Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation

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A strength of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is its ability to resolve and investigate the abundance of several thousand proteins in a single sample. This enables identification of the major proteins in a tissue or subcellular fraction by mass spectrometric methods. In addition, 2D PAGE can be used to compare quantities of proteins in related samples, such as those from altered environments or from mutant and wild type, thus allowing the response of classes of proteins to be determined. Those proteins showing a correlated difference in expression may participate in related processes, and this subsequently helps to define protein function. Although there are many limitations of the 2D gel technology that mean it will never be comprehensive in protein coverage, its use for the identification of relatively abundant proteins is now widespread. However, there are still surprisingly few examples of quantitative analysis of changes in protein abundance. In this review we highlight recent advances towards true quantitative analysis of 2D gels that will lead to better prediction of protein function. Despite the development of promising alternatives, 2D PAGE is likely to remain in extensive use for the foreseeable future, because the technology is now simple and readily available to many laboratories.

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Abbreviations

2D PAGE	two-dimensional polyacrylamide gel electrophoresis
DIGE	difference gel electrophoresis
GPI	glycosylphosphatidylinositol
pI	isoelectric point

Introduction

Numerous proteins have now been predicted from genome sequence data. In a recent review[1••], these proteins were arranged into three categories: those of known function; those with recognisable motifs and, hence, a vague idea of function; and those with no sequence similarities with any protein and therefore unknown function. Many proteins reside in this latter 'functional vacuum', which for *Arabidopsis* is 30% of the predicted proteins [2]. Determining protein expression and any change in defined circumstances is key to understanding cellular mechanism. Although the genes expressed in specific tissues, or in response to a given biological perturbation, can be gleaned from mRNA profiling studies, it is misleading to assume that these changes in transcript levels also imply corresponding changes in either protein amount or activity within cells [1••]. Even when the above is true, such data provide no information about subcellular localisation and post-translational modification, knowledge of the latter being critical to our understanding of physiological protein function [3]. To comprehend fully, we need integrated data sets from protein expression studies that encompass relative abundances, subcellular location, profiling of isoforms generated by mRNA splicing variations and post-translational modifications, and information on protein complex formation. Much of this information can be derived from two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in combination with mass spectrometric identification.

2D PAGE, best described in the review of Görg *et al.* [4••], is not without numerous technical difficulties and inadequacies. There are problems associated with reproducibility, resolution, proteins with extremes of pI (isoelectric point), recovery of hydrophobic proteins and large molecular weight proteins, poor representation of low abundance proteins, visualisation methods, analysis and normalisation of images and, finally, compatibility with mass spectrometric techniques that are generally employed in the identification of protein species of interest. Below, we chart some of the many recent advances in 2D PAGE and describe how these have aided the definition of protein function in biological systems.

Protein fractionation

For comprehensive proteome analysis, every protein would ideally be resolved as a discrete, detectable spot by 2D PAGE. However, as maybe 90% of the total protein of a typical cell is made up of only 10% of the 10 000–20 000 different protein species [1••,5••], many low-abundance proteins may not be detectable by 2D PAGE, and will therefore be unintentionally omitted from any subsequent analysis. Moreover, as a maximum of 3000 spots can be observed on a typical 2D PAGE by standard visualisation methods, many proteins will inevitably co-migrate to the same spot position, thus confounding their accurate quantitation and mass spectrometric identification. In one example, Gygi *et al.* [6•] found that the products of six genes migrated to the same faint spot on a silver-stained, narrow pH range gel of a yeast cell extract.

One method of achieving visualisation of lower-abundance proteins and greater resolution of protein species has been to use narrow-range immobilised pH gradient (IPG) strips. Although it is estimated that at least 1000 copies of a protein have to be present in a cell for them to be detected by 2D PAGE [6•], Hoving *et al.* [7] demonstrated that proteins down to 300 copies per cell could be detected in a β-lymphoma cell line using single unit pH zoom strips. In

addition, Tonella *et al.* [8•] estimated that they could display 70% of the entire *Escherichia coli* proteome using a combination of six different pH range gels. A device that enables isoelectric prefocusing in solution prior to 2D PAGE can improve on this further still. It consists of chambers of different pH ranges delineated by membranes, where proteins of similar pI migrate to the same chamber. This separation technique was applied to *E. coli* and human plasma extracts prior to 2D PAGE [9••]. The authors noted that as well as a reduction in smearing, many more spots were visible because much larger amounts of the prefractionated proteins could be loaded onto the gels. Similar conclusions were reached by Zuo *et al.* [5••] using a miniaturised version of this apparatus.

Pre-electrophoretic subfractionation has proved a successful approach towards the simplification of protein mixtures. For instance, Butt *et al.* [10•] employed non-denaturing, anion-exchange chromatography, a very attractive approach to take when attempting to ascribe function to proteins. As well as demonstrating a 13-fold enrichment of lower-abundance proteins from *E. coli* cell extracts and improved spot resolution, this technique also enabled the functional grouping of proteins. An alternative subfractionation approach used for membrane proteins utilises sucrose density centrifugation of solubilised mitochondrial membrane complexes [11]. Stevanovic and Bohley [12] were able to prefractionate dual-labelled rat liver cytosolic proteins by hydrophobic interaction chromatography in order to look at the turnover of proteins. Glycosylphosphatidylinositol (GPI)-anchored proteins, which may have a function in the cell wall of *Aspergillus fumigatus*, can be enzymically released from membrane preparations and purified by a combination of liquid chromatography and 2D PAGE [13•]. Finally, Stancato and Petricoin [14•] combined selectively pre-enriched for phospho-tyrosine-containing proteins using a panel of anti-phospho-tyrosine antibodies. These prefractionation approaches demonstrate that simplification of a sample can greatly improve the chances of identification and assignment of function to lower-abundance proteins.

Membrane proteins are scarcely soluble in the buffers used during isoelectric focussing, leading to a partial extraction of certain species, or complete absence from the final gel [15]. Prefractionation of proteins with Triton-X114, chloroform/methanol or sodium carbonate washes, and sequential extraction by detergents can partly resolve this problem. In this way, Santoni *et al.* [16••] were able to partition proteins from *Arabidopsis* according to their hydrophobicity, and solubilise them for 2D PAGE using a new set of zwitterionic detergents. They thus provided a novel way to isolate classes of membrane proteins. In another example, novel rat liver Golgi complex proteins were identified by sequential fractionation using Triton-X114, anion exchange chromatography and 2D PAGE [17•], although very few of the proteins identified on the gels were integral membrane proteins, suggesting that solubilisation was still a problem. A combination of two detergents, ASB14 and Triton-X100 has been used to solubilise the outer-membrane proteins from bacteria in a way compatible with 2D PAGE, and Molloy *et al.* [18•] used this to study conservation of such proteins within bacterial families. Another study compared solubilisation of total membrane proteins from *Haemophilus influenzae* using standard detergents and strong detergents such as sodium dodecyl sulfate (SDS) and lithium dodecyl sulfate (LDS), and concluded that no single detergent led to visualisation of every protein, but that stronger detergents helped detection of certain membrane proteins [19].

Protein detection

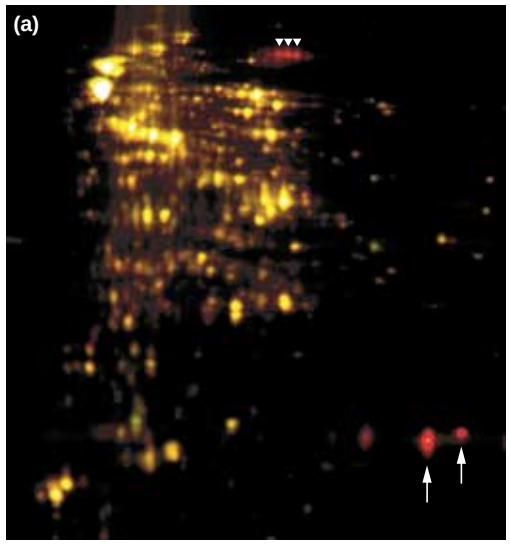
Silver staining, being more sensitive than Coomassie staining methods, has been widely used for high sensitivity protein visualisation on 2D PAGE. It is unsuitable for quantitative analysis, as it has a limited dynamic range, and the most sensitive of silver staining methods are also incompatible with protein identification methods based on mass spectrometry. More recently, the Sypro post-electrophoretic fluorescent stains (Molecular Probes, Eugene, Oregon, USA) have emerged as alternatives, offering a better dynamic range, and ease of use [20•,21•]. Sypro Ruby has been shown to be more sensitive than silver, and is compatible for subsequent peptide mass mapping [22•]. A careful analysis of the mass spectrometry compatibility of several Sypro dyes, colloidal Coomassie and silver stains concluded that Sypro orange and red give the best results [23••]. A drawback has been the cost of Sypro Ruby from commercial suppliers, and Rabilloud *et al.* [24••] kindly provided a protocol to synthesise a very similar preparation that works as successfully as the commercial version.

Quantitation

The next phase of 2D PAGE analysis involves quantitative studies of changes in protein abundance in samples. The more sensitive and representative staining of 2D PAGE, now easily achievable, should facilitate this analysis of protein expression. However, image analysis remains a bottleneck within most proteomics laboratories. In addition, there are inherent problems associated with the quantitative comparison of 2D gels that can be attributed to the irreproducibility of the technique. For example, during gel analysis, difficulties may be encountered in the accuracy of spot boundary assignment, in the normalisation of both gel background and spot intensity variation between gels, and finally in the matching of spots between gels. The difficulty in spot matching between large numbers of silver-stained gels has been highlighted by Voss *et al.* [25]. Providing hope for the future, Smilansky [26] describes a novel system for gel matching by raw-image-based registration technology that is incorporated into the Compugen analysis package.

There are numerous commercial software packages available that are able to perform these tasks. In general, however, all require substantial user intervention, which consequently leads to increased loss of reproducibility.

Figure 1



A demonstration that quantitation of DIGE spots is accurate and linear over a range of protein abundances. **(a)** 2D DIGE overlay image of Cy3- (green) and Cy5- (red) labelled test-spiked *Erwinia carotovora* proteins. The protein test spikes were three conalbumin isoforms (arrowheads) and two myoglobin isoforms (arrows). Spots that are of equal intensity between the two channels appear yellow in the overlay image. As spike proteins were eight times more abundant in the Cy5 channel, they appear as red spots in the overlay. Gels are oriented with the acidic end to the left. **(b)** A plot of the $\log_{10}$ Cy3 normalised volumes against $\log_{10}$ Cy5 normalised volumes for each spot shows that the majority of protein spots cluster around the 1:1 spot volume ratio. Quantitative analysis with Phoretix/ImageMaster (Nonlinear/APBiotech) was used to assess differences in protein abundances between the two channels. The majority of protein spots cluster around a ratio of 1:1. The average increase of the five spike proteins in the Cy5 channel was estimated as being 7 ± 1.4 (SD) fold. This is close to the true eightfold quantity of spike added to the Cy5 labelling reaction and therefore confirms the quantitative analysis of DIGE spots using the Phoretix/Image Master software. In addition, the measurements were linear for each of the five spike proteins. This observation demonstrates that quantitation of DIGE spots can be linear over at least a threefold range of protein abundances, a property that is vital for true quantitative analyses of 2D PAGE.

Studies that use quantitation often aim to identify proteins with an increase or decrease in expression beyond a significance threshold [27–31]. However, more informative correlations could be deduced if true values for changes in expression were studied. A prerequisite for these true values is that quantitative measurements are linear over a wide range of protein abundances. Importantly, Phoretix/ImageMaster (Nonlinear/APBiotech) provides a linear measure of change in protein abundance over three orders of magnitude for essentially every spot analysed in colloidal Coomassie-stained gels [32••]. Mahon and Dupree describe some pitfalls of using this program that can lead to irreproducible data. It will be important to see how other software packages and staining methods fare in similar tests of data quality.

Difference gel electrophoresis (DIGE), first described some time ago, has the potential to overcome many issues described in this and the previous section [33]. The technique relies on pre-electrophoretic labelling of samples with one of three spectrally distinct fluorescent dyes, Cyanine- 2 (Cy2), Cyanine-3 (Cy3) or Cyanine-5 (Cy5). The labelled samples are then run in one gel and viewed individually by scanning the gel at different wavelengths, thus circumventing problems with spot matching between gels. The labelling takes place via lysine residues and is carried out at stoichiometries such that only a small proportion of the protein is labelled, and is therefore compatible with in-gel digestion and mass spectrometric analysis. Although DIGE is as sensitive as staining with either silver or any Sypro dye, with a detection limit of somewhere in the region of 100–200 pg of protein, labelling has a linear dynamic range over five orders of magnitude (KS Lilley, A Razzaq, unpublished data).

Two studies employing DIGE have appeared since the beginning of 2001. Kernec *et al.* [34] investigated changes in the heart mitochondrial proteome in mice deficient in creatine kinase. Changes in protein spot intensity between the Cy3 and Cy5 images of the same gel were detected by studying a looped two-frame movie. This was a good demonstration of the simplicity of the technique, but is probably not the best method for assessing changes in protein quantity as the lack of normalisation of background intensity and spot intensities between the two images can be misleading. A second study, looking for changes in protein expression in mouse liver after administration of paracetamol, demonstrated the robustness of the DIGE method in terms of its sensitivity and reproducibility [35••]. Using Phoretix/ImageMaster software for quantitative analysis, Tonge *et al.* assigned significance thresholds for quantitative changes and showed that this threshold was dependent upon normalised spot volumes, with lower volumes having significantly higher thresholds. They also found that there was more variation between individual mice than variation contributed by error associated with the DIGE process. Current analysis of DIGE data in our laboratory suggests that quantitation of spots using such programs as Phoretix/ImageMaster provides a linear measure of changes in protein abundance. Using 1D PAGE we have shown a five-fold range of linearity (data not shown). Figure 1 represents a study in which equal amounts of soluble protein extracted from *Erwinia carotovora* were labelled with Cy3 and Cy5, but were spiked with a known

amount of conalbumin and myoglobin, with eight times more in the Cy5 labelling reaction. Quantitative analysis of the data reveals a linear relationship of the spots corresponding to various isoforms of conalbumin and myoglobin over three orders of magnitude and an average increase of 7 ± 1.4 (SD) between the two channels. These data thus demonstrate the robustness of this DIGE methodology.

It is noteworthy that normalisation of such data requires careful consideration. When there are substantial changes in abundance of numerous spots, especially the more intense species, current normalisation procedures, which are based on global normalisation, result in skewed data.

Conclusions

Several new techniques such as multi-dimensional-liquid-chromatography-based technology [36] and isotope-coded affinity tagging used in conjunction with several chromatography steps [37], dispense with gel electrophoresis as a separation method altogether. However, these new techniques have some limitations and can more realistically be viewed as complementary approaches to 2D PAGE.

By employing a combination of protein prefractionation and subfractionation to produce a reduced subset of species, zoom strips to improve resolution and loading capacity and quantitative analysis in conjunction with fluorescent detection methods, 2D PAGE is likely to remain a technique central to the study of the proteome.

Update

Since the submission of this manuscript, several additional papers have been published that further describe some of issues raised in this view. Westbrook *et al.* [38] describe the use of combinations of very-narrow pH range IPG strips to visualise the human heart proteome and demonstrated a reduction in number of co-migrating species, and hence more reliable database search results when compared with identifications of species run on broader-range IPG strips. The authors reported visualising novel proteins as well as new isoforms of previously identified proteins on such gels.

A successful and simple approach to subcellular fractionation of rat liver into organelle-enriched fractions has been achieved by Murayama *et al.* [39] using Nycodenz gradients created by freeze-thawing.

Another approach to quantitative proteomics has been taken by Consoli and Damerval [40], who have established linear relationships between different amounts of individual zein proteins from maize using colloidal Coomassie staining and used the this information to assess the genetic variability of expression of isoforms.

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