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REVIEW

Quantitative proteomics: assessing the spectrum of in-gel protein detection methods

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Abstract Proteomics research relies heavily on visualization methods for detection of proteins separated by polyacrylamide gel electrophoresis. Commonly used staining approaches involve colorimetric dyes such as Coomassie Brilliant Blue, fluorescent dyes including Sypro Ruby, newly developed reactive fluorophores, as well as a plethora of others. The most desired characteristic in selecting one stain over another is sensitivity, but this is far from the only important parameter. This review evaluates protein detection methods in terms of their quantitative attributes, including limit of detection (i.e., sensitivity), linear dynamic range, inter-protein variability, capacity for spot detection after 2D gel electrophoresis, and compatibility with subsequent mass spectrometric analyses. Unfortunately, many of these quantitative criteria are not routinely or consistently addressed by most of the studies published to date. We would urge more rigorous routine characterization of stains and detection methodologies as a critical approach to systematically improving these critically important tools for quantitative proteomics. In addition, substantial improvements in detection technology, particularly over the last decade or so, emphasize the need to consider renewed characterization of existing stains; the quantitative stains we need, or at least the chemistries required for their future development, may well already exist.

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Abbreviations

2DE	two-dimensional electrophoresis
BisANS	4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid
BSA	bovine serum albumin
C16-F	C-16 fluorescein
CA	carbonic anhydrase
CBB	Coomassie Brilliant Blue
cCBB	colloidal Coomassie Brilliant Blue
CCD	cooled charge device
Cy2	Cyanine 2
Cy3	Cyanine 3
Cy5	Cyanine 5
cys	cysteine
DIGE	differential gel electrophoresis
DMSO	dimethyl sulfoxide
DP	deep purple
DTT	dithiothreitol
EBT	erichrome black T
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
EZ	ethyl violet and zincon
HAc	acetic acid
HSA	human serum albumin
ICy	iodoacetylated cyanine dye
IEF	isoelectric focusing
LC	liquid chromatography
LDR	linear dynamic range
LLD	lowest limit of detection
lys	lysine

MALDI-ToF-MS	matrix-assisted laser desorption ionization-time of flight-mass spectrometry
mBB	monobromobimane
MDPF	two-methoxy-2,4-diphenyl-3(2H)-furanone
MeOH	methanol
MS	mass spectrometry
MW	molecular weight
OPA	*o*-phthalaldehyde
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PhosB	phosphorylase b
PMF	peptide mass fingerprinting
RuBPS	ruthenium (II) tris (bathophenanthroline disulfonate)
SA	Stains All (1-ethyl-2-{3- [1-ethylnaphtho (1,2d) thiazolin-2-ylidene]-2-methyl-propenyl}-naptho (1,2d) thiazolium bromide)
SDS	sodium dodecyl sulfate
SNR	signal-to-noise ratio
sp.	species
SR	SYPRO Ruby
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
Trp	tryptophan
UV	ultraviolet

Introduction

Proteins are the primary functional agents of biological systems; they underlie and regulate metabolic processes, signal transduction, small molecule/ion transport, cell replication, and apoptosis [1, 2]. Examining the proteome (the entire complement of proteins expressed by a genome in a given biological sample—whole organism, tissue, fluid, cell, or organelle) is thus a critical way to analyze how a cell responds to its environment [3]. The pursuit of this endeavor has spanned over half a century and involved innovations in a range of different methodologies that have made the study of organisms across an array of molecular levels possible. Such a breadth of 'omics analyses (i.e., genomics, proteomics, lipidomics, metabolomics, etc) has led to a Systems Biology approach that is now gradually enabling the integrated understanding of cell and organismal physiology [4]. At the protein level, although newer technologies for resolution and identification are regularly introduced, polyacrylamide gel electrophoresis (PAGE) remains the most accepted, widespread, and successfully implemented technique for the quantitative, high-resolution separation, and characterization of these critical molecules [5].

Utilization of PAGE as a single (native or detergent based PAGE) or second dimension of separation following isoelectric focusing (2D PAGE), has been shown to deliver very high resolution and reproducibility for protein separation [3, 6–12]. In a single analysis, 2D electrophoresis (2DE) provides information on protein charge, abundance, localization, isoforms, and post-translational modifications. The interface between PAGE and downstream mass spectrometry (MS) has provided perhaps the most important modern innovation for protein identification as well as more detailed analysis of post-translational modifications [13]. Although it was initially thought that 2DE was not ideal for resolving some proteins (i.e., membrane, very acidic, low abundance, and so forth), modern methodological optimizations minimize such suggested limitations; this is the power and versatility of a well-characterized, mature technology [14, 15]. Examples of these technical enhancements include the use of (narrow range) immobilized pH gradient strips or (extra) large gel formats to accommodate additional resolving area [14]. The complexity of the protein milieu can also be reduced using pre-/sub- and/or post-fractionation of samples [3, 13, 16]. With these techniques, protein resolution has been substantially and routinely improved. Taken together, all of these developments indicate that the main limitation to the amount of proteomic information obtained from 2DE is unlikely to be its capacity to resolve proteins but rather to be one of protein detection (i.e., stain sensitivity) [17].

Even though 2DE separates proteins such that their individual isoelectric points (*pI*) and subsequent electrophoretic mobility act as physical coordinates on a gel, none of this information can be assessed until the protein map has been visualized. This is achieved through the use of protein stains which generally bind to proteins in situ, within the polyacrylamide gel matrix. As might be expected of such a mature technology, there exists a diversity of such reagents including densitometric stains such as (colloidal) Coomassie Brilliant Blue or Silver, fluorescent stains including Sypro Ruby (SR), Deep Purple (DP), and the reactive CyDyes and Alexa Fluors [differential gel electrophoresis (DIGE) dyes] [10, 18–21]. Despite this variety of available stains, the greatest challenge with regard to protein detection is to identify a protein stain that has the following characteristics:

1. a routine and reproducible lowest limit of detection with optimal signal-to-noise ratio (SNR);
2. a wide dynamic range, with a linear relationship between protein quantity and staining intensity;

3. compatibility with downstream microchemical characterization techniques;
4. ease of use;
5. inexpensive, high throughput rates of use.

There is no protein stain currently available that possesses all of these desired properties.

This paper will thus review and critically analyze some common stains used for quantitative protein detection in an effort to identify stains that may currently best satisfy the breadth of proteomic applications. Specific criteria chosen as relevant measures of effectiveness include: (1) a reported lowest limit of detection (LLD; standard error noted when given), defined as the lowest concentration that delivers a pixel volume three standard deviations greater than that of the measured background [22]—the smaller this value, the more sensitive the stain; and (2) linear dynamic range (LDR), a measure of the total capacity of a stain for accurate quantification—defining a strictly linear relationship between quantity and signal with minimal deviation. Whenever such information is available stains will also be evaluated in terms of (3) inter-protein variability; (4) total number of spots detected (i.e., after 2DE); and (5) MS compatibility. Evaluation of quantitative performance will focus on those studies presenting a minimum of three of the above defined criteria for 1D and/or 2DE analyses.

Densitometric stains

Coomassie Brilliant Blue

Prior to the 1960s, the separation of protein mixtures was commonly performed using filter paper, cellulose acetate strips, starch, or agarose support mediums. Polyacrylamide was introduced as an alternative due to its superior physical properties [23–25]. The stain most commonly utilized at this time for mainly qualitative in-gel protein detection was amido black. It was not until the mid 1960s that researchers prioritized the detection sensitivity of protein stains and described Coomassie Brilliant Blue (CBB) as the preferred stain for quantitation [18, 26]. CBB is thought to bind to proteins through electrostatic interactions between the sulfonic groups of the dye and the basic side groups of amino acids [18, 27–29]. It has also been suggested that CBB binds to proteins through interactions with aromatic residues as well as hydrophobic interactions [27, 29].

Over the years, there have been a remarkably large number of studies dedicated to improving solvent composition, changing dye concentration/type and developing strategies for staining protein/destaining gel matrix in 1D PAGE or isoelectric focusing (IEF) gels to potentially achieve higher levels of sensitivity [30–42]. This continuous drive for improvement, however, was not able to enhance the detection sensitivity of CBB below ~30 ng of bovine serum albumin (BSA) or actin [33, 43–45]. The year 1981 saw mini-gel staining with CBB R-250 detect as little as 10 ng of protein (BSA, carbonic anhydrase (CA), α–lactalbumin) via microdensitometry [46]. This method also delivered a LDR between 10 and 200 ng for the aforementioned proteins. Inter-protein variability was reported as a percentage of protein weight successfully stained and was determined to be acceptable (percentages ranged from 11.5% to 26.7% with 5.4% standard deviation). Perhaps the most significant contribution to CBB staining was made by Neuhoff et al. [47] when the colloidal state of CBB (in particular the G form) was utilized to improve detection sensitivity from 10–30 ng to 1 ng of BSA. It was also claimed as little as 0.1 ng (BSA) could be detected [47]. This move from the traditional CBB formulation in organic solvents which produced high background staining to an alternate formulation marked a significant advance in CBB staining. The use of colloidal CBB permitted free coomassie molecules to penetrate the gel and bind to protein while the remainder was in large colloidal particles that were excluded from the gel [47, 48]. Additional advantages of this colloidal CBB (cCBB) formulation included reduced background staining of polyacrylamide gels and a simplified, lower cost procedure. Comparative evaluation of the available literature concerning CBB sensitivity yielded little published quantitative information that fulfilled the criteria for analysis in this review. Although, single aspects of CBB sensitivity have been previously addressed in specific studies, recent work clearly indicates that the sensitivity related characteristics of a stain, such as LLD and LDR, must both be analyzed to ensure confidence in the capacity of a stain to quantitatively represent the amount of protein in a given band or spot.

A well-designed study utilized a commercial formulation of cCBB (Pierce Chemical Company) and determined the LLD to be between 8 and 16 ng for broad-range molecular weight (MW) standards (Bio-Rad; nine proteins, 6.5–200 kDa) and a LDR for all proteins between 30 and 250 ng with high correlation (R=0.9883) [20]. It was also shown that cCBB staining for four standards, in comparison to the fluorescent stain SR, produced low inter-protein variation. Furthermore, similar peptide mass profiles from matrix assisted laser desorption ionization–time of flight–mass spectrometry (MALDI–ToF–MS) were obtained following the use of either stain [20]. In comparison, the use of the Neuhoff CBB formulation [49] yielded a LLD of 4–8 ng protein (five standard proteins), LDR between 30 and 500 ng for BSA (R=0.985), 8–500 ng for phosphorylase b (PhosB), ovalbumin (OVA) and peroxiredoxin (R=0.987, 0.992, 0.983, respectively) and 15–500 ng for CA (R=0.981); and there was similar MALDI–ToF–MS sequence coverage for all proteins and protein loads (4–125 ng/band)

[49]. However, it was noted that sequence coverage was detrimentally affected when CBB stained gels were not destained.

While the staining characteristics of standard proteins are informative, the real application of a stain is the sensitive detection of proteins obtained from complex native samples. The Neuhoff formulation of cCBB was applied to a 2D separation of a total soluble protein extract derived from *Arabidopsis thaliana* to determine detection sensitivity [17]. Here, it was shown that the LLD for the total protein extract, consisting of a complex mixture of proteins with varying mobilities and abundance, was 2 μg of the protein loaded. The LDR for two proteins, one of high and the other of low abundance with relatively similar mobility, was linear (15.6 ng–4 μg) with a correlation value of 0.99. Resolution of this native extract by 2DE resulted in 250 detectable spots, the lowest total spot number obtained in comparison to all stains tested in the study [17].

Notably, it has recently been demonstrated that the imaging mode of cCBB stained gels may contribute to reduced sensitivity. Analysis of low MW protein standards (GE Healthcare) revealed that using the scanning transparency mode (as opposed to the standard reflection mode) for imaging cCBB stained gels improved the sensitivity of low MW proteins by as much as eight- to 29-fold [50]. This study reported a LDR from the individual protein LLD up to 1,000 ng, with no observed change in MS compatibility; the sequence coverage for all analyzed protein groups (three groups based on molecular weight, for a total of 24 proteins) ranged between 47% and 60% [50]. Therefore, it may be useful to explore alternative imaging modes before the densitometric use of CBB is completely dismissed as a tool for protein quantification.

As noted above, although several other published improvements to CBB staining protocols reportedly also deliver increased sensitivity, the assessments carried out in those studies unfortunately do not allow for their evaluation in this review [21, 51–55]. Similarly, alternate staining strategies have also been developed to improve protein detection sensitivity by combining CBB with other stains. Limited quantitative characterization, and in some cases failure to offer a quantitative advantage have limited their use in proteomics [45, 56, 57]. Commercialization of CBB has become widespread and the stain is available as a stable and ready to use product at low cost. Most commercial CBB stains, usually the G form, are marketed for densitometric detection. However, CBB may be re-visited as a sensitive stain once again since recent literature has indicated that near-infrared fluorescence detection of proteins by CBB offers improved sensitivity [21, 54]. Even though the densitometric use of CBB is relatively insensitive, use of this stain may be reborn in proteomics for fluorescent protein detection.

Silver stain

The search for a new stain was warranted by the principle limitation to CBB staining: its apparently inadequate sensitivity for protein detection. In 1979, a silver stain procedure for in-gel protein detection was suggested to be 100-fold more sensitive than CBB [19]. This marked improvement in detection sensitivity heralded a period of studies aimed at perfecting the original silver stain method and/or developing alternate silver stains [58–71]. Even though there are numerous methods for silver staining described in the literature, most comprise the same main steps—fixation, pretreatment/sensitization, impregnation (saturating the gel with silver ions), development (change in pH reduces silver ions to metallic silver) and cessation of development. In 1981, a rapid silver stain method was reported that still delivered reproducibility and high sensitivity, yielding a LLD of 0.1–0.2 ng and LDR between 1 and 30 ng (for three standard proteins) [46]. The authors described inter-protein variability in terms of percentage weight of proteins in the sample that were successfully stained. The stained fraction ranged from 5.2% to 28.2% (16.7±9.7%; mean ± SD), suggesting that proteins such as PhosB, BSA, and OVA were not stained uniformly by this silver staining protocol. A different silver stain method was utilized to optimize quantitative detection of BSA, OVA, CA, soybean trypsin inhibitor, and α-lactalbumin detection in 2D gels [72]. While the rapid method above clearly indicated the temperatures at which staining should be undertaken [46], this second protocol was less attentive to temperature [72], focused on stain components and duration of exposure as well as employing a twofold stronger developer to deliver a LLD of 27 pg/mm^3. The LDR values were markedly different for each of the proteins tested and ranged from the reported LLD up to 5–50±16.3 ng/mm^3 [72]. While numerous similar studies for the optimization of silver staining have been published, most do not meet the quantitative requirements for this review.

In 2000, it was shown that the acidic silver nitrate stain (Investigator Silver Stain Kit, Genomic Solutions) was more sensitive than CBB (and cCBB), detecting as little as 2–4 ng protein, but was limited by a narrow LDR (4–60 ng protein or 1 order of magnitude) [20]. This silver stain was susceptible to high inter-protein variability and could only be compatible with MS when glutaraldehyde was omitted from the process. Application of this silver stain kit (without glutaraldehyde fixation) to 2DE analysis of rat fibroblast whole soluble cell lysate revealed a small dynamic range—231.1 pixels within the spot perimeter (calculated as the difference between the most intense and the least intense matched spot) and a slightly lower total spot number (82%) relative to staining with SR [73]. While comparable between silver and SR stained protein at higher

loads, MS coverage for two standard proteins (PhosB and OVA) was decreased at lower protein loads for silver stain (without glutaraldehyde). A silver nitrate staining method developed by Mortz et al. [74] was applied to *A. thaliana* total soluble protein extract separated by 1D and 2D PAGE [17]. The LLD was determined to be 1 ng (i.e., more sensitive than cCBB), but showed lower correlation for two unspecified protein bands arbitrarily selected by the authors (band 1, R=0.82; band 2, R=0.88). Also, total spot detection in 2D gels using silver nitrate was superior to that of cCBB but not equivalent to SR (cCBB ~300; Silver ~600; SR ~800). Since the introduction of silver stain, there have been numerous attempts to enhance staining intensity. However, the addition of blue toning (i.e., incubation of a silver stained gel in a solution containing ferric chloride/potassium hexacyanoferrate III/oxalic acid which turns protein bands blue), Stains All (SA) or CBB staining prior to or following silver staining has demonstrated no substantive improvement in sensitivity of detection and/or quantitation [75–82]. Thus, while it is generally accepted that silver staining provides slightly better sensitivity than CBB, one of its major limitations is its incompatibility with MS [83]. This incompatibility is believed to be due to the strong chemical reagents employed in the silver staining process which results in chemical modification or destruction of proteins. Chemical modification can involve glutaraldehyde cross-linking with proteins and blockade of trypsin digestion. This reduces the number of available peptides, sequence coverage and thus quality of protein identification [67, 84, 85].

MS compatibility is improved when glutaraldehyde/formaldehyde is omitted and/or ammoniacal silver staining is used, but this compromises detection sensitivity [68, 86, 87]. Due to this less-than-ideal compromise, there have been attempts to sustain sensitivity and increase MS compatibility by other means. It was shown that calcon-carboxylic acid introduced as a silver ion sensitizer produced better sensitivity over the traditional silver stain method (0.05–0.2 ng/band) [88]. The LDR for standard proteins (SDS–6H, a mixture of the six standard proteins BSA, CA, PhosB, β-galactosidase, albumin, myosin; Sigma) covered a variety of ranges all with a narrow spread of linearity and exhibited high inter-protein variation (based on visual examination of the LDR plots shown) [88]. Application of this alternate silver stain to *Escherichia coli* BL-21 total soluble protein extract yielded a 2D protein map with a greater number of spots than seen with the traditional silver stain (as indicated by authors, and visual comparison of the images shown). Although not yet demonstrated, the assumption of MS compatibility is based on the fact that the stain does not appear to covalently modify protein. Another protocol utilizes erichrome black T (EBT) as the silver ion sensitizer [89]. Here, the LLD of EBT–Ag was 0.05–0.2 ng/band (SDS-6H; Sigma) and the stain was also capable of qualitatively detecting lower loads of total soluble protein extracted from *E. coli* in comparison to silver staining without glutaraldehyde. 2DE analysis of *E. coli* total soluble protein also supported the superior performance of EBT-Ag over traditional silver staining with 16% more detected spots [89]. The staining of standard proteins demonstrated that MS compatibility and sequence coverage were relatively similar for each protein tested between loads of 6–100 ng/band. Some proteins were also identified from as little as 3 ng/band [89]. Another MS compatible silver stain made use of the counter–ion dyes, ethyl violet and zincon (EZ). The LLD of standard proteins was 0.2 ng/band (SDS–6H; Sigma), the LDR was between 4 and 50 ng, and sequence coverage was relatively similar for each protein tested between loads of 6–100 ng/band and occasionally at 3 ng/band [90].

Although it is clear that silver staining can be altered to achieve specific requirements and high-detection sensitivity, there are various drawbacks to the use of this method. Silver staining requires various reagents to be prepared fresh with high quality water, can be laborious and tedious, tends to produce large inter-gel variation in intensities as it is without a staining endpoint and exhibits poor linear dynamic responses. Other potential drawbacks to silver staining that may also interfere with qualitative and quantitative analyses arise due to the fact that silver stain does not specifically stain proteins—it also detects nucleic acids and lipopolysaccharides [66, 91–94] and is not sensitive for detection of all types of proteins [95]. Thus, a range of potential problems need to be carefully considered when choosing silver stain for in-gel protein detection and quantitation.

Negative stains

While various approaches to negative staining of proteins in-gel had previously been developed [96, 97], it was not until the introduction of heavy metal salts that wider interest in the application was piqued. Copper chloride was initially utilized for negative staining of protein bands [98]. Although copper chloride was found to be useful for negative staining, it was later shown that zinc chloride detected proteins with even greater sensitivity [99]. Alternative zinc chloride staining could also be achieved by exploiting the pH dependence of zinc chloride complex precipitation [100]. However, zinc staining by these methods was not homogeneous. The introduction of imidazole for zinc staining overcame this problem in SDS-PAGE applications [101] and was further modified

for application to non-SDS gels [102]. These marked improvements to zinc staining resulted in a large number of studies aiming to further enhance the method and demonstrate that proteins within zinc stained gels were still biologically active, could be recovered with high yields and were compatible with MS technologies [103–110]. Since this process leaves proteins presumably untouched, significant effort was invested in highlighting its qualitative potential and studies examining its quantitative capacity have been less prevalent. The quantification of protein using zinc staining is controversial in itself as the protein is not stained and protein concentration can only be quantified by pixel inversion. For this reason, few studies have characterized the potential for zinc staining as a quantitative in-gel protein detection method.

The zinc staining procedure developed by Fernandez-Patron et al. [111] showed the LLD of broad-range MW standards (Bio-Rad) to be between 1 and 2 ng protein, similar to that of SR [20]. Although the LDR ranged from 250 to 2,000 ng (standard proteins; R=0.9843) it was obvious that the main limitation of zinc staining was the detection of low abundance proteins. Using a rapid zinc stain kit (Visual Protein, Taipei, Taiwan) it was shown that the LLD of low MW standards (GE Healthcare) ranged between 1.8 and 4.0 ng/band [50]. Although the use of scanning mode, rather than reflective mode, for imaging did not improve detection sensitivity it did, however, provide images with greater contrast. The LDR for this zinc procedure was very narrow (no greater than 140 ng) and may have been influenced by the rapid nature of the procedure used, as described by the manufacturer. MS analysis of soluble proteins from human hepatoma cell (hepG2) revealed that for 24 selected proteins (divided into three groups based on MW: (1) >45 kDa, (2) 30–45 kDa, and (3) <30 kDa), sequence coverage was roughly uniform (51.3–56.4%) [50].

The most significant disadvantage of negative staining is that protein quantification is only approximate given the nature of the staining. Also, zinc staining is not specific to protein and can also effectively detect nucleic acids and lipopolysaccharides [112, 113]. Since this stain has no definitive endpoint, its use also involves the risk of overdevelopment. Given these limitations and the sparse literature investigating the quantitative capacity of zinc staining, it seems questionable whether the application of this method will make a significant contribution to quantitative proteomics.

Reactive densitometric dyes

The application of Uniblue A capitalized on the staining capacity of amines. When reacted pre-electrophoretically at a ratio of 3 mg dye for every 10 mg protein (40°C for 3 h–pH 10.5), the 600 nm densitometrically measured LLD was reported to be 0.5 μg of protein [114]. While lysozyme staining was not consistent with the other standard proteins tested, the relationship between quantity and stain intensity for the majority remained linear from 0.5 to 25 μg (LDR) [114]. A high pH was chosen to accelerate the pre-labeling reaction; however, this may not be optimal in some cases. The authors advised that the reaction was feasible at lower pH levels and that this may be preferable if a universal approach to staining proteins of different pH lability was required. Another moiety specific stain, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide in combination with fast black K, was used to densitometrically detect sulfhydryl groups. An LLD of 0.25 μg of lysozyme and 1 μg for all other tested proteins was reported [115]. However, since the sensitivity of this dye combination depends on sulfhydryl content, inter-protein variability is to be expected.

Additional densitometric stains

While CBB (colloidal or traditional) and silver staining have remained the strongest contenders for densitometrically detecting proteins, alternatives have been explored. However, the only other densitometric stain that has been quantitatively assessed and adheres to the criteria of this review, is based on the counter-ion dye couple of EZ. This EZ stain was first introduced for in-gel protein staining in 2002 and was slightly less sensitive than cCBB [116]. Two years later, the same research group used longer fixing times and EtOH instead of MeOH as the solvent to successfully demonstrate EZ staining comparable to cCBB [49]. For five standard proteins (PhosB, BSA, chicken OVA, bovine CA, and human peroxiredoxin I) the LLD was between 4 and 8 ng protein. The LDR for PhosB, OVA, and peroxiredoxin spanned between 8 and 1,000 ng (R=0.997, R=0.996, and R=0.987, respectively); 4–1,000 ng for CA (R=0.991) and 8–500 ng for BSA (R=0.983) [49]. It was also shown that MS compatibility of this stain was comparable to cCBB for all loads between 4 and 125 ng/band [49]. Alternate densitometric stains have not gained popularity within the proteomic research community because these stains have been unable to compete with the performance of fluorescent staining and imaging. Also, most of these alternate densitometric stains have, at best, had limited characterization or have similar detection sensitivity to cCBB and hence have not been pursued for further application in proteomics [54, 117–126].

Fluorescent dyes

Unlike densitometric stains which absorb light, fluorescent stains are detected by the light they emit. Emission is a result of excitation with a particular wavelength of light

which elicits an energy shift in the fluorophore. When the fluorophore returns to its ground state, this energy can be emitted as a measurable photon, thereby enabling detection of protein when it is associated with the fluorophore [127]. New fluorescent stains have seen increasingly widespread use as they address some of the limitations of densitometric stains: they are sensitive, are measured using light emission rather than absorbance, have a broad LDR, produce minimal background interference and are compatible with MS analysis.

Reactive fluorescent dyes

The reactive dyes used in proteomics, so called as their labeling of proteins involves a chemical reaction, form permanent covalent bonds with proteins. Typically, this reaction targets specific moieties on an amino acid residue such as an amine, thiol or carboxyl side chain. In the presence of surplus dye (and/or permissive pH conditions), this reaction can become non-specific and label any susceptible amino acid. The majority of approaches using reactive dyes implement pre-labeling, whereby the reactive dye is attached to proteins in an extract prior to their resolution by electrophoresis. Some amine reactive dyes that have been tested include dansyl and dabsyl chloride and remazol; although these reactive dyes have been utilized for pre-labeling, their LLD, LDR, and degree of inter-protein variability have not yet been reported in great detail [128–130].

Amine groups are a valid target choice for pre-labeling as they are present on almost every protein in the form of N-terminal, α- and ε amines. They are highly reactive and produce a strong amide bond [131]. In particular, labeling lysine (lys) residues facilitates near complete coverage of a proteome given the prevalence of lys in proteins [132]. This may, however, affect the efficiency of subsequent trypsin treatment if the reactive dye masks the lys residues [132, 133]; nonetheless, there are a range of alternative reagents available for the controlled digestion of proteins to defined peptides, as is required prior to MS analysis [134–136].

Two-methoxy-2,4–diphenyl–3(2H)–furanone (MDPF) is a fluorescent alternative that also reacts with amines. When used to pre–label proteins, MDPF has been shown to yield a LDR between 1 and 500 ng for CA, myoglobin, catalase, and BSA [137]. An LLD of 1 ng was reported for this reactive dye and non-uniform staining for different proteins was identified [137]. For labeling, 20 μg of protein dissolved in 0.01 M borate buffer (pH 9.5) was mixed with 60 μg of MDPF in dimethyl sulfoxide (DMSO). In an independent study, MDPF was also applied to IEF, 1D and 2D post-electrophoretic gel staining [138]. For post-IEF staining, gels were shaken with 0.02 moles of MDPF in MeOH/0.2 M sodium borate buffer (pH 9.5) and washed in MeOH and water prior to second-dimension separation [138]. For post-SDS-PAGE staining proteins were fixed and stained with 3.8 mM MDPF for 1D gels and 0.95 mM MDPF with longer staining time for 2D gels [138]. An LDR between 50 and 300 ng (soluble human lymphoid cell line IM9 protein) was identified but this is clearly only a portion of that determined using the pre-electrophoretic MDPF method, and as such does not indicate a quantitative advantage over pre-labeling [138].

The quantitative potential of *o*-phthalaldehyde (OPA), a compound that reacts with primary amines, has also been reported [139]. Protein concentrations ranging from 0.1 to 50 μg/ml in 0.05 M sodium phosphate (pH 8.5) were dosed with 0.19 μmol OPA (in MeOH) and incubated in the dark. Also, the addition of β-mercaptoethanol prior to the labeling treatment was used to increase the fluorescent signal sevenfold; the reason for this was not clearly established but the authors suggested some augmentation of –SH group reactivity by β-mercaptoethanol as the cause. When used to detect transferrin, the LLD was ~10 ng while the LDR was optimal for higher loads of protein, 0.1–50 μg/ml. These values were not consistent for all proteins tested, and the authors acknowledged that inter-protein variability was an impacting factor for detection [139].

Similar limits of sensitivity were achieved by pre-labeling with fluorescamine. Protein at concentrations up to 1.0 mg/ml in 0.04 M borate buffer (pH 9.0), were labeled with 0.5 mg of fluorescamine (in DMSO), however, no information regarding the conditions of incubation were provided. This amine reactive fluorophore could detect a minimum of 6 ng of myoglobin; and the LDR for myoglobin, chymotrypsinogen and OVA were 0.5–7, 0.5–9, and 0.5–12.5 μg, respectively [140]. Again, this stain was unable to deliver a uniform interaction with all protein standards as shown by the different LDR plots for the tested proteins [140]. Furthermore, it would be impossible to reproduce these results without details concerning the incubation conditions used. Fluorescamine has also been tested as a post-electrophoretic stain of myoglobin; while this application showed potential, only the LDR (1–7 μg), was reported [141].

The staining performance of the aforementioned reactive dyes applied prior to electrophoresis, however, was not optimal in terms of sensitivity, LDR, and inter-protein variability. A significant contribution to pre-labeling methods was achieved through the introduction of the mass and charge matched lys-targeting CyDyes. These dyes are used to label only a minimal number of lys residues (1–3%) at the ε-amine of each protein [132]. A proportional representation of proteins in a sample is attempted by maintaining low dye/protein ratios. DIGE is the primary technique that applies these dyes and involves tagging proteins pre-electrophoretically with fluorescently distinct labels known

as cyanine 2 (Cy2), cyanine 3 (Cy3), and cyanine 5 (Cy5) [10, 142]. The use of these three spectrally distinct dyes allows for multiple samples to be resolved and then individually imaged on a single gel, although at substantially reduced total protein loads per sample. Alexa Fluor dyes 555 and 647 have been shown to be spectrally similar to Cy3 and Cy5, respectively, with an added advantage over the Cy dyes since they exhibit reduced photobleaching and self-quenching especially with more extensive labeling [143]. The application of these Alexa Fluor dyes for pre-labeling, however, has not been extensively tested and it remains to be seen whether they can deliver similar sensitivity.

Proteins to be labeled with CyDyes were prepared in a buffer containing 4–7 M urea/2 M thiourea/2% CHAPS (pH 8.5). Additional components include 30 mM Tris–HCl [10], 2% SB3–10 and 0.5% Triton X–100 [142]. Proteins were pre-labeled on ice for 30 min as recommended by GE Healthcare, the firm predominantly involved in marketing the DIGE technique [10, 142]. The fluorophores are used at doses between 200 and 400 pmol/50 μg of total protein and the reaction is incubated in the dark. Low rates of labeling were required to prevent reduced sample solubility and protein spot chains on gels that result from the labeling of multiple lys residues on any given protein. Pre-labeling is said to reduce inter-gel variability and improve protein detection with a LLD as low as 0.25 ng and a LDR of up to three to four orders of magnitude (determined using protein standards supplied by Sigma) [142]. Gels can also be imaged immediately following electrophoresis. While lys residues are almost ubiquitous in proteins, inter-protein variability can be expected as proteins do not have uniform amino acid content and lower abundance proteins are less likely to be labeled [10, 132]. In addition, it has also been reported that gel-to-gel variation still contributes most of the inherent variability to DIGE [144]. It is also important to consider the ramifications of selectively labeling a sample and subsequently loading only a fraction of this sample for electrophoresis; the total complement of detectable protein is thus reduced by the very design of the protocol used for detection.

When used to pre-label soluble mouse liver homogenate for 2DE separation, Cy2 detected 414±0.21 spots, Cy3 detected 289±1.09 spots, and Cy5 detected 398±0.81 spots [142]. Pre-labeling soluble proteins from *Pirellula* sp. Strain 1 for 2DE separation as described by the manufacturer (GE Healthcare), showed that Cy2 detected 399 spots, Cy3 387 spots and Cy5 418 spots [10]. In comparison, SR was found to detect more spots (443, no error provided). Unfortunately, the pre-labeled proteins themselves cannot be identified using MS techniques. However, as only a fraction of the total amount of protein per sample is thought to be labeled, the unlabeled protein is, in theory, available for MS identification. These 'unlabeled' spots can be picked by estimating the shift in gel mobility that dye labeling causes and then selecting the unlabeled gel spot that is thought to correspond to the labeled fraction [10, 145]. It is, however, unclear how this estimation is done without knowing the number of potentially labeled lys residues in any given spot representing an unknown protein. Both studies reported that the CyDye pre-labeling approach is compatible with MS identification; however, the *Pirellula* sp. strain study did not present any data to support this claim [10]. The mouse liver homogenate protein spot identities were assigned using MALDI–ToF–MS and while sequence coverage was not quoted, the authors described their process for validating MS data and cross reference the calculated characteristics of candidate proteins with reported *pIs* and MW [142].

Another approach to reactive labeling is to target the thiol groups presented by cysteine (cys) residues. While cys is not as abundant or widespread in proteins as lys [132], these thiol groups are readily reactive and thus effective targets for labeling. For this reason, cys residues can also be labeled to saturation without the substantial loss of protein solubility encountered when lys residues are targeted [146]. Cys residues are also less likely to be at trypsin cleavage sites. As such, proteins labeled at cys residues can still be identified by MS following standard digestion protocols [133, 146, 147]. Focusing on thiols also allows the *pIs* of labeled proteins to be maintained since the dyes are neutrally charged. Recently, it has been demonstrated that saturation labeling of thiols can also be successfully carried out using the BODIPY dyes (FL–*N*–(2–aminoethyl) maleimide; FL C1–idoacetamide) by optimizing the labeling reaction conditions [148]. The BODIPY dyes demonstrate a LLD of 10 fmol (without a reported error) and a LDR of three orders of magnitude for yeast enolase I. Inter-protein variation was not referred to but can be inferred from the thiol specific nature of these dyes [148].

A recent study using monobromobimane (mBB) to label cysteine residues in proteins reported a LDR of 32–1,000 ng [20]. The LLD of this stain was between 4 and 32 ng (broad-range MW standards) and when compared to silver, cCBB and SR staining, mBB demonstrated the greatest inter-protein variability [20]. Also, mBB did not demonstrate strong MS compatibility since both BSA and soybean trypsin inhibitor could not be correctly identified by MS when labeled to saturation [20]. Proteins were pre-labeled at a concentration of 2 mg/ml and following denaturation, samples were cooled before the addition of mBB (6 mM final concentration). Samples were incubated in the dark, before excess cys was used to quench the reaction. Another older study used recombinant proteins to examine mBB quantification of proteins with published cys content [149]. The average LLD of protein was 86.2±14 ng with an outlier of 212 ng for p21, a value much higher and

thus representing lower sensitivity than the more recent study. Additionally, LDR maxima themselves ranged from 110 ng (recombinant protein p49) to 8.47 mg (recombinant protein p21); however, these are the extremes of detection across all of the tested recombinant proteins. LLD and LDR were also expressed in terms of cys content; mBB was capable of detecting as little as 6.3±1.1 pmol of cys per band and delivered a linear response between 25 and 400 pmol [149]. A possible explanation for the increased sensitivity observed in the results of the more recent study was improved methods of detection. The advent of the cooled charge-coupled device (CCD) camera may have been a definitive factor in the difference in sensitivity observed between the two studies. Although mBB can be used to pre-label protein prior to IEF for 2DE, like other reactive dyes, it only detects a specific reactive group, so those proteins lacking thiols go undetected. Pre-labeling may also affect the mobility of some low MW proteins [150].

In 2003, a saturation approach to labeling protein cys residues with the commercially available CyDye maleimides (Amersham Biosciences/Invitrogen) reported a LLD of 0.1–5 ng of protein per band and LDR of three to four orders of magnitude [132]. Proteins were dissolved in lysis buffer, pH adjusted to 7.5, and reduced with Tris (2–carboxyethyl) phosphine hydrochloride (TCEP). Fluorophore was then added at a ratio 20 nmol/50 μg of protein before incubation, and quenched with 2×2D sample buffer prior to analysis by 2D gel electrophoresis. The main difference between minimal labeling and this protocol is the final ratio of dye to protein. Labeling of cys residues to saturation allows for the use of much smaller masses of tissue and is thus useful when working with human surgical, biopsy, post-mortem, and other limited tissue sources [151]. Application of saturation labeling to samples from bacteria, mouse liver, cancer cells, and feline brains have also been successful. Maleimide CyDyes have also been used to examine the 2D separation of pancreatic intra-epithelial neoplasia cell proteins [152]. This method was consistent with the protocol mentioned previously; however, the proteins were prepared from laser micro-dissected tissue and were quantified in terms of cell number [132]. The 1,000 cells used for optimal labeling yielded 2.3 μg of total protein; this was then labeled with 4 nmol of fluorophore, resulting in a much higher fluorophore to protein ratio than used previously [132]. This study detected ~2,500 spots using micro-dissected cells and as a test-of-concept MALDI–ToF–MS was used to identify trypsin cleaved γ-actin with 38.7% sequence coverage [152].

Additional maleimides include the newly developed DY-680 and DY-780 dyes which are infrared thiol-reactive dyes; these were used to compare newborn and adult murine brains [153]. Proteins (2.5 mg) were solubilized, reduced and desalted before being treated with 200 μg of DY-680 or DY-780 in DMF. While this labeling procedure reduces the initial protein pool during the desalting and concentration steps, DY-680 was shown to be highly sensitive with a LLD of 10 fg (labeled tubulin, with no reported standard deviation) [153]. Once again, however, proteins without accessible cys residues were not detected by these maleimides, indicating a level of inter-protein variability that will be sample dependent. For 2D analysis of soluble mouse brain proteins, the LLD was 5 μg total protein (labeled and unlabeled). Although this labeling procedure has great potential sensitivity, the interference of these dyes with subsequent MS analysis will limit their implementation. This interference was described as a decrease in the MALDI–ToF–MS MASCOT scores of proteins labeled with DY-dyes compared with those stained with cCBB. Recently, the application of other fluorescently distinct unpatented DY-maleimides in protein staining has also been explored [154]. Pre-labeling for both 1D and 2D gels began with 20 μg total protein (human serum albumin (HSA) or Human keratinocytes for 2D) in 30 mM Tris–HCl (pH7.5). Samples were reduced with TCEP before being treated with 8 nmol of a DY-maleimide dye and pre-labeled in the dark. While the 1D sample reaction was quenched with sample buffer in preparation for loading, 2D samples were quenched with stop buffer [154]. Using this method, DY-maleimides 505 and 635 made the detection of 0.13–1 ng HSA possible, and yielded a LDR of three orders of magnitude. 2D application of DY–maleimides 505 and 635 identified 1,212±124 and 1,050±28 protein spots, respectively, on 200×250×1.5 mm gels [154]. A subset of these spots was also submitted to MS analysis resulting in 22–60% sequence coverage for 17 DY-maleimide-labeled proteins.

Iodoacetylated cyanine dyes (ICy3 and ICy5) are a variation of the commercially available maleimides that were originally synthesized non-commercially [147]. The only reported LLD value was for BSA, a protein with 35 cys residues (5.8% of the total amino acid complement), at 2 ng [20]. While a LDR of three to four orders of magnitude is comparable with the current staining benchmark, SR, this range was only applicable to thiol containing proteins. An additional indication of inter–protein variability includes the 1–8% of proteins that are preferentially bound by one of the two iodoacetylated dyes [147]. Standard proteins, including BSA, chicken OVA and equine myoglobin, at 2 pmol final concentration were dosed with an ICy fluorophore. Soluble proteins extracted directly from cells (HMLEC line HB4a and its ErbB2-overexpressing derivative C3.6) were labeled concurrently with lysis to limit thiol modification during/after cellular disruption. Iodoacetylated fluorophores were used at 80 pmol/μg of total protein and reactions were incubated on ice for 60 min before being quenched with DTT [147]. The main goal of this method was to achieve

saturation labeling of all thiol groups and this was reflected in the 20-fold increase in fluorophore concentration used by the authors compared with succinimidyl ester cyanine dyes discussed previously. These dyes were used in a comparative study of the detection delivered by 'traditional' and more contemporary stains in 2DE. When compared with lysine targeting CyDyes, silver and cCCB staining, ICy dyes detected 1,034±245 spots, 85% of the total number of spots detected on the same gel with MS compatible silver stain. To further characterize the iodoacetylated cyanine dyes, soluble proteins extracted from mammary luminal epithelial samples were pre-labeled and the detected protein spots were tested for MS compatibility. Of these spots, 89 were submitted for identification by MALDI–ToF–MS and 51 proteins were identified. Identity assignment was made with a range of 25–68% sequence coverage [146, 147]. The IC–OSu ethyl–Cy3 and –Cy5 *N*–hydroxysuccinimide ester cyanine dyes (IC3 and IC5; Dojindo Laboratories) were also recently developed [155]. These dyes were directly compared with the commonly used CyDyes (GE Healthcare) by identifying protein spots displaying two- or five-fold differences in volume ratio and dividing this number by the total number of identified spots to give a percentage of similarity. Based on this, the authors judged the two labeling strategies to be equal in suitability for protein detection, multiplexing, and proteome quantitation [155].

Non-reactive fluorescent dyes

Post-electrophoretic staining with fluorescent non-covalent dyes is the most widely applied technique in proteomics for in-gel protein detection. These stains are not reactive and can commonly be removed from the gel and resolved proteins through extensive washing. SR is the most commonly employed fluorescent stain; not only is its use as simple as CBB staining, it is also reported to have a high level of sensitivity and wide LDR [20]. The manufacturer's staining procedures used for 1D and 2D gels differ slightly. 2D gels are fixed in MeOH/HAc before SR staining whereas 1D gels are immersed in stain immediately following electrophoresis. A LLD between 1 and 2 ng (broad-range MW standards; Bio-Rad) was identified and quantification was linear from the LLD up to 1,000 ng of protein [20]. In comparison to the other stains tested (i.e., variations of silver, zinc/imidazole and CBB), SR was reported to have the lowest level of inter–protein variability but no quantitative measure was provided for this claim. SR also exhibited MS compatibility with sequence coverage greater than 36% for all proteins tested.

Recombinant proteins (rhuMAb, tPA, hGH) were serially diluted to characterize the staining potential of SR [156]. Following electrophoresis of these proteins, 1D gels were fixed in a solution of MeOH/HAc as were 2D gels. 2D gels were then incubated in stain overnight whereas 1D gels were stained for 3-4 h. 1D gel destaining was conducted using MeOH/HAc and a less concentrated solution was used for 2D gels. Subsequently, both gel types were rinsed with water. This report noted a LLD value between 0.5 and 5 ng of recombinant protein and the LDR of each of these three proteins spanned from 200- to 1,000-fold. This indicated a degree of inter-protein variation not suggested in the study mentioned previously [20] and implied that linearity is strongly dependent on the type of protein. 2DE analysis of a soluble *E. coli* protein fraction yielded an LDR of about a 1,000-fold [156]. This was determined using Progenesis or PD Quest generated histograms and plotting protein amount versus staining intensity.

Independent studies using the SR staining method detailed previously [20] were also carried out [157, 158]. It was reported that SR again yielded a LLD of 1–2 ng (SDS-6H marker proteins; Sigma) and a LDR of 2–500 ng [158]; little inter-protein variation was suggested by the LDR plot shown. Also, MALDI–ToF–MS analysis of standard proteins (BSA and CA) displayed sequence coverage between 29–34% and 48–58%, respectively (for protein loads between 4 and 64 ng) [157]. A comparison of this and the previous studies suggests that the LLD values are consistent. However, despite using similar protein standards, the LDRs reported in these studies show a twofold difference, the source of which may be the limited experimental range of protein concentrations tested by Cong et al. [158].

Using the manufacturer's SR protocol, another study analyzed a total soluble protein extract from *A. thaliana*; the LLD was determined to be 500 ng and a linear relationship was revealed for both high and low abundance proteins (R=0.96 and 0.97, respectively) [17]. In this study, SR provided the best overall protein spot detection in comparison to all other tested stains (i.e., DP, cCBB, silver nitrate and C16-F) and the highest staining reproducibility across triplicate 2D gels [17]. The significant potential of SR for 2D analysis was further highlighted using rat fibroblast-soluble lysate (50 μg protein per gel) [73]. SR detected 1,290±34 spots, the greatest number in comparison to all other tested stains (including silver stain and cCBB) and was shown to have a dynamic range 680-fold greater than cCBB detected densitometrically, demonstrating superior capacity for protein detection. MALDI–ToF–MS analysis of PhosB and OVA (1D–PAGE) reinforced the advantages of using SR since reasonable sequence coverage was obtained even from loads below 9.3 ng/band [73]. 2D separation of pre-reduced standard proteins (Bio-Rad 161–0320) stained with SR according to the manufacturer's staining instructions revealed the presence of 34 spots [detected by eye from an image captured using a 532 nm excitation and a 610±30 nm band-pass emission filter

(Typhoon 9200; GE Healthcare)] [159]. Among these, soybean trypsin inhibitor and myoglobin were only minimally detected due to an apparent ineffective staining capacity of these proteins. While this indicates a level of inter-protein variability, the majority of resolved spots were identifiable by MALDI–ToF–MS with minimum sequence coverage of 40% [159]. It has also been suggested that to overcome the high cost of SR for proteomic application, dilution or re-use of the stain could be considered; however, such use of SR was not optimal [160, 161]. Reported LLD values for BSA were detrimentally affected by SR re-use or dilution with water. Either treatment produced a twofold reduction in LLD from 1–2 to 2–4 ng. LDR values were also affected by up to sixfold with any attempt at economizing SR use [161].

Since the introduction of SR at its relatively high cost, there has been interest in developing equivalently performing yet inexpensive fluorescent ruthenium based alternatives. Ruthenium (II) tris (bathophenanthroline disulfonate), RuBPS (also known as RuBPSA and RuBTS) was introduced as an economical alternative to SR in 2000 [162]. Although it was suggested that RuBPS delivered superior sensitivity in comparison to SR [163], it was later demonstrated that there was no quantitative advantage over the original or optimized SR formulation [164]. It is, however, definitely more cost-effective and has thus been used in various proteomic investigations [165–170]. It has been shown that the limit of sensitivity of RuBPS is approximately 10 ng protein/band (broad-range MW standards, Bio-Rad), as reported previously [164] and determined qualitatively from published data [163]; however, a more detailed evaluation of RuBPS staining indicated this sensitivity threshold to be much lower, at ~2 ng protein/band (CA, soybean trypsin inhibitor, OVA, albumin, PhosB) [168]. Similarly to SR, the LDR for RuBPS was between 2 and 2,500 ng for BSA [168]. Another fluorescent ruthenium based stain, ASCO_Ru—commercially available from Sigma as bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidyl ester-bis(hexafluorophosphate), was explored and shown to detect and quantify as little as 80 pg of glutamate dehydrogenase [171]. It was also noted that approximately 12% more spots were detected by ASCO_Ru in comparison to SR (total soluble protein extracted from human colon carcinoma cells HCT 116) [171]. Rubeo fluorescent protein stain (G Biosceinces) was shown to not only detect fewer spots (mouse liver total soluble protein) but was subject to high inter-gel variation in comparison to SR and other fluorescent protein stains [21]. Thus, it seems that the search will continue for a fluorescent protein stain that outperforms SR, and is without its substantial expense.

Deep Purple (DP) is a fluorescent dye based upon the natural compound epicocconone, originally isolated from the fungus *Epicoccum nigrum* [172]. In 2003, a study comparing Lightning Fast (later renamed DP) to SR showed that the LLD of DP (~64 pg protein/band; no error given) was eightfold superior to that of SR [173]. Additionally, 18–19% more spots in a 2DE separated sample of soluble rat microsomal proteins were detected using this alternate fluorescent stain in comparison with SR. The staining protocol consisted of agitated fixation in HAc followed by two water washes and a light protected 0.5% (*v*/*v*) DP staining step. Background was reduced by performing three short water washes [173]. Most proteins tested had a wide LDR of ~102 pg–1,024 ng when detected with DP, and subsequent sequence analysis by MALDI–ToF–MS (>23%) was similar to that obtained following CBB or SR staining. It was also noted that the number of spots detected was greater when using a non-linear immobilized pH gradient strip regardless of the stain used [173]. Efforts to streamline the manufacturer's protocol by consolidating washes with the pH changes necessary for effective staining were also assessed for their effect on the quantitative capacity of DP [174]. Here, they determined that pH played a critical role in DP staining and the enhanced method maintained sensitivity and LDR with fewer steps and less handling time [174]. Application of DP to a native soluble protein sample derived from *A. thaliana* demonstrated a LLD of 0.5 μg total loaded protein and a linear staining relationship was found for both high and low abundance proteins (R=0.84) [17]. However, use of the manufacturer's protocol resulted in only 75% of the spots being detected by DP in comparison to SR.

The performance of DP (a.k.a. LavaPurple), using the manufacturer's protocol, was also assessed using 2D protein standards (Bio–Rad 161–0320); 41 protein spots were detected, comparable to the number seen using SR (38) [159]. These proteins were amenable to MALDI–TOF–MS analysis, with identifications being made with as much as 58.5% sequence coverage [159]. DP is suggested to have a slight advantage over SR when applied to peptide mass fingerprinting (PMF) as it is less likely to result in tested spot identification failure and provides consistent PMF for lower abundance spots [175]. It is also compatible with MALDI–ToF–MS and Liquid chromatography (LC)-MS methods of protein sequencing [176, 177]. One known disadvantage to DP staining is its photoinstability. After 6 min of light exposure, DP signal suffered a 50% reduction while 19 min of exposure to the same light intensity was required to produce a 44% reduction in SR signal [178]. Even with these disadvantages, DP and SR are available at similar prices. Direct comparison of DP and SR using fractions of total soluble and total membrane proteins extracted from mouse liver, revealed that the SNR of DP was closer to that of densitometrically detected CBB than SR, as well as demonstrating inferior detection of acidic,

membrane, and low MW proteins [21]. Unfortunately, the separate testing of soluble and membrane protein fractions is not otherwise routinely carried out in the field. This raises the question of how many stains, largely characterized with soluble protein extracts, might be found to underperform in full proteomic analyses that naturally include membrane proteins. Additionally, the DP staining protocol requires more hands-on time than that of SR, even using the consolidated protocol [174]. Although the binding mechanism for DP with proteins has not been clearly defined it has been noted that DP does undergo a unique reversible reaction with primary amines [179].

C-16 fluorescein (C16-F) has been recently explored as an alternative to SR. 1D gels were fixed and stained in 1 μM dye dissolved in EtOH/HAc (twice), followed by two water washes and a brief rinse in HAc [180]. The same procedure was applied to 2D gels without the water washes. Using this C16-F staining procedure, the LLD identified for BSA was 0.12 ng, the LDR was 7.8–125 ng protein/band (based on four standard proteins) and some degree of inter-protein variability was indicated [180]. The staining method for C16-F [180] was also applied to a native soluble protein sample derived from *A. thaliana* [17]. Here, the LLD using C16-F for detection was equivalent to that of silver stain and DP but not as sensitive as SR. The linear relationship of C16-F staining for a pair of high and low abundance proteins with similar MW was equivalent to that of SR (R=0.99). Although 2D protein detection using C16-F was poorer than with all stains tested other than cCBB, staining reproducibility across multiple gels was high.

Other fluorescent stains available in the commercial market include Krypton and Krypton IR protein stains (Pierce) and the family of LUCY dyes—LUCY® 506, LUCY® 565, LUCY® 569 (Sigma-Aldrich). The characteristics for protein sensitivity, however, have not yet been independently tested by researchers other than the manufacturer and their collaborators. Authors examining the LUCY dyes reported LLDs for BSA at 2 ng for LUCY® 506 and 5–10 ng for the remaining LUCY dyes [176]. All of the LUCY dyes stain α1-acid glycoprotein poorly, indicating some inter-protein variability.

Additional fluorescent stains

Since the introduction of fluorescent stains for in-gel protein detection, there have been various developments for alternate fluorescent detection methods. Most, however, do not reach the level of sensitivity achieved by SR, have complicated protocols or have not been extensively characterized for widespread use in proteomic applications [163, 181–190]. Recently, there have been a few candidate stains that show equivalent staining characteristics to SR. In 2008, palmatine was shown to have similar detection sensitivity to cCBB [190], but was subsequently optimized to achieve sensitivity comparable to that of SR [158]. Originally, the stain was dissolved in EtOH/HAc, however, an alternate solvent containing SDS and HAc enhanced the performance of palmatine [158]. The LLD was 2 ng for all marker proteins tested (SDS-6H; Sigma); since the protein concentration range tested was narrow (2–500 ng), the resultant LDRs were the same for both palmatine and SR [158]. It was also shown that for five protein standards, sequence coverage achieved by MS analysis was similar for all loads between 4 and 125 ng/band. Additionally, sequence coverage for 12 palmatine-stained spots from neuroblastoma SH-SY5Y total soluble protein extract was comparable to that obtained by SR staining (ranging from 31% to 69%). The other advantages of using this stain are the low cost, reduced labor and environmental friendliness (i.e., harmful organic reagents are not necessary).

Another possible alternative to rival SR is 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (BisANS). Significant changes to the original staining method for BisANS have recently achieved comparable protein detection sensitivity to that seen with SR [157]. The original BisANS staining protocol according to Horowitz and Bowman [184] washed gels in water, stained with 20 μm BisANS (in water), washed in 2 M KCl, and rinsed briefly in water. The method developed by Cong et al. [157] differed significantly; gels were first fixed in EtOH/HAc, washed in water, stained with 0.0002% BisANS in EtOH/HAc and rinsed briefly with water before imaging. The LLD of standard protein markers (SDS–6H2; Sigma) was 1 ng/band and the LDR between 1 and 250 ng; both values were comparable to those obtained using SR [157]. MS analysis (MALDI–ToF) showed similar sequence coverage for both BisANS and SR for two standard proteins. Although palmatine and BisANS are simple and inexpensive to use (almost 100 times cheaper than SR), more research will be required to further develop the full extent of their detection capacity relative to that of SR.

Unlike exogenous stains, native fluorescence relies on the amino acid composition of proteins to facilitate in-gel detection. The ultraviolet (UV)-induced fluorescence of amino acid residues, tryptophan (Trp) and tyrosine, is the basis of native fluorescence. Since the introduction of native protein detection in gels, there have been various attempts to capitalize on and improve this process [185, 191–193]. Native fluorescence has demonstrated protein detection comparable to that achieved with silver staining [194]. The LLD of PhosB, CA and glyceraldehyde 3-phosphate dehydrogenase was 5 ng and 1 ng for BSA. The LDR was between 1 and 500 ng of protein and 2D analysis of EA.hy 926 soluble proteins showed that native fluores-

cence detected 86% of spots relative to silver staining [194]. This procedure for in-gel protein detection is, however, very lengthy and requires up to two weeks for completion. Yet with the addition of 2,2,2-trichloroethanol to the gel matrix, native fluorescence protein detection could be carried out immediately after electrophoresis at 300 nm [195]. The LLD for low MW protein standards (Pharmacia) was 200 ng (Trp content 0.8–2.3%) and 20 ng protein (4.5% Trp). The LDR was between 0.2 and 2 μg protein/band (R=0.99) and between 0.7 and 100 ng of Trp mass/protein [195]. It was undeniable that sensitive protein detection was highly dependent on the Trp content of proteins and hence resulted in a high degree of inter-protein variability.

Detection by native fluorescence was improved again with microelectrophoresis, where proteins are resolved with 3.5×8 cm slab gels (unlike mini-gels which are usually 6×8 cm). Detection of standard proteins (Sigma) was generally possible with as little as 0.1 ng of protein and based on this it was determined by the authors that the absolute limit of detection was 0.04 ng protein [196]. The LDR was between 0.1 and 20 ng of protein but the fluorescence intensities of each protein differed greatly from one another. This was not unexpected considering the varied number of Trp and tyrosine residues across the spectrum of proteins. Analysis of *E. coli* soluble protein extract (commercial preparation) by 2D analysis revealed that ~300 spots could be detected (10 μg total protein load) [196]. The application of UV laser side entry excitation for native fluorescence protein detection has been shown to be even more sensitive in mini-gel applications [197]. The average LLD of the six protein standards used in this study (Sigma) was 5 pg per band. For these six standards, the LDR was between 20 pg and 16 ng protein and notable variation in fluorescence intensity was again revealed [197]. 2D analysis of a soluble sample prepared from *E. coli* (Bio-Rad) showed that limiting total protein loads to 1 and 0.25 μg did not alter the number of spots detected (280), but decreasing protein loads did negatively impact detection success [197]. UV–laser side entry excitation may be a more effective detection method than standard UV excitation and CCD camera detection as comparable spot numbers could be detected even with lower protein loads [196, 197].

Specific protein stains

This section will constitute a general overview of stains that are specific for particular protein moieties; however, most of the following stains have not been characterized extensively enough to fulfill all of the criteria established here for review. Like some of the techniques reviewed above, inter-protein variability is high with these stains, and indeed that is the key to their functional success; stain performance should correlate with the specific amount of a given moiety per protein, and is thus not uniform across a proteome. The cationic carbocyanine dye, 1-ethyl-2-{3- [1-ethylnaphtho (1,2d) thiazolin-2-ylidene]-2-methyl-propenyl}-naptho (1,2d) thiazolium bromide, for example, commonly known as SA, was one of the first stains used to distinguish proteins from RNA in bacterial polyribosomes [198]. In addition, by 1973, SA had been shown to differentially stain phosphorylated proteins blue and non-phosphorylated proteins red [199]. However, as studies continued with the use of SA it was determined that specificity could only be achieved if the sample components were known since both glycosylated and calcium binding proteins also stained blue [200–202].

Glycosylated proteins

For the detection of glycosylated proteins in acrylamide gels, the periodic acid-Schiff stain (PAS) method was first introduced in 1964 and by 1969 had been further refined [203, 204]. Other modifications made to the PAS staining method have included the capacity for quick detection [205] and applicability to proteins resolved by native PAGE [206]. Alternative applications of the PAS stain for detection of glycoproteins include combining the PAS reaction with alcian blue or dansyl chloride staining [207–210]. These procedures have not become widespread since their staining success depends on carbohydrate content with a minimum sugar requirement of 1 μg [209]. Although the thymol–sulfuric acid glycoprotein detection method is twofold more sensitive than the PAS method (limit of detection 0.05 μg carbohydrate), it is not stable and stained protein zones have been shown to fade and diffuse within a few hours at room temperature [211].

In addition to the fundamental staining demonstrated by PAS methods, the recent prevalence of fluorescence-based detection methods has led to the development of a sensitive fluorescent dye, Pro-Q Emerald 300, which has since become the stain of choice for glycoprotein detection [212]. Not only can this stain be useful in studying this protein modification in a sample but it can also be applied to quantification. Broad-range MW standards (Bio-Rad) were serially diluted and separated via 1D PAGE in order to assess the performance of Pro-Q Emerald 300 [212]. The manufacturer's staining protocol delivered LLD values at or below the nanogram range for α1-acid glycoprotein (300 pg), glucose oxidase (300 pg) and avidin (1–2 ng) [212]. The LDR for α1-acid glycoprotein and glucose oxidase was demonstrated to be between ~9 and 600 ng/lane and all other glycoproteins between ~1.2 or 2.3–1,200 ng/lane (as derived from LDR figures and information provided on standard dilutions). Pro-Q Emerald 300 does not bind to non–glycosylated proteins but can detect lipopolysaccharides

at concentrations of 2–4 ng, which may complicate crude protein extract analysis [212]. The main limitation to the use of Pro–Q Emerald 300 is that it cannot be used with laser-based gel scanners. This disadvantage led to the development of a new fluorescent glycoprotein stain, Pro-Q Emerald 488 [213]. Detection sensitivity of glycosylated protein depends greatly on carbohydrate content (α_2-macroglobulin (9–10% CHO), glucose oxidase (12–13% CHO) and fetuin (22% CHO)–9.4 ng; α_1-acid glycoprotein (38–42% CHO)–4.7 ng; avidin (7% CHO) and OVA (3–4% CHO)–18.8 ng). All glycoproteins were readily quantified over a 128–225-fold linear range, except avidin and OVA (64-fold, due to their carbohydrate content). It must also be noted that non-specific staining is observed when gels are heavily loaded with proteins that are not glycosylated (i.e., 250–1,000 ng). Pro-Q Emerald 488 is compatible with 2DE, however, since total protein stains used subsequently, such as SR, quench its fluorescence, simultaneous visualization is not possible and the stains must be used and detected serially [213].

Commercial glycoprotein stains available from Sigma (Glycoprotein detection kit) and Pierce (GelCode Glycoprotein Stain) detect glycoproteins based on modified versions of the PAS method, and have been successfully employed to reveal the glycosylation state of proteins [214, 215]. Although these colorimetric stains are useful, fluorescent detection of proteins is more sensitive. Glycoprofile III fluorescent kit (Sigma) and Krypton Glycoprotein staining kit (Pierce) also employ the periodate–oxidate chemistry to react with glycoproteins. The stains mentioned above detect total glycoprotein profiles of protein samples; however it is possible to detect sub-categories of glycosylation. Invitrogen has developed the Click-It™ *O*-GlcNAc Enzymatic Labeling System for detection of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) residues on target proteins. This system utilizes the enzymatic labeling of *O*-GlcNAc residues to azido-modified galactose via β-1,4-galactosyltransferase [216, 217]. This azide-labeled protein can then be fluorescently labeled with any of the Click-It™ detection regents, tetramethylrhodamine–alkyne (TAMRA; 300 nm UV illumination or 532 nm laser) or dapoxyl-alkyne (300 or 365 nm UV illumination) dyes. Determination of which alkyne dye to utilize may depend on the intended proteomic application. Both dyes are compatible with SR, but TAMRA can also be multiplexed with Pro-Q Emerald 300 glycoprotein gel stain and western detection with anti–TAMRA antibody, and Dapoxyl only with the Pro-Q Diamond phosphoprotein gel stain. Information available in regard to sensitivity of the above stain has only been stated by the manufacturer but has yet to be validated independently.

Phosphorylated proteins

Historically, phosphoproteins have been detected on polyacrylamide gels by radioactive means but this method of detection relies on radioactive phosphate being incorporated into proteins metabolically (preferably to equilibrium) and thus requires living cells [218]. In 1973, however, it was demonstrated that phosphorylated proteins could also be detected specifically via entrapment of liberated phosphate [219]. This method initially relies on the hydrolysis of phosphoester bonds under alkaline conditions in the presence of calcium ions to produce an insoluble calcium phosphate complex. This trapped phosphate is then treated with ammonium molybdate in dilute nitric acid to produce an insoluble nitrophosphomolybdate complex. Detection of this blue complex is then enhanced by staining with methyl green. This method is specific toward phosphoproteins, with a LLD of ~3 μg (1 nmol of protein-bound phosphate) [219]. The GelCode Phosphoprotein staining kit (Thermo Scientific) and Phosphoprotein Stain kit (Ameresco) detect phosphoproteins based on this densitometric method developed by Cutting and Roth [219].

An alternate method for visualizing phosphoproteins is based on trivalent metal chelation [220]. In this method, aluminum ions are added to an acidic CBB stain solution. This promotes the formation of metal–protein chelates in which the aluminum reduces the negative charge and acts as a bridge between the dye and phosphate residue. This method could detect as little as 40 ng of apo-phosvitin, equivalent to 0.13 nmol of phosphate [220]. Like glycoproteins, a fluorescent dye for phosphoprotein detection is also now available. Pro-Q Diamond preferentially binds to phosphate moieties of proteins (weak non-specific binding to unphosphorylated protein was noted), can be used in conjunction with total protein stains and is MS compatible [221]. Pro-Q Diamond detects phospho-serines, -tyrosines, and -threonines with similar sensitivity [222]. Staining with Pro-Q Diamond can detect 1–2 ng of β-casein (five phosphate residues) and 8 ng of pepsin (one phosphate residue) [221]. As might be expected, it was also shown that total phosphate content (i.e., the total number of phosphorylated residues) influenced the detection limit for a particular protein. Non-specific detection of sulfonated moieties and others can also result in background issues. An advantage to the use of Pro-Q Diamond is that it can be diluted threefold without compromising sensitivity, fluorescence intensity or the LDR, thus substantially lowering the cost of use [223]. A new range of fluorescent phosphoprotein stains have been manufactured by PerkinElmer; the Phos–tag™ phosphoprotein stains are based on a metal (II) ion chelator that is highly selective for phosphomonoester residues of phospho-serine, -tyrosine, -threonine, -histidine, and -aspartate [224–226]. This stain is also available in two forms to enable detection with a variety of gel imagers—Phos-tag™ 540 with maximum excitation at 540 nm and Phos-tag™ 300/460 with dual excitation at 300 and 460 nm.

Other proteins (iron-bound proteins, lipoproteins, protein tags)

Stains to detect other components of protein have also been developed. Ferene S {3–(2–pyridyl)–5,6–bis [2–(5–furylsulfonic acid)]–1,2,4–triazine, disodium salt} was introduced to detect non–heme protein–bound iron [227]. Although this method of staining with 0.75 mM Ferene S and 15 mM thioglycolic acid in HAc was rapid, it was relatively insensitive and the detection limits for the three proteins tested (ferritin, hemoglobin, and cytochrome c) varied greatly. A slightly more sensitive method for detecting non-heme iron proteins was developed and relied on the reaction of potassium ferricyanide with protein bound iron atoms [228]. This method yielded a LLD of 1 μg of ferritin, 2 μg of cyanobacteria extracted ferredoxin, and 100 μg human transferrin. The most sensitive iron stain to date, however, is based on a chemical reaction in which iron catalyzes the H_2O_2 oxidation of diaminobenzoate to an insoluble colored complex [229]. The detection limit was based on the amount of iron present per band, which was approximately 5 ng iron in 0.5 μg ferredoxin protein (spinach) and 4.7 ng iron for 1.9 μg of iron-containing superoxide dismutase (*E. coli*). Although this stain was sensitive and specific for the detection of protein bound iron, it could not differentiate between heme and non-heme protein-associated iron.

The detection of lipoproteins has commonly been carried out with Sudan Black B [230–233] or Oil Red O [234, 235], however, these dyes are relatively insensitive. In 1994, a new dye for lipoprotein identification after native PAGE separation was demonstrated [236]. Filipin, a fluorescent stain could detect approximately 5 ng of unesterified cholesterol/band (based on pure low density lipoprotein) after 12 h of staining. This stain was suggested as part of a dual staining approach whereby lipoproteins were detected initially with filipin followed by total protein detection using CBB.

Proteomic stains have ventured still further and can now also be used for the selective staining of protein tags. Invision™ His-Tag In-gel stain (Invitrogen) is based on a fluorescent dye conjugated to a Ni^{2+}/nitriloacetic acid complex. The Ni^{2+} metal binds selectively to the oligohistidine domain of His-tagged fusion proteins and as expected, detection varies and depends on the individual protein. A similar commercial His-tag protein stain is also available from Thermo Scientific (6xHis GelCode Protein Tag Staining Kit) and has been successfully applied [237]. Proteins can also be modified with the addition of a tetracysteine peptide (Cys–Cys–X–X–Cys–Cys—where X is a noncysteine amino acid). This reporter probe can be identified in the presence of FlAsH (a small, synthetic, membrane–permeable biarsenical ligand) where the interaction of the arsenic compound with the pair of thiol groups results in fluorescence [238]. This tetracysteine motif was optimized revealing that Cys–Cys–Pro–Gly–Cys–Cys had enhanced stability, the highest affinity and most rapid binding to biarsenical compounds [239]. This detection system for fused tetracysteine peptides is the foundation of the Lumino™ Green detection kit (Invitrogen) and detection sensitivity has not yet been proven outside the manufacturing labs. An overview of the published LLD values of all stains evaluated throughout this review is provided in Table 1.

Equipment innovations

In addition to considering the physiochemical properties of a stain it is important to remember that detection will also be influenced by our ability to quantitatively assess the stain. Detecting stained protein depends as much on the instruments and equipment that are available as on the stain itself. When the innovations in CBB staining that fathered quantitative staining as it is known today were made, densitometry was measured on a recording strip scanner with a film attachment [18]. While this machine only had a 4.65-fold level of magnification, it also performed the integrations necessary to measure protein amount. For the detection of CBB, amido black and silver stain during this era, densitometers (with varying degrees of automation) were the main form of imaging equipment. In 1968, for example, a Joyce Loebl Chromoscan recording and integrating densitometer was used to quantify amido black [240]. Two years later, there was improved automation with the use of a plexiglass cartridge driven by a motor that passed below a Gilford Model 220 absorbance indicator [241]. This apparatus was interfaced with a Gilford Optical Density Converter and Healthkit Servo Recorder. The sensitivity of this system was based on the recorder having four known ratio settings, thereby enabling adjustment to different band intensities. A spectrophotometer was used to densitometrically detect Drimarene and Uniblue A stained proteins; this instrument required the excision of gel pieces for measurement, including an unstained gel slice of the same thickness to serve as a blank [114]. The use of the spectrometer for detection, transport for more rapid sample exchange, and a recorder (or similar equipment), dominated densitometric imaging techniques for most gel assessment until the mid-to-late 1970s [43, 76, 118, 199, 200, 205, 242].

For silver-stained proteins, it was more common to photograph gels and scan these images for analysis, although densitometers were used as well [59, 62–64]. One study used a Cohu Model 7120, 525 line, black and white camera to image silver stained proteins, and pictures were digitized using a Colorado Video Model 270 digitizer with 512×480 pixels and a 0 to 255 gray density value scale [72]. The consensus for zinc–imidazole-stained gels

Table 1 Comparison of protein stains

Stain Details	1D SDS-PAGE			2D SDS-PAGE	Mass spectrometry compatibility	Reference
	LLD	Protein	LDR	Spot number[a]		
Coomassie Brilliant Blue	30 ng	BSA/actin	0.5-20 μg/cm	–	Yes	[33], [43–45]
CBB R-250 Mini gel staining microdensitometry	10 ng	BSA, CA, β-lactalbumin	10-200 ng	–		[46]
Colloidal CBB	0.1-1 ng	BSA	30-500 ng	250 (*A. thaliana* total protein)		[17], [47]
Commercial cCBB Neuhoff formulation	8-16 ng 4-8 ng	Broad-range MW standards	30-250 ng	–		[20]
Rapid Silver Stain with careful temp. control	0.1-0.2 ng	Standard Proteins	1-30 ng	–	No	[46]
Silver staining with EtOH used as primary solvent	27 pg/mm^3	Soybean trypsin inhibitor	27 pg/mm^3-5-50 ng/mm^3	1,800 (mouse liver cytosolic proteins)	No	[72]
Silver Nitrate	1 ng	*A. thaliana* total soluble extract	–	–	Yes	[74]
Acidic silver nitrate	2-4 ng	Broad-range MW Std	4-60 ng	–	Not without compromising LLD	[20]
Alkaline silver diamine	2-4 ng	Broad-range MW Std	8-60 ng	–	N0	[20]
Silver staining preceded by calconcarboxylic acid, sensitization	0.05-0.2 ng/band	CA, Phos B, β-galactosidase, ovalbumin, myosin	0.8-100 ng (myosin, B-galactosidase, Phos B) and 0.2-25 ng (ovalbumin, CA)	–	Yes	[88]
Silver Stain with ethyl violet zincon	0.2 ng/band	BSA, CA, Phos B, β-galactosidase, albumin, myosin	4-50 ng	–	Yes	[90]
Ethyl violet Zincon–MeOH solvent	> 4-8 ng	PhosB, BSA, chicken OVA, bovine CA and human peroxiredoxin I	Between 8 and 1,000 ng	–	–	[116]
Ethyl Zincon, longer fixation and EtOH solvent	4-8 ng	Phos B, BSA, Chicken OVA, bovine CA	8-1,000 ng	–	Yes	[49]
MDPF 60 μg MDPF: 20 μg protein	1 ng	CA, myoglobin, catalase, BSA	1-500 ng	–	–	[138]
OPA 0.19 μmoles OPA: up to 50 μg/ml protein	~10 ng	Transferrin	0.1-50 μg/ml	–	–	[139]
Fluorescamine 0.5 mg fluorescamine: Up to 1 mg/ml protein	6 ng	Myoglobin	0.5-12.5 μg	–	–	[140]
DIGE 200-400 pmol/50 μg protein	0.25 ng	MLH and Pirella Strain	3-4 orders of magnitude	367 (av.) (*Pirellula* sp. Culture) 401 (av.) (APAP exposed livers)	Yes	[10], [142]
BODIPY dyes	10 fmol	Yeast enolase 1	3 orders of magnitude	–	Yes	[148]

mBB	4-32 ng	Broad range MW Standards	32-100 ng	–	Weak (BSA, soybean trypsin inhibitor misidentified)	[20]
mBB	86.2±14 ng	Recombinant Proteins	86.2 ng-8.47 mg (largest range)	–		[149]
CyDye maleimides	0.1-5 ng	Albumin	3-4 orders of magnitude	~2,500 (microdissected pancreatic cancer cells)	Yes	[132], [152]
DY680 and DY780 Dyes 200 μg fluorophore: 25 mg protein	10 fg	Labeled tubullin	–	–	Yes	[153]
DY505 and DY635 Dyes 20 μg total protein	0.13-1 ng	Human serum albumin	3 orders of magnitude	1,212±124 1,050±28, respectively (keratinocyte cell lysate)	Yes	[154]
ICy3 and ICy5 80 pmol fluorophore: 1 μg protein	2 ng	BSA	3-4 orders of magnitude	1,034±245 (Erb2 overexpressing cells)	Yes	[147]
SR (manufacturer's protocol)	1-2 ng 500 ng	Broad range MW Std *A. thaliana* total protein	1-100 ng	1,290±34 (rat fibroblast total cell lysates)	Yes	[20], [73], [157, 158]
SR, MeOH/HAc fixation, stain from 3 h to overnight, destain in MeOH/HAc, water washed.	0.5-5 ng	Recombinant protein	200-1,000-fold	1,324±142 (using *E.coli* total protein and PDQuest)	Yes	[156]
RuBPS	10-20 ng	Broad range MW Std	2-2,500 ng	~1,800 (thyroid cell extract)	Yes	[168]
ASCQ_Ru	80 pg	Glutamate dehydrogenase	4 orders of magnitude	1,537 (Human colon carcinoma cells)	Not fully with trypsin	[171]
DP	64 pg/band	Low MW Std	4 orders of magnitude	1,102 (mouse/rat liver tissue)	Yes	[173]
C-16 fluorescein	0.12 ng	BSA	7.8-125 ng/band	–	Yes	[180]
LUCY® Dyes	2-10 ng	BSA	–	–	–	[176]
Palmatine	2 ng	BSA, CA, PhosB, β–galactosidase, albumin, mysoin	2-500 ng	–	Yes	[158]
BisANS (new protocol)	1 ng/band	BSA, CA, PhosB, β–galactosidase, albumin, mysoin	1-250 ng	–	Yes	[157]
Pro-Q Emerald (488)	300 pg	α1-acid glycoprotein and glucose oxidase	~9-600 ng/lane	–	–	[212]
Pro-Q Diamond	1-2 ng	α-casein	500-100-fold	–	Yes	[221]
Zinc staining	1-2 ng	Broad-range MW Std	250-2,000 ng	–	–	[20]
Rapid zinc stain kit	1.8-4 ng/band	Low MW Std	1.8-140 ng/band	~350 (TCA precipitated human hepatocytoma cells)	Yes	[50]
Trivalent metal chelation	40 ng	Apo-phosvitin	–	–	–	[220]
Ferene S	1 μg	Ferritin	–	–	–	[228]
Iron and diaminobenzoate oxidation of H_2O_2	0.5 μg	Ferredoxin	–	–	–	[229]
Filipin	5 ng/band	Unesterified cholesterol	–	–	–	[236]

av. Average of all three cyanine dyes

[a] Only reported if a quantitative number is provided.

was also to take images, against a black background in this case, usually using a Polaroid camera or an automated imager, and follow with analysis [20, 100, 101, 104, 107, 108, 243].

Fluorescence posed a different challenge in terms of detection given the specific excitation and emission requirements of different fluorophores. In 1969, the fluorescence of proteins pre-labeled with anilinonaphthalene sulfonate and separated on a polyacrylamide gel were detected in two stages; it was necessary to first excite the fluorophore with a long wave UV lamp before capturing the image with an everyday camera fitted with appropriate filters [181]. This dual imaging process was widespread. In one study, a desk lamp was adapted by lining the shade with foil and fitting it with a UV bulb to visualize dansyl chloride-tagged proteins [244]. Following fluorophore excitation, the gel image was once again recorded with a 'filtered' camera. This practice for detecting fluorescence continued throughout the 1970s and was used in conjunction with a variety of fluorophores, including OPA and fluorescamine [139, 140, 245]. Even in the mid-1970s, Gilford instruments still required adjustment to facilitate the measurement of fluorescence [137, 140]. Not only was a filter (Corning 051) required to remove excitation energy but since the Gilford was designed to read absorbance, it was necessary to calculate the antilogarithm of peaks to obtain a measurement of signal. It gradually became more common to transilluminate gels with UV light to detect fluorescently stained proteins [20, 157, 158, 182, 184, 186, 190, 191, 195, 246, 247]. Subsequently, photographs of transilluminated gels had to be scanned by a densitometer before quantitative analysis could be undertaken; again, all without the aid of local computers that are now considered standard lab equipment.

During this time, the use of CCD cameras began to be introduced in electrophoresis literature. In 1988, the use of the CCD 2200 Imaging System which boasted 385× 578 pixels was reported [138]. The maximum signal detectable by this camera was 10^5 electrons while the read noise or minimum signal was 1 electron. The range of detection afforded by this system presented an important development in the routine quantification of proteins and the application of CCD based systems for imaging gels still remains one of the most prevalent technologies in use today [193, 194, 196, 197, 236, 247–249].

In the 1990s, specialized equipment for automated densitometric and fluorescent detection of proteins was marketed. While the transilluminator/camera option remained in use due to its accessibility [149], an Elsie 5 computer analysis system was also available [250]. It could be described as the end product of some 30 years worth of multistep gel handling in pursuit of quantitative analysis. Elsie 5 was a system designed specifically for the analysis of 2D gels. Not only did the Elsie 5 image gels but it was also designed to facilitate quantification and image manipulation including comparison with other gel images. The major advantage to using such systems for detection, as they are the basis for most current analyses, is their ability to reduce human error and bias and increase throughput; this is the cornerstone of all large-scale analyses of specific molecules or 'omics'.

By 2001, a CCD camera with 1,024×1,024 pixel resolution was being used to detect proteins stained with SR and tagged with the fluorescent reporter gene, β-glucuronidase [251]. Despite being able to identify signal by eye in this study, the authors recommended the use of a CCD camera for the most optimal and accurate analysis. A comparison of three different in-gel protein detection approaches using MDPF, mBB, and SR was also carried out [252]. The CCD camera used in this study was automated and fully accessorized with the necessary filters and UV transilluminator to visualize each of these stains. A similar instrument, the Typhoon 8600 was also in fairly widespread use during this decade [253, 254].

Although CCD cameras revolutionized the in-gel detection of stained proteins and are thus in extensive use in the proteomic field, new technologies are emerging which may displace their prevalence. Complementary-metal-oxide-semiconductor devices allow for ultrasensitive detection through signal amplification [255]. These devices can be applied to many different detection systems including gel imaging which may present great potential in terms of decreasing detection limit.

Just as the ability to image gels affects the capacity for analysis, so too do the programs used to quantify and determine the signal associated with stained protein. A number of programs are available for gel image analysis and these have developed from hardware intensive systems that lacked a visual user interface to sophisticated gel analysis software that can be run from a desktop computer [256]. Currently, Delta2D (DECODON) and Progenesis SamesSpots (Nonlinear Dynamics) utilize an approach that first warps the images and then matches spots. This minimizes the problems associated with matching spots from gels that may have slight variations. While this approach is faster, the approach used by PDQuest (Bio-Rad), Decyder 2D (GE Healthcare), Melanie III (Genebio) and Dymension (Syngene), of detecting spots first and then following with image warping remains valid. Each of these programs also offers a differing array of options for editing images, statistical analysis and user interaction depending on the users' needs. A comparison of different analytical programs rated PDQuest (Bio-Rad) as best able to deal with spot overlap but described Melanie III (Genebio) and Progenesis SameSpots (Nonlinear Dynamics) as "all-rounders" in terms of accuracy and coping with low S/N ratios [133]. A comparison of Delta2D and Proteomweaver (Bio-Rad)

showed that Delta2D outperformed the Bio-Rad analysis program in spot detection, automatic spot matching and manual correction after warping [257]. Further, the use of a consensus gel image compiled from each gel image in an experimental series economizes spot detection and editing steps in analysis using Delta2D.

The advent of MS was a huge leap forward in protein identification [258]; this was and remains especially powerful as it links and promotes analysis using the vast number of available databases, including SwissProt, TrEMBL and NCBInr. In this review it is clear that most of the cited papers used MALDI–ToF for protein identification. In recent years, however, electrospray ionization (ESI) has been widely applied with MS. As ESI ionizes proteins from solution it is easily amenable to liquid chromatography MS (LC-MS) [259]. Liquid chromatographic separations of tryspin digested samples have increased sensitivity since few peptides elute at any one time. This can be valuable in those instances in which multiple proteins resolve to a single spot after 2DE. Not only does LC-MS achieve greater protein sequence coverage (particularly when iterative analyses are used), it is also a useful technique for analyzing complex protein mixtures when additional electrophoretic separation would otherwise be required [259, 260].

Coupled with these advances has been the further development of mass analyzers. Together, specific ionization and the development of ion trapping mass analyzers were able to achieve greater sensitivity and sequence coverage. A continuation of advances in ionization includes the relatively new Fourier transform-ion cyclotron resonance (FT-ICR) MS which can now measure protein quantities at low to sub-ppm ranges [261]. The new orbitrap mass analyzer also demonstrates this level of sensitivity and resolution but separates ions in an oscillating electric field. Also, while traditional MS utilizes collision-induced dissociation ions, alternatives which produce more uniform fragmentation, such as electron capture dissociation and electron transfer dissociation have been developed. Higher levels of sensitivity in MS methods would allow for the successful analysis of low copy number proteins that have proven difficult to identify from 2DE gels without sample enrichment; although in current circumstances serendipity still plays a role here, and even if a suspected low abundance protein is found, identification more often than not relies on a single peptide.

Although MALDI–ToF and ESI-MS are among the leading techniques for protein identification, advances have been made towards new MS proteomic strategies. These include so-called ‘shotgun’ approaches in which whole protein extracts are digested prior to to MS analysis; advances in new mass analyzers (the performance of these new instruments have been summarized in detail by Han et al. [262]; Table 1) as well as new quantitative strategies employing metabolic amino acid labeling (stable isotope labeling with amino acids in cell culture, isotope-coded affinity tagging, and isobaric tags for relative and absolute quantification) have demonstrated sub-femtomole sensitivity [260, 262–265]. Nonetheless, these approaches have their own inherent limitations in terms of extent and reproducibility of the labeling reactions or breadth of applicability; ongoing refinements of these tools and their application will almost certainly overcome most difficulties, as has been the case with the maturing of gel-based proteomics.

It is clear that when detection technology moved forward, not only was there an increase in sensitivity but also a more comprehensive integration of the multiple tools required for detecting and identifying proteins; we’ve gone from jury-rigged UV illuminators and hand held cameras to the fully integrated imager, such as the Fuji LAS–3000, with illumination, filters, and camera all inclusive in the design. Data is also more easily accessible as images are already formatted for quantitative analysis. Furthermore, these advances in detection and imaging technology may well mean that some previously characterized stains are worth re-evaluating. Given the decreasing protein masses that are now routinely detectable, with the equipment now available it is possible that the quantitative values (LLD and LDR) originally reported one-to-four decades ago no longer accurately reflect the true detection capacity of these stains. Examples of stains already demonstrating improved LLDs with modern detection technology include the IR fluorescent detection of cCBB and the 2.5-fold improvement in the sensitivity of mBB detection between the years 1994 and 2000. Furthermore, without MS and its integration with databases to aid protein identification, a significant proportion of proteomic investigation would be little more than large-scale exercises in electrophoresis. It is the convergence of these concepts and techniques in pursuit of comprehensive, large scale analyses of native proteins that has made modern proteomics possible. Indeed, quantitative image analysis and ‘hyphenated’ PAGE–MS are perhaps the most important developments and techniques in our modern pursuit of proteomics. A continued drive for greater sensitivity and thus better overall detection will impel the pursuit of additional improvements and thus further, ‘deeper’ dissection of proteomes.

Discussion

Despite a moderate amount of success, historically, in detecting and quantifying proteins in-gel, each staining revolution also brings forth its own limitations. The effectiveness of some of these staining techniques must, in some cases, be questioned. For example, pre-labeling

with cyanine succinimidyl esters and maleimides is reported to deliver subnanogram detection sensitivity rapidly and with minimal background. Practitioners also claim that low abundance proteins are easily accessed for detection and that detection is linear over up to five orders of magnitude. However, there are a few inherent complications in the process. For instance, labeling alters the molecular weight and *pI* of proteins and both the protein itself and its labeled derivative (s) have to then be resolved. Two (or more) resolved protein spots for each protein species doubles the total number of spots and reduces the effective resolution of the gel [20]. Labeling can also disrupt the structure and physical characteristics of proteins [266]. This means that excising these proteins for MS application requires estimation of the difference in migration to locate the unlabeled protein. However, post-electrophoretic staining is not without its own limitations; these gels usually have higher background levels and none have been reported to have LDR values of more than four orders of magnitude [267]. Also, even the most rapid staining techniques require washing and fixing in addition to the staining step. This makes post staining a more time intensive process although one that does not alter the native proteome and can provide for quantitative analysis. Low molecular weight proteins of low abundance continue to be poorly detected by any method, and its limitation still plagues even the most innovative staining approaches. At this stage, according to the criteria used here, SR likely remains the benchmark post-electrophoretic stain in the compromise between performance, ease of use and cost. DIGE, however, is also somewhat popular due to its claims of high sensitivity and multiple sample comparisons. Nonetheless, it remains that this method relies on loading less sample in an effort to effect multiple separations within a single gel; it has still not been effectively established that this approach is in any way superior to resolving full protein loads on separate gels and using powerful, widely available imaging systems/packages to most comprehensively compare the resolved proteomes.

Prior to beginning this review, it was necessary to identify the characteristics of a stain that would indicate its protein quantification efficacy. What was its minimum detection limit (i.e., LLD)? How broad was the linear relationship between signal and protein quantity (i.e., LDR)? How uniform was staining between different proteins (i.e., inter-protein variability)? These were the most widely reported characteristics, with LLD and LDR commonly used as determinants of the staining sensitivity. One of the problems associated with LLD reporting is that between different papers, units are very rarely uniform and there exists no standard unit of measurement for sensitivity. This becomes a impediment to the unequivocal comparison of stain performance. In this review, these criteria were minimally required to facilitate quantitative and comparative evaluation of stains used in proteomics. Unfortunately, some definitive criteria were consistently omitted from studies. Inter–protein variability was rarely addressed. Another frequent omission was reference to lowest limit of quantification (LLQ). In addition to LDR, this characteristic describes the functional 'window' of a stain; the concentration range in which a stain can be used quantitatively. An LLD does not guarantee a quantifiable signal that relates to protein quantity. If anything, LLQ is a more useful reporter of stain performance as it indicates the limit of useful quantitative capacity. Very few papers reported LLQ, likely further emphasizing the critical and growing need for better and wider interactions between proteomics and physical chemistry (for example, see International Congress on Analytical Proteomics) [268].

In addition to regularly including supplementary quantitative criteria, such as LLQ and inter-protein variability, when evaluating stain performance we must also actively assess the equipment used in any given evaluation. When reviewing stains last evaluated decades ago, the question of how large an improvement contemporary technology might bring to sensitivity assessments cannot be avoided. Certainly the increased sensitivity demonstrated by CBB when imaged using IR light [21] suggests that other older stains may also benefit from examination with higher resolution imaging equipment. The quantitative stains we need, or at least the chemistries required for their future development, may well already exist.

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