps should be carried out at 4 °C to minimize degradation of skeletal muscle proteins.

1. Thaw frozen skeletal muscle on ice. Rinse muscle in PBS to remove blood/hair and trim away any fat (*see* **Note 1**). For comparative biomedical studies of a neuromuscular disorder, weigh the muscle and use an equal amount for control and diseased samples. A relatively large amount of tissue is necessary for subcellular fractionation procedures. While the amount required will have to be optimized by the user, between 0.75 and 1 g is sufficient for subsequent DIGE analysis and verification by immunoblot analysis (*see* **Note 2**).
2. Using a sharp razor blade, finely chop the tissue into small pieces.
3. Transfer diced tissue to a round-bottomed 15 ml falcon tube (*see* **Note 3**) and add 10 volumes of homogenization buffer (*see* **Note 4**).
4. Using a hand-held homogenizer, blend the tissue at 30 s intervals until the mixture is homogenous. Avoid over-heating of the homogenizer and sample. Allow the liquid to settle to the bottom of the tube and then transfer to a 1.5 ml microcentrifuge tube (*see* **Note 5**).
5. Extract proteins by incubating samples at 8 °C at 300 rpm for 2 h on a Thermomixer.
6. Centrifuge the solution at 14,000 × *g* for 20 min at 4 °C. Retain the protein-containing supernatant and transfer to a

fresh microcentrifuge tube. Discard the pellet and the uppermost fatty layer.

7. If samples are not to be used immediately, dispense into 50 µl aliquots and store at −20 °C for short-term or −80 °C for long-term storage.

3.2 Preparation of the Crude Microsomal Fraction

1. Precool the rotor in a 4 °C room at least 1 h before ultracentrifugation.
2. Fill OptiSeal 4.9 ml tubes to the bottom of the stem (these tubes must be completely filled for ultracentrifugation) with the crude skeletal muscle homogenate. Retain a portion of the crude homogenate for later analysis. Close tube with a tube plug.
3. Weigh each tube with its spacer and adaptor. Ensure that tubes that will be opposite each other during ultracentrifugation are exactly the same weight to three decimal places. Adjust volumes of liquid accordingly to ensure that the tubes are balanced.
4. Dry tubes with tissue paper and then load into the precooled rotor. Place the appropriate spacer on the top of the tube, followed by the rotor plug (gasket side down). Screw the plugs into place and leaving the screw in place tighten with a wrench to 120 ounces. Remove screw and ensure all tubes are flush with the top of the rotor.
5. Rub some vacuum grease along the edges of the rotor. Place the rotor into the ultracentrifuge and close the door.
6. Centrifuge samples at $100{,}000 \times g$ at $4^{\circ}C$ for 1 h.
7. Loosen the rotor plugs with a wrench and then a screw, remove the tubes from the rotor. Aspirate the supernatant (this represents the cytosol). Resuspend the pellet in an appropriate buffer, for example DIGE lysis buffer for subsequent 2D-DIGE analysis (*see* **Note 6**).
8. Quantify the protein concentration of the original crude skeletal muscle homogenates and the crude microsomal fraction using a reliable assay (*see* **Note 7**).

3.3 Verification of Enrichment of Membrane Protein Fraction

Immunoblot analysis can be used to verify the large-scale removal of the acto-myosin apparatus and the concomitant enrichment of membrane and membrane-associated proteins in the crude microsomal fraction.

1. Run one-dimensional gels using either precast or hand-made polyacrylamide gels (*see* **Note 8**). For precast gels refer to manufacturer's instructions as to the setup of the gel apparatus. For most routine immunoblotting experiments 10–20 µg protein per lane is sufficient. Reduce samples by adding a reducing buffer, such as Laemmli-type buffer, and heat at 97 °C for

7 min. Allow the samples to cool and then load onto the gel. Electrophorese samples at 60 V until they pass through the stacking gel and then increase the voltage to 120 V and run until the Bromophenol Blue dye front just runs off the end of the gel.

2. Remove the gel from between the glass plates and incubate in transfer buffer for 20 min.
3. Prepare for membrane transfer by cutting filter paper and nitrocellulose membranes to the size of the gel to be transferred.
4. Soak a sponge in the transfer buffer, squeeze to remove excess liquid and place on the anode side of the transblot holder. Place two pieces of presoaked filter paper on the top of this, followed by the nitrocellulose membrane that should also be rinsed in transfer buffer so that it is evenly wet. Carefully place the gel on the top of the membrane, place two more pieces of filter paper on the top, followed by a sponge. Using a 15 ml falcon tube or a roller, gently roll the assembled gel sandwich to remove any air bubbles which would otherwise affect the transfer of proteins to the membrane.
5. Place the cathode side of the transblot holder on the gel sandwich and transfer to the electrophoretic transfer unit. Fill the unit with transfer buffer and put an ice-pack into the unit also (*see* **Note 9**). Place the entire unit in a container of ice or run electrophoresis in a 4 °C room. Carry out electrophoretic transfer at 100 V for 70 min.
6. Verify the efficacy of the transfer using Ponceau staining solution. Rinse in Ponceau until protein bands can be visualized, typically 1 min. Rinse in distilled water to remove excess pink dye. Label the nitrocellulose membrane with a black marker while protein lanes are still visible.
7. Remove Ponceau stain by rinsing nitrocellulose membranes in PBS for 10 min. Repeat three times.
8. Block nitrocellulose membranes with a milk protein solution for 1 h at room temperature (*see* **Note 10**). Briefly rinse with PBS before incubating with appropriately diluted primary antibodies overnight at 4 °C with gentle agitation (*see* **Note 11**).
9. Wash nitrocellulose membranes twice with the blocking buffer for 10 min each time.
10. Incubate nitrocellulose membranes with appropriately diluted peroxidase-conjugated secondary antibodies for 90 min at room temperature with gentle shaking (*see* **Note 11**).
11. Block nitrocellulose membranes twice with the blocking buffer for 10 min each time.

12. Wash nitrocellulose membranes twice with PBS for 10 min each time.
13. Visualize immuno-decorated protein bands by the enhanced chemiluminescence method (ECL) in a dark room.
14. Wash nitrocellulose membranes with 5 ml of ECL solution, containing 50 μl ECL starting solution and 5 ml of luminescence substrate. If the signal is intense bands may be seen "glowing." If not then incubate nitrocellulose membranes in the ECL solution for between 1 and 5 min.
15. Place the nitrocellulose membrane in between two acetate sheets in an exposure cassette.
16. Place a piece of X-ray film on the top and incubate for as long as required. This will need to be optimized by the user as strong signals require much less time than very weak signals which could take hours (*see* **Note 12**).
17. Place X-ray film into a container of commercially available developer until clear bands can be seen.
18. Transfer X-ray film to a container of commercially available fixer and rinse briefly (*see* **Note 13**).
19. Allow X-ray film to dry. They can then be used for densitometric analysis (*see* **Note 14**). For verifying the removal of the actomyosin apparatus from the microsomal fraction, protein markers such as myosin light chain isoforms and tropomyosin can be used. To verify the enrichment of membrane proteins, antibodies against membrane-associated proteins, such as the SERCA isoforms of the Calcium-ATPase from the sarcoplasmic reticulum or beta-dystroglycan from the sarcolemma, may be employed.

3.4 Comparative DIGE Analysis

Once immunoblotting analysis has been used to verify the success of the subcellular fractionation, proceed to comparative DIGE analysis [11]. Briefly, proteins arising from the control sample are labeled with one of the CyDyes and proteins arising from the test sample are labeled with the other CyDye [10]. A pooled internal standard is labeled with Cy2 [32]. CyDye labeled proteins (control, test, and standard) can be pooled together and the protein constellation is first separated by isoelectric focusing and subsequently by two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis [33]. 2D gels are then scanned with fluorescence scanning, where each of the samples can be visualized independently by choosing the unique excitation and emission wavelengths for each CyDye [34]. A preparative gel must also be run, such gels are stained, for example with silver stain, Coomassie stain or deep purple total protein stain, and protein spots of interest are excised from these gels, digested and analyzed by mass spectrometry.

4 Notes

1. Areas of fatty and connective tissue in skeletal muscle can be difficult to homogenize and so excess fat should be trimmed off.
2. The exact amount of tissue to be used should be optimized by the end user. It will largely depend on the downstream analyses required by the user. For example, larger amounts of tissue will be required for gel-based mass spectrometric proteomic work as opposed to label-free liquid chromatography mass spectrometry methodologies.
3. Use round-bottomed tubes instead of traditional 15 ml falcon tubes as the tissue tends to sink to the bottom of the tube and it is easier to homogenize at the bottom of a round-bottomed tube rather than a pointed end.
4. To limit the degradation of muscle-associated proteins during sample homogenization the homogenization buffer was supplemented with a protease inhibitor cocktail, using one tablet of Roche's "Complete Mini" for every 10 ml of buffer.
5. Samples tend to foam up a little during homogenization so allow the liquid to settle to the bottom of the tube before transferring it to a 1.5 ml microcentrifuge tube. Keep the original 15 ml tube and continue to transfer the remaining liquid as it settles.
6. After aspirating the supernatant, use a scissors to cut off the top of the OptiSeal tube in order to be able to resuspend the pellet. Resuspend by gently pipetting up and down. Transfer the crude microsomal fraction to a fresh microcentrifuge and vortex to resuspend fully.
7. A number of commercially available protein quantification assay kits may be used for this purpose. In our lab we use the Bradford method to quantify protein concentrations. However in situations where detergents such as Triton X-100 have been included in the buffers an alternative assay that is compatible with detergents must be used, for example the BCA protein assay.
8. Depending on the proteins of interest, different acrylamide percentages can be used. For large proteins a low acrylamide percentage of 3–4% is recommended, while smaller proteins require a higher acrylamide percentage. Gradient gels, for example 4–12% gels, may also be used to optimize the separation of a large number of proteins of different sizes. Gradient gels are commercially available and can also be poured with the aid of a gradient maker.

9. Transfer buffer should be stored at 4 °C.
10. Since milk contains phospho-proteins a milk-based blocking buffer is not recommended for the detection of phospho-proteins. In this case alternative blocking buffers such as those containing serum or BSA may be used.
11. Appropriate dilutions for primary and secondary antibodies will have to be determined by trial and error. This will depend on the amount of antigen present and the specificity of the antibodies. Refer to the suppliers' recommendations for starting dilutions.
12. Leave the first piece of X-ray film down for a relatively short period of time; 30 s to 1 min and use this immunoblot to judge whether you need to increase exposure time (if signal is weak or absent) or reduce (if signal is very strong or if there is high background appearing).
13. Ensure X-ray is not exposed to light until after it has been fixed.
14. Routine densitometric analyses of developed X-ray films can be performed with a suitable scanner and Imagequant analysis software. Statistical analyses can then be performed using Prism analysis software.

Acknowledgments

Research in the author's laboratory has been supported by project grants from the Irish Health Research Board and Muscular Dystrophy Ireland, as well as a Hume Scholarship from Maynooth University.

References

1. Lander ES, Linton LM, Birren B et al (2001) Initial sequencing and analysis of the human genome. Nature 409:860–921
2. C. elegans Sequencing Consortium (1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. Science 282:2012–2018
3. Adams MD, Celniker SE, Holt RA et al (2000) The genome sequence of Drosophila melanogaster. Science 287:2185–2195
4. Bensmail H, Haoudi A (2003) Postgenomics: proteomics and bioinformatics in cancer research. J Biomed Biotechnol 2003:217–230
5. Gregorich ZR, Ge Y (2014) Top-down proteomics in health and disease: challenges and opportunities. Proteomics 14:1195–1210
6. Rifai N, Gillette MA, Carr SA (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 24:971–983
7. Deshmukh AS, Murgia M, Nagaraj N et al (2015) Deep proteomics of mouse skeletal muscle enables quantitation of protein isoforms, metabolic pathways, and transcription factors. Mol Cell Proteomics 14:841–853
8. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021
9. Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26:231–243
10. Lilley KS, Friedman DB (2004) All about DIGE: quantification technology for

differential-display 2D-gel proteomic. Expert Rev Proteomics 1:401–409

11. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077
12. Oliva K, Barker G, Rice GE et al (2013) 2D-DIGE to identify proteins associated with gestational diabetes in omental adipose tissue. J Endocrinol 218:165–178
13. Doran P, O'Connell K, Gannon J et al (2008) Opposite pathobiochemical fate of pyruvate kinase and adenylate kinase in aged rat skeletal muscle as revealed by proteomic DIGE analysis. Proteomics 8:364–377
14. Rong Y, Jin D, Hou C et al (2010) Proteomics analysis of serum protein profiling in pancreatic cancer patients by DIGE: up-regulation of mannose-binding lectin 2 and myosin light chain kinase 2. BMC Gastroenterol 10:68
15. Ummanni R, Mundt F, Pospisil H et al (2011) Identification of clinically relevant protein targets in prostate cancer with 2D-DIGE coupled mass spectrometry and systems biology network platform. PLoS One e16833:6
16. Lewis C, Ohlendieck K (2010) Mass spectrometric identification of dystrophin isoform Dp427 by on-membrane digestion of sarcolemma from skeletal muscle. Anal Biochem 404:197–203
17. Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1:845–867
18. Coenen-Stass AM, McClorey G, Manzano R et al (2015) Identification of novel, therapy-responsive protein biomarkers in a mouse model of Duchenne muscular dystrophy by aptamer-based serum proteomics. Sci Rep 5:17014
19. Hortin GL, Jortani SA, Ritchie JC (2006) Proteomics: a new diagnostic frontier. Clin Chem 52:1218–1222
20. Pieper R, Gatlin CL, Makusky AJ et al (2003) The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Proteomics 3:1345–1364
21. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiol Rev 91:1447–1531
22. Ohlendieck K (2011) Skeletal muscle proteomics: current approaches, technical challenges and emerging techniques. Skelet Muscle 1:6
23. Gygi SP, Corthals GL, Zhang Y et al (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc Natl Acad Sci U S A 97:9390–9395
24. Fonslow BR, Carvalho PC, Academia K et al (2011) Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. J Proteome Res 10:3690–3700
25. Rabilloud T, Lelong C (2011) Two-dimensional gel electrophoresis in proteomics: a tutorial. J Proteome 74:1829–1841
26. Tu C, Rudnick PA, Martinez MY et al (2010) Depletion of abundant plasma proteins and limitations of plasma proteomics. J Proteome Res 9:4982–4991
27. O'Connell K, Ohlendieck K (2009) Proteomic DIGE analysis of the mitochondria-enriched fraction from aged rat skeletal muscle. Proteomics 9:5509–5524
28. Wallace DC (1999) Mitochondrial diseases in man and mouse. Science 283:1482–1488
29. Wallace DC (2000) Mitochondrial defects in cardiomyopathy and neuromuscular disease. Am Heart J 139:S70–S85
30. Carberry S, Zweyer M, Swandulla D, Ohlendieck K (2014) Comparative proteomic analysis of the contractile-protein-depleted fraction from normal versus dystrophic skeletal muscle. Anal Biochem 446:108–115
31. Maughan DW, Henkin JA, Vigoreaux JO (2005) Concentrations of glycolytic enzymes and other cytosolic proteins in the diffusible fraction of a vertebrate muscle proteome. Mol Cell Proteomics 4:1541–1549
32. Alban A, David SO, Bjorkesten L et al (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3:36–44
33. Malm C, Hadrevi J, Bergström SA et al (2008) Evaluation of 2-D DIGE for skeletal muscle: protocol and repeatability. Scand J Clin Lab Invest 68:793–800
34. Lilley KS, Razzaq A, Dupree P (2002) Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. Curr Opin Chem Biol 6:46–50

Chapter 19

DIGE Analysis of Immunodepleted Plasma

Paul Dowling and Kay Ohlendieck

Abstract

This chapter will focus on upstream immunodepletion of high abundant proteins from plasma samples and subsequent analysis by difference gel electrophoresis (DIGE). The abundances of proteins in biofluid proteomes, such as serum, plasma, saliva, and bronchoalveolar lavage fluid (BALF), can exceed 10 orders of magnitude. This substantial dynamic range is problematic for the detection of medium and low abundance proteins by DIGE analysis. To increase the detection, quantification, and identification of medium-low abundant proteins, the targeted depletion of known abundant proteins with antibody columns has been successfully employed. From the literature, it is clear that the performance of abundant protein depletion with immunodepletion columns has been successful in broadening the coverage of the biofluid proteome and facilitating the identification of disease-specific biomarkers. The task for a successful biomarker strategy involves the combination of a reproducible and robust fractionation method, coupled with a highly accurate quantitative method, a task that is exemplified by combining both immunodepletion and DIGE together to discover significant proteins associated with the disease phenotype.

Key words Antibodies, Immunodepletion, Low abundant, Plasma, Serum

1 Introduction

In recent years, the discovery of disease biomarkers has become a major focus of cancer research [1]. When searching for novel biomarkers, biofluids such as serum and plasma are potentially the most valuable biological samples, because they contain many thousands of different protein species [2]. These are also the most easily accessible, minimally invasive, and widely collected samples. Unfortunately, using serum or plasma (and other types of biofluids such as saliva or urine) for biomarker discovery studies is challenging due to the broad dynamic range of protein abundances (over 10 orders of magnitude) and the fact that 22 proteins make up around 99% of the total protein mass [3, 4]. This large dynamic range greatly exceeds the analytical capabilities of traditional proteomic methods, making the detection of lower abundance proteins, residing in the ng/ml and below, extremely challenging. It is very likely that most potential biomarkers are among those low-abundance

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_19,

proteins, secreted or shed into the bloodstream by tissues or cells because of the disease area [5, 6]. To detect such low-abundance proteins using currently available technologies, it is logical to remove the most abundant proteins first [7]. A wide range of fractionation methods are now available for the removal of albumin and other high-abundance proteins [8, 9].

Protein depletion is becoming a standard practice as the routine sample preparation strategy in proteomics used prior to protein identification and quantitation [10–12]. Depletion of plasma/serum proteins can be performed using different approaches, with the ultimate end-point of separating the high abundance proteins from their low abundance counterparts. Many systems have been developed to deplete albumin, immunoglobulins, and other high-abundance proteins from human plasma/serum samples [13]. Most of the commercially available immunodepletion kits are comprised of an immunoaffinity column packed with affinity-purified antibodies that have been developed with highly purified antigens to target high-abundance proteins. In addition, a set of complementary proprietary buffers are used to minimize protein-protein interactions so that nonspecific binding is minimized prior to loading samples onto the column. This allows low-abundance proteins to be enriched in the flow-through fractions obtained from the system with minimal contamination from the high-abundant fraction [14], as diagrammatically outlined in Fig. 1.

There are currently several methods for depleting these higher abundance proteins from plasma/serum. IgG-based systems remove human serum albumin (HSA), immunoglobulin G (IgG), immunoglobulin A (IgA), haptoglobin, transferrin, and alpha-1-antitrypsin from human serum samples [15, 16]. An IgY-antibody-based technology has also been developed and successfully used by many research groups [17]. This approach is based on chicken IgY antibodies, covalently cross-linked to beads so that it can be used with either LC systems or spin column devices. The system can deplete human albumin, IgG, IgA, IgM, fibrinogen, and transferrin, and appears to have a less species specificity for reactivity of immunoglobins than is obtained using affinity-purified rabbit polyclonal IgGs.

A range of commercially produced LC columns/cartridges are now available for immunodepletion of various biofluids. These include the Multiple Affinity Removal System (MARS) columns, removing 6, 7, or 14 abundant proteins depending on the column/cartridge employed (Agilent), the ProteoPrep20 that removes 20 proteins (Sigma) and the Proteome Purify™ Immunodepletion Kits, 2 Serum Protein Immunodepletion Resins/10 Serum Protein Immunodepletion Resins (R&D Systems) removing 2 and 12 abundant proteins respectively.

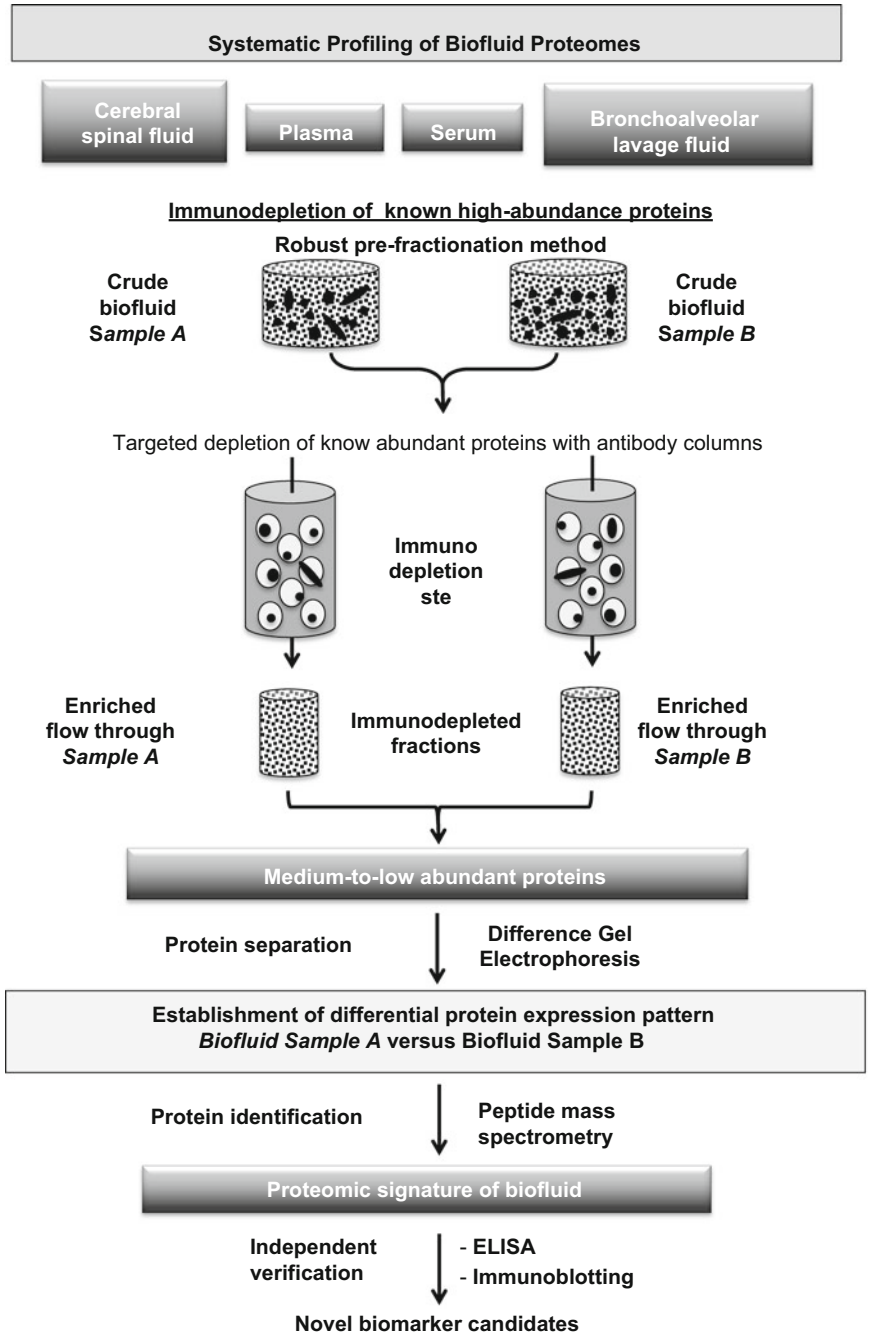

Fig. 1 Overview of immunodepletion method to enrich medium-to-low abundant proteins in biofluids prior to difference gel electrophoretic analysis. The targeted depletion of known abundant proteins with antibody columns drastically increases the detection, quantification, and identification of low abundant proteins

For the purposes of this chapter, we will be discussing the MARS system for the removal of high abundant proteins from plasma samples.

2 Materials

2.1 Equipment

1. IPGphor IEF unit (GE Healthcare).
2. IPG DryStrip reswelling tray (GE Healthcare).
3. Manifold (GE Healthcare).
4. Sample loading cups (GE Healthcare).
5. Gel casting box (GE Healthcare).
6. Cassette racks (GE Healthcare).
7. Ettan DALTsix multiple vertical slab gel unit (GE Healthcare).
8. Electrophoresis power supplies (GE Healthcare).
9. Glass plates suitable for fluorescence analysis.
10. Typhoon Trio variable mode imager (GE Healthcare).
11. Vortex Genie-2 (Scientific Industries).
12. Stuart SSL4 shaker (Lennox Laboratory Supplies Ltd.).
13. Heto speedvac concentrator.
14. Eppendorf Model 5417R centrifuge.
15. Sonicating water bath.

2.2 Reagents

1. Multiple Affinity Removal Spin Cartridge, Hu-14 0.45 ml pack (product number: 5188-6560)-contains Spin Cartridge, 1 each, Luer-Lok Adapters, 1 pack (2/pack), Cap & Plug, 1 each (Agilent) or alternative spin cartridges (*see* **Note 1**).
2. Multiple Affinity Removal Spin Cartridge, Starter Kit (product number: 5188-5254)-contains Buffer A, 1 bottle (1 L), Buffer B, 1 bottle (1 L), Spin filters, 2 packs (25/pack), Spin concentrators, 5 kDa MWCO 4 ml, 1 pack (25/pack), Luer-Lok adapters, 1 pack (2/pack), Luer-Lok syringes, 5 ml, 1 pack (2/pack), Microtubes, 6 packs (100/pack), Caps and plugs, 1 pack (6/pack), PTFE Luer-Lok needles, 1 pack (10/pack). (Agilent) or alternative spin cartridges (*see* **Note 2**).
3. LavaPurple Protein Kit (Fluorotechnics Pty Ltd).
4. CyDye DIGE fluor minimal dye Cy2 (GE Healthcare).
5. CyDye DIGE fluor minimal dye Cy3 (GE Healthcare).
6. CyDye DIGE fluor minimal dye Cy5 (GE Healthcare).
7. Laemmli-type slab gel electrophoresis buffer system (GE Healthcare).
8. Immobilized linear pH gradient (IPG) strips (GE Healthcare).

9. pH 3–11 ampholytes (GE Healthcare).
10. IPG buffer (GE Healthcare).
11. Precast 12.5% polyacrylamide DIGE gel (GE Healthcare).
12. Bradford assay (Bio-Rad, 500-0205).
13. Bovine serum albumin (BSA) solution (Sigma, A9543).
14. PepMix4 (LaserBio Labs).

2.3 Solutions

All solutions should be prepared with analytical grade chemicals and ultrapure water.

2.3.1 Sample Preparation and DIGE Labeling

1. DIGE lysis buffer: 30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, pH 8.5.
2. Sample reducing buffer: 4% CHAPS, 9.5 M urea, 2% IPG buffer pH 3–11, 130 mM dithiothreitol.
3. Stock solution: Dissolve dyes in 5 µl of fresh Dimethyl Formamide (DMF). Concentration is now 1 nmol/µl. Store at −20 °C for 3 months.
4. Working solution: Dilute 1 µl of dye with 4 µl of DMF (5 µl total—concentration is now 200 pmol/µl). Keep on ice during use.

2.3.2 Gel Electrophoresis

1. Rehydration Buffer: 8 M Urea, 2% CHAPS, 20 mM DTT, 0.5% IPG Buffer, trace amount of bromophenol blue (*see* **Note 3**).
2. Equilibration Buffer 1: 6 M Urea 30% Glycerol 2% SDS 50 mM Tris–HCl pH 8.8-add 1% DTT.
3. Equilibration Buffer 2: 6 M Urea 30% Glycerol 2% SDS 50 mM Tris–HCl pH 8.8-add 2.5% Iodoacetamide.
4. 10× SDS buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS (*see* **Note 4**).
5. Sealing solution: 1% agarose in 1× SDS buffer, and Bromophenol Blue dye. Heat solution until agarose has properly dissolved.

2.3.3 Protein Visualization Using Lava Purple

1. Gel-fixing Solution: mix 850 ml HPLC-grade water, 150 ml Ethanol, and 10 g Citric Acid powder.
2. Staining Solution: mix 1 L HPLC-grade water 6.2 g Boric Acid powder 3.85 g NaOH and 5 ml purple stain concentrate.
3. Washing Solution: mix 850 ml HPLC-grade water and 150 ml Ethanol.

2.3.4 Reduction, Alkylation, and In-Gel Digestion

1. 50 mM Ammonium bicarbonate: 0.0396 g in 10 ml of HPLC-grade water.
2. Trypsin solution: 20 µg of trypsin in 200 µl of 50 mM ammonium bicarbonate buffer.

3. Digestion buffer: 2.5 ml 100% acetonitrile, 45.5 ml HPLC-grade water, 2 ml 1 M ammonium bicarbonate.
4. Reducing solution: 10 mM stock solution of DTT (dithiothreitol) in 50 mM ammonium bicarbonate.
5. Alkylation solution: 55 mM iodoacetamide in 50 mM ammonium bicarbonate.
6. Peptide extraction solution A: 5% formic acid in HPLC-grade water.
7. Peptide extraction solution B: 5% formic acid in 50:50 HPLC-grade water: acetonitrile.

3 Methods

3.1 Preparation and Enrichment of Low Abundant Plasma Proteins

1. Dilute 6–8 μl human plasma sample to 200 μl with Buffer A and filter through 0.22 μm spin filter.
2. Remove cartridge cap and plug and remove buffer from top of resin bed with transfer pipette (*see* **Note 5**).
3. Add 200 μl diluted plasma sample. Cap cartridge loosely or leave open. Place in 1.5 ml collection tube labeled F1 (Flow-through fraction 1). Centrifuge for 30 s at 200 × *g*.
4. Add 400 μl Buffer A. Centrifuge for 1 min at 200 × *g*. Collect in F1 tube.
5. Place spin cartridge in new collection tube labeled F2 (Flow-through fraction 2). Add 400 μl Buffer A. Centrifuge for 1 minute at 200 × *g*. Collect in F2 tube.
6. Remove spin cartridge from F2 tube and attach the luer-lock adapter securely to top of cartridge.
7. Fill the 5 ml Luer-Lok™ plastic syringe with 2 ml of Buffer B and attach to the Luer-Lok adapter. Very slowly push Buffer B through cartridge to elute bound proteins into a new collection tube. Discard eluate.
8. Fill a new 5 ml plastic syringe with 4 ml of Buffer A and attach to the Luer-Lok adapter. Very slowly push Buffer A through the cartridge to re-equilibrate for the next sample or store wetted with Buffer A (at 4 °C). Re-cap both the ends for storage.
9. Combine fractions F1 and F2 together.

3.2 Precipitation of Low Abundant Proteins and Protein Quantification

1. Prior to analysis by proteomic methods such as 2D-DIGE, precipitate low abundant proteins using TCA precipitation.
2. For TCA/acetone precipitation of the proteins add a sufficient volume of 100% TCA to the "low-abundant fraction" to reach a final TCA concentration of 10%. The sample should turn

milky white if proteins are present. Vortex and incubate on ice for 30 min (*see* **Note 6**).

3. Centrifuge the sample at 4 °C for 30 min at 16,000 × *g* until the supernatant is clear and there is a white pellet of protein at the bottom of the tube. Discard the supernatant.
4. To the pellet add 1 ml of −20 °C acetone. Vortex the sample and incubate overnight −20 °C. Centrifuge at 4 °C for 10 min at 16,000 × *g*. Discard the supernatant (*see* **Note 7**).
5. To the pellet, add a fresh aliquot of −20 °C acetone, vortex and incubate at −20 °C for 30 min and centrifuge as above. Discard the supernatant and repeat. After the final centrifugation, discard the acetone and dry the pellet completely.
6. Solubilize protein pellet in DIGE lysis buffer with sonication.

3.3 Protein Quantification

1. Check the pH of the sample by spotting 3 µl onto a pH indicator strip to ensure that it lies between pH 8.0–9.0.
2. Use a small aliquot of sample for protein estimation. Divide the sample into smaller aliquots to reduce freeze-thaw steps and store at −80 °C until use.
3. Prepare dilutions of BSA stock for 0.125, 0.25, 0.5, and 1.0 mg/ml to generate a protein standard curve.
4. Add 240 µl/well of Bradford protein assay reagent to the wells of a 96-well plate.
5. Add 10 µl of protein standard dilution or sample (diluted 1:10) to the relevant wells of the 96-well plate.
6. Run all samples in triplicate. Incubate the samples for 5 min with gentle shaking on a rocking platform to ensure even mixing of sample with protein assay reagent.
7. Read the absorbance of each standard and sample at 595 nm using a plate reader.
8. The concentration of the protein samples is determined from the plot of the absorbance at 595 nm versus the concentration of the protein standard.

3.4 DIGE Labeling

1. Add a volume of protein sample equivalent to 50 µg to a microfuge tube. For control v test of immunodepleted serum/plasma samples, label 50 µg of each control sample using Cy3 dye, and 50 µg of each test sample using Cy5 dye. Reverse labeling should also be included where—label 50 µg each control sample using Cy5 dye, and 50 µg of each test sample using Cy3 dye.
2. For the internal standard, label 50 µg of pooled samples using the Cy2 dye for each sample included in the DIGE experiment. For example, if ten control and ten test samples are examined,

including reverse labeling, then 20 × 50 μg Cy2 labeled pooled samples will need to be prepared (50 μg × 10 control and 50 μg × 10 test—all combined and labeled with Cy2).

3. Add 1 μl of diluted CyDye to the microfuge tube containing the protein sample (i.e., 50 μg of protein is labeled with 400 pmol of dye for the labeling reaction).
4. Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 min in the dark.
5. Add 1 μl of 10 mM lysine to stop the reaction. Mix by pipetting and spin briefly in a microcentrifuge.
6. Leave for 10 min on ice in the dark.
7. Pool the samples together to give 150 μg of total (50 μg Cy2-labeled internal pool + 50 μg Cy3-labeled control/test + 50 μg Cy5-labeled control/test).
8. Add an equal volume of sample reducing buffer and leave on ice for 10 min. Samples are now ready to be separated by first and second dimension electrophoresis.
9. For pick gels, run 1 mg of unlabeled protein by pooling samples from control and test.

3.5 Rehydration of First-Dimension Gel Strips

1. Place 450 μl of rehydration buffer into the individual slots on the Ettan IPGphor DryStrip reswelling tray (depending on how many samples are to be run).
2. Remove protective cover from DryStrip (24 cm long IPG strips of pH 3–11 NL) before placing gel side down into strip holder starting at the anode (pointed end) and laying strip down to the cathode (blunt end).
3. Move strip back and forth in order to spread out rehydration buffer along the length of the strip.
4. Remove all air bubbles from underneath the DryStrip before adding DryStrip Mineral Oil Cover Fluid.
5. Add cover fluid from both ends so that the fluid meets in the middle.
6. Rehydrate strips overnight.

3.6 First Dimension Isoelectric Focusing

1. Place wicks, wet with deionized water, onto ends of strips.
2. Place sample-loading cups onto strips and load sample. Running conditions for 24 cm strips: 6 h step at 100 V, 2 h gradient step to 1000 V, 4 h gradient step to 8000 V and a 3 h step at 8000 V (all steps at 20 °C).
3. Move onto equilibration step.

3.7 Equilibration of IEF Strips

1. Prepare 10 ml of fresh equilibration buffers 1 and 2 for each strip.
2. Wash strips with HPLC-grade water before placing into equilibration buffer 1.
3. Incubate each strip in 5 ml of equilibration buffer 1 for 15 min.
4. Remove strip and rinse with HPLC-grade water before placing into equilibration buffer 2.
5. Incubate each strip in 5 ml of equilibration buffer 2 for 15 min.
6. Remove strip and rinse with HPLC-grade water, allow excess water to drain from strip.

3.8 Second Dimensional Slab Gel Electrophoresis

1. Lay strip across the top of the precast 12.5% polyacrylamide DIGE gel, remove any bubbles between the strip and the top of the gel.
2. Add warm sealing solution, allow the agarose to cool and solidify and move to the electrophoresis apparatus.
3. Add 1× running buffer to the upper and lower buffer chambers and place gel inside apparatus.
4. Run gels using the EttanDalt 6 apparatus (GE Healthcare) at 2.5 W/gel for 30 min and then 100 W total at 10 °C until the dye front had run off the bottom of the gels.
5. Scan gels or remove gel from plates to stain.

3.9 Lava Purple Staining of Pick Gels

1. 1 mg of low/medium abundant proteins (immunodepleted fraction) should be run on the prep gel. Following gel electrophoretic separation, gel is washed in distilled water.
2. Fix gels in fix/acidification solution for 2 h.
3. Prepare staining solution with thawed stain concentrate and stain for 2 h.
4. Wash with washing solution for 1 h.
5. Acidify gels in fix/acidification solution for 1 h.
6. Image gels on Typhoon with 610BP30 emission filter and 532 nm laser (*see* **Note 8**).
7. Store gels in acidification solution in the dark.

3.10 Image Analysis of Protein Spot Patterns

1. DIGE analysis: Obtain gel images for the analysis using a Typhoon 9400 Variable Mode Imager using the following settings: Cy2 (488 nm excitation laser and 520BP40 emission filter); Cy3 (532 nm excitation laser and 580BP30 emission filter); and Cy5 (633 nm excitation laser and 670BP30 emission filter). Perform statistics and quantitation of protein expression in DeCyder software (GE Healthcare).

2. Process the resulting images using DeCyder Differential Analysis Software v 5.0.
3. Perform matching between gels and calculate Student's *t*-test for every matched spot set (comparing the average and standard deviation of protein abundance for a given spot between the normal and test groups).
4. Generate a list of proteins of interest and match to the prep gel using landmark proteins. Every protein of interest now has a unique *x* and *y* coordinates. Lava purple stained gel: Import the subsequent gel image into the BVA module of DeCyder software and match to images generated from DIGE analysis.
5. Send *x* and *y* coordinate data to the Ettan spot picker to capture gel plugs containing the proteins of interest.

3.11 Excision of Protein Spots from Two-Dimensional Gels and Destaining

1. Use the robotic Ettan spot picker to capture protein spots from the 2D prep gels and transfer the picked proteins into 96-well microplates.
2. Add enough water to cover gel pieces for 15 min, then remove water.
3. Destain three times by adding 200 μl of 50 mM ammonium bicarbonate in 50% acetonitrile, incubate for 30 min, and discard the liquid with each wash.
4. Remove destaining solution.
5. Add enough 100% acetonitrile to fully cover gel piece, incubate for 5 min, and discard acetonitrile.
6. Dry gel pieces using Speed Vac for 5 min.

3.12 Reduction and Alkylation of Preparative Protein Spots

1. Add 100 μl of 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate to the dried gel piece and incubate for 30 min at 37 °C, make sure all the gel pieces are covered.
2. Let the gel spots cool to room temperature and discard the DTT solution.
3. Add 100 μl of 55 mM iodoacetamide in 50 mM ammonium bicarbonate until gel pieces are covered.
4. Incubate for 30 min in the dark at room temperature.
5. Remove iodoacetamide/ammonium bicarbonate solution and discard to waste.
6. Dehydrate with 100 μl of 100% acetonitrile. Remove and discard solution when gel pieces are white/opaque (repeat this step to ensure gel pieces fully dehydrated).
7. Re-swell in 100 μl of 50 mM ammonium bicarbonate for 5 min. Remove and discard solution.

8. Dehydrate with 100 µl of 100% acetonitrile. Remove and discard solution when gel pieces are white/opaque (repeat this step to ensure gel pieces fully dehydrated).
9. Use the Speed Vac to evaporate remaining solvents for 5 min.

3.13 Digestion of Low Abundant Proteins for Mass Spectrometric Analysis

1. Add enough Trypsin solution (trypsin: protein ratio of 1:20, w/w) to re-swell gel pieces completely at 4 °C for 15 min.
2. If after 15 min at 4 °C, gel pieces are uncovered, add more buffer to cover gel pieces.
3. Digest overnight at 37 °C (16–18 h).
4. Add 50 µl of peptide extraction solution A to the gel spots and incubate at RT for 5 min. Transfer the solution to a new labeled vial.
5. Add peptide extraction solution B to the gel spots (enough to cover gel pieces) and incubate for 30 min at RT. Transfer the solution to the new labeled vial and combine with solution A (**step 4**).
6. Add enough acetonitrile to cover gel pieces and dehydrate, transfer the solution to the labeled vial, and combine with solutions A and B (**steps 4** and **5**).
7. Dry the solution using Speed Vac and store at −80 °C.

3.14 C18 Zip Tipping

1. Condition each ZipTip with 15 µl of 90% acetonitrile with 0.1% TFA, aspirate and dispense, repeat two more times.
2. Equilibrate with 15 µl of 2% acetonitrile, 0.1% TFA, aspirate and dispense, repeat four more times.
3. Load sample to tip, slowly aspirating and dispensing ten times (do not pass air through sorbent bed).
4. Wash tip with 20 µl of 2% acetonitrile, 0.1% TFA, aspirate and dispense, repeat four more times.
5. Elute peptides with 2 µl of 90% acetonitrile, 0.1% TFA (analyte).

3.15 Mass Spectrometric Identification of Individual Protein Species

1. Make a saturated solution of 10 mg α-cyano-4-hydroxy-cinnamic acid in 1 ml 60% acetonitrile/0.1%TFA. Vortex and centrifuge.
2. Mix the analyte (1 µl) and the matrix (3 µl) in the ratio of 3:1 (v:v). Vortex.
3. Use 1 µl of this to spot on the target plate.
4. Record mass spectra using the MALDI TOF instrument operating in the positive reflector mode using the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500).

5. Perform internal and external calibration using trypsin autolysis peaks at *m/z* 842.50, *m/z* 2211.104 and Pep4 mix, respectively.
6. Analyze mass spectra using MALDI evaluation software (GE Healthcare), and protein identification using the PMF ProFound search engine.

4 Notes

1. Other company provides immunodepletion kits, such as R&D Systems or Sigma-Aldrich.
2. Other company provides immunodepletion kit reagents, such as R&D Systems, Sigma-Aldrich.
3. DTT, IPG Buffer and bromophenol blue to be added just before use.
4. Store this solution at room temperature.
5. Never let frits or resin bed run dry.
6. Use fresh TCA. TCA degrades with time to chloroform which will result in incomplete precipitation.
7. The acetone used must be HPLC grade. Poor quality acetone will result in poor recovery of proteins.
8. For DIGE applications, the violet excitation filter (390/20 nm) with the orange emission filter (595/25 nm) avoids cross talk (e.g., with the Cy2 and Cy3 signal).

References

1. Huang Z, Ma L, Huang C, Li Q, Nice EC (2016) Proteomic profiling of human plasma for cancer biomarker discovery. Proteomics. doi:10.1002/pmic.201600240
2. Dowling P, Meleady P, Henry M, Clynes M (2010) Recent advances in clinical proteomics using mass spectrometry. Bioanalysis 2 (9):1609–1615. doi:10.4155/bio.10.69
3. Zhang Q, Faca V, Hanash S (2011) Mining the plasma proteome for disease applications across seven logs of protein abundance. J Proteome Res 10(1):46–50. doi:10.1021/pr101052y
4. Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1(11):845–867
5. Surinova S, Schiess R, Hüttenhain R, Cerciello F, Wollscheid B, Aebersold R (2011) On the development of plasma protein biomarkers. J Proteome Res 10(1):5–16. doi:10.1021/pr1008515
6. Teng PN, Bateman NW, Hood BL, Conrads TP (2010) Advances in proximal fluid proteomics for disease biomarker discovery. J Proteome Res 9(12):6091–6100. doi:10.1021/pr100904q
7. Kim MR, Kim CW (2007) Human blood plasma preparation for two-dimensional gel electrophoresis. J Chromatogr B Analyt Technol Biomed Life Sci 849(1–2):203–210. doi:10.1016/j.jchromb.2006.11.046
8. Polaskova V, Kapur A, Khan A, Molloy MP, Baker MS (2010) High-abundance protein depletion: comparison of methods for human plasma biomarker discovery. Electrophoresis 31 (3):471–482. doi:10.1002/elps.200900286
9. Gundry RL, White MY, Nogee J, Tchernyshyov I, Van Eyk JE (2009) Assessment of albumin removal from an immunoaffinity spin column: critical implications for proteomic examination of the albuminome and albumin-depleted

samples. Proteomics 9(7):2021–2028. doi:10.1002/pmic.200800686

10. Fernández-Costa C, Calamia V, Fernández-Puente P, Capelo-Martínez JL, Ruiz-Romero C, Blanco FJ (2012) Sequential depletion of human serum for the search of osteoarthritis biomarkers. Proteome Sci 10(1):55. doi:10.1186/1477-5956-10-55
11. Timms JF, Arslan-Low E, Kabir M, Worthington J, Camuzeaux S, Sinclair J, Szaub J, Afrough B, Podust VN, Fourkala EO, Cubizolles M, Kronenberg F, Fung ET, Gentry-Maharaj A, Menon U, Jacobs I (2014) Discovery of serum biomarkers of ovarian cancer using complementary proteomic profiling strategies. Proteomics Clin Appl 8(11–12):982–993. doi:10.1002/prca.201400063
12. Pieper R, Su Q, Gatlin CL, Huang ST, Anderson NL, Steiner S (2003) Multi-component immunoaffinity subtraction chromatography: an innovative step towards a comprehensive survey of the human plasma proteome. Proteomics 3(4):422–432. doi:10.1002/pmic.200390057
13. Gourin CG, Zhi W, Adam BL (2009) Proteomic identification of serum biomarkers for head and neck cancer surveillance. Laryngoscope 119(7):1291–1302. doi:10.1002/lary.20279
14. Rajpal R, Dowling P, Meiller J, Clarke C, Murphy WG, O'Connor R, Kell M, Mitsiades C, Richardson P, Anderson KC, Clynes M, O'Gorman P (2011) A novel panel of protein biomarkers for predicting response to thalidomide-based therapy in newly diagnosed multiple myeloma patients. Proteomics 11(8):1391–1402. doi:10.1002/pmic.201000471
15. Fahiminiya S, Roche S, Gérard N (2012) Improvement of 2D-PAGE resolution of human, porcine and canine follicular fluid: comparison of two immunodepletion columns. Reprod Domest Anim 47(5):e67–e70. doi:10.1111/j.1439-0531.2011.01939.x
16. Patel BB, Barrero CA, Braverman A, Kim PD, Jones KA, Chen DE, Bowler RP, Merali S, Kelsen SG, Yeung AT (2012) Assessment of two immunodepletion methods: off-target effects and variations in immunodepletion efficiency may confound plasma proteomics. J Proteome Res 11(12):5947–5958. doi:10.1021/pr300686k
17. Corrigan L, Jefferies C, Clive Lee T, Daly J (2011) Evaluation and optimization of IgY spin column technology in the depletion of abundant proteins from human serum. Proteomics 11(16):3415–3419. doi:10.1002/pmic.201000587

Part VI

DIGE Verification Analysis

Chapter 20

Elucidating Cellular Metabolism and Protein Difference Data from DIGE Proteomics Experiments Using Enzyme Assays

Andrew Dowd

Abstract

Assays for measuring enzyme activity can be useful tools for proteomics applications. Enzyme testing can be performed to validate an experimental system prior to a Difference Gel Electrophoresis (DIGE) proteomic experiment and can also be utilized as an integral part of multifaceted experiment in conjunction with DIGE. Data from enzyme tests can be used to corroborate results of DIGE proteomic experiments where an enzyme or enzymes are demonstrated by DIGE to be differentially expressed. Enzyme testing can also be utilized to support data from DIGE experiments that demonstrate metabolic changes in a biological system. The different types of enzyme assays that can be performed in conjunction with DIGE experiments are reviewed alongside a discussion of experimental approaches for designing enzyme assays.

Key words Difference gel electrophoresis, DIGE, Enzymology, Enzyme, Enzyme assay

1 Introduction

Two-dimensional gel electrophoresis (2-DE) remains the principal method for separation of undigested (intact) proteins and is a fundamental technique in the field of quantitative intact proteomics (QIP) [1]. Difference Gel Electrophoresis (DIGE) technology that features mass- and charge-matched cyanine dyes (Cy™2, Cy™3, and Cy™5) enables multiplexing of gel images with a maintained super-imposable protein separation pattern. This matching therefore allows the correction of experimental variation by means of a pooled internal standard and is a key step in improving the quantitative aspects and overall statistical power of the 2-DE technique [1].

DIGE technology can also be adapted to measure protein modifications in order to quantify changes in cellular metabolism. Hurd et al. [2] developed a customized DIGE method to identify thiol proteins that are modified by low levels of endogenous

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_20,

reactive oxygen species, which may occur due to upregulation of metabolic enzymes due to cellular stress. This method, termed redox DIGE, was used to identify the redox state of mitochondrial proteins involved in fatty acid and carbohydrate metabolism. The effect of reactive oxygen species (ROS) on proteins was assessed using enzyme activity assays, namely on propionyl-CoA carboxylase and pyruvate dehydrogenase kinases, to support their redox DIGE results [2].

DIGE can also be modified to facilitate measurement of enzyme activities in situ. Morek et al. [3] developed a novel method called differential activity-based gel electrophoresis (DABGE) for comparative analysis of lipolytic and esterolytic activities in mouse adipose and liver tissues. The authors developed a set of three fluorescent suicide inhibitors and these probes had the same substrate analogous structures but possessed different cyanine dyes (Cy3 maleimide, and Cy5 maleimide) as reporter fluorophores. Analysis of the fluorescence intensities from the DABGE gel showed that the enzyme ratios that were found very closely reflected the relative amounts of the labeled enzymes that were used for spiking [3].

Notwithstanding the examples outlined above that use variations of DIGE technology to either measure the potential effects of protein-modification on enzyme levels or to measure enzyme activities in a more direct fashion, most DIGE experiments use the commercially available Cy2, Cy3, and Cy5 dyes [1]. When the individual spots of interest are identified from the DIGE gel, excision of these spots and Mass Spectrometry are subsequently performed in order to identify these proteins.

Experiments such as RT-PCR, Western blotting, or enzyme assay are either desirable or necessary as a follow-up to a DIGE experiment, and this depends on the nature of the investigation being carried out. Where the results of a DIGE experiment show differences in levels of enzymes these results can be validated using enzyme assays as described by O'Connell and Ohlendieck [4], Bentaib et al. [5], and Faure et al. [6], for example. When a spot of interest exhibits low identity to known proteins the use of additional method(s) is a necessary post-DIGE step, however [7]. Follow-up enzyme assays are also used to determine the function in a case of a protein that is considered hypothetical and may potentially exhibit enzyme activity and the mass spectrometry identification of this enzyme is ambiguous [8].

The literature shows many examples of enzyme assays used to validate DIGE results as depicted in Table 1, and in this chapter I will attempt to review the various ways that enzyme assays are used to achieve this.

Table 1
Summary of assays described in the text

Ref.	Author	Year	Enzyme assays	Notes
[9]	Boone et al.	2016	Aconitase and GAPDH	Lidocaine is a pro-oxidant. 2D-DIGE enzyme activities (abundance, carbonylation) of aconitase and GAPDH were measured
[2]	Hurd et al.	2007	Propionyl-CoA carboxylase, pyruvate dehydrogenase kinases	Redox Difference Gel Electrophoresis
[10]	Mandili et al.	2015	Numerous enzymes	Three key enzymes of carbohydrate, lipid, and amino acid catabolism
[4]	O'Connell and Ohlendieck	2009	NADH dehydrogenase, Aldolase and succinate dehydrogenase, Citrate synthase activity	Measured enzyme activity of select marker proteins, using established assay systems
[11]	Fekkar et al.	2012	*N*-Acetyl-β-D-Glucosaminidase Release Assay	BEAS-2B bronchial epithelial cell line degranulation in response to the fungal pathogen *Aspergillus fumigatus* was measured by enzyme assay
[12]	Overgaard et al.	2014	Glycogen Phosphorylase (GP) activity	Rapid cold hardening response of insect *Drosophila melanogaster*. Validation of DIGE experiments as four of seven differentially regulated proteins were variants of GP enzymes
[13]	Winkler et al.	2010	Caspase assay	Effect of phosphorothioate antisense oligonucleotides on 607B melanoma cells
[5]	Bentaib et al.	2015	Pyruvate Kinase/LDH isoenzymes zymography/ Glutamine synthetase	Used enzyme assay and zymography to document and confirm metabolic reprogramming in transformed mouse astrocyte cells
[14]	Fanjul-Fernández et al.	2013	In vitro cleavage proteolysis assays on SDS-PAGE	Used DIGE to investigate that the absence of Mmp-1a hampers tumor progression. Follow up protease cleavage assays
[15]	Wendelboe-Nelson and Morris	2012	Catalase activity assay, lipoxygenase activity assay, peroxidase activity, glutamine synthetase activity, thioredoxin reductase assay	Comparison of drought-resistant and normal barley strains under drought and normal conditions.

(continued)

Table 1
(continued)

Ref.	Author	Year	Enzyme assays	Notes
				Enhanced stress response in regulation of ROS production and breakdown by resistant strains
[6]	Faure et al.	2013	Acetyl CoA dehydrogenase, cytochrome c oxidase, citrate synthase/Complex I activity	Key enzymes of Krebs cycle and mitochondrial respiration. Used Dipstick Assay Kit to measure Complex I activity
[16]	He et al.	2016	Superoxide dismutase, glutathione reductase	Assays performed prior to DIGE, Drought effect on Sea Buckthorn
[17]	Schröeder et al.	2013	Reductase assays under anoxic conditions, products measured by HPLC	Conversion of Daidzein and Genistein by the Equol-Forming Bacterium *Slackia isoflavoniconvertens*
[18]	Jindahl et al.	2012	Glycogen phosphorylase, lactate dehydrogenase, Pyruvate dehydrogenase, Succinate dehydrogenase, Cytochrome c oxidase	Glycogen phosphorylase measured by liquid scintillation counting. Other assays used commercially available kits
[19]	Lennicke et al.	2017	NAPH dehydrogenase, glutathione peroxidase, Superoxide Dismutase (SOD), MnSOD, thioredoxin reductase, Glutathione S-transferase and protein tyrosine peroxidase	Effects of different selenocompounds on the hepatic proteome and energy metabolism of mice. Enzyme activities performed prior to DIGE
[20]	He et al.	2016	Superoxide dismutase, glutathione reductase	Assays performed prior to DIGE, Similar to [16] above—drought stress responses of *Hippophae rhamnoides*
[21]	Menazza et al.	2013	Succinate Dehydrogenase, Electron Transfer Flavoprotein-Ubiquinone Dehydrogenase, Malate-Aspartate Shuttle (MAS) Activity	Absorbance and NADH formation Proteomic findings did not match assay findings
[22]	Pieper et al.	2015	Various digestive enzymes: alpha amylase, Lipase, Trypsin, Chymotrypsin, elastase, antioxidative activity	Colorimetric assays, e.g. lipase activity, and antioxidant capacity measured before DIGE analysis
[23]	Li et al.	2014	Total amylase/alpha amylase/beta amylase/limit dextrinase	Crude extract analyses

[24] García-Lorenzo et al.	2012	Alkaline phosphatase, maltase	Colorimetric assay enzyme measurement of known markers of enterocytic differentiation
[25] Marín-Buera et al.	2015	GAPDH assay/Pyruvate dehydogenase	Commercial kit (GAPDH) and PDH enzyme assay dipstick kit
[26] Gutiérrez-Sánchez et al.	2012	Caffeine-demethylase assay	HPLC detection
[27] Ray et al.	2016	Patient ALT, AST and ALP were measured using automated chemical analyzers, SOD activity assay	Commercially available kit (Cayman Chemicals USA). Colorimetric measurement of SOD activity
[28] Daneshvar et al.	2012	Trypanothione reductase/Trypanothione-dependent peroxidase/Alanine aminotransferase	Assays on frozen cell pellets (Alanine amino transferase is control) spectrophotometric assays
[29] Drüppel et al.	2014	Amino acid degradation (tryptophan, phenylalanine, methionine, leucine, isoleucine, valine, histidine, lysine and threonine) and TCA cycle enzymes assayed	The catabolic network [excluding tricarboxylic acid (TCA) cycle] was reconstructed from 71 genes using omics and enzyme assay approaches Enzyme assays were performed to measure Units of activity
[30] Jia et al.	2015	Superoxide dismutase (SOD) activity assay Chitinase activity assay, ATPase activity assay	Colorimetric detection. Enzyme assays used to validate DIGE results
[31] von Löhneysen et al.	2012	Pyruvate Kinase/G6PD/Hexokinase/GPI/TPI/ Glutathione peroxidase/Reduced glutathione	Colorometric assays—Beutler E (1984) Red Cell Metabolism: A Manual of Biochemical Methods. New York, NY: Grune & Stratton, Inc.
[32] DePalma et al.	2013	Citrate synthase/NADH dehydrogenase (complex I)/ succinate dehydrogenase activity (complex II)	Assay kit/spectrophotometric assays
[33] Capitanio et al.	2012	NADH dehydrogenase/succinate dehydrogenase/ citrate synthase/	Spectrophotometric assays
[34] Dorts et al.	2014	Citrate synthase/lactate dehydrogenase	Spectrophotometric assays—initial assays performed before DIGE
[35] Kube et al.	2013	Enzymes: (1) pyrophosphatase, (2) glycerophosphodiesterase, (3) anion-activated carboxyl esterase, (4) unspecified esterase and (5) dihydroorotate oxidase	Substrates: (1) 0.5 mM pyrophosphate, (2) 12 mM bis-p-nitrophenyl phosphate, (3) 2 mM α-naphthyl acetate, (4) 2 mM α-naphthyl propionate, and (5) 4 mM dihydroorotate and 1 mM K-ferricyanide

1.1 Enzyme Assays as Support for DIGE Experiments

1.1.1 Experimental Approaches

There are two main ways that enzyme testing can be used in the context of a DIGE experiment. One experimental approach is to perform the assays prior to a DIGE experiment to validate the experimental system that is being tested [16, 17, 19, 20, 22, 24, 26, 27, 29, 31–35] (Table 1). The second approach is to use enzyme testing to validate differential expression of an enzyme following an initial DIGE experiment in a similar manner to Western blotting, for example (Table 1). This approach will be outlined in Subheading 1.2.

1.1.2 Pre-DIGE Enzyme Assays to Validate an Experimental System

The more usual approach for enzyme testing prior to a DIGE experiment is to use targeted enzyme assays to validate the experimental system prior to committing to a DIGE experiment. For example, He et al. [16] performed superoxide dismutase (SOD) and glutathione reductase assays on sea buckthorn leaf homogenates to measure physiological and biochemical characteristics during drought stress prior to DIGE analysis. Another example of this approach is by Schröeder et al. [17] who performed an initial time-course experiment to measure the conversion of daidzein and genistein by a cell extract of *Slakia isoflavoniconvertens* prior to DIGE. He et al. [20], in a separate paper, measured the activities of two major antioxidant enzymes, SOD and glutathione reductase, to measure drought-stress responses of *Hippophae rhamnoides* prior to performing DIGE. In an interesting example, Gutiérrez-Sánchez et al. [26] utilized a caffeine-demethylase assay to determine if the induction phase in a two-step fermentation process of *Aspergillus tamarii* to produce caffeine degrading enzymes was successful prior to DIGE analysis of induced and noninduced mycelium extracts. García-Lorenzo et al. [24] utilized alkaline phosphatase and maltase assays to assess enterocytic differentiation of an adenocarcinoma cell line prior to setting up the DIGE analysis.

1.1.3 Pre-DIGE Enzyme Assays in Clinical Studies

Where the experiment involves patient samples some investigators perform enzyme assays prior to DIGE. In a study of the global proteome of red cells of patients with hereditary anemia, von Löhneysen et al. [31] measured kinase, glucose-6-phosphate dehydrogenase, hexokinase, glucose phosphate isomerise, triose phosphate isomerise, glutathione peroxidise and reduced glutathione to evaluate the blood samples of patients with hereditary nonspherocytic hemolytic anemias prior to DIGE analysis. Ray et al. [27] used 2D-DIGE of serum from Plasmodium vivax malaria patients to reveal oxidative stress and cytoskeletal proteins as possible markers for severe Vivax malaria. They assessed aspartate transaminase, alanine aminotransferase, and alkaline phosphatise levels in patients prior to the DIGE experiments [27].

1.1.4 DIGE and Enzyme Assays in Multifaceted Experiments

Enzyme activity assays can be part of a large experimental system which includes enzyme activity assays and DIGE among other methods. In a complex set of experiments by Pieper et al. [22], the impact of high dietary zinc on various enzyme activities (α-amylase, lipase, trypsin, chymotrypsin, and elastase) and also zinc accumulation and proteomic profiles as measured by DIGE experiments was assessed. Lennicke et al. [19] measured glutathione peroxidise and thioredoxin reductase activities to determine hepatic Se status following long-term supplementation with selenite, selenate, or selenomethionine for 20 weeks. They also performed several activity assays to determine whether or not the redox-sensitive transcription factor Nrf2 was increased in the selenium-deficient group. Furthermore, they performed DIGE analysis to show Se compound-specific differentially expressed proteins. Some agreement of glutathione s-transferase (GST) assay results with experimental findings was found [19]. Capitano et al. [33] utilized proteomics, biochemical and enzymatic assays, and bioinformatics to characterize protein alterations in hind limb (gastrocnemius) and fore limb (triceps) muscles in an amyotrophic lateral sclerosis (ALS) (SOD1G93A) mouse model. Nicotinamide adenine dinucleotide (NADH) dehydrogenase, succinate dehydrogenase, and citrate synthase assays were performed. The authors used enzyme assays (plus Western blotting and biochemical assays) to augment their proteomics data, and found some differences between the results of the different methods used [33]. Drüppel et al. [29] used a combination of "omics" and enzymatic approaches to elucidate the catabolic routes of nine selected amino acids (tryptophan, phenylalanine, methionine, leucine, isoleucine, valine, histidine, lysine, and threonine) in substrate-adapted cells of *Phaeobacter inhibens* DSM 17395. The study by DePalma et al. [32] identified metabolic and protein phenotypic alterations in gastrocnemius, tibialis anterior and diaphragm muscles of Col6a12/2 mice, a model of human collagen VI myopathies. Citrate synthase and NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) activities supported the proteomics data and further suggested that gastrocnemius and tibialis anterior of Col6a12/2 mice were attempting to maintain the energetic homeostasis by increasing complexes I and II activities [32].

According to Dorts et al. [34] increased water temperature consistent with climate change predictions may modulate the response of ectotherms to chemical insults. They used DIGE and enzyme assay (citrate synthase—the first enzyme in the Krebs cycle and Lactate dehydrogenase (LDH) the terminal enzyme of anaerobic glycolysis) to determine these effects. Their data highlighted complex interaction patterns of elevated temperature and Cadmium exposure on the LDH activity and protein expression profiles in gill tissue of an ecologically relevant species with few genomic

sequences available in databases. No change in citrate synthase activity was found [34].

Kube et al. [35] reported on the genome sequence analysis of the marine, oil degrading bacterium *Oleispira antarctica* RB-8- having a leading role in cold and deep marine environments. Genome analysis and genome-based functional studies were carried out including DIGE and enzyme assays (pyrophosphatase, glycerophosphodiesterase, anion-activated carboxyl esterase, unspecified esterase, and dihydroorotate oxidase) which revealed insights into its ability to degrade alkanes, to produce siderophores, to scavange for micronutrients and to cope with various habitat-specific stress factors [35].

1.2 Post-DIGE Validation Using Enzyme Assays

There are numerous examples of follow-up enzyme activity assays that were carried out to support the results of 2D-DIGE experiments in the literature [4–6, 9–15, 18, 21, 23, 25, 28, 30] (Table 1). Researchers have adopted a variety of experimental approaches for these follow-up assays. The simplest method is by validating differentially regulated proteins demonstrated by DIGE experiment using enzyme assays on the material that was subjected to DIGE analysis (Fig. 1). The second approach is to use follow-up enzyme assays to validate a hypothesis demonstrated by the DIGE data, for example performing assays post-DIGE on oxidative stress enzymes where the proteomics data demonstrated changes of proteins that regulate oxidative stress response in a cell. This is a more complex undertaking, however, and it involves using several enzyme assays to demonstrate a change in a metabolic process.

Apart from these differences in approach there are also some instances where a demonstration of changes in protein expression in a DIGE experiment does not necessarily result in a change in enzyme activity. The potential reasons why this might occur depend on the metabolic system being studied and supporting enzyme assays need to be backed up by a thorough understanding of the pathways that are involved in the system that is being studied.

1.2.1 Experimental Approaches to Post-DIGE Validation Using Enzyme Assays

1.2.2 Enzyme Assays to Validate DIGE Differential Enzyme Expression

The paper by Mandili et al. [10] illustrated a functional enzyme assay approach to validation of their DIGE experiments and a total of nine enzyme assays were used to validate the DIGE experiments [10]. O'Connell and Ohlendieck measured changed biochemical activity of DIGE-identified mitochondrial proteins with a differential expression pattern in senescent muscle fibers. They also used immunoblotting and confocal microscopy to validate the DIGE data [4]. The secretome of human bronchial cells infected by the fungal pathogen *Aspergillus fumigatus* was studied and it was found that infected cells secreted *N*-Acetyl-β-D-Glucosaminidase (NAGase) which was subsequently demonstrated by enzyme assay [11]. Overgaard et al. investigated the rapid cold hardening (RCH) response of *Drosophilia melanogaster*. DIGE analysis of RCH-

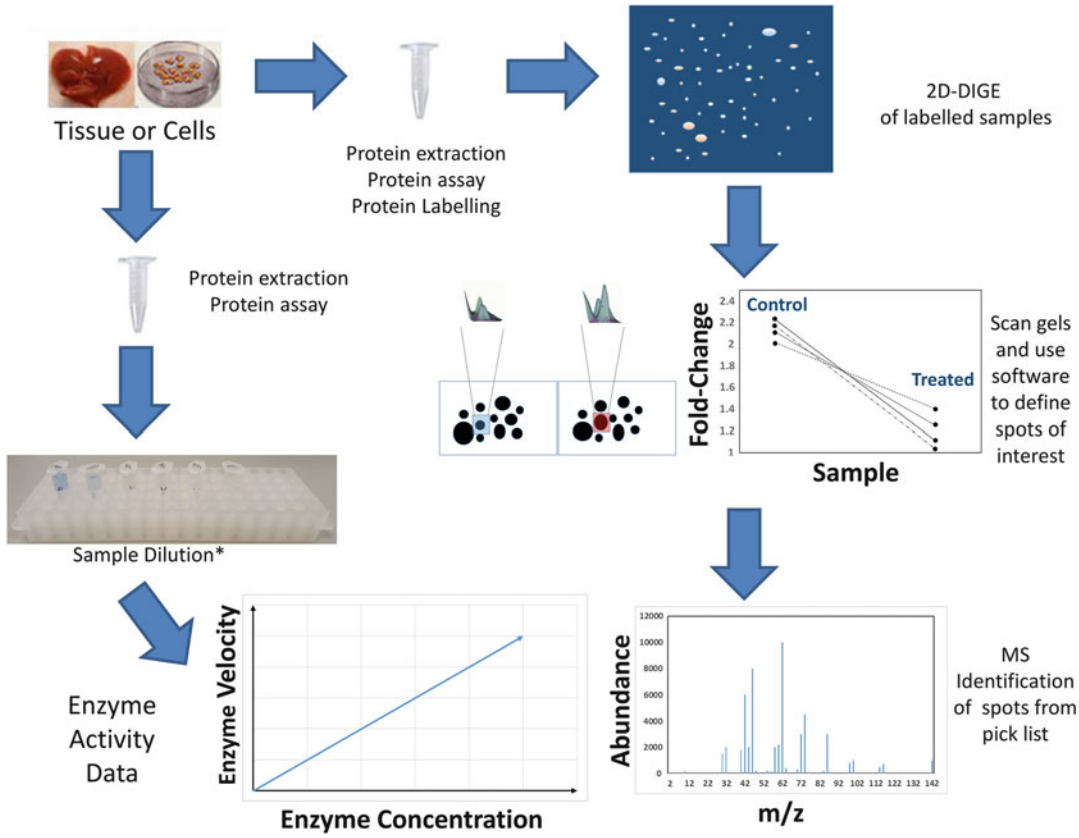

Fig. 1 Flowchart demonstrating the process of performing a parallel enzyme assay on a biological sample which has been processed for DIGE analysis

treated insect extracts identified changes in the levels of glycogen phosphorylase isoforms [12]. They performed enzyme assays on these extracts but did not see activity changes and discussed these findings in detail [12]. The authors stressed the necessity of combining "omics" experiments with subsequent targeted validations across numerous levels of the biological organization [12].

Li et al. [23] performed comparative proteomic analyses between malts produced by malting processes I and II of the Dan'er barley cultivar. The activities of several key enzymes (α-amylase, β-amylase, and limit dextrinase) were compared between the two malts. No differentially regulated spots were identified for limit dextrinase but enzyme activity assays did show differences in limit dextrinase activities. Both α-amylase I and β-amylase exhibited more abundance in malt II than in malt I. However, enzyme assay results indicated that the malt II extract exhibited higher total amylase and β-amylase activity but lower α-amylase activity. The authors concluded that the lower diastatic power (DP) of malt I (i.e., the ability of malt to reduce starch to sugar) was the result of

lower β-amylase despite the higher activity of limit dextrinase and α-amylase [23]. This paper again demonstrates that some contradictions between proteomic and enzyme activity data can occur, but proteomics data showing differential expression of enzymes ultimately need to be validated by enzyme activity measurements and where there may be contradiction, the authors usually rely on enzyme activity data to support their experimental findings.

1.2.3 Enzyme Assays to Validate Metabolic Changes Found by DIGE Analyses

Boone et al. [9] investigated lidocaine-induced protein and pathway alterations in *Saccharomyces cerevisiae*. They performed DIGE using amine reactive dyes and carbonyl reactive dyes to measure protein abundance and protein oxidation, respectively [9]. Follow-up aconitase and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) assays were performed in order to determine the effect of carbonylation on proteins. These results suggested that oxidative damage of aconitase directly affected its activity [9].

Winkler et al. [13] assessed the effects of phosphorothioate antisense oligonucleotides on 607B melanoma cells using DIGE and tested for caspase activity to determine unspecific toxicity and apoptosis induction in human melanoma cells [13].

Bentaib et al. used DIGE to allow a global approach to investigate metabolic reprogramming in transformed mouse astrocyte cells [5]. DIGE analysis of normal and transformed astrocytes allowed the investigators to identify and measure relative abundance of reprogrammed enzymes in carbohydrate and glutamate metabolism [5]. Using follow-up enzyme assays and zymography, they documented and confirmed numerous alterations in abundance and activity of various isoenzymes that are expected to be involved in metabolic programming [5]. Fanjul-Fernández et al. [14] used DIGE to reveal decreased levels of chitinase-3-like 3 (CHI3L3) and accumulation of the receptor for advanced glycation end-products and its ligand S100A8 in lung samples from *Mmp1* (Matrix metalloproteinase-1) deficient mice compared with those from wild-type [14]. In vitro-cleavage protease assays demonstrated that MMP-1 can cleave the large form of S100A8, similar to that produced by Matrix metalloproteinase-2 (MMP-2), used as a control. By contrast, S100A9 and CHI3L3 were not cleaved by this collagenolytic enzyme [14].

Faure et al. [6] investigated Citrulline (Cit) actions on muscle metabolism on Tibialis muscles from male Sprague-Dawley rats using a proteomic approach. They followed up their DIGE analysis with enzyme assays on key enzymes of mitochondrial metabolism (β-Hydroxyacyl-CoA dehydrogenase, citrate synthase, cytochrome c oxidase and NADH dehydrogenase Complex I) and these enzymes were not significantly affected, except for Complex I which was significantly increased in the Cit-refed rats, whereas the protein content of some NADH Complex 1 subunits was

significantly decreased [6]. The authors' personal data showed that NADH complex 1 activity is increased when cellular redox status is improved due to decreased reactive oxygen species (ROS) production and/or improved antioxidant protection, which was supported by data showing that Cit decreased the level of carbonylated proteins [6].

Jindahl et al. [18] performed DIGE proteomic analyses of Long QT syndrome (LQTS) transgenic rabbit LQT1 and LQT2 hearts. DIGE results showing upregulation of the expression of the enzymes associated with ATP generation was validated by the results of enzyme assays, which revealed increases in the activities of glycogen phosphorylase, lactate dehydrogenase, pyruvate dehydrogenase, and succinate dehydrogenase in LQT1 and LQT2 hearts, compared to littermate controls [18].

Menazza et al. [21] characterized the metabolic changes in mouse hearts lacking the mitochondrial chaperone Cyclophilin D, which regulates the mitochondrial permeability transition pore (MPTP) opening and is a major determinant of mitochondrial dysfunction and cardiomyocyte death during ischemia/reperfusion (I/R) injury [21]. DIGE analysis demonstrated decreased levels of succinate dehydrogenase and electron transfer flavoprotein but follow-up enzyme assays showed no alteration in activities, however. These data indicate the importance of follow-up assays as noncorroboration of DIGE data by enzyme assays could indicate a more complex process than first anticipated, which may prompt further corroborating experiments to be performed [21].

Marín-Buera et al. analyzed the cellular pathways and metabolic adaptations that occur in primary skin fibroblasts from patients with mutations in the *BCS1L* gene [25]. 2D-DIGE identified 36 proteins with altered expression that included alterations in energy metabolism, cell signaling and expression regulation, cytoskeleton formation, and intracellular stress response. The authors used GAPDH assays to validate the increased expression of GAPDH and a pyruvate dehydrogenase (PDH) assay to validate the upregulation of dihydrolipoyl dehydrogenase (DLD) which was found in the proteomic data [25].

Daneshvar et al. [28] used 2D-DIGE to elucidate the molecular basis of their attenuated *Leishmania infantum* line. They found 18 proteins that had significant changes in expression, and these proteins were components of the thiol-redox control system in this parasite. Further investigation of the attenuated strain to oxidative stress showed that the attenuated *Leishmania* parasite was significantly more susceptible to hydrogen peroxide. Enzyme activity assays for tryptathione-dependent peroxidase showed lower activity in the attenuated line, and further, the parasite demonstrated lower trypathione reductase in the attenuated line also [28].

The proteomic and physiological responses of *Brassicanapus* leaves under salt stress were investigated by Jia et al. [30].

A comparative proteomic analysis of seedling leaves exposed to 200 mM NaCl for 24, 48, and 72 h was conducted. Forty four protein spots were differentially accumulated upon NaCl treatment and of these, 42 were identified, including several novel salt-responsive proteins [30]. SOD and chitinase activities were significantly increased at 72 and 48 h of salt stress treatment, respectively, while the adenylpyrophosphatase (ATPase) activity was significantly reduced at all three time points, thus supporting the DIGE data [30].

Wendelboe-Nelson and Morris [15] utilized enzyme assays for glutamine synthase, thioredoxin reductase, class III peroxidise, catalase, ascorbate peroxidise, and lipoxygenase activities to support DIGE comparison studies of drought-resistant and susceptible barley strains which were grown under normal and drought conditions. The enzyme assay data showed similar patterns of activity or abundance as that determined by the original DIGE analysis [15].

1.3 Experimental Approaches for Enzyme Testing

DIGE analysis and enzyme testing have been performed on a variety of sources including animal tissue [4, 6, 14, 18, 22, 32–34], plant tissue [15, 16, 20, 23, 30], insect extracts [12], cell culture samples [5, 13, 24, 25], fungi [9, 26], bacteria [17, 29, 35, 36], parasite [28], and patient samples [27, 31].

1.3.1 Sources of Samples for DIGE Proteomics and Enzyme Testing

1.3.2 Sample Preparation Methods

A variety of methods for extracting proteins for DIGE analysis have been utilized and this is dependent on sample origin. For example, the preparation of culture supernatants for subsequent DIGE analysis by Fekkar et al. [11] involved the progressive depletion of fetal calf serum from culture of the bronchial cell line under study, concentration of cell culture media through a 3-KDa membrane filter, precipitation of the proteins using a clean-up kit, and subsequent suspension in isoelectric focusing buffer. Cell culture supernatants were used in the follow-up *N*-Acetyl-β-D-Glucosaminidase assays [11]. For more complex samples, such as animal tissue sample preparation for DIGE analysis and follow-up enzyme assays may involve numerous steps. For example, O'Connell and Ohlendieck [4] measured potential changes in the enzyme activity of select marker proteins from DIGE analysis of muscle mitochondria on crude muscle extracts. For the DIGE experiments mitochondrial pellets were combined and resuspended in DIGE lysis buffer and kept at pH 8.5 to ensure successful labeling by the Cyanine dyes [4]. The preparation process for the muscle tissue extract for enzyme assay involved grinding liquid-nitrogen frozen muscle tissue into fine powder with sonication followed by a centrifugation step [4].

1.3.3 Experimental Approaches for Enzyme Testing in DIGE Proteomics

Although there are many experimental approaches linking DIGE analysis and enzyme testing, it is possible to summarize a common experimental approach which is depicted in a schematic diagram in Fig. 1. In this scheme separate protein extractions steps are shown for DIGE and for enzyme assay. This does not necessarily indicate that the protein extraction step is performed on separate sample as outlined in the experiments by O'Connell and Ohlendieck [4]. Alternatively, there may be a common protein extraction step, which feeds into a separate labeling step for DIGE and separate processing steps for the enzyme activity assays.

1.3.4 Considerations when Preparing Samples for Enzyme Testing Experiments

Although extracts for enzyme assays and DIGE experiments need to be as similar as possible in order to produce meaningful data, consideration also needs to be made to maintaining optimal conditions for the enzyme activity assays, such as pH, temperature, ionic strength, cofactors, and coenzymes [37]. Maintaining enzyme stability is also important and also protection from protease activity may also be necessary, and this depends on the source of material for the DIGE experiment.

Overgaard et al. [12] utilized phenylmethylsulfonyl fluoride (PMSF) to prevent unwanted proteolysis from *Drosophila* fly material for enzyme assay but did not require inhibitors for the DIGE samples. When extracting muscle tissue for DIGE analysis, Capitanio et al. [33] added phenylmethane sulfonyl fluoride (PMSF) to the DIGE buffer, and for enzyme assays extracted the proteins using a Dounce homogenizer in a HEPES buffer containing phosphate, sucrose, and potassium chloride but not PMSF. The homogenate was immediately assayed [33]. Reducing agents, such as 2-mercaptoethanol, are often included to maintain activity and stability and to prevent the formation of aggregates [37, 38]. Low molecular weight additives such as salts, polyols and sugars and inert polymers such as polyethylene glycol may also be useful in stabilizing enzymes for activity measurement [37, 39]. Occasionally, proteins such as bovine serum albumin are added to the reaction buffer to stabilize enzymes [37]. In addition individual assay systems, such as for NADH Complex I of the respiratory chain, require bovine serum albumin as a critical component [40].

1.3.5 Optimization of Assay Conditions When Enzyme Testing

Once the extraction and buffering conditions have been satisfied the concentration of substrate in the assay also requires some consideration. The concentration of all substrates and cofactors directly involved in the enzyme reaction should be saturating in order that none of the reactants can be rate limiting [37]. Regarding substrate concentration it is recommended that this value should be considerably above the Michaelis constant (K_M) of the target enzyme for the substrate so as to ensure that all of the enzyme is saturated with substrate and the reaction proceeds at the maximum rate [41]. Once this is achieved enzyme activity is

linearly dependent on the amount of enzyme used [41]. This situation is attempted to be reached in enzyme activity measurements as far as practically possible, although this depends on substrate solubility, background interference, and also the possibility of a particular component acting on an enzyme in an unspecific manner, including as a substrate inhibitor [37]. Thus, a saturating concentration of $100K_M$ substrate is used as far as possible but generally a value of at least $10K_M$ can be used [37].

Co-substrates with a central role in metabolism, such as acetyl-CoA, ATP, or AMP, may also affect the rate of reaction by allosteric regulation [41]. Allosterically regulated enzymes, such as pyruvate dehydrogenase, have a quaternary structure and are composed of two or more structurally similar or identical subunits (protomers), each with a binding site for the substrate and another for the co-substrate [41, 42]. Cooperation of substrate and effector regulates the overall catalytic activity of the enzyme, which is dependent on metabolite concentration [41]. Allosteric enzymes do not exhibit classical Michaelis-Menten kinetics and produce a sigmoidal plot of initial rate vs. substrate concentration rather than the hyperbolic plot produced by classical Michaelis-Menten kinetics [41].

In addition, some of the assays are difficult to detect directly and require to be coupled to a more easily observed reaction [37]. One example of a coupled reaction is the assay for SOD, which relies on the ability of this enzyme to inhibit the reduction of nitro blue tetrazolium which was used by Jia et al. [30] in their study of the physiological responses to short-term salt stress in *Brassica napus* leaves.

Once an assay method has been chosen the final considerations are the amount of enzyme to add to the reaction mixture, incubation time and whether or not an activation step is required. As part of the assay development process the preliminary assays should be performed to determine the amount of enzyme sample added to the reaction mixture to produce a linear response. These experiments may show that the enzyme sample requires dilution prior to the addition to the reaction mixture. Alternatively, the assay incubation time may be reduced (Fig. 2a). Regarding incubation time, most assays are incubated at a set temperature for a defined incubation time before stopping by the addition of acid, base, or inhibitor and the amount of product formed or substrate converted is read afterwards (Fig. 2b) [37]. Alternatively, for some assays a signal is recorded over a set period of time usually in a spectrophotometer, typically with a thermostated cuvette holder in order to ensure a constant temperature during the assay (Fig. 2b). In addition, prior to substrate addition some enzymes require an activation phase, e.g., by interaction with a co-factor and this needs to be considered during the design of an enzyme assay [37].

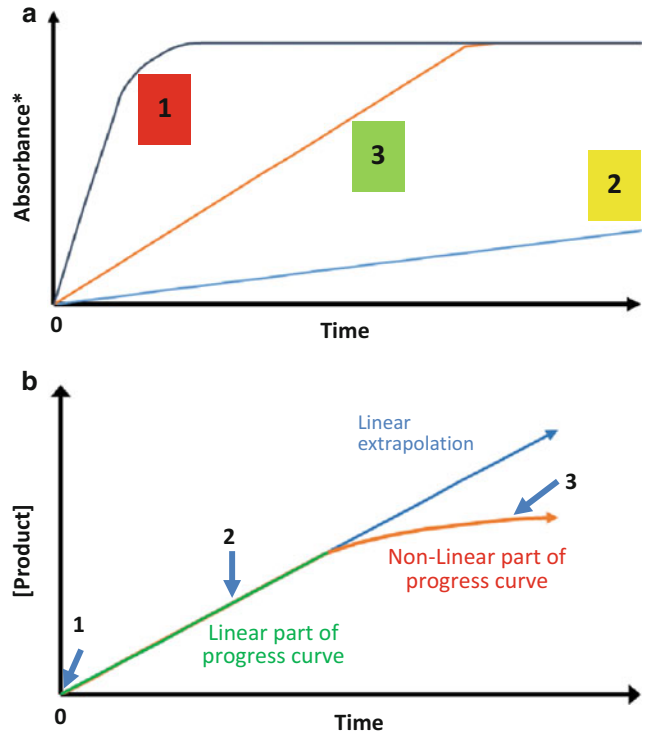

Fig. 2 Effect of enzyme addition (**a**) and incubation time (**b**) on enzyme assay data. (**a**) Enzyme activity is dependent on the amount of enzyme added to a reaction. When too much enzyme is added, the reaction becomes nonlinear after a short incubation time (*1*). When insufficient enzyme is added, the reaction is linear but the signal is low, leading to measurement errors due to background interference (*2*). When sufficient enzyme is added, the reaction is linear for a significant amount of time and generates a measurable signal (*3*). (**b**) Assays can either be continuous or stopped (depicted by *blue arrows*). For stopped assays the measurement points need to be chosen carefully. At time $T = 0$ (prior to enzyme addition) a blank reading is obtained (*1*). Subsequent measurement points need to be taken during the linear part of the enzyme reaction (*2*) and not during the nonlinear part of the progress curve (*3*). *Absorbance at λ_{max}

1.3.6 Other Enzyme Testing Methods

Most of the discussion so far has been focused on enzyme activity assays that are performed in solution. However, it may be possible in some instances to use zymography to measure enzyme activity such as the LDH zymograms performed by Bentaib et al. [5] and the novel use of DIGE by Morak et al. [3] to measure lipolytic and esterolytic activities in situ in 2D gels. Some investigators have developed methods to excise spots from a 2D gel which were subsequently re-natured and tested for enzyme activity but this method is beyond the scope of this chapter and is not discussed here [8].

1.4 Conclusions

This review has covered the different ways that enzyme testing can be used in DIGE proteomics. Enzyme assays are useful in the validation of an experimental system prior to a DIGE experiment and also as a vital part of multifaceted experiments that utilize DIGE proteomics. Enzyme activity data can be utilized to corroborate results of DIGE experiments where an enzyme has been demonstrated to be differentially expressed and can also be employed to support DIGE assay results demonstrating metabolic changes in biological systems. Finally, there are several experimental approaches for designing enzyme assays in a DIGE context, and due consideration should be given to the enzyme behavior with respect to its kinetic properties and stability in the test environment.

References

1. Sandberg AS, Buschmann V, Kapusta P et al (2016) Use of time-resolved fluorescence to improve sensitivity and dynamic range of gel-based proteomics. Anal Chem 88(6): 3067–3074. doi:10.1021/acs.analchem.5b03805
2. Hurd TR, Prime TA, Harbour ME et al (2007) Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis: implications for mitochondrial redox signaling. J Biol Chem 282 (30):22040–22051. doi:10.1074/jbc.M703591200
3. Morak M, Schmidinger H, Krempl P et al (2009) Differential activity-based gel electrophoresis for comparative analysis of lipolytic and esterolytic activities. J Lipid Res 50 (7):1281–1292. doi:10.1194/jlr.M800566-JLR200
4. O'Connell K, Ohlendieck K (2009) Proteomic DIGE analysis of the mitochondria-enriched fraction from aged rat skeletal muscle. Proteomics 9(24):5509–5524. doi:10.1002/pmic.200900472
5. Bentaib A, De Tullio P, Chneiweiss H et al (2014) Metabolic reprogramming in transformed mouse cortical astrocytes: a proteomic study. J Proteome 113:292–314. doi:10.1016/j.jprot.2014.09.019
6. Faure C, Morio B, Chafey P et al (2013) Citrulline enhances myofibrillar constituents expression of skeletal muscle and induces a switch in muscle energy metabolism in malnourished aged rats. Proteomics 13(14): 2191–2201. doi:10.1002/pmic.201200262
7. Lubec G, Afjehi-Sadat L, Yang JW, John JPP (2005) Searching for hypothetical proteins: theory and practice based upon original data and literature. Prog Neurobiol 77(1–2): 90–127. doi:10.1016/j.pneurobio.2005.10.001
8. Afjehi-Sadat L, Lubec G (2007) Identification of enzymes and activity from two-dimensional gel electrophoresis. Nat Protoc 2(10): 2318–2324. doi:10.1038/nprot.2007.317
9. Boone CHT, Grove RA, Adamcova D et al (2016) Revealing oxidative damage to enzymes of carbohydrate metabolism in yeast: an integration of 2D DIGE, quantitative proteomics, and bioinformatics. Proteomics 16(13): 1889–1903. doi:10.1002/pmic.201500546
10. Mandili G, Alchera E, Merlin S et al (2015) Mouse hepatocytes and LSEC proteome reveal novel mechanisms of ischemia/reperfusion damage and protection by A2aR stimulation. J Hepatol 62(3):573–580. doi:10.1016/j.jhep.2014.10.007
11. Fekkar A, Balloy V, Pionneau C et al (2012) Secretome of human bronchial epithelial cells in response to the fungal pathogen Aspergillus fumigatus analyzed by differential in-gel electrophoresis. J Infect Dis 205(7):1163–1172. doi:10.1093/infdis/jis031
12. Overgaard J, Sørensen JG, Com E, Colinet H (2014) The rapid cold hardening response of Drosophila melanogaster: complex regulation across different levels of biological organization. J Insect Physiol 62(1):46–53. doi:10.1016/j.jinsphys.2014.01.009
13. Winkler J, Stessl M, Amartey J, Noe CR (2010) Off-target effects related to the phosphorothioate modification of nucleic acids. ChemMedChem 5(8):1344–1352. doi:10.1002/cmdc.201000156
14. Fanjul-fernández M, Folgueras AR, Fueyo A et al (2013) Matrix metalloproteinase Mmp-1a is dispensable for normal growth and

fertility in mice and promotes lung cancer progression by modulating inflammatory responses. J Biol Chem 288(20):14647–14656. doi:10.1074/jbc.M112.439893

15. Wendelboe-Nelson C, Morris PC (2012) Proteins linked to drought tolerance revealed by DIGE analysis of drought resistant and susceptible barley varieties. Proteomics 12(22): 3374–3385. doi:10.1002/pmic.201200154
16. He C, Gao G, Zhang J et al (2016) Proteome profiling reveals insights into cold-tolerant growth in sea buckthorn. Proteome Sci 14 (1):14. doi:10.1186/s12953-016-0103-z
17. Schröder C, Matthies A, Engst W et al (2013) Identification and expression of genes involved in the conversion of daidzein and genistein by the equol-forming bacterium slackia isoflavoniconvertens. Appl Environ Microbiol 79 (11):3494–3502. doi:10.1128/AEM.03693-12
18. Jindal HK, Merchant E, Balschi JA et al (2012) Proteomic analyses of transgenic LQT1 and LQT2 rabbit hearts elucidate an increase in expression and activity of energy producing enzymes. J Proteome 75(17):5254–5265. doi:10.1016/j.jprot.2012.06.034
19. Lennicke C, Rahn J, Kipp AP et al (2017) Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice. Biochim Biophys Acta 1861(1 Pt A):3323–3334. doi:10.1016/j.bbagen.2016.08.015
20. He CY, Zhang GY, Zhang JG et al (2016) Physiological, biochemical, and proteome profiling reveals key pathways underlying the drought stress responses of Hippophae rhamnoides. Proteomics 16(20):2688–2697. doi:10.1002/pmic.201600160
21. Menazza S, Wong R, Nguyen T et al (2013) CypD−/− hearts have altered levels of proteins involved in Krebs cycle, branch chain amino acid degradation and pyruvate metabolism. J Mol Cell Cardiol 56:81–90. doi:10.1016/j.yjmcc.2012.12.004
22. Pieper R, Martin L, Schunter N et al (2015) Impact of high dietary zinc on zinc accumulation, enzyme activity and proteomic profiles in the pancreas of piglets. J Trace Elem Med Biol 30:30–36. doi:10.1016/j.jtemb.2015.01.008
23. Li X, Jin Z, Gao F et al (2014) Comparative proteomic analysis of Dan'er malts produced from distinct malting processes by two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE). J Agric Food Chem 62:9310–9316. doi:10.1021/jf5030483
24. García-Lorenzo A, Rodríguez-Piñeiro AM, Rodríguez-Berrocal FJ et al (2012) Changes on the Caco-2 secretome through differentiation analyzed by 2-D differential in-gel electrophoresis (DIGE). Int J Mol Sci 13 (11):14401–14420. doi:10.3390/ijms131114401
25. Marín-Buera L, García-Bartolomé A, Morán M et al (2015) Differential proteomic profiling unveils new molecular mechanisms associated with mitochondrial complex III deficiency. J Proteome 113:38–56. doi:10.1016/j.jprot.2014.09.007
26. Gutiérrez-Sánchez G, Atwood J, Kolli VSK et al (2012) Initial proteome analysis of caffeine-induced proteins in Aspergillus tamarii using two-dimensional fluorescence difference gel electrophoresis. Appl Biochem Biotechnol 166(8):2064–2077. doi:10.1007/s12010-012-9634-7
27. Ray S, Patel SK, Venkatesh A et al (2016) Clinicopathological analysis and multipronged quantitative proteomics reveal oxidative stress and cytoskeletal proteins as possible markers for severe vivax malaria. Sci Rep 6:24557. doi:10.1038/srep24557
28. Daneshvar H, Wyllie S, Phillips S et al (2012) Comparative proteomics profiling of a gentamicin-attenuated Leishmania infantum cell line identifies key changes in parasite thiol-redox metabolism. J Proteome 75 (5):1463–1471. doi:10.1016/j.jprot.2011.11.018
29. Drüppel K, Hensler M, Trautwein K et al (2014) Pathways and substrate-specific regulation of amino acid degradation in Phaeobacter inhibensDSM 17395 (archetype of the marine Roseobacter clade). Environ Microbiol 16 (1):218–238. doi:10.1111/1462-2920.12276
30. Jia H, Shao M, He Y et al (2015) Proteome dynamics and physiological responses to short-term salt stress in Brassica napus leaves. PLoS One 10(12). doi:10.1371/journal.pone.0144808
31. von Löhneysen K, Scott TM, Soldau K et al (2012) Assessment of the red cell proteome of young patients with unexplained hemolytic anemia by two-dimensional differential in-gel electrophoresis (DIGE). PLoS One 7(4). doi:10.1371/journal.pone.0034237
32. De Palma S, Leone R, Grumati P et al (2013) Changes in muscle cell metabolism and mechanotransduction are associated with myopathic phenotype in a mouse model of collagen VI deficiency. PLoS One 8(2). doi:10.1371/journal.pone.0056716
33. Capitanio D, Vasso M, Ratti A et al (2012) Molecular signatures of amyotrophic lateral sclerosis disease progression in hind and

forelimb muscles of an SOD1(G93A) mouse model. Antioxid Redox Signal 17(10): 1333–1350. doi:10.1089/ars.2012.4524

34. Dorts J, Kestemont P, Thézenas ML et al (2014) Effects of cadmium exposure on the gill proteome of Cottus gobio: modulatory effects of prior thermal acclimation. Aquat Toxicol 154:87–96. doi:10.1016/j.aquatox.2014.04.030
35. Kube M, Chernikova TN, Al-Ramahi Y et al (2013) Genome sequence and functional genomic analysis of the oil-degrading bacterium Oleispira antarctica. Nat Commun 4:2156. doi:10.1038/ncomms3156
36. Junghare M, Spiteller D, Schink B (2016) Enzymes involved in the anaerobic degradation of ortho-phthalate by the nitrate-reducing bacterium Azoarcus sp. strain PA01. Environ Microbiol 18(9):3175–3188. doi:10.1111/1462-2920.13447
37. Bisswanger H (2014) Enzyme assays. Perspect Sci 1(1–6):41–55. doi:10.1016/j.pisc.2014.02.005
38. Trivedi M, Laurence J, Siahaan T (2009) The role of thiols and disulfides on protein stability. Curr Protein Pept Sci 10(6):614–625. doi:10.2174/138920309789630534
39. Fágáin CÓ (1995) Understanding and increasing protein stability. Biochim Biophys Acta 1252(1):1–14. doi:10.1016/0167-4838(95)00133-F
40. Janssen AJM, Trijbels FJM, Sengers RCA et al (2007) Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. Clin Chem 53(4): 729–734. doi:10.1373/clinchem.2006.078873
41. Aehle W (2007) Enzymes in industry: production and applications. Wiley, Weinheim. doi:10.1002/9783527617098
42. Linn TC, Pettit FH, Reed LJ (1969) Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. Proc Natl Acad Sci U S A 62:234–241. doi:10.1073/pnas.62.1.234

Chapter 21

Enzyme Assay Methods to Validate DIGE Proteomics Data

Andrew Dowd

Abstract

Enzyme activity assay methods can be used to corroborate the results generated by Difference Gel Electrophoresis (DIGE) proteomic experiments. Two assay methods were chosen to demonstrate how this can be achieved. Assays for determining the activity of superoxide dismutase and NADH dehydrogenase are outlined in detail in this paper. These methods were chosen as examples because they are frequently used in conjunction with DIGE proteomics.

Key words Difference gel electrophoresis, DIGE, Enzymology, Enzyme, Enzyme assay, Superoxide dismutase, NADH dehydrogenase, Fluorescence spectrophotometry, Spectrophotometry

1 Introduction

In the area of quantitative intact proteomics (QIP), two-dimensional gel electrophoresis (2-DE) is the most important technique for the separation of intact proteins [1]. Difference Gel Electrophoresis (DIGE) enables multiplexing of gel images with a maintained super-imposable protein separation pattern. The DIGE method achieves this through the application of mass- and charge-matched cyanine dyes (Cy™2, Cy™3, and Cy™5) which allow the correction of experimental variation through the use of a pooled internal standard. The correction of experimental variation is a key step in improving the quantitative aspects and overall statistical power of the 2-DE technique [1].

There are many examples of enzyme assays used to directly validate DIGE results, for example, DIGE experiments as described by O'Connell and Ohlendieck [2], Mandili et al. [3], and Li et al. [4]. Alternatively, enzyme assays can also be utilized to validate the metabolic changes found by DIGE analyses as described by Bentaib et al. [5], Faure et al. [6], and Jia et al. [7].

In this chapter, two relatively simple enzyme activity assay methods which can be carried out in parallel to a DIGE experiment are described. The first assay is used to test for superoxide dismutase

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_21,

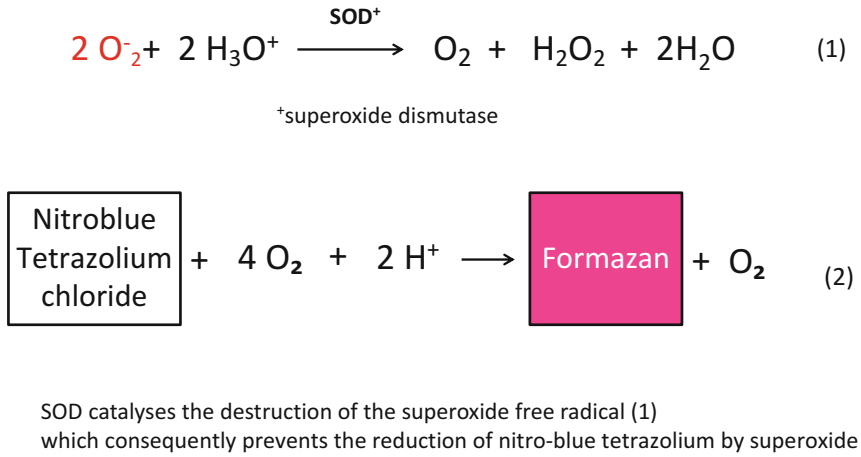

Fig. 1 Reaction scheme for SOD assay. SOD catalyzes the destruction of the Superoxide free radical (*1*) which consequently prevents the reduction of nitro-blue tetrazolium by superoxide (*2*) (adapted from Naraginti et al. [8])

(SOD) activity (Fig. 1) and has been selected because increase in SOD expression is linked to oxidative stress [7, 9] and is also an example of a stopped colorimetric assay. The second assay selected is for reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (Fig. 2) which is a continuous assay and was used to directly validate DIGE data by O'Connell and Ohlendieck [2]. The NADH dehydrogenase assay in this example uses fluorescence detection as a comparison to the technically simpler SOD activity assay [2].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Superoxide Dismutase (SOD) Assay

1. Potassium phosphate buffer pH 7.8: Prepare 1 L of 100 mM monobasic anhydrous potassium phosphate by adding 13.6 g of powder to a 1 L beaker and adding 900 mL of water. Mix till dissolved and make up to the mark in a 1 L graduated cylinder. Prepare 1 L of 100 mM dibasic potassium phosphate by adding 17.4 g of anhydrous powder to a 1 L beaker and adding 900 mL of water. Mix till dissolved and make up to the mark in a 1 L graduated cylinder. Add 750 mL of the dibasic potassium phosphate solution to a 1 L beaker and with a pipette add sufficient volume of monobasic potassium phosphate solution to achieve a pH of 7.8 as measured by a pH meter (e.g. Mettler SevenGo^{TM} portable pH meter) (*see* **Note 1**).
2. Riboflavin 100× stock (130 mM): Add 1.2 mg of Riboflavin in 25 mL of 100 mM potassium phosphate buffer.

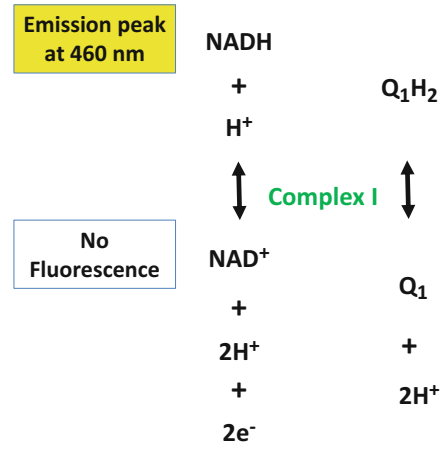

Fig. 2 Reaction scheme for measurement of NADH dehydrogenase activity by mitochondrial complex I. Under normal physiological conditions, mitochondrial complex I catalyzes the two-electron oxidation of NADH (*1*) and reduction of Coenzyme Q_1 (Q_1) (*2*) (adapted from Hirst [10])

3. Nitro Blue Tetrazolium (NBT) Stock solution: dissolve one NBT tablet in 1 mL of ultrapure water (*see* **Note 2**).
4. SOD substrate: Prepare the SOD substrate by adding 49.4 mL of 100 mM phosphate buffer, 0.5 mL of 100× riboflavin stock, 96.5 mg methionine and 106.4 µL of NBT stock solution to a 50 mL Falcon® tube (*see* **Note 3**).
5. Glass Test tubes (round bottomed 12 × 75 mm, Borosilicate) plus suitable wire test tube rack to allow access to light.
6. Semi-Micro glass cuvette 1 cm light path, 1.4 mL volume (e.g. Hellma® Analytics type 104-OS) (*see* **Note 4**).
7. Thermostated water bath set at 25 °C.
8. Fluorescent lamp mounted on a suitable retort stand.
9. Box lined with Aluminum foil to cover both lamp and water bath assembly (*see* **Note 5**).

2.2 NADH Dehydrogenase Assay

1. Homogenization Buffer (2×) (1.0 M HEPES sodium salt, pH 7.4, 400 mM ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*,*N*′,*N*′-tetraacetic acid (EGTA), 20% w/v sucrose, 6 mM magnesium chloride, 0.2% w/v sodium azide): Add 6.0 g Hepes (*see* **Note 6**), 3.8 g EGTA, 5 g sucrose, 14.3 mg anhydrous magnesium chloride and 50 mg sodium azide to 20 mL of water. Mix to dissolve and adjust to 25 mL in a volumetric flask (*see* **Note 7**).
2. Buffer A (50 mM sodium phosphate pH 6.5). Prepare 1 L of 50 mM monobasic sodium phosphate by adding 6 g of anhydrous powder to a 1 L beaker and adding 900 mL of water. Mix till dissolved and make up to the mark in a 1 L graduated

cylinder. Prepare 1 L of 50 mM dibasic sodium phosphate by adding 7.1 g of powder to a 1 L beaker and adding 900 mL of water. Mix till dissolved and make up to the mark in a 1 L graduated cylinder. Add 600 mL of the dibasic potassium phosphate solution to a 1 L beaker and with a pipette add sufficient volume of monobasic potassium phosphate solution to achieve a pH of 6.5 as measured by a pH meter (e.g. Mettler SevenGo™ portable pH meter) (*see* **Note 1**).

3. Incubation buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM Coenzyme Q_1, 2 mM potassium cyanide (KCN) and 0.2 mM NADH in 50 mM sodium phosphate pH 6.5): Add 14.6 mg EDTA, 1.3 mg Coenzyme Q_1, 6.5 mg KCN and 7.1 mg NADH (disodium salt) to 50 mL Buffer A (*see* **Note 8**).
4. Pepstatin stock (100 μM): Add 3.4 mg pepstatin to 50 mL of water.
5. Aprotinin stock (10 μM): Add 1.3 mg aprotinin to 20 mL of water.
6. E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane) stock (60 μM): Add 1.1 mg E-64 to 50 mL of water.
7. Leupeptin stock (200 μM): Add 1 mg leupeptin to 10 mL of water.
8. Soybean trypsin inhibitor (100 μM): Add 2 mg of soybean trypsin inhibitor to 1 mL of water.
9. Protease cocktail (2×) (2 mM EDTA, 0.4 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 2.8 μM pepstatin, 0.3 μM aprotinin, 0.6 μM E-64, 2 μM leupeptin and 1.0 μM soybean trypsin inhibitor): Add 7.4 mg Disodium EDTA, 1 mg AEBSF, 280 μL pepstatin A stock solution, 30 μL aprotinin stock solution, 60 μL E-64 stock solution, 100 μL of leupeptin stock solution and 100 μL of soybean trypsin inhibitor stock solution to 9.4 mL of water (*see* **Note 9**).
10. Semi-micro quartz cuvette 1 cm path length suitable for fluorescence measurements (*see* **Note 10**).
11. Ultrasonic homogenizer (e.g. Sonopuls® HD 2200 apparatus from Bandelin, Berlin, Germany).
12. Fluorescence Spectrophotometer (e.g. Agilent® Cary Eclipse).

3 Methods

3.1 Superoxide Dismutase (SOD) Activity Measurement

1. Grind 0.5 g of leaf sample in an ice-chilled mortar with 5 mL potassium phosphate buffer pH 7.8 (*see* **Note 11**).
2. Transfer to a 15 mL Falcon® tube and centrifuge the homogenate at 12,000 × *g* for 20 min at 4 °C.

3. Add 50 μl of the supernatant from **step 2** to SOD substrate (usually 1–2 mL) prepared as described in Subheading 2.1 to a clean glass test tube (*see* **Note 12**).
4. Gently mix using a vortex mixer.
5. Immediately place the tube in a rack in the water bath.
6. Place the lamp so that the samples are fully exposed to light and cover the assembly with the light-proof box (*see* **Note 13**).
7. Switch on the luminescent lamp and illuminate the mixture for 15 min.
8. Switch off the lamp and remove the tubes from the waterbath.
9. Pipette 1 mL of sample into a cuvette.
10. Measure absorbance in a spectrophotometer set at 560 nm.
11. One unit of SOD was defined as the amount of enzyme that induced 50% inhibition of the NBT reduction, and SOD activity was expressed as unit g fresh weight^{-1} (*see* **Note 14**).

3.2 NADH Dehydrogenase Activity Measurement

3.2.1 Preparation of Muscle Extracts

1. Grind frozen muscle specimens in the presence of liquid nitrogen into a fine powder using a mortar and pestle (*see* **Note 15**).
2. Place the resulting powder into 0.5 mL of 2× homogenization buffer.
3. Add 0.5 mL of 2× protease cocktail to the mixture from **step 2**.
4. Add 0.2 μL of DNase I solution (*see* **Note 16**).
5. Homogenize for 10 s with an ultrasonic homogenizer.
6. Incubate the suspensions on ice, with gentle vortexing every 10 min for 10 s, for 4 h.
7. Centrifuge the suspensions for 20 min at 20,000 × *g* in a refrigerated microcentrifuge.
8. Retain the supernatant fraction and determine the protein concentration by the method of Bradford [11].

3.2.2 Measurement of NADH Dehydrogenase Activity

1. Pre-incubate an aliquot of 20 μL of supernatant fraction from **step 6** in 960 μL incubation buffer at room temperature in a 1.4 mL semi-micro cuvette (*see* **Note 12**).
2. Place the cuvette containing the buffer and sample into a fluorescence spectrophotometer set at an excitation wavelength of 340 nm and an emission wavelength and 460 nm (*see* **Note 17**).
3. After 3 min, add 20 μL of 10 mM NADH stock solution prepared as described in Subheading 2.2, above and mix with a pipette (*see* **Note 8**).
4. Record the fluorescence intensity for 10 min.

5. The oxidation of NADH to NAD^+ by NADH dehydrogenase is monitored by reduction of the emission peak 460 nm (*see* **Note 18**).
6. Activity units can be expressed as (μmol/min/mg at 25 °C).

4 Notes

1. Add water to a beaker with a stirring bar and add small amounts of powder to avoid caking.
2. We use NBT tablets for the sake of convenience (Sigma Catalogue number N5514 which contain approximately 25 mg per NBT per tablet). The tablets are stored between 2 and 8 °C.
3. Prepare fresh for each assay.
4. Using a glass cuvette provides more consistent readings.
5. Use a suitably sized box lined with aluminum foil and a fluorescence lamp (e.g. Phillips MLL 500 W Hg) [9, 12].
6. This may require adjustment to pH 7.4 with concentrated HCl (4 M) solution.
7. Sodium azide is known to be explosive when heated near its decomposition temperature or when it comes in contact with certain metals. It is safe to use when diluted so it is advisable to make a stock solution to be stored in a toxic cabinet for subsequent dilution into the homogenization buffer. It is imperative to follow the directions in the safety data sheet for this chemical.
8. Prepare fresh for each assay as NADH is unstable.
9. For convenience, a 2× protease inhibitor cocktail stock can be made from commercially available tablets [13]. For example, Sigma Complete™ Ultra Tablets can be used (Sigma catalogue number 05892970001).
10. For example, a Hellma®, semi Micro Suprasil® quartz, limit 200–2500 nm spectral range, pathlength 10 × 4 mm, chamber volume 1400 μL fluorescence cuvette can be used.
11. For validation of DIGE experiments, it is important to match samples as closely as possible. In this instance, leaf samples need to be matched by weight prior to protein extraction.
12. It is crucial to ensure that sufficient sample is added to the assay to achieve a linear reaction rate. The test sample may also be diluted to ensure that excess enzyme has not been added to the reaction. Dilution scouting experiments may be necessary to optimize the reaction conditions. If possible a commercially sourced enzyme should be used as a positive control for each reaction to ensure reproducibility.

13. Identical tubes which have not been exposed to the fluorescence light can be used as blanks [9].
14. To express activity as units per mg protein, the protein concentration of the supernatant from **step 2** can be estimated using the Bradford method [11].
15. Equal quantities of 100 mg wet weight of young and aged gastrocnemius muscle were used.
16. DNase I is available as a commercially available stock solution or as a lyophilized powder. Two units of DNase I is added per 1 mL of homogenate. DNase I is added to reduce the interference by excess DNA in the assay.
17. The use of fluorescence to determine NADH concentrations allows an order of magnitude greater sensitivity than absorbance.
18. To convert from fluorescence to NADH construct a standard curve of fluorescence v NADH concentration in the 0–20 μM range. The rate of conversation of NADH to NAD+ by NADH dehydrogenase can be calculated as a reduction in NADH over the incubation period.

References

1. Sandberg AS, Buschmann V, Kapusta P et al (2016) Use of time-resolved fluorescence to improve sensitivity and dynamic range of gel-based proteomics. Anal Chem 88 (6):3067–3074. doi:10.1021/acs.analchem.5b03805
2. O'Connell K, Ohlendieck K (2009) Proteomic DIGE analysis of the mitochondria-enriched fraction from aged rat skeletal muscle. Proteomics 9(24):5509–5524. doi:10.1002/pmic.200900472
3. Mandili G, Alchera E, Merlin S et al (2015) Mouse hepatocytes and LSEC proteome reveal novel mechanisms of ischemia/reperfusion damage and protection by A2aR stimulation. J Hepatol 62(3):573–580. doi:10.1016/j.jhep.2014.10.007
4. Li X, Jin Z, Gao F et al (2014) Comparative proteomic analysis of Dan'er malts produced from distinct malting processes by two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE). J Agric Food Chem 62:9310–9316
5. Bentaib A, De Tullio P, Chneiweiss H et al (2014) Metabolic reprogramming in transformed mouse cortical astrocytes: a proteomic study. J Proteome 113:292–314. doi:10.1016/j.jprot.2014.09.019.
6. Faure C, Morio B, Chafey P et al (2013) Citrulline enhances myofibrillar constituents expression of skeletal muscle and induces a switch in muscle energy metabolism in malnourished aged rats. Proteomics 13(14):2191–2201. doi:10.1002/pmic.201200262
7. Jia H, Shao M, He Y et al (2015) Proteome dynamics and physiological responses to short-term salt stress in Brassica napus leaves. PLoS One 10(12):e0144808. doi:10.1371/journal.pone.0144808
8. Naraginti S, Li Y, Wu Y et al (2016) Mechanistic study of visible light driven photocatalytic degradation of EDC 17[small alpha]-ethinyl estradiol and azo dye Acid Black-52: phytotoxicity assessment of intermediates. RSC Adv 6(90):87246–87257. doi:10.1039/C6RA20702B
9. Bailly C, Benamar A, Corbineau F, Côme D (1996) Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. Physiol Plant 97(1):104–110. doi:10.1111/j.1399-3054.1996.tb00485.x
10. Hirst J (2010) Towards the molecular mechanism of respiratory complex I. Biochem J 425 (2):327–339. doi:10.1042/BJ20091382

11. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1–2):248–254. doi:10.1016/0003-2697(76)90527-3

12. Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol 59(2):309–314

13. Doran P, Dowling P, Lohan J et al (2004) Subproteomics analysis of Ca2+-binding proteins demonstrates decreased calsequestrin expression in dystrophic mouse skeletal muscle. Eur J Biochem 271(19):3943–3952. doi:10.1111/j.1432-1033.2004.04332.x

Chapter 22

Immunoblot Analysis of DIGE-Based Proteomics

Martin Landsberger and Heinrich Brinkmeier

Abstract

Proteins can be separated according to their size by gel electrophoresis and further analyzed by Western blotting. The proteins can be transferred to a membrane made of nitrocellulose or polyvinylidene fluoride (PVDF), which results in a replica of the proteins' separation patterns. The proteins on the membrane can be detected by specific antibodies followed by visualization either on the membrane itself, on film or by CCD cameras. Western blotting is a sensitive technique to verify data obtained from difference gel electrophoresis (DIGE)-based proteomics.

Key words Electrophoresis, Denatured proteins, Polyacrylamide gels, Molecular weight, Antibodies, Immunoblotting, Enhanced chemiluminescence (ECL)

1 Introduction

Western blotting is the transfer of proteins to a membrane support and the subsequent immunological detection [1]. There are several arguments to use Western blotting in combination with difference gel electrophoresis (DIGE)-based proteomics; two of them should be mentioned. First, it is a generally good concept in science to verify obtained data with an independent method. Second, modern proteomic techniques are getting increasingly sensitive and allow the detection of low abundance proteins. If such techniques are applied to complex mammalian tissues, such as liver or skeletal muscle, hits of low abundance could originate from blood, vessels, or nerves instead from the intended target tissues. To avoid misinterpretations of proteomic results, Western blotting and, in continuation, immunofluorescence microscopy can be valuable subsidiary methods. Western blotting is routinely used to verify findings of DIGE-based proteomics [2]. Proteins, e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates, are separated on polyacrylamide gels under denaturing conditions and are then transferred to membranes, in most cases made of nitrocellulose or polyvinylidene difluoride resulting in a replica of the

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_22,

Table 1
Separation range of gels under denaturing conditions

Acrylamide concentration (%)	6	8	10	12	15
Separation range (kDa)	50–200	30–95	20–80	12–60	10–43

original gel pattern [3]. Transfer can be performed by diffusion, capillary action, or electrophoresis with the latter being the most widely used technique due to simplicity, performance, duration of the transfer, and cost-effectiveness [3]. Electrophoretic transfer of proteins to nitrocellulose membranes was first described by Towbin and coworkers [1] whereas Burnette continued "with due respect to Southern [..], the established tradition of "geographic" naming of transfer techniques ("Southern," "Northern")" and introduced the term "Western" blotting [4].

Electrophoresis of proteins is usually performed in vertical polyacrylamide gels under either native or denaturing conditions. Though larger gels provide more separation area and thus better resolution for complex samples, small format mini-gels are typically used because they are easy to use and less material is needed for the whole Western blotting experiment as compared to large gels. Proteins that are separated by polyacrylamide gel electrophoresis (PAGE) respond to an electrical field and migrate through pores in the gel matrix. The higher the concentration of the monomeric acrylamide, the smaller the pore size will be within the polyacrylamide gel. The migration rates of the proteins to be separated are dependent on gel pore size and protein charge, size, and shape.

In the following, we describe how to perform one-dimensional gel electrophoresis under denaturing conditions using 0.1% sodium dodecyl sulfate (SDS)—first described by Laemmli [5]—how to transfer the proteins to a membrane support and how to detect proteins by specific antibodies. Typically, the polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. The separating gel is often called resolving or running gel. PAGE of proteins as described here works for a wide molecular weight range from 10 to 200 kDa (Table 1).

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Follow safety recommendations and waste disposal regulations when handling toxic and hazardous substances and disposing waste.

2.1 Equipment

1. 25 ml Erlenmeyer side-arm flasks.
2. Vacuum pump with cold trap.

3. Electrophoresis apparatus, e.g., *PROTEAN II xi Cell* (Bio-Rad Laboratories), *miniVE Vertical Electrophoresis System* (GE Healthcare) or equivalent, with pressure cams, glass plates, casting stand, and buffer tank (*see* **Note 1**).
4. 0.75 or 1.00 mm spacers.
5. 0.45 μm filters (used in stock solution preparation).
6. 0.75 or 1.00 mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth.
7. Tray.
8. PVDF membrane (*see* **Note 2**).
9. Film, e.g., *Hyperfilm ECL* (Amersham) or equivalent (*see* **Note 3**).
10. X-ray film cassette.
11. Film developing solution.
12. Film fixing solution.
13. Clamps, clothes-pegs, or equivalent.
14. Software for densitometry, e.g., *ImageJ*, *Scion Image*, or *Image Lab™ Software* (Bio-Rad Laboratories) etc., and statistical analysis, e.g., *GraphPadPrism* or *SigmaStat*.

2.2 Reagents

All chemicals used should be of analytical grade.

1. Protein molecular weight standard.
2. Primary antibody (specific to the protein of interest).
3. Secondary antibody, species-specific anti-Ig conjugated to horseradish peroxidase.

2.3 Solutions

All solutions should be prepared with analytical grade chemicals and ultrapure water.

2.3.1 Preparation of SDS Polyacrylamide Gels

1. Acrylamide solution: 30% (29.2% acrylamide/0.8% bisacrylamide solution): *Caution*: Wear a safety mask and gloves while handling the neurotoxic acrylamide. Weigh 29.2 g acrylamide and 0.8 g *N*,*N*′-methylenebisacrylamide. Dissolve in H_2O. Adjust volume to 100 ml. Filter the solution through a 0.45 μm filter. Store in the dark at 4 °C (*see* **Note 4**).
2. 4× Tris–HCl/SDS, pH 8.8 (1.5 M Tris–HCl, 0.4% SDS): Dissolve 182 g Tris base in 600 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 1000 ml total volume. Filter the solution through a 0.45 μm filter. Add 4 g sodium dodecyl sulfate (SDS) and swirl. Store up to 3 months at 4 °C.
3. 1× Tris–HCl/SDS, pH 8.8 (0.375 M Tris–HCl, 0.1% SDS): Dilute 4× Tris–HCl/SDS, pH 8.8 with H_2O.
4. 4× Tris–HCl/SDS, pH 6.8 (0.5 M Tris–HCl, 0.4% SDS): Dissolve 6.05 g Tris base in 60 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the

solution through a 0.45 μm filter. Add 0.4 g SDS and swirl. Store up to 3 months at 4 °C.

5. 1× Tris–HCl/SDS, pH 6.8 (0.125 M Tris–HCl, 0.1% SDS): Dilute 4× Tris-HCl/SDS, pH 6.8 with H_2O.
6. Ammonium persulfate solution (10%): Dissolve 1 g ammonium persulfate in 10 ml H_2O. Store at 4 °C for several weeks.
7. Separation gel solution (10% acrylamide): Prepare immediately before use. Use a 25 ml side-arm flask. Add 5 ml of 30% acrylamide solution, 3.75 ml of 4× Tris-Cl/SDS, pH 8.8, and 6.25 ml of H_2O. Swirl gently to mix. Degas under vacuum for approximately 5 min (*see* **Note 5**). Add 50 μl of 10% ammonium persulfate and 10 μl of *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED). Swirl gently to mix. Use immediately for pouring the separating gel. Required volumes for separating gels with different acrylamide concentrations are shown in Table 2.
8. Stacking gel solution (3.9% acrylamide): Prepare immediately before use. Use a 25 ml side-arm flask. Add 0.65 ml of 30% acrylamide solution, 1.25 ml of 4× Tris–HCl/SDS, pH 6.8, and 3.05 ml H_2O. Degas under vacuum for approximately 5 min (*see* **Note 6**). Add 25 μl of 10% ammonium persulfate and 5 μl TEMED. Swirl gently to mix. Use immediately for pouring the stacking gel.
9. H_2O-saturated isobutyl alcohol: Put 50 ml of isobutyl alcohol and 50 ml of H_2O in a glass separatory funnel. Shake, release pressure, and wait until the suspension is clear. Repeat three to five times. Discard lower phase and collect upper phase in a glass bottle. Use the collected upper phase, i.e., H_2O-saturated isobutyl alcohol, for overlaying the polymerized separating gel. Store indefinitely at room temperature.
10. Protein molecular weight standards (*see* **Note 7**): Dissolve protein molecular weight standards in 1× SDS sample buffer

Table 2
Required volumes for separating gels with different acrylamide concentrations

Acrylamide concentration (%)	6	8	10	12	15
30% Acrylamide solution (29.2% acrylamide/0.8% bisacrylamide)	2.0	2.7	3.3	4.0	5.0
4× Tris pH 8.8	2.5				
H_2O (sterile)	5.3	4.6	4.0	3.3	2.3
10% (w/v) SDS	100 μl				
10% (w/v) Ammonium persulfate	100 μl				
TEMED	10 μl				

Unless otherwise stated, all volumes are given in ml and refer to a final volume of separating gel solution of 10 ml

according to supplier's instructions. Use these standards as a control.

11. 6× SDS sample buffer: Mix 7 ml 4× Tris–HCl/SDS, pH 6.8 and 3 ml glycerol (30% final concentration). Add 1 g SDS (10% final), 0.93 g DTT (0.6 M final), 1.2 mg Bromophenol Blue (0.012% final) and mix. Adjust volume to 10 ml with H_2O. Prepare 0.5 ml aliquots and store at −70 °C.
12. 2× and 1× SDS sample buffer: Dilute 6× SDS sample buffer with H_2O.
13. 10× SDS electrophoresis buffer: Add 30.2 g Tris base (0.125 M final), 144 g glycine (0.96 M final), and 10 g SDS (0.5% final) to 800 ml H_2O. Mix until the solution is clear. Add H_2O to 1000 ml (*see* **Note 8**).
14. 1× SDS electrophoresis buffer (working solution for running the gel): Dilute 10× SDS electrophoresis buffer to 1× with H_2O.
15. Protein sample(s), on ice: Dilute a portion of the protein sample to be analyzed 1:1 (v/v) with 2× SDS sample buffer and heat at 100 °C for 3–5 min in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, dissolve the protein in 50–100 µl of 1× SDS sample buffer and incubate at 100 °C for 3–5 min.

2.3.2 Transfer to Membrane

1. 10× Transfer buffer: Dissolve 144.1 g glycine (1.92 M) and 30.3 g Tris (0.25 M) in H_2O. Bring volume to 1000 ml with H_2O. The pH of the resulting solution will be ~8.3–8.4.
2. 1× transfer buffer: Dilute one volume of 1× transfer buffer with 9 volumes of H_2O. Do not adjust the pH.
3. 10× Tris-buffered saline (TBS): Dissolve 60.6 g Tris (0.5 M) and 87.7 g NaCl (1.5 M) in 900 ml H_2O. Bring pH to 7.6 with HCl. Add H_2O to 1000 ml.
4. 10% (v/v) Tween 20: Mix 10 ml Tween 20 with 90 ml H_2O.
5. 1× TBS/Tween 20 (TBST): Dilute 10 ml 10× TBS with 89 ml H_2O and add 1 ml of 10% (v/v) Tween 20. Mix gently.
6. Blocking solution: Dissolve 5 g nonfat milk powder in 100 ml 1× TBST (*see* **Note 9**).
7. 1× TBS: Mix 10 ml 10× TBS with 90 ml H_2O.

2.3.3 Immunoprobing with Primary Antibody and a Directly Conjugated Secondary Antibody

1. Primary antibody against protein of interest: Dilute primary antibody according to the manufacturer's instructions or use a starting dilution of 1:1000 in 1× TBS.
2. Secondary antibody, species-specific anti-Ig conjugated to horseradish peroxidase: Dilute in 1× TBS according to the manufacturer's instructions. Prepare 25 µl aliquots and store at −20 °C.

2.3.4 Visualization by a Luminescence Substrate, Luminol

1. Luminescent substrate buffer: Dissolve 6.1 g Tris in 900 ml H_2O. Adjust pH to 7.5 with HCl. Bring volume to 1000 ml with H_2O.
2. 10× luminol stock solution: Dissolve 40 mg luminol in 10 ml dimethyl sulfoxide.
3. 10× p-iodophenol stock solution: Dissolve 10 mg p-iodophenol in 10 ml dimethyl sulfoxide.
4. Luminol working solution: Mix 0.5 ml 10× luminol stock, 2.5 ml 100 mM Tris-Cl, pH 7.5, 0.5 ml 10× p-iodophenol stock and 25 µl 3% H_2O_2. Add H_2O to 5 ml. Prepare just before use in a dark bottle (*see* **Notes 10** and **11**).

3 Methods

3.1 Preparation of SDS Polyacrylamide Gels

1. Use gloves when handling the glassware and pouring the gel. Wash the glass plates, spacers, and teflon combs with warm water, rinse in deionized water, and let it air dry.
2. Lay the larger plate flat on the bench and arranges spacers on both sides parallel to the two edges.
3. Put the smaller (front) plate on the spacers and align both plates resulting in the gel cassette.
4. Put pressure cams over the glass plates. Make sure that glass plates and spacers are aligned, and then lock the pressure cams. Secure the gel cassette into the gel casting stand (*see* **Note 12**).
5. Prepare the separating gel solution in a clean beaker with spout and swirl carefully.
6. Pour separating gel solution slowly between the plates to avoid formation of air bubbles.
7. Using a pipette carefully overlay the separating gel solution with H_2O-saturated isobutyl alcohol.
8. Allow the acrylamide to polymerize at room temperature for 30–60 min. Should a Schlieren pattern be visible just beneath the layer of H_2O-saturated isobutyl alcohol, polymerization will be complete.
9. Pour off the H_2O-saturated isobutyl alcohol and rinse the gel with water.
10. Prepare the stacking gel solution in a clean beaker with spout and swirl carefully.
11. Pour stacking gel solution slowly between the plates to avoid formation of air bubbles. Immediately insert the comb with the required number of teeth so that the tops of the teeth are slightly higher than the top of the glass. Should air bubbles have become trapped under the teeth, remove comb carefully and remove air bubbles using a pipette. Insert comb again.

12. Allow the stacking gel to polymerize at room temperature for 20–30 min. Should a Schlieren pattern and/or gel wells be visible just beneath the teeth of the comb, polymerization will be complete.
13. Remove glass plates, i.e., the gel cassette, from the casting stand (*see* **Note 13**).
14. Assemble electrophoresis module according to the manufacturer of the electrophoresis apparatus you are using.
15. Pour 1× running buffer in the upper (inner) and lower (outer) buffer reservoir. Do not overfill upper buffer reservoir.
16. Remove the comb without disturbing the separating gel between the wells. Immediately rinse the wells with 1× running buffer to remove any amounts of acrylamide.
17. Prepare protein samples (*see* **Note 14**). Heat at 100 °C for 2 min and immediately place on ice.
18. Using a pipette with a long tip, put the appropriate volume of each protein sample in a separate well. Use one or more wells for running the molecular weight standard.
19. Place lid with cables on the gel cassette.
20. Connect the electrodes to a power pack. Run at a current of 15 mA until the samples have entered the gel, then increase current to 25 mA. Continue electrophoresis until the Bromophenol Blue has migrated to the bottom of the gel.
21. Turn the power supply off and disconnect the electrophoresis chamber from the power supply.
22. Detach the gel cassette from the electrophoresis chamber and discard the running buffer.

3.2 Assembly of the Transfer Sandwich

1. Wear gloves when handling gels and membranes. Handle PVDF membrane pieces with blunt forceps only. Wash gel briefly in demineralized water. Discard water and incubate gel in 1× transfer buffer for 30 min at room temperature under constant gentle shaking.
2. Measure length and width of the gel with a ruler.
3. Cut a piece of PVDF membrane from the role that has the exact dimensions as the gel.
4. Cut four pieces of filter paper, e.g., Whatman 3MM, with identical dimensions as the membrane.
5. Put PVDF membrane into methanol for 60 s, remove the membrane, wash it in demineralized water, and put it into 1× transfer buffer.
6. Soak a piece of flat sponge with 1× transfer buffer.
7. Fill an additional tray with 1× transfer buffer and put the open transfer cassette into the tray.

8. Put the soaked flat sponge on the upper side of the transfer cassette (*see* **Note 15**).
9. Put two pieces of filter paper onto the sponge.
10. Place the gel gently onto the filter paper. Carefully remove any air bubbles trapped under the gel.
11. Put the piece of PVDF membrane onto the gel so that PVDF membrane and gel will be aligned (*see* **Note 16**).
12. Remove any trapped air bubbles by gently rolling a 10 ml pipet across the membrane.
13. Put two pieces of pre-wetted filter papers onto the gel and place a pre-wetted flat sponge onto the filter paper. Align all filter papers and sponges with the gel (Fig. 1) (*see* **Note 17**).
14. Close the transfer cassette and put it into the transfer chamber with the black side of the cassette facing the negative electrode.

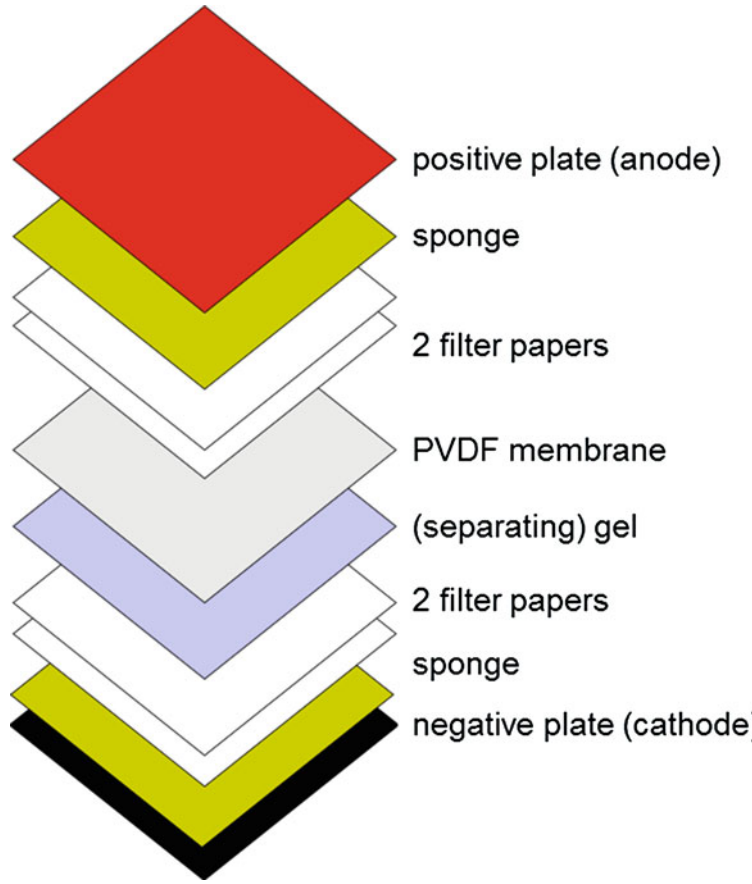

Fig. 1 Schematic representation of the assembly of a sandwich for Western blotting. The stacking gel has been removed prior to assembly of the sandwich

15. Put a magnetic sizzle stick in the transfer chamber and place the transfer chamber onto a magnetic stirrer.
16. Put the cooling units provided with the electrophoresis/transfer chamber into the transfer chamber and carefully half-fill the transfer chamber with 1× transfer buffer (*see* **Note 18**).
17. Turn magnetic stirrer on and set speed to slow.
18. Fill transfer chamber with 1× transfer buffer up to the mark.
19. Watch the correct orientation, place lid on the transfer chamber, and connect cables to the power supply.
20. Turn power supply on and set current to 350 mA (*see* **Note 19**). Start the protein transfer and let it continue for 1 h.
21. Turn the power supply off and disconnect the cables from the power supply.
22. Remove lid from transfer chamber and take out transfer cassette.
23. Open cassette and move PVDF membrane to a filter paper.
24. Mark the molecular weight standards with a pencil and cut off the upper right corner.

3.3 Immunoprobing with Antibodies

1. Place PVDF membrane into blocking solution and incubate under constant shaking for 60 min. Discard the blocking solution.
2. Dilute primary antibody in a total volume of 5 ml blocking solution according to the manufacturers' instructions, e.g., use 5 µl antibody solution for 5 ml blocking solution to obtain a dilution of 1:1000.
3. Incubate the PVDF membrane with the primary antibody under gentle rocking at room temperature for 2 h (*see* **Note 20**).
4. Pour off primary antibody solution and store at −20 °C (*see* **Note 21**).
5. Wash PVDF membrane with 100–150 ml 1× TBST under heavy shaking for 10 min. Discard the washing solution. Repeat washing step three times.
6. Dilute secondary antibody coupled to horse radish peroxidase in blocking solution according to the manufacturers' instructions, e.g., use 1 µl of antibody in 10 ml blocking solution to obtain a 1:10,000 dilution of the secondary antibody.
7. Incubate PVDF membrane with the secondary antibody under gentle rocking at room temperature for 60 min. Discard secondary antibody solution (*see* **Note 22**).
8. Wash PVDF-membrane with 100–150 ml 1× TBST under heavy shaking for 10 min. Discard washing solution. Repeat three times.

3.4 Visualization by the Luminescence Substrate, Luminol

1. Wear gloves when performing ECL signal detection. Use blunt forceps when handling the PVDF membrane. Equilibrate PVDF membrane twice in 50 ml luminescent substrate buffer. Discard luminescent substrate buffer.
2. Use 0.1 ml/cm^2 of membrane area of luminol working solution.
3. Pipet the luminol working solution onto the PVDF membrane and incubate for 5 min at room temperature. Remove PVDF membrane.
4. Wrap PVDF membrane in transparent film. Remove any excess liquid with filter paper. Remove any air bubbles trapped between PVDF membrane and transparent film.
5. Place the wrapped PVDF membrane into an X-ray film cassette with protein-side up.
6. The following steps are performed in a dark room.
7. Switch off white light and switch on safe light (*see* **Note 23**).
8. Open box with the ECL films. Take out pouch containing the films. Open the pouch, take out one film, close pouch, and put it back into the box. Close box.
9. Cut ECL film to desired size or use whole film for large pieces of PVDF membrane.
10. Open film cassette, put ECL film onto the PVDF membrane and close the film cassette. Set timer to desired exposition time (*see* **Note 24**).
11. After the exposition time has passed, open the film cassette and remove the ECL film.
12. Put ECL film into developing solution for 5 min and then wash the film with water.
13. Put ECL film into fixing solution until signals are clearly visible and the film coating has become transparent.
14. Wash ECL film with a sufficient amount of tap water, then wash film briefly with demineralized water and let it air-dry.
15. Switch off safe light, switch on white light.
16. Wet PVDF membrane with 1× TBS, wrap in transparent film and store at 4 °C (*see* **Note 25**).
17. Digitalize signals on ECL film and perform densitometry on the band of interest using appropriate software (*see* **Note 26**).

4 Notes

1. A list of commercial suppliers of Western Blotting Transfer Systems can be found at, e.g., www.labtimes.org/labtimes/product/j2016/lt_2016_04_prods.pdf.

2. PVDF membranes are available with different membrane pore sizes, e.g., 0.2 or 0.45 μm. Choice of membrane pore size is dependent on the size of the protein of interest. For most standard analyses, e.g., for proteins with a molecular weight of 20 kDa or larger, a PVDF membrane with a pore size of 0.45 μm should perform well. For proteins smaller than 20 kDa, a PVDF membrane with a pore size of 0.2 μm is recommended.
3. Alternatively, a CCD camera system can be used for visualization of the enhanced chemiluminescence signals. Commercially available systems include *ChemiDoc™ XRS+ System* (Bio-Rad Laboratories) or *Amersham Imager 600* (GE Healthcare), for example. Then the steps describing exposition and development of the film do not apply.
4. Monomeric acrylamide and *N*,*N*′-methylenebisacrylamide 2× crystallized grades are recommended. Discard after 30 days for acrylamide gradually hydrolyses to acrylic acid and ammonia. To avoid handling acrylamide in powder or crystalline form, use ready-prepared 29.2% acrylamide and 0.8% bisacrylamide solutions.
5. This step is optional.
6. This step is optional.
7. Commercially available. Consist of various either unstained or prestained proteins of different molecular weights.
8. Do not adjust the pH of the stock solution. The pH will be pH 8.3 when diluted. Store up to 6 months at 4 °C.
9. Alternatively, 5% (weight per volume) bovine serum albumin (BSA) or blocking agents on polymeric basis such as ROTI®-Block (Carl Roth, Karlsruhe Germany) may be used. Blocking the remaining surface of the membrane will reduce nonspecific binding of the detection antibodies during subsequent steps. Different proteins may require a blocking buffer other than milk or BSA. For the detection of phosphorylated proteins milk is not recommended as a blocking agent. Milk has a high content of casein, an abundant phosphoprotein, which will give high background signals because the phosphor-specific antibody will (also) detect casein.
10. Recipe is from Schneppenheim et al. [6]. Premixed luminol substrate mixes that are commercially available may also be used.
11. Alternatively, put aluminum foil around a glass beaker and on the top. Luminol working solution will be stable for approximately 8 h at room temperature.
12. Alternatively, you can seal the gel cassette with SDS-stable tape by binding the entire length of the two sides and the bottom of the plates with the tape. Put gel cassette in a tray.

13. Should you have sealed the gel cassette with SDS-stable tape, remove sealing tape from the bottom of the plates using a scalpel or a razor blade.
14. For a 1.0 mm thick gel, 20–25 μg of a complex protein mixture, e.g., a total protein extract from tissue or cell culture, in a volume not exceeding 40 μl is recommended for loading per slot.
15. I.e. the black side in case of *Protean* cells (BioRad Laboratories).
16. Once the piece of PVDF membrane has been placed onto the gel, do not remove the membrane from the gel.
17. It is best to work under 1× transfer buffer. This helps in preventing the gel to rupture.
18. Should you have no suitable cooling units for your transfer chamber, perform the transfer in a cool room at 4 °C.
19. A 1-hour transfer can be performed using a constant current of 700–1600 mA for large gels and 350 mA for small gels. In general, the transfer time is inversely correlated to protein size. For proteins larger in size than 150 kDa, the transfer time should be increased to 2 h. For overnight transfer, the settings are 100 mA for large gels and 90 mA for small gels.
20. Alternatively, the PDVF membrane may be incubated with the primary antibody solution overnight at 4 °C.
21. Often, the primary antibody solution can be stored at −20 °C and be reused. Follow manufacturer's instructions for storage.
22. Secondary antibody solution can be stored at −20 °C and may be reused once.
23. The following steps must be performed under safe light conditions or in complete darkness.
24. Exposition times can vary between 15 s and 1 h or longer depending on the abundance of the protein of interest.
25. The PVDF membrane may be stripped from bound antibodies and can be reused for probing with a different antibody.
26. Densitometry can be performed using the public domain software *ImageJ* (download available at imagej.nih.gov/ij/download.html) or commercially available software, e.g., BIORAD, etc. Statistical analysis may be performed online using the free site *VassarStats: Website for Statistical Computation* (http://www.vassarstats.net/index.html) or commercially available software such as *SigmaPlot* and *SigmaStat* (both from Systat Software San Jose, USA), or *GraphPad Prism* (GraphPad Software, La Jolla, USA), for example.

Acknowledgments

Research in the authors' laboratory has been supported by project grants from the European Commission (FP7-REGPOT-2010, grant no. 264143 to H.B.) and *Deutsche Duchenne Stiftung der aktion benni & co. e.V* (to M.L. and H.B.).

References

1. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354
2. Holland A, Henry M, Meleady P, Winkler CK, Krautwald M, Brinkmeier H, Ohlendieck K (2015) Comparative label-free mass spectrometric analysis of mildly versus severely affected mdx mouse skeletal muscles identifies annexin, lamin, and vimentin as universal dystrophic markers. Molecules 20:11317–11344. doi:10.3390/molecules200611317
3. Kurien BT, Scofield RH (2006) Western blotting. Methods 38:283–293
4. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195–203
5. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
6. Schneppenheim R, Budde U, Dahlmann N, Rautenberg P (1991) Luminography—a new, highly sensitive visualization method for electrophoresis. Electrophoresis 12:367–372

Chapter 23

Immunofluorescence Microscopy for DIGE-Based Proteomics

Rustam R. Mundegar, Margit Zweyer, and Dieter Swandulla

Abstract

Alterations in the proteome of a tissue in different settings, as assessed by difference gel electrophoresis, can be verified for single proteins using immunohistochemistry. In fluorescence immunohistochemistry, an antibody to a particular antigen is applied to tissue sections, and fluorophores conjugated to a secondary antibody allow for the detection of target antigen with fluorescent microscopy. Visual comparison is sufficient for the detection of significant alterations in the abundance of a certain protein in different settings. Additionally, unlike large-scale proteome analyses and Western blot methods, expression of target protein can be analyzed at the cellular level by immunohistochemistry. In this chapter, a protocol for the application of fluorescence immunohistochemistry for the detection of dystrophin in skeletal muscle sections is outlined, including sample preparation, tissue sectioning, and immunostaining.

Key words Immunohistochemistry, Dystrophin, Skeletal muscle, Antibodies, Fluorescence

1 Introduction

In comparative proteomics, alterations in the abundance of proteins are often substantiated by Western blotting and immunohistochemistry for a protein of interest. Immunohistochemistry is a powerful corroborative method for identification of proteome alterations at the cellular level. In immunohistochemistry an antibody (Ab) which specifically reacts with a target protein is applied to tissue sections. Conjugation of the antibody with a fluorophore allows for light microscopic detection of Ab binding through fluorescence [1]. Drastic alterations in the abundance of a protein are easily detected by visual inspection. In contrast to difference gel electrophoresis (DIGE) analysis of the proteome or Western blotting, fluorescent immunohistochemistry or immunofluorescence (IF) microscopy additionally yields information on the pattern of expression of a protein and its assignment to a particular cell type in tissues, especially in diseased states, such as skeletal muscle dystrophies [2, 3].

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5_23,

Duchenne muscular dystrophy (DMD) is a severe progressive disease of childhood resulting in early loss of ambulation and with a high mortality in the first three decades of life. Mutations in the dystrophin gene result in an absence of the Dp427 isoform of dystrophin in skeletal muscle. Dystrophin is localized at the cytoplasmic aspect of the sarcolemma and links the cytoskeleton with the extracellular matrix by association with several glycoproteins [4]. This dystrophin–glycoprotein complex is disrupted by the absence of dystrophin resulting in a reduction of sarcolemmal stability and contraction-induced damage to the sarcolemma resulting in increased myofiber leakiness. Dystrophin-deficient myofibers have abnormal calcium handling [5] and elevated cytosolic Ca^{2+} levels [6] show increased rates of proteolysis [7] and are more susceptible to osmotic shock [8]. Dystrophic muscles are characterized by skeletal muscle degeneration and myofiber necrosis, fat replacement, increased connective tissue, and fibrosis. Regenerating myofibers and inflammatory cells contribute a further subset of proteins to the already vast and complex muscle proteome repertoire. Disease progression results in increased fibrosis and severe myofiber depletion. The proteome of the skeletal muscle of the *mdx* mouse, the murine homolog of the human disease has been extensively investigated [9, 10]. In the *mdx* mouse, muscular dystrophy is most pronounced in the diaphragm and milder dystrophic alterations are present in the limb muscles. The proteomic profile of dystrophin-deficient skeletal muscles of *mdx* mice show increased fibrosis markers and extracellular matrix proteins and a decrease in contractile proteins [2, 11].

In this chapter, we describe the detection of dystrophin in mouse skeletal muscle with IF microscopy (Fig. 1). Dystrophin is expressed at low levels in skeletal muscle and the sarcolemmal localization of dystrophin complicates identification if high extracellular background is present. The method used [12] is a two-step method whereby a primary unconjugated anti-dystrophin Ab is first applied to skeletal muscle tissue and the bound primary Abs are then detected with a secondary Ab, conjugated with fluorophore, directed to the primary Ab. This two-step method is performed without amplification (e.g., biotin-streptavidin) and should be applicable for most validation studies where significant alterations of the proteome are present. Although the method described below is optimized for skeletal muscle, a general discussion of the various steps is provided.

2 Materials

2.1 Equipment

Fine Forceps, small scissors, micro-scissors.

Dewar (vacuum) flask.

Plastic beaker (100 ml).

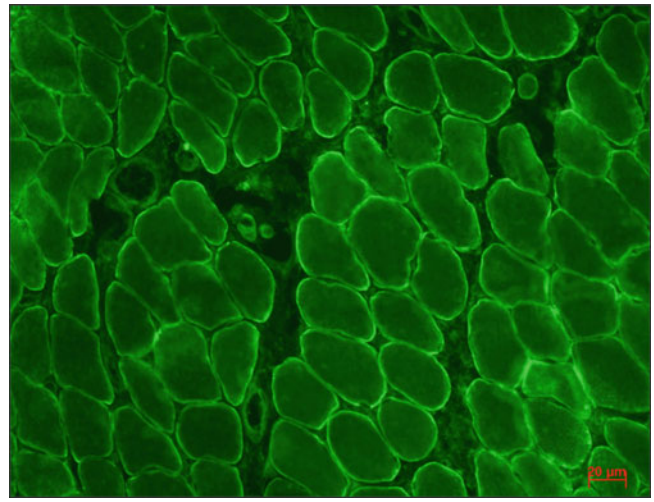

Fig. 1 Immunofluorescence microscopy of dystrophin labeling in mouse *tibialis anterior* muscle. The sarcolemma of skeletal muscle fibers shows positive dystrophin immunostaining

Cryovials.

Cryostat.

SuperFrostPlus microscopic slides.

Plastic slide storage box.

Glass beaker (300 ml).

Hot plate.

Staining jars (glass Coplin or Hellendahl jars).

Laboratory Shaker.

Bench top centrifuge.

Moist incubation chambers.

Epifluorescence microscope with digital camera for image acquisition.

2.2 Sera and Antibodies

Normal goat serum (NGS).

Novocastra™ lyophilized mouse monoclonal antibody to Dystrophin (C-terminus) (Leica Biosystems).

Fluorescein-conjugated goat anti-mouse Ab (Jackson ImmunoResearch Laboratories).

Reconstitute lyophilized antibodies according to the manufacturers' instructions. For long-term storage, add an equal volume of glycerol and store 20 µl aliquots at −20 °C. Avoid repetitive freeze–thawing of antibody solutions. Dilute antibody and serum in PBS. Antibody solutions should be centrifuged briefly (~12,000 × *g*) before use.

2.3 Solutions

All chemicals should be of analytical grade and ultrapure water should be used.

1. Phosphate Buffered Saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl. Dissolve 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 8 g NaCl and 0.2 g KCl in 800 ml of H_2O and adjust pH to 7.4 with HCl at room temperature (RT). Add water to 1 L. Store at room temperature for 2 weeks.
2. Hoechst 33342 solution: For a 1 μg dye/ml solution, dissolve 10 mg of bisBenzimide H 33342 trihydrochloride in 10 ml of PBS at RT. Dilute 1:000 (v/v) before usage. Store at −4 °C.
3. Sudan Black B solution: For a 0.3% (w/v) dye solution, add 150 mg Sudan Black B to 50 ml 70% ethanol in a beaker and stir in the dark for 2 h at RT. Store in the dark at RT.

3 Methods

3.1 Preparation of Muscle Tissue Samples

1. In a fume hood add 2-methylbutane to a plastic beaker (approximately 50 ml) and submerge in a dewar flask containing liquid nitrogen. The lower third of the beaker should be covered by liquid nitrogen (*see* **Note 1**).
2. Label cryovials and cool on dry ice or in liquid nitrogen.
3. Euthanize a mouse (C57BL/6) according to institutional and governmental regulations. Lay the mouse on the dissecting board and secure the mouse by pinning the extremities through the paws. Spray the hind limb with 70% ethanol.
4. Lift skin above the knee with forceps and make an incision from the knee toward the digits. Retract the skin to expose the *tibialis anterior* (TA) muscle.
5. Cut the distal tendon of the muscle; gently lift the muscle from the leg toward the knee. The overlying fascia can be removed with a pair of forceps or a scalpel. Cut the proximal tendon (*see* **Note 2**).
6. Snap-freeze the skeletal muscle specimen by immersing the muscle with forceps in 2-methylbutane (*see* **Note 3**).
7. Transfer the muscle to cooled cryovials and store at −80 °C or in liquid nitrogen.

3.2 Transverse Sectioning of Muscle Specimen

1. Transfer cryovials with TA muscle into a cryostat pre-cooled to −20 °C (*see* **Note 4**).
2. Add a drop of cryoprotective tissue embedding medium (TissueTek or OCT) to a specimen disc at RT and place in the cryostat.

3. When the immersion medium starts freezing, lower the TA muscle specimen (perpendicular to the plane of the disc) with forceps into the embedding medium with gentle pressure (*see* **Note 5**).
4. Allow the embedding medium to freeze completely before sectioning (*see* **Note 6**).
5. Transfer the specimen disc to the chuck and start sectioning at 6–8 μm thickness (*see* **Note 7**).
6. Press a glass slide gently on the section. Remove slide immediately from the cryostat (*see* **Note 8**).
7. Allow sections to dry for at least 30 min at RT (*see* **Note 9**).

3.3 Immunofluorescence Microscopy of Dystrophin

1. Place a glass beaker (300 ml) filled with PBS on a hot plate. Heat until the PBS starts to boil.
2. Submerge slides in the heated PBS such that the muscle section faces the bottom of the beaker (*see* **Notes 10–12**). A minimum of two slides are required.
3. After boiling for 5 min remove slides from the beaker with forceps and submerge slides in a staining jar with PBS at RT.
4. Drain off excess PBS and wipe around sections with tissue paper.
5. Circle the tissue section with a hydrophobic (PAP) pen without touching the section and dry briefly (*see* **Note 13**).
6. Apply NGS diluted 1:20 with a pipette to the sections. Sections must be covered with NGS solution. Place slides in a moist chamber at RT for 30 min. From this step forward until immunofluorescence microscopy is completed, sections must not be allowed to dry out. All incubations with sera or antibodies must be carried out in moist chambers (*see* **Note 14**).
7. Drain off excess NGS from slides and cover tissue section on one slide with monoclonal anti-dystrophin Ab diluted 1:40. To the other slide add PBS only. This serves as a control for unspecific binding of secondary antibodies (*see* **Note 15**). Incubate both slides at RT in a moist chamber for 60 min.
8. Remove the slide to which antibody solution was added from the moist chamber. Pipette off excess antibody from the tissue section and immerse the slide in a staining jar with PBS and shake gently on a shaker for 5 min. Repeat twice.
9. Remove slide from the jar and control slide from the moist chamber and drain off PBS. Cover sections with secondary anti-mouse antibody diluted 1:200 and incubate in a moist chamber for 45 min.
10. Wash all slides 3× in PBS for 15 min.

11. Add Hoechst solution to sections for 5 min for nuclear staining.
12. Wash slides in PBS briefly. Dry around sections and place a drop of anti-fade reagent (Fluoromount or any other commercially available anti-fade reagent) and place a coverslip on the sections.
13. View sections under a fluorescence microscope equipped with appropriate filters (*see* **Note 16**).
14. If sections show autofluorescence then repeat the procedure and after **step 12** add Sudan Black B solution to sections for 5 min and wash extensively in PBS before microscopy (*see* **Note 17**).

4 Notes

1. Wear safety glasses and protective gloves. 2-Methylbutane will start to freeze at the bottom of the beaker. Do not allow all of the liquid to freeze.
2. Avoid stretching the muscle. Muscle bundles can be dissected from larger thigh muscles such as the vastus muscle.
3. Swirl the muscle in 2-methylbutane. Freezing is complete within seconds and the muscle will turn pale.
4. TA muscle should be transferred in a dewar with liquid nitrogen. Place the cryovial in the cryostat before labeling sections to allow for temperature equilibration of the muscle (5 min). Sectioning will be difficult if the muscle sample is too cold. All metal instruments used to handle muscle samples should also be placed in the cryostat. Label slides before sectioning.
5. The embedding medium will start freezing from the periphery and the bottom and the muscle sample should be placed in the immersion medium at this stage. If the embedding medium is not cold enough then this will result in thawing artifacts.
6. Embedding medium must freeze completely (2–3 min) or the muscle sample will fall off the disc during sectioning. Enough embedding medium should be used to secure the specimen. However, excess embedding medium may result in poor quality sections.
7. The muscle sample may be trimmed with a blade till larger sections are obtained.
8. Glass slides should be at RT. Suitable microscopy slides would be SuperFrostPlus slides from Menzel-Gläser (Braunschweig, Germany).
9. Sections can be stored at −20 °C for a fortnight or at −80 °C for longer periods.

10. The slides should be in an oblique position. If several slides are to be processed then avoid crowding of slides and repeat boiling with fewer slides. Frozen sections must be allowed to thaw at RT for at least 5–10 min. This method of background reduction should only be used if monoclonal mouse antibodies are used on mouse sections. This background is caused by reaction of secondary anti-mouse antibodies to endogenous immunoglobulins and binding of the Fc-fragment of secondary antibodies by tissue components resulting in high intercellular background. This background is abolished by boiling sections. The primary antibodies used on boiled sections must also bind to the denatured target protein.
11. Importantly, background staining differs between mouse strains and between organs. Brain tissue has negligible antibody binding compared to skeletal muscle and it is best to test for secondary antibody binding before boiling sections. Furthermore, not all target proteins are detectable after boiling of sections either because epitopes are irreversibly destroyed or target proteins are leached out of tissue on boiling. Mouse on mouse blocking kits are commercially available for such proteins or, if available, polyclonal antibodies or rabbit monoclonal antibodies should be used. Washing of sections in 1% Triton-X-100 in PBS for 30 min with gentle shaking at RT may restore immunoreactivity of some epitopes.
12. Boiling of sections is also a physical method for antigen fixation and if this method is not used then sections must be fixed first. There is no single optimal method for tissue fixation. Ice cold methanol or acetone or acetone–methanol mixtures may be used. Skeletal muscle may be fixed in 4% paraformaldehyde, which also conveniently reduces background staining. However, not all antigens (such as dystrophin) are detected by conventional staining methods, even after heat-induced epitope retrieval.
13. Do not make the hydrophobic barrier too close to the section or apply to slides before boiling.
14. Non-immune or "normal serum" from the animal species in which the secondary antibody was raised should be used to block sections. Albumin may also be used to block such unspecific protein–protein interactions, may however increase autofluorescence.
15. Controls for the specificity of staining with primary antibody may be carried out by replacing primary antibody with normal serum from the animal species in which the antibody was raised or by using commercially available antibody isotype controls.
16. Images are captured by a digital camera and processed with Adobe Photoshop (Adobe System). Only linear adjustments

for contrast should be made. Ideally, a bright green staining of the sarcolemma is present. Nuclei are stained blue. The control section (secondary antibody control) should be negative. High background is not encountered if this protocol is adhered to. High background present when using other anti-dystrophin antibodies or antibodies to a different target protein may be reduced by:

- Optimizing dilution of primary and secondary antibodies (reduction in the concentration of secondary antibodies may even increase specific staining intensity).
- Increasing the number of washes with PBS.
- Decreasing incubation times.
- Incubation at 4 °C overnight with (optional) higher dilutions of primary antibody.
- Using monoclonal antibodies instead of polyclonal antibodies.
- Using cross-absorbed polyclonal antibodies.
- Using affinity purified monoclonal antibodies.

17. Autofluorescence is the natural fluorescence of tissues and shows a broad emission spectrum. It may be difficult to distinguish between autofluorescence background and specific spectra of fluorophores. Lipofuscins (especially in brain tissue), flavins, elastins, and collagens may contribute to autofluorescence. Aldehyde fixation of tissues increases background autofluorescence especially if glutaraldehyde is used. Boiling of sections as described here tends to decrease autofluorescence. Oxidative skeletal muscle fibers show a dull green autofluorescence and the bright fluorescence signal of green fluorophores is normally easily distinguishable, thus obviating the need for quenching with Sudan Black. Low background autofluorescence is almost always present and muscle fascicles, blood vessels, and nerves can be easily identified and aid in orientation.

References

1. Coons AH, Kaplan MH (1950) Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med 91:1–13
2. Murphy S, Henry M, Meleady P et al (2015) Simultaneous pathoproteomic evaluation of the dystrophin-glycoprotein complex and secondary changes in the mdx-4cv mouse model of Duchenne muscular dystrophy. Biology (Basel) 4:397–423
3. Murphy S, Dowling P, Zweyer M et al (2016) Proteomic analysis of dystrophin deficiency and associated changes in the aged mdx-4cv heart model of dystrophinopathy-related cardiomyopathy. J Proteome 11(145):24–36
4. Matsumura K, Campbell KP (1994) Dystrophin-glycoprotein complex: its role in the molecular pathogenesis of muscular dystrophies. Muscle Nerve 17:2–15
5. Allen DG, Gervasio OL, Yeung EW et al (2010) Calcium and the damage pathways in

muscular dystrophy. Can J Physiol Pharmacol 88:83–91

6. Mallouk N, Jacquemond V, Allard B (2000) Elevated subsarcolemmal Ca^{2+} in mdx mouse skeletal muscle fibers detected with Ca^{2+}-activated K^+ channels. Proc Natl Acad Sci U S A 97:4950–4955
7. Kumamoto T, Fujimoto S, Ito T et al (2000) Proteasome expression in the skeletal muscles of patients with muscular dystrophy. Acta Neuropathol 100:595–602
8. Menke A, Jockusch H (1991) Decreased osmotic stability of dystrophin-less muscle cells from the mdx mouse. Nature 349:69–71
9. Holland A, Carberry S, Ohlendieck K (2013) Proteomics of the dystrophin-glycoprotein complex and dystrophinopathy. Curr Protein Pept Sci 14:680–697
10. Carberry S, Zweyer M, Swandulla D et al (2013) Application of fluorescence two-dimensional difference in-gel electrophoresis as a proteomic biomarker discovery tool in muscular dystrophy research. Biology (Basel) 2:1438–1464
11. Carberry S, Zweyer M, Swandulla D et al (2012) Proteomics reveals drastic increase of extracellular matrix proteins collagen and dermatopontin in the aged mdx diaphragm model of Duchenne muscular dystrophy. Int J Mol Med 30:229–234
12. Mundegar RR, Franke E, Schäfer et al (2008) Reduction of high background staining by heating unfixed mouse skeletal muscle tissue sections allows for detection of thermostable antigens with murine monoclonal antibodies. J Histochem Cytochem 56:969–975

INDEX

Kay Ohlendieck (ed.), *Difference Gel Electrophoresis: Methods and Protocols*, Methods in Molecular Biology, vol. 1664,
DOI 10.1007/978-1-4939-7268-5,

E

F

G

H

I

L

M

N

P

R

S

T

U

W