

REVIEW

Protein stains for proteomic applications: *Which, when, why?*

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This review recollects literature data on sensitivity and dynamic range for the most commonly used colorimetric and fluorescent dyes for general protein staining, and summarizes procedures for the most common PTM-specific detection methods. It also compiles some important points to be considered in imaging and evaluation. In addition to theoretical considerations, examples are provided to illustrate differential staining of specific proteins with different detection methods. This includes a large body of original data on the comparative evaluation of several pre- and post-electrophoresis stains used in parallel on a single specimen, horse serum run in 2-DE (IPG-DALT). A number of proteins/protein spots are found to be over- or under-revealed with some of the staining procedures.

Received: April 28, 2006

Revised: June 14, 2006

Accepted: June 14, 2006

Keywords:

Compatibility / Detection limit / Dynamic range / Fluorescence / Visible light (spectro)-photometry

1 Introduction

Figure 1 draws a sketchy flow sheet of the various steps in a proteomic investigation. 'Detection' precedes and is prerequisite to obtaining qualitative and quantitative data on the proteins in a sample, as much as to comparing the protein composition of different samples. 'Detection' is often synonymous to staining, *i.e.*, the reversible or irreversible binding by the proteins of a colored organic or inorganic chemical; however, various approaches, based on diversified principles, have been optimized for different purposes.

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Abbreviations: **AB**, Amido Black; **BPSA**, bathophenanthroline disulfonate; **MDPF**, 2-methoxy-2,4-diphenyl-3(2H)furanone; **PAS**, periodic acid-Schiff; **PR**, Ponceau Red; **RuBP**, ruthenium II tris (bathophenanthroline disulfonate); **TMB**, monobromobimane

In this manuscript we aim to review the main procedures outlined in Fig. 1, while calling attention to the rationale for each alternative selection. We also focus on the performance of various stains, both by listing literature records and evaluating quantitative data (sensitivity, reproducibility) produced in our laboratories when processing one sample in parallel through different staining protocols.

Figure 1 contains some reference to immunological procedures. These will not be dealt with in this review but need to be mentioned as the main alternative and complement to staining with organic dyes. Immunological detection may be directed towards the protein moiety as a whole (when using polyclonal antibodies) or towards an amino acid stretch or 3-D structural motif (epitope-specific mAb) or towards a PTM [either in the context of a given protein (protein variant specific) or irrespective of the context (group specific)]. These various combinations fulfill most of the requirements for identification, quantitation and characterization of individual proteins of known sequence and of their PTM variants.

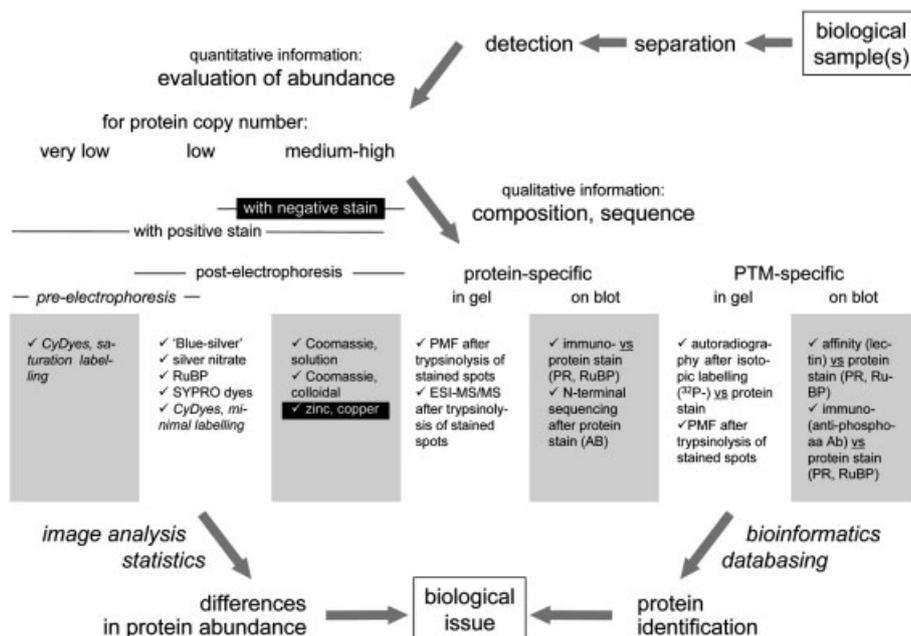


Figure 1. Overview on protein detection procedures in a proteomic experiment based on 2-DE. The relationships between qualitative and quantitative assessment of a sample's components are emphasized. Examples are given for the main alternative procedures. aa, amino acid; AB, amido black; PR, Ponceau Red, RuBP, ruthenium II tris (bathophenanthroline disulfonate) or other reversible stain.

If 'detection' is taken in a still broader sense (Fig. 2), radioactive labeling, *in vivo* or *in vitro*, is another general approach to pattern imaging (top panels). The *in vivo* protocols using isotopically labeled amino acids selectively reveal *de novo* synthesized polypeptide chains. High specific activity is easier to attain with abundant amino acids; β -emission by ^3H and ^{14}C , however, is weak (slow), so that fluorographic

procedures or (costly) storage phosphor screen technology are required for data acquisition. ^{35}S is a harder emitter but the percent abundance of sulfur-containing amino acids in proteins is very low. Most PTM may be monitored through radiolabeling, but due to safety (and safety-related economic) considerations, alternative protocols to the use of radiochemicals are currently preferred whenever possible.

A number of chemical (bottom panels) and biochemical (middle panels) procedures for the assessment of various PTM rely on the comparison of physicochemical properties (*pI* and *pI* microheterogeneity, M_r) of the test proteins after the modification has been prevented by specific *in vivo* inhibition or removed by specific *in vitro* cleavage versus reference conditions.

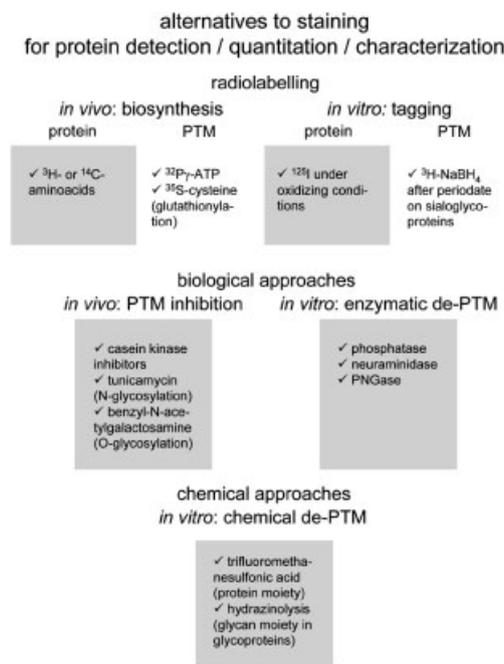


Figure 2. Overview on protein detection procedures other than staining in a proteomic experiment based on 2-DE. Examples are given for each of the alternative options.

2 General protein stains

2.1 Staining in gels

Table 1 compiles the most commonly applied general protein stains and their sensitivity ranges.

2.1.1 Post-electrophoresis, positive stain, visible light

2.1.1.1 Coomassie Blue and Fast Green protocols

2.1.1.1.1 CBB solutions with solvents

CBB was introduced for protein detection in 1963 [1] and is today still frequently used in electrophoresis. CBB is a disulfonated triphenylmethane textile dye of which two mod-

Table 1. Selected staining methods in 2-DE

Staining method	Detection mode	Detection limit (ng)	Linearity range (orders of magnitude)	MS compatibility
<i>Post-electrophoretic stains:</i>				
CBB-R	Colorimetry	8–10 [7] 50–100 [6]	1–1.3	+
CBB-G (colloidal)	Colorimetry	8–10 [95] 10–20 [6] 30–100 [7]	3 [95] 1–1.3 [7]	+
Silver nitrate (acidic methods)	Colorimetry	1 [7, 95] 3–5 [95]	2 [95], 1 [7]	+ (if without glutaraldehyde) [6]
Silver ammonia (alkaline methods)	Colorimetry	<1 [6] 5–10 [6]		– [6] + [55]
Zinc imidazole	Colorimetry	10 [85, 95] 1 [86]		+ [85]
SYPRO® Ruby	Fluorescence	1 [6, 95] 1 [7]	3 [95] >3 [7]	+
SYPRO® Orange, Red, Tangerine	Fluorescence	4–8 [7] 30 [95]	3 [95] 3 [7]	+ [61]
Epicoccone (Lightning Fast®, Deep Purple®)	Fluorescence	<1 [80]	4 [80]	+ [80]
<i>Pre-electrophoretic stains:</i>				
CyDyes® minimal labeling	Fluorescence	0.1–0.2 [95] 2 [7]	3–5 [95]	+
CyDyes® saturation labeling	Fluorescence	0.005–0.01 [95]	3–5 [95]	+ [95]
FlaSH ^{Pro} ® Dyes	Fluorescence	2–3 ^{a)}		+

a) http://www.raytest.de/bio_imaging/products/reagents/flashpro.html.

ifications exist: Coomassie R-250 (reddish tint) and G-250 (greenish tint, dimethylated form). In acidic solutions the dye sticks to the amino groups of the proteins by electrostatic and hydrophobic interactions. The original protocols use stains with up to 1% CBB in the presence of an alcohol (methanol, ethanol, or isopropanol, up to 45%) and acid (acetic acid, trichloroacetic acid, for the latter in cases of IEF sometimes with addition of sulfosalicylic acid). Destaining is performed with a similar solution devoid the dye [2–5]. The proteins have a higher affinity for the dye than the gel matrix, therefore destaining can be carried out until the background is low and protein bands are clearly visible. CBB staining is easy to use, linear over at least one order of magnitude and MS compatible. In the early days of MS one used to say that CBB-stained spots (detection limit 10–100 ng [6]) contained just the right amount of protein needed for identification. For quantification, it provides a linear response with protein amounts over a 10–30-fold concentration range [7].

CBB R was the first dye to be used in early proteomics applications, e.g., the *E. coli* proteins separation by O'Farrell [8] and the human serum protein map [9]. It is still a valuable tool in applications where protein concentrations are high

enough, as some selected recent examples show [10–12]. Reproducibility of staining was determined in a study on mouse liver proteins by evaluating 137 spots: almost half of them had a variation coefficient of less than 10%, more than three quarters had less than 15% [13]. Comparable results were found investigating CBB R stained rat serum protein patterns of healthy animals [14]. In the latter case, 2-DE evaluation was only performed on gel series which had been processed in the same batch (*i.e.*, from IPG to 2-DE, staining and scanning) to keep variation levels as low as possible. This also prevents potential problems arising from staining/destaining, which are processes difficult to control. Proteins destain to varying extents along with the gel matrix, but with slower kinetics. The exception are some proteins, e.g., collagen, which in the presence of solvent fade more quickly than gel background [15].

2.1.1.1.2 Colloidal CBB

Diezel *et al.* [16] introduced CBB G, dissolved in TCA, for protein staining, intensified by storage in dilute acetic acid. They observed less background staining and explained this

fact by the apparent conversion of CBB G in 12.5% TCA into a colloidal state. A very extensive study by Neuhoff *et al.* [17] on more than 600 variations arrived at a protocol that is the basis of almost all colloidal CBB G staining methods, including a number of commercial kits. Staining of proteins on a clear background can be performed with phosphoric acid and CBB G in the presence of ammonium sulfate. During staining equilibrium is achieved between colloidal particles and freely dispersed dye in solution. The low concentrated free dye enters the gel matrix and preferentially stains the proteins, while the colloidal dye particles are excluded, avoiding background staining. By transferring the dye completely into its colloidal form, it is possible to stabilize the protein-dye complex, allowing prolonged storage without any loss of protein staining (leaching is known from CBB gels stained in the presence of solvent). The original recipe was later modified by reintroducing methanol in the staining solution, to shift the equilibrium from the colloidal form to the molecularly dispersed dye, to facilitate and speed up the staining process [18]. A similar protocol was also tested for CBB R, but is less sensitive [18]. In general, colloidal CBB G staining is regarded as more sensitive than CBB R in solvent solutions (see Table 1).

Colloidal CBB G according to Neuhoff was also applied by Anderson *et al.* [19] for creating a rat liver protein 2-DE database, as a prerequisite for gene regulation and drug effects studies. After fixing and washing steps, gels were kept in staining solution for 4 days, to achieve optimal staining. Similar staining recipes, sometimes slightly shortened, have also been used for establishing protein maps [20–22] or for monitoring protein level changes [23].

The “blue silver” staining described by Candiano *et al.* [24] was reported to be more sensitive than the colloidal CBB G stains, with a detection limit of 1 ng for BSA. It differs from the original Neuhoff stain [18] by a 20% increase in dye and a 5-fold concentration of phosphoric acid. Staining occurs faster and is said to be more intensive due to lowering the pH, which enables more comprehensive protonation of Asp and Glu residues, followed by considerable hydrophobic association with the aromatic and hydrophobic residues along the polypeptide backbone. Despite the name, it is not as sensitive as silver staining.

Recently, it has been suggested that CBB fluorescence could be utilized by scanning gels with an infrared laser scanner. An increase in sensitivity of two orders of magnitude has been reported, thus making CBB at least twice as sensitive as SYPRO Ruby [25].

The binding of CBB to proteins and protein complexes led to another application, the “blue native electrophoresis”. CBB included into the cathode buffer (in modified systems of Laemmli [26], Swank and Munkres [27] or Schaeffer and von Jagow [28]) visualizes the migration/separation of protein zones during the run. The method is especially useful for protein complexes and allows subsequent elution of separated complexes from the gel [29]. Combination with a second dimensional run with a different separation criterion (*e.g.*, SDS-PAGE) is possible [30].

2.1.1.1.3 Fast Green FCF

Another “old generation” stain is the organic dye Fast Green FCF. In contrast to the at that time frequently used amido black (AB), it does not stain proteins multichromatically. It is of similar sensitivity and can be used for protein detection after native PAGE, SDS-PAGE and IEF, also for alkaline proteins [31, 32]. It also belongs to the triphenylmethane dye family, but, unlike CBB R, it does not bind to ampholytes and can, therefore, be used as a stain for IEF gels also without separate protein fixation and ampholyte removal step. In quantitation, Fast Green proved slightly less sensitive than CBB, but showed a broader linear range (two orders of magnitude [32, 33]).

2.1.1.2 Silver nitrate protocols

The first silver stains go back to the early ‘80s [34–36]. 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.

Today, more than 100 different variants of silver-staining protocols exist for proteins separated in polyacrylamide gels. However, in general, there are two large categories: alkaline and acidic silver stains, depending on the conditions used for silver impregnation. Alkaline methods work with a diamine complex of silver nitrate in a highly alkaline environment (ammonia and sodium hydroxide). Patterns are then developed in dilute acidic solutions of formaldehyde ([37] and <http://us.expasy.org/ch2d/protocols/protocols.fm4.html>). Acidic methods use silver nitrate in water (weakly acidic solutions) for gel impregnation and a development step in formaldehyde solutions at alkaline pH [38–40]. Silver-staining protocols are multi-step procedures which have four main steps in common (apart from numerous washing steps): (i) fixation, to insolubilize the proteins and to remove interfering compounds present in the gels, (ii) sensitization, to increase the subsequent image formation, (iii) silver impregnation with silver nitrate or ammoniacal silver, and (iv) image development in dilute carbonate (for acidic stain) or citric acid (for alkaline stain), both with minor but essential amounts of formaldehyde. Image formation is stopped with appropriate solutions and preserved.

Different protocols exist and selection among them will largely depend on specific needs (reproducibility, speed, uniformity of staining/multi-color, post-separation analyses, see [6, 41]). Influence of different compounds and variations in the protocols on the outcome of the staining is discussed in detail in [42]. Ammoniacal staining is said to be more sensitive for basic proteins [43], but ammonia concentration (storage) is a crucial point for reproducibility. It is not compatible with all SDS gel systems and works best with piperazine diacrylamide as a cross-linker and thiosulfate polymerized into the gel [41, 44].

Acidic silver nitrate staining may also be used for IEF and SDS-PAGE applications that use plastic-backed gels. Direct comparisons of alkaline and acidic approaches are rare [43].

Because it is not an endpoint procedure, the staining intensities can vary from gel to gel. Depending on the protocol, 100 pg to 1 ng protein can be detected per spot [15]. Unfortunately, silver staining shows only a narrow dynamic range (one to two orders of magnitude) and thus is not very reliable for quantification. Furthermore, the poor linearity of classic silver-staining technique means that, unless carefully controlled, saturation and negative staining (“doughnut effect”) of highly abundant spots is commonly observed [43, 45]. These saturation effects can be challenging for subsequent evaluation (see Section 4.2.3). Nevertheless, with careful optimization of the 2-D system, even quantitative evaluation is possible (a 30% RSD of normalized values was reported for a chicken brain embryo homogenate using a strictly standardized protocol [46]). Even better results were shown for an ammoniacal silver stain of human leukocyte proteins. Reproducibility between replicate patterns was below 10% for 27–30% of the spots, and below 15% for about 50% [47]. Devices have been constructed for automated staining, to help to reduce error level [48]. Recently, a time-based analysis method that negates the saturation effects inherent with silver staining has also been described [49], and results in an improved dynamic range.

In general, silver staining detects the proteins mainly on the gel surface. This is also the reason why further analysis of silver-stained proteins is still possible. There are specific protocols for acidic silver staining to make the procedure compatible to MS applications (generally, by omission of glutaraldehyde in the sensitizing solution and formaldehyde in the silver nitrate solution [50], sometimes with more formalin in the developer). Classical silver staining with formaldehyde in the developer gives a much lower sequence coverage than the less sensitive CBB, reverse Zn-imidazole stain or ruthenium II tris (bathophenanthroline disulfonate, BPSA) (RuBP) [51, 52], even when a silver-destaining protocol with ferricyanide [53] or hydrogen peroxide [54] is used prior to MS. There has been an attempt to replace formaldehyde by carbonylhydrazide or related substances, but these methods gave high background staining [52]. Interference by formaldehyde increases with time between silver staining and gel processing, even upon storage in water, probably by cross-linking between reactive amino acid side chains (lysine, cysteine, to a lesser extent also serine and threonine [52]). Ammoniacal silver stains have been said to be not MS compatible [6], but recently a modified procedure has been described, working with naphthalene disulfonic acid in the fixing solution and formaldehyde only in the developer. For selected samples, peptide numbers found with acidic and ammoniacal silver stains, RuBP and the Shevchenko method have been compared [55].

2.1.2 Post-electrophoresis staining with fluorescent dyes

Several dyes have been reported for fluorescence staining of proteins either before or after electrophoresis (reviewed in the introduction of [56]). Fluorescamine was utilized more than 25 years ago to stain 2-D patterns of nonhistone chromatin proteins [57]. Its sensitivity has been found to be comparable to CBB, but, as it detects free amino groups, it may produce different patterns. A related compound, 2-methoxy-2,4-diphenyl-3(2H)furanone (MDPF), is more sensitive and more stable [58], and has also been used for 2-DE gels [56].

Today, there are a number of noncovalent fluorescent dyes for electrophoretic applications, mainly the SYPRO dyes developed by Molecular Probes (Eugene, OR, USA). The early SYPRO dyes, *e.g.*, SYPRO Red and Orange, bind to the detergent coat surrounding proteins in SDS denaturing gels; thus, staining in such gels is not strongly selective for particular polypeptides [59, 60]. Staining is a one-step procedure after electrophoresis, but dye can also be included in the running buffer so that only a short destaining step is required after the run. Excitation is either with UV light (approx. 300 nm) or at visible wavelengths, allowing a variety of detection devices and combination with successive glyco-, phospho- or general protein stains [61]. Depending on running conditions and dye, 1–10 ng may be detected, and staining is reversible. Coomassie Fluor Orange, SYPRO Orange, and SYPRO Red are chemically similar and need dilute acetic acid for staining. SYPRO Tangerine can also be used in non-fixative solutions, thus permitting subsequent electroblotting, electroelution, and zymography [61].

SYPRO Ruby is a ruthenium metal chelate that binds to the basic amino acids in proteins. In contrast to other SYPRO dyes, there is no intercalation into SDS micelles; binding is via direct electrostatic interaction with basic amino acid residues by a mechanism similar to colloidal CBB. The staining procedure is quite simple and allows high-throughput and large-scale proteomic applications. Excitation is achieved either with UV light or with laser scanners. It is as sensitive as the best silver-staining methods, but superior to them in term of ease of use, linear dynamic range (three orders of magnitude) and compatibility with downstream microchemical characterization techniques [62–64]. The original formulation of SYPRO Ruby required fixation in acetic acid/methanol; a later developed formulation allowed more flexibility and showed an increased signal strength [65]. Critical aspects of fixation, washing, and staining have also been investigated by other groups [66, 67].

Lopez *et al.* [68] have found an almost 700-times larger integrated intensity range than with silver-stained gels, but similar staining mechanisms. Only 10% of protein spots from rat fibroblast lysates differed markedly in staining intensities/properties. A detection limit of 0.5–5 ng was reported for purified recombinant proteins, depending on

the particular protein [69]. Similarly, the linearity of the relationship between protein level and SYPRO Ruby staining intensity is protein dependent, with observed linear dynamic ranges of 200-, 500-, and 1000-fold for proteins analyzed by SDS-PAGE [69].

SYPRO Ruby can be used for multiplexed staining, in combination with fluorescence detection of glyco- or phosphoproteins (see Sections 3.1 and 3.2 [62, 70]) or metabolic labeling with ^{35}S [71]. It can also be used for protein staining on blots (see Section 2.2).

Besides the commercially available stain SYPRO Ruby, the synthesis and application of the metal chelate RuBP has been described and tested in detail [72]. Discussion about similarity/differences and performance of both stains is slightly controversial [65, 72] and also complicated by the fact that the original SYPRO Ruby formulation has been improved. In the meantime, a commercial RuBP stain is currently marketed, for which an optimized protocol has been reported, with a better signal-to-background ratio and improved baseline resolution [73].

None of the SYPRO dyes contains superfluous chemicals (formaldehyde, glutaraldehyde, Tween-20) that frequently interfere with peptide identification in MS and proteins are not chemically modified during staining. Proteins can thus be successfully identified by peptide mass profiling using MALDI-TOF and LC-MS/MS, with even better sequence coverage than using the conventional colorimetric stains [63, 64, 74–77].

Bell and Karuso [78] isolated a compound from the fungus *Epicoccum nigrum* useful as a fluorescent stain for polyacrylamide gels. Formerly named Lightning Fast, it is now distributed by GE Health Care under the trade name Deep Purple Total Protein Stain. The reactive component of the Deep Purple stain is epicocconone, a non-fluorescent azophilone that becomes fluorescent when it interacts with protein [79]. The stain is very sensitive, gives less speckles than SYPRO Ruby, and is linear over four orders of magnitude [80]. Smejkal *et al.* [25] report that its photostability is lower than that of SYPRO Ruby, resulting in a loss of 83% after 19 min under UV transillumination (compared to 44% of SYPRO Ruby). The primary mechanism of epicocconone binding is thought to be through hydrophobic interaction with the lipophilic tail of SDS bound to protein [80]. Differences in staining of various proteins have been reported and are explained by different SDS binding (of proteins at their *pI*, or of glycoproteins [25]).

2.1.3 Post-electrophoresis staining with negative stains

Negative or reverse stains are characterized by transparent colorless protein bands on a semi-opaque to white gel background. Lee *et al.* [81] reported negative images of this kind after immersing SDS-PAGE gels for 5 min in copper chloride, and, to a lesser degree, in copper sulfate. Sensi-

tivity was reported to be between that of CBB and silver, with very similar patterns. In nondenaturing gels, patterns were reversed and staining less sensitive. Proteins are not permanently fixed within the gels, therefore they can be quantitatively eluted after chelation of copper ions with EDTA. Similar properties were reported also for other heavy metal salts (*e.g.*, cobalt, nickel, zinc) with a sensitivity and reaction speed with zinc chloride higher than with copper chloride [82].

A reverse staining using imidazole and zinc salts for protein detection in electrophoresis gels was introduced a few years later [83]. It exploits the ability of biopolymers (in particular, proteins and protein-SDS complexes) to bind Zn^{2+} and that of imidazole to react with unbound Zn^{2+} to produce insoluble zinc imidazolate (seen as deep white-stained background); bound Zn^{2+} (on proteins) does not stain. Proteins with low affinity to CBB may be detected. Other Zn^{2+} binding biopolymers, like peptides, glycolipids, oligonucleotides, multimolecular complexes, may be visualized as well. The association of zinc to negatively charged or polarizable groups may occur through His, Cys, Glu and Asp in proteins, phosphate and amino groups in nucleic acids and polyhydroxyls (from the sugar moieties) and carboxylate (from sialic acid) and phosphate (from lipid A) in lipopoly-/lipooligosaccharides [84]. The introduction of a preincubation step in SDS solution allows application to non-SDS containing and agarose gels. Staining is limited to the gel surface, with minimal risk of modification of the proteins. The method is compatible with microsequencing or MS and should offer high sensitivity (1–10 ng protein/band in SDS-PAGE gels [84, 85]). Reverse-stained proteins can be efficiently eluted and used in biological and enzymatic assays. Before any of these procedures, staining should be fully reversed by incubating the gel or gel band in a chelating agent (EDTA, glycine, DTT, citric acid) [84, 86]. Although for MS good sequence coverage is reported for reverse-stained gels, some limitations concerning band detection of glycoproteins (*e.g.*, fetuin) or smaller proteins in PAGE have been shown (*e.g.*, myoglobin) [87].

Zinc-imidazol reverse-stain gel patterns can be transformed into permanently stained gels by a “toning” procedure. By a redox reaction with ferricyanide/*o*-toluidine the formerly white background turns into a deep blue, leaving the protein bands transparent and colorless. Gels may be scanned and protein amounts quantified in the range 10–100 ng, independent of the protein nature [88].

2.1.4 Pre-electrophoresis staining

Proteins are modified prior to electrophoresis by attaching a (usually fluorescent) label in a defined chemical way. If dyes with similar structure but different spectral properties are available, combination of these fluorophores in one gel is possible, thus allowing differential imaging (DIGE). This new technique is discussed in the next section.

2.1.4.1 Modified lysine residues

As already described in Section 2.1.2, some of the very first fluorophores were used to stain proteins either before or after the electrophoretic separation. One of these is MDPF. It reacts irreversibly with the ϵ amino-group of lysine residues and protein N-terminal groups, giving a very stable and sensitive fluorescent signal. It can be applied prior to electrophoresis (SDS-PAGE [58]) as well as after the IEF step or after 2-DE [56].

Today, cyanine-based dyes have found wide use as the commercially available CyDyes[®] (Amersham Biosciences, now GE Health Care). There are three dyes (Cy2, Cy3 and Cy5) that covalently label lysine residues of proteins via an amide linkage. Although structurally similar, they are spectrally quite distinct [89]. The dye/protein ratio is kept deliberately low (at 3% or below, “minimal labeling”) to ensure that only a single lysine residue in each protein is labeled. Multiple dye additions on each molecule would create multiple vertical spots per protein on a 2-DE gel and also reduce sample solubility [90]. With three dyes of this kind, a multiplexed system is possible, allowing differential images (DIGE) to be created, as first described by Uenlue *et al.* [91]. Up to three samples, each labeled with one of these fluorophores, may be mixed and separated electrophoretically in only one run. Subsequent scanning at appropriate wavelengths generates an image for each sample, all run under identical conditions, without interference of run-to-run differences. For a larger number of samples the experimental setup of each gel comprises two unknown specimens and one internal reference, used throughout all gels [92, 93]. This internal standard should be a pool of all samples of the respective experiment to ensure representation of all proteins on all the gels analyzed. Alban *et al.* [92] described spiking experiments of *E. coli* lysate with four different known proteins in different ratios and improved recovery when using this internal standard approach.

The dyes are charge-matched, therefore the *pI* of the investigated proteins should stay constant upon labeling. In molecular mass, slight mobility differences between labeled and unlabeled species of the same protein have been reported: the unlabeled bulk of the respective protein is slightly faster than the labeled (about 0.5 kDa), a change that may be noticed for smaller proteins [92]. While this does not seem to have any major influence on the evaluation of patterns (the shift is equivalent with each cyanine dye), it can be problematic for subsequent MS analysis. As a consequence, post-staining with SYPRO Ruby or colloidal CBB is recommended prior to spot-picking from minimally labeled DIGE gels [89, 90, 94].

The general idea behind DIGE is that with multiplexing fewer gels are needed to enable reliable evaluation of a given sample set [89]. Besides a lower number of gels and replicates necessary for statistical analysis, this new method is supposed to allow smaller differences to be accurately detected and quantified with statistical confidence. DIGE

should exert its full strength in settings comprising highly similar but not identical biological conditions [95]. This includes comparison of treated/untreated or healthy/pathological states, *e.g.*, normal and cancerous tissues [94, 96], protein patterns in transgenic mice [97], but also more complex experiments like time courses and dose-response experiments [95]. Some other recent applications include the validation of sample preparation methods [98], the study of pattern changes induced by treatment, *e.g.*, phosphorylation [99], and the search for possible contaminants [100] or for disease markers [101].

As previously discussed, post-staining with other dyes is recommended prior to spot picking from minimally labeled DIGE gels. However, differences in individual staining properties of single proteins, mean that some proteins may be missed/not detected in post-staining. Such a case is described in [89], where about 40% of the differentially regulated spots could not be reliably detected after post-staining with colloidal CBB, either due to lower sensitivity of this stain or to the proteins' different staining properties. Better results were reported for SYPRO Ruby [94].

Tonge [89] reports one protein spot that was differently stained with Cy3 and Cy5 in the same sample. Further examples are shown in Section 5.2. To avoid errors arising from this, it is recommended that samples are randomly labeled and distributed on the gels, and that reverse labeling (running samples in duplicate, with both labels) are employed.

DIGE technology requires specialized equipment and evaluation software. For imaging, specific fluorescence scanners are necessary, usually point scanners with laser light sources with different wavelength and a wide dynamic range. Experiments produce large amounts of data, which need to be normalized to the internal standard. Dedicated algorithms have been developed, similar to those from microarrays, to eliminate dye influences [102–104]. Differentially expressed proteins are extracted by means of statistical tests, like t-test, ANOVA or cluster analysis [95, 105].

2.1.4.2 Modified cysteine residues

Cysteine is a less prevalent amino acid; it is chemically modified through a reaction with its thiol group. The compound monobromobimane (TMB) has been used to fluorescently label sulfhydryl groups in protein solutions prior to electrophoresis and to study lymphoid cell line patterns in comparison to silver stain with equal protein loads [106]. Small changes in *pI* and *M_r* have been found for TMB-labeled proteins as well as variations in relative spot intensities.

More recently, a very sensitive type of cyanine dyes has been developed that also allows multiplexing, although with only two fluorophores. The dyes CyDye DIGE Fluor Cy3[®] and CyDye DIGE Fluor Cy5[®] contain maleimide reactive groups that covalently bind to the cysteine residues on proteins via a thioether linkage. The general mass shift is about

0.7 kDa, the dyes have the same spectral qualities as minimal CyDyes [107]. This saturation labeling reaction has to be carefully optimized for each type of sample to ensure that all accessible cysteine residues contained within a protein are labeled (“saturation labeling”). Excess dye may give side reactions with lysines [90]. These dyes offer increased sensitivity over their minimal-labeling counterparts (the method is also propagated as “scarce sample labeling”). As staining is based on labeling cysteines, the resultant spot patterns cannot be expected to resemble those generated on silver, SYPRO Ruby or minimally labeled CyDye gels [90]. Spots may be picked directly from saturation-labeled gels without post-staining, if enough protein is present for MS analysis. The dyes have been found useful for multiplexing in an example with mouse liver of treated/control animals; preferential labeling of proteins with one dye over another were noticed but controlled for through the experimental design. In the same investigation, little evidence was found for significantly altered migration of a given spot due to cysteine saturation labeling [90]. Most applications described are in the field of cancer research, in search of potential biomarkers, using samples obtained by laser capture microdissection [108, 109]. Being very sensitive, saturation labeling can be performed on protein lysates obtained from as little as 1000 microdissected cells [110]. It has also been described for comparing protein expression profiles of human hepatocellular carcinoma cell lines with primary culture hepatocytes [111].

Another set of cysteine-specific fluorescent dyes on the market are the FlaSH^{Pro} dyes (Fuji, Raytest, Straubenhardt, Germany). They also react with the cysteine residues via their maleimide group. An example of these dyes is included in Section 5.

2.1.4.3 Other applications

Mayrhofer *et al.* [112] used CyDyes to label the surface proteins of intact cells. Upon lysis and 2-DE, membrane proteins (Cy-labeled) could be distinguished from other cellular proteins, by comparing fluorescent and silver stain patterns.

Birner-Gruenberger *et al.* [113] reported application of fluorescent inhibitors for the in-gel detection of esterolytic or lipolytic enzymes. Various enzymes in crude porcine pancreatic lipase preparations were reacted with covalently binding fluorescent inhibitors (fluorescently labeled alkyl- or dialkylglyceryl-phosphonates) and separated by electrophoresis. Fluorescent bands indicated the positions of the respective enzymes in the gel.

2.2 Staining on blots

Electroblotting drives protein molecules from the whole thickness of a polyacrylamide (or less commonly, of an agarose) gel to the surface of a binding membrane. This allows for increased availability of the sites with affinity for both general and specific protein reagents: in principle, total pro-

tein stain should be more sensitive on blots than in gels. Actually, protein staining on blots is seldom performed *per se*, as a complex but effective detection protocol. Rather it most often represents the preliminary step for specific detection/characterization procedures. N-terminal sequencing requires permanent stains; CBB has been routinely used with NC, and AB with PVDF. Conversely, immunological and affinity reagents require reversible stains. An overview on the different methods is given in Fig. 3.

The detection limits for the most commonly used stains are listed in Table 2.

	non-reversible stains	reversible stains
stains	✓ AB on NC, CBB on PVDF	✓ colorimetric, low-medium sensitivity (e.g. PR) ✓ fluorescent high sensitivity (e.g. RuBP)
further processing	✓ sequencing	✓ affinity reagents with group-specificity ✓ immunological reagents with protein or epitope specificity

Figure 3. Overview on blot protein staining procedures.

Table 2. Selected general staining methods on blot membranes

Staining method	Detection limit (ng)	
	NC	PVDF
Ponceau Red	1000	1000 [194]
Coomassie BB R-250	500–1000 [195]	100–200 [194]
Amido Black 10B	500–1000 [195]	250 [194]
Indian ink	100–500 [196]	
silver stain	4–10 [197]	
SYPRO Ruby [®]	2–4 [64]	
AuroDye [®]	1–2 [118]	

2.2.1 Non-reversible procedures

Indeed, a few irreversible procedures have been devised for maximal sensitivity detection of blotted proteins. In one such procedure, transferred proteins are derivatized by reaction with sulfosuccinimidobiotin, and are then incubated sequentially with streptavidin, rabbit anti-streptavidin, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. After zymography, the procedure detects less than 5 ng of transferred protein per band [114]. Other high sensitivity protocols involve the use of colloidal metal particles. AuroDye[®] staining [115, 116] is based on the hydrophobic and ionic interaction between colloidal gold particles and proteins at pH 3; dark red bands/spots develop. Sensitivity is equal or higher than with silver stain in gels [117], and 1–100 ng protein can be quantified by scanning densitometry [116, 118]. The procedure is compatible with NC and PVDF

membranes. If using a non-ionic detergent as blocking agent, total protein stain with AuroDye® may be applied after immunogold/silver staining of specific antigens [119]. Ferri-Dye® requires the incubation in a cationic iron (hydrous) oxide sol and subsequent intensification with potassium hexacyanoferrate in acidic medium (Perls' reaction). Ferri-Dye is of intermediate sensitivity, but can be used also on nylon-based membranes [118, 120].

2.2.2 Reversible procedures, colorimetric and fluorescent dyes

Direct Blue 71 stains proteins with a sensitivity of 5–10 ng on NC and 10–20 ng on PVDF. The dye may be removed from the developed bands by changes in pH and solvent hydrophobicity [121].

In highly alkaline conditions copper iodide forms on proteins a reddish-brown precipitate that may be removed without loss of immunoreactivity by washing the membrane for 15 min. On NC blots, the sensitivity is 100–150 pg/mm² [122]. A proprietary formulation (MemCode®) also contains copper, as a part of an organic complex that interacts non-covalently with proteins. The stain is easily reversible (within 2 min for NC and 10 min for PVDF) using a commercial 'stain eraser' reagent. The manufacturer claims unprecedented sensitivity [123].

Metal chelates such as ferrozine/ferrous complex and ferrocyanide/ferric complex bind to blotted proteins at acidic pH; nonspecific sites on the membranes need to be saturated with polyvinylpyrrolidone-40. The protocol is compatible with both microsequencing and immunoblotting. Staining is reversible by incubation at neutral to basic pH in the presence of 20 mM EDTA [124]. The double-metal chelate (DMC) stain, *i.e.*, ferrozine followed by ferricyanide complex, affords similar sensitivity to colloidal gold stain [124, 125]. Another reversible metal chelate stain for detection of blotted proteins makes use of pyrogallol red-molybdate [126].

Whereas colorimetric stains are indeed really "reversible", *i.e.*, to be removed with destaining, fluorescent stains are usually not. Different stains on the same blot (without stripping of the membrane) are possible because the dyes need different scanning conditions where they show little cross-talk.

Among fluorescent dyes, the detection sensitivity of SYPRO Ruby® (0.25–1 ng protein/mm²) is superior to that of common colorimetric stains and nearly matches colloidal gold staining, with the advantage of a rapid detection procedure and of a wider linear dynamic range [64].

BPSA forms a luminescent europium (Eu) complex that reversibly binds at acidic pH to proteins and nucleic acids. Upon epi-illumination with UV light the phosphorescence of BPSA-Eu is measured at 590–615 nm. The linear dynamic range of the stain is 476- and 48-fold for protein and DNA, respectively. A strong chelating agent such as EDTA combined with a shift to basic pH (pH 8–10) elutes BPSA-Eu from the membrane [127]. SYPRO® Rose Plus is an

improved Eu-based metal chelate formulation. It exhibits exceptional photostability, allowing long exposure times for maximum sensitivity. The staining is fully compatible for subsequent biochemical analyses [128].

Many of these protein stains have also been assessed as alternative procedures for the quantitation of total protein content in liquid samples with dot- or slot-blot microfiltration set-ups to avoid chemical interference in the assay: Ponceau Red (PR) 3R or AB 10B on NC [129], ferrozine/ferrous metal-chelate stain on PVDF membranes [125, 130], Eu on both [127].

3 Specific stains (PTM specific)

3.1 Phosphoproteins

Phosphorylation is a reversible PTM widely used by cells for activation/inhibition of specific pathways both in basic energy metabolism and in specialized signal transduction processes. Phosphorylation results in a shift of the protein *pI* to more acidic values (the extent of the shift inversely depending on the buffering power of the protein at *pI*); discrepancy between computed and experimental *pI* is strongly suggestive of the occurrence of some PTM, but inconclusive as for the nature of PTM. In some cases (multiple) phosphorylation results in a shift in *M_r* with band thickening and even band doubling in SDS-PAGE. Incorporation of ³²P from γ -ATP ('in' step) may be easily monitored *in vitro*; the use of specific inhibitors in the same experimental set-up may address the question of which protein kinase is involved in the process. In the analysis of *in vivo* samples, the 'out' step may be inferred from the shift in *pI* and/or *M_r* after phosphatase treatment. Specialized procedures in MS avoid hydrolysis of phosphate groups and identify presence and location of PTM residues [131–133]. Immunochemicals specific for P-tyrosine, P-serine and P-threonine have been developed as much as for the P-variants of individual proteins. The former should allow surveying the whole phosphoproteome [134, 135], the latter assessing the ratio between/among phosphorylation isoforms; some aspecific binding of the antibodies may be a confounding factor in these investigations. Sample enrichment in phosphoproteins using immunological (anti-phosphoserine immobilized onto agarose [136]) or affinity (Fe³⁺-laden IMAC resin [137], metal oxide/hydroxide affinity chromatography [138]) procedures may be a valuable alternative.

Detection of phosphoproteins in gels after 1-DE and 2-DE may also be achieved with specific stains. An old procedure [139] depends on the hydrolysis of the phosphoester linkage of phosphoserine and phosphothreonine (but not of phosphotyrosine) using 0.5 N NaOH in the presence of calcium ions. Treatment with ammonium molybdate in dilute nitric acid shifts insoluble calcium phosphate to insoluble nitrophospho-molybdate complex, which is stained with the basic dye, Methyl Green. The procedure takes 3 h, and is

compatible with later staining with CBB. The limit of detection depends on several factors, including molar amount of loaded phosphate and accessibility to hydrolysis of the phosphoester groups. Two model phosphoproteins, phosphovitin and β -casein, are detected in the 40–80 ng/band and 80–160 ng/band range, respectively. Current applications of this staining protocol appear limited to the study of vitellogenin in different species [140–142].

A recently developed [143] proprietary fluorescent stain, Pro-Q Diamond[®] by Molecular Probes, affords wider specificity, higher sensitivity, and a straightforward protocol. Phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins are detected, without sequence or context specificity. With excitation/emission maxima around 555/580 nm protein patterns stained with this dye may be recorded with visible-light-scanning instruments and visible-light or 300-nm transilluminators. Multiplexed proteomics analysis on a single gel is made possible by the compatibility of Pro-Q Diamond with other fluorescent dyes (SYPRO Ruby, see above; Pro-Q Emerald, below). Its readily reversible interaction with the proteins also allows for MS analysis (MALDI-TOF-MS) or Edman sequencing on the stained gels and blots. Conditions to reduce the costs connected with the use of the proprietary formulation without curtailing sensitivity have been detailed [144]. Pro-Q Diamond allows the detection of 1–16 ng phosphoprotein per band, depending on the phosphorylation state of the protein. The limit is 1–2 ng for β -casein, a pentaphosphorylated protein, and 8 ng for pepsin, a monophosphorylated protein. The Pro-Q Diamond signal for individual phosphoproteins is linear over approximately three orders of magnitude (*i.e.*, 500–1000-fold concentration range) and correlates with the number of phosphate groups [143]. On NC and PVDF blots, the detection limit is 2–4 ng phosphoprotein with a linear dynamic range of approximately 15-fold [145]. It should be cautioned, however, that a comparative assessment concluded that there is not perfect correlation between radiolabeled and Pro-Q Diamond-stained phosphoproteins [146].

A very interesting example of the application of Pro-Q Diamond has been reported with the PTM characterization of immunopurified oxidative phosphorylation mitochondrial complexes [147].

3.2 Glycoproteins

Alternative detection procedures are listed in Fig. 4. The most classical procedure for glycoprotein staining is periodic acid-Schiff (PAS). When treated with periodic acid, vicinal diols are oxidized to aldehydes, able to react with pararosaniline, a triphenylmethane derivative, and sodium metabisulfite (together, the Schiff's reagent) and to form an adduct, pink to magenta in color. The procedure was devised as a histochemical stain and has been adapted to glycoprotein detection [148] in gels and on blots [149–151]. Sensitivity is low; 25–100 ng carbohydrate or 1–10 μ g highly glycosylated proteins.

Staining with Alcian Blue, a copper phthalocyanine dye, was first devised for electron microscopy. After electrophoresis [152], it is most useful for the detection of acidic polysaccharides, for which the limit is 15–40 ng on cellulose acetate strips and 50–150 ng on agarose plates [153]. The metachromatic carbocyanine dyes, Ethyl-Stains-all, a triethyl dye, and Stains-all, a diethyl methyl dye, stain apoprotein red, phosphoproteins and sialic acid-rich glycoproteins blue to blue-green [154]. Silver enhancement of these traditional staining methods results in a two- to fivefold increase in sensitivity [155, 156]. With a model glycoprotein, the range of linearity for densitometric quantitation moves from 10–50 ng down to 0.25–10 ng [156].

A still higher sensitivity, approximately 50-fold more than PAS, is afforded by proprietary stains, Pro-Q Emerald[®] by Molecular Probes. Detection limit is as low as 300 pg for α_1 -acid glycoprotein (40% carbohydrate) and 1 ng for glucose oxidase (12% carbohydrate) or avidin (7% carbohydrate). As for all fluorescent stains, linear range of detection spans two to three orders of magnitude. Two variants of the stain are

procedures for glycoprotein detection and characterization

	presence/absence vs control	detection	analysis of composition	analysis of structure
cell culture	✓ inhibition of N- or O-glycosylation		✓ metabolic incorporation of ³ H-sugars	
sample preparation	✓ chemical deglycosylation ✓ enzymatic deglycosylation (endoglycosidase)		✓ (exo)glycosidase treatment	
separation by electrophoresis	after glycosylation inhibition or deglycosylation ✓ mobility shift		✓ lectin affinity electrophoresis - crossed immunoelectrophoresis	
post-electrophoresis procedures		stain of untreated proteins ✓ <i>Alcian Blue</i> ✓ <i>Stains-All</i> reaction after periodate oxidation ✓ PAS ✓ <u>derivatized hydrazides (biotin, digoxigenin)</u> ✓ <i>Pro-Q Emerald[®]</i>	✓ lectin affinity blotting	on the sugar chains chemically or enzymatically removed from the protein ✓ sequential enzymatic degradation and HPLC ✓ MS

Figure 4. Outline of the procedures for glycoprotein investigation in a proteomic experiment based on 2-DE. Under 'detection', italics for reagents to be used on gels, underlined for reagents to be used on blots, italics underlined for reagents to be used both on gels and on blots.

commercially available, dubbed '300' and '480' on the basis of their excitation maxima, and used for detection with UV-light and visible-light sources, respectively. Both chemicals are fluorescent hydrazides, which may be conjugated to glycoproteins by a PAS mechanism without the need for reduction with sodium metabisulfite or sodium borohydride to stabilize the product; both can be used for either gel or PVDF blot staining. Pro-Q Emerald 300 dye-labeled gels and blots may be post-stained with SYPRO dyes, allowing sequential two-color detection (green, red) of glycosylated and nonglycosylated proteins [157, 158].

Other procedures for labeling glycoproteins via periodate oxidation/reductive amination utilize biotin hydrazide as tag [159] and streptavidin–horseradish peroxidase with ECL zymography as detector [160, 161], or digoxigenin hydrazide [162, 163] followed by an anti-digoxigenin antibody conjugate of alkaline phosphatase [164]. Both labeling procedures may be used for glycoprotein detection on blots and for affinity purification but are seldom utilized in practice.

Biospecific reagents to be used on blots are lectins, proteins of non-immune origin that specifically interact with sugar molecules. Their restricted binding capacity is the basis not only for recognition of glycoproteins but also an indirect way for assessing the composition of their glycan moieties. Concanavalin A from *Canavalia ensiformis* binds to internal and nonreducing terminal α -mannosyl groups, detecting most glycoproteins. Lectins from *Sambucus nigra* and from *Maackia amuriensis* show affinity for specific types of sialic acid linkages: the former recognizes the sequence NeuAc(α 2,6)GalNAc, the latter reacts with the sequence NeuAc(α 2,3)Gal(β 1,4)GlcNAc. Together they can detect most glycoproteins of animal origin (all antennae of the glycans from these sources ending with sialic acid residues).

Concanavalin A is a tetramer and after its interaction with the blotted glycoprotein is able to bind horseradish peroxidase, itself a glycoprotein. Chromogenic zymogram should then be applied, e.g., according to [165], as ECL under these conditions results in an extremely high background. For concanavalin, as well as for all other lectins, covalent conjugates with either peroxidase or phosphatase are commercially available. For many, fluorescent adducts are also available on the market or may be synthesized [166]. The latter have a detection limit in the range of 5–25 μ g glycoprotein, and may be used directly on polyacrylamide gels.

Biospecificity is the basis also of mobility-shift assays, which detect glycoproteins susceptible to specific deglycosylating enzymes. By removing sialic acid, neuraminidase affects the pI of the target proteins; all enzymes do change their M_r although the shift may be negligible for large proteins. A countercheck of the effect of a given deglycosylation enzyme may come from loss of interaction with the relevant lectin. For complete removal of the glycan chains, peptide: *N*-glycosidase F (PNGase F), an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins, is effective on whole antennae. *O*-Glycosidases

on the contrary are limited in scope as they only act on Gal-GalNAc as a substrate, and further substitutions have to be trimmed in advance with the appropriate exoglycosidases. Specific inhibitors may be used in cell cultures to prevent *N*- and *O*-glycosylation, such as tunicamycin and benzyl-*N*-acetylgalactosamine. Examples of the combined use of all the above approaches may be found in [167–170].

Deglycosylation of the protein sample, with the above enzymatic treatments or with trifluoromethanesulfonic acid, reduces the complexity of gel patterns on 1-DE and 2-DE gels, and enhances the protein identification of some proteins via MALDI-TOF-MS [171]. Protocols for in-gel deglycosylation prior to MS analysis with reference to standard patterns with untreated samples have been optimized, for the identification of the (apo)proteins, for the recognition of the glycosylation sites [172] and for the characterization of the glycan moieties [173, 174].

4 Imaging and evaluation

Quantitative analysis of visualized proteins is achieved through comparative statistical interpretation of digitized representations of the 2-D gel or blot using dedicated image analysis software solutions. This section provides a brief guide to the range of different image acquisition devices currently in use for 2-D gel and blot applications, and defines some of the important technical factors required to generate digital images of a quality suitable for automated image analysis.

4.1 Image capturing devices

For image analysis to be performed on a 2-D gel, it must first be converted into digital data. Image acquisition for 2-DE applications can be achieved using a variety of devices, broadly categorized into three major types: laser-based detectors, CCD camera systems and flatbed scanners. The choice of imaging system is largely dependent on the type of protein stain utilized.

Laser-based scanners and CCD camera systems are the two most sophisticated and widely used image acquisition devices to date [175–178]. Laser-based systems operate by scanning the gel, point by point, with a powerful laser. The resultant emission energy is detected by high voltage PMTs, and converted into digital signal (pixels). Laser-based image capture devices are commonly used to detect some of the more recently developed fluorescent dyes such as the CyDyes, SYPRO, Deep Purple and FlaSH^{Pro} Dyes. Multiple lasers and emission filters can be used to accommodate the wide variety of fluorophores available. They can also be used with the common visible protein stains such as silver and CBB, and for phosphor-imaging of radioactive labeling. CCD camera image acquisition systems can also be used with both visible dyes and fluorescent stains. These instruments operate with visible or UV illumination for visible

protein stains, and fluorescent or Xenon lamps for fluorescent applications. The emitted light is captured by high sensitivity cooled area array CCD sensors and converted into digital signal. The CCD cameras can be either fixed or scanning. The latter are used to compensate for the relatively low dimensions of high quality camera chips (typically less than 2000×2000 pixels), and function by generating a series of overlapping images, which are assembled to form the final image. Detailed reviews on both laser-based scanners and CCD camera systems have been published by Miura [178, 179]. Standard commercial document scanners are also often used as densitometers [180]. In general, the scanners used for 2-DE applications differ from commercial office scanners in that they are sealed units to protect against wet samples and their optical path is modified to accommodate the gel assembly. In newer scanners, the light source is either a cold cathode fluorescent lamp (CCFL) or a xenon lamp, while older scanners may have a standard fluorescent lamp. Reflected or transmitted light is detected, line by line, as electrical current by linear array CCD sensors and subsequently converted into digital information. Flatbed scanners can be used for imaging visible dyes and to scan autoradiographs or blots. Table 3 compares the main imaging technologies for the detection of multiple stain types in proteomics.

4.2 General considerations for image capture for quantitative analysis

Good image capture is critical to guarantee optimal performance of automated image analysis packages and generate reliable quantitative data. There are therefore a number of important considerations that must be taken into account during acquisition. These include bit depth, spatial resolution and dynamic range. Inadequate attention to any of these factors may not only cause sub-optimal detection but will also compromise quantitative results when using any image analysis software.

4.2.1 Bit depth and dynamic range

The term 'bit depth' describes the number of bits used to define each pixel of a digitized image, and determines how many levels of gray can be generated. Greater bit depth allows a greater range of tones (grayscale) to be represented by a pixel, *e.g.*, an 8-bit grayscale image file has 256 (2^8) possible shades of gray for each pixel, while a 16-bit image file stores 65536 (2^{16}) values. In reality, the images displayed on the computer screen will only be represented in 256 shades of gray, and so, by eye, the same image acquired at 8-bit and 16-bit will appear identical. Image analysis software, however, can distinguish between the different levels of gray. As a rule, the more levels of gray represented in an image, the better the ability to differentiate low abundance spots from background, and the greater the quantitative accuracy. Dynamic range (or gray-level resolution) defines the actual range of grayscales that are utilized within a digital image. When scanning, it is beneficial to optimize the dynamic range so that the majority of the available grayscale range is represented. A limited dynamic range can result in limited sensitivity for low abundance proteins, impact on the total number of spots detected, and compromise quantitative results when comparing data between images.

4.2.2 Image resolution

Image (or spatial) resolution relates to the number of pixels displayed per unit length of a digital image, and is often measured in dpi (dots per inch) or in micrometers (the size of the area each pixel represents). Images with a higher spatial resolution are composed of more pixels and have more image detail than those of lower resolution. Variations in spatial resolution will not only affect the final appearance of the image (the image may appear pixelated), but will also impinge on the quality of spot detection and the accuracy of any subsequent quantitative measurements (Fig. 5). At low resolutions, there will be fewer pixels available to represent

Table 3. Comparison of the main imaging technologies for the detection of multiple protein stains in proteomics

Criteria	Laser scanners	Document scanners	CCD devices	
			Scanning	Fixed
Image resolution (μm)	10–250	20–250	50–200	>120
Dynamic range (orders of magnitude)	5	4–5	3–4	3–4
Scan speed	Slow	Fast	Slow	Medium
Wavelength accuracy	High	Low	High	High
Silver, Coomassie, autoradiography	Yes	Yes	Yes	Yes
Storage phosphor	Yes	No	No	No
Single color fluorescence (CyDyes, Deep Purple, ProQ, FlaSH)	Yes	No	Yes	Yes
Multicolor fluorescence (DIGE)	Yes	No	Yes	Limited
Chemiluminescence	Yes	No	Yes	Yes
Cost	££££	£	£££	££

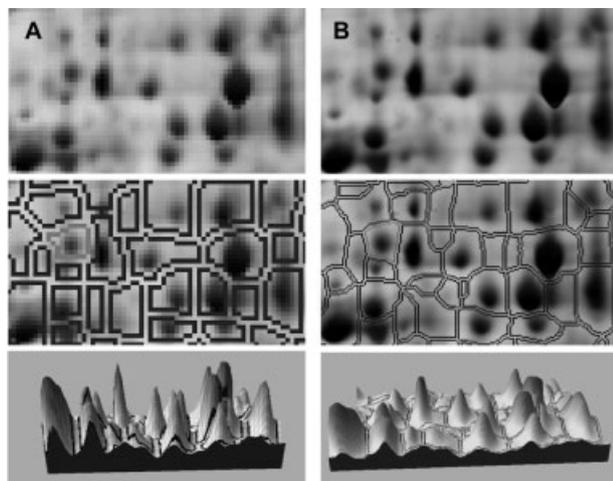


Figure 5. Spot detection on a 20-cm 2-D gel image, captured at a resolution of (a) 100 dpi and (b) 300 dpi. At 100 dpi, the reduced pixel content means that spot outlines cannot be accurately placed in highly populated areas. Spots here are only separated from one another by a single pixel, and, as a result, one spot may end-up “losing” material to its neighbor, as illustrated in the 3-D representation. Increasing the resolution to 300 dpi increases the number of pixels available to represent each spot, thereby negating these problems. Data for this figure was acquired using Progenesis PG240 (Nonlinear Dynamics, Ltd).

each spot, and as a result, spot detection and quantitative accuracy will be compromised. Increasing the resolution enhances the number of pixels available, thereby improving quantitative measurements. However, there is a maximum resolution that, once exceeded, produces minimal additional information. Once you have sufficient resolution to adequately represent the smallest features, any further increases in spatial resolution will simply increase the accuracy with which you can represent the noise in the system. In addition, every doubling in spatial resolution quadruples the amount of data that has to be processed, which can cause problems in processing speed and memory management.

4.2.3 Saturation effects

When optimizing the dynamic range, it is important to prevent signal saturation. Saturation occurs when gray levels exceed the maximum available. When a spot becomes saturated, any differences in high pixel intensities cannot be resolved and it cannot be accurately quantified. To avoid saturation, the usual recommendation is to ensure that, during scanning, the more abundant protein spots are represented by pixels slightly below the maximum intensity available, while keeping the dynamic range as high as possible. However, in practice, this compromise often means that the fainter less abundant spots, which are often the more biologically relevant, are too faint to be accurately quantified. There are a number of ways that this can be circumvented. Pre-fractionation procedures can be performed prior to 2-DE

to reduce the complexity of the sample and to enrich the content of the low abundance proteins [181]. Utilizing protein stains that exhibit a high linear dynamic range can reduce saturation problems associated with abundant spots, and acquiring images at 16-bit rather than 8-bit resolution will result in a larger dynamic range, enabling improved allocation of pixel intensity values to less abundant spots, and increased quantitative accuracy. If the abundant proteins within a gel image are not experimentally important or biologically interesting, then it may be possible to allow these spots to saturate to achieve sufficient sensitivity for the lower abundant spots. Sacrificing resolution of “unimportant” spots could be the difference between seeing a result and not. However, saturated (and abundant) spots have the potential to bias normalization methods that are based on total volume normalization, particularly if their variance is a significant proportion of the total spot volume, therefore measures should be taken to exclude these from the normalization calculation if required.

4.2.4 Scanning recommendations

Table 4 summarizes several useful recommendations for producing good quality digital images from 2-DE gels or blots, with the aim of providing an optimal starting point for image analysis and interpretation.

4.3 Image analysis

Computerized analysis of 2-D gel digitized images is required to interpret the complex data generated by 2-DE into valid biological information. A number of different software packages are commercially available for this purpose. The following provides a brief overview of the steps involved, with specific reference to protein staining related issues that can affect image analysis of 2-D gels.

Many software packages use ‘preprocessing’ filters to clean up the gel images prior to analysis. These algorithms are used to eliminate technical noise that can originate from the image acquisition process, *e.g.*, low level Gaussian distributed sensor noise, generated by any capturing device which utilizes an electrical current. They can also reduce the more visually obvious random noise, for example speckling from the crystallization of certain stains (*e.g.*, SYPRO), dust particles and other such artifacts. Noise as defined here is not to be confused with the image’s general background intensity caused by the staining technique employed; this is handled in a separate ‘background subtraction’ operation.

4.3.1 Background subtraction

Background subtraction eliminates fluctuations in the gel background intensity level caused by overexposure during the capture process and nonspecific protein staining. Background removal is required so that background signal does not contribute to protein signal. Some protein stains and

Table 4. Scanning recommendations

Recommendation	Reason
Scan at the best resolution for your images	The active area of the gel (the area of spot material) should fall in the range 1000–1800 pixels in both horizontal and vertical directions. This range provides a good trade-off in information content and analysis performance. For most images, this will correspond to 300 dpi or 100 μm . If your gels are small, then you may need to increase the resolution to achieve this
Scan at 16-bit rather than 8-bit	The bit depth of a 16-bit image compared to an 8-bit image results in enhanced sensitivity and accuracy of quantification for less abundant proteins
Optimize the dynamic range to maximize use of available grayscale values	Aim for the maximum gray levels in the image to be 5–10% less than the maximum available by adjusting the exposure time for a CCD camera, or altering the PMT voltage
Avoid signal saturation when scanning	Saturated spots cannot be accurately measured and have the potential to bias normalization
Try to only scan the active area of the gel	Crop during scanning to remove blank parts of the scanner plate, labels etc. These areas provide no useful information, can ‘steal’ dynamic range, distort image statistics and increase storage requirements
Try to scan gel images using the same orientation	If you do need to rotate, flip or mirror images after scanning use the tools provided within your 2-D software package. Other packages may alter the integrity of the original data
Do not perform any post-processing of 2-D gel images in any general image processing software	These do not maintain the integrity of your original data, and you will almost certainly lose any calibration information contained in the image file
If possible, choose GEL or IMG/INF files formats, rather than generating TIFF files	The former often contain additional grayscale calibration information, which will not be included in the TIFF version
Do not use JPEG files for image analysis	The JPEG format is what is called a “lossy” compression system; while the images may look the same they are not. A great deal of smoothing and averaging may have taken place within the compression process and this will affect the underlying raw pixel data. Converting a JPEG image back to a TIFF is not a solution; once the image has been compressed in this way, the data have been lost and cannot be retrieved

imaging systems are more predisposed to higher, non-uniform backgrounds than others, *e.g.* CBB [45]. Background fluorescence from acrylamide can also be problematic when using fluorescent protein stains. A number of different background subtraction algorithms exist, ranging from simple boundary-based methods to more advanced mathematical modeling techniques.

4.3.2 Spot detection

Spot detection involves the location and boundary definition of individual protein spots resolved on each 2-D image. Generally, to minimize user bias, the more advanced algorithms perform spot detection automatically. Data quality is key to the performance of many of the spot detection algorithms, where very noisy gels and images with a poor dynamic range will all yield sub-optimal and inconsistent spot detection. For example, low abundance spots may not be accurately defined in images that have been captured at a low dynamic range and/or bit depth; noise spikes occurring within a spot boundary may result in over-splitting and will affect quantitation if included in the spot volume measurements. The choice of a reduced linear dynamic range protein stain will also impact on the ability to reliably detect quanti-

tative differences in protein expression [68]. Saturated spots are generally not detected well, neither are negatively stained silver spots. Such inconsistencies in spot detection can often be dealt with manually using the tools available for editing provided within each software package.

4.3.3 Warping and matching

The main objective of 2-D gel analysis is the identification of differentially expressed proteins between different samples run on different gels. In the majority of cases, this is achieved by a combination of image warping and gel matching. Gel matching is used to relate corresponding proteins in samples across different gels. All images are matched to a common reference (or master) gel, which is either a representative image, or a synthetic gel. Each protein spot in the experiment is mapped into the reference gel, which is then used as a basis for matching. In this way, related spots on different gels can be compared. In practice, however, cross-gel matching is often complicated by experimental noise inherent in the system, which causes geometric distortion of protein patterns common to different gels [182]. The major source of this noise includes differences in sample preparation, gel composition, experimental conditions, staining

procedures, gel storage and the positioning of the gel (or autoradiograph) during image acquisition. An increasing number of software packages now incorporate advanced image warping algorithms, which can compensate for distortions of protein spot patterns arising from gel-to-gel variation, thereby improving the speed and accuracy of matching between gel images.

5 Experimental (“different stains stain differently”)

This section gives examples from our labs illustrating what was discussed in theory in the first part of the review. The main focus is on comparison of one single, complex sample (horse serum) processed in 2-DE in a similar way, but with different stains (Section 5.1). However, also the modern and nowadays “fashionable” DIGE dyes are not without problems, as is shown in a few examples in Section 5.2. Finally, Section 5.3 refers to some other papers where results from different staining procedures were compared.

5.1 Staining one sample with different dyes

The same serum sample of a clinically healthy mare (Norican breed) was used for all 2-DE experiments. Classical 2-DE was performed in accordance to existing protocols ([21] and <http://www.expasy.org/ch2d/>), followed by different staining/detection methods and scanning under appropriate conditions (Table 5). Protein amounts were adapted to the staining technique to be applied, being in the range of 0.17–5 μ L original serum (Table 5).

Digitized 2-D gel images of each type of stain (10 gels) were aligned by geometric correction in TT900 S2S (Nonlinear Dynamics Ltd) and subsequently analyzed using Progenesis™ PG240 (Nonlinear Dynamics Ltd). SameSpots was then applied; this propagates the same spot outline across all gel images within the experiment. Background was removed using the Progenesis background subtraction method. Spot volumes were normalized and expressed as a percentage of the total spot volume for that gel, therefore allowing direct quantitative cross-gel comparisons to be made, regardless of sample load variation and different imaging techniques.

The evaluation and comparisons described here are all based on data obtained from single gels; as a result, we can only comment on trends observed between the same proteins under different staining conditions. Nevertheless, all observations are well in accordance with our own experiences using different stains and more comprehensive sample sets and/or with peer-reviewed literature, as detailed.

As already seen in Fig. 6, patterns obtained with different methods vary in spot number and their intensities, although for this display, they were adjusted to approximately the same intensity by Corel Photo-Paint. An even clearer picture is obtained when evaluating spot intensities. A total of 411 spots were detected on all images following the implementation of SameSpots. Figure 7A shows the normalized volumes of spots in the CBB G-stained pattern, which was chosen as reference gel, when they are sorted according to size. Of the spots, 248 had volumes of less than 0.1% of total volumes each, 388 were below 1%. Only the last spot, the main albumin spot, was well above 10%. The other parts of this figure (Fig. 7B-I) compile normalized spot volumes obtained with other dyes: for easier comparison, spot volume

Table 5. Sample amounts, staining procedures and scanning conditions in experimental data shown^{a)}

Stain	Serum volume (μ L)	Method according to:	Scanning parameters ^{b)}
Colorimetric			
CBB R	5	0.05% CBB R-250 in ethanol/water/acetic acid	Red filter
CBB G	5	Colloidal [21]	Red filter
Fast Green silver	5	[32]	Red filter
	0.17	Acidic silver nitrate [198]	Green filter
Fluorescent			
RuBP	0.5	[73], with home-made fluorophore [72]	488/610 nm
Deep Purple	0.5	Manufacturer's instructions ^{c)}	532/560 nm
Cy3 minimal	0.42	Manufacturer's instructions ^{c)}	532/580 nm
Cy5 minimal	0.42	Manufacturer's instructions ^{c)}	633/670 nm
FlaSH ^{Pro} Red	0.17	Manufacturer's instructions ^{d)}	633/670 nm

a) Colorimetric stains were detected on a Sharp JX-330 flatbed scanner operated by a Sun SPARCstation 4 (Sun Microsystems, Mountain View, CA, USA), using appropriate filter settings. Images were extracted from PDQuest V5.1 (PDI, Huntington Station, NY, USA) as tif files. Fluorescent staining was performed on a Typhoon 9400 imager (GE Health Care). Images were extracted from DeCyder (GE Health Care) V5.02 as tif files.

b) For fluorophores: excitation (laser)/emission (filter).

c) GE Health Care.

d) Fuji, Raytest; two ratios of protein/dye, differing by a factor of 5 (4 μ L or 0.8 μ L dye, respectively, for 10 μ g protein).

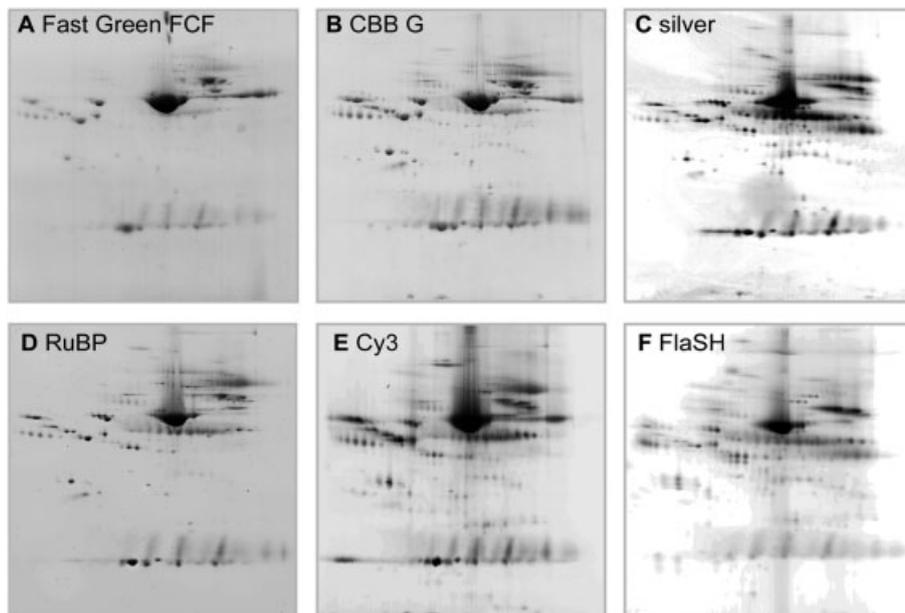


Figure 6. Multi-panel figure with 2-DE separation of horse serum (obtained from a clinically healthy animal), with different stains. Separation conditions are identical except for different sample load; the latter was adjusted to the detection sensitivity. (A) Fast Green FCF: 5 μ L serum. (B) CBB G (colloidal): 5 μ L serum; similar patterns were obtained for CBB R. (C) silver: 0.17 μ L serum. (D) RuBP: 0.5 μ L serum; similar patterns were obtained with Deep Purple. (E) 0.42 μ L serum pre-labeled with Cy3 (on the same gel also 0.42 μ L serum pre-labeled with Cy5 was run; due to filtering, only the Cy3-channel was recorded in this image); similar patterns were obtained with Cy5. (F) FlaSH^{Pro} Red: 0.17 μ L serum labeled with 4 μ L dye. For more details see Section 5.1. After scanning, images were extracted as tif files; they were adjusted for the present figure to approximately the same intensity by Corel Photo-Paint.

changes were expressed as percentages of the corresponding spot in the CBB G gel. The obtained curves show clearly that results from different staining procedures may vary considerably, depending on the respective spot. Variation is usually larger for very small spot volumes (left part of the curves), but cannot explain the majority of changes noticed. For most spots, dyes can be grouped: triphenylmethane dyes react more similarly to each other than to RuBP and Deep Purple, silver, CyDyes or even FlaSH^{Pro}. Changes range from less than 100% to several thousand percent.

Figure 8 compiles histograms of some spots or spot chains, most of them already identified [21]. They show different staining behavior, as illustrated, which may sometimes be explained by the amino acid composition of the molecules. Albumin, for instance, stains very well with Fast Green, but also with Deep Purple and FlaSH^{Pro}. The latter may be explained by the high number of disulfides present in the molecule (17 disulfide groups in HSA, see [183]), which are well detected in cysteine-specific labeling. Similar preferential effects are noticed with spots from the IgG heavy chain: they are nicely seen with FlaSH^{Pro}, but also with CyDyes, silver and the fluorescent dyes, but stain very badly with CBB and Fast Green (see also Fig. 6). They contain alkaline amino acids and cysteines, which form the bonds between their subunits and within different stretches of the molecule (for human IgG, see [184]). The contrary is seen for apolipoprotein A-I which is almost absent in staining with

this dye (see Fig. 6F), as it contains no cysteines in its sequence (Swiss-Prot accession number P02648). Good detection of BSA and low reactivity of IgG, both with CBB G, have already been noticed in the Bradford [185] protein assay, and selection of the first or the latter to prepare the standard curve for the photometric assay gives different results for a given sample.

Proteins consisting of different spots or spot rows may show similar or different behavior of those with the same dye, as seen for haptoglobin β -chain, proteins A (a moderately acidic protein of about 60 kDa, only 3 spots out of 6 are shown) and B (55 kDa; *pI* approx. 5), and later on in Section 5.2. There are also proteins that stain very badly with

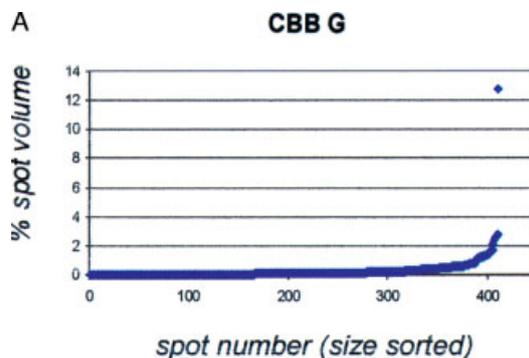


Figure 7

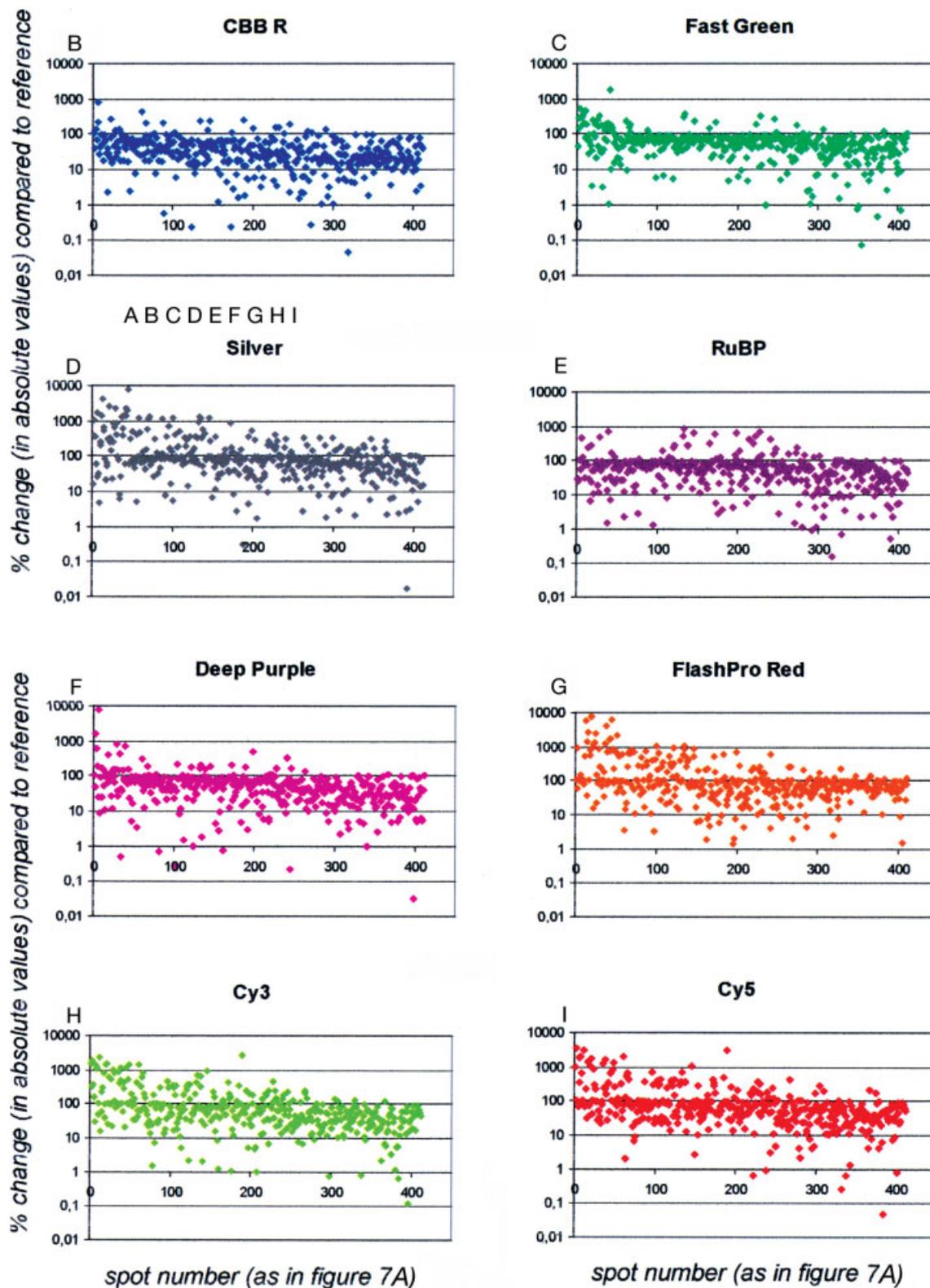


Figure 7. Comparison of all spots in the overall pattern: normalized volumes (expressed as percentage of overall volume) of the detected 411 spots in the CBB G gel are plotted. (A) Resulting curve when they are sorted according to size. (B–I) Spots from other gels corresponding to those in (A) are displayed. Percent changes of normalized spot volumes are plotted (as absolute values and on a logarithmic scale) in the same order as in (A), with one graph for each dye.

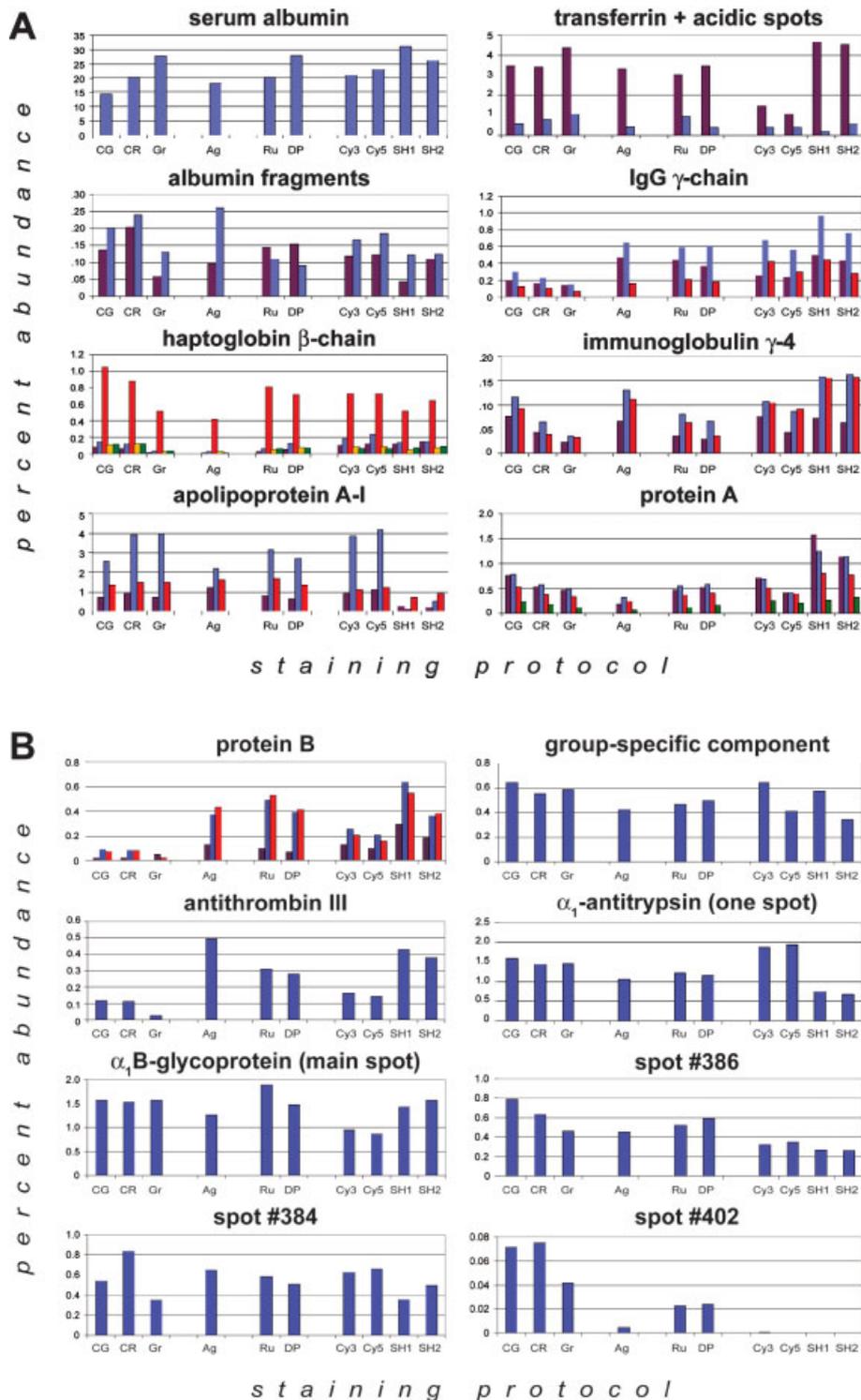


Figure 8. Histograms of selected spots/proteins, comparing normalized spot volumes obtained for the same spots with different staining methods. CG, CBB G; CR, CBB R, Gr, Fast Green; Ag, silver; Ru, RuBP; DP, Deep Purple; Cy3, Cy3 minimal dye; Cy5, Cy5 minimal dye; SH1, FlaSH^{Pro} Red (4 μ L dye/10 μ g protein); SH2, FlaSH^{Pro} Red (0.8 μ L dye/10 μ g protein).

one or the other dye (*e.g.*, anti-thrombin III, spot 402), even if they belong to the same dye family (group-specific component with Cy3 and Cy5, for more examples see Section 5.2) or are only coupled with different ratios (FlaSH^{Pro} and group-specific component or protein B).

Different dyes mean also different sensitivity and for each dye there is an optimal concentration range. In the present experiment, concentrations vary over a range of 30 (see Table 5), which may give rise to different kinds of artifacts (*e.g.*, overloading of the gel).

Saturation of abundant spots is a general problem in 2-DE evaluation, in particular when working with complex samples like serum, which have a very uneven protein distribution. In the presented examples, saturation was avoided by careful selection of protein load specific for each staining method and, for fluorescence measurements, by adapting the photomultiplier voltage for the scanning process. For stains with a narrow linear range, like silver and CBB G, this proved challenging, and some spots were slightly saturated or close to saturation.

In this study, we explored the effect of the 14 most abundant spots per gel image on the normalization procedure; some of those spots exhibited signal saturation in silver staining. Data obtained showed that, while the exclusion of these spots did alter the normalized volumes obtained, the general trend in fold-change across the gel images for a representative number of spots chosen were not appreciably affected, *i.e.*, that normalization was not being biased by the large volume spots. Despite this observation, it is important to emphasize that these data represent a controlled set, constituting a comparison of the same sample, albeit visualized by different staining procedures. In real biological applications where different experimental states are being compared for expression changes representative of a particular condition, then the inclusion of highly to medium abundant and saturated spots will have the potential to bias normalization, in particular if they have a variance that is a significant proportion of the total spot volume. For example, serum protein distribution changes markedly in inflammation and several highly abundant proteins are either positive or negative acute-phase proteins [14, 186]. A possible bias can be determined by reviewing the normalized volumes to see if a minority of spots comprise a large proportion of the total spot volume. Once identified, these spots can either be excluded from the normalization calculations or investigated to see if they vary consistently across the gel series.

The use of TT900 S2S incorporating SameSpots ensured that the same spot in every gel could be measured and used in the investigation, thereby eliminating the influence of missing values attributable to variations in spot patterns and intensities associated with different protein stains. Furthermore, the use of the same spot outline afforded the unique ability to be able to measure the same area from each image.

5.2 Single proteins and their behavior in labeling with CyDyes

DIGE has become very fashionable during the last few years, and first reports were enthusiastic about the similarity of the dye sets used (see Section 2.1.4). With growing numbers of users, increasing experience and improved equipment, “strangely behaving” proteins are sometimes mentioned. Despite the fact that the dyes are charge-matched and checked by the suppliers, dyes may behave differently in particular cases. This short section is intended to highlight potential problems and to avoid them with appropriate strategies.

Figure 9A shows the spot train of apolipoprotein A-I, a prominent serum protein in all animal species and humans. In a study on dog serum protein patterns (manuscript in preparation), we noticed differential behavior of the spots in minimal labeling with CyDyes. Eight serum samples (of 5 individuals with different health status) were minimally labeled both with Cy3 and Cy5, respectively, spot volumes quantitated, and ratios Cy3/Cy5 determined. Patterns were characteristic: the two acidic spots 406 and 408 bound more Cy5, the main spot 386 and the pro-form 401 more Cy3. Variation was only within a minimal range. According to the literature, the more acidic spots derive from stepwise deamidation [187], but in this process only asparagine and glutamine should be involved. Both Cy minimal dyes react preferentially with lysine (see Section 2.1.4.1), but it seems that these amino acids are not equally well accessible for them. Interestingly, no such behavior has been noticed for equine apolipoprotein A-I (see Fig. 8).

In a study on rat mitochondria of controls and LPS-challenged animals [188], two spots identified as cytochrome c oxidase were among potentially interesting candidates for differentially expressed spots. An experiment with reverse-labeled samples showed that this was not a true difference: spots gave higher values in Cy3 labelling and scanning with

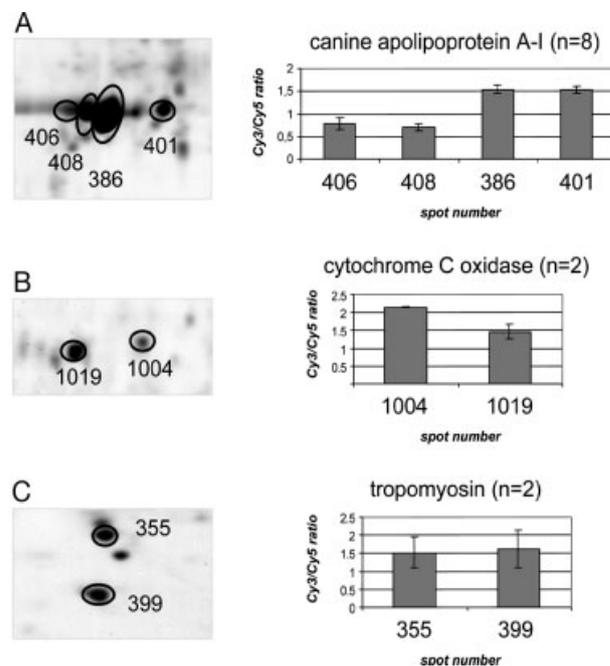


Figure 9. Examples for protein spots with differential labeling with CyDyes: close-ups and Cy3/Cy5 ratios for (A) canine apolipoprotein A-I in dog serum (identification by immunoblot, own unpublished results); (B) cytochrome c oxidase in rat liver mitochondria preparations: spots were identified by MS [188] with accession numbers P11240 for spot 1019 (cytochrome c oxidase polypeptide subunit Va) and P12075 for spot 1004 (subunit Vb); (C) tropomyosin in transfected cat kidney cells (spots identified by MS [189] with accession numbers P07951 and P09493 for spot 355 and Q5VU66 and P67936 for spot 399).

setting appropriate for this fluorophore (Fig. 9B). A similar case was also found for tropomyosin spots when investigating MMTV transfection of a cell line (cat kidney cells [189]). Here, two tropomyosin spots were markedly better detected with Cy3 (Fig. 9C). Therefore, tropomyosin spots could clearly be excluded from the list of potentially interesting spots in this experiment. Tropomyosin has been reported in literature to be an interesting protein in oncology. For instance, Friedman *et al.* [93] found tropomyosin levels changed in colon cancer patterns when comparing patient samples of Cy3-labeled normal mucosa and Cy5-labeled tumor tissue. Changes are in a range similar to ours. On the other hand, with CyDye saturation labeling, Kondo *et al.* [190] report differences in tropomyosin levels in normal intestinal epithelium *vs.* adenoma tissue in Min mice, using only Cy5 for samples (and Cy3 for standard mix). Literature data are on human or mouse homologues, our own on feline.

The explanation for preferential staining of rat cytochrome c oxidase and feline tropomyosin seems to lie in the spectral properties of the respective proteins. Their self-fluorescence adds to the one from the red fluorophore Cy3.

An example for preferential labeling of bacterial proteins from *Erwinia carotovora* separated in 2-DE DIGE is mentioned and shown also in [103, 191].

5.3 Examples from literature (for comparison of different dyes)

There are few papers or examples in the literature dealing with different staining properties of specific proteins or samples (some examples are already referred to in Sections 2–4). Metabolic labeling ($[^{35}\text{S}]\text{Met}$ and $[^{32}\text{P}]\text{ATP}$, detected by autoradiography or fluorography) has been compared to pre-electrophoretic fluorescence labeling with monobromobimane, silver staining and blotting of *S. cerevisiae* proteins, separated by CA (carries ampholyte) and IPG-DALT [192]. Differences in patterns were noticed, especially with cysteine labeling. For wool traits, CBB (esp. CBB G) proved to give higher sensitivity than the silver patterns stained in parallel. Those gave negatively stained spots for intermediate filament proteins, the major high sulfur proteins and the high glycine tyrosine proteins [193]. Lopez *et al.* [68] found about 90% similar proteins in silver- and SYPRO Ruby stained rat fibroblast cell lysates, whereas Chevallet *et al.* [55] showed differential images obtained with acidic, ammoniacal and the Shevchenko silver stains, and RuBP, for human erythrocyte membrane and mouse macrophage cell line proteins. 2-DE *E. coli* protein separations displayed overall pattern similarity, but closer examination revealed regions with marked differences between the new fluorescent dyes SYPRO Ruby and Deep Purple, CBB and silver [25]. An evaluation of *Arabidopsis* total protein extract patterns obtained by SYPRO Ruby, Deep Purple, C16-F (5-hexadecanoylamino fluorescein), colloidal CBB, and silver gave best results (spot numbers and reproducibility) for the new fluorescent dyes [45].

6 Summary

The examples listed in Section 5 and the basics compiled in Sections 2 and 3 show clearly that, although there is a large number of different staining methods, the patterns obtained may differ, depending on the detection mechanisms of the applied methods. Reactions are not always to be predicted, but may be sample specific and need careful evaluation, and probably a few practical experiments. Some points to be aware of when selecting a stain for a particular experiment are: (i) composition of the proteins of interest (presence/absence of single amino acids), (ii) availability of sample (protein amounts and concentrations, expected evenness of protein distribution, additives), (iii) post-staining of the gels (*e.g.*, MS, sequencing), and (iv) equipment.

Ingrid Miller gratefully acknowledges funding by GEN-AU (BM: BWK GZ 200.069/2-VI/1/2003). The project was also supported by the Research Focus Programs "Proteomics in veterinary medicine" and "Biomarker" of the University of Veterinary Medicine Vienna. Proteomic investigation at DSF is supported by grants from MIUR (FIRB) and from Association Française contre les Myopathies.

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