2D protein electrophoresis: can it be perfected?

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23 years after O'Farrel developed two-dimensional gel electrophoresis we still debate if the technique can be improved, or if there are other alternative separation technologies that can challenge its central position in proteomic projects. These questions are relevant as the pharmaceutical industry expects proteomic studies to provide novel protein targets for drug discovery and diagnostics. In our opinion, there are various aspects of the technology that can be improved, including resolution, sample preparation and detection, but so far there is no alternative technique(s) available, or any under development, that can replace it.

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Abbreviations

2D PAGE	two-dimensional polyacrylamide gel electrophoresis
IPG	immobilized pH gradient
pI	isoelectric point

Introduction

For the past 23 years, high resolution two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been the technique of choice for analysing the protein composition of cells, tissues and fluids, as well as for studying changes in global patterns of gene expression elicited by a wide array of effectors (see [1,2,3••,4••] and references therein). The technique, which was originally described by O'Farrell *et al.* [5,6] and Klose [7], provides the highest resolution for protein analysis and, therefore, plays a central role in 'proteomics', an area of functional genomics that deals with the global analysis of gene expression at the protein level. This novel field of research combines a plethora of techniques to resolve (high resolution 2D PAGE), identify (peptide sequencing by Edman degradation, mass spectrometry, Western immunoblotting), quantitate (scanners, phosphorimagers) and characterize proteins, as well as to store (image analysis and 2D PAGE databases), communicate, and interface protein and forthcoming DNA sequence and mapping information from genome projects ([2,3••,8,9•]; http//biobase.dk/cgi-bin/celis). Proteomics, together with genomics, cDNA arrays, phage antibody libraries, transgenics and knockouts, as well as bioinformatics, provide an impressive array of technologies for studying gene expression both in health and disease.

Presently, a great deal of effort is being devoted to the development of high-throughput proteomic technology ([10•] and references therein), a technical development that is expected to facilitate the study of diseases and expedite the process of drug discovery by providing hundreds, or even thousands of putative protein targets [11,12]. The questions still remain, however, whether the current 2D PAGE technology can be perfected, or if there are alternative separation technologies that can challenge its central position in proteomic projects. In this review, we describe the increasing challenges being faced by the 2D gel technology and give an appraisal of its current status and perspectives.

Why 2D PAGE?

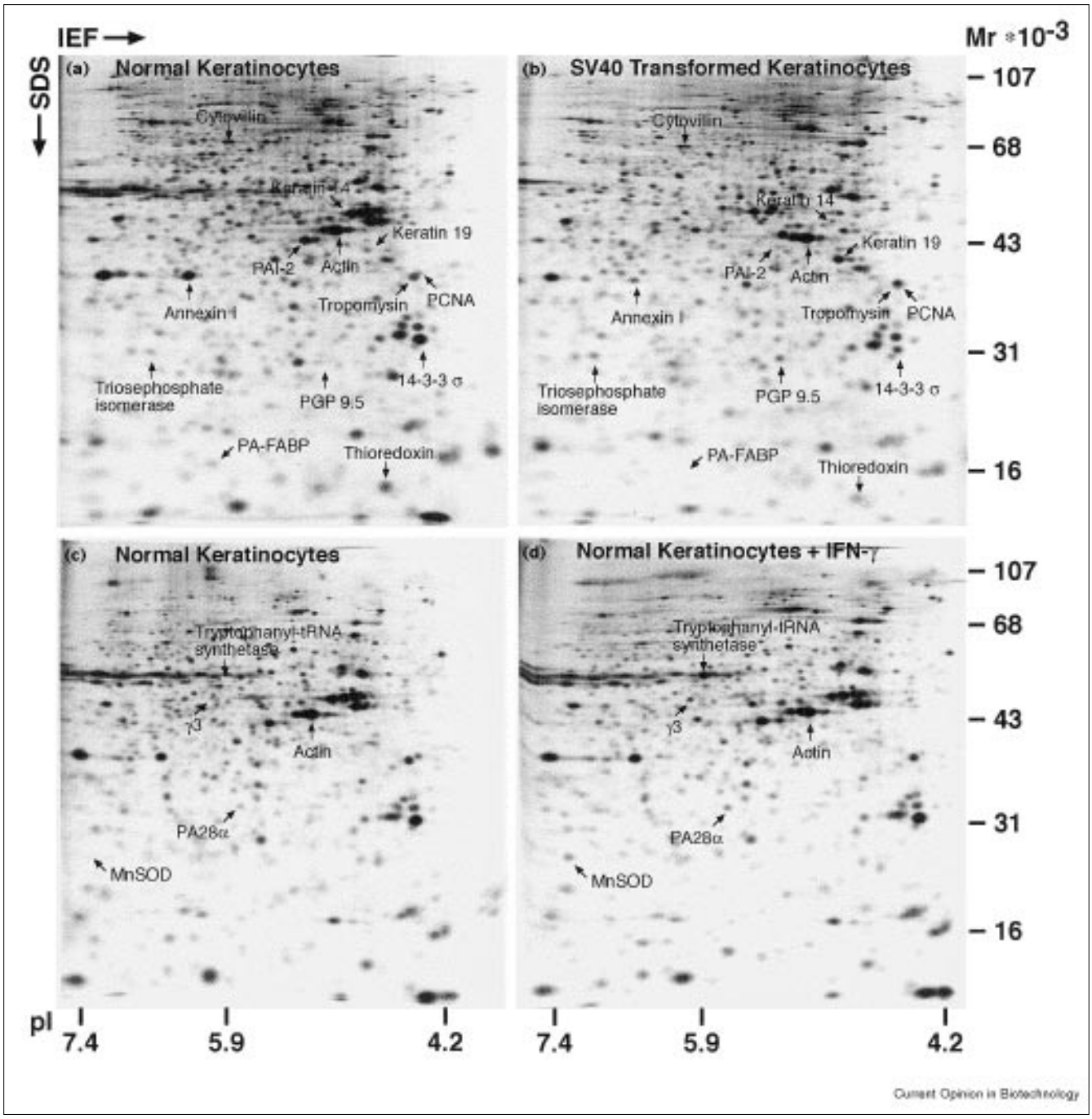
2D PAGE separates proteins both in terms of their isoelectric point (pI) and molecular weight (Mr), and accordingly, its resolving power is unsurpassed when compared to one dimensional gel separation techniques [1,3••,13]. The current 2D PAGE technology can be used for several applications: firstly, separation of complex protein mixtures into their individual polypeptide components; secondly, comparison of protein expression profiles of sample pairs (normal versus transformed cells, cells at different stages of growth or differentiation, etc.); and thirdly, choosing a condition of interest, for example, the addition of a cytokine or a drug to a given cell type or tissue, and allowing the cell or tissue to reveal the global protein behavioral response under conditions where all of the detected proteins can be analyzed, both qualitatively and quantitatively in relation to each other (see examples in Figure 1).

Proteome profiles can be scanned and quantitated to search for protein differences (changes in the levels of preexisting proteins, induction of new products, co-regulated polypeptides), and interesting targets can be identified using additional proteomic technologies, such as peptide sequencing by Edman degradation [14–16], mass spectrometry [17,18,19••], and 2D PAGE Western blotting [20]. Furthermore, by carriyng out these studies in a systematic fashion one can store the information in comprehensive 2D PAGE databases that record how genes are regulated in health and disease (http://biobase.dk/cgi-bin/celis and http://expasy.hcuge.ch/ch2d/2d-index.html). As these databases achieve critical mass of data, they will become indispensable sources of information for expediting the identification of signaling pathways and components that are affected in diseases [2,9•].

How many proteins do we expect in a complex biological sample? The proteome complement of a eukaryotic cell

The usefulness of the 2D PAGE technique for large-scale proteomic projects depends very much on the number of

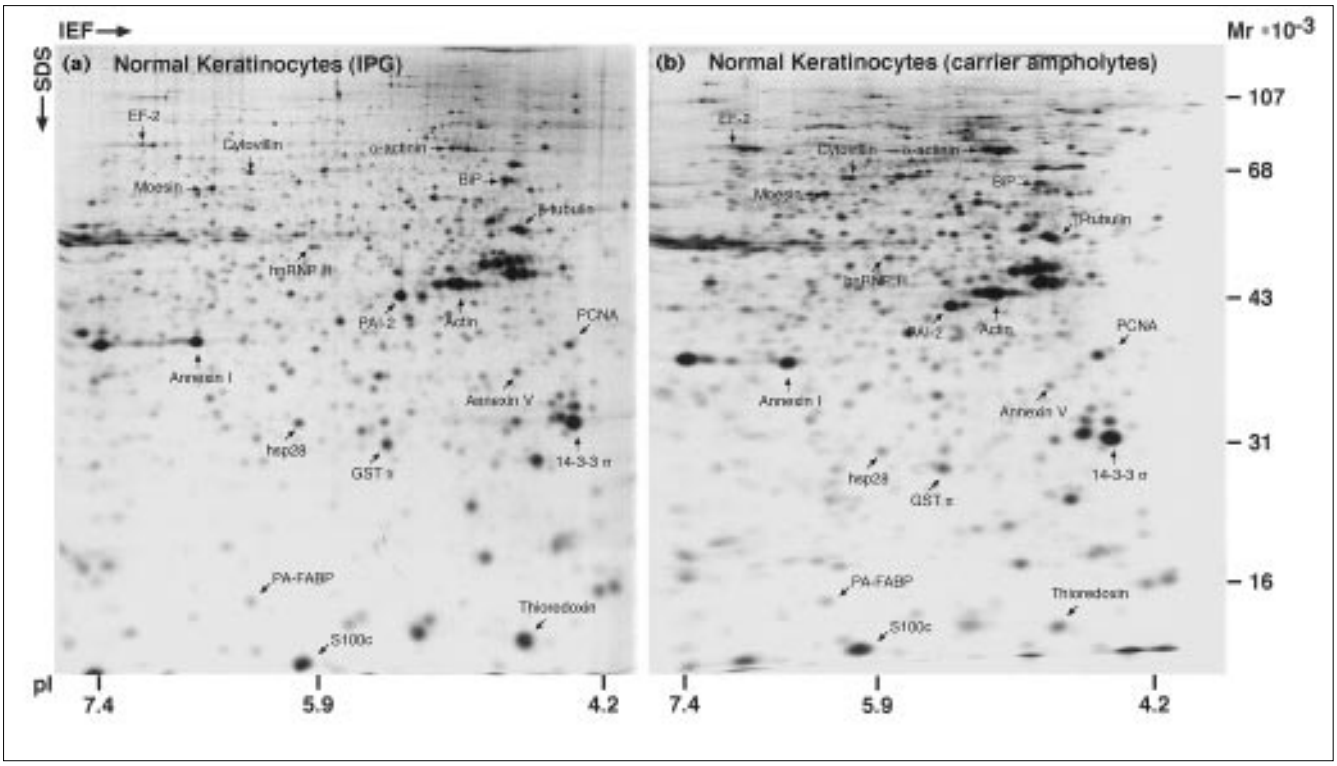
Figure 1



Isoelectric focusing (IEF) 2D PAGE using carrier ampholytes of [^{35}S]methionine labeled proteins from human keratinocytes. **(a,c)** Noncultured normal human keratinocytes. **(b)** SV40 transformed keratinocytes (K14). **(d)** Noncultured human keratinocytes treated with IFN-γ. The increased levels of the DNA replication protein PCNA in the transformed cells (b), as compared to the normal keratinocytes (a), clearly reflect differences in the rate of cell proliferation. Only some protein changes are indicated.

proteins to be resolved in a complex protein mixture, for example, a eukaryotic cell. In yeast, for which the entire genome has been sequenced, one would expect a total of about 6000 proteins [21] of which not all may be expressed at any given time. In the case of human cells, however, the situation is much more complex as there is no concensus as to the number of genes contained in the genome. Numbers from 60,000–100,000 genes have been reported, although the issue is still unresolved [22]. Proteome profile data from a few laboratories, including our own, have indicated that only a fraction of the human genes are switched on in a given cell type [2,23,24] and extensive analysis of

Figure 2



Noncultured normal human keratinocyte proteins separated using **(a)** IPG 3–10 and **(b)** carrier ampholytes (pH 3.5–10). A few proteins are indicated for reference. Figure (a) is reproduced from [32] with permission.

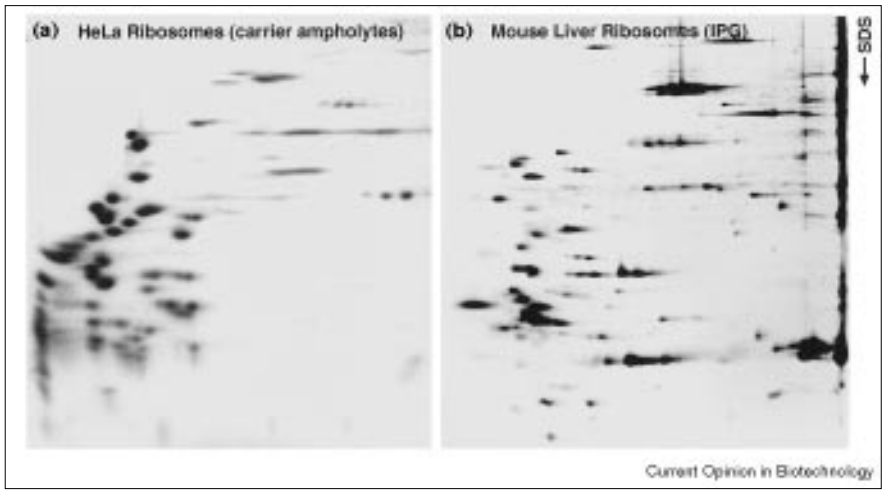
whole-cell extracts and organelles as well as partially purified subcellular fractions, suggest that individual cells may not express more than 6000 primary translation products at any given time. On top of that, however, one has to add the post-translational processing and chemical modifications (phosphorylation, glycosylation, methylation, acetylation, myristoylation, palmitoylation, sulfation, ubiquination, etc.), the latter being rather common and extensive in the case of some proteins [25,26•]; the modified variants can be distinguished from the primary translated products by 2D PAGE as post-translational modifications may lead to changes in molecular weight (protein processing) and/or pI (chemical modifications). As much as 80–90 % of the proteins may represent house-keeping genes (i.e. components of metabolic pathways, cytoarchitectural components, etc.) that are expressed — albeit in variable amounts — by most cell types [2,25]. Considering that there are at least 250 different cell types in the human body and a total of about 100,000 genes [22], one can argue that on average individual cell types may not differ from each other in more than 400 unique proteins.

The current 2D PAGE technology: can it be improved?

For many years the 2D PAGE technology relied on the use of carrier ampholytes to establish the pH gradient [5–7], but this technique has proven to be difficult in the hands of many as it is labour intensive and because of the lack of reproducibility created by uncontrolable variations in the batches of ampholytes used to generate the pH gradients. Lately, however, with the introduction of immobilized pH gradients (IPGs) [27,28,29••,30], which are an integral part of the polyacrylamide matrix, it has been possible to obtain more reproducible focusing patterns. IPGs avoid some of the problems associated with carrier ampholytes, such as cathionic drift, allow a higher loading capacity for micropreparative runs, and provide increased resolution when narrow pH gradients are used (approximately 0.05 pH/cm) ([31] and references therein). In our hands, broad range IPGs (pH 3–10; see Figure 2a) and carrier ampholytes (pH 3.5–10; see Figure 2b) resolve a similar number of [^{35}S]methionine-labeled polypetides (about 2500) as illustrated in Figure 2 with the separation of whole protein extracts from human keratinocytes [32]. Even though the carrier ampholyte and IPG-based 2D gels look similar overall, confirmation of the identity of proteins exhibiting similar mobility requires the use of standard protein identification techniques, a fact that has also been emphasized by Nawrocki *et al.* [33•]. In the case of carrier ampholytes, an additional 1500 polypeptides, mostly basic, can be separated by non-equilibrium pH gradient electrophoresis (NEPHGE) [6], some of which have pIs of up to 12 (http://biobase.dk/cgi-bin/celis and also Figure 3a). Recently, Görg *et al.* [30] described a

Figure 3

Separation of **(a)** HeLa and **(b)** mouse liver ribosomes using carrier ampholytes (7–9.5) and IPG (9–12) respectively. Figure (b) was kindly provided by A Görg and W Weiss.



wide-range immobilized pH 4–12 gradient that yields highly reproducible protein patterns with focused spots up to pH 12. For comparison, Figure 3 shows examples of Hela and mouse liver ribosomal proteins resolved using carrier ampholytes (Figure 3a) and IPGs (Figure 3b) [34•], respectively.

Clearly, as it stands today one could expect to visualize a maximum of about 4,000–5,000 proteins using broad pH gradients, a fraction that is still short of the total number of proteins that may be present in a eukaryotic cell. Missing polypeptides are either not resolved by the pH gradient (too basic or too acidic), do not enter the gel due to solubilization problems or molecular weight size, precipitate and streak during the run, or simply are not detected due to limitations in the sensitivity of the current procedures, which are based mainly on isotope incorporation (autoradiography, fluorography), and/or staining with either silver nitrate or Commassie Brilliant Blue. Some of these limitations can be partly overcome by applying prefractionation procedures to enrich for the desired protein, protein fraction, or subcellular component, although in our experience the more one manipulates a sample the higher the chance of degradation and/or introducing artificial protein modifications.

Even though larger format gels may be expected to yield better resolution [9•,35], we do not believe that it will be possible in the near future to resolve the proteome complement of any given cell type in a single 2D gel. In the long run, IPGs offer the best way ahead as the resolution can be improved substantially by using narrow pH gradients which also allow increased sample loading. Currently, dehydrated IPG strips are available commercially in narrow and broad pH gradients (except for separation of very basic proteins), a development that has assured reproducibility, and that has greatly stimulated the intra- and inter-laboratory comparison of gel images. There have been reports, however, concerning smears and precipitation when using the IPG strips, but these are expected to be solved by adopting a more strict quality control process on the part of the manufacturers [31].

At present, there is some discussion about the possibility of replacing the 2D gel technology as the core technique in high-throughput proteomics [10•,11,36]. Alternatives being considered include on-line combination of capillary isoelectric focusing with electrospray ionization mass spectrometry, integrating innovative chromatographic separations with MALDI-TOF mass spectrometry, as well as the use of an antibody chip. None of these technologies, however, offer the resolution currently provided by 2D PAGE, and we foresee that at least for the near future its pivotal role in proteomic projects will remain unchallenged. We are aware, on the other hand, that there is much room for improvement, in particular with respect to sample preparation and detection.

Can sample preparation and detection be improved?

As proteome projects develop, it has become more and more evident that some proteins cannot be properly dissolved by the lysis solution originally developed by O'Farrell [5], and so it has become a matter of urgency to devise protocols for optimizing sample solubilisation. Rabilloud and co-workers [37•,38] have made use of the high loading capacity of IPGs to resolve membrane proteins for structural analysis, and in doing so have improved their solubility by using a combination of detergents and chaotropes. For example, they have shown that the addition of thiourea, CHAPS and sulphobetain surfactants to the lysis solution containing urea results in a much improved solubilisation, as well as transfer to the second dimension SDS gel. As far as nuclear proteins are concerned, Görg and co-workers have improved considerably the separation of very basic proteins by first precipitating the samples with acetone prior to solubilisation

in the lysis solution (A Görg, personal communication). The problems associated with the extraction of tissue samples, on the other hand, are much more complex and have not been addressed yet in a systematic fashion, this is because tissues are composed of various cell types, including epithelia, connective tissue material, muscle and fat.

Even if we were to optimize the sample preparation, we still have to deal with the problem of detection as many of the interesting proteins are present in very low copy numbers. For known low abundancy proteins, for which there are antibodies available, detection does not pose a problem as we have shown that 2D PAGE immunoblotting in combination with enhanced chemoluminiscence can detect as few as 100–500 molecules per cell in unfractionated cellular extracts [25]. Similarly, using blot overlay procedures it may be possible to detect low abundancy proteins that bind to a particular radiolabeled ligand [39]. Everyday research, however, requires detection techniques that can be applied to a large number of resolved protein whose abundance may span through seven or eight orders of magnitude. Clearly, the sensitivity of silver nitrate and Coomassie Blue staining is inadequated, and only metabolic labeling with specific isotopes may reveal enough proteins to warrant proteomic projects. Furthermore, using phosphor-imaging technology it is possible to enhance the sensitivity and linearity of detection. There are limitations to the radiolabeling approach: firstly, lack of labeling of some proteins due to low (slow) turn-over; secondly, problems associated with safety regulations and disposal; and finally, difficulties in obtaining fresh human biopsy material for labeling experiments. Ideally, one would like to have a highly sensitive fluorescence-based protein detection technique able to support all types of studies irrespective of the sample, or the end point of the analysis. Preferably, the dye should not alter the molecular weight and pI of the proteins if it is to be added prior to electrophoresis, and should support quantitative studies involving proteins having extreme differences in their copy numbers. Unfortunately, no such ideal dye is available on the market yet, although Oxford GlycoScience has developed fluorescent IPG-PAGE (http://www.ogs.com/proteome/ home.html), a technology not available to the scientific community. Fluorescent compounds, such as SYPRO Orange and SYPRO Red, have been used to analyse whole-protein lysates from bacterial and mammalian cells but their sensitivity is not high enough [40]. Advantages over silver staining include short staining time and the fact that the gels do not need to be fixed prior to staining.

Conclusion

Today, there is no technology in sight that matches the resolving power of 2D PAGE, a technique we believe will continue to enjoy a central position in proteomic projects for some time. There is considerable room for improvement, however, in particular as far sample preparation, choice of pH gradient and detection methods are concerned. In general, we expect researchers first to use wide IPG gradients to obtain an overview of the proteome profiles, and then proceed with a more detailed analysis using narrow pH gradients, which provide high resolution and sample loading, thus increasing the possibility of visualizing the lesser abundant proteins.

There are still many additional challenges that will have to be addressed before complete proteomic projects can be implemented. These include, to name a few, the heterogeneity of biopsy material, the lack of procedures for quantitating protein changes, as well as the need to develop better image analysis systems for supporting gel comparisons and databasing.

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- •• of outstanding interest

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