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Journal of Chromatography B, 771 (2002) 3–31

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Detection technologies in proteome analysis

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Abstract

Common strategies employed for general protein detection include organic dye, silver stain, radiolabeling, reverse stain, fluorescent stain, chemiluminescent stain and mass spectrometry-based approaches. Fluorescence-based protein detection methods have recently surpassed conventional technologies such as colloidal Coomassie blue and silver staining in terms of quantitative accuracy, detection sensitivity, and compatibility with modern downstream protein identification and characterization procedures, such as mass spectrometry. Additionally, specific detection methods suitable for revealing protein post-translational modifications have been devised over the years. These include methods for the detection of glycoproteins, phosphoproteins, proteolytic modifications, *S*-nitrosylation, arginine methylation and ADP-ribosylation. Methods for the detection of a range of reporter enzymes and epitope tags are now available as well, including those for visualizing β-glucuronidase, β-galactosidase, oligohistidine tags and green fluorescent protein. Fluorescence-based and mass spectrometry-based methodologies are just beginning to offer unparalleled new capabilities in the field of proteomics through the performance of multiplexed quantitative analysis. The primary objective of differential display proteomics is to increase the information content and throughput of proteomics studies through multiplexed analysis. Currently, three principal approaches to differential display proteomics are being actively pursued, difference gel electrophoresis (DIGE), multiplexed proteomics (MP) and isotope-coded affinity tagging (ICAT). New multiplexing capabilities should greatly enhance the applicability of the two-dimensional gel electrophoresis technique with respect to addressing fundamental questions related to proteome-wide changes in protein expression and post-translational modification. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Proteomics; Detection

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PII: S1570-0232(02)00043-0

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1. Introduction

Review articles providing overviews of significant milestones in the development of assorted protein detection techniques, as applied to gel electrophoresis, have already been published in recent years [1–10]. After briefly updating these topics, this review will primarily focus upon current issues in protein visualization as they directly relate to modern proteomics investigations. Since two-dimensional (2D) gel electrophoresis is increasingly being used in the micropreparative isolation of proteins destined for characterization by mass spectrometry, it is now expected that protein visualization methods be validated with respect to their compatibility with this technology. So, in addition to sensitivity, linear dynamic range and reproducibility, visualization methods should be fully capable of interfacing with the modern analysis tools of proteomics. Key issues addressed in this review article include detection methods for determining total protein, protein post-translational modifications and epitope-tagged fusion proteins, as well as strategies for detecting changes in protein profiles by differential display proteomics.

2. General protein detection methods

2.1. Organic dye- and silver stain-based methods

Though a range of organic dyes have been used to visualize proteins in polyacrylamide gels, Coomassie Blue dyes (R and G types) have certainly enjoyed the most popularity due to their low cost, ease of use and unexpectedly good compatibility with downstream microchemical characterization methods, such as mass spectrometry [11]. Typically, Coomassie Blue R-250 dye is used in a regressive staining approach where gels are saturated with dye that has been dissolved in an aqueous solution containing methanol and acetic acid, followed by destaining in a similar solution devoid of dye. Since the proteins have a higher affinity for the dye molecules than the gel matrix, a point is reached where the background staining is minimal but protein bands are well labeled. Gel-to-gel reproducibility in staining is difficult to control, however, as proteins do destain to varying extents along with the gel matrix, albeit with slower kinetics. Later, progressive staining approaches were introduced where proteins are gradually stained to an endpoint, without significant staining of the gel matrix [12]. These methods are

predicated upon the formation of colloidal dye particles. During staining an equilibrium is achieved between colloidal particles and freely dispersed dye in solution. The low concentration of free dye penetrates the gel matrix and preferentially stains the proteins, but the colloidal dye particle is excluded from the gel, thus preventing matrix staining. The limits of detection for conventional Coomassie Blue and colloidal Coomassie Blue stains are 8–10 and 30–100 ng, respectively. Both dyes provide a linear response with protein amount over a 10–30-fold range of concentration. Thus, the principal limitations associated with Coomassie Blue-based staining methods is the poor detection sensitivity and small linear dynamic range obtained with the dyes.

Silver staining techniques are based upon saturating gels with silver ions, washing the less tightly bound metal ions out of the gel matrix and reducing the protein-bound metal ions to form metallic silver [13–15]. Most commonly, silver staining is accomplished using silver nitrate in combination with formaldehyde developer in alkaline carbonate buffer or using an ammonia–silver complex in combination with formaldehyde developer in citrate buffer. Silver stain methods permit detection of as little as a single nanogram of protein, but the linear dynamic range of the stain is restricted to a 10-fold range. Silver staining methods are quite complex, multi-step procedures that must be stopped at some arbitrary time point in order to avoid over development. Consequently, gel-to-gel reproducibility is problematic and variations of 20% in spot intensity have been documented in the literature [16]. Robotic staining devices offer the potential for minimizing the variability of silver staining, but end-point stains provide consistency without investing in expensive machinery [17]. Though the best silver stain methods use aldehyde-based fixatives prior to silver impregnation, this prevents subsequent microchemical analysis by Edman sequencing or mass spectrometry. Thus, alternative silver staining methods must be employed that omit aldehydes in the fixatives [18,19]. Generally, detection sensitivity is poorer and background staining less uniform with the modified staining techniques. Additionally, destaining methods are often employed to improve the compatibility of silver staining with peptide mass profiling methods [20].

2.2. Radioactive labeling methods

Radiolabeling is commonly accomplished by incorporating ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{33}P, or ^{125}I into proteins [21,22]. After electrophoresis, signal detection may be accomplished using film, by direct autoradiography for the γ-emitting isotopes or by fluorography for the β-emitting isotopes. Fluorography employs fluorescent enhancers impregnated into the gel matrix that improve detection of the radioactive emissions through the generation of light. For certain high-energy isotopes, such as, ^{32}P or ^{125}I, intensifying screens may be used to enhance the signal. These screens absorb the radioactive emissions that pass through the film, fluoresce and generate light that exposes the film. Over the past decade, storage phosphor imagers and microchannel plate analyzers have begun to replace autoradiography film for detecting radiolabeled proteins. The newer technologies offer a wider dynamic range and better detection sensitivity than film.

Radiolabeling has been used extensively for in vivo metabolic experiments. Organic dye or silver stain methods are commonly used in conjunction with radiolabeling techniques to simultaneously assess total protein expression levels along with protein synthesis rates, as well as to provide visual landmarks for the localization of low abundance proteins in 2D gels [23–25]. However, the general utility of radiolabeling as a tool for the assessment of protein synthesis rates has been called into question recently [26–28]. Metabolically incorporating radiolabels such as [^{35}S]methionine, using standard doses and time-courses, has been shown to induce DNA fragmentation, elevate p53 tumor suppressor protein levels, alter cell/nuclear morphology and lead to cell cycle arrest or apoptosis.

Though sensitive detection is readily achieved, radiolabeling is both hazardous and expensive [29,30]. In 1995, the United States National Institutes of Health alone spent more than $4 million disposing of radioactive waste [31]. Additional expenditures were required for monitoring exposure to radioactivity, inventory control and training laboratory personnel. During that same year, a pregnant cancer researcher was deliberately contaminated with at least 600 μCi of ^{32}P in one of the Bethesda, Maryland NIH research laboratories, as detailed in

US National Institutes of Health Case Number 1-96-033; released 09/17/97, entitled ''Wrongful administration of P-32''. An additional 26 staff members were exposed to lesser amounts of the isotope from a water cooler at this facility. Reducing reliance on radioactivity in biomedical research has become a high priority in many laboratories over the past several years as it makes economic good sense and promotes a better quality of life by providing a safer work environment for laboratory personnel.

2.3. Reverse stain methods

A number of reverse-staining methods for visualizing proteins in SDS–polyacrylamide gels have been described over the years including methods that employ potassium chloride [32], copper chloride [33], zinc chloride [34], cobalt acetate [34], nickel chloride [34] and zinc chloride–imidazole [35,36].

The three reverse stain techniques that have been used most extensively are the potassium chloride, copper chloride and zinc chloride methods. Copper chloride staining is slightly more sensitive, while potassium chloride staining is significantly less sensitive than Coomassie Blue staining [37]. Zinc chloride–imidazole staining is the most sensitive reverse-stain developed to date [38,39]. In the presence of imidazole, free or weakly bound zinc ions are readily precipitated as zinc–imidazole, while tightly bound ions associated with the proteins are refractory to precipitation. This generates clear protein zones on a semi-opaque gel background. Careful attention to the duration of staining is necessary as over-staining of gels can obscure bands and on occasions complex patterns of negative and positive staining are obtained using zinc–imidazole [38]. The limit of detection for the zinc–imidazole stain is roughly 10–20 ng per band for protein separated by SDS–polyacrylamide gel electrophoresis, 40–80 ng per band for protein separated on native polyacrylamide gels, and 100–500 ng per band for proteins separated in carrier ampholyte or immobilized pH gradient isoelectric focusing gels [38,39].

Proteins visualized in zinc–imidazole-stained gels can be evaluated by densitometry, though the linear dynamic range for protein detection is restricted to 10–100 ng [40,41]. Zinc–imidazole-stained gels can be electroblotted or electroeluted by addition of a chelating agent to the transfer buffer [42–45]. Proteins stained with zinc–imidazole are also compatible with Edman-based microsequencing and in-gel tryptic digestion followed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry for determination of peptide masses and sequences [41,42,46].

2.4. Fluorescence-based staining methods

Fluorescent detection of proteins in electrophoresis gels is gaining popularity, particularly with laboratories engaging in large scale proteomics research. Several recently developed fluorescent stains are suitable for incorporation into integrated proteomics platforms for global analysis of protein regulation. The linear dynamic range of detection using fluorescent stains is usually superior to colorimetric or reverse stains.

Nile Red dye has excitation maxima of about 300 and 540 nm and an emission maximum of roughly 640 nm [47]. The detection sensitivity of 20–100 ng for Nile Red staining is slightly better than detection obtained with Coomassie Blue staining, and the dye is compatible with standard Edman sequencing and immunodetection procedures [48–51]. Nile Red staining is not stable, however, and the dye is prone to precipitation in aqueous solution, consequently making it more difficult to handle than commercialized fluorescent stains. The photolabile nature of Nile Red staining makes quantitation of proteins by image analysis difficult. Nile Red dye can be used to pre-stain gels prior to electroblotting them to polyvinylidene difluoride (PVDF) membrane [50,51].

SYPRO Red, SYPRO Orange and SYPRO Tangerine dyes can detect proteins in SDS–polyacrylamide gels using a simple, one-step staining procedure that requires 30–60 min to complete and does not involve a destaining step [52–55]. As little as 4–8 ng of protein can be detected, rivaling the sensitivity of rapid silver staining techniques and colloidal Coomassie Blue staining methods. Since all three dyes excite at roughly 300 nm, documentation of stained gels can readily be achieved by photography on a standard laboratory UV transilluminator. Alternatively, the dyes may be quantified with commercially available CCD camera-based image analysis workstations or a variety of laser scanners,

providing a linear dynamic range of three orders of magnitude. In addition to their UV excitation peak, SYPRO Orange, Red and Tangerine dyes may be excited by visible light at approximately 470, 550 and 490 nm, respectively. The three dyes emit maximally at 570, 630 and 640 nm, respectively. Thus, SYPRO Orange and Tangerine dyes are well suited for imaging systems with 473 nm second-harmonic generation (SHG) or 488 nm argon-ion (Ar) lasers as well as those that use 470 nm blue-LEDs or 460 nm blue fluorescent light sources. SYPRO Red dye is most suitable for 532-nm frequency-doubled neodymium–yttrium–aluminum–garnet (Nd–YAG) laser sources, 543-nm helium–neon (He–Ne) laser sources or 550-nm green-light transilluminators [56].

Both SYPRO Orange and SYPRO Red dyes require 7% acetic acid in the staining solution, which is problematic when electroblotting, electroeluting or measuring enzyme activity is indicated. SYPRO Tangerine dye is an environmentally benign alternative to conventional protein stains that does not require solvents such as methanol or acetic acid for effective protein visualization [55]. Instead, proteins can be stained in a wide range of buffers, including phosphate-buffered saline or simply 150 m*M* sodium chloride. Since proteins may be stained using SYPRO Tangerine dye without the need for harsh fixatives, they are readily eluted from gels or used in zymographic assays, provided that SDS present during electrophoresis does not inactivate them. SYPRO Tangerine dye is also suitable for staining proteins in gels before they are transferred to membranes by electroblotting. Using a 7% acetic acid staining solution, SYPRO Tangerine dye-stained gels have a slightly higher background fluorescence than SYPRO Orange and SYPRO Red dyes. Thus, the latter dyes are preferred choices for routine staining in a fixative solution.

SYPRO Ruby dye is a proprietary ruthenium-based metal chelate stain, which side-by-side comparisons demonstrate is as sensitive as the best silver stains available (Fig. 1) [57,58]. SYPRO Ruby dye allows one-step, low background staining of proteins in polyacrylamide gels without resorting to lengthy destaining steps. The linear dynamic range of the dye extends over three orders of magnitude, thus surpassing both silver and Coomassie blue stains in performance. The dye can be excited using a standard

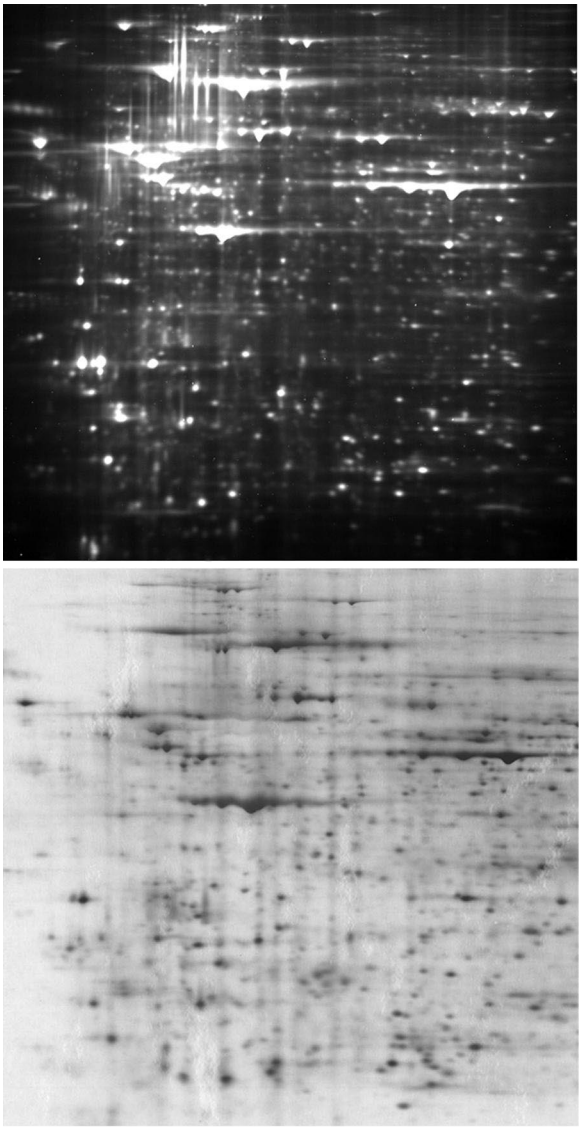

Fig. 1. Fluorescent staining compared with silver staining of two-dimensional gels. Proteins from a cultured fibroblast cell line (Rat-1) were fractionated by 2D gel electrophoresis and then stained using SYPRO Ruby protein gel stain (top image) or silver stain (bottom image), as previously described [9,58]. The comparison demonstrates similar detection sensitivity between these techniques. It should be noted that the silver stain method employed in this comparison is optimized for protein detection, but not compatible with subsequent analysis by mass spectrometry. Mass spectrometry-compatible silver stains are significantly less sensitive than the mass spectrometry-friendly fluorescent stain. In addition, the silver stain provides only qualitative information about the proteins due to its narrow 10-fold linear dynamic range, while the fluorescent stain provides quantitative capabilities extending over 3-logs. Figure courtesy of Drs. Birte Schulenberg and Jill Hendrickson, Molecular Probes.

300-nm UV transilluminator or using imaging systems equipped with 450-, 473-, 488- or even 532-nm lasers. Though more sensitive than SYPRO Orange, SYPRO Red and SYPRO Tangerine dyes, optimal staining is somewhat slower, requiring about 3 h to complete. Similar to colloidal Coomassie Blue stain but unlike silver stain, SYPRO Ruby dye is an end-point stain. Thus, staining times are not critical and staining can be performed over night without gels overdeveloping.

Side-by-side comparisons of silver staining (minus glutaraldehyde) and SYPRO Ruby dye staining have been performed, and SYPRO Ruby stain was found to be superior with respect to possessing a much broader linear dynamic range of quantitation (1000-fold), ability to visualize a 20% greater number of proteins, better reproducibility in quantifying the lowest intensity proteins in a 2D gel profile, and ability to identify lower amounts of protein with higher sequence coverage by peptide mass profiling (75–100 fmol) [57]. This clearly demonstrates that SYPRO Ruby dye is superior to silver staining for modern proteomics applications.

2.5. Chemiluminescence-based staining methods

While commonly employed for the detection of proteins after electroblotting, chemiluminescence had not been used to directly detect proteins in polyacrylamide gels until very recently [59,60]. A chemiluminescent acridan phosphate labeling reagent (maleimide derivative) was used to pre-derivatize bovine serum albumin prior to subjecting the protein to SDS–polyacrylamide gel electrophoresis [59]. Gels were then treated with acidic and basic buffer containing a peroxide, which initiated a rapid generation of light. Roughly 1–650 ng of serum albumin could be detected by the procedure. This should be considered a best-case scenario, however, as serum albumin is unusual in containing a very large number of cysteine residues (35 total) when reduced. Detection sensitivity for proteins with average numbers of cysteine residues would be closer to 10 ng, or the level of sensitivity achieved by colloidal Coomassie Blue stain. Currently, the chemiluminescence detection method has only been validated for feasibility using a single model protein. It remains to be seen whether the labeling technique will permit detection of proteins in complex biological specimens with a detection sensitivity that is competitive with fluorescence stain, reverse-stain or silver stain methods.

The specific detection of an antigen directly in SDS–polyacrylamide gels has also been demonstrated recently, obviating the need to electroblot gels [60]. The method is simply based upon old procedures for immunodetection in gels that were in routine use prior to Towbin's seminal paper on electroblotting [61]. The availability of highly sensitive chemiluminescence methods in recent years has made the in gel technique sensitive enough for certain applications.

2.6. Immobilized pH gradient/MALDI-TOF mass spectrometry and surface-enhanced laser desorption–ionization mass spectrometry (SELDI-MS)

Laser desorption of proteins by direct MALDI-TOF mass spectrometry-based surface scanning of carrier ampholyte isoelectric focusing gels, immobilized pH gradient isoelectric focusing gels, native polyacrylamide gels, and SDS–polyacrylamide gels has been demonstrated, with sub-picomolar detection sensitivities achieved [62–66]. In a preferred embodiment, ''virtual'' 2D gels may be created by desorbing proteins directly from immobilized pH gradient gels using MALDI-TOF mass spectrometry, in effect substituting mass spectrometry for SDS–polyacrylamide gel electrophoresis [65,66]. Analytical data may be presented as a computer-generated image that is similar to a classical 2D gel in appearance. The ''virtual'' 2D gel approach appears to detect lower-molecular-mass proteins ($<$30 kDa) better but higher-molecular-mass proteins ($>$30 kDa) more poorly than conventional 2D gels [65]. Currently, a number of artifacts must be contended with using the method, including horizontal streaks due to variations in baseline slope, artifacts caused by the presence of protein multimers and matrix adducts on the proteins that generate duplicate or triplicate spots, higher molecular masses than predicted due to difficulty in desorbing proteins from the gel matrix, and difficulty in quantifying the amounts of the proteins due to ion suppression phenomenon, a well-known problem associated with mass spectrometry

[65,66]. The procedure is also currently quite slow, requiring a day to run the gel, 2 days to dry it down and 2 days to acquire spectra [65].

Surface-enhanced laser desorption/ionization (SELDI) involves retaining proteins on a solid-phase chromatographic surface (ProteinChip Array) and direct detection of the retained proteins by time-of-flight mass spectrometry (TOF-MS) [67–69]. Typically, cationic, anionic, hydrophobic, hydrophilic, mixed charge, or immobilized metal ion chemistries are employed on SELDI ProteinChip surfaces, as well as antibodies, DNA, enzymes or receptors. Surfaces are incubated with a crude cell lysate or biological fluid, and then washed at various stringency levels to remove nonspecifically associated components. After this sample purification, the retained components are evaluated by TOF-MS. Quantitation of specifically captured TNF-α over a 100-fold concentration range has been demonstrated, though more complex samples are likely to be susceptible to ion suppression phenomenon [68]. Though excellent for rapid profiling of minute samples, the ability to identify proteins from SELDI profiles remains challenging, and conventional peptide mapping must be employed [69].

3. Specific detection of protein post-translational modifications

Though genomics provides comprehensive databases of sequence information, DNA and mRNA provide no information concerning the activities and post-translational modifications of proteins. The elucidation of protein post-translational modifications is perhaps the most important justification for proteomics as a scientific endeavor. The number of documented protein co- and post-translational modifications now exceeds 400 (http://abrf.org/index.cfm/dm.home). The most common modifications include phosphorylation, glycosylation, lipidation, sulfation and proteolytic modifications. These chemical modifications of proteins are crucial to modulating their function, but are not directly coded for by genes. Even in the simplest self-replicating organism, *Mycoplasma genitalium*, there are 24% more proteins than genes due to post-translational modifications, and in humans there are estimated to be at least three times as many proteins as genes [70]. Post-translational modifications modulate the function of proteins and thus directly impact their capacity to participate in cellular regulatory events.

Mass spectrometry has become a powerful research method for the characterization of protein post-translational modifications, since modifications either increase or decrease the molecular mass of the affected amino acid residue [71]. Generally, some method for the rapid proteome-wide screening of proteins with respect to the presence or absence of a particular modification is required prior to micro-characterization by mass spectrometry. Currently, heavily modified proteins, such as highly glycosylated proteins, can prove difficult to identify by standard mass spectrometry-based methods such as peptide mass profiling, since derivatized proteins cannot be matched to calculated peptide masses derived from genome databases. Finally, the presence of certain post-translational modifications, such as phosphorylation and sialoglycosylation, results in peptide signal suppression, complicating identification of peptides bearing these groups.

The detection of three prominent protein post-translational modifications: glycosylation, phosphorylation and proteolytic modifications are summarized below. In addition, three lesser known post-translational modifications, *S*-nitrosylation, arginine methylation and ADP-ribosylation are discussed. Proteomic investigations regarding certain of these post-translational modifications are only just beginning, as new methodologies allowing their detection have only recently been introduced.

3.1. Glycoprotein detection methods

Protein glycosylation is a key post-translational modification relevant to a range of biological phenomenon. For example, the metastatic spread of tumor cells in malignant progression is known to be a major cause of cancer mortality. Protein glycosylation is increasingly being recognized as one of the most prominent biochemical alterations associated with malignant transformation and tumorigenesis [72–75]. Not only is the total amount of protein-associated carbohydrate of relevance to cancer, but alterations in the branching patterns of the attached glycans also play important roles in malignancy.

Glycosylation changes in human carcinomas appear to contribute to the malignant phenotype observed downstream of certain oncogenic events [72].

Glycoproteins can be detected by autoradiography after incorporation of ^{3}H or ^{14}C sugars into cultured cells or tissues [76–78]. Though sensitive detection is achieved, radiolabeling is slow, hazardous and expensive. Nonradioactive detection of glycoproteins is usually performed using a periodic acid Schiff base procedure that employs the dye, acid fuchsin (pararosanaline) [79,80]. The principal limitation of this colorimetric method is that it has poorer detection sensitivity than Coomassie Blue staining, thus making the technique difficult to implement in the context of modern proteomics research. Similarly, Alcian Blue has been used as a colorimetric stain for glycoproteins [81,82]. Sensitivity of this method is similar to the acid fuchsin dye method and neutral glycoproteins, such as horseradish peroxidase and transferrin, are poorly detected by the staining method.

Dansyl hydrazine is a fluorescent alternative to acid fuchsin dye that can be used for the detection of glycoproteins in polyacrylamide gels [83]. Glycoproteins are subjected to periodate oxidation in gels and the resulting aldehydes are condensed with dansyl hydrazine to form hydrazones. These are in turn reduced to stable hydrazine derivatives with sodium borohydride. The coupling reaction requires incubating gels in acidified dimethylsulfoxide at 60 °C for 2 h and this harsh treatment has undoubtedly prevented widespread adoption of the method. Very high concentrations of dye are required for the dansyl hydrazine labeling procedure (0.5–2.0 g/l) and newer methods offer better specificity and sensitivity of detection.

Three new fluorescence-based methods for detecting glycoproteins have been introduced recently [84–86]. All techniques are based upon periodic acid Schiff base chemistry. Fluorescein semicarbazide hydrazide and 8-aminonaphthalene-1,3,6-trisulfonate have been employed for the detection of glycoproteins electroblotted to PVDF membranes, but the techniques are not suitable for detection of glycoproteins directly in polyacrylamide gels [84,86]. To date, the fluorescein semicarbazide labeling method has not been characterized extensively [86]. However, the method employing ϵ-aminonaphthalene-1,3,6-trisulfonate requires heating at 60 °C for 45–60 min, and limits of detection sensitivity range from 50 to 200 ng [84]. A sensitive green-fluorescent glycoprotein-specific stain, referred to as Pro-Q Emerald 300 dye, was recently reported, that allows detection of glycoproteins on PVDF membranes or directly in polyacrylamide gels [85]. Pro-Q Emerald 300 dye may be conjugated to glycoproteins by a periodic acid Schiff's (PAS) mechanism using very mild reaction conditions at room temperature. Pro-Q Emerald 300 dye-labeled gels may be post-stained with SYPRO Ruby dye, allowing sequential two-color detection of glycosylated and nonglycosylated proteins (Fig. 2). As little as 300 pg of α1-acid glycoprotein (40% carbohydrate) and 1 ng of glucose oxidase (12% carbohydrate) or avidin (7% carbohydrate) are readily detected in gels after staining with Pro-Q Emerald 300 dye, and a 500–1000-fold linear dynamic range of detection is obtained using the labeling method [85]. A UV transilluminator is required to detect the stain, but recently Pro-Q Emerald 488 dye was introduced for detection of glycoproteins using laser-based gel scanners.

A general antibody-based method for detecting glycoproteins involves periodic acid oxidation of the carbohydrate moieties, reaction with digoxigenin hydrazide, and detection with alkaline phosphatase-conjugated anti-digoxigenin antibody [87]. Similar methods have also been devised using biotin hydrazide and alkaline phosphatase-conjugated or horseradish peroxidase-conjugated streptavidin [88,89]. The methods are unsuitable for glycoprotein detection in gels, however, and the introduction of contaminating reporter proteins complicates target identification by peptide mass profiling. Methods for the determination of carbohydrates in proteins after gel electrophoresis by covalent derivatization with fluorophores such as 2-aminobenzoic acid have been introduced, but require band excision and protein hydrolysis in strong acids at high-temperature [90]. Pulsed amperometric detection procedures employing similarly harsh reaction steps have also been devised [91].

3.2. Phosphoprotein detection methods

Though the study of reversible protein phosphorylation reactions has become central to biochemistry

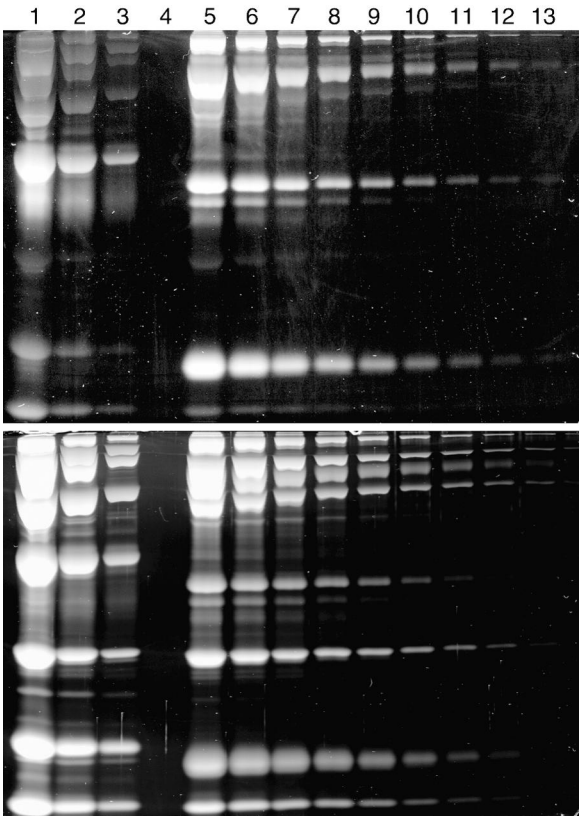

Fig. 2. Serial dichromatic detection of glycosylated and unglycosylated proteins in the same polyacrylamide gel. Detection of glycoproteins using Pro-Q Emerald 488 glycoprotein stain kit (Molecular Probes) (Top image). This PAS-based chemistry generates green fluorescent bands in gels. Detection of total protein profile using SYPRO Ruby protein gel stain (Molecular Probes) (Bottom image). This colloidal metal chelate staining procedure generates orange–red fluorescent bands in gels. Both images were collected using an FLA-3000 laser-based gel scanner (Fuji Film Scientific Imaging). Lanes 1–3: 2-fold serial dilution of broad range molecular mass markers containing the glycoprotein ovalbumin (3% carbohydrate). Lanes 5–13: 2-fold serial dilutions of CandyCane molecular mass markers (Molecular Probes) containing a mixture of glycosylated and unglycosylated proteins. Figure courtesy of Ms. Courtenay Hart, Molecular Probes.

since its discovery in the 1950s, by Nobel Prize winners Drs. Edwin Krebs and Edmond Fischer, phosphoproteomics, the systematic parallel analysis of the phosphorylation status of large sets of proteins involved in the regulatory circuitry of cells and tissues, is a relatively new field destined to drive research in the post-genomics era for many years to come [92,93]. Reversible protein phosphorylation plays a central role in biological regulation, and this is especially true in the context of carcinogenesis. The role of protein phosphorylation cascades in malignant transformation is exceedingly well established. Malignant transformation occurs through successive mutations in specific cellular genes, leading to the activation of oncogenes and the inactivation of tumor suppressor genes [94–96]. The cellular genes that are altered are normally involved in maintaining cellular homeostasis through participation in signaling cascades that are tightly regulated to maintain the functional integrity of the cell [96]. Oncogenic signaling pathways lead to the activation of tyrosine or serine/threonine kinases. Tyrosine kinases that are often found to be mutated in human tumors include the cytoplasmic kinases Src and Abl, as well as the integral plasma membrane kinases EGFR and PDGFR [96]. Serine/threonine kinases that are mutated or activated in human cancer include Raf, Akt and Tpl-2 [96]. These protein kinases are involved in signaling pathways that lead to cell proliferation, or inhibition of apoptosis through activation of transcription factors, inhibition of pro-apoptotic molecules, or deregulation of cell cycle control. A tyrosine phosphatase associated with metastasis in colorectal cancer has recently been identified [97].

Phosphoproteins are most often detected by autoradiography after incorporation of ^{32}P or ^{33}P into cultured cells or after incorporation into subcellular fractions by protein kinases [98]. Such approaches are restricted to a limited range of biological materials, such as tissue culture samples. Needless to say, analysis of clinical samples would require in vivo labeling of patients, which is not feasible. Several alternatives to radiolabeling have also been developed over the years. Phosphoproteins can be detected by immunoblotting [99,100]. Though high quality antibodies to phosphotyrosine are commercially available, antibodies that specifically recognize phosphoserine and phosphothreonine residues have been more problematic, often being sensitive to amino acid sequence context. Immunoblotting is best performed after blocking unoccupied sites on the solid-phase support with protein solutions, which interferes with microchemical analysis. Removal of the antibody and stain require relatively harsh treatments (i.e., heating to 65 °C, incubation with 0.1%

SDS and 1 m*M* DTT). This also poses problems with subsequent use of the protein for sequencing and mass spectrometry.

The cationic carbocyanine dye ''Stains-All'' (1-ethyl-2-[3-(3-ethylnaphtho[1,2*d*] thiazolin-2-ylidene)-2-methylpropenyl]-naphtho [1,2*d*]thiazolium bromide) stains RNA, DNA, phosphoproteins and calcium-binding proteins blue while unphosphorylated proteins are stained red [101,102]. Stains-All is not routinely used to detect phosphoproteins due to poor specificity and low sensitivity. Stains-All is at least 10 times less sensitive than Coomassie Brilliant Blue and several orders of magnitude less sensitive than ^{32}P autoradiography.

Phosphoproteins may be specifically detected in gels through alkaline hydrolysis of phosphate esters of serine or threonine, precipitation of the released inorganic phosphate with calcium, formation of an insoluble phosphomolybdate complex and then visualization of the complex with a dye such as Methyl Green, Malachite Green or Rhodamine B [103,104]. The Methyl Green dye approach has recently been commercialized as the GelCode Phosphoprotein detection kit (Pierce Chemical, Rockford, IL). The detection sensitivity of the staining method is considerably poorer than Coomassie Blue staining, with 80–160 ng of phosvitin, a protein containing roughly 100 phosphoserine residues, being detectable by the commercialized kit. The staining procedure is fairly complex (involving seven different reagents) and alkaline hydrolysis requires heating gels to 65°C, which causes the gel matrix to hydrolyze and swell considerably. Since phosphotyrosine residues are not hydrolyzed by the alkaline treatment, proteins phosphorylated at this amino acid residue escape detection by the method. Gels may be destained and then post-stained with colloidal Coomassie Blue dye in order to visualize the total protein profile.

Several instrument-based methods are available for the determination of protein phosphorylation such as ^{31}P NMR [105,106], mass spectrometry [71,107–109] and protein sequencing [110]. While these procedures accurately characterize the phosphorylation status of proteins and peptides, they are unsuitable for high-throughput screening of phosphorylated substrates. The techniques are generally used after a phosphoprotein has been identified by autoradiography or immunoblotting with anti-phosphotyrosine antibody. Though methods have recently been introduced to directly quantify the relative abundance of phosphoproteins in two different samples by mass spectrometry through culturing different cell populations in ^{15}N-enriched and ^{14}N-enriched medium, the linear dynamic range of such methods has explicitly been demonstrated over only a 10-fold range [109]. In addition, an isotope-coded affinity tag approach to the differential quantitation of phosphopeptides by β-elimination and subsequent addition of biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine containing either four alkyl hydrogen or four alkyl deuterium atoms has been reported [111]. Ion suppression phenomena associated with mass spectrometry prevents stoichiometric comparison of different phosphoproteins by such techniques.

Recently, two methods for the chemical derivatization and enrichment of phosphopeptides have been described [112,113]. The first method requires proteolytic digestion of the sample, reduction and alkylation of cysteine residues, N-terminal and C-terminal protection of the peptides, formation of phosphoramidate adducts at phosphorylated residues by carbodiimide condensation with cystamine, capture of the phosphopeptides on glass beads coupled to iodoacetate, elution with trifluoroacetic acid and evaluation by mass spectrometry [112]. The second method involves oxidation of cysteine residues with performic acid, alkaline hydrolysis to induce β-elimination of phosphate groups from phosphoserine and phosphothreonine residues, addition of ethanedithiol, coupling of the resulting free sulfhydryl residues with biotin, purification of phosphoproteins by avidin affinity chromatography, proteolytic digestion of the eluted phosphoproteins, a second round of avidin purification and then analysis by mass spectrometry [113]. Both procedures were validated using the phosphoprotein β-casein. Both procedures identified the monophosphorylated trypsin peptide fragment from the test protein, but both failed to detect the tetraphosphorylated peptide fragment [112,113].

Fluorescence detection methods appear to offer the best solution to global protein quantitation in proteomics. However, currently, there is no satisfactory method for the specific and reversible fluorescent detection of gel-separated phosphoproteins from complex samples. Derivatization and fluorophore

labeling of phosphoserine residues by blocking free sulfhydryl groups with iodoacetate or performate, alkaline β-elimination of the phosphate residue, addition of ethanedithiol, and reaction of the resulting free sulfhydryl group with 6-iodoacetamidofluorescein has been demonstrated in capillary electrophoresis using laser-induced fluorescence detection and similar reactions have been performed on protein microsequencing membranes [114,115]. Neither publication demonstrates detection of phosphoproteins directly in gels, but the detection of cysteine residues by similar chemistry is shown, indicating that the pre-derivatization of phosphoproteins by this chemistry should be feasible [115]. One problem with the approach is that a delicate balance must be struck between the base and the ethanedithiol in order to achieve elimination of the phosphate group from the serine residue and addition of the ethane dithiol to the resulting dehydroalanine residue without hydrolysis of the peptide backbone [114]. Though the quest for a Coomassie Blue-like stain allowing the simple and selective detection of phosphoproteins in gels dates back to the 1960s, this holy grail remains lost for the time being.

3.3. Proteolytic modification detection methods

Gel-based methods for the detection of proteolytic activity are too numerous to enumerate in this review article [116,117]. Zymographic assays for serine proteinases, cysteine proteases, aspartate proteases and metalloproteinases have been developed over the years. For example, a fluorescence-based protease assay was recently developed for the detection of different members of the papain family of cysteine proteases within a complex cell lysate [118]. Four different fluorescent BODIPY dye conjugates of the general cysteine protease inhibitor *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane were synthesized, allowing multiplexed analysis of papain family proteases. Covalent modification of the proteases through the active site cysteine residue allows analysis of changes in enzyme activity. For example, a specimen can be divided in two, and one sample incubated with the red-fluorescent BODIPY 588/616 conjugate. The other sample can then be incubated with a specific protease inhibitor, along with the blue-fluorescent BODIPY 493/503 conjugate. Next, the samples are combined and the proteins separated on a single 2D gel. Specific inhibition of particular cysteine proteases is readily identified through comparison of the resulting red-fluorescent and blue-fluorescent images. Gels can subsequently be stained with a high sensitivity total protein stain, the protein isoforms of interest excised and then identified by standard mass spectrometry-based techniques.

Tools for the specific proteome-wide detection of natural substrates of proteolytic enzymes are not currently available. Individual proteins can be incubated with specific proteases to determine whether they are potential substrates, but the physiological relevance of such measurements is not assured. Standard immunodetection techniques may be used to monitor the proteolysis of specific proteins after electroblotting, as long as the epitope used for detection is not proteolysed. In the realm of 2D gel electrophoresis, spot-by-spot identification of the majority of proteins resolved in a particular sample generally leads to the identification of subsets of proteins related to one another through proteolytic processing events.

3.4. S-Nitrosylation detection method

Nitric oxide has been implicated in a variety of physiological and pathophysiological conditions, including vasodilation, proliferation, differentiation, NMDA receptor activation, septic shock, atherosclerosis, apoptosis and hypoxia-induced pulmonary hypertension [119,120]. The intracellular reaction of the higher oxides of nitric oxide (NO_2, N_2O_3, $OONO^-$) with reactive cysteine groups leads to the generation of protein *S*-nitrosothiols. Like protein phosphorylation, protein *S*-nitrosylation could potentially represent a fundamental mechanism for the reversible post-translational control of protein activity and by extension cellular functions [119–121].

The difficulty in devising specific gel-based assays for particular post-translational modifications should be readily apparent from the preceding discussion of phosphorylation detection systems. To date, radiolabeling with ^{32}P provides the bulk of the data on protein phosphorylation, with anti-phosphotyrosine antibodies and most recently monitoring 80-Da shifts in peptide mass by mass spectrometry serving as very useful adjunct detection technologies. Until

recently, the evaluation of protein *S*-nitrosylation in biological systems has been hampered by the lack of a detection technology to conveniently monitor this labile post-translational modification. Radioactive isotopes of nitrogen or oxygen, the atoms forming the nitric oxide adduct are not available, and the half life of protein *S*-nitrosylation is in the few second to few minute range. Protein *S*-nitrosylation is not stable enough to survive the rigors of denaturing SDS–polyacrylamide gel electrophoresis, so while anti-*S*-nitrosocysteine antibodies have been generated and applied in immunohistochemistry studies, they are of little use in the context of proteomics investigations.

Recently, the Biotin Switch method was established to allow detection of protein *S*-nitosylation after SDS–polyacrylamide gel electrophoresis and electroblotting [122]. The procedure allows specific biotinylation of *S*-nitrosylated cysteine residues in proteins. First, free thiols are blocked by incubation with a thiol-specific methiolating agent (methyl methanethiosulfonate or MMTS) in the presence of sodium dodecyl sulfate to ensure access to buried cysteine residues. The methiolating agent does not react with nitrosothiols or disulfide bonds under the reaction conditions employed. Excess MMTS is removed using a spin column or by acetone precipitation. Next, nitrosothiol bonds are selectively decomposed to thiols with ascorbate. Finally the free thiol groups are reacted with the sulfhydryl-specific biotinylation reagent, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). The labeled proteins may then be separated by SDS–polyacrylamide gel electrophoresis, electroblotted and detected by standard immunoblotting methods using anti-biotin antibodies or streptavidin. Labeled proteins may be selectively enriched using streptavidin affinity chromatography as well. The Biotin Switch method has permitted new proteomic investigations into the fundamental role of protein *S*-nitrosylation in cellular signalling [121,122].

3.5. Arginine methylation detection methods

Arginine methylation was first noted more than 30 years ago, and study of this post-translational modification has benefited from renewed interest due to the completion of several genome sequencing programs [123,124]. A consensus sequence for arginine methylation has been identified, the RGG box RNA-binding motif, allowing identification of numerous proteins as potential substrates of arginine methylation [125]. Other arginine methylation motifs have been identified as well, such as RXR, DRG and GRA [126,127]. Unlike *S*-nitrosylation, arginine methylation is essentially an irreversible post-translational modification, as the generated aminoalkyl bond is quite stable and no dimethylarginine demethylase has been identified to date [126]. Arginine methylation has been noted in proteins involved in signal transduction, nuclear RNA processing, ribosome biogenesis, nucleocytoplasmic transport, and gene silencing [125,128].

As with protein *S*-nitrosylation, investigations into the role of protein arginine methylation have been fettered somewhat by a lack of technologies facilitating the detection of this post-translational modification on a proteome-wide scale [129]. Yeast cells, in contrast to the majority of other cell types, actively import the biological methyl donor *S*-adenosylmethionine, allowing radiolabeling of proteins in intact cells under physiological conditions [128]. Consequently, radiolabelling with [methyl-^{3}H] *S*-adenosyl-L-methionine, followed by electrophoresis and fluorography, is a commonplace approach to the study of arginine methylation in this organism [125,128]. In eukaryotic organisms lacking an active import mechanism, such as mammals and insects, cultured cells are incubated in medium containing protein synthesis inhibitors and then in methionine-free medium containing [methyl-^{3}H] *S*-adenosyl-L-methionine for several hours [130,131]. Conventional gel electrophoresis and fluorography are then employed to detect the modified proteins.

Mass spectrometry is playing an increasingly important role in the characterization of arginine methylation sites [126,127,132–134]. For example, the 24-kDa high-molecular-mass fibroblast growth factor-2 has been demonstrated to contain seven to eight dimethylated arginine residues (28 Da per residue) using a combination of MALDI-TOF mass spectrometry and Edman sequencing [127]. Such studies are performed on a protein by protein basis, with candidates suspected to contain the modification.

An immunological approach to the global de-

tection of arginine methylation has recently been reported that does not require radiolabeling of specimens [129]. At the heart of the procedure are monoclonal antibodies specific for mono- and asymmetric dimethylated arginine residues (Abcam, Cambridge, UK). Proteins are first immunoprecipitated with antibodies to methylarginine, the immunoprecipitated proteins are separated by SDS–polyacrylamide gel electrophoresis, transferred to membranes by electroblotting and specific proteins are then detected by immunoblotting or total protein staining. Unfortunately, while the anti-methylarginine antibodies are suitable for immunoprecipitation, they do not appear to be suitable for Western blot analysis, leaving assignment of the post-translationally modified proteins somewhat tentative. Control experiments are suggested in order to validate the assignment of the post-translational modification [129]. Recombinant protein substrates may be methylated in vitro using *S*-adenosyl-L-methionine and used to monitor the effectiveness of the immunoprecipitation step. Furthermore, preincubation of the antibodies with unmethylated or in vitro methylated protein as competitors can serve as an additional control for the specificity of the immunoprecipitation.

3.6. ADP-ribosylation detection methods

Mono-ADP ribosylation is a reversible post-translational modification of proteins catalyzed by enzymes that transfer the ADP-ribose moiety of NAD to specific amino acid residues, especially arginine residues, on protein substrates [135]. Among the known protein substrates for ADP-ribosyltransferases are small RAS-like membrane GTP-binding proteins. Nonenzymatic ADP ribosylation of cysteine residues in certain proteins, such as nuclear GTP-binding proteins has also been observed [136]. ADP ribosylation is known to regulate membrane trafficking and actin cytoskeleton arrangement [137].

Mono-ADP ribosylation of proteins is typically monitored by incubating cells or membranes with [^{32}P]NAD [138–142]. Protein labeling in the presence of [^{32}P]NAD may arise from several different reactions, however. Two important ones to consider in experiments of this type are poly-ADP ribosylation and nonenzymatic mono-ADP ribosylation [139]. Mono-ADP ribosylation is confirmed if labeled proteins that have been electroblotted and treated with snake venom phosphodiesterase, generate [^{32}P]5′-AMP, as identified by TLC or HPLC analysis. Nonenzymatic mono-ADP ribosylation can be excluded as a possibility if incubation of samples with [^{32}P]ADP ribose does not cause labeling of the proteins.

An alternative approach to detecting ADP ribosylation is the use of anti-ADP ribose antibodies by standard immunoblotting methods [143]. Since ADP ribose-conjugated serum albumin results in 5′-AMP-specific antibodies due to hydrolysis, an ADP ribose analog resistant to enzymatic degradation at the pyrophosphate group was coupled to bovine serum albumin in order to generate an antigen appropriate for the production of anti-ADP ribose antibodies. The antibody is reported to detect as little as 1 pmol of ADP ribosylated proteins. Additionally, 1,6-etheno NAD, a fluorescent analog of NAD, may be employed to measure ADP ribosylation [144–146]. The fluorophore excites maximally at 292 nm and emits maximally at 413 nm.

4. Detection of reporter enzymes and epitope tags

Several recombinant DNA-based strategies for appending affinity tags and reporter enzymes to proteins have been developed as tools for protein purification and detection. These include fusion tags of maltose-binding protein, hemagglutinin, β-galactosidase, β-glucuronidase, green fluorescent protein, glutathione *S*-transferase, c-myc oncoprotein, FLAG peptide or oligohistidine peptide. β-Galactosidase, β-glucuronidase, the oligohistidine peptide tag and green fluorescent protein are discussed in more detail below:

4.1. β-Glucuronidase

The *Escherichia coli* β-glucuronidase-encoded gene, gusA, is commonly used as a reporter gene for nonvertebrate organisms that minimally express endogenous β-glucuronidase activity such as lower and higher plants, most bacteria, yeast, fungi and insects

[147]. As plants express significant amounts of β-galactosidase endogenously, β-glucuronidase is an especially useful reporter enzyme for molecular biology studies focusing upon these organisms. Naturally, β-glucuronidase protein levels can be determined by standard immunoblotting procedures using anti-GUS antibody [148,149]. By measuring β-glucuronidase activity directly in polyacrylamide gels, time-consuming immunoblotting steps and costly antibodies are avoided, however. β-Glucuronidase activity can be detected directly in gels using colorimetric detection systems such as naphthol-AS-BI β-D-glucuronide coupled to Fast Garnet GBC, 6-bromo-2-naphthyl β-D-glucuronide coupled to Fast Blue B or 5-bromoindol-3-yl β-D-glucuronide in combination with nitroblue tetrazolium [116,150]. Gels are incubated in the stain solution until colored bands appear and then fixed in an acidified aqueous solution of 7% acetic acid or 10% trichloroacetic acid. The fluorogenic substrate 4-methylumbelliferyl β-glucuronide is more commonly used to measure β-glucuronidase activity in gels [116]. Typically, the substrate is applied to the gel surface soaked into filter paper. After incubating the gel at 37 °C for 90 min, the filter paper is removed and fluorescent bands are detected using long wavelength ultraviolet (UV-A) illumination in combination with a 420-nm long-pass emission filter. The hydrolytic product of this substrate does not precipitate at the site of enzyme activity, however, making the compound less than ideal for localization studies in polyacrylamide gels due to a tendency to diffuse after a period of time. Additionally, the emission of the product, 4-methylumbelliferone, is maximal at 456 nm, which overlaps with the emission of polyester backings commonly used to support pre-cast polyacrylamide gels. An analogous fluorogenic substrate, fluorescein mono-β-D-glucuronide was recently employed in microchip capillary electrophoresis for rapid enzyme assay [151].

A fluorescent serial staining method based upon detection of total protein after SDS–polyacrylamide gel electrophoresis using SYPRO Tangerine dye in saline solution, followed by enzyme renaturation in 0.1% Triton X-100 and detection of β-glucuronidase activity using ELF 97 β-D-glucuronide was described recently, but unfortunately, simultaneous detection of total protein and enzyme activity was not possible as SYPRO Tangerine dye must intercalate into SDS micelles for protein detection while measuring β-glucuronidase activity requires prior removal of SDS from the gels [55]. Combining ELF 97 β-D-glucuronide with SYPRO Ruby dye does permit convenient dichromatic detection of β-glucuronidase activity and total protein profiles using a single gel, however. Unlike SYPRO Tangerine dye, SYPRO Ruby dye interacts directly with basic amino acids in proteins. This electrostatic binding mechanism allows staining of proteins after renaturation of enzyme activity through removal of SDS from the gels [147].

4.2. β-Galactosidase

The β-galactosidase gene (lacZ) is commonly used as a reporter enzyme for the evaluation of gene regulation, the detection of recombinant fusion genes, monitoring cell transfection efficiencies and the detection of protein–protein interactions by the two-hybrid system [152]. In a parallel manner as with β-glucuronidase, β-galactosidase protein levels may be determined by standard immunoblotting procedures [153]. This enzyme may also be detected directly in gels using a number of assays based upon enzyme activity [116]. Similar to β-glucuronidase, colorimetric and fluorogenic substrates for β-galactosidase, including 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, alizarin-β-D-galactoside, *p*-naphtholbenzein-β-D-galactoside, cyclohexenosculetin-β-D-galactoside, 4-methylumbelliferyl β-D-galactopyranoside, 3-carboxyumbelliferyl β-D-galactopyranoside, resorufin-β-D-galactopyranoside or fluorescein mono-β-D-galactopyranoside, have been developed for detecting β-galactosidase activity in agar-based colonies or in gel-based zymography [116,154–157]. In an alternate coupled enzyme detection system, gels are incubated in lactose, which is hydrolyzed to D-glucose and D-galactose by β-galactosidase [158]. Galactose dehydrogenase subsequently converts D-galactose to D-galactono-1,4-lactone, utilizing the cofactor NAD. The resulting NADH is coupled to a phenazine methosulfate (PMS)/methyl thiazolyl blue (MTT) chromogenic system to form the colored product formazan. Finally, in yet another method, salicyl β-D-galactoside generates D-galactose and salicylic acid when hydrolyzed by β-galactosidase. Addition of terbium chlo-

ride and EDTA produces a ternary luminescent lanthanide chelate complex with excitation maximum of roughly 280 nm and emission maximum of 515 nm [159].

4.3. Oligohistidine tags

The oligohistidine domain is a transition metal-binding peptide sequence comprising a string of four to 10 histidine residues. Recombinant proteins are often fused to oligohistidine affinity tag sequences to aid in their purification by immobilized nickel-ion affinity chromatography. The detection of oligohistidine-tagged fusion proteins after polyacrylamide gel electrophoresis typically requires many time-consuming steps, including the transfer of the gel to a membrane, the blocking of unoccupied sites on the membrane with protein or detergent solutions, incubation with an oligohistidine-binding agent (primary antibody or biotin-nitrilotriacetic acid), incubation with a secondary detection agent (antibody–reporter enzyme conjugate, streptavidin–reporter enzyme conjugate), and incubation with a visualization reagent (colorimetric, fluorogenic or chemiluminescent reagent). Specifically, biotinylated nitrilotriacetic acid has been used in combination with streptavidin–alkaline phosphatase or streptavidin–horseradish peroxidase conjugates and colorimetric or chemiluminescent substrates to allow detection of the oligohistidine-tagged fusion proteins after electroblotting [160–162]. Additionally, direct reporter enzyme–nitrilotriacetate–nickel conjugates have been used to detect oligohistidine-tagged fusion proteins on electroblots [163,164]. Likewise, colloidal gold–nitrilotriacetic acid conjugates have been used to detect oligohistidine-containing fusion proteins on blots after a silver enhancement step [165]. Lastly, though oligohistidine is not very immunogenic, several high affinity monoclonal antibodies specific to the epitope have been produced that allow detection of fusion proteins by standard immunoblotting techniques [166–168].

Two novel fluorophore–nitrilotriacetic acid conjugates, Pro-Q Sapphire 365 and Pro-Q Sapphire 488 oligohistidine gel stains (Molecular Probes, Eugene, OR), allow for the fluorescence detection of oligohistidine-tagged fusion proteins directly in sodium dodecyl sulfate–polyacrylamide gels, without requiring immunoblotting, reporter enzymes or secondary detection reagents. Pro-Q Sapphire 365 oligohistidine gel stain emits maximally at 450 nm (blue fluorescence) when illuminated with 300–365 nm ultraviolet light. Pro-Q Sapphire 488 oligohistidine gel stain emits maximally at 515 nm (green fluorescence) when illuminated with visible light from a laser-based gel scanner equipped with a 470–488-nm laser source. Roughly 25–65 ng of oligohistidine-tagged fusion protein is detectable using either stain. After recording the fluorescence signal from the Pro-Q Sapphire dyes, gels may be post-stained with the red-fluorescent SYPRO Ruby protein gel stain in order to reveal the total protein profile (Fig. 3).

4.4. Green fluorescent protein

Wild-type green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, as well as its differently colored variants, are useful tools commonly employed to monitor gene expression and protein localization when expressed as chimeric fusion proteins [169]. Wild type GFP displays excitation maxima of 395 and 475 nm and an emission maximum of 509 nm. Mutants of the GFP protein have been devised with visible excitation maxima of 490 nm, providing excellent compatibility with fluorescein filter sets and argon ion laser sources. From a proteomics perspective, it should be noted that the introduction of a single GFP reporter gene, used to monitor cell metabolism, can profoundly effect a host of cellular processes, including carbon metabolism, protein synthesis and protein translation [170]. Using pH 4–7 isolectric focusing gels and 12% T SDS–polyacrylamide gels, it has been demonstrated that the introduction of GFP into *Escherichia coli* strain JM105 using pBluescript expression vector (Stratagene, La Jolla, CA) leads to the expression of 68 new proteins (including 50S ribosomal protein L9, serine methylase, aerobic glycerol-3-phosphate dehydrogenase and amino acid ABC transporter binding protein) and the loss of 44 others (including sulfate starvation-induced protein 7, histidine-binding periplasmic protein and glucose-permease IIA component) [170]. Numerous quantitative protein changes were observed as well.

Recent studies indicate that GFP may be isolated with high yield and purity employing serial copper

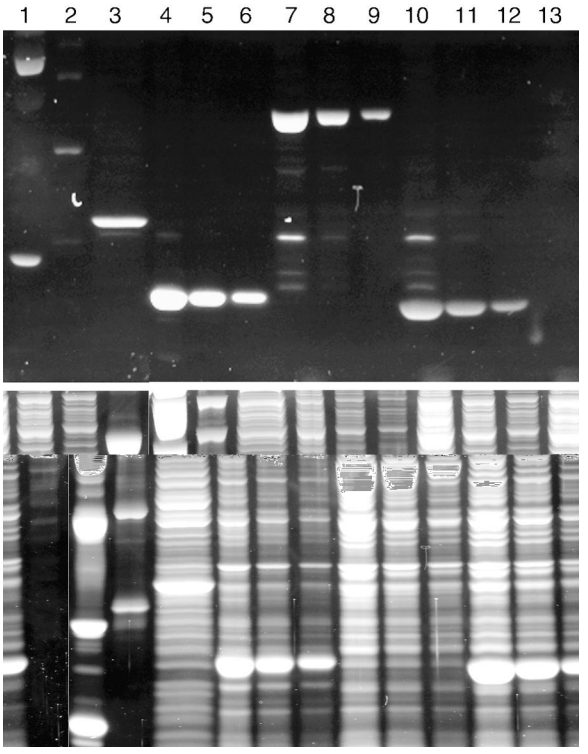

Fig. 3. Serial dichromatic detection of oligohistidine-tagged recombinant proteins and total proteins in the same polyacrylamide gel. Detection of oligohistidine-containing fusion proteins using Pro-Q Sapphire 365 oligohistidine gel stain kit (Molecular Probes) (top image). This nitrilotriacetate-based detection system generates blue fluorescent bands in gels. Detection of total protein profile using SYPRO Ruby protein gel stain (Molecular Probes) (bottom image). This colloidal metal chelate staining procedure generates orange–red fluorescent bands in gels. Lanes: (1) broad range molecular mass markers; (2) 6Xhis protein ladder (Qiagen); (3) protein lysate containing hexahistidine-tagged urate Oxidase (Pierce Corporation); (4–6) serial dilutions of *Escherichia coli* lysate containing the recombinant oligohistidine fusion protein, OSCP; (7–9) serial dilutions of *Escherichia coli* lysate containing the recombinant oligohistidine fusion protein, α-subunit of ATPase; (10–12) serial dilutions of *Escherichia coli* lysate containing the recombinant oligohistidine fusion protein, d subunit of ATPase; (13) bovine serum albumin. Both images were collected using A LumiImager CCD-camera-based gel scanner (Roche Biochemicals). Figure courtesy of Ms. Courtenay Hart, Molecular Probes.

(II) and zinc (II) immobilized metal-ion affinity columns, using diafiltration between the two chromatography steps [171]. Though more complex than the standard nickel (II) immobilized metal-ion affinity column used for isolation of oligohistidine-tagged fusion proteins, the two-step chromatography procedure for GFP is relatively straightforward and likely to be applicable in many instances to GFP-fusion proteins as well. In the context of electrophoretic separations methodologies, the native fluorescence of GFP has had limited utility, as its signal is destroyed by conventional denaturing SDS–polyacrylamide gel electrophoresis. Consequently, overexpressed GFP fusion proteins are typically detected by Coomassie Blue staining, silver staining or by immunoblotting techniques using anti-GFP antibodies [172–175]. GFP may be visualized directly in SDS–polyacrylamide gels if a nonreducing sample buffer is used to prevent complete protein denaturation [176,177].

A GFP-protein A fusion protein has been constructed for use as a single-step detection reagent for Western blotting [178,179]. As with any direct fluorophore conjugate of an antibody, protein A or streptavidin, sensitivity is limited by the overall brightness of the probe, and reporter enzyme constructs employing alkaline phosphatase, horseradish peroxidase or β-galactosidase are generally much more sensitive due to the signal amplification afforded by rapid turnover of large numbers of substrate molecules.

Certain specialized applications of GFP fluorescence in gel electrophoresis have been reported. For instance, a GFP–calmodulin fusion protein has been generated, permitting rapid, nonradioactve detection of interacting proteins in gel overlay assays (Far-Western blots) [176]. Proteins are fractionated by conventional SDS–polyacrylamide gel electrophoresis, electroblotted to poly(vinylidene difluoride) (PVDF) membrane, blocked with 2% nonfat milk protein and incubated with 2–5 μg/ml of the GFP fusion protein over night. After washing the membrane several times, as little as 100 ng of calmodulin-binding proteins may be visualized by UV epi-illumination. Detection sensitivity using biotinylated calmodulin, horseradish peroxidase-conjugated streptavidin and luminol was found to be vastly superior to that obtained by the direct GFP–calmodulin fusion protein, though the conventional detection method required more steps.

In another example, native gel electrophoresis and affinity capillary electrophoresis with laser-induced fluorescence have been employed to monitor the

interaction of a GFP-cyclophilin fusion protein (''bait'' protein) with cyclosporin A [177]. The native gel band-shift assay was performed using Tris–borate–EDTA electrophoresis gels and fluorescence signal was visualized using a UV transilluminator/CCD camera system. Roughly 1 μg of purified recombinant protein was required to obtain a band of moderate intensity with the approach. The affinity capillary electrophoresis band-shift assay allowed detection of the protein–protein interaction in phosphate buffer without addition of detergent. Consequently, weak and moderate interactions could be monitored using 60-fold lower amounts of protein and significantly shorter analysis times. Subsequently, both band shift assays were applied to extracts of *Drosophila melanogaster* embryos, and a putative high affinity endogenous ligand of cyclophilin was detected. Since GFP is larger than its fusion partner, it is necessary to establish in control experiments to ensure that any observed interactions arise from the bait component of the fusion protein.

5. Differential display proteomics: approaches and options

Proteomics is often based upon the comparison of different protein profiles. The central objective of differential display proteomics is to increase the information content of proteomics studies through multiplexed analysis. Currently, three principal approaches to differential display proteomics are being actively pursued, difference gel electrophoresis (DIGE), multiplexed proteomics (MP) and isotope-coded affinity tagging (ICAT). The basic principles of these three methods in the context of 2D gel electrophoresis are outlined in Figs. 4, 5 and 7.

5.1. Difference gel electrophoresis (DIGE)

Succinimidyl esters of the cyanine dyes, Cy2, Cy3 and Cy5, may be employed to fluorescently label as many as three different complex protein populations prior to mixing them together and running them simultaneously on the same 2D gel using a method referred to as difference gel electrophoresis (DIGE) (Fig. 4) [180,181]. Recently, a similar procedure utilizing Alexa Fluor dyes has been demonstrated as well [182]. Images of the 2D gels are acquired using as many as three different excitation/emission filters, and the ratio of the differently colored fluorescent signals is used to find protein differences among the samples. DIGE allows two to three samples to be separated under identical electrophoretic conditions, simplifying the process of registering and matching the gel images. Most often, the technology is used to examine differences between two samples (e.g., drug-treated versus control cells or diseased versus healthy tissue). Though seductive in concept, it should be noted that a few problems afflict DIGE technology, especially when endeavoring to perform quantitative proteomics analyses.

One requirement of DIGE is that only ~1–2% of the lysine residues in the proteins be fluorescently modified, so that the solubility of the labeled proteins is maintained during electrophoresis [180]. The solubility of the proteins is decreased considerably when higher degrees of substitution are employed, resulting in the detection of many fewer protein spots on 2D gels. This limited covalent labeling step sets the lower limit for fluorescence detection to 1–2% of its intrinsic limit, given an alternate fluorescence imaging technology that does not have this solubility constraint. In experimental terms, optimized DIGE technology is capable of detecting about half as many proteins as conventional silver staining, or about four times as many proteins as colloidal Coomassie Blue staining [181]. It is almost certain that the proteins missed by DIGE staining represent the lowest abundance species in the sample. DIGE technology is more sensitive than modified silver stain formulations optimized for mass spectrometry, though SYPRO Ruby dye staining detects 40% more protein spots than the Cy dyes [183].

While matched in charge, the dye-labeled proteins produced by DIGE technology migrate with slightly higher mass than the bulk of the unlabeled protein, due to the addition of the fluorophores [180,181]. This produces registration errors between the labeled and unlabeled proteins in 95% of the spots, and consequently complications arise when spots are to be excised and analyzed by mass spectrometry [181]. As a result, gels must be post-stained to aid in protein cutting for spot identification by mass spectrometry [181,183,184]. Regrettably, since colloidal Coomassie Blue staining is less sensitive than the

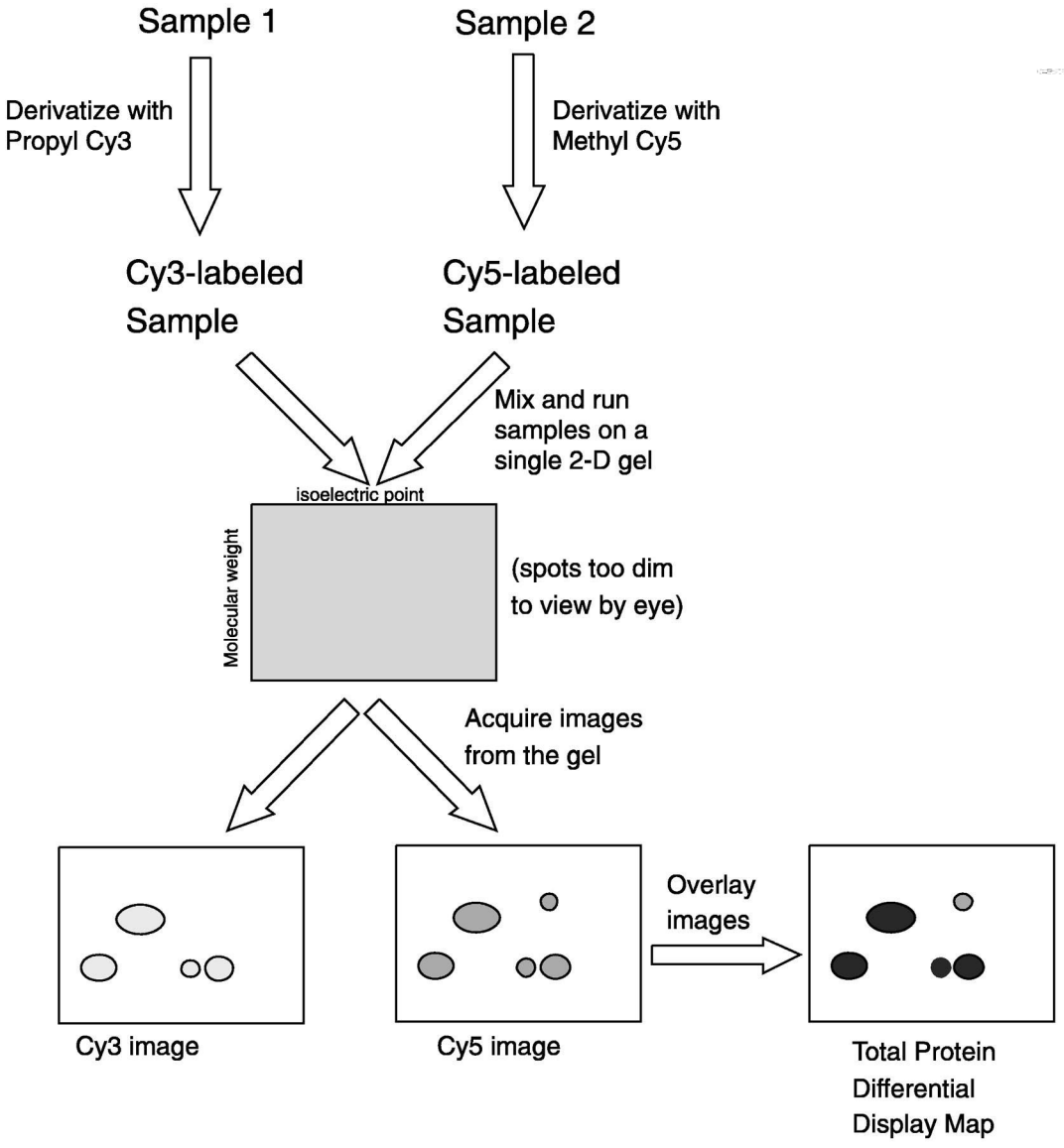

Fig. 4. Schematic illustration of the difference gel electrophoresis (DIGE) technology platform [180]. Two different samples are derivatized with two different fluorophores, combined and then run on a single 2D gel. Proteins are detected using a dual laser scanning device or xenon arc-based instrument equipped with different excitation/emission filters in order to generate two separate images. The images are then matched by a computer-assisted overlay method, signals are normalized, and spots are quantified. Differences in protein expression are identified by evaluation of a pseudo-colored image and data spreadsheet. DIGE technology can maximally evaluate three different samples using Cy2-, Cy3- and Cy5-based chemistries.

DIGE technology, 40% of the proteins of interest cannot be identified directly from the Coomassie Blue-stained gel [181]. Consequently, SYPRO Ruby dye has been adopted as the post-stain dye of choice for DIGE analysis [183,184]. Another issue to be aware of that can effect sensitivity of detection is that fluoresence cross-talk between excitation/emission band-pass filters may cause Cy5-labeled proteins to emit when illuminating at the optimal Cy3 excitation wavelength [185].

It has been demonstrated recently that 20% changes in very abundant proteins and 800% changes in scarce ones can be quantified reliably at the 90% confidence level using DIGE technology [181]. Thus, though the DIGE technology can be applied to the detection of qualitative changes in protein expression, quantitative changes in lower abundance proteins are likely to be overpowered by dye-dependent photophysical effects. A concrete illustration of the qualitative nature of DIGE technology may be found with regard to the study of protein changes in heart mitochondria of knockout mice lacking sarcomeric mitochondrial creatine kinase [186,187]. Mitochondrial creatine kinase plays a central role in cellular energetics of muscle, catalyzing the reversible transfer of the phosphoryl group between phosphocreatine and ADP [188,189]. Deletion of such a vital enzyme is expected to have proteome-wide repercussions, as cells are forced to adapt to the metabolic crisis. Conventional Western blot analysis has demonstrated loss of the target kinase, as well as 2–4-fold changes in a number of proteins, including mitochondrial cytochrome c oxidase, inorganic phosphate carrier, adenine nucleotide translocator and voltage-dependent anion channel proteins, consistent with the impaired cardiac energetics observed in these animals [187]. Proteome-wide analysis of the mitochondria by DIGE technology confirmed the complete absence of sarcomeric mitochondrial creatine kinase in the knockout mice, as well as a minor difference in the precursor form of aconitase, but surprisingly, no other large-scale changes in heart mitochondrial proteins were revealed by the technique [186]. Presumably, the quantitative changes identified in this model system by Western blotting could not be differentiated from background fluctuations associated with the DIGE labeling methodology.

Recent DIGE studies comparing the difference between normal and cancerous tissue were more successful in demonstrating changes in protein expression levels [183,184]. In a study comparing esophageal carcinoma cells with normal epithelial cells, statistical analysis revealed that 56 proteins were up-regulated by more than 3.5-fold and 39 were down-regulated by more than 4-fold [184]. Two changes, protein gp96 and annexin I, were confirmed by Western blotting. It should be noted that both validated changes were qualitative, with gp96 being absent from normal tissue and annexin I absent from tumor tissue. In a study comparing an ErbB-2-receptor tyrosine kinase transformed luminal epithelial cell line to its untransformed counterpart, 135 proteins with a 1.3-fold average difference in abundance were identified. Though many of these proteins were identified by mass spectrometry, none of the expression changes were confirmed by an independent method, such as Western blotting.

Another important issue with respect to the DIGE technology is that it is predicated upon an overly simplistic tenet that all of the information required in an analysis can be distilled down into a single 2D gel using a simple control versus treated state experimental design. However from a biology perspective, the analysis of drug dose–response curves or the kinetics of changes in tissue/cell proteomes require that multiple 2D gels be quantified. The assessment of different stages of cancer progression and epidemiological studies of global protein expression patterns in populations also require many more than the two to three sample capacity of the DIGE technique. Therefore, multiple 2D gels almost always must be quantified and referenced to one another, regardless of the method employed to detect the proteins. This necessity to quantify multiple 2D gels effectively contradicts the whole premise behind the DIGE detection technology. This shortcoming has been recognized by some, and an innovative method for linking multiple 2D gels generated by DIGE technology has recently been devised [183]. Cy2-labeled proteins are used as internal standards with all Cy3- and Cy5-labeled sample pairs to aid in quantitative comparisons between gels in a database.

Even if the biological problem can realistically be framed as a simple binary experimental/control study, it should be noted that DIGE technology cannot reliably identify differences between two samples using a single 2D gel. In an analysis of six two-color DIGE gels comparing toxin-treated to control liver homogenates, a total of 138 protein changes were identified [181]. Of these 138 differences, however, only 4.4% (eight differences) were observed in all six gels. Thirty percent (41 differences) were finally nominated as real changes in protein abundance, as they occurred in three or more gels. The authenticity of the changes was not corroborated using any independent measurement technique such as Western blotting, however.

Radiolabeling is probably the only method that can compete with fluorescence in terms of linear dynamic range and detection sensitivity. However, radio-imaging procedures for *differential display* such as the $^{125}I/^{131}I$ multi-photon detection (MPD) method devised by BioTraces (Herndon, VA) suffer from the same experimental design limitations as the fluorescence DIGE technology. The inherent slowness of radioactive 2D gel imaging also severely limits this technology. Currently, 2.5 days are required to collect an image with attomole detection sensitivity. Throughput might be improved in a proteomics work environment by employing multiple imaging devices, but the instruments are expensive to manufacture, costing around US $100 000 apiece. By contrast, a typical 2D gel fluorescently labeled with a total-protein dye, such as SYPRO Ruby protein gel stain, may be imaged in 0.1–2 s on a wide range of imaging platforms, approximately 200 000 times faster than radiolabel imaging.

5.2. Multiplexed proteomics (MP)

The multiplexed proteomics (MP) platform is designed to allow the parallel determination of protein expression levels as well as certain functional attributes of the proteins, such as levels of glycosylation, drug-binding capabilities or drug-metabolizing capabilities (Fig. 5). The MP technology platform utilizes the same fluorophore to measure proteins across all gels in the database, and employs additional fluorophores with different excitation and/or emission maxima to accentuate specific functional attributes of the specimen. As stated previously, biological investigations usually require large numbers of 2D gels and methods are required that facilitate the rigorous and reliable matching of proteins across a database. A clear advantage of MP technology over DIGE technology is that it provides a much broader bandwidth of information. A limitless number of gels may be evaluated with respect to a number of distinct attributes.

With the MP platform, a set of 2D gels is fluorescently stained and imaged to reveal some functional attribute of the proteins, such as drug-binding capability, or a particular post-translational modification. Then, protein expression levels are revealed in the same gels using SYPRO Ruby protein gel stain as the foundation staining technology. Differential display comparisons are made by computer, using image analysis software, such as Compugen's Z3 program [190]. All gels are imaged using the same excitation/emission filter sets and resulting images are then automatically matched, with the option of adding some manual anchor points to facilitate the process. Any two images can then be re-displayed as a single pseudo-colored map, permitting the visual inspection of differences in an identical manner as performed with DIGE technology. In addition, quantitative information can readily be obtained in tabular form, with differential expression data calculated. One of the strengths of the platform is that similar profiles, such as total protein patterns, are matched by computer from different gels, while dissimilar ones, such as total protein patterns and glycoprotein patterns, are matched by computer from the same gel. Thus, though few landmarks are shared between total protein profiles and the assayed functional property, the images may be matched by superimposition anyway.

A recently published study demonstrates the feasibility of the MP platform, describing methods to assay for the presence of glycans, followed by the detection of the total protein profile [85]. Pro-Q Emerald 300 dye, a sensitive green-fluorescent glycoprotein-specific stain is employed, having non-overlapping spectral properties with the red-fluorescent total protein stain, SYPRO Ruby dye [85]. This hydrazide dye may be affixed to glycoproteins using a periodic acid Schiff's (PAS) reaction under very mild coupling conditions. Proteins may then be post-stained with SYPRO Ruby dye, allowing sequential two-color detection of glycosylated and non-glycosylated proteins, an unprecedented new capability in proteomics research.

Other functional attributes of protein samples that may be detected in 2D gels with subsequent post-staining by SYPRO Ruby dye include the detection of β-glucuronidase activity using ELF 97 β-D-glucuronide, the detection of penicillin-binding proteins using fluorescent Bocillins, and the detection of oligohistidine-tagged proteins using Pro-Q Sapphire dye [147,191]. These examples illustrate four areas in which the MP platform can find application: analysis of post-translational modifications, enzymatic activity, small ligand binding and epitope tags. Other procedures for detecting hydrolase activi-

ty, and cell surface proteins are actively under development.

5.3. Isotope-coded affinity tagging (ICAT)

Isotope-coded affinity tag (ICAT) peptide labeling is predicated upon distinguishing between two populations of proteins using isotope ratios [192–196]. Isotope-coded affinity tags are reagents consisting of a protein-reactive group, an ethylene glycol linker region and a biotin tag. Currently, the commercialized ICAT reagent employs a reactive functionality specific for the thiol group of cysteine residues in proteins and peptides. Two different isotope tags are generated by using linkers that contain either eight hydrogen atoms (d0, light reagent) or eight deuterium atoms (d8, heavy reagent). A reduced protein mixture from one protein specimen is derivatized with the isotopically light version of the ICAT reagent, while the other reduced protein specimen is derivatized with the isotopically heavy version of the ICAT reagent. Next, the two samples are combined, and proteolytically digested with trypsin or Lys-C to generate peptide fragments. The combined sample is then fractionated by avidin affinity chromatography, thus retrieving only the cysteine-containing peptides. The affinity-purified mixture of peptides contains roughly 10-fold fewer peptides than the original sample, thus simplifying analysis. The peptides may be further separated by microcapillary reversed-phase liquid chromatography, followed by mass spectrometry. The ratio of the isotopic molecular mass peaks that differ by 8 Da, as revealed by mass spectrometry, provides a measure of the relative amounts of each protein from the original samples. Typically, nanoscale liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) provides quantitative information based upon the relative abundances of the heavy and light peptides, while nanoscale liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) provides qualitative information based upon the peptide molecular mass and amino acid sequence information [196].

Though quantitation by mass spectrometry is often unreliable, due to ion suppression phenomena, ICAT reagents are chemically identical and differ in mass by only eight neutrons. Nevertheless, it has been suggested that the background of co-eluting peptide pairs does vary in composition and concentration and thus quantification of the peptides occurs in different matrix environments [197]. The deuterated and non-deuterated peptides often separate from each other during liquid chromatography, and are ionized at different times in different matrixes. This can strongly impact the ionization efficiency of peptides across a chromatographic peak, leading to errors in quantitation. In addition, the ICAT tag is a relatively large molecule compared to the size of some small peptides and it has been suggested that the tag may interfere with peptide ionization, leading to complications in the interpretation of mass spectra [198].

One significant obstacle to the routine implementation of ICAT technology in the study of biological systems is that proteolytic digests of cells or tissues are exceedingly complex, with literally hundreds of thousands of peptides generated in a sample [196]. Though isolation of cysteine-containing peptides reduces sample complexity perhaps 10-fold, other fractionation methods are usually required to reduce the number of peptides delivered to the mass spectrometer further. Recently, the microsomal fraction of control versus differentiated HL-60 human myeloid leukemia cells were evaluated by the ICAT method [199]. After labeling with heavy and light ICAT reagents and combining the samples, the peptide mixture was subjected to cation-exchange chromatography, followed by avidin affinity chromatography, a multidimensional chromatographic approach. Finally, peptides were separated and analyzed by LC–ESI-MS. A total of 491 proteins were unambiguously identified and the ratio of each protein between control and differentiated cultures was determined.

One weakness of the current ICAT method is that it requires that the proteins contain cysteine residues flanked by appropriately spaced protease cleavage sites [198]. The problem has recently been highlighted in the study of the multi-subunit membrane protein, *Escherichia coli* F_1–F_0–ATP synthase [200]. This enzyme consists of distinct extramembranous and transmembranous components of known subunit stoichiometry, and thus provides a good model for the combination of hydrophobic and hydrophilic proteins normally encountered in proteomics studies. The membrane-associated F_0 component is composed of three subunits, a, b, and c, polypeptides with five, one and two transmem-

brane domains, respectively. Labeling with the cysteine-reactive fluorophore, monobromobimane, failed to detect four of the eight subunits of ATP synthase (Fig. 6). None of the membrane-embedded proteins in the F_0 complex were visualized. Genome-wide analysis of cysteine distribution in *Escherichia coli* demonstrates that the cysteine-labeling problem does not arise from a peculiar choice of model protein complex. In the *Escherichia coli* genome, the number of proteins containing no cysteine residues is high (~10–15%), obviating the use of any cysteine-specific technology as a total-protein indicator.

This cysteine-labeling problem may be overcome by devising different ICAT reagents, reactive for other amino acid residues in proteins. Indeed, several alternative isotope tags have been devised that label all proteolytic fragments in a sample. A method based upon the growth of cells in either ^{14}N- or ^{15}N-enriched medium has been described for quantifying changes in protein expression by 2D gel electrophoresis and mass spectrometry [199]. Likewise, regular water ($H_2^{16}O$) and heavy water ($H_2^{18}O$) have been employed as the solvent during Glu-C proteolysis of samples, leading to the incorporation of two ^{18}O or two ^{16}O atoms in the C-terminal moiety of each proteolytic fragment [201]. This results in a 4-Da difference in mass between paired peptides. Acetate (d0) and trideuteroacetate (d3) have been employed to acetylate primary amino groups in peptides [202]. Peptide digests are subsequently simplified using copper-immobilized metal affinity chromatography to selectively capture histidine-containing peptides. Alternatively, lectin affinity chromatography is employed to selectively analyze glycoproteins. Similarly, methyl esterification of aspartate and glutamate residues using regular methanol (d0) or trideuteromethanol (d3) has been used as an isotope tagging strategy [203]. Finally, standard 1,2-ethanedithiol (d0) and tetraalkyl deuterated 1,2-ethanedithiol (d4) have been employed to measure differences between *O*-phosphorylation sites in samples using β-elimination chemistry [111]. The pendant sulfhydryl group is then reacted with biotin iodoacetamidyl-3,6-dioxaoctanediamine. Just as with the original cysteine-reactive ICAT reagent, the phosphate-labeling procedure allows enrichment of target peptides using avidin affinity chromatography.

Recently, it has been demonstrated that the advantages of 2D gel electrophoresis and ICAT labeling technology can be combined into a single differential display platform (Fig. 7) [194]. This method closely parallels the DIGE methodology, with some im-

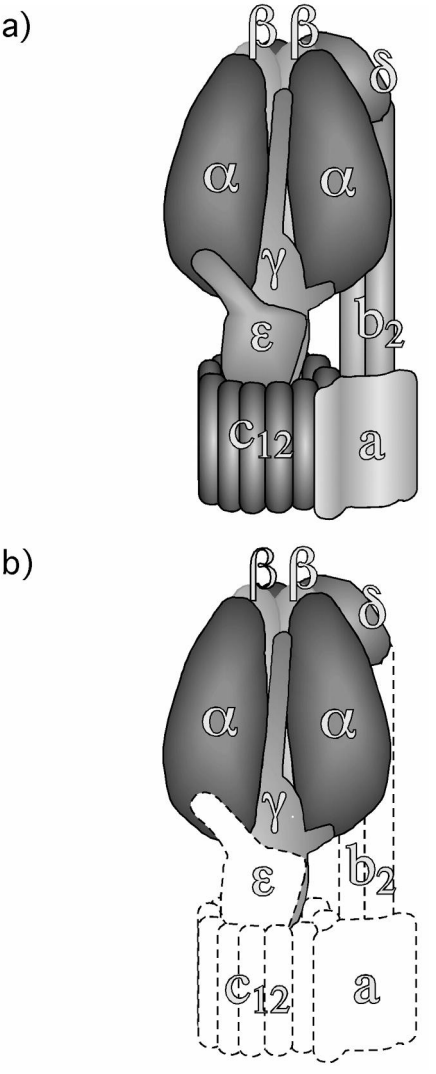

Fig. 6. Subunits of F_1F_0–ATP synthase revealed by two different detection methods [200]. (A) Subunits revealed using a basic amino acid binding dye, SYPRO Ruby protein gel stain. (B) Subunits detected using a thiol-reactive fluorophore, monobromobimane. Thiol-reactive chemistries, such as monobromobimane and ICAT labeling do not globally detect all proteins in a cell or tissue. Roughly 10–15% of the polypeptides escape detection. Secreted proteins and certain plant lectins tend to have a higher than average number of cysteine residues, while polypeptides that are components of multi-subunit protein complexes tend to have lower than average number of cysteine residues. Figure courtesy of Dr. Birte Schulenberg, Molecular Probes.

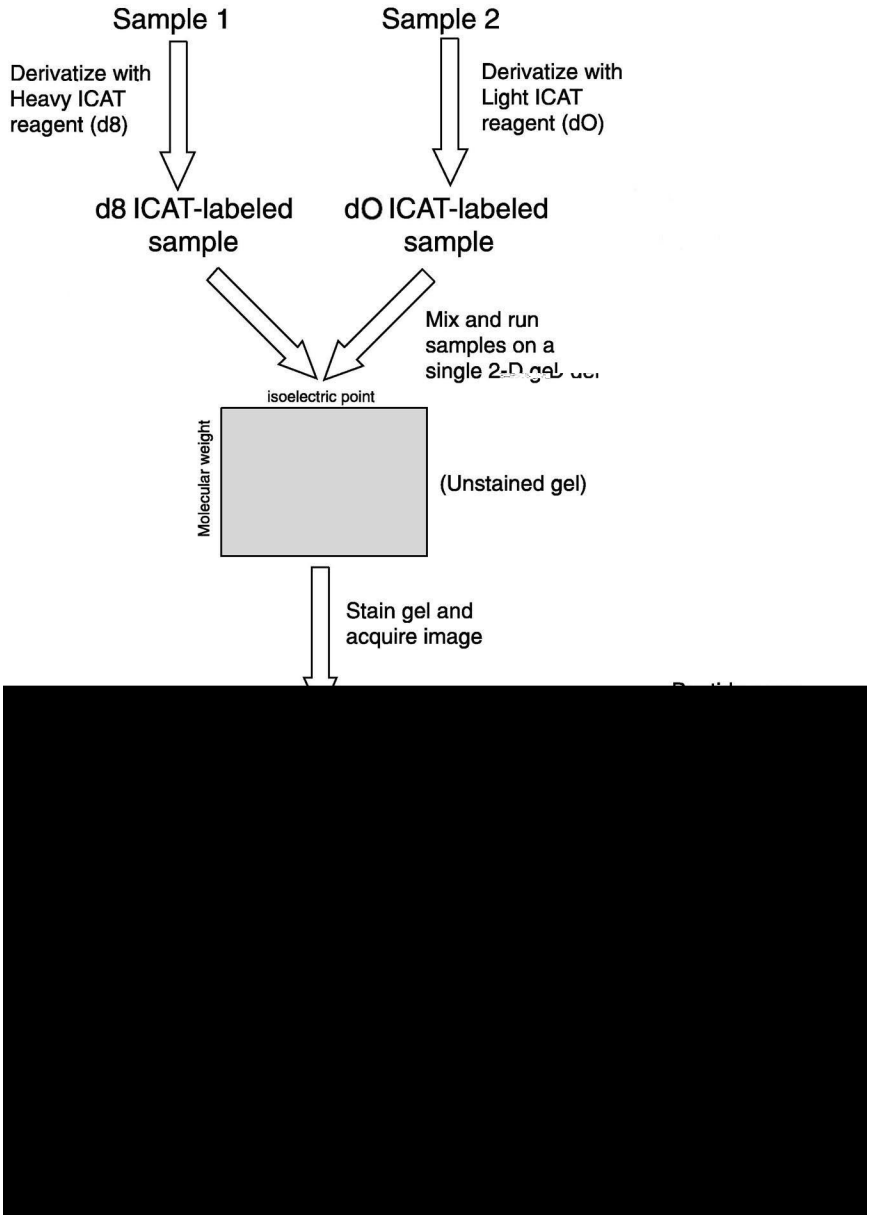

Fig. 7. Schematic illustration of the iosotope-coded affinity tagging (ICAT) technology platform [194]. Two different samples are derivatized with two different ICAT reagents; heavy (deuterated) and light (normal). The samples are combined and then run on a single 2D gel. Proteins are detected using a sensitive mass spectrometry-compatible protein stain, such as modified silver stain or SYPRO Ruby protein gel stain. Proteins are excised from the gel, enzymatically digested in the gel matrix, extracted and analyzed by mass spectrometry. Proteins are identified by peptide mass fingerprinting using a single stage mass spectrometer or by collision-induced dissociation of selected peptides and database sequence searching. Differences in protein expression between individual proteins in the two samples are determined by evaluation of the ratio of signal intensities for the isotopically normal and heavy forms of a specifically tagged peptide. As illustrated, every spot in the profile must be evaluated by mass spectrometry and cysteine-containing peptides identified. Alternatively, three gels can be run containing heavy isotope-labeled material, light isotope-labeled material and a mixture of the two. The first two gels are used to generate difference maps using a sensitive protein stain and computer-based matching software. The third gel is then used to excise differences identified from the first two gels.

portant improvements. It is also quite similar to the $^{14}N/^{15}N$ labeling procedure [109]. Unlike the $^{14}N/^{15}N$ labeling scheme, the ICAT method is a post-isolation isotopic labeling approach that does not require cells to be cultured in specialized media. Proteins from two different samples are labeled with heavy and light ICAT reagents, combined and then separated by 2D gel electrophoresis. The gel-separated proteins are detected with a sensitive protein stain, excised, treated with protease and identified by peptide mass profiling. Additionally, selected peptides can be evaluated further using collision-induced dissociation (CID) and sequence database searching. As with DIGE, the ICAT labeling decreases the electrophoretic mobility of proteins, due to addition of 422 Da at every cysteine residue. Since cysteine residues are modified with a neutral ICAT group, the isoelectric point is preserved for all but the most basic proteins. While DIGE requires minimal labeling with the hydrophobic cyanine dyes, ICAT labeling must be performed quantitatively and to completion in order to avoid generating molecular mass ladders of spots with varying degrees of substitution [184,185]. This is readily accomplished using excess labeling reagent and since the ICAT reagent is relatively hydrophilic, migration problems do not arise during electrophoresis. Of course, the biotin component of the tag is superfluous in this application and smaller cysteine-reactive reagents could be employed, as long as a normal and deuterated species are used. One important application of ICAT differential display in 2D gels is for the assessment of the relative abundances of protein isoforms that arise from post-translational modification. This utilizes a similar strategy as originally employed with the $^{14}N/^{15}N$ labeling procedure [109].

6. Conclusions

The pace of technological innovations in protein detection has markedly accelerated in the past 4 years. Undoubtedly, this is partly due to a shift in the focus of the research enterprise from genomics to proteomics, spurred by the completion of the human genome project. Methods for the global analysis of protein abundance, protein post-translational modifications and changes in these two parameters as a function of the biological state of the cell or tissue are being developed utilizing electrophoresis, chromatography, and/or mass spectrometry as key components of the detection platforms. Methods for the differential display analysis of protein populations that parallel the capabilities of mRNA-based approaches are actively under development. Gel electrophoresis remains a formidable analytic tool that has found wide application in a variety of fields, ranging from toxicology, and cancer discovery research to quality control and clinical screening of samples. Though efforts to displace gel-based electrophoretic techniques with alternatives, such as solution-phase, microarray, capillary electrophoresis and microfluidic devices are underway, gel electrophoresis-based technology remains attractive due to its simplicity, reliability, higher information content and ready accessibility to researchers [204].

Acknowledgements

The author thanks Richard Haugland, Joseph Beechem, Tom Steinberg, Kiera Berggren, Courtenay Hart, Birte Schulenberg, Karen Martin, Brad Arnold, Mark Lim, Negin Shojaee and Lily Lam for numerous contributions to the development of metal chelate detection strategies over the past 8 years.

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