REVIEW

Two-dimensional gel electrophoresis and mass spectrometry for biomarker discovery

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The proteome project, initiated in 1995, was made possible by 2-DE combined with MS. The project main objective was and remains the identification of all proteins expressed by a cell, tissue or organism in a given time and condition. Following this objective, the global profiling of proteins in health *versus* pathological state by the 2-DE/MS-based proteomic approach has contributed to the elucidation of the basic mechanisms of disease by discovering candidate disease biomarkers and disease targets for new drug development. This review will briefly summarize the historical evolution of 2-DE up to today, and review 2-DE/MS technology and its specific methods of study of immunoresponse (immunoproteomics), PTM of proteins, complex protein–protein interactions (interactome), the proteome of cell membrane and intracellular proteome turnover in disease biomarker discovery.

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1 2-DE historical evolution

Global profiling of proteins in healthy *versus* pathological states is a strategy to discover biomarkers for early diagnostics, disease progression, treatment response and novel targets for therapeutic drugs development. The idea of making an inventory of all the proteins in a cell, tissue or organism with normal or abnormal physiology, was (re)initiated in the middle of the 1990s with the proteome project [1]. Since then, many technologies to make the analysis of the proteome a reality have been united.

The perfect technological 'marriage' that made the proteome project feasible was between high resolution 2-DE for separation of large numbers of proteins and MS for identification and characterization of the proteins displayed on this gel [2]. However, before this happy union occured, science and technology devoted to protein study had a long journey until 2-DE and MS combination was possible (Fig. 1). It started in the last century, in 1937, when Tiselius [3] introduced electrophoresis technique as a modification of Reiner's early concept (1927) [4] to analyse a complex mixture of proteins such as serum proteins. Only 20 years later, in 1959, electrophoresis on a gel support formed by crosslinked polymerization of Cyanogum 41, a commercial name for two organic monomers, acrylamide and the crosslinking agent, *N*,*N*1-methylenebisacrylamide, was shown to be useful and was named PAGE by Raymond and Weintraub [5]. The adaptation of polyacrylamide gel to IEF in a stable pH gradient, developed by Svensson in 1962 [6], was possible by the end of the 1960s [7–9]. About this time, the 2-D by combining IEF to separate undenatured proteins according to their charge and gradient SDS-PAGE for a second separation, according to their molecular mass, was for the first time introduced by Kenrick and Margolis (1970) [10]. Only in 1975, was IEF in denaturing conditions, as known today, used to follow the 2-D PAGE and it was described in three independent works [11–13]. The most powerful demonstra-

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Abbreviations: BN, blue native; **CN,** clear native; **Co-IP,** co-immunoprecipitation; **CTAB,** cetyl trimethyl ammonium bromide; **ICAT,** isotope-coded affinity tags; **MAP1B,** microtubule-associated protein 1B; **NBS,** 2-nitrobenzenesulphenyl chloride; **SILAC,** stable isotope labelling with amino acids in cell culture

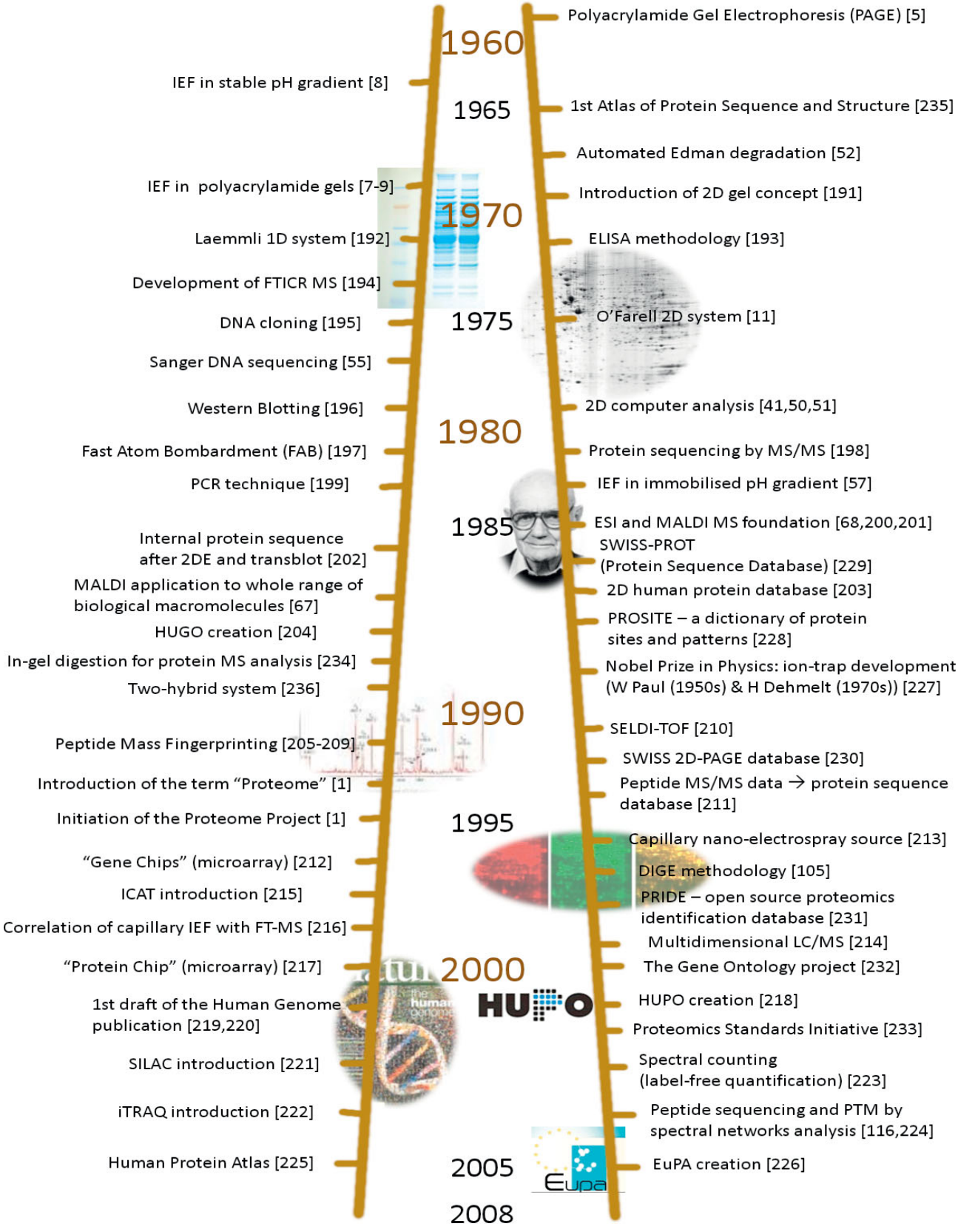

Figure 1. A Proteomics Timeline: Indicates important scientific contributions to the proteomics development.

tion was O'Farrell's [11] system showing over thousands of *Escherichia coli* proteins resolved on a single 2-D gel. For the next 20 years, O'Farrell's system was the method of choice for separation and analysis of complex protein mixtures, leading us to say that the analysis of proteome expression started in that middle of the 1970s. 2-DE patterns of proteins from cells, tissues or whole organisms were described [14–24]. Turnover of protein precursors, such as for actin and tubulin into isoforms as result of PTM, using pulse-chase technique and 2-DE were demonstrated (examples [25–32]). Protein synthesis during the cell growth/cycle [33–40] and modulation of protein expression in response to molecules [41–45], drugs or environmental conditions revealing induction of proteins such as heat shock proteins were extensively demonstrated [46–49].

Facing the complexity of dealing with 2-D gel patterns, investigators soon developed (1979) the first computerized scanning programs for comparing 2-D gels quantitatively [41, 50, 51]. Because automated protein sequencing by the Edman degradation, introduced in 1967 [52], required hundreds of picomoles of protein, more than that purified by the O'Farrell system, the proteins were identified, on a limited scale, by analysis of their comigration with known proteins. Prepurification methods, such as immunoprecipitation, Western blotting, developed in 1979 [53] or peptide mapping [54] were used. Although recognized as a powerful method, the traditional O'Farrell procedure was laborious and very challenging for the majority of investigators and there was low reproducibility in comparisons within and between laboratories. Poor sample loading capacity with IEF carrier ampholyte pH gradients was another limitation.

When the analysis of DNA/RNA became the crowning interest, with the introduction of new methodologies like DNA sequencing (Sanger *et al.* 1977) [55] and the PCR by Mullis and coworkers in 1983 [56], enthusiasm for protein analysis using time consuming techniques such as 2-DE decreased. In 1980s, 1-D SDS-PAGE combined with mRNA analysis by Northern blotting or PCR was the method of choice for the study of gene expression.

The study of proteins by 2-DE was again brought to the front page of the journals in the middle of 1990s thanks to the introduction of IPG in gels supported by plastic backing instead of the traditional tubes of carrier ampholyte pH gradients by Bjellqvist *et al.* [57] followed with subsequent optimizations [58–66]. The introduction of IPG brought superior resolution, reproducibility and loading capacity to 2-DE. The availability of commercial precast IPG gels in different combinations, from narrow to broad IPG ranges (see below), together with dedicated equipment and friendly computer programs (see Table 2) made possible the rapid production and analysis of highly reproducible 2-D maps. However, the biggest impact on 2-DE came with the ability to get information on the separated protein spots (Fig. 1). For example, in-gel digestion of protein with specific endoproteases such as trypsin, gave protein fingerprints, which could be analysed further by MALDI-MS, developed by Karas and Hillenkamp's laboratory [67] or ESI-MS developed by Fenn and coworkers [68], in 1985. A protein spot can be identified by comparing the mass spectrometric peptide map with that theoretically calculated in a database. The door to building up several extensively annotated 2-D gel databases (see studies in 'World-2DPAGE Index', www.expasy.com) was opened by the idea of database searching using PMF and/or peptide MS/MS fragmentation spectra (short partial sequence or tag) carried out later in ESI/MS/MS [69] or in MALDI-TOF MS by PSD [70] or in MALDI-TOF-TOF [71]. Having the complete human genome sequences, the first steppingstones to the most challenging project, the human proteome project in health and disease, were followed (see HUPO projects www.hupo.org).

Today, there have been dramatic improvements in both 2-DE and MS due to the development of improved apparatus and refinements of sample preparation and quantification methods (see below) associated with new biostatistics and bioinformatics tools that have increased the capability of 2-DE and MS in biomarker discovery.

Although the use of gel-free technologies are rapidly growing, 2-DE in combination with MS still remains the most popular and versatile procedure for proteome analysis.

2 2-DE today

Advances in 2-DE made it very friendly to use. Comprehensive 2-DE protocols can be easily found in academic books [72–77] or in recent publications [78, 79], websites of companies or tutorials in the websites of recognized institutions (see Table 1). Basic or advanced practical courses in 2-DE have been currently provided (see HUPO or EuPA websites www.hupo.or or www.eupa.org for updated courses announcements). Since 2-DE and MS are independent technologies, any laboratory without an MS facility can undertake a 2-DE-based proteomic study, either through collaboration with laboratories having an MS facility or using paid services of institutions or companies, making 2-DE an affordable proteomics technology.

Although the main advantage of 2-DE technology is its capacity to provide a global view of a sample proteome at a given time by resolving hundreds to thousands of proteins simultaneously on a single gel, one must take into account limitations in the technology before planning to use it for biomarker discovery. The limitations are associated with the inability of 2-DE to resolve all proteins present in a sample because of: (i) extreme differences in their solubility (highly hydrophobic proteins, see below for membrane proteins), (ii) a wide range in their expression levels (low abundance proteins), (iii) the presence of extremely basic or acidic p*I* values that exceed the gel IPG range capacity or (iv) upper or lower molecular size gel limits.

In 2-DE, the proteins are separated (in the first dimension during IEF) according to their p*I* value (the point at

Table 1. 2-DE website tutorials

Tutorial administrator	Link
Angelika Görg	http://www.weihenstephan.de/blm/deg/manual/manfrm.htm
Australian Proteome Analysis Facility	http://www.proteome.org.au/2D-Gel-Electrophoresis/default.aspx
By James Jefferies	http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html Section%204
Expasy	http://biophilessurf.info/proteomics.html
Wellcome Trust	http://genome.wellcome.ac.uk/doc_WTD021045.html

which their charge is the same as the surrounding pH) and (in the second dimension during SDS-PAGE) by their molecular mass.

However, as in Fig. 2 shows, the whole method comprises six steps: (i) sample collection, handling and preparation, (ii) first dimension (IEF), (iii) second dimension (SDS-PAGE), (iv) 2-DE protein pattern visualization, (v) 2-DE pattern computer-assisted analysis and (vi) selection and digestion of protein spots for further MS identification.

Variations in one of these steps can enable the procedure to be adapted for a specific sample or biomarker discovery study (for a recent review see Görg *et al. Proteomics* 2004 [80]).

Since, there is not yet a fully automated 2-DE apparatus and the biological and technical variations require the use of biological and technical replicates to produce meaningful results, one is concerned with the availability of sample in any experimental design. The number and type of replicates required is influenced by the reproducibility, the optimal sample size and the advantages and limitations of pooling biological samples in proteomic study have been recently addressed [81–86].

The use of minigels [87] or technical variations such as the DIGE system (see below) in which the number of gels and/or replicates are reduced, are alternatives to be considered.

The first crucial step of every proteome analysis for biomarker discovery is to consider the sample set, handling and preparation. This has been recently extensively reviewed [84].

Prefractionation of complex cell/tissue mixtures leads to a reduction in sample complexity, advantageous for the subsequent preparation, 2-DE separation and quantification of the sample. A search for disease biomarkers in tissue or blood would be more successful if individual cell type components rather than whole tissue were investigated. Laser capture microdissection microscopy [88], and fluorescence-activated cell sorting (FACS) [89] are very specific methods for cell isolation. One must be aware of possible alterations in the protein profile during cell, tissue or blood manipulation, especially when cells are kept alive during the procedure.

Cell components such as nuclei, mitochondria, lysosomes, microbodies or any other organelles can be subfractionated by differential centrifugation methods to decrease the complexity of the sample (methods reviewed in refs. [90, 91]). Other possibilities are fractionation of proteins according to their solubilities or chromatographic or electrophoretic separation (for reviews see [80, 92]).

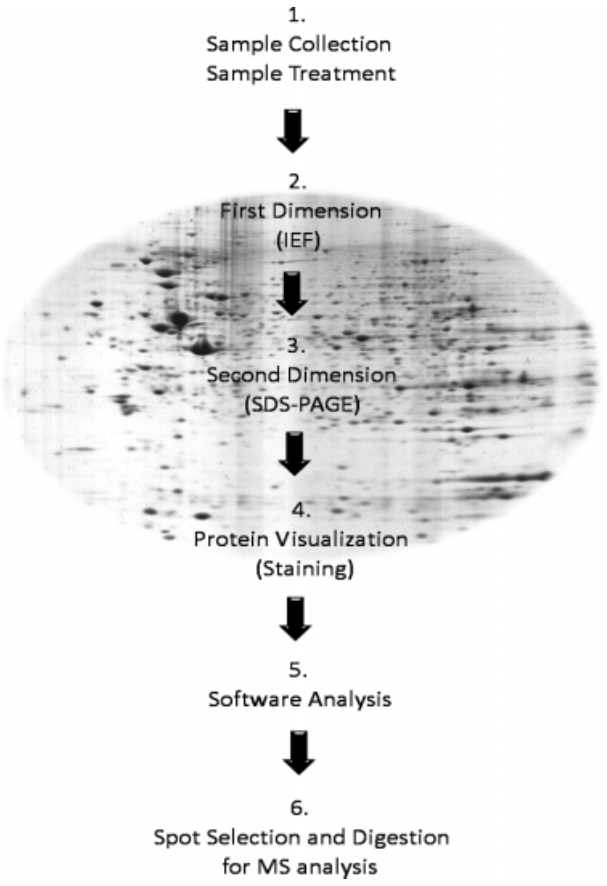

Figure 2. 2-DE workflow.

2.1 Sample preparation

For every type of sample cell, tissue or body fluid, sample preparation involves fundamentally, cell disruption, inactivation or removal of interfering substances and solubiliza-

tion to ensure that the majority of proteins in the sample will be 2-D displayed for further quantitative or qualitative computer-assisted analysis and identification by MS. Therefore, sample preparation should be adequately optimized according to the type of sample and the aim of the study. However, it is possible that a completely different set of proteins might be obtained when using a different method. More detailed considerations have been discussed elsewhere (for review see [80, 93]).

Different methods of cell disruption can be applied individually or in combination but for every case the workout must be robust and equally well controlled among the samples in terms of duration and temperature to avoid protein degradation and variations among the samples. Protease inhibitors, high concentrations of chaotropic agents for denaturing proteolytic enzymes and an appropriated buffer with low pH must be used during disintegration, typically at 4°C.

Substances which interfere with electrophoretic separation, like high concentration of salt ions, nucleic acids (DNA/RNA), lipids, polysaccharides, charged metabolites and impurities in the sample should be completely removed or minimized (for review see [80, 93].

For sample solubilization, the consensual best buffer to start with is 7 M w/v urea, 2 M w/v thiourea, 2–4% w/v CHAPS, 1% w/v dithioerythritol (DTE)/DTT and 2% v/v carrier ampholytes. To optimize the solubilization of particular samples such as those containing larger and more hydrophobic proteins, variations in the concentration of chaotropes and reducing agents and utilization of zwitterionic detergents or reducing agents such as tributylphosphine (TBP) has been recommended [94–96].

2.2 The first dimension analysis

Although IPG could be home-made in the laboratory, the introduction of commercial dry IPG strips in a variety of lengths (usually from 7 to 24 cm) and pH gradient ranges, from linear or nonlinear wide (*e.g.* IPG 3–10 or pH range 3–12), medium (*e.g.* IPG 4–7), narrow (*e.g.* IPG 4.5–5.5) or ultra-narrow (*e.g.* IPG 4.9–5.3), made 2-DE easier to handle. With these IPGs, the reproducibility of the gels was increased and the comparison of data among laboratories improved. These advances contributed to the widespread application of 2-DE in proteomics.

General guidelines for IEF with IPGs suggesting appropriate protein amounts and voltage programmes among other things have been published ([80] books, companies and websites (Table 1). Usually, the linear or nonlinear wide or medium pH gradient range with 18 or 24 cm length are the first choice for analysis of a simple proteome to have a global overview of proteins present in a complex mixture. The necessity of exploration of other IPG will depend on the type and the aim of the study as well as the specificity or availability of the samples. For example, very alkaline proteins corresponding to approximately 30% of all proteins are better resolved using IPGs 4–12 [80]. The use of narrow overlapping IPGs (IPG 4–5, 4.5–5.5, 5.0–6.0, *etc.*) known as 'zoom-in' gels has proved useful to achieve optimal resolution in complex protein mixtures. Spots (10–40%) detected on a broader pH range are usually visualized as a single spot or as a spot cluster, since there are many proteins in a sample with similar proprieties of p*I* and molecular weight such as isoforms resulting from PTM. The use of narrow, overlapping IPGs alone or in combination with prefractionation can improve gel resolution and therefore the unambiguous protein pattern analysis, protein identification and characterization by MS.

2.3 Second dimension

After IEF is run, the IPG strip is equilibrated in a suitable buffer to reduce and alkylate proteins before application to the top of a SDS-PAGE gel, usually a Laemmli standard system for molecular weight separation. Again, there are many opinions in terms of polyacrylamide gel type (home- or commercial precast gels; vertical, the most commonly used or horizontal) and gel matrix (10, 12 or 8–16% polyacrylamide gel gradient the most popular ones) that should be chosen according to the sample and the purpose of the study. However, not all proteins are equally well detected. For very small, $<$15 kDa or very high proteins, $>$150 kDa that escape conventional SDS-PAGE, other nonstandard systems like tris-glycine buffer or a combination of several gels are recommended (see below for alternatives approaches in 'membrane protein').

2.4 Protein visualization

Since, most pathological processes are associated with qualitative or quantitative changes to an individual protein species, proteins once displayed on the 2-D gel must be visualized in a valuable form to enable their accurate qualitative/quantitative analysis by dedicated software. Post-electrophoretic protein visualization is most frequently obtained by colorimetric and fluorescent staining methods. The critical factors of staining methods are their sensitivity, reproducibility and the linear range of detection (for a review see [97, 98]). Colorimetric methods include CBB and variants, such as Colloidal (C)-CBB or blue silver (C-CBB modified), silver-staining (there are more than 100 variants) and zinc or imidazole–zinc staining. Most of them have linear responses over a very limited range (maximum two orders of magnitude) due to saturation effects that make them unable to cover the great variation in protein concentration of a sample (see discussion reviewed by [97–100]). CBB-based stainings are simple to use and compatible with MS with a detection limit of around 8–100 ng/spot or 1–100 ng/spot for blue silver [101]. Silver staining is more sensitive, $<$1–10 ng/spot but has a low reproducibility, a more complex time-consuming protocol and the most sensitive methods are not MS compatible [99, 102].

A higher sensitivity and better linear dynamic range of detection are obtained with fluorescent dye compounds. Fluorescence staining methods are being increasingly used due to their comparatively wide linear dynamic range ($>10^3$), good sensitivity (1–2 ng protein/spot) and ease of use (for recent reviews [97, 98]). The overall quality and relative cost of five commercially available fluorescent stains, Krypton, Deep Purple, Rubeo, Flamingo and the most popular used stain, Sypro Ruby dye were recently evaluated by Harris *et al.* 2007 [97]. The authors also reported that C-CBB was found to be comparable to Syrpo Ruby when detected using an infrared fluorescence imaging system rather than standard densitometry [97].

Preelectrophoretic protein visualization, with a higher dynamic range ($>10^3$–10^5 order magnitude) and sensitivity ($<0.1/0.01$ ng) is possible by using either lysine with its ε-amino group or cystein with its thiol groups *in vitro* covalently labelled with different fluorescent cyanine dyes with various excitation and emission wavelengths (CyDye2, CyDye3 or Cydye5), or by *in vivo* or *in vitro* isotopic radiolabelling of the proteins (*e.g.* ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I or ^{131}I). The advantages of these methods are discussed below in more detail, in 'DIGE' and 'Turnover Proteomics', respectively.

The use of isotope-coded affinity tags (ICAT) or 2-nitrobenzenesulphenyl chloride (NBS) isotope-labelling technology in combination with 2-DE and MS for quantitive proteome analysis have been recently proposed [103, 104]. Both labelling methods are conceptually similar since they involve the binding of light and heavy (ICAT or NBS) moieties. ICAT is traditionally used in combination with LC-MS/MS and comprises a biotin-affinity tag and a thiol-specific reactive group that labels peptides at cysteine residues. NBS binds to tryptophan residues, resulting in a 6 Da mass difference between light (^{12}C) and heavy (^{13}C) NBS-labelled tryptophan peptides.

After labelling, a mixture of light and heavy labelled samples is co-analysed in the same 2-DE. For any spots on the gel, the relative differences in protein quantity can then be determined by measuring the ratio intensity of these light/heavy paired ICAT/NBS peptide peaks in a MS. However, the selection of spots for excision is usually based on a prior analysis using conventional 2-DE gels.

ICAT/2-DE/MS was applied for example to quantify proteomic alterations induced by a metabolic shift in yeast *Saccharomyces cerevisiae* and to compare 20S proteasome subunit compositions from different cells [103]. NBS/2-DE/MS was used to profile proteomic alterations in breast cancer cells induced by tamoxifen, the most commonly used selective estrogen receptor modulator [104].

2.5 DIGE

DIGE, introduced by Unlu *et al.* 1997 [105] is a modification of 2-DE in which controls and disease samples are labelled preelectrophoresis using different fluorescent CyDyes. After labelling, equal amounts of disease and control samples are mixed and analysed on a single 2-DE gel. The respective 2-DE patterns on the gel are visualized and discriminated from each other by their own unique excitation and emission wavelength signals, detected using a fluorescence imaging scanner. Comigrating samples on the same gel enable a perfect match of pairs of spots and thus the accurate analysis of differences in protein abundance between samples.

Two DIGE procedures are commercially available, known as minimal and saturation labelling. In the minimal-labelling procedure, all three CyDyes are used; the individual disease or control sample is usually labelled with Cy3 or Cy5, while Cy2 is used to label an internal standard consisting of a pooled mixture of all samples (*i.e.* disease samples plus controls) in the experiment. In the saturation-labelling procedure, two different CyDyes-labelled samples, typically a sample and the internal standard, are co-run on the same gel. Although the number of gels is higher using saturation labelling, the amount of protein needed is ten times lower, giving the advantage that more proteins are detectable than with the minimal-labelling procedure [106]. Whatever labelling procedure is used, the analysis of one or two different samples on a single gel together with an internal standard, which is used in all gels within a series of experiments, reduces experimental variation and the need of gel replicates. DIGE is a sensitive technique, capable of detecting as little as 0.5 fmol of protein, and this detection system is linear over a 410 000-fold concentration range. Data reproducibility and normalization among gels, as well as confidence in matching and quantifying different samples are also dramatically improved [107]. Although the CyDyes are very expensive, the advantages of DIGE over standard 2-DE has made their increasing use as recommended method for disease biomarker discovery in proteomics [80, 106]. An optimized protocol of DIGE was recently reported [78].

2.6 2-D pattern computer analysis

The visual image of a protein pattern on a 2-D gel must be captured in a digital format before computer-based image analysis. The objective of computerized 2-D image analysis is to identify, in crowded gels, protein spots that have increased or decreased in size and intensity, by comparison with control gels. The critical parameter in computer-based image analysis is the quality of the images. Good quality requires high quality and reproducible 2-D gels, as above discussed, and also high quality acquisition of the image with image capturing devices (*e.g.* fluorescent or phosphor imagers, laser densitometers, CCD cameras, *etc.*) (for more detailed considerations and recommendations see [98]). For 2-D image analysis there are several commercially available software programs such as those listed in Table 2. Basically, the software is designed for automated spot detection, matching, normalization and quantification. However, a totally automatic image analysis system is still required, since correction by user intervention is usually necessary.

Table 2. 2-DE softwares

Company	Link	Availability
Alpha-GelFox 2-D (Alpha Innotech, USA)	http://www.alphainnotech.com/	Commercially available
Delta 2-D (Decodon, Germany)	http://www.decodon.com	Commercially available
Dymension (SYNGENE, USA)	http://www.syngene.com	Commercially available
ImageMaster2D/Platinium	http://www1.gelifesciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&parentid = 366139&moduleid = 166672&zone = Proteomics	Commercially available
Ludesi 2-D gel image analysis	http://www.ludesi.com/	Commercially available
PDQuest/Proteomweaver (BioRad, USA)	http://discover.biorad.com	Commercially available
Progenesis/Phoretix/ MODAS (Nonlinear, UK)	http://www.nonlinear.com	Commercially available
REDFIN (Ludesi, USA)	http://www.ludesi.com	Freely available
Shimadzu Phoretix 2D Evolution (Shimadzu Biotech, Japan)	http://www.shimadzu-biotech.net	Commercially available

In order to facilitate the analysis, dissemination and exchange of 2-DE data, standards have been developed by the HUPO–Proteomics Standards Initiative (PSI), namely by the subgroup, Gel Analysis Workgroup of the PSI. The workgroup develops reporting requirements, data formats and controlled vocabularies for experimental gel electrophoresis, and informatics performed on gel images. A tutorial on how such resources can be used was recently published by Jones and Gibson 2007 [108]. This includes the use of guidelines called MIAPE (Minimum Information About Proteomics Experiments) gel electrophoresis (http://psidev.sourceforge.net) that act as checklists of the essential information that should be reported about particular experimental techniques.

2.7 MS identification

Protein spots on the gel can be systematically isolated and identified by MS as major proteins present in a sample. In the case of biomarker discovery, the spots showing differential expression in the disease sample are selected as candidates for potential novel drug targets or diagnostic disease markers.

Proteins spots are excised from the gels or from NC membranes (see below 'Immunoproteomics'), digested, usually with trypsin, and the resulting mixture of peptides is introduced into a mass spectrometer for protein identification. The most common systems for doing this are MALDI-TOF MS and ESI-MS (for a review see [109, 110].

Methods have been proposed to speed up the reactions involved in the steps of sample handling, to give better in-gel protein identification by PMF using MALDI-TOF [111, 112].

From MS analysis, two specific types of data can be obtained, the PMF which involves determination of the masses of all the peptides in the digest and the amino acid sequences of the peptides (MS/MS), also called peptide-sequence tags [109, 110, 113].

The MS or MS/MS spectra data are used to search a predicted mass-map or protein sequence within a database to identify the protein of interest using algorithms such as SEQUEST or MASCOT [109, 110, 113] or without a database so-called spectral networks that was recently developed by Bandeira *et al.* [114–116]. Using a database search, candidate proteins are ranked from a list of most closely matched candidates using various scoring algorithms. If there is no match with any known sequence, enough sequence information can be obtained to identify new proteins, *i.e.* new genes. In spectral network, similar spectra are aligned and connected by relationship into network leading tryptic and nontryptic overlapping fragments or from modified and unmodified versions of the same peptide to be singled out. The alignment into a matrix, similar to the matrix used in the classical Smith–Waterman algorithm for DNA sequence alignment, accurately infers peptide sequencing and PTMs [114–116]. This spectral networks program was designed to analyse ESI-MS/MS data and is freely available at www.cse.ucsd.edu/groups/bioinformatics/software.html.

3 2-DE specific applications

3.1 Immunoproteomics

Immunoproteomics is defined as the large-scale study of proteins involved in the immune response. Since, humoral immune response to diseases in humans is diverse, the analysis of an antigen/antibody repertoire by immunoproteomics constitutes a powerful means to understand disease pathogenesis including various cancerous diseases that may be suitable for immunotherapy [117, 118].

The antigen/antibody signature as a biomarker may identify subsets of patients with certain clinical phenotypes, prognostic outcomes or differences in therapeutic responses.

Among the current proteomic techniques available, 2-DE combined with Western blotting (2-D blot) and MS, also

known as serological proteome analysis (SERPA) has been chosen as a powerful tool in immunoproteomics application. An optimized SERPA protocol was recently described [119].

Cell lines derived from patients, tissues or body fluid are separated by 2-DE, transferred to NC filter (2-D blot) and used as source of (auto)antigens for the screening of candidate binding antibodies present in the serum from patients [119]. Typically, the number of spots and the degree of immunoreactivity are significantly higher in 2-D blots screened with patients sera in comparison with normal sera. The identity of these sets of antigens is established by the isolation of corresponding spots from a preparative stained 2-D gel and further analysis by MS.

The information provided using this proteomic approach has indicated new deregulated autoantigen/autoantibody expression patterns, not only in classical autoimmune diseases such as lupus, Behç ets and arthritis [120] that are characterized by disorders of immune responses and autoimmune impairment, but also in cancer [121–123] and some chronic diseases like allergic rhinitis, chronic hepatitis, idiopathic cardiomyopathy, male infertility and cystic fibrosis [124, 125].

Therefore, the autoantigens/autoantibodies identified are not only the hallmarks of autoimmune diseases but are also involved in chronic pathogenesis accompanied by continuous cell/tissue injury, sufficient to cause autoimmune response. Knowing the target profile of these autoantibodies may be potentially useful for the serodiagnosis of these diseases as well as opening opportunities for the development of personalized immunotherapies.

This is also true for cancer from which a growing number of tumour-specific or tumour-associated antigens have been identified by immunoproteomics as promising for the development of early stage detection tests and possibility for the isolation of tumour antigens for immunotherapy [126–129].

Immunoproteomics has been successfully applied to the discovery of antigens from infectious microorganisms such as *Helicobacter pylori*, *Chlamydia trachomatis* and *Borrelia garinii* that may shortly become an important tool in vaccinology [130, 131].

3.2 Post-translational modification

PTMs are responsible for protein folding, stability, cellular localization, recognition and immune reactions, all of which are necessary for preservation or degradation, restoration and regulation of protein function in normal biological processes [132]. Therefore, for successful disease biomarker and therapeutic target discoveries, it is advantageous to be able not only to identify differentially expressed proteins globally but also to assess eventual aberration in the level of protein PTMs.

Studies have shown that glycosylation and phosphorylation, the major PTMs in proteins, are usually altered in many diseases states (for recent review [133]). The extension and distribution of aberrant phospho- and glyco-proteins in biological systems can be detected on 2-DE gels. Three possible methods are briefly summarized:

(i) 2-DE gel autoradiography or phosphor-imaging after *in vivo* incorporation of $^{32}P/^{33}P$ orthophosphate or ^{14}C-labelled sugar into proteins to detect phosphorylated and glycosylated proteins, respectively. The radiolabelling is performed *in vivo* during cell culture. Therefore, it could be applied either in established cell lines in culture or in primary cell culture of fresh collected human tissue, as model systems of a given disease.

(ii) Using specific PTM fluorescent stains on 2-DE such as Pro-Q Diamond phophoprotein dye and Pro-Q Esmerald 488, a glycoprotein stain (available from Molecular Probes, Eugene, OR, USA). These stains offer broad dynamic ranges (500–1000-fold) for quantification, and do not interfere with MS analyses for protein identification [134–137].

(iii) 2-DE immunoblotting with specific antibodies such as antiphospho-tyrosine/-seryl/-threonyl for mapping proteins phosphorylated on either tyrosine, serine or threonine residues, respectively (for review [138]). A combination of at least two antibodies can be used, one specific for the total (phosphorylated and unphosphorylated) forms of a given protein and the other, specific for the phosphorylated form. The balance between these two forms of the protein could be associated, for example, with a disease. Both types of phospho-antibodies (the latter exist for several different types of proteins) are commercially available.

Based on the Western blotting principle, sugar-binding proteins, so-called lectins with different carbohydrate specificities that are labelled with appropriate reporting groups such as peroxidase or phosphatase, are commercially available for glycoprotein detection. The differentiation would depend on the specificity of the lectin (for recent reviews [139–141]). Using this technique, glycosylation patterns of proteins associated with cancer have been described [142, 143].

3.3 Membrane proteome

One-third of the human genome codes for membrane proteins [144, 145]. Besides providing a physical barrier between the cell and its environment and the various intracellular compartments, membrane proteins also regulate cell maintenance, cell–cell and organelle–cytosol interaction and communication *via* signalling mechanisms and the mediation of transport of ions, metabolites and peptides/proteins. The biological importance of membrane proteins as well as their being the main targets for most drugs make membrane proteome exploration extremely relevant to the discovery of targets for novel drug design [146]. However, the proteomic study of membranes, especially by conventional 2-DE gel has been challenging, due to the amphiphilic nature of membrane proteins that is incompatible with IEF separation.

Nevertheless, several improvements in the technique have been developed to overcome this, and apparently successful analyses of purified or enriched plasma membrane

proteins by 2-DE have been published (for recent review [144]). The 2-DE analysis of human erythrocyte membrane proteins [147–150], human liver plasma membrane [151] and cell envelope or outer membrane proteins from bacteria [152, 153] are recent examples. To increase the number of membrane proteins separated on the 2-D gel, approaches involve differential fractionation with the use of organic solvents and/or detergents such as Triton X-100, Triton X-114, dodecylmaltoside or ASB14 in the lysis buffer to enrich and improve the solublization of membrane proteins. Other approaches include delipidation that has shown to be beneficial for 2-DE separation and the use of soft IPG strips (<4% of polyacrylamide pores) to reduce protein aggregation, among others (for recent review [80, 95, 96, 144, 154]). Biotinylation of proteins at the cell surface, followed by highly specific purification of membrane proteins using avidin, is another strategy that has been used successfully for the enrichment and separation of surface membrane proteins by 2-DE [155].

Despite these advances, most plasma membrane proteins identified by conventional 2-DE and MS have been integral proteins with one or two membrane-spanning regions or pore-like proteins (*i.e.* less hydrophobic) and peripheral membrane proteins (*i.e.* more hydrophilic) of which the interaction with the cell membrane is transient or covalent and made directly or *via* integral membrane, since they do not contain membrane-spanning domains. Highly hydrophobic integral membrane proteins comprising several membrane-spanning regions are markedly depleted on conventional 2-DE.

To overcome this limitation and maximize the inclusion in a membrane proteome, alternative methods for performing 2-DE have been introduced. These involve replacing IEF with electrophoresis using cationic detergents namely SDS, 16-benzyldimenthyl-*n*-hexadecylammonium (16-BAC) or cetyl trimethyl ammonium bromide (CTAB) for a complete solubilization of membrane proteins (for recent review [144]). In the 'first dimension', the proteins are separated according to their molecular mass, *i.e.* by 1-D PAGE using SDS, BAC or CTAB ionic denaturing agents. After electrophoresis the whole lane containing proteins is cut out and laid on top of the second SDS-PAGE. In the 2-D SDS/SDS-PAGE, variations in the acrylamide concentrations in the first as well as in the second dimension, the addition of glycerol or urea to the gel buffer in one of the two dimension, the addition of different ions with distinct mobilities (*e.g.* glycine, tricine or bicine) in electrophoresis buffers, among others, could result in higher resolution of membrane proteins (for recent review [144]). For 2-DE using 16-BAC or CTAB-PAGE in the first dimension, variations in the technique have been suggested to improve the resolution [156].

Another unconventional alternative to 2-DE analysis of membrane proteins is the application of mild-nonionic methods namely blue native (BN) or clear native (CN)-PAGE, developed by Schagger *et al.* [157, 158]. Both these methods use detergents such as Triton X-100, dodecyl matoside or digitonin that permit the solubilization of native membrane proteins as well as native membrane protein complexes. Proteins are separated by their inherent negative charge (with p*I*<7) in CN-PAGE or by a negative charge shift provided by the CBB G-250, a negatively charged protein-binding dye in BN-PAGE (for review [144]). The combination of BN/CN-PAGE for the first dimension and denaturing SDS-PAGE in the second dimension has been successfully applied to the analysis of the mitochondrial protein complexes for which the method was designed [157–159], as well as to several other membrane preparations [160–162]. In-gel activity assays of proteins have been possible, since proteins are kept in their native and enzymatic active state [157, 163].

The combined application of conventional 2-DE with denaturing/nondenaturing, unconventional 2-DE approaches has been suggested to be sufficient to provide a repertory of all membrane proteins of a given sample [144, 164].

3.4 Interactome

The functional interpretation of protein networks or 'interactome' maps in cellular pathways has important implications in biomarker and drug discovery.

A protein–protein or protein–DNA/RNA/metabolites network can be deduced by screening experimental literature using computer-assisted network data analyses that are now free or commercially available (for recent reviews [165, 166]. In essence, the inference on the existence of an interaction between two proteins is based typically either on automated 'text mining', of PubMed articles including those that do not correspond to experimentally verified protein interactions or on data derived from high-throughput experimentation, for example yeast 2-hybrid screen test (Y2H) performed in model organisms such as Yeast [167], fly *Drosophila melanogaster* [168] and the worm *Caenorhabditis elegans* [169], in which false positives for interactions are over 50% [170, 171]. Only recently, two independent human protein–protein maps were generated using a large-scale screening based on Y2H [172] and affinity purification-MS approaches, respectively [173]. Although generated for a small fraction of human proteome (7000 and 5500 human proteins, respectively) these maps revealed similarities to other protein networks and to new interactions, some of which are relevant to diseases (for a recent review [166]). However, only 5–10% of pair-wise interactions of human interactome have been charted. It has been proposed that multiple coverage approaches are required to complete a human interactome. Also, to elucidate a pair-wise interaction which affects a particular disease or has therapeutic potential, approaches should be performed as much as possible in an appropriate patient cell/tissue of the disease in question. The contribution of small-scale screening experiments could be decisive for a high confidence of deduction of given protein interactions.

One of the biochemical strategies to investigate native protein–protein interactions in a cellular environment is direct or affinity purification (*e.g.* by co-immunoprecipitation

(co-IP) technique) of a stable protein complex and further elucidation of the components by direct MS, as above mentioned and/ or by 2-DE and MS analysis (for method review: [174]).

Co-IP is a popular technique in which a protein complex is 'coprecipitated' in a mild condition using an antibody to one of the complex's components. The whole complex is captured on the protein A or G gel support. The assumption is that proteins that coprecipitate are related to the function of protein used as the target antigen to the antibody. Although this assumption is usually subject to further verification, Co-IP associated with MS or 2-DE/MS enables direct and quantitative detection of interactions between active proteins therefore is an important proteomic-based method for interactome study.

As an example, Te *et al.* (2007) [175] provided new insights into human Hsp90 interactome, a potential target protein for anticancer drugs using Co-IP and 2-DE/MS-based proteomics. From the novel Hsp90-binding partners discovered, the authors revealed new Hsp90 subunits that may be involved with the assembly, regulation or exploitation of the tubulin-based cytoskeleton network, particularly the mitotic spindle. Several proteins were recently identified as part of microtubule-associated protein 1B (MAP1B) interactome, a major cytoskeletal protein during brain development, by Cueille *et al.* [176] using 2-DE gel-based proteomic approach. One of these components, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which directs binding with MAP1B was also confirmed by an *in vitro*-binding assay and it may be essential in the local energy provision of cytoskeletal structures. MAP1B may help to keep this key enzyme close to the cytoskeleton.

Methods for the mapping of interaction networks involving membrane proteins have also been published (for a recent review [177]). The successful application of 2-DE NB/CN/SDS-PAGE for analysis of protein–membrane complexes has been reported as already discussed above in membrane proteins session [144, 178].

3.5 Proteome turnover

To fully understand the proteome expression, it is important to ascertain protein turnover, *i.e.* the balance between synthesis and degradation rate that is responsible for the maintenance or alteration of intracellular protein concentration [179]. Abnormalities in this balance are associated with diseases.

The combination of 2-DE/MS and cell metabolic labelling by pulse-chase experiments with [^{35}S]-methionine, [^{35}S]-cysteine, [^{14}C]-leucine, [^{3}H]-leucine and/or [^{32}P]-phosphotyrosine, have provided investigators with information about protein turnover on a proteome-wide scale. For example, using this approach, protein turnover in response to a certain condition (*e.g.* drug treatment or stress response) can be studied. A proteins destiny through the cell, *i.e.* from the protein's synthesis to its final cellular destination, can be tracked.

In a pulse-chase experiment, cells are grown in a radioactive medium for a brief period (the pulse) for incorporation of radioactive isotopes and then transferred to a non-radioactive medium for a longer period (the chase). 2-DE gels were performed for each cell culture replicate at different times in the 'pulse' and 'chase', and proteins were visualized by conventional autoradiography or fluorography or by phosphorimaging using specific devices. By densitometric analysis of images, the protein turnover was measured and quantified comparing the level of incorporation or loss of radioactive tracer through the time-course of the labelling. The MS identification is usually performed in 2-DE of non-radioactive samples.

Pulse-chase experiments and 2-DE has been taken to study protein turnover in adipocyte-derived cell cultures [180], skeletal muscle-derived cells in culture [181] and in articular cartilage proteins [182].

With [^{35}S] labelling of the atrial trabeculae tissue, obtained from patients undergoing selective cardiac open-heart surgery and subsequent 2-DE, Lampert *et al.* [183], demonstrated a broad spectrum of protein synthesis with the complexity of the entire heart proteome. The protein synthesis in terminally differentiated cardiomyocytes was not confined just to the synthesis of those structures needed for the postmitotic house-keeping functions, leading the authors to conclude that this model, might serve as a valid experimental system to measure and to monitor physiological and biochemical variables of the heart tissue [183].

Although, metabolic labelling is routinely used in cell and tissue culture, it has been already applied in a whole-animal to study differentiation of synthesis of tripsinogen isoforms in rat pancreas [184]. In complex multicellular organisms, like animals, besides the health and safety implications of high radiation there are technical difficulties in controlling protein turnover conditions.

In vitro radiolabelling using iodination with ^{131}I or ^{125}I is also possible but only as a high sensitivity detection method, since it does not provide information about protein turnover.

Recently, metabolic labelling with stable (nonradioactive) isotopes (deuterium, ^{13}C or ^{15}N, the most common) have been used to trace protein turnover (for recent reviews see: [179, 185].

Stable isotope labelling with amino acids in cell culture (SILAC) is ultimately amenable to gel free LC/MS or LC/MS/MS analysis [186–188]. Traditionally, the 2-DE analysis is used just to confirm that SILAC does not interfere with the pattern of protein expression. However, Jiang and English [189] developed a SILAC method associated with 2-DE and MS for both qualitative and quantitative comparison of yeast proteomes in whole-cell lysates based on stoichiometric incorporation of perdeuterated leucine (D_{10}-Leu) into the organism's proteins. The yeast cultures (H_{10}-Leu) or (D_{10}-Leu), are mixed prior to protein separation by 2-DE, and the proteomes are compared by isotope-ratio quantitation using MALDI-TOF MS. The relative abun-

dances of H_{10}- and D_{10}-proteins were determined from the intensities of their peptide peaks, and D_{10}-Leu labelling provided an effective internal calibrant for quantitation by MS. The number of Leu residues *per* peptide yielded an additional parameter for peptide and protein identification. The investigators have used this method to study the response of the yeast proteome to oxidative stress over an extended period of time (45–225 min) following the challenge [190]. As pointed out by those authors, the advantages in using stable isotopes *versus* radioisotopes in investigating a proteome's response to stress, are the ease of obtaining large quantities of stable isotope-labelled proteins at very low cost, the avoidance of radioactive contamination during 2-DE and MS analysis and finally the lack of injury of SILAC to cells.

4 Concluding remarks

The use of 2-DE separation combined with MS detection to profile a complex mixture of proteins was the foundation stone for the initiation of the Proteome Project in the middle 1990s. Recent advancements have significantly enhanced the versatility of 2-DE technology allowing the whole procedure to be designed or adapted to suit a specific sample or biomarker discovery study. The new methods for sample fractionation and PTM analysis and the introduction of labelling methodologies like DIGE or radio/stable isotopes associated with advanced biostatistic/bioinformatics tools has improved considerably the capabilities of 2-DE particularly for low abundance protein identification and quantification, providing more reliable proteome comparison and characterization. Further technical improvements are still needed to increase the throughput of the methodology. However, the growing developments in gel-free MS-based approaches (see reviews of this journal issue), mostly due to new instruments and bioinformatics tools, have made researchers wonder about the future of 2-DE. In spite of this, it is still likely that for a while 2-DE/MS-based approaches, alone or in combination with gel-free MS approaches, will remain the most popular technology in proteomics.

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