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Review

An evaluation of the use of two-dimensional gel electrophoresis in proteomics

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Abstract

With whole genomes being sequenced almost routinely, the next logical step towards a better understanding of cellular mechanisms lies in studying the functional units of gene expression—proteins. One of the fundamental approaches in proteomics is the use of two-dimensional gel electrophoresis as a mode of separation and visualization of complex protein mixtures. Despite several limitations of the method, its ability to separate large numbers of proteins, including their post-translationally modified forms, ensures that it will continue to be popular in several well-defined areas of proteomics. In this article, we discuss the merits and drawbacks of two-dimensional gels and compare them with alternative systems such as one-dimensional gels and liquid chromatography-based separation methods. In the wake of recent advances in mass spectrometry and related areas, we outline areas where two-dimensional gels can best be utilized as the preferred separation method in proteomic strategies. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the 25 years since two-dimensional gel electrophoresis (2-DE) was first described by O'Farrell [1] and Klose [2], 2-DE has been used in a diverse range of applications. In this technique, proteins are resolved in the first dimension along a pH gradient according to their isoelectric points. The gel is then transferred to the second dimension, which is typically sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in which proteins are separated on the basis of their molecular weights. Of late, 2-DE has become quite popular in the field of proteomics for separating proteins. 2-DE is presently unparalleled in its ability to separate and array complex protein mixtures; hence the reliance of many experimenters on this technology, despite its many pitfalls and difficulties. Although many criticisms of the use of this method are quite valid, it can be judiciously used in specific proteomic applications.

2. 2-DE—the early days

In the early days of 2-DE, the mere possibility of being able to display the protein content of a sample in a manner that gave an approximation of two important physical characteristics, the isoelectric point and molecular weight of proteins, was reason enough to use this technology. The idea of cataloging the proteome of a cell, and that one could identify proteins only on the basis of pI and molecular weight might have been possible in organisms with small genomes (e.g.

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Escherichia coli [3]) but, for obvious reasons, has not been realized in more complex organisms. Although there are several 2-DE databases [4,5] presently in existence, these appear to serve more of an archival function rather than acting as an extensive resource (e.g. it is almost impossible to extrapolate the identity of a human protein based on its annotation in the Swiss 2D-PAGE, one of the more established 2-DE databases, simply by comparing gel images).

3. 2-DE as a tool for monitoring gene expression

Gene expression profiling is the survey of a large number of genes and/or their expression products, typically in an effort to identify differentially expressed genes, or broad patterns of gene expression under different experimental conditions. The utility of gene expression profiling for understanding molecular processes, elucidation of drug–target interactions, clinical diagnosis, etc., cannot be overstated. Several DNA-based methods for profiling of gene expression have been previously described. These include serial analysis of gene expression [6], representational difference analysis, differential display polymerase chain reaction (PCR) or conventional cDNA microarrays. Such methods have seen tremendous progress because of the availability of human genomic data and will undoubtedly benefit from the sequencing of other genomes. Laboratories in academic settings can even build their very own microarray platforms [7]. The general applicability of these methods ensures that these DNA-based methodologies will continue to produce valuable data for the scientific community.

Why then would there be an interest to monitor global gene expression on the protein level? The fact remains that, in spite of the numerous successes of DNA-based approaches to monitoring gene expression [8], the key differences between DNA and protein-based methods (see Table 1) still make a global analysis of proteins a prerequisite in certain situations. For instance, there have been several studies that indicate that mRNA abundances show a poor correlation to protein abundance in the cell [9,10]. Some differences in protein amounts were as large as 20-fold where the respective mRNA levels were similar. Indeed, the most complete

Table 1
A comparison of DNA-based (e.g. cDNA microarray) and protein-based (e.g. 2-DE followed by mass spectrometry) approaches

	DNA	Protein
Level of detection/ sensitivity	Amplification of DNA is possible with PCR. The lower limit of detection is about 30 transcripts/sample [35]	Proteins cannot be amplified
Method of detection	Highly specific	Non-specific to specific
	The ability of DNA to hybridize with its complementary sequence (DNA/RNA) lends itself easily to immobilization on targets and probing with fluorescent probes. Dedicated scanners specifically designed to meet the demands of detecting signal in high-density arrays are available	Proteins may be detected by non-specific methods such as protein stains or more specific agents like antibodies
Stability of molecule	DNA is very stable and does not lose biological function even at elevated temperatures	Proteins are notoriously difficult to work with. Native function of protein is lost on denaturation of molecule, requiring extra care to maintain native conformation.
Reproducibility	Quite reproducible	Less reproducible
	The maturity of the field has resulted in improved methods to immobilize DNA on surfaces. Coupled with the inherent stability of DNA, reproducibility is good	Reproducibility can be finicky due to reasons related to the stability of the molecule
Cost-effectiveness	Although initial investment may be significant. (microarrays, scanner/modified confocal microscopes), the amount of data generated is quite high in short period of time	Present methods to analyze proteins are dependent on high cost of equipment (e.g. Mass spectrometers, 2-DE apparatus)
Expertise	Methods are now quite standardized	Difficulties with working with protein, purification methods and mass spectrometry imply that only trained operators and laboratories can perform a majority of these analyses
Time/automation	High-throughput screening tools are available both as commercial and open-source options	High throughput is currently limited to industry
Post-translational modifications	Cannot be studied	The only way to study post-translational modifications is to study the proteins themselves
Dynamic range/level of Expression	Five orders of magnitude	Seven to 12 orders of magnitude

picture of molecular processes occurring in the cell has to include changes that arise from post-translational processing of proteins. Most proteins within the cell undergo some form of post-translational modification (PTM) and such modifications can affect the function, half-life or localization of the protein. The differences in protein abundance arising from alternative splicing, PTMs, and differing protein/mRNA half-lives means that there is a clear significance of monitoring gene expression at the protein level.

At this point in time, however, obtaining a global overview of protein expression is not a trivial matter. In addition, the drive towards gel-to-gel comparisons for the purposes of profiling gene expression has led to a need for greater reproducibility and larger statistically significant pools of gels for purposes of comparison.

The improvements to 2-DE methodology coupled with those in the field of biological mass spectrometry make this potent combination a tool that biologists have not had general access to before, i.e. the ability to separate a complex mixture of proteins, to visualize these differences and thereafter to identify these proteins. Naturally, with the ability to characterize proteins that are differentially displayed on separate or even on the same 2-DE gel [11], there have been several laboratories that have been able to demonstrate its efficacy in answering a wide range of biological questions [12–14].

4. Recent advances in 2-DE technology

4.1. Immobilized pH gradient strips

Before immobilized pH gradients (IPG) were described in 1982 by Bjellqvist et al. [15], classic 2-DE was performed with rod gels with pH gradients generated with carrier ampholytes. The pH gradients generated by such procedures in the first dimension were inherently variable [16] and made it difficult to reproducibly manufacture 2-DE gels in large numbers. After the advent of IPG strips stabilized on a stiff plastic support, the practicality of utilizing the technique to resolve complex protein mixtures in a quick and reproducible manner became apparent [17]. At the same time, improvements in Edman micro-sequencing and eventually mass spectrometry (MALDI and ESI), as well as efforts to marry the two technologies [18,19], contributed greatly to the strengths of the approach that is the core of most proteomics efforts today.

4.2. Gel formats for visualizing more proteins

One of the main advantages of 2-DE is that it allows the experimenter to get an overview of changes in protein abundances. As shown in Fig. 1, depicting a 2-DE map of a human hepatocellular carcinoma cell line SK-Hep1, it is possible to separate protein isoforms as distinct spots in addition to a large proportion of the proteins in a given sample [20]. With the separation of proteins on the basis of molecular weight and isoelectric point, one can, for example, determine whether there are many basic or acidic proteins in a protein sample. In addition, if you have the option to identify your protein of interest from immunoblotting a membrane derived from a two-dimensional gel, it might be useful to first run a 2-DE gel that would include the majority of the proteins in the sample, albeit at a lower resolution. For such uses, broad-range IPG strips from pH 3 to 10, pH 4 to 7 (acidic), pH 6 to 12 (basic), and even wider range IPG strips from pH 3 to 12 have been developed. With these pH 3–12 gels, it has been shown that it is possible to increase the uptake of basic proteins over that of the pH 6–12 gels because of the reduction in reverse endo-osmotic flow [21].

Although a typical 18 cm 2-DE gel is generally able to resolve up to 2000 spots routinely, this is not sufficient to resolve all the proteins (along with their post-translationally modified variants) in a given protein mixture. Work by several groups to increase the separation distance from 18 cm (generally available commercially) to as much as 40 cm, have shown that this is a feasible way to increase the number of proteins resolved. A study of the mouse proteome employing pre-fractionation of proteins with different buffer systems and organ-specific samples with a large 2-DE gel format yielded an estimated 27 752 unique protein spots in total, with the largest number of proteins resolved on a single gel numbering above 10 000 [22].

4.3. Narrow pI strips (zoom gels)

Along somewhat similar lines, Wildgruber et al. have shown that by using overlapping narrow immobilized pH gradients of 1 pH unit from pH 3.5 to 6.7, they were able to increase the total number of observed yeast proteins within that pH range from 755 to 2286 protein spots [23]. The discrepancy in the number of proteins observed is due primarily to the fact that on a 2-DE gel (e.g. 18 cm pH 4–7), spots that appear to be single, well defined, spots can often be a mixture of two or even more proteins migrating together. This can be seen clearly from MS analyses of 2-DE gel spots [24] and from comparing narrow range gels with broad range gels [23].

4.4. Development of new protein solubilization cocktails

In addition to changes to pH ranges in the first dimension gel, there has been considerable effort to extend the use of 2-DE for integral membrane and other hydrophobic proteins. Perhaps the main reason

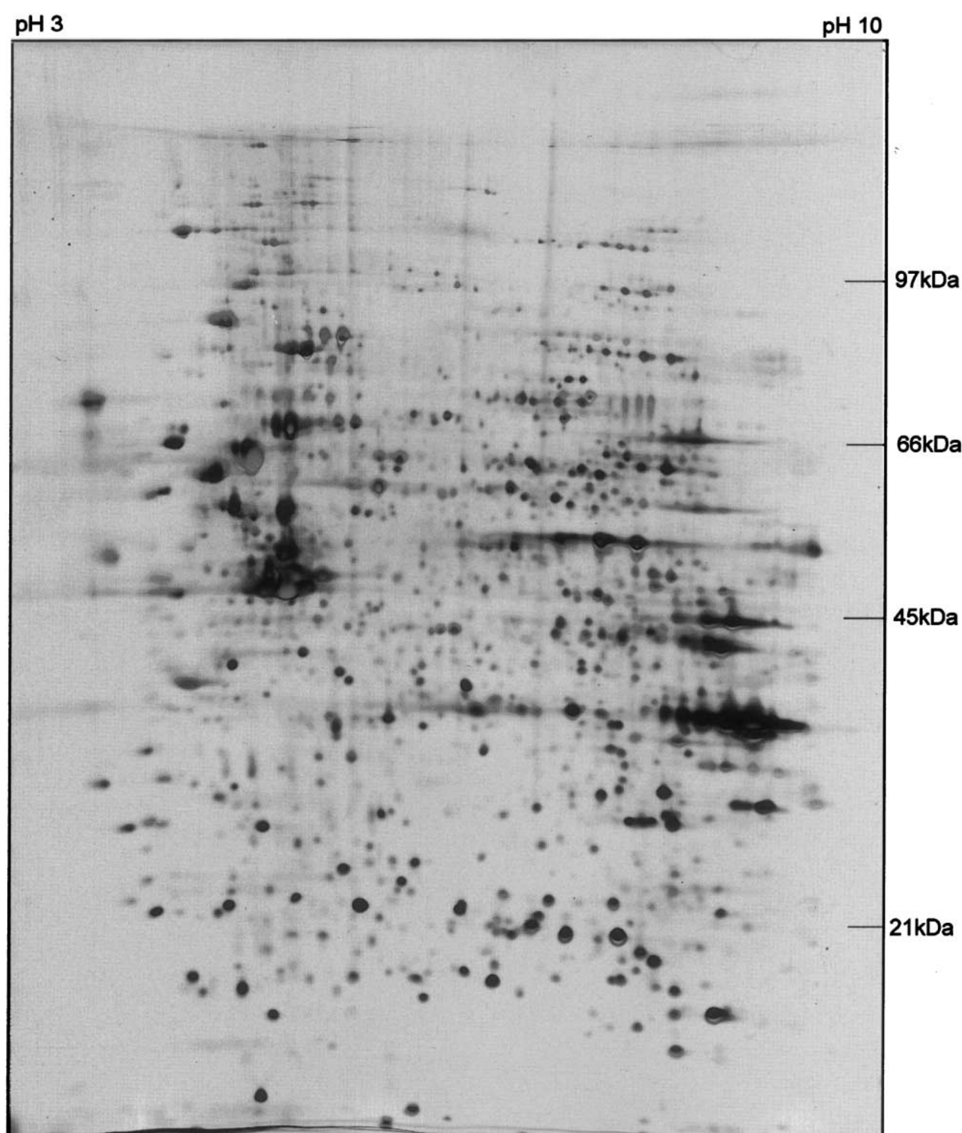

Fig. 1. A lysate of the human liver adenocarcinoma cell line, SK-Hep-1, run on a broad range pH 3–10 NL IPG strip with a 10%T SDS-PAGE in the second dimension. Protein spots were visualized by silver staining. The gel shown demonstrates 2-DE's ability to resolve complex protein mixtures, including protein isoforms, which differ only slightly in isoelectric points.

for this interest is the fact that membrane proteins (by virtue of their location at the interface of the lipid membrane and the cytoplasmic/extracellular matrix) often serve important functions for the cell in signal transduction, cell adhesion, import and export of biomolecules, etc. Although many early 2-DE studies have shown that membrane (and hydrophobic) proteins rarely enter the gel, new solubilization cocktails with the use of chaotropic agents, new detergent formulations, or organic solvents, have improved the situation considerably. On an encouraging note, a recent study comparing gel separated proteins with genome predicted proteins of the outer membrane of *E. coli* shows that up to 80% of the predicted proteins were resolved on the 2-DE gel [25].

4.5. Staining and visualization methods

Most commonly employed methods to visualize proteins are Coomassie and silver staining with limits of detection being in the mid-picomole range and the mid-femtomole range, respectively. Interestingly enough, several recent 2-DE/MS proteomics studies have used Coomassie stain despite the fact that it is not as sensitive as other presently available stains [26,27]. Indeed, for the purpose of producing 2-DE databases, the use of the Coomassie stain may prove to be more practical as it is easy to use. Silver staining is approximately 20–100 times more sensitive than staining with Coomassie, and is also compatible with mass spectrometric analyses [19]. Although the protocol for silver

staining is lengthy and prone to lab-to-lab variability, the sensitivity obtained with affordable reagents makes it one of the most widely used protein stains in the proteomic laboratories today.

Silver staining, however, is not reliable as a method of quantitation as the relationship between protein amount and the stain has a narrow linear dynamic range. The development of fluorescent protein dyes [28], shown to be compatible with mass spectrometric analyses, with comparable sensitivity to silver stains and a larger linear dynamic range, should help improve the comparison of protein amounts in 2-DE gels. At present, the high cost of such dyes and the equipment required to visualize them may be the factors limiting their widespread use in proteomic analyses.

By far the greatest sensitivity can be obtained using radioisotope labeling and autoradiography to visualize proteins on the gel. It can be extremely effective in 'pulse-chase' experiments with ^{35}S [13] or in phosphoprotein detection with ^{32}P or ^{33}P. Although the sensitivity of radioisotope labeling is higher than with protein stains, it cannot be used for non-living samples as they cannot be labeled in vitro (e.g. human tissue samples).

4.6. 2-DE image analysis

Large-scale comparisons of differential protein expression in proteomics approaches would not be possible without the capacity to handle the large sets of data generated by multiple gel runs. Indeed, the need for image analysis software was already recognized in the early days of 2-DE [29]. Trying to track the multitude of protein spots migrating to variable locations in a pair of 2-DE gels may be manageable but having to do this for several sets of gels would be quite unthinkable. In order to minimize the variability of the 2-DE method, most of the image analysis software packages available today offer the user the option of creating a 'synthetic master' gel; in effect, a gel image comprising all the protein spots believed to be representative of the sample, although not necessarily present in all members of the analysis set. The eventual task of comparing two conditions is essentially reduced to a comparison of the respective 'master' gels.

In order to effectively implement an approach that couples 2-DE and mass spectrometric analyses, it would be ideal if the information from 2-DE image analyses and the corresponding protein identification data were linked as seamlessly as possible. Several commercial products available today offer integrated solutions combining image analysis and library information management systems. However, many such packages require the commitment to a particular vendor's equipment due to the implementation of proprietary data formats, etc., and the commitment to one particular product line among several competing products may not be in the best interests of the user at this present time.

4.7. Throughput and automation

Attempts to study protein expression using 2-DE in a high-throughput fashion would not be possible if the process were not amenable to automation. Several multinational pharmaceutical companies and biotechnology-based companies have made great commitments to the 2-DE proteomics approach and placed a great deal of their research efforts towards enhancing their in-house methods and developing enabling technologies in order to be competitive.

Steps to automating the process have largely been in the realm of sample preparation. Gel spot excision with robots, liquid-handling stations for enzymatic in-gel digestions in 96/384-well formats, and automated MALDI-time of flight mass spectrometry (MALDI-TOF MS) analyses are fairly commonplace in most facilities. Although the equipment is available presently to run several large format gels in parallel, many find that 2-DE image analysis hinders the throughput of the whole process. The difficulties of 2-DE image analysis will be discussed later. There has also been considerable interest in automated liquid chromatography (LC)-MS and LC-MS/MS systems for the analysis of protein mixtures. Although these methods allow one to avoid 2-DE as the method of separation, they are relatively new technologies with their inherent limitations.

5. Limitations of 2-DE

Although it is quite tempting to imagine 2-DE as the solution to detect differential protein expression, one must first be acquainted with the variety of problems that accompany such an analysis.

5.1. Poor design of the upfront experimental set-up

A 2-DE gel is a 'snapshot' of a cell that only provides data that is as qualitative and quantitative as the methods employed to obtain that protein sample. For example, if one wants to obtain 2-DE gel maps of tumors from patients in order to identify proteins whose abundance is directly correlated with tumor progression, it would be necessary to consider differences arising from patient-to-patient heterogeneity as well as the multiple cell types that exist within the tumor. In a recent paper where 2-DE was employed for the proteomic analyses of human tumors, Emmert-Buck et al. [12] ran 2-DE gels with proteins extracted from 50 000 cells obtained by laser-capture microdissection (LCM). The quality of the data obtained from such differential display experiments using protein extracts from relatively pure popu-

lations of relevant cells would obviously be superior to comparisons made with samples from interfering cell types. Although LCM is a powerful method that has been effectively used for the extraction of DNA and RNA [30,31] for subsequent analyses, it should be noted that the effort required to obtain sufficient protein amounts for 2-DE proteomic analyses is considerable.

5.2. Standard operating procedures in 2-DE?

In addition to the complexities of obtaining comparable cell populations, there are several other considerations in a 2-DE run. Sample preparation is a critical step in 2-DE and naturally, if one is attempting to make a comparison of protein abundance in two samples, close attention to how sample preparation was performed should be foremost in the experimenter's mind. It is worth remembering that sample preparation includes all the preliminary steps of procuring and preparing the sample in the chosen solubilization cocktail. Furthermore, it would be necessary to load comparable amounts of sample across comparison sets, and if sample preparation protocols are not standardized this could lead to errors if overlooked. 2-DE protocols should be standardized within a particular laboratory but subtle differences can arise from the type of strips used, different lots of reagents used, the sample loading methods employed (cup loading versus in-gel rehydration), and even the operator.

In the literature, there exist several formulations of protein solubilization cocktails and sample preparation protocols that may be suited to some applications more so than others. There is no attempt to standardize these, and perhaps no practical way to do so, as the success of one protocol over another is highly variable and operator dependent.

These instances are not cited with the intention of prejudicing the reader against 2-DE as a scientific method. Indeed, it is quite possible to standardize 2-DE procedures within the laboratory and have reproducible runs between different operators. These issues only become more of a consideration if lab-to-lab comparisons of 2-DE gels and 2-DE image databases are envisaged.

5.3. Inherent variability of 2-DE

For any individual with the right equipment and the correct disposition wishing to run 2-DE, it is relatively straightforward to obtain a reasonable pattern of protein spots in their first run. However, if the same individual were required to run the same sample again, it would not be trivial to be able to reproduce the protein pattern exactly in subsequent runs. Depending on the sample (amount of salt, contaminating lipids, etc.), considerable amounts of time may have to be spent on optimizing the 2-DE run conditions in order to obtain optimal separation of proteins across the pH gradient. Even in situations where you might obtain maximal resolution, run-to-run variability (especially in alkaline pH where reverse electro-endo-osmostic flow may result in streaky patterns) will typically result in a small number of differences in the 2-DE pattern (e.g. the edges of some gels may have better separation when compared with others that were even run in the same focusing run). It is also important to bear in mind that the gel itself can change in size, depending on what solution is used to store the gel in. Some groups have minimized this warping of the 2-DE gel image by preparing gels for the second dimension backed with a solid support like glass or stiff plastic [32]. Ideally, the variability of the method will be reduced to a minimum as a better understanding of the variables that govern the run is reached. Until then, it is difficult to imagine how comparisons of 2-DE gel maps between different laboratories can be possible although there already exists a web-browser enabled java program, FLICKER, that attempts to do just that [33].

One other serious criticism of the 2-DE approach is the way image analysis is presently carried out. Despite the availability of commercial software designed specifically for 2-DE image analysis, it is still a time-consuming exercise that has not been possible to fully automate because of several problems: If one were to consider the pattern of protein spots in terms of $X-Y$ co-ordinates, it would be necessary to track all these changes in 2-DE gel patterns in order to match these gels for statistical significance. It quickly becomes obvious that although one can routinely observe a large number of protein spots in a 2-DE gel map, it may be much harder to come to a firm conclusion on whether all these protein spots appear consistently in all gels. The apparent amount of the protein (often correlated to how darkly it stains with a protein stain) can also vary from gel to gel despite the greatest care employed in the staining steps. Often enough, to come to a reasonable conclusion about the statistical relevance of each spot requires a judicious eye. Clearly this dependence on a human decision is subject to bias and hence introduces yet another variable. In addition, the spot detection software that specify a 'spot' are not robust enough to be able to unambiguously identify the borders of overlapping protein spots. This requires a great deal of user intervention in terms of redefining the actual spots as determined by the software. These considerations play a central role in the process of creating 'synthetic master' gels described earlier. In practice, the process of creating a master gel is not trivial; it is very much

dependent on the uniformity of the gels that are used to create it. Imagine having to decide whether a differentially expressed spot is a 'real' difference if it appears definitively in 60% of the gels and is ambiguous in the remainder. Indeed, it is almost a Catch-22 situation, where in order to increase confidence in the 'master' gel, it is necessary to run more gels, which requires more time and effort, may lead to greater ambiguity and is contrary to the concept of a high throughput method. Whereas reasonable throughput from multiplexing has been realized in running multiple 2-DE gels and MALDI-TOF MS analyses on 96/384-well sample targets, limitations in current image analysis software and difficulties referred to previously has led to the criticism of image analysis as the major bottleneck in 2-DE proteomics approaches

5.4. Dynamic range

In addition to the complications already mentioned, it is claimed that it has a limited dynamic range and that low copy number proteins are rarely visualized and identified on 2-DE gels [34]. In present mRNA-based approaches, the dynamic range spans five orders of magnitude [35]. This is seriously limiting when one considers that the actual dynamic range of proteins in a cell may be as great as seven to eight orders of magnitude [36] (i.e. for a particular species of abundant protein that may exist in a cell at the level of 10^8 copies/cell, there are other proteins that could be expressed at ten copies in the same cell). The issue of dynamic range is not trivial; many differing opinions exist about any particular method. Take 2-DE in proteomics as an example; if you were to compare a Coomassie-stained 2-DE gel and a radioisotope-labeled gel for an equivalent protein load, the difference in the number of visualized spots would be obvious. At this point in time, however, some of the low abundance proteins visible only with radioisotope labeling would not be detectable by even the best mass spectrometric methods. You might then think that, by increasing the amount of protein loaded on the gel, one could then obtain a mass spectrometric readout with the low abundance proteins. However, by doing so, you might exceed the load capacity of the IPG strip, obtain very poor resolution of proteins and, in doing so, fail to visualize the proteins of interest. Hence, it may be more practical to think of dynamic range in the 2-DE/MS method in terms of what can be observed in a single experiment (a silver-stained 2-DE gel)—this is typically about three orders of magnitude. In other case, it would be possible to state that the dynamic range of a method is only limited by the amount of enrichment steps, pre-fractionations, and separate experiments that one is prepared to perform.

5.5. Loss of certain protein species

If one is to think of 2-DE as a truly global approach, it should ideally be able to resolve representative protein populations in the cell without selective bias towards any protein types or characteristics. The loss of proteins in the 2-DE analysis (specifically hydrophobic as well as large proteins above 100 kDa) remains a serious limitation to the concept of 2-DE as a global approach at present.

It is recognized that the large majority of 2-DE analyses of cell extracts have reported mostly cytosolic (and mostly hydrophilic) proteins and that membrane proteins are an underrepresented minority. Briefly summarized, the difficulties of resolving membrane (hydrophobic) proteins include the following: membrane proteins are typically not present in the cell in large amounts, and without a special preparation to enrich for these membrane components, it would be difficult to identify these proteins out of a crude cell extract. Although such a 'membrane-only' preparation is possible through sedimentation followed by extraction with detergents or pH, etc., there still remains the problems of other non-protein components of the membrane (e.g. lipids) and of having the proteins of interest remain soluble in the first dimension gel, which is necessarily less hydrophobic for the purposes of isoelectric focusing. Another problem relates to the fact that the class of membrane proteins can have varying hydrophobicities and some may have very hydrophobic regions (transmembrane domains) while others have just a few hydrophobic patches spanning the primary sequence—this makes the development of a generic 2-DE strategy for resolving all membrane proteins very difficult.

In addition, proteins whose pI values fall at the extremities of the pH gradients (very acidic or basic proteins) are difficult to resolve on a gel. Although there have been modifications to 2-DE that demonstrate improved resolution of basic proteins [37], some groups have also had considerable success with the use of techniques like non-equilibrium pH gradient electrophoresis-2D-PAGE [38]. It appears that, at least at present, there is no general 2-DE method that could claim to meet the requirements of every experimenter.

6. Specific applications for 2-DE in proteomics

With all the apparent limitations of 2-DE, why is it still so popular as a fundamental tool in proteomics? It is the most powerful procedure presently available to resolve a complex protein mixture. In many ways, it is the most straightforward way to search for and subsequently identify unknown proteins and their post-translationally modified forms. If the same protein is identified unambiguously in multiple locations on a

2-DE gel, the migration in multiple locations on the gel could partially be explained by PTMs. A recent publication in *Nature* clearly demonstrated the efficacy of 2-DE as a way to observe changes in protein phosphorylation of actin in *M. pudica* [14]. It is clear that if one already has some familiarity with their proteins of interest, the ability to 'zoom in' on the region of interest with narrow range pH IPGs would potentially provide a lot of useful information.

However, it must be said that performing a 2-DE run still requires considerable effort and the results may vary depending on salts and other contaminants that may be present in the sample. Therefore, it is entirely conceivable that if one intends to follow up the gel run with identification using mass spectrometric approaches (MS/MS sequencing of tryptic peptides or MALDI-TOF), it might be wholly sufficient to depend on a SDS-PAGE run instead. (For a comparison of the salient features of 2-DE and SDS-PAGE, see Table 2.)

We anticipate that the merits of 2-DE are most easily realized in the following applications.

6.1. Analyzing immunoprecipitates and protein complexes

In our laboratory, we have used antibodies that recognize tyrosine-phosphorylated states of proteins to enrich for these molecules from growth factor treated cells [39,40]. With this enrichment step, we are able to get more information from one- or two-dimensional gels when compared with gels of protein lysates. In another example, Neubauer et al. purified the human spliceosome complex using a pre-mRNA substrate followed by separation by 2-DE and mass spectrometric identification [41].

6.2. Identifying components of organelles

Enriched extracts of organelles such as mitochondria or nucleus can be prepared by a number of protocols employing ultracentrifugation and analyzed by 2-DE. A large number of organelle-specific proteins can thus be identified from a single experiment. To cite some examples, components of the human placental mitochondrial proteome [42] and thylakoid proteins isolated from pea chloroplasts were recently published [43]. Of course, one of the disadvantages of such experiments is that not all of the proteins are derived from the organelle being studied [44] due to the imperfect nature of the biochemical fractionation procedure itself.

6.3. Metabolic and toxicological studies

An obvious application of 2-DE is in cases where monitoring broad changes in protein expression profiles (i.e. in the more abundant proteins) provides valuable information. Clear examples include quality control in biochemical engineering where cell lines are developed and continually monitored for production of recombinant proteins. 2-DE can also be used to examine the effects of drugs on the expression of proteins by cell lines or explants. By closely monitoring changes in

Table 2
A comparison of 2-DE with 1-DE as protein separation techniques

	2-DE	1-DE (SDS-PAGE)
Basis of separation	First dimension: isoelectric points Second dimension: molecular weight	Molecular weight
Protein load limit	100–500 μg for most applications Resolution of protein spots becomes poor if the gels are overloaded	Large amounts of proteins can be loaded—sufficient for all practical analyses
Reproducibility	Considerable variability even with commercially available reagents	Good. Commercially available pre-cast 1-DE gels with good consistency of gel composition allows good reproducibility
Ability to resolve protein mixtures	Presently the most powerful method available (includes non-gel-based approaches)	Fair, separation on the basis of molecular weight
Large molecular weight proteins	Limited entry of proteins above 100kDa	Able to handle even protein complexes. Agarose-polyacrylamide gels can even accommodate particles as large as polyribosomes
Highly hydrophobic proteins	Difficult to solubilize and therefore harder to observe in 2-DE	Possible to resolve in 1-DE
Very acidic and basic proteins	Often difficult to resolve in 2-DE, prone to smearing	Can be easily resolved
Post-translational modifications/protein isoforms	Separation is dependent on charge so negatively charged phospho groups in phosphoproteins are generally seen as separate protein spots	Is generally difficult to detect phosphorylated species merely by a difference in molecular weight. Glycosylation may lead to smearing

protein expression in response to drug treatment, researchers can obtain relevant information about the mechanism of drug action, the cytotoxic effects of a drug, etc. For example, the effect of cyclosporine A (CsA) was studied in several animal models and a protein, calbindin-D 28 kDa, was found to be a potential marker for CsA-mediated nephrotoxicity [45]. In another study, a drug-induced increase in hepatocellular rough endoplasmic reticulum in rat livers was examined using 2-DE [46].

6.4. Analyzing whole proteomes of simpler organisms

One application of 2-DE that is already well established and where its use is likely to expand is the study of the proteomes of simpler organisms. The advantages of working with simpler organisms include the decreased complexity of the genome, as well as the fact that one is not limited by the amount of sample that can be obtained. The proteomes of several organisms including *Mycobacterium tuberculosis* [4,47], *Plasmodium falciparum* [48], *Candida albicans* [26,49], *Helicobacter pylori* [50], and *Haemophilus influenzae* [27] have now been studied by the 2-DE approach. In addition, the ability to compare pathogenic and non-pathogenic strains of bacteria and to test the drug response of such strains is obviously of great value to the scientific community.

6.5. Immunoblotting—Western blots and Far-Western blots

A common technique in laboratories today exploits the fact that proteins immobilized on a membrane such as nitrocellulose can still bind antibodies that recognize a particular epitope. In a clinical setting, this principle may be used for the detection of new allergens by immunoblotting with patients' sera [51]. Making use of the high resolving power of 2-DE, Soskic et al. identified 300 phosphoserine-containing proteins and 260 phosphotyrosine-containing proteins by immunoblotting with the respective anti-phospho antibodies [52]. Although not all proteins were present in amounts detectable by mass spectrometric methods, this approach demonstrates its ability to potentially detect large numbers of phosphoproteins in a single experiment. Because of its high sensitivity and specificity, immunoblotting methods have seen widespread use even before the concept of proteomics existed. Similarly, in a Far-Western approach, the membrane can be incubated with tagged proteins, protein domains or peptides. Binding to the membrane is revealed directly if the tag is fluorescent or by other indirect procedures that detect the tag. The proteins of interest that are identified by this method can then be analyzed by mass spectrometry.

6.6. Other technologies—LC-MS systems

Although the resolving power of 2-DE is presently unparalleled, there are efforts to find other ways to resolve complex mixtures of proteins and peptides. The use of liquid chromatography (high-performance liquid chromatography, gel filtration, ion exchange) systems for the separation of proteins and peptides is common in most biochemical laboratories. However, in the past several years, the coupling of micro, capillary or nano-LC systems to mass spectrometers in LC-MS systems has made it possible to perform online protein identification without having to run a single gel.

In-solution digests of crude protein mixtures are prepared and then loaded onto microcapillary columns packed with reverse-phase (usually C_{18}) material. Peptides are eluted from the reverse-phase material and electrosprayed directly into the inlet of a mass spectrometer. Columns with small internal diameters (typically 75–100 μm) and low flow rates ($<$ 300 nl/min) have greatly increased the sensitivity of this approach in the past few years. If necessary, further separation of peptides can be performed by placing an ion-exchange column in-line with the reverse-phase column. Using a similar set-up, Link et al. were able to identify proteins from the large protein complexes like the yeast and human ribosomal subunits directly [53].

In addition to protein identification, there are also newer methods to compare protein amounts with LC-MS approaches. In one of these methods, proteins from the two states to be compared are digested and the cysteines labeled with different versions (heavy and light) of a 'tag' carrying a biotin moiety. The samples are then mixed and the tagged peptides are affinity purified using immobilized avidin. Peaks corresponding to the same peptide can be identified as doublets in mass spectra due to the difference in the molecular weight of the heavy and light versions of the tag. In this method, the peak intensities correlate directly with the relative abundance of the protein in the two states. Gygi et al., who developed this method, have termed it 'isotope coded affinity tag' [54]. Munchbach et al. have developed a similar method to tag the N terminus of peptides for quantitation purposes [55].

7. Summary

With the recent advances in biological mass spectrometry, 2-DE has seen a resurgence in popularity because of its ability to separate protein mixtures. 2-DE can be judiciously coupled to several types of biological experiments to provide meaningful data. However, the 2-DE approach is still largely refractory to high-throughput methods due to a number of reasons. Indeed, efforts to improve reproducibility have largely

centered on robotics, improved visualization methods as well as better spot-detection algorithms. All of these require financial investment on a scale that is rarely available in most academic settings. Nevertheless, as we have outlined in the article, 2-DE-based approaches can still be effectively used when applied with a clear understanding of its strengths and limitations.

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