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Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives

Haleem J. Issaq and Timothy D. Veenstra

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The recent trend in science is to assay as many biological molecules as possible within a single experiment. This trend is evident in proteomics where the aim is to characterize thousands of proteins within cells, tissues, and organisms. While advances in mass spectrometry have been critical, developments made in two-dimensional PAGE (2D-PAGE) have also played a major role in enabling proteomics. In this review, we discuss and highlight the advances made in 2D-PAGE over the past 25 years that have made it a foundational tool in proteomic research.

INTRODUCTION

The last 25 years, and particularly the last decade, has witnessed an increased effort to develop technologies capable of identifying and quantifying large numbers of proteins expressed within a cell system (i.e., the proteome) in the hope of detecting disease biomarkers, mapping protein circuitry, or identifying novel phosphorylation sites, for example. The complexity of the proteome has made developing methods for efficient separation and sensitive detection of proteins a critical component of this effort. Continued advances in mass spectrometry (MS) technology have enabled the detection of proteins with much greater speed and sensitivity than previously possible. Even cutting-edge MS, however, is unable to characterize all of the components within a complex proteome. Scientists take a “divide and conquer” approach to characterizing proteomes, in that they attempt to temporally limit the number of proteins that the mass spectrometer is asked to analyze. By spreading out the proteome, more proteins will ultimately be analyzed within an individual experiment.

To separate proteomes, scientists have used electrophoretic and chromatographic technologies, separately and in combination, and both offline and online. Although these efforts can result in the separation and identification of thousands of proteins, no single method can resolve

all the proteins in a proteome, due to their large number and concentration dynamic range. Single-dimension separations are inadequate for effectively resolving complex protein mixtures. This fact was acknowledged over half a century ago by Smithies and Poulik (1), who recognized that a combination of two electrophoretic processes on a gel at right angles should give a much greater degree of resolution than is possible with either separately. The two electrophoretic processes are resolution by molecular size and free solution mobility on a starch gel. Their prediction continues to be proven true and has formed the basis for developing orthogonal multidimensional methodologies for the separation of complex mixtures not only by gel electrophoresis but also by chromatography and capillary electrophoresis.

To properly understand the advances made in two-dimensional PAGE (2D-PAGE), one needs to go back much further than a quarter of a century. In 1930 Tiselius introduced the moving boundary method as an analytical tool for studying the electrophoresis of proteins (2). Since his pioneering work, various forms of electrophoresis have been used for the separation of complex mixtures of proteins, each with improved resolution. As early as 1962, Raymond and Aurell (3) demonstrated the significant nonlinear effects of gel concentration on the electrophoretic mobility of proteins by employing 2-D electrophoresis using different acrylamide gel concentra-

tions to separate serum proteins. Two years later, Raymond (4) demonstrated the superiority of flat slab gels compared with cylindrical tube gels. For example, the flat slab provides maximum surface area for cooling the gel; the resulting patterns are easier to quantify in standard recording densitometers; a large number of samples can be processed using a single gel plate, facilitating the direct comparison of specimens processed under identical conditions; and, most importantly, the flat slab permits the application of 2-D separations. These insightful preferences have been proven true and are practiced today in many bioanalytical laboratories.

Another advancement in 2-D gel separations was introduced in 1972 by Wright (5), who used a 4.75% (2% cross-linkage) polyacrylamide gel column in the first dimension, which was then removed from the glass cylinder and laid on the upper edge of a 2% gradient slab. Following electrophoresis, the gel slab was placed in a staining solution, resulting in the visualization of 112 resolved human serum proteins.

These novel approaches resolved only a small number of proteins, primarily the most abundant proteins of a cell or serum proteome. The introduction of 2D-PAGE in 1975 by O’Farrell (6) for separating cellular proteins under denaturing conditions enabled the resolution of hundreds of proteins. The principle applied was very simple: proteins were resolved on a

gel using isoelectric focusing (IEF), which separates proteins in the first dimension according to their isoelectric point, followed by electrophoresis in a second dimension in the presence of sodium dodecyl sulfate (SDS), which separates proteins according to their molecular mass. O'Farrell's method is truly the basis of modern 2D-PAGE, which was quickly adapted and widely accepted by other researchers. Anderson and Anderson (7) used 2D-PAGE for the analysis of human plasma proteins. They were able to separate and detect approximately 300 distinct protein spots upon staining. Unlike O'Farrell, Manabe (8) separated human plasma proteins using 2D-PAGE without denaturing agents. About 230 protein spots could be observed on the gel; however, the spots were smeared and not well-resolved.

Introduction of Immobilized pH Gradients

As mentioned above, 2D-PAGE comprises IEF in the first dimension, followed by SDS-PAGE in the second dimension. Since its introduction by Kolin in 1954 (9), IEF has undergone several advances. The first dimension is carried out in polyacrylamide gel rods that are formed in glass or plastic tubes and contain ampholytes that form a pH gradient in an electric field. These rods were historically irreproducible, unstable, and hard to work with. The introduction of immobilized pH gradients (IPGs) by Bjellqvist et al. (10) had a significant impact on the use of IEF to separate complex mixtures over a wide pH range. The IPGs enabled the formation of stable and reproducible pH gradients capable of focusing acidic and basic proteins on a single gel prepared with broad pH gradients. In IPGs, the carrier ampholytes are attached to acrylamide molecules and cast into the gels to form a fixed pH gradient. Fixing the gradient prevents drift in the gel and also ensures that they can be cast in an efficient and reproducible manner. Using narrow-range IPG strips allowed a larger number of proteins to be separated than had been possible with standard 2D-PAGE because a narrower pH range was spread out over a greater physical distance. This spread allowed proteins with similar isoelectric point (pI) values to be separated with higher resolution. To illustrate this point, Hoving et al. developed a 2D-PAGE method in which they applied narrow-

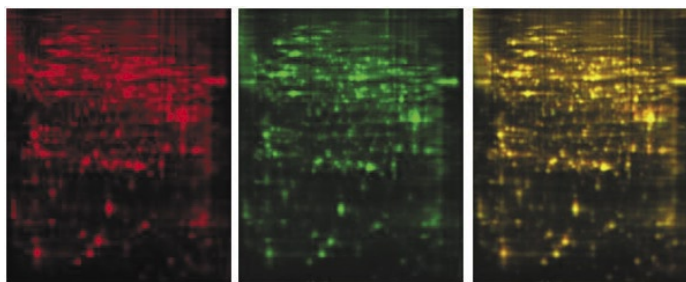


Figure 1. Two-dimensional differential in-gel electrophoresis (2D-DIGE) fluorescence images of insecticide-treated insect *Spodoptera sf*-21 cells (resistant Cy3-labeled and sensitive Cy5-labeled). The right-hand panel is an overlay of the two images. Equal amounts of protein in the latter appear yellow, whereas if a protein is only present in one sample, the spot appears green (resistant) or red (sensitive). The relative protein quantity within the samples is given by the Cy3: Cy5 ratio. Adapted from: www.liv.ac.uk/science_eng_images/biology/DIGE.jpg

range IPG strips in the first dimension (11). The IPG strips were typically 1–3 pH U wide and overlapped one another by at least 0.5 pH U. Six IPG strips covering the pH range of 3.5 to 10 were used. Proteins from a B-lymphoma cell line were applied to each strip and separated using IEF. Each strip was then applied to an individual SDS-PAGE gel plate and proteins were separated in the second dimension based on their molecular weight. Approximately 5000 distinct spots were detected using the six IPG strips, compared with 1500 spots detected using a single IPG strip with a pH range of 3–10 and a single standard 2D-PAGE gel plate. Wildgruber et al. (12) compared the use of 3 IPG strips with pH ranges of 4–5, 5–6, and 5.5–6.7 against gels run with IPG strips with pH gradient ranges of 3–10 and 4–7. They were able to detect 2.3 and 1.6× more protein spots using three narrow-range IPG strips than with the two wider gradient-range IPG strips (3–10 and 4–7), respectively.

While the higher resolution obtainable using multiple overlapping narrow IPGs enables the identification of more proteins, each narrow strip requires a separate gel plate and a certain amount of the same sample to be loaded on each. This requirement means that if the sample volume or concentration is limited, such an experiment may not be possible. For limited samples, a wider pH range or a minimum number of IPGs should be considered.

Two-dimensional Differential In-gel Electrophoresis (2D-DIGE)

The objective of separating proteins using 2D-PAGE is twofold: (i) identifying

new proteins and (ii) measuring their relative abundance between comparative samples. One advantage of 2D-PAGE as a separation technique is it not only resolves large numbers of proteins, but staining these proteins enables the relative abundances of the proteins to be quantified. For example, proteins extracted from two serum samples (healthy and diseased) are each loaded on a separate gel plate. After staining, the protein spots are aligned and scanned to measure their individual intensities. While many advances in software alignment tools have been made, it has been challenging to ensure direct spot-to-spot comparison between two separate gels. The development of 2-D differential in-gel electrophoresis (DIGE) in 1997 overcame this limitation by allowing up to three distinct protein mixtures to be separated within a single 2D-PAGE gel (13). In a typical 2D-DIGE experiment, proteins extracted from three different samples, healthy, diseased, and internal control (a pooled sample formed from mixing equal amounts of the proteins extracted from the healthy and diseased samples), are covalently labeled, each with a cyanine fluorescent dye that has a different excitation and emission wavelength. The samples are migration matched, so that the same protein labeled with any of the dyes will migrate to the same position on the gel. The cyanine dyes that have been used are: 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy3); 1-(5-carboxypentyl)-1'-methylindocarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5); and 3-(4-carboxymethyl)phenylmethyl-3'-ethyloxycarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2). Equal concentrations of the differentially labeled

proteomes and the control sample are mixed, applied to a single gel plate, and separated using 2D-PAGE. The control sample serves as an internal standard, enabling both inter- and intra-gel matching. The control sample should contain every protein present across all samples in an experiment. This means that every protein in the experiment has a unique signal in the internal standard, which is used for direct quantitative comparisons within each gel and to normalize quantitative abundance values for each protein between gels. Scanning the gel at the specific excitation wavelengths of each dye, using a fluorescence imager, allows visualization of the differentially labeled proteins (Figure 1). The images are then merged and analyzed using imaging software, which enables differences between the abundance levels of proteins to be compared. The value in DIGE eliminates any error related to gel misalignment and ensures accurate quantitation (14).

Proteins of interest are excised from the gel, proteolytically digested, and identified using MS. Since it is performed using a single gel plate, 2D-DIGE requires 50% fewer gels, making it more economical and differences in protein expression between two different samples of proteins easier to compare and more accurately imaged. In addition, less time is required to detect the protein spots because the labeling reaction in DIGE is faster than visualization using staining methods. When there is a need to compare the protein expression levels of two different samples, DIGE is the method of choice (15).

Strengths and Weaknesses of 2D-PAGE

Electrophoresis is an established technique that has undergone several advances that have enhanced resolution, detection, quantitation, and reproducibility. The 2-D SDS-PAGE and 2D-DIGE approaches to protein profiling are accessible and economical methods that possess high resolving power and enable the detection of hundreds of proteins on a single gel plate. Although reproducibility has been an issue with 2D-PAGE, especially when profiling two protein mixtures, it has been greatly improved with the use of 2D-DIGE. Resolution has been enhanced by the introduction of IPGs, which enable the analyst to tailor the

pH gradient for maximum resolution using ultrazoom gels with a narrow pH gradient range. With modern 2D-PAGE, it is not unusual to resolve two proteins that differ in pI by 0.001 U.

Although 2D-PAGE has been limited by its inability to resolve proteins that are too basic or too acidic, too large or too small, this limitation is continuously diminishing. For example, the separation of basic proteins can be analyzed using IPGs in the pH range of 4–12. Separation science is always evolving, and it will not be long before the remaining issues of gel electrophoresis are adequately resolved.

The introduction of 2D-DIGE contributed immensely to solving problems of reproducibility and quantitation. The use of imagers and computers allows not only fast data mining, acquisition, and analysis but also spot detection, normalization, protein profiling, background correction, and reporting and exporting of data. As a separation, detection, and quantitation technique, 2D-DIGE is an important tool, especially for clinical laboratories involved in the determination of protein expression levels and disease biomarker discovery. When absolute biological variation between samples is the main objective, as in biomarker discovery, 2D-DIGE is the method of choice.

While there has been significant progress in nongel (or solution-based) methods for coupling fractionation methods directly online with MS analysis, 2D-PAGE has remained a popular technique for conducting proteomic studies. Though 2D-PAGE, like any fractionation scheme, has its advantages and disadvantages, there is no doubt that it will remain an essential technique for the characterization of proteomes for many years to come.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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