Review

Current two-dimensional electrophoresis technology for proteomics

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Two-dimensional gel electrophoresis (2-DE) with immobilized pH gradients (IPGs) combined with protein identification by mass spectrometry (MS) is currently the workhorse for proteomics. In spite of promising alternative or complementary technologies (*e.g.* multidimensional protein identification technology, stable isotope labelling, protein or antibody arrays) that have emerged recently, 2-DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates. 2-DE enables the separaration of complex mixtures of proteins according to isoelectric point (p*I*), molecular mass (M_r), solubility, and relative abundance. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications. This is in contrast to liquid chromatography-tandem mass spectrometry based methods, which perform analysis on peptides, where M_r and p*I* information is lost, and where stable isotope labelling is required for quantitative analysis. Today's 2-DE technology with IPGs (Görg *et al., Electrophoresis* 2000, *21*, 1037–1053), has overcome the former limitations of carrier ampholyte based 2-DE (O'Farrell, *J. Biol. Chem*. 1975, *250*, 4007–4021) with respect to reproducibility, handling, resolution, and separation of very acidic and/or basic proteins. The development of IPGs between pH 2.5–12 has enabled the analysis of very alkaline proteins and the construction of the corresponding databases. Narrow-overlapping IPGs provide increased resolution (ΔpI = 0.001) and, in combination with prefractionation methods, the detection of low abundance proteins. Depending on the gel size and pH gradient used, 2-DE can resolve more than 5000 proteins simultaneously ($\sim$2000 proteins routinely), and detect and quantify $<$ 1 ng of protein *per* spot. In this article we describe the current 2-DE/MS workflow including the following topics: sample preparation, protein solubilization, and prefractionation; protein separation by 2-DE with IPGs; protein detection and quantitation; computer assisted analysis of 2-DE patterns; protein identification and characterization by MS; two-dimensional protein databases.

Keywords: Immobilized pH gradient / Mass spectrometry / Prefractionation / Protein detection / Protein identification / Proteomics / Review / Sample preparation / Two-dimensional gel electrophoresis / Two-dimensional protein databases / Two-dimensional image analysis

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Abbreviations: CA, carrier ampholyte; **FFE**, free flow electrophoresis

Contents

1 Introduction

2-DE with IPGs [1, 2] combined with protein identification by MS [3] is currently the workhorse for proteomics. In spite of promising alternative/complementary technologies (*e.g.* multidimensional protein identification technology, stable isotope labelling, protein arrays) [3–9] that have emerged recently, 2-DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates. Whatever technology is used, proteome analysis is technically challenging, because the number of different proteins expressed at a given time under defined biological conditions is likely to be in the range of several thousand for simple prokaryotic organisms and up to at least 10 000 in eukaryotic cell extracts. Moreover, current proteomic studies have revealed that the majority of identified proteins are abundant housekeeping proteins that are present in numbers of 10^5 to 10^6 copies *per* cell, whereas proteins such as receptor molecules that are present in much lower concentrations (typically $<$100 molecules *per* cell) are usually not detected. Consequently, improved methods for enrichment of low-abundance proteins are required, such as prefractionation procedures, as well as more sensitive detection and quantitation methods.

2-DE couples IEF in the first dimension with SDS-PAGE in the second dimension, and enables the separaration of complex mixtures of proteins according to p*I*, M_r, solubility, and relative abundance. Depending on the gel size and pH gradient used, 2-DE can resolve more than 5000 proteins simultaneously ($\sim$2000 proteins routinely), and can detect $<$ 1 ng of protein *per* spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or PTM. This is in contrast to LC-MS/MS based methods, which perform analysis on peptides, where M_r and p*I* information is lost, and where stable isotope labelling is required for quantitative analysis. One of the greatest strengths of 2-DE is its capability to study proteins that have undergone some form of PTM (such as phosphorylation, glycosylation or limited proteolysis) and which can, in many instances, be readily located in 2-DE gels as they appear as distinct spot trains in the horizontal and/or vertical axis of the 2-DE gel. In addition, 2-DE not only provides information on protein modifications and/or changes in their expression levels, but also permits the isolation of proteins in mg amounts for further structural analyses by MALDI-TOF MS, ESI-MS or Edman microsequencing [10–16]. The former limitations of carrier ampholyte (CA) based 2-DE [17–19] with respect to reproducibility, resolution, separation of very acidic and/or very basic proteins, and sample loading capacity have been largely overcome by the introduction of IPGs for the first dimension of 2-DE [1]. Narrow-overlapping pH gradients provide increased resolution (ΔpI = 0.001) [1, 20] and detection of low abundance proteins [21–22], whereas alkaline proteins up to pH 12 have been separated under steady-state conditions [23–29]. The major steps of the 2-DE-MS workflow include: (i) sample preparation and protein solubilization; (ii) protein separation by 2-DE; (iii) protein detection and quantitation; (iv) computer assisted analysis of 2-DE patterns; (v) protein identification and characterization; (vi) 2-D protein database construction [30].

2 Sample preparation and protein solubilization for 2-DE

2.1 Sample preparation

To take advantage of the high resolution of 2-DE, proteins of the sample have to be denatured, disaggregated, reduced and solubilized to achieve complete disruption of molecular interactions and to ensure that each spot represents an individual polypeptide. The major problems

concerning the visualization of proteins from total cell or tissue extracts lie in the high dynamic range of protein abundance, and the diversity of proteins with respect to M_r, p*I* and solubility. Although a one-step procedure for protein extraction would be highly desirable with regard to simplicity and reproducibility, there is no single method of sample preparation that can be universally applied to all kinds of samples analyzed by 2-DE [30]. Although a large number of standard protocols has been published, these protocols have to be adapted and further optimized for the type of sample (*e.g.* microbial cells or mammalian tissue) to be analyzed, as well as for the proteins of interest (*e.g.* soluble cytosolic or highly insoluble membrane proteins, respectively). Some general recommendations, however, can be made: sample preparation should be as simple as possible to increase reproducibility, and protein modifications during sample preparation must be minimized, because they might result in artifactual spots on 2-D gels. In particular, proteolytic enzymes in the sample must be inactivated. Samples containing urea must not be heated, to avoid charge heterogeneities caused by carbamylation of the proteins by isocyanate formed in the decomposition of urea [30]. The three fundamental steps in sample preparation are cell disruption, inactivation or removal of interfering substances, and solubilization of the proteins (reviewed in [30–34]).

Cell disruption can be achieved by osmotic lysis, freeze-thaw cycling, detergent lysis, enzymatic lysis of the cell wall, sonication, grinding with (or without) liquid nitrogen, high pressure (*e.g.* French press), homogenization with glass beads and a bead beater, nitrogen cavitation, or a rotating blade homogenizer. These methods can be used individually or in combination. Typically, microbial cells or plant tissues require rather harsh conditions for the cell lysis due to the robustness of their cell walls, whereas more gentle methods can be applied for mammalian tissues. Gentle cell disruption procedures (*e.g.* enzymatic lysis) are also required for the preparation of intact organelles (*e.g.* mitochondriae) for subsequent subproteome analysis (see Section 2.3). During or after cell lysis, interfering compounds such as proteolytic enzymes, salts, lipids, nucleic acids, polysaccharides, plant phenols and/or highly abundant proteins have to be removed or inactivated. The two most important parameters are salt and proteolysis.

Proteases must be inactivated to prevent protein degradation that otherwise may result in artifactual spots and loss of high M_r proteins. Protease inhibitors are usually added, but they may modify proteins and cause charge artifacts [30]. Other remedies are boiling the sample in SDS-buffer (without urea!), or inactivating proteases by low pH (*e.g.* precipitating with ice-cold TCA). However, it should be kept in mind that it may be rather difficult to completely inactivate all proteases [35]. TCA/acetone precipitation is very useful for minimizing protein degradation and removing interfering compounds, such as salt, or polyphenols. Attention has to be paid, however, to protein losses due to incomplete precipitation and/or resolubilization of proteins. Moreover, a completely different set of proteins may be obtained by extraction with lysis buffer depending on whether or not there was a preceding TCA precipitation step. On the other hand, this effect can be used for the enrichment of very alkaline proteins (such as ribosomal or nuclear proteins) from total cell lysates [2].

Salt ions may interfere with electrophoretic separation and should be removed if their concentration is too high ($>$ 100 mM). This is particularly a problem when samples are applied by sample in-gel rehydration, whereas higher salt concentrations are better tolerated by cup-loading. In this case, however, rather low voltages (approximately 150 V) have to be applied for several hours; otherwise proteins may precipitate at the site of sample application. Salt increases the conductivity of the IEF gel, thereby prolonging the time required to reach the steady-state. In extreme cases, IEF may virtually stop due to salt fronts. Salt removal can be achieved by spin dialysis, or precipitation of proteins with TCA or organic solvents (*e.g.* cold acetone). One alternative is the use of 2-D clean-up kits (*e.g.* Amersham Biosciences, Uppsala, Sweden). Another is dilution of the sample below a critical salt concentration followed by application of a larger sample volume onto the IPG gel. The sample is desalted in the gel by applying low voltages (100 V) at the beginning of the run for up to several hours and replacing the filter paper pads beneath the electrodes (where the salt ions have collected) several times [2]. High amounts of lipids may interact with membrane proteins and consume detergents. Delipidation of lipid-rich biological material (*e.g.* brain tissues) can be accomplished by extraction with organic solvents (*e.g.* cold ethanol or acetone). However, severe losses in proteins may be experienced, either because certain proteins are soluble in organic solvent, or because the precipitated proteins do not always resolubilize. Alternatively, high-speed centrifugation [36] and subsequent removal of the lipid-layer is employed.

Polysaccarides (especially the charged ones) and nucleic acids can interact with carrier ampholytes and proteins, and give rise to streaky 2-D patterns. Moreover, these macromolecules may also increase the viscosity of the solutions and obstruct the pores of the polyacrylamide gels. Unless present at low concentrations, polysaccharides and nucleic acids should be removed. A common method is precipitation of proteins with acetone

or TCA/acetone, but losses in proteins cannot always be avoided due to insufficient resolubilization of proteins. Other recommendations for the removal of nucleic acids are digestion by a mixture of protease-free (!) RNAses and DNAses, or by ultracentrifugation and addition of a basic polyamine (*e.g.* spermine) [32]. Phenols are present in plant materials, especially in plant leaves, and may interact with proteins, giving rise to horizontal streaks in 2-D gel patterns. Polyphenolic compounds can be removed either by binding to polyvinylpolypyrrolidone, or by protein precipitation with TCA and subsequent extraction of phenols with ice-cold acetone [37–39]. Sometimes, highly abundant proteins present a problem since they impair separation and detection of lower abundance proteins by limiting the amount of these proteins to be loaded onto the 2-D gel and/or by masking them on the 2-D pattern. In particular albumin, which constitutes up to 60% of the bulk protein in plasma, is a major problem [40]. There are several albumin removel kits on the market, but due to nonspecific binding, one has to be aware that most of these kits remove proteins other than albumin, too (reviewed by Simpson [41]).

2.2 Protein solubilization

After cell disruption and/or removal of interfering compounds, the individual polypeptides must be denatured and reduced to disrupt intra- and intermolecular interactions, and solubilized while maintaining the inherent charge properties. Sample solubilization is usually carried out in in a buffer containing chaotropes (*e.g.* urea and/or thiourea), nonionic and/or zwitterionic detergents (*e.g.* Triton X-100 or CHAPS), reducing agents, CA and, depending on the type of sample, protease inhibitors. The most popular sample solubilization buffer is based on O'Farrell's lysis buffer [17] and modifications thereof (9 M urea, 2–4% CHAPS, 1% DTT, and 2% v/v CA. Unfortunately, urea lysis buffer is not ideal for the solubilization of all protein classes, particularly for membrane or other highly hydrophobic proteins. Improvement in the solubilization of hydrophobic proteins has come with the use of thiourea [42] and new zwitterionic detergents such as sulfobetaines [43–45]. Merits and limits of these new detergents, chaotropes, and reducing agents have been reviewed recently [31, 46, 47] (see also section 3.1.6).

2.2.1 Chaotropes

Urea is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and and denaturation. In contrast, thiourea, introduced by Rabilloud [42], is better suited for breaking hydrophobic interactions, but its usefulness is somewhat limited due to its poor solubility in water. However, it is more soluble in concentrated urea solutions. Currently the best solution for solubilization of hydrophobic proteins is a combination of 5–7 M urea and 2 M thiourea, in conjunction with appropriate detergents. The major problem associated with urea in aqueous solutions is that urea exists in equilibrium with ammonium (iso)cyanate, which can react with the α-amino groups of the *N*-terminus and the ε-amino groups of lysine residues, thereby forming artefacts such as blocking the *N*-terminus and introducing charge heterogeneities (altered p*I*s). To prevent this carbamylation reaction, temperatures above 37°C have to be avoided under all circumstances, and CA (2% v/v), which act as cyanate scavengers, should be included in the urea solution. Given that these precautions have been complied with, it has been demonstrated that protein carbamylation is negligible for a period of at least 24 h, which is sufficiently long for most protein extraction and solubilization protocols [48]. Similar experiments have shown that carbamylation is not a problem during electrophoresis in the presence of urea, even with prolonged run-times, since the urea breakdown products are electrophoretically removed [49].

2.2.2 Detergents (surfactants)

Detergents are utilized to prevent hydrophobic interactions between the hydrophobic protein domains to avoid loss of proteins due to aggregation and precipitation. Since the anionic detergent SDS is one of the most efficient surfactants, solubilization of proteins in (boiling) SDS solution has been recommended for protein solubilization (for details see [50, 51]). However, horizontal streaks in the 2-D pattern are observed if samples initially solubilized in 1% SDS are not diluted with at least 4-fold excess of (thiourea/urea) lysis buffer, to displace the anionic detergent SDS from the proteins and to replace it with a nonionic or zwitterionic detergent to decrease the amount of SDS below a critical concentration (0.2%). Additionally, obtaining sufficient dilution may constitute a major problem when micropreparative protein loads in 2-DE are employed, since the amount of sample volume that can be applied onto an IPG strip is limited. Therefore, nonionic or zwitterionic detergents are currently favoured for protein solubilization. The most popular nonionic detergents are NP-40, Triton X-100 and dodecyl maltoside. Regrettably, NP-40 and Triton X-100 are not very effective in solubilizing very hydrophobic membrane proteins. In contrast, zwitterionic detergents such as CHAPS, and sulfobetaines (*e.g.* SB 3-10 or ASB 14) perform better, and have been shown to solubilize, in combination with urea and thiourea chaotropes at least several integral membrane proteins [46, 47]. More detailed investigations revealed that the efficiency of zwitterionic detergents for

solubilizing hydrophobic proteins not only depends on the nature of the protein itself, but also on the presence and nature of other compounds, in particular on the lipid content of the sample [44]. Besides these chaotropes and detergents, organic solvents have also been successfully applied for solubilization and IEF of very hydophobic proteins [52–53]. Regardless of recent advances, these studies demonstrated that there does not exist a single solution for the complex solubility problem of membrane proteins. Most membrane proteins cannot be solubilized adequately with a single nonionic or zwitterionic detergent, and empirically testing and optimizing the composition of sample solubilization buffer to improve the solubility of membrane proteins still remains important [45].

2.2.3 Reducing agents

Reduction and prevention of re-oxidation of disulfide bonds is also a critical step of the sample preparation procedure. Reducing agents are necessary for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding. The most commonly used reductants are DTT or dithioerythritol (DTE) which are applied in excess, *i.e.* in concentrations up to 100 mM. Unfortunately, these agents are weak acids with pK values between 8.5 and 9, which means that they will ionize at basic pH, and therefore, run short in the alkaline gel area due to migration to the anode during IEF. Moreover, DTT and DTE are not well suited for the reduction and solubilization of proteins which contain a high cysteine content, such as wool keratins. Herbert *et al.* [54] have proposed tributylphosphine (TBP) as an alternative to DTT. TBP is applied in quite low concentrations (2 mM) due to its stoichiometric reaction. However, this reagent has also several disadvantages, the major of which are its low solubility in water and its short half-life. Moreover, TBP (and its solvent dimethylformamide, respectively) is toxic, volatile, and has a rather irritating odor. Alternatively, tris(2-carboxyethyl)phosphine (TCEP) is used in the saturation labelling procedure in DIGE. In conclusion, in certain cases TBP and TCEP have several advantages over DTT and DTE, but the choice of reductant is predominantly sample specific [55]. For additional information see section 3.1.5.

2.3 Prefractionation procedures

Since there is no amplification step for proteins analogous to the PCR method for amplifying nucleic acids, and due to the high dynamic range and diversity of expressed proteins, particularly in eukaryotic tissues, it is often preferable to carry out a prefractionation step to reduce the complexity of the sample, enrich for certain proteins such as low-copy number proteins or alkaline proteins, and to get some information on the topology of the proteins. This can be accomplished by: (i) isolation of specific cell types from a tissue, *e.g.* fluorescence activated cell sorting (FACS), or laser capture micro dissection (LCM), (ii) isolation of cell compartments and/or organelles, *e.g.* by sucrose gradient centrifugation, or free flow electrophoresis, (iii) selective precipitation of certain protein classes (*e.g.* TCA/acetone precipitation for ribosomal proteins), (iv) sequential extraction procedures with increasingly powerful solubilizing buffers, for example, aqueous buffers, organic solvents (*e.g.* ethanol or chloroform/methanol), and detergent-based extraction solutions, (v) chromatographic or electrokinetic separation methods, such as column chromatography, affinity purification, electrophoresis in the liquid phase and/or IEF in granulated gels.

The major problem with diseased (*e.g.* cancerous) mammalian tissues is their heterogenous nature. It is, therefore, of utmost importance to obtain targeted populations (*e.g.* tumor cells) from tissue specimens [10, 56]. Hence, several microdissection techniques are applied to enrich for targeted cell populations, such as LCM technology that allows the isolation of pure cell populations [57]. Banks and coworkers [58] have demonstrated that samples which had been enrichend with LCM can be analyzed with 2-DE. The drawback of this technology, however, is that it is rather time-consuming because a large number of cells must be dissected to obtain sufficient protein for 2-DE analysis. Another possibility for recovering specific subpopulations of cells from clinical samples is FACS of antibody-bound cells (reviewed by [59]). However, it is not clear to what extent stress is exerted on the cells by this kind of treatment, and whether their protein expression profiles are affected.

Subfractionation of cell components, *e.g.* organelles such as mitochondria by centrifugation in a sucrose density gradient is the most effective method for organelle isolation (reviewed by Huber and coworkers [60, 61]). Other, although less common techniques, are free flow electrophoresis (FFE) or immunoaffinity binding methods [62, 63]. Whereas these procedures can be applied for mammalian cells with relative ease since they do not possess a cell wall, access to organelles is complicated for most microorganisms because in this case a lysis method is required which is both efficient in disrupting the cell wall and gentle enough to guarantee that organelles remain intact. For example, sheroblasts are prepared for the isolation of intact organelles from yeast by digesting the cell wall with polysaccharide-cleaving enzymes prior to liberating the cell content by gentle lysis conditions such as hypotonic solutions and/or mechanical treatment [64]. The quality of these preparations is not always sufficient for proteome analysis, but Pfanner and co-workers [65] have estab-

lished an improved purification protocol for yeast cell mitochondriae which has been successfully applied for 2-DE/MS analysis of the yeast mitochondrial proteome. Due to the limitations in sample loading capacity on the first dimension IEF gel, a whole cell lysate may not yield sufficient quantities of lower abundance proteins to be displayed on a 2-DE gel. Precipitation procedures allow increased loading of particular proteins while keeping the total protein load constant. In particular, TCA/acetone precipitation has been found to be very valuable for the enrichment of alkaline proteins such as ribosomal proteins from total cell lysates [2]. Additional benefits of TCA/acetone precipitation are inactivation of proteases to minimize protein degradation, and removal of interfering compounds (see section 2.1). However, attention has to be paid to protein losses due to incomplete precipitation and/or resolubilization of proteins.

Sequential extraction of proteins from cells or tissues on the basis of their solubility properties is another possibility to enrich for certain protein classes, and to simplify the 2-DE pattern for subsequent image analysis and protein identification by MS. *e.g.* plant proteins from barley and wheat seeds were extracted sequentially with Tris-HCl buffer, aqueous alcohols and urea/NP-40/DTT lysis buffer and then analyzed by 2-DE [66, 67]. In a similar manner, Tris-base was used to solubilize cytosolic *Escherichia coli* proteins [68]. The resultant pellet was then subjected to conventional solubilizing solutions (urea/CHAPS/DTT), and, finally, the membrane protein rich pellet was partially solubilized using a combination of urea, thiourea, and zwitterionic surfactants. Eleven membrane proteins from this pellet could be identified, including two outer membrane proteins that had previously been known only as an open reading frame in *E. coli*. One approach for improved solubilization of membrane proteins is to treat isolated membrane preparations (*e.g.* obtained by ultracentrifugation) with sodium carbonate at alkaline pH [69] or chaotropic salts (*e.g.* potassium bromide) to remove carry over cytoplasmic proteins and only loosely attached peripheral membrane proteins (membrane washing, membrane stipping). Other procedures to enrich for hydrophobic proteins are based on the differential extraction of membrane proteins by organic solvents [52, 53], *e.g.* chloroform/methanol mixtures [70]. The major limitation of these procedures is cross-contamination between the individual fractions. Several different chromatographic procedures have been used successfully to enrich for low-abundance proteins, including hydrophobic interaction chromatography, hydroxyapatite and heparin chromatography and chromatofocusing [71].

Other approaches are based on electrophoretic prefractionation according to p*I* in the liquid phase, such as preparative IEF [72], IEF in a rotating, multichamber device [73], FFE, or the use of a multifunctional electrokinetic membrane apparatus [74] in which samples are separated by charge and/or size. A multicompartment electrolyzer with isoelectric membranes has been developed by Righetti and coworkers [75]. Zuo *et al.* [76, 77] have simplified this device for prefractionation of various kinds of samples. These procedures are particularly useful if the prefractionated proteins are then applied onto narrow-range IPG gels (zoom gels). It has been reported that this type of prefractionation allows higher protein load (6- to 30-fold) on narrow IPG gels without protein precipitation and allows detection of low abundance proteins because major interfering proteins such as albumin have been removed [76, 77]. The major drawbacks of most chromatographic and electrophoretic prefractionation procedures in the liquid phase are that (i) sophisticated instrumentation is required, (ii) the sample is usually diluted during or after the separation process, and (iii) protein precipitation cannot always be avoided.

Recently, a simple, cheap, and fast prefractionation procedure based on flat-bed IEF in granulated gels has been devised by Görg and coworkers [78]. Flatbed IEF in granulated gels for the separation of enzymes was described by Radola as early as 1973. Görg *et al.* [78] have adapted this method for sample prefractionation before loading on 2-D gels with narrow pH ranges. Complex sample mixtures, *e.g.* mouse liver proteins, were prefractionated in flat-bed Sephadex gels containing urea, thiourea, zwitterionic detergents, DTT, and CA. After IEF, up to ten Sephadex fractions alongside the pH gradient can be removed with a spatula and directly applied onto the surface of the corresponding narrow-range IPG strips for the first dimension of 2-D PAGE. Proteins in the Sephadex gel fraction are transported electrophoretically into the IPG gel with high efficiency and without any sample dilution. In conclusion, prefractionation procedures have many advantages in terms of protein enrichment, and visualization of low-abundance proteins. The major disadvantage of most prefractionation procedures lies in cross-contamination between individual fractions and in the fact that they are either time consuming, complicated to handle, require concentration steps due to elution/dilution precedures, and/or do not allow more than a few samples in parallel to be processed.

3 2-DE with IPG (IPG-Dalt)

The prerequisite, but also the challenge, for proteome analysis is to separate proteins from complex biological samples with high reproducibly and high resolution. Despite all the merits of O'Farrell's [17] CA based 2-DE technology, which set a world-wide standard for the

separation of complex protein samples by adding urea and detergents for protein solublization and IEF under denaturing conditions, in contrast to Scheele's [18] native IEF/SDS-PAGE and Kloses's [19] native IEF/PAGE approach (based on Stegemann's method [79]) for water-soluble proteins, it is, however, often difficult to obtain reproducible results even within a single laboratory, let alone between different laboratories. The problem of limited reproducibility is largely due to the synthetic CA used to generate the pH gradient required for IEF, for reasons such as pH gradient instability over time, cathodic drift, and batch-to-batch variability of CAs [80–81]. In practice, CA-generated pH gradients rarely extend beyond pH 7.5, with resultant loss of alkaline proteins. For the separation of these alkaline proteins, O'Farrell *et al.* [82] developed an alternative procedure, known as nonequilibrium pH gradient electrophoresis, however at the expense of reproducibility, since this procedure is extremely difficult to control and to standardize.

The above mentioned difficulties of 2-DE have been largely overcome by the development of IPG [83], based on the use of the bifunctional Immobiline reagents, a series of ten chemically well defined acrylamide derivatives with the general structure CH_2 = CH-CO-NH-R, where R contains either a carboxyl or an amino group. These form a series of buffers with different pK values between pK 1 and 13. Since the reactive end is copolymerized with the acrylamide matrix, extremely stable pH gradients are generated, allowing true steady-state IEF with increased reproducibility, as has been demonstrated in several inter-laboratory comparisons [84, 85]. Other advantages of IPGs are increased resolution by the ability to generate (ultra)narrow pH gradients (Δ pI = 0.001) [1, 20], reproducible separation of alkaline proteins [23–29] and increased loading capacity [86]. Consequently, IEF with IPGs is the current method of choice for the first dimension of 2-D PAGE for most proteomic applications.

The original protocol of 2-DE with IPG-Dalt was described by Görg *et al*. (1988 [1], updated in 2000 [2] and 2004 [87]), and summarized the critical parameters inherent to IEF with IPGs and a number of experimental conditions. The first dimension of IPG-Dalt, IEF, is performed in individual, 3 mm wide and up to 24 cm long IPG gel strips cast on GelBond PAGfilm (laboratory-made or commercial Immobiline Dry-Strips). Samples can be applied either by cup-loading or by in-gel rehydration. IPG-IEF has been simplified by use of an integrated system such as the IPGphor [27, 88] where rehydration with sample solution and IEF can be performed in a one-step automated procedure. After IEF, the IPG strips are equilibrated with SDS buffer in the presence of urea, glycerol, DTT and iodoacetamide, and applied onto horizontal or vertical SDS gels in the second dimension. After electrophoresis, the separated proteins are visualized by Silver-staining, organic or fluorescent dyes, or autoradiography (or phosphor-imaging) of radiolabelled samples. Limitations (not only of 2-DE, but of almost all current proteome analysis technologies) remain in the field of the analysis of very hydrophobic and/or membrane proteins, as well as in the lack of highly sensitive and reliable techniques for detection and quantitation of low abundant proteins. Yet, the recent introduction of more powerful chaotropes and detergents such as thiourea and sulfobetaines [42–45], as well as the advent of sensitive fluorescent dyes, in particular of dual label techniques for the visualization of differentially expressed proteins [89] have contributed to improve the situation considerably.

3.1 First dimension: IEF with IPGs

Linear or nonlinear [90] wide pH range (*e.g.* IPG 3–12), medium (*e.g.* IPG 4–7), narrow (*e.g.* IPG 4.5–5.5), and/or ultra-narrow (*e.g.* IPG 4.9–5.3) IPGs can be cast in different pH ranges between pH 2.5 and pH 12, as well as in different lengths, usually from 7–24 cm (however, IPG strips up to 54 cm long have been applied [91]). Besides laboratory-made IPG gels, a variety of commercial IPG dry strips can now be purchased from different suppliers. Ready-made IPG dry strips are increasingly popular due to easier handling, better comparability of results and exchange of data, and have significantly contributed to the widespread application of 2-DE in proteomics.

3.1.1 IPG gel casting

Although narrow (one pH unit) and ultra-narrow ($<$ 1 pH unit) linear IPG can be calculated with the help of the Henderson-Hasselbalch equation with relative ease, or by using nomograms [92], computer assisted programs [93, 94] are mandatory for wider ($>$ 1 pH unit) and/or more complex (*e.g.* nonlinear) IPGs. IPG gels are formed by mixing two immobiline starter solutions in a gradient mixer according to the gradient casting technique for ultrathin gels described by Görg *et al.* [95]. IPG gels are 0.5 mm thick and cast on GelBond PAGfilm. The gel casting mold, which is typically loaded in a vertical position from the top, consists of two glass plates, one covered with the GelBond PAGfilm, whereas the second one bears a 0.5 mm thick U-frame as spacer. Besides Immobiline chemicals, the gel casting solutions contain an acrylamide/bisacrylamide mixture (typically 4%T / 3%C). For narrow-range alkaline IPG gels (*e.g.* IPG 9–12), acrylamide may be substituted by *N,N*-dimethylacrylamide for improved stability of the gel matrix [24]. After polymerization, the IPG gel is washed with deionized water, impreg-

nated in 2% w/v glycerol, and dried. The surface of the dry IPG gel is protected with a sheet of plastic film before it is stored in a sealed plastic bag at −20°C. Prior to use, the dried gel is cut into 3 mm wide IPG dry strips with a paper cutter. For detailed information on IPG gel casting see [96].

3.1.2 IPG strip rehydration and sample application

Prior to IEF, the IPG dry strips must be rehydrated (usually overnight) to their original thickness of 0.5 mm with a rehydration buffer containing 8 M urea (or, alternatively, 2 M thiourea and 6 M urea), 0.5 – 4% nonioninc or zwitterionic detergents (*e.g.* 2% CHAPS), a reductant (typically 0.4% DTT) and 0.5% v/v CA (*e.g.* IPG buffer or Pharmalyte 3-10). Rehydration buffer should be prepared fresh before use, or stored frozen in aliquots at −70°C. It is important to deionize the urea with an ion exchange resin prior to adding the other components, because urea in aqueous solution exists in equilibrium with ammonium cyanate which can react with protein amino groups and introduce charge artifacts, giving rise to additional spots on the IEF gel. CA are added for improved protein solubility, but also as a cyanate scavenger. IPG dry strips are either rehydrated with sample already dissolved in rehydration buffer (sample in-gel rehydration), or with rehydration buffer without sample, followed by sample application by cup-loading. Alternatively, rehydrated IPG strips can be stored at −80°C for instantaneous use (Görg *et al.*, in preparation).

For sample in-gel rehydration [97–99], the extract (1–10 mg protein/mL) is directly solubilized in an defined volume of rehydration buffer. For 240 mm long and 3 mm wide IPG dry strips, 450 μL of this solution is pipetted into the grooves of the reswelling tray or into the IPGphor strip holder (Amersham Biosciences). For shorter IPG strips, the rehydration volume has to be adjusted accordingly. The IPG strips are applied, gel side down, into the grooves without trapping air bubbles. The IPG strip, which must still be moveable and not stick to the tray, is then covered with silicone oil or DryStrip cover fluid and rehydrated overnight at approximately 20°C. Higher temperatures (> 37°C) hold the risk of protein carbamylation, whereas lower temperatures (< 10°C) should be avoided to prevent urea crystallization on the IPG gel. Improved entry of higher M_r proteins (M_r > 100 kDa) into the IPG gel matrix is facilitated by active rehydration, *i.e.* by applying low voltages (30–50 V) during reswelling [2].

Sample in-gel rehydration is not recommended for samples containing very high M_r, very alkaline and/or very hydrophobic proteins, since these are taken up into the gel only with difficulty, *e.g.* due to hydrophobic interactions between proteins and the wall of the tray, or because of size-exclusion effects of the gel matrix. The latter phenomenon is particularly pronounced if the sample volume significantly exceeds the calculated volume of the IPG strip after reswelling, since higher M_r proteins preferably remain in the excess reswelling solution instead of entering the IEF gel matrix. Cross-contamination is another problem; hence the reswelling tray must be thoroughly cleaned between different experiments. In conclusion, sample in-gel rehydration is less reliable than cup-loading, in particular for quantitative analyses. For cup-loading, IPG dry strips are reswollen in rehydration buffer, either in a reswelling cassette or, more conveniently, in a reswelling-tray, however without sample. After IPG strip reydration, samples (20–100 μL) dissolved in lysis buffer are applied into disposable plastic or silicone rubber cups placed onto the surface of the IPG strip. Best results are obtained when the samples are applied at the pH extremes, *i.e.* either near the anode or cathode. Sample application near the anode proved to be superior to cathodic application in most cases. When using basic pH gradients such as IPGs 6–10, 6–12 or 9–12, anodic application is mandatory for all kinds of samples investigated [2].

3.1.3 General guidelines for IEF with IPGs

The amount of protein which can be loaded onto a single IPG gel strip for optimum resolution, maximum spot numbers and minimum streaking/background smearing depends on parameters such as the pH gradient used (wide or narrow), separation distance, and protein complexity of the sample. For analytical purposes, typically 100 μg of protein can be loaded on an 18 cm long, wide pH range gradient, and 500 μg on narrow range IPGs. For micropreparative purposes, five to ten times more protein can be applied. For micropreparative 2-DE, in-gel rehydration is often preferred, but up to several mg of protein may also be applied by (repeated) cup-loading. The optimum sample volume for cup loading is 20 μL–100 μL, whereas volumes less than 20 μL are not recommended because of the increased risk of protein aggregation and precipitation at the point of sample application, resulting in loss of proteins and/or horizontal or vertical streaking. For the same reason, the protein concentration of the sample solution should not exceed 10 mg/mL.

Settings are usually limited to 50 μA *per* IPG strip and 150 V to avoid Joule heating, because the conductivity is initially high due to salts. As the run proceeds, the salt ions migrate to the electrodes, resulting in decreased conductivity and allowing high voltages to be applied. Samples with high salt concentrations can be desalted directly in the IPG gel by restricting the voltage to 50–

100 V during the first 4–5 h with several changes of the electrode filter paper strips [2, 100]. Likewise, voltage should be limited to 100 V overnight for improved sample entry in the case of large sample volumes (micropreparative runs and/or narrow IPGs) prior to continuing IEF at higher voltages ($>$ 3500 V). Final settings of up to 8000 V are particularly useful for zoom-in gels and alkaline pH gradients (see sections 3.1.4 and 3.1.5). The longer the IPG strip and the narrower the pH gradient, the more vh that are required to achieve steady state separation for high reproducibility. If the second dimension cannot be performed directly after IEF, the IPG strips should be immediately frozen and stored at -70°C between two plastic sheets.

Too short focusing times will result in horizontal streaking, but severe over-focusing should also be avoided. In contrast to the classical O'Farrell [17] method, over-focusing does not result in migration of proteins towards the cathode (cathodic drift), but will result in excess water exudation at the surface of the IPG gel due to active water transport (reverse electroendosmotic flow). This leads to distorted protein patterns, horizontal streaks at the basic end of the gel, and loss of proteins. The optimum focusing time must be established empirically for each combination of protein sample, protein loading and the particular pH range and length of IPG gel strip used. Detailed protocols including optimum focusing parameters for a number of different wide and narrow pH range IPGs have been published by Görg *et al.* [2, 87] and are also available at http://www.wzw.tum.de/proteomik.

Temperature during IEF has an important effect on the resulting 2-DE patterns, since spot positions vary along the pH axis with different applied temperatures. For enhanced reproducibility it is thus extremely important to run the separations at an actively controlled temperature, where 20°C proved to provide the optimal conditions [101]. Recently, an integrated instrument, named the IPGphor (Amersham Biosciences), has been developed to simplify the IPG-IEF dimension of 2-DE [88]. This instrument features a strip holder that provides rehydration of individual IPG strips with or without sample, as well as optional sample cup-loading, and subsequent IEF without handling the strip after it is placed in the strip holder. The instrument can accommodate up to twelve individual strip holders, or a multiple strip holder (manifold), and incorporates Peltier cooling with precise temperature control between 19.5 and 20.5°C and a programmable power supply. The IPGphor saves about a day's worth of work by combining sample application and rehydration, as well as by starting the run at preprogrammed times, and by running the IEF at rather high voltages (up to 8000 V).

3.1.4 Narrow overlapping IPGs (zoom-in gels) and extended separation distances

The choice of pH gradient primarily depends on the sample's protein complexity. Wide or medium range IPGs, such as IPGs 3–12, 4–9, or 4–7, are typically used to analyze simple proteomes (small genome, organelle, or other subfraction), or to get an overview of a more complex proteome, respectively (Fig. 1). Although at first glance the resolution of 2-DE seems impressive, it is, however, still not sufficient compared to the enormous diversity of proteins from higher eukaryotic proteomes, where extensive cotranslational modifications and PTM of proteins and differential gene splicing lead to expression of more proteins than the total number of genes in their genomes. With samples such as total lysates of eukaryotic cells or tissues, 2-DE on a single wide-range pH gradient reveals only a small percentage of the whole proteome. The best remedy, preferably in combination with prefractionation procedures, is to use multiple narrow overlapping IPGs (zoom-in gels, *e.g.* IPG 4–5, IPG 4.5–5.5, or 5.0–6.0) and/or extended separation distances (up to 24 cm, or even longer) to achieve an optimal resolution to avoid multiple proteins in a single spot for unambiguous protein identification and to facilitate the application of higher protein amounts for the detection of minor components (Fig. 2).

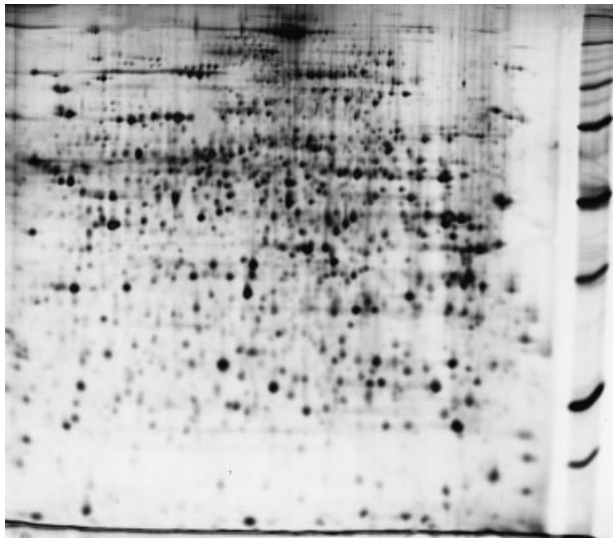

Figure 1. 2-DE of a TCA-acetone extract of mouse liver proteins, separated by IEF in a 24 cm long IPG strip containing a wide-range nonlinear pH gradient 3–11, followed by SDS-PAGE in a vertical 12.5% gel. Protein detection was by Silver-staining. (Reproduced by permission of Wiley from Westermeier, R., Görg, A.: Two-Dimensional Electrophoresis in Proteomics. *in:* Janson, J. C. (Ed.), *Protein Purification, 3rd Edition*, John Wiley and Sons, New York, USA 2004, *in press*) (Reference [170]).

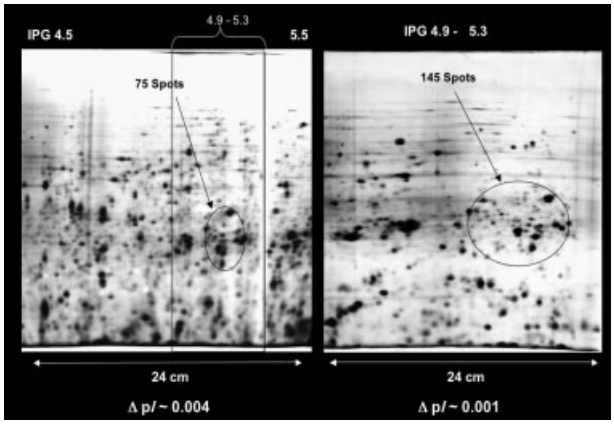

Figure 2. 2-DE of mouse liver proteins, separated by IEF in 24 cm long IPG strips containing narrow pH gradient 4.5–5.5 (left hand side), and ultra-narrow pH gradient 4.9–5.3 (right hand side), respectively, followed by SDS-PAGE in vertical 12.5% gels. Protein detection was by Silver-staining.

Wildgruber *et al.* [21] have demonstrated the improved separation and visualization of the *Saccharomyces cerevisiae* proteome by using narrow overlapping IPGs. Combining IPGs 4–5, 4.5–5.5, 5–6, 5.5–6.7 and 6–9 and excluding the overlap, so that every protein was counted only once, it was possible to detect three times more protein spots than in the IPG 3–10. Interestingly, not only were spots further apart, but several single spots divided into two or more protein spots. This observation is in perfect agreement with the detection by MS of different proteins in a single spot in about 10–40% of all spots detected on broader pH range IPG gels. Similarly, Westbrook *et al*. [22] demonstrated not only improved resolution and higher spot numbers by using narrow-range IPGs, but also the identification by MS of additional protein species and isoforms of proteins from apparent single spots, or unresolved spot clusters, on broader-range IPGs. Nevertheless, caution must be exercised in comparing the number of proteins resolved with the number of ORFs. First, not all ORFs are expressed; second, not all proteins are expressed in the (growth) conditions used; third, not all proteins are soluble under the conditions used for gel electrophoresis; and last but not least, low copy number proteins may be present in quantities below the detection limit of the stain. Gygi *et al*. [102] were able to visualize more than 1500 protein spots (by Silver-staining) on a IPG 4.9–5.7 2-DE gel in which 0.5 mg of unfractionated yeast protein had been separated. Despite this relatively high sample load, proteins from genes with codon bias values of < 0.1 (*i.e.* lower abundance proteins) were not detected. However, proteins from genes with codon bias values of < 0.1 were found, if proteins were prefractionated prior to analysis on narrow-range IPGs. These results indicate that low abundance proteins can be detected if larger starting amounts of prefractionated proteins are used. This result is endorsed by the findings of Speicher and coworkers [76, 77] and Görg *et al.* [78] by applying mg quantities of prefractionated protein samples on narrow-range IPG strips.

Zoom gels in the acidic and neutral pH range between pH 4 and 7 (*e.g.* IPG 4–5, IPG 4.5–5.5, IPG 5–6) work with in-gel rehydration or cup-loading. These gels are typically used for micropreparative purposes with sample loads up to several milligrams. In order to avoid protein precipitation and horizontal streaking, low voltages (approximately 50–100 V) should be applied during the initial stage of IEF, in particular when samples are applied by cup-loading. Because of the high number of Vh ($> 100\,000$) and concomitant long focusing time (up to 24 h) required for focusing to steady-state, the surface of the IPG strips has to be protected by a layer of silicone oil to prevent them from drying out. The paper strips beneath the electrodes should be removed after several hours and replaced by fresh ones. This is of particular importance when the sample contains high amounts of salt, but also to remove proteins with p*I*s outside the chosen pH interval [2]. Much better results, however, are obtained when prefractionated samples are separated on zoom gels [76–78]. Very long separation distances (> 30 cm) for maximum resolution of complex protein patterns have been described [103, 104]. However, size, stability, and handling of the fragile tube gels used in CA 2-DE is often a problem. This is in contrast to IPG gel strips, which are cast on plastic backings so that they can neither stretch nor shrink, which contributes significantly to improved reproducibility. Meanwhile, 24 cm long IPG gel strips [27] are routine. Recently, 54 cm long IPG strips were successfully applied [91].

3.1.5 IEF of very alkaline proteins

Theoretical 2-D maps calculated from sequenced genomes indicate that approximately 30% of all proteins possess alkaline p*I*s up to pH 12 [28, 105]. Wide range IPGs 3–12 [27] and 4–12 [25] are ideally suited to provide an overview of the proteome of a cell or tissue. In particular, the IPG 4–12 which is flattened between pH 9–12, proved to be a most useful gradient for the separation of very alkaline proteins. Prefractionation procedures such as TCA/acetone precipitation of proteins are recommended for enrichment and visualization of basic proteins exceeding p*I* 10, since these are usually not included in lysis buffer extracts of eukaryotic organisms

[2]. Strongly alkaline proteins such as ribosomal and nuclear proteins with closely related p*I*s between 10.5 and 11.8 were focused to the steady state by using IPGs 3–12, 6–12 and 9–12 [28, 29] (Fig. 3). For highly resolved 2-D patterns, different optimization steps with respect to pH engineering and gel composition were necessary, such as the substitution of dimethylacrylamide for acrylamide, the addition near the cathode of a paper strip soaked with DTT providing a continuous influx of DTT to compensate for the loss of DTT (which is a weak acid and migrates out of the basic part of the gel) [100, 106], and the addition of isopropanol to the IPG rehydration solution in order to suppress the reverse electroendosmotic flow which causes highly streaky 2-D patterns in a narrow pH range IPGs 9–12 and 10–12 [24]. With the advent of the IPGphor, the procedure was greatly simplified by applying high voltages (8000 V) to shorten run times considerably, which permits these IPGs to be run under standard conditions without isopropanol, at least with analytical sample amounts [28, 29].

For optimized separation, cup-loading at the anode is mandatory, and the use of high voltages (final settings up to 8000 V) is strongly recommended [2, 28, 29]. With IPGs above pH 7, horizontal streaking due to DTT depletion can occur at the basic end. To avoid streaking, cysteines should be stabilized as mixed disulfides by using hydroxyethyl-disulfide (HED) reagent (DeStreak; Amersham Biosciences) in the IPG strip rehydration solution instead of a reductant. Besides eliminating streaking, the use of HED results in a simplified spot pattern and improved reproducibility [107, 108].

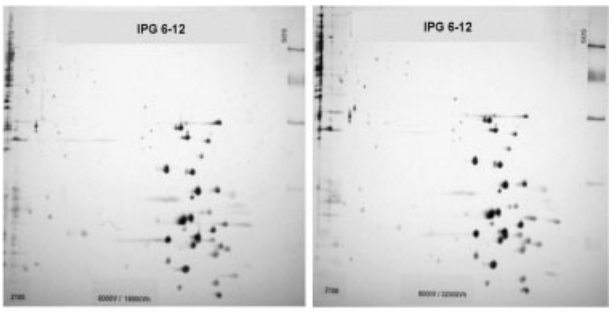

Figure 3. Steady state IEF of *Lactococcus lactis* proteins in IPG 6–12. Gels are Silver-stained. For IEF, the same protein quantities were loaded by cup-loading and utilizing the IPGphor. After sample entry, 18 kVh (left hand side) or 32 kVh (right hand side) 8000 V were applied to reach steady state. (Reproduced by permission of Wiley-VCH from Drews, O., Reil, G., Parlar, H., Görg, A.: Setting up standards and a reference map for the alkaline proteome of the Gram-positive bacterium Lactococcus lactis. *Proteomics*, 2004, *4*, 1293–1304) (Reference [29]).

3.1.6 Analysis of very hydrophobic membrane proteins

Membrane proteins, which constitute a significant proportion (approximately 30%) of the cell's proteins, are of particular interest since they play key functions in various important cellular processes including cell adhesion, signal transduction, ion or metabolite transport and are, therefore, important targets for drug development. Despite their importance, recent proteomics studies have demonstrated that very hydrophobic proteins and, in particular, membrane proteins are an extremely underrepresented group on 2-D gels and that the analysis of very hydrophobic proteins such as integral membrane proteins remains a challenge for both 2-DE and LC-based proteomic approaches. This under-representation may be attributed to several factors: besides their low solubility and their tendeny to aggregate and precipitate in aqueous media, many membrane proteins possess basic p*I*s and/or are expressed in low copy numbers [109, 110]. Certain loss of membrane proteins on 2-D gels may also be attributed to the fact that these proteins, once solubilized, may in fact enter the IPG strips and also focus properly, but do not elute during the transfer step from first to second dimension (see section 3.2) [111, 112]. Although some progress has been made towards improving solubilization and separation of membrane proteins, *e.g.* by the introduction of thiourea and novel zwitterionic detergents which make it possible to display at least some membrane proteins on 2-DE gels, currently the best strategy is the combination of SDS-PAGE analysis of membrane fractions in combination with LC-MS/MS. This method has been termed geLC-MS/MS [113].

3.2 Equilibration of IPG gel strips

Before the second-dimension separation, it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with SDS. Due to the observation that the focused proteins bind more strongly to the fixed charged groups of the IPG gel matrix than to CA gels, relatively long equilibration times (10–15 min), as well as urea and glycerol to reduce electroendosmotic effects are required to improve protein transfer from the first to the second dimension [1]. Thiourea is sometimes recommended for more efficient transfer of hydrophobic proteins [111], but may cause vertical streaks in the 2-D pattern. The best protocol by far is to incubate the IPG strips for 10–15 min in the buffer originally described by Görg *et al.* [1] (50 mM Tris-HCl (pH 8.8), containing 2% w/v SDS, 1% w/v DTT, 6 M urea and 30% w/v glycerol]. This is followed by a further 10–15 minute equilibration in the same solution containing 4% (w/v) iodoacetamide instead of

DTT. The latter step is used to alkylate any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel, resulting in an artifact known as point-streaking that can be observed after Silver-staining [114]. More importantly, the iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation; this step is highly recommended for subsequent spot identification by MS. After equilibration, the IPG strips are applied onto the surface of the second-dimension horizontal or vertical SDS-PAGE gels. Loss of proteins during the equilibration step and subsequent transfer from the first to the second dimension has been reported and is primarily due to (i) proteins which remain in the IPG strip because of adsorption to the IPG gel matrix and/or insufficient equilibration times, and (ii) wash-off effects. Experiments with radioalabelled proteins have shown that up to 20% of the proteins get lost during equilibration [115, 116]. The majority of these proteins (most probably, those located near the surface of the IPG strip) are lost during the very first minutes of equilibration. This is quite reproducible for a given sample, whereas protein losses in the second equilibration step are only marginal.

3.3 Second dimension: SDS-PAGE

SDS-PAGE can be performed on horizontal or vertical systems [100]. Horizontal setups [117] are ideally suited for ready-made gels (*e.g.* ExcelGel SDS; Amersham Biosciences), whereas vertical systems are preferred for multiple runs in parallel, in particular for large-scale proteome analysis which usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels for higher through-put and maximal reproducibility [118]. The most commonly used buffers for the second dimension of 2-DE are the discontinuous buffer system of Laemmli [119] and modifications thereof, although for special purposes other buffer systems are employed, such as borate buffers for the separation of highly glycosylated proteins [120]. Typically, gel sizes of 20×25 cm^2 and a gel thickness of 1.0 mm are recommended. In contrast to horizontal SDS-PAGE systems, it is not necessary to use stacking gels with vertical setups, as the protein zones within the IPG strips are already concentrated and the nonrestrictive, low polyacrylamide concentration IEF gel acts as a stacking gel [30, 34].

3.3.1 Analysis of low and/or high M_r proteins

The analysis of low M_r (< 15 kDa) and high M_r (> 150 kDa) proteins is somewhat intricate since there is no standard 2-DE system which effectively allows separation of proteins over the entire M_r range between 5 kDa and 500 kDa. A common approach is to combine several gels optimized for the approximate M_r ranges 5–30 kDa, 15–200 kDa, and > 150 kDa instead of using a single standard 2-DE system. Conventional Tris-glycine gels do not allow efficient separation of proteins below 15 kDa, but Schägger and von Jagow [121] have described a Tris-tricine buffer systems for the separation of low M_r (3–30 kDa) polypeptides. Fountoulakis *et al*. [122] have improved this gel system using two urea concentrations in the second dimension, which permitted efficient and reproducible separation of *Haemophilus influenzae* proteins with M_r between 5 kDa and 20 kDa. The major problem associated with high M_r proteins is that a significant proportion of these proteins are rather hydrophobic, and, consequently, will not readily dissolve in standard urea lysis and/or rehydration solutions used for sample solubilization and IEF. Even though these proteins were solubilized, they will not always enter the IEF gel matrix, or are not transferred from the first to the second dimension. Several strategies have been proposed to overcome at least some of these obstacles. For example, it has been demonstrated that sample application of high M_r proteins to IPG gels *via* cup-loading is more efficient than passive sample application by sample in-gel rehydration (see section 3.1.2). If samples are applied by in-gel rehydration, active reswelling by applying low voltages (30–50 V) during the rehydration step is superior to passive loading and improves the entry of high M_r proteins into the polyacrylamide matrix [2]. The transfer of high M_r proteins from the IPG strip onto the SDS gel is enhanced by sufficiently long equilibration steps (2×15 min; see section 3.2). The same holds true for application of low voltages during the transfer step, *i.e.* 50 V for vertical SDS gels, and 100 V for horizontal SDS-PAGE systems, respectively [2].

4 Protein detection and quantitation

After 2-DE, the separated proteins have to be visualized, either by universal or by specific staining methods. Since the concentrations of individual proteins in a single cell differ between six or seven orders of magnitude, ranging from several millions of copies/cell for some highly abundant proteins (*e.g.* glycolytic enzymes) to a few copies/cell for low abundant proteins, these enormous variations in protein concentrations are a major challenge for almost all currently available protein detection methods [109, 110]. The most important properties of protein visualization methods are high sensitivity (low detection limit), high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with post-electrophoretic protein identification procedures, such as MS. Unfortunately, currently no staining method meets all requirements for proteome analysis.

Universal detection methods of proteins on 2-D gels include staining with anionic dyes (*e.g.* Coomassie Blue), negative staining with metal cations (*e.g.* zinc imidazole), Silver-staining, fluorescence staining or labelling, and radioactive isotopes, using autoradiography, fluorography, or Phosphor-imaging. For most of these staining procedures, the resolved polypeptides have to be fixed in solutions such as in ethanol/acetic acid/ H_2O for at least several hours (but usually overnight) before staining to remove any compounds (*e.g.* CA, detergents) that might interfere with detection. Specific staining methods for detection of PTM (glycosylation, phosphorylation *etc.*) are employed either directly in the 2-DE gel or, more frequently, after transfer (blotting) onto an immobilizing membrane. The blotted proteins can be probed with specific antibodies (*e.g.* against phosphotyrosine residues) or with lectins (agains carbohydrate moieties). The pros and cons of most protein detection and quantitation methods applied in 2-DE for proteome analysis have recently been reviewed by Patton [123, 124] and Rabilloud [125, 126]. These methods are, therefore, only briefly discussed in the following section.

4.1 Universal protein detection and quantitation methods

CBB staining methods have found widespread use for the detection of proteins on 2-DE gels, because of their low price, ease of use and compatibility with most subsequent protein analysis and characterization methods such as MS. However, in terms of the requirements for proteome analysis, the principal limitation of CBB stains lies in their insufficient sensitivity, which does not permit the detection of low abundance proteins (the detection limit of CBB stains is in the range of 200–500 ng protein *per* spot). Hence, typically no more than a few hundred protein spots can be visualized on a 2-DE gel, even if milligram amounts of protein had been loaded onto the gel. CBB in colloidal dispersions according to Neuhoff *et al.* [127] and modifications thereof [128] have been reported to be more sensitive than the classical CBB stain, but are still less sensitive than the majority of chemical stains employed in 2-DE for proteomics.

Reverse staining exploits the fact that protein-bound metal cations (*e.g.* potassium, copper or zinc) are usually less reactive than the free salt in the gel. Thus, the speed of precipitation of free or only weakly bound ions to form an insoluble salt is slower on the sites occupied by proteins than in the protein-free background. This generates transparent protein zones or spots, while the gel background becomes opaque due to the precipitated, unsoluble salt. Compared to the other reverse staining methods, but also CBB, the zinc stain offers some distinct advantages as it can be completed in 15 min for most applications, and is more sensitive than CBB, KCl or copper stains [129]. Hence, zinc or imidazole-zinc stains [130, 131] are currently the most sensitive reverse (or negative) staining methods applied in 2-DE. Zinc-imidazole staining is rapid, simple, and sensitive, and has a detection limit of roughly 20–50 ng of protein *per* spot. Moreover, it is compatible with subsequent protein identification by MS, making the stain quite popular for detection of proteins separated on micropreparative 2-DE gels. The major disadvantage of zinc-staining is its rather restricted linear dynamic range, which makes this staining procedure unsuitable for detecting quantitative differences on 2-DE gels.

Silver-staining methods [132, 133] are far more sensitive than CBB or imidazole-zinc stains (detection limit is as low as 0.1 ng protein/spot). They provide a linear response with over a 10- to 40-fold range in protein concentration, which is slightly worse than with CBB staining. However, Silver-staining methods are far from stoichiometric, and are much less reproducible than CBB stains due to the subjective end-point of the staining procedure which makes them less suitable for quantitative analysis. Silver staining methods are quite laborious and complex, although some progress with respect to automation has been made [134, 135]. Silver-staining methods using aldehyde-based fixatives/sensitizers are the most sensitive ones, but prevent subsequent protein analysis (*e.g.* by MS) due to protein cross-linkage. If aldehydes are omitted in the fixative and in the subsequent gel impregnating buffers (except in the developer), microchemical characterization by PMF is possible, [136, 137], however at the expense of sensitivity.

Better and more confident results in terms of sensitivity and linear dynamic range of detection are obtained by protein detection methods relying on fluorescent compounds, or by radiolabelling of proteins combined with highly sensitive electronic detection methods. Prior to the advent of highly sensitive silver staining methods, detection of proteins labelled with radioisotopes was the only method of sensitive detection for proteins separated on 2-DE gels. Radiolabelling can be accomplished by incorporating radioactive isotopes (*e.g.* ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I, or ^{131}I) into proteins. *In vivo* metabolic radiolabelling of samples by the incorporation of radioactive amino acids (such as (^{35}S)-methionine, (^{14}C)-leucine, and/or (^{32}P)-phosphotyrosine) has been extensively used for proteome analysis of microorganisms and cell culture systems, *e.g.* for studying the stress response of organisms by pulse labelling. *In vitro* radiolabelling, *e.g.* of human tissue proteins, is also possible by using iodination with ^{131}I or ^{125}I, however at the risk of formation of artifacts

[30]. The radiolabelled 2-DE separated proteins can be detected by autoradiography or fluorography using X-ray films which are exposed to the dried gels and which can be quantified by densitometry [138]. However, these film-based techniques require long exposure times (up to several weeks) if high sensitivity is desired. Moreover, due to the limited dynamic range of the X-ray film, multiple film exposures combined with computer-aided image processing are required to quantitate high as well as low abundant proteins present in a sample. Nonetheless, even with multiple film detection only a limited dynamic range ($< 10^3$) is achievable.

To overcome the limitations of X-ray film-based autoradiography, several electronic methods for the detection of radiolabelled proteins in 2-D gels have been developed. The most popular is phosphor-imaging, where X-ray films have been replaced by so-called storage-phosphor screens that contain a thin layer of special crystals doped with a europium salt. Radioactive radiation excites electrons in the crystals and a latent image is formed on the plate. Scanning the plate with a He-Ne laser results in the emission of a blue luminescence proportional to the original amount of radiation which is then quantified with a photomultiplier. The advantages compared to autoradiography using X-ray films are the possibility to detect very low levels of radioactivity in a considerably shorter time, and the high linear dynamic range (up to five orders of magnitude) [139]. The major disadvantage (besides the well-known general shortcomings associated with radiolabelling, such as use of hazardous and expensive radiochemicals, waste disposal, safety considerations) lies in the rather high costs for equipment (phosphor-imager, imaging screens). Another method for the detection of radiolabelled proteins, initially described for detection of radiolabelled DNA [140] is multi photon detection (MPD) [141]. Although MPD technology has several advantages over conventional autoradiography such as a very high linear dynamic range (up to 7–8 orders of magnitude), high sensitivity, and the possibility of dual isotope detection for multiplexed differential display (provided that the two radiation energies are sufficiently different, which is the case for ^{125}I and ^{131}I labelled proteins) [142]. However, the technology suffers from several shortcomings, such as low resolution, low throughput (image capture of a single high resolution 2-DE gel takes up to several days), and high costs of equipment, which have to be solved before MPD technology can be routinely applied in proteome analysis.

Due to the shortcomings of organic dyes, silver staining or radiolabelling for visualization and quantitation of proteins, fluorescent detection of proteins has increasingly gained popularity for proteome analysis. Two major approaches for the fluorescent detection of proteins on 2-DE gels are currently practiced. These are: (i) covalent derivatization of proteins with fluorophores prior to IEF, and (ii) post-electrophoretic protein staining by intercalation of fluorophores into the SDS micelles coating the proteins, or by direct electrostatic interaction with the proteins [123]. Mid-labelling, *i.e.* fluorescently labelling the focused proteins while still present in the IEF gel, prior to transfer to the second-dimensional SDS gel [143], is also possible, but this method seems to be applied only rarely. The best known examples for pre-electrophoretic fluorescent labels are monobromobimane [144] and the cyanine-based dyes [89] that react with cysteinyl residues and lysyl residues, respectively. The latter dyes are commercially available as CyDyes (Amersham Biosciences), and their properties will be discussed in more detail in section 4.3 on DIGE. The major problem of pre-electrophoretic labelling is the occurence of protein size and/or protein charge modifications which may result in altered protein mobilities alongside the M_r and/or p*I* axis. Alternatively, proteins can be stained with a fluorescent dye molecule after the electrophoretic separation has been completed. The most prominent example is the ruthenium-based dye SYPRO Ruby [145]. Staining is accomplished within a few hours in a single step procedure which may be easily adapted for use with automated instrumentation. The detection limit is approximately 1–2 ng protein/spot, and the linear dynamic range of quantitation is about three orders of magnitude. A cost efficient alternative to SYPRO Ruby staining, which is based on ruthenium II tris (bathophenanthroline disulfonate), has been developed by Rabilloud *et al.* [146]. Recently, the staining protocol has been considerably improved with respect to sensitivity by optimizing reagent concentration, pH and solvent composition [147]. In conclusion, protein detection and quantitation methods based on fluorescent staining and/or labelling are rather promising. They have a comparatively wide linear dynamic range ($> 10^3$) and are relatively easy to use. Furthermore, most fluorescent staining procedures are compatible with subsequent protein identification methods such as MS. The major limitation of most fluorescent staining methods is their lower sensitivity compared to electronic detection methods of radiolabelled proteins. Typically, only proteins expressed at greater than 10^3 copies/cell can be detected on standard 2-DE gels by using fluorescent dye technologies, whereas, at least in theory, less than a dozen copies of a protein/cell can be visualized with the most sensitive electronic detection methods for radiolabelled proteins.

4.2 Methods for the analysis of protein PTMs

Through genome sequencing no information can be gained on PTMs of proteins. Protein phosphorylation is a key PTM, crucial in the control of numerous regulatory

pathways, enzyme activities, and degradation of proteins, whereas glycosylation is associated with biochemical alterations, developmental changes and pathogenesis, *e.g.* tumorgenesis. Hence, detection and characterization of PTMs are a major task in proteomics. One of the strenghts of 2-DE is its capability to readily locate post-translationally modified proteins, as they frequently appear as distinct rows of spots in the horizontal and/or vertical axis of the 2-DE gel. Up to now, several hundred PTMs, including phosphorylation, glycosylation, acetylation, lipidation, sulfation, ubiquination, or limited proteolysis have been reported. Various methods for the analysis of PTMs have been reviewed recently [148–152]. The analysis of the most important PTMs, phosphorylation and glycosylation, is briefly summarized below.

Phosphoproteins can be detected on 2-DE gels by autoradiography or phosphor-imaging after *in-vivo* incorporation of ^{32}P or ^{33}P orthophosphate into proteins. However, this method is restricted to cell cultures, and cannot be applied for clinical samples obtained from patients. There are other shortcomings of this method: *e.g.* radioactive phosphate is incorporated not only in proteins, but also in DNA and RNA, and can result in severe background staining [153, 154]. Another drawback is that rapid events, such as protein phosphorylation after application of external stress, cannot always be visualized due to the fact that phosporylation happens in the first minutes after the onset of stress, whereas the time-frame of incorporation of ^{32}P or ^{33}P orthophosphate is much longer in order to achieve sufficient uptake of radioactive phosphate in the cells [154]. To avoid dephosphorylation during sample preparation, phosphatase inhibitors should be added. An alternative method for phosphoprotein detection is immunostaining with phosphoamino acid-specific poly- or monoclonal antibodies after transfer (blotting) of the 2-DE separated proteins onto an immobilizing membrane. Anti phospho-tyrosine specific antibodies, which are also commercially available, are quite specific, whereas antibodies directed against phosphoseryl and phosphothreonyl residues are more problematic, and often sensitive to the context of a larger epitope [155].

A technique that is particularly useful for the characterization of phosphorylation sites is MS of 2-DE separated proteins, preferably in combination with alkaline or enzymatic hydrolysis of the phosphate esters, after phosphoprotein detection by autoradiography of radiolabelled phosphoproteins, or after immunodetection (reviewed by Mann and Jensen [150]). Recently, a fluorescent detection method for gel-separated phosphoproteins using Pro-Q Diamond phosphoprotein dye (available from Molecular Probes, Eugene, OR, USA) has been introduced. It has been reported that the procedure is simple, rapid, and MS compatible. The detection limit is 1–2 ng of beta-casein. The method is also suitable for multiplexed proteomics (section 4.3) [156]. However, the specificity of the stain, in particular with complex protein samples such as in 2-DE applications, has sometimes been questioned, since highly abundant, nonphosporylated proteins may also be stained, albeit less intense than the phosphorylated ones.

Glycoproteins separated by 2-DE are usually detected after blotting onto an immobilizing membrane, although several (though less sensitive) methods can also be applied directly in the electrophoresis gel (reviewed by Packer [152]). Besides detection with autoradiography after incorporation of ^{14}C labelled sugar, a method that is applied only rarely, two major principles prevail: one is detection of glycoproteins after periodate oxidation of vicinal hydroxyls, by coupling a carbonyl reactive group (usually a subsituted hydrazine) to the aldehyde groups generated in the carbohydrate part of the glycoproteins. Visualization of the glycoproteins depends on the kind of reporter group attached to the hydrazine, and is achieved by UV illumination in the case of fluorescent molecules (*e.g.* dansyl hydrazine), or through the reaction product (insoluble colour, chemiluminescence *etc.*) in the case of hydrazine-conjugated enzymes. Recently, Pro-Q Emerald 488, a glycoprotein stain that reacts with periodic acid oxidized carbohydrate groups, and which generates a green-fluorescent signal on glycoproteins has been described. The stain permits detection of approximately 5–20 ng of glycoprotein per band/spot, depending upon the nature and the degree of protein glycosylation [157]. The second principle for glycoprotein detection is based on sugar binding proteins, so-called lectins. A wide range of lectins with different carbohydrate specificities is commercially available, either unlabelled, or labelled with appropriate reporter groups such as enzymes (peroxidase, phosphatase *etc.*), fluorescent dye molecules (*e.g.* FITC), or colloidal gold. Whereas it is not possible to discriminate between different carbohyrate moieties of glycoproteins when detection methods based on periodate oxidation have been employed, lectins permit a certain degree of differentiation, depending on the specificity of the lectin. However, for the detailed analysis of the saccharide composition of glycoproteins, HPLC- or MS-based methods are usually preferred.

4.3 DIGE and related differential display techniques

A bottleneck for high throughput proteomic studies is image analysis. In conventional 2-D methodology, protein samples are separated on individual gels, stained, and quantified, followed by image comparison with computer-

aided image analysis programs. Because multistep 2-DE technology often prohibits different images from being perfectly superimposable, image analysis is frequently very time consuming. To shorten this laborious procedure, Ünlü *et al.* [89] have developed a method called DIGE, in which two samples are labeled *in vitro* using two different fluorescent cyanine minimal dyes (CyDyes; Amersham Biosciences) differing in their excitation and emission wavelengths, then mixed before IEF and separated on a single 2-D gel. After consecutive excitation with both wavelengths, the images are overlaid and subtracted (normalized), whereby only differences (*e.g.* up- or down-regulated, and/or PTM proteins) between the two samples are visualized (Fig. 4). Due to the comigration of both samples, methodological variations in spot positions and protein abundance are excluded, and, consequently, image analysis is facilitated considerably.

A third cyanine dye is now available, which makes it possible to include an internal standard, which is run on all gels within a series of experiments. This internal standard, typically a pooled mixture of all the samples in the experiment labelled with this dye, is used for normalization of data between gels thereby minimizing experimental variation and increasing the confidence in matching and quantitation of different gels in complex experimental designs [158]. Nevertheless, even this approach is still dependent on accurate matching and comparison of large sets of 2-DE gels in order to generate meaningful data on differential protein expression between sets of samples [159]. Applications that profit from the DIGE system include the investigation of differential protein expression of samples generated under various prespecified conditions, the comparison of extracts, and the analysis of biological variance. In short, all analyses in which 2-D gels need to be compared are simplified and accelerated by this method.

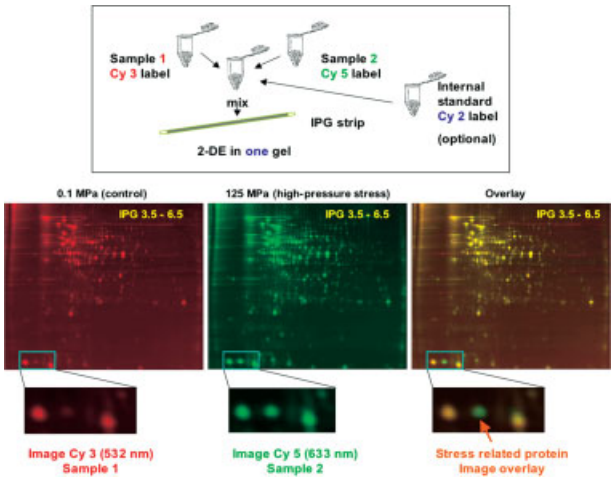

Figure 4. DIGE of high-pressure inducible *Lactobacillus sanfranciscensis* proteins. Samples (Control (grown at atmospheric pressure = 0.1 Mpa) and high-pressure stressed (at 125 MPa)) were labelled *in vitro* with two different fluorescent cyanine dyes (Cy3 and Cy5, respectively) differing in their excitation and emission wavelengths. The samples were mixed, and the mixture was separated on a single 2-DE gel. After consecutive excitation with both wavelengths, the resultant gel images were overlayed to visualize differences (*e.g.* up- or down-regulated proteins) between the samples. It is also possible to include an internal Cy2-labelled standard, which is run on all gels within a series of experiments. This internal standard, typically a pooled mixture of all the samples in the experiment, is used for normalization of data between gels, thereby minimizing experimental variation and increasing the confidence in matching and quantitation of different gels [87].

The p*I*s of the cyanine dye labelled proteins remain unaffected, because the cyanine dyes compensate for the loss of the positive charge of the lysyl residues. However, the M_r increases by 434–464 Da (depending on the dye molecule) *per* labelled lysyl residue. Consequently, labelling of more than one lysine residue *per* protein molecule must be avoided; otherwise labelling with CyDyes would result in multiple spots in the vertical axis of the 2-DE gel. In practice, approximately only 3–5% of protein is labelled (hence, this procedure is also referred to as minimal labelling). Since the bulk of the protein remains unlabelled, the slight increase in M_r, sometimes presents a problem for spot excision for MS analysis, particularly with lower M_r proteins [160]. This off-set has to be taken into accout when automatic spot pickers are used. One alternative is to stain the separated proteins additionally with SYPRO Ruby or CBB prior to spot picking from micropreparative gels. Another is so-called saturation labelling with similar cyanine dyes which label cysteine (instead of lysine) residues to saturation l160]. An additional advantage of saturation labelling is the greater sensitivity of the stain (detection limit is approximately 0.1 ng of protein, compared to 1 ng with minimal labelling [161]). However, saturation labelling has also several drawbacks: (i) the labelling reaction must be carried out at a defined protein/dye ratio to obtain an optimal 2-DE spot pattern with a minimal number of spot trains in the vertical (*i.e.* M_r) dimension; (ii) the dye ratio must be assessed for different types of samples, depending on the percentage of cysteines, which is a time-consuming and laborious procedure; (iii) care must be taken to avoid side-rections, such as lysine labelling; (iv) moreover, currently only two different dyes are available, which excludes the use of an internal standard; (v) up to 25% of the protein material may precipitate during the labelling reaction due to the introduction of the hydrophobic dye molecule, and (iv) the 2-DE spot pattern is significantly altered compared to that of unlabelled or minimal-labelled proteins [160].

DIGE is not the only method for the visualization of multiple samples separated on a single 2-DE gel. These differential display (or multiplexing) methods are based on radiolabelling of different samples with distinguishable isotopes. However, in contrast to DIGE they can be applied in cell cultures only, albeit $^{125}I/^{131}I$ MPD technology [140] (section 4.1) can be also used for *in-vitro* labelling of samples. However, this technique is not fully mature yet and suffers from several shortcomings such as slowness of image capture and costly instruments. A quantitative double-label autoradiography of 2-DE gels, based on *in vivo* metabolic labelling of samples with (^{14}C) and (^{3}H) leucine and using color negative film and computer analysis has been described more than 20 years ago [162]. Cultures of *E. coli* were labeled with (^{3}H)-leucine and (^{14}C)-leucine, respectively. These samples were mixed, and proteins were separated on a single 2-DE gel. Spatial and quantitative data for both radionuclides were recorded on color negative film by autoradiographic exposure. Using different filters, these two data sets were then analyzed with a computer program for analysis of 2-DE gels. This method has further evolved and has been termed differential gel exposure [163]). After 2-DE of *in vivo* metabolic labelled samples on a single gel, the separated proteins are transferred onto an immobilizing PVDF membrane and then exposed to two different types of phosphor imaging screens which are sensitive to $^{14}C/^{3}H$, and ^{14}C, respectively. Since both samples have been coseparated on the same 2-DE gel, the images are perfectly super-imposable and image analysis is considerably simplified. The major drawback of this method lies in the low specific activity of commercially available (^{14}C)- and (^{3}H)-labelled amino acids which requires much longer exposure times and loading higher amounts of protein compared to ^{35}S labelling experiments. A third differential display approach, termed dual channel imaging, has been developed by Bernhardt *et al.* [164]. By pulse-labelling with (^{35}S)-methionine, the protein synthesis pattern (*e.g.* of stressed cells) can be directly compared with the Silver-stained protein pattern on the same 2-D gel. Because matching of different gels is avoided, this technique is useful for the rapid search for proteins induced or repressed by stress.

5 Computerized 2-D image analysis and database construction

One of the key objectives of proteomics is to identify the differential expression between control and experimental samples run on a series of 2-D gels. That is, the protein spots that have been inhibited (disappeared), induced (appeared) or have changed abundance (increased or decreased in size and intensity). Once these gel features have been found, the proteins of interest can be identified using MS. This goal is usually accomplished with the help of computerized image analysis systems [159]. The first step in computerized image analysis of 2-DE protein patterns is capture of the gel images in a digital format. A range of devices, including modified document scanners, laser densitometers, CCD cameras, and fluorescent and phosphor imagers, are available for the acquisition of 2-D gel images. The saved images are then subjected to computer assisted image analysis. The traditional workflow for a 2-DE software package is (i) preprocessing of the gel images, *i.e.* image normalization, cropping and background subtraction; (ii) spot segmentation, detection and expression quantification; (iii) landmarking, *i.e.* an initial user guided pairing of a few spots between the reference and sample gels. The sample gel is then warped to align the landmarks; (iv) matching, *i.e.* automatic pairing of the rest of the spots; (v) identification of differentially expressed spots; (vi) data presentation and interpretation; and (vii) creation of 2-D gel databases [159, 165, 166].

Currently, several 2-D image analysis software packages are commercially available. These programs have been continuously improved and enhanced over the years in terms of faster matching algorithms with lesser manual intervention, and with focus on automation and better integration of data from various sources. New 2-D software packages have also emerged which offer completely new approaches to image analysis and novel algorithms for more reliable spot detection, quantitation and matching. Several programs include options such as control of a spot cutting robot, automated import of protein identification results from MS, superior annotation flexibility (*e.g.* protein identity, mass spectrum, intensity/quantity, links to the Internet), and/or multichannel image merging of different images to independent color channels for fast image comparison. However, despite these improvements, we are still a long way from totally automatic image analysis systems that do not require user intervention [159, 166, 167].

5.1 Computer assisted 2-D image analysis

Briefly, the digitized image is first subjected to several clean-up steps to reduce background smear and to remove horizontal and/or vertical streaks. This procedure is usually quite fast and does not require much user interaction. The individual spots on the 2-D pattern are then detected and quantified. This step is also performed automatically. Regrettably, most image analysis programs do not identify all spots correctly, particularly when the overall quality of the electrophoretic separation is low (*e.g.* when crowded areas and overlapping spots due to improper sample preparation or insufficient spatial resolution are present on the gel). Hence, manual spot editing with reference to the original stained 2-DE gel (or image) is usually necessary. Depending

upon the number of spots, the quality of the 2-DE separation and the algorithms used for spot detection, this process may be quite laborious and time-consuming. After spot editing, each spot on one 2-DE gel must be matched to its counterpart on the other gels, usually by means of a reference (master) gel. For this, in most computer aided 2-D image analysis programs, several landmark spots (which should be evenly distributed over the entire gel area) must be manually identified on each gel by the operator. Starting from these landmark spots, the program proceeds to match the other spots automatically. Again, mismatches must be carefully checked and edited manually. Typically, at least two 2-D gel patterns are matched (*e.g.* a diseased *versus* a control) and then compared to each other with respect to qualitative and/or quantitative differences between the 2-D patterns (*e.g.* induced or repressed, up- or down-regulated proteins). In most cases, however, many gels from different experiments have to be compared, usually by establishing a hierarchical 2-D pattern database [166].

5.2 2-DE databases

Once the 2-D gel database has been established, information stored in it can be exploited by addressing questions such as "Can particular proteins be identified that are associated with a certain disease or disease state (*e.g.* disease markers)?", or "What is the function of a particular protein?" Currently, enormous efforts are being undertaken to display and analyze with 2-DE the proteomes from a large number of organisms, ranging from organelles such as mitochondriae, nuclei or ribosomes to simple prokaroytes including *E. coli*, *Bacillus subtilis*, *H. influenzae*, *Mycobacterium tuberculosis* and *Helicobacter pylori*, to single-celled eukaryotes such as the yeast *S. cerevisiae*, to multicellular organisms, *e.g. Caenorhabditis elegans,* plants such as rice (*Oryza sativa*) or *Arabidopsis thaliana*, and mammalian cells and tissues including rat and human heart, mouse and human liver, mouse and human brain, different cancer cell lines, HeLa cells, human fibroplasts, human keratinocytes, rat and human serum *etc.* Most of these and many other studies in progress are summarized at www.expasy.org/ch2D/2d-index.html ("WORLD-2DPAGE Index to 2D PAGE databases"). The Proteomics Standards Initiative aims to define community standards for data representation in proteomics to facilitate data comparision, exchange and verification (http://psidev.sourceforge.net/).

6 Protein identification from 2-D gel spots

MS has become the technique of choice for identification of proteins from excised 2-D gel spots as these methods are very sensitive, require small amounts of sample (femtomole to attomole concentrations) and have the capacity for high sample throughput (reviewed by [3, 142]). Recent advances in MS also allow the investigation of PTMs including phosphorylation and glycosylation (reviewed by [148, 150]). PMF is typically the primary tool for protein identification. This technique, which is user-friendly and quite fast, is based on the finding that a set of peptide masses obtained by MS analysis of a protein digest (usually trypsin) provides a characteristic mass fingerprint of that protein. The protein is then identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated *in silico* using protein and nucleotide sequence databases. This approach proves very effective when trying to identify proteins from species whose genomes are relatively small, completely sequenced, and well annotated, but is not so reliable for organisms whose genomes have not been completed. A second problem is to identify proteins that are extensively post-translationally modified, since the peptides generated from these proteins may not match with the unmodified protein in the database [13]. A third problem is that PMF does not work very well if several different proteins are present in the same spot. Although search engines such as ProFound [168] have been developed that enable identification of the correct protein(s) even when the data quality is relatively low or when the sample consists of a simple mixture of proteins, it may prove impossible to identify a protein based on PMF alone. In these cases it is then essential to obtain amino acid sequence information. This is most readily accomplished using either MALDI-MS with PSD or chemically assisted fragmentation, or by MS/MS. MS/MS takes advantage of two-stage instruments, MALDI-TOF-TOF-MS/MS or ESI-MS/MS triple-quadropole, ion-trap, or Q-TOF machines to induce fragmentation of peptide bonds. One approach is to generate a short partial sequence or tag which is used in combination with the mass of the intact parent peptide ion to provide significant additional information for the homology search. A second approach uses a database searching algorithm SEQUEST to match uninterpreted experimental MS/MS spectra with predicted fragment patterns generated *in silico* from sequences in protein and nucleotide databases. The major drawback of MS/MS based protein identification methods is that the process cannot be readily automated, and that considerable time and expertise are required for interpreting the MS/MS spectra.

7 Automated procedures

Due to the large number of samples which have to be analyzed in high-throughput proteomic studies, there is an increasingly urgent need for automated procedures [169]. Despite earlier improvements such as the possibil-

ity to run up to twenty second dimension SDS gels in parallel, 2-DE has long been a laborious and difficult to automate procedure. Only recently, the situation has changed due to: (i) the availability of ready-made gels (IPG DryStrips, in particular) on stable plastic supports, (ii) the introduction of the IPGphor and similar devices for automated first dimensional IEF, (iii) semi-automated Silver-staining devices which allow staining of up to ten gels in parallel, (iv) one-step post-electrophoretic fluorescent protein staining methods which can be easily automated, (v) for simplified image comparison and improved quantiation due to the possibility of including an internal standard, (vi) by improved algorithms and better computer programs for easier gel image analysis, and (vii) automation of spot excision and protein digestion for mass spectrometric analysis. In particular, the advent of the IPGphor, in combination with ready-made IPG strips, was an important step towards automation of the 2-D PAGE procedure. However, despite these improvements, there is currently no fully automated system available that is capable of performing the entire 2-D PAGE process.

8 Concluding remarks

Although we have today a diversity of emerging proteomic platforms, there is still no generally applicable method that can replace 2-DE in its ability to simultaneously separate and display several thousand proteins from complex samples such as microorganisms, cells and tissues. 2-DE using IPGs in the first dimension (IPG-Dalt) has proven to be extremely flexible with respect to the requirements of proteome analysis. Although by no means perfect, IPG-Dalt coupled with MS remains the core technology for separating and identifying complex protein mixtures in proteomic projects at least for the foreseeable future.

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