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Fluorescence detection of proteins in sodium dodecyl sulfate-polyacrylamide gels using environmentally benign, nonfixative, saline solution

SYPRO Tangerine stain is an environmentally benign alternative to conventional protein stains that does not require solvents such as methanol or acetic acid for effective protein visualization. Instead, proteins can be stained in a wide range of buffers, including phosphate-buffered saline or simply 150 mM NaCl using an easy, one-step procedure that does not require destaining. Stained proteins can be excited by ultraviolet light of about 300 nm or with visible light of about 490 nm. The fluorescence emission maximum of the dye is approximately 640 nm. Noncovalent binding of SYPRO Tangerine dye is mediated by sodium dodecyl sulfate (SDS) and to a lesser extent by hydrophobic amino acid residues in proteins. This is in stark contrast to acidic silver nitrate staining, which interacts predominantly with lysine residues or Coomassie Blue R, which in turn interacts primarily with arginine and lysine residues. The sensitivity of SYPRO Tangerine stain is similar to that of the SYPRO Red and SYPRO Orange stains - about 4–10 ng per protein band. This detection sensitivity is comparable to colloidal Coomassie blue staining and rapid silver staining procedures. Since proteins stained with SYPRO Tangerine dye are not fixed, they can easily be eluted from gels or utilized in zymographic assays, provided that SDS does not inactivate the protein of interest. This is demonstrated with in-gel detection of rabbit liver esterase activity using α-naphthyl acetate and Fast Blue BB dye as well as *Escherichia coli* β-glucuronidase activity using ELF-97 β-D-glucuronide. The dye is also suitable for staining proteins in gels prior to their transfer to membranes by electroblotting. Gentle staining conditions are expected to improve protein recovery after electroelution and to reduce the potential for artifactual protein modifications such as the alkylation of lysine and esterification of glutamate residues, which complicate interpretation of peptide fragment profiles generated by mass spectrometry.

Keywords: Protein stain / Electrophoresis / Zymography / Mass spectrometry / Phosphate-buffered saline / Fluorescence / Environment EL 3788

1 Introduction

Increasingly, the health hazards of working with toxic solvents are being recognized as well as the economic considerations of skyrocketing costs that accompany appropriate handling and disposal of mixed waste. Conventional protein staining procedures in polyacrylamide gels generate large volumes of toxic solvents [1, 2]. Coomassie Blue R (CBB-R) is a nonpolar, sulfonated aromatic dye typically utilized in methanol-acetic acid or methanol-trichloroacetic acid solutions. The acidic environment is essential for interaction of sulfonate residues of the dye with basic amino acids in proteins [1]. Staining with colloidal Coomassie Blue G (CBB-G; a dimethylated derivative of CBB-R) is usually performed in concentrated trichloroacetic acid, perchloric acid, or phosphoric acid, often in combination with methanol or ethanol. Studies of protein modifications by mass spectrometry indicate that staining proteins in CBB-G solutions containing trichloroacetic acid and alcohol leads to irreversible acid-catalyzed esterification of glutamic acid side-chain carboxyl groups [3]. This can complicate interpretation of peptide mapping data from mass spectrometry.

Silver staining provides the requisite sensitivity for most protein detection problems, but the procedure requires many steps and employs numerous toxic solvents and chemicals [1, 2, 4]. Different silver stain protocols employ a range of solvents and reagents, including ethanol, methanol, acetic acid, glutaraldehyde, dichromate, silver

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nitrate, formaldehyde, periodate, thiosulfate, ammonium hydroxide, methylamine, and sodium hydroxide. Conventional silver staining is not compatible with Edman-based protein sequencing from gels, though mass spectroscopic analysis has successfully been applied utilizing modified approaches [5–8]. Silver staining of polyacrylamide gels allows detection of low nanogram amounts of protein by mass spectrometry but special care must be taken to avoid metal-catalyzed protein oxidation by chilling silver solutions to 4°C [7]. Fixation steps with glutaraldehyde should be avoided and only low concentrations of formaldehyde should be employed during pattern development to minimize alkylation of α- and ε-amino groups of proteins. Recent studies indicate that peptide mass fingerprint sequence coverage for such modified silver staining procedures is poorer than that obtained with CBB-R staining [8].

The SYPRO Red and SYPRO Orange stains are well suited for sensitive fluorescent detection of proteins in SDS-polyacrylamide gels [9–12]. SYPRO Red and SYPRO Orange dyes can detect proteins with a simple, one-step staining procedure using 7% acetic acid in water, and no subsequent destaining step is needed. As little as 4 ng of protein can be detected, rivaling the sensitivity of standard, rapid silver staining techniques and surpassing detection levels obtained with colloidal CBB-G. Since staining appears to be due to intercalation of dye in the SDS micelle, little protein-to-protein variability is observed in SDS-polyacrylamide gel electrophoresis compared with amine-directed stains such as CBB-R.

SYPRO Orange and SYPRO Red dyes should be used in well ventilated areas due to the strong odor of 7% acetic acid in the stain formulation. In addition, acetic acid is a protein fixative, though certain proteins such as histones and glutenin are acid-soluble. Consequently, recovery of proteins from gels can be compromised, particularly when retention of enzyme activity is required. SYPRO Tangerine dye, a new addition to the SYPRO dye family, eliminates the need for either methanol or acetic acid in staining protocols. Though SYPRO Tangerine dye effectively stains proteins in 7% acetic acid or in 12.5% trichloroacetic acid solution, staining can be performed in phosphate-buffered saline solution or even simply 150 mM NaCl. SYPRO Orange and SYPRO Red dyes do not effectively stain proteins in phosphate-buffered saline or simple saline. Like SYPRO Red stain and SYPRO Orange stain, SYPRO Tangerine dye interacts primarily with SDS-protein micelles during gel staining. SYPRO Tangerine dye is suitable for applications such as zymography, electroelution, gel staining prior to electroblotting, and matrix-assisted laser desorption mass spectrometry.

2 Materials and methods

2.1 Materials

Amino acid homopolymers, heteropolymers, bromophenol blue, rabbit liver esterase (EC 3.1.1.1) and *Escherichia coli* β-glucuronidase (EC 3.2.1.31) were from Sigma (St. Louis, MO). Bacteriophage λ DNA was from Boehringer-Mannheim Corporation (Indianapolis, IN). Carrier ampholytes (pH 3–10), SDS, ammonium persulfate, TEMED, Tris base, glycine and Duracryl were from Genomic Solutions (Chelmsford, MA). Nitrocellulose membranes, 0.45 μm pore size, were from Bio-Rad Laboratories (Hercules, CA). Dithiothreitol, SYBR Gold, SYPRO Tangerine, SYPRO Red, and SYPRO Orange gel stains were from Molecular Probes (Eugene, OR). Broad-range molecular mass standards for SDS-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories and contained approximately equal weight mixtures of rabbit skeletal muscle myosin (molecular mass 200 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), chicken egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), chicken egg white lysozyme (14.4 kDa), and bovine aprotonin (6.5 kDa).

2.2 Fluorescence spectroscopy

Excitation/emission profiles of SYPRO Tangerine dye were obtained by incubating a 1:5000 dilution of dye stock with 2% SDS prepared in either 7% acetic acid or 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0, for 30 min. Measurements were made in a quartz cuvette using a Hitachi F4500 fluorescence Spectrometer (Hitachi, Tokyo, Japan).

2.3 Electrophoresis and staining

SDS-polyacrylamide gel electrophoresis was performed as previously described utilizing 4%T, 2.6%C stacking gels, pH 6.8, and 15%T, 2.6%C separating gels, pH 8.8 [13]. The total monomer concentration, %T, is expressed in g/100 mL; %C is the percentage cross-linker. The mechanically strong gel matrix, Duracryl, was utilized in all electrophoresis gels [14]. For two-dimensional (2-D) gel electrophoresis, whole cell lysates of rat fibroblasts were extracted according to the procedure for mammalian cells given in the Investigator 2-D Electrophoresis System manual (Genomic Solutions). Approximately 50 μg protein were applied per gel and large-format 2-D gel electrophoresis was performed as described previously [15]. SYPRO Tangerine staining solution was prepared by diluting the stock reagent 1:5000 in either 7.5% acetic

acid or 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0, while mixing vigorously. Alternatively, a wide range of buffers was found to be compatible with the SYPRO Tangerine stain including formate, pH 4.0, citrate, pH 4.5, acetate, pH 5.0, MES, pH 6.0, imidazole, pH 7.0, HEPES, pH 7.5, Tris acetate, pH 8.0, Tris-HCl, pH 8.5, Tris borate, 20 mM EDTA, pH 9.0, and bicarbonate, pH 10.0. Buffers should be prepared as 50–100 mM solutions containing 150 mM NaCl. Approximately 50–75 mL of SYPRO Tangerine staining solution was used for a typical minigel (5 cm × 9 cm × 1 mm). The gel was placed into the staining solution and the container was covered with aluminum foil to protect the dye from bright light. The gel was gently agitated for at least 30 min at room temperature using an orbital shaker (50 rpm). After staining, the gel was briefly dipped in water and visualized on a UV light box (see below). Silver staining was performed using a sensitive, nitrate-based procedure capable of detecting as little as 125–300 pg/mm^2 of protein (Table 2, 3rd column, from [4]). Coomassie Blue-R staining was performed by standard procedures as described [10]. Colloidal Coomassie Blue-G staining was performed using GelCode Blue reagent according to the manufacturer's instructions (Pierce Chemical, Rockford, IL).

2.4 Detection of proteins

Though SYPRO Tangerine stain is readily visualized by eye on a standard UV light box, a computerized CCD camera- or laser scanner-based image analysis system should be utilized for data acquisition to realize its full potential, since fluorescence intensity can be integrated and quantitative information can be obtained using image processing and analysis software. The use of a photographic camera, CCD camera or laser scanner is essential to obtain the greatest sensitivity. The instrument's integrating capability can make bands visible that can not be detected by eye. Images are best obtained utilizing a cooled CCD camera by digitizing at a resolution of about 1024 × 1024 picture elements (pixels) with 12- or 16-bit gray-scale levels assigned per pixel. Typically, image acquisition and analysis were performed on a Fluor-S Max Multi-Imager (Bio-Rad Laboratories), Lumi-Imager (Roche, Indianapolis, IN), FLA-3000G (Fuji, Tokyo, Japan) or BioImage (Genomic Solutions, Ann Arbor, MI) system. Image acquisition and analysis were performed as described previously [16]. SYPRO Tangerine dye is spectrally well matched to standard Texas Red emission filters due to its emission at approximately 640 nm and also has significant absorption in the UV region of the spectra, making it compatible with standard UV transilluminators operating at 302 nm (UV-B) such as the Foto/UV 450 transilluminator (Fotodyne, Hartford, WI) or the Polaroid transilluminator (UVP, Upland, CA). SYPRO Tangerine stain is best visualized on a UV transilluminator using a 490 nm long pass filter such as Kodak Wratten gelatin filter No. 9 (S-6656; Molecular Probes). The dye also has a 490 nm absorbance peak, allowing excitation by blue light. SYPRO Tangerine dye can thus be visualized on a blue light box (Dark Reader™; Clare Chemical Research, Denver, CO). With this instrument, gels were viewed in a darkened room. The stained gel was placed on the Dark Reader and an amber sheet of plastic (supplied with the device) was placed on top of the gel. Gels were photographed at *f*-stop 4.5 with Polaroid 667 black-and-white print film. Exposure times were generally 3–8 s.

2.5 Staining of amino acid polymers

Homopolymers of L-arginine, asparagine, histidine, lysine, aspartate, glutamate, alanine, glycine, isoleucine, leucine, methionine, serine, threonine, tryptophan, tyrosine and proline were copolymerized into 15%T, 2.6%C polyacrylamide gels at a concentration of 1 μg/mL. Except when indicated, 0.1% sodium dodecyl sulfate was included in the polyacrylamide solution as well. The amino acid heteropolymers, poly(glutamate, alanine, tyrosine), poly(glutamate, tyrosine), poly(lysine, tyrosine), poly(glutamate, lysine, tyrosine), and poly(arginine, tyrosine) were copolymerized into polyacrylamide gels in an identical manner as the homopolymers. Blank gels containing no polymer were prepared for reference. The copolymerized gels were cut into discs using the large end of a 1000 μL pipet tip (Sorenson Bioscience, West Salt Lake City, UT) and stained in Falcon 48-well flat-bottom tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ) on an orbital shaker operating at 50 rpm. Gel discs were quantified on a Roche Lumi-Imager system and classified into three groups based upon staining intensity (strong, moderate, or weak staining).

2.6 Detection of DNA

Twofold serial dilutions containing 20 ng–40 pg ΦX174 RF DNA were prepared in formamide loading buffer, heat-denatured at 95°C, quick-cooled on ice, loaded onto 0.75 mm thick 5%T, 5%C polyacrylamide gels containing 7 M urea and separated by electrophoresis for 3–4 h in 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (TBE buffer) at 60 V as previously described [17]. SYBR Gold stain was diluted 1:10 000 into fresh electrophoresis buffer and gels were stained as previously described [17]. Alternatively, gels were stained utilizing the standard SYPRO Tangerine dye protocol for protein staining.

2.7 Electroblotting and immunodetection

Twofold serial dilutions of β-tubulin ranging from 1000 to 1 ng were applied and separated by SDS-PAGE utilizing a 4.25%T, 2.6%C stacking gel, pH 6.8, and 13%T, 2.6%C separating gel, pH 8.8. The 0.75 mm thick, 6 × 10 cm gels were subjected to electrophoresis using the Bio-Rad mini-Protean II system according to standard procedures. After electrophoresis gels were stained, photographed, and then incubated in a transfer buffer consisting of 10 mM Tris, 96 mM glycine, 10% methanol, 0.1% SDS, pH 8.3 [18]. Proteins were electroblotted to poly(vinylidene difluoride) (PVDF) membranes with the same transfer buffer for 120 min using the Investigator semidry blotter (Genomic Solutions) according to the manufacturer's instructions. Addition of SDS to the transfer buffer was essential to obtain the most complete transfer of tubulin from the gel. We found that many other proteins present in broad-range molecular weight markers were efficiently transferred to membranes without inclusion of SDS in the transfer buffer. Immunoblotting was performed by standard methods and colorimetric procedures were employed to detect the tubulin using bromochloroindoyl phosphate/nitroblue tetrazolium (BCIP/NBT).

2.8 Zymography

For enzyme activity gels, protein samples were prepared in standard sample buffer, except the reducing agent (dithiothreitol or 2-mercaptoethanol) was omitted [13]. Samples were not heated in order to avoid protein denaturation. Esterase activity was measured in 12%T, 2.6%C SDS-polyacrylamide gels after staining with SYPRO Tangerine dye in 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0. Gels were incubated in two 10 min changes of 0.1% Triton X-100 in phosphate-buffered saline and then in an esterase staining solution prepared just prior to use. The esterase staining solution was prepared by adding 1 mL of 1% α-naphthyl acetate prepared in acetone and 25 mg Fast Blue BB salt to 50 mL of 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0. The esterase staining solution should be bright yellow and is light-sensitive. Gels were incubated in the staining solution at 37°C until purple-black bands appeared, generally after 30–45 min. The colored product results from a conjugate generated between the azo moiety of the dye and the hydrolysis product produced by cleavage of the naphthyl ester by the esterase. β-Glucuronidase activity was detected in a similar manner as the esterase activity [19]. After SYPRO Tangerine dye staining, gels were incubated in two 10 min changes of 0.1% Triton X-100 in phosphate-buffered saline. Gels were then incubated in 25 μM ELF 97-β-D-glucuronide (Molecular Probes) in phosphate-buffered saline at 37°C for 30–60 min. The resulting green-fluorescent product was visualized utilizing a transilluminator/Polaroid system (UVP) with 302 nm transillumination and photographed with Polaroid 667 black-and-white print film using a SYBR Green/Gold gel filter (Molecular Probes).

2.9 Handling and disposal of SYPRO Tangerine stain

Though removing methanol and acetic acid from staining solutions makes the SYPRO Tangerine staining protocol environmentally friendly, no data are available on the toxicity of SYPRO Tangerine dye itself. For disposal, SYPRO Tangerine stain should be captured by pouring stain solutions through activated charcoal or other compatible combustible material and then the adsorbant should be burned in a chemical incinerator to destroy the dye. All Federal, state and local environmental regulations should be observed when disposing of the dye.

2.10 Mass spectrometry

The samples were separated under reducing conditions using 1 mm thick 10–20% gradient gels (Novex, San Diego, CA) and stained with SYPRO Tangerine dye by standard methods. After staining SDS-polyacrylamide gels with SYPRO Tangerine stain, proteins were subjected to protease digestion and mass spectrometry as previously described [20]. Briefly, the stained gel bands were cut into 1 mm^2 pieces, placed into a 0.5 mL siliconized microcentrifuge tube. The gel pieces were incubated in two washes of 50% methanol for 15 min each while shaking on an orbital rotator set at 200 rpm at room temperature. The gel pieces were then incubated in two washes of 50% acetonitrile, 25 mM ammonium bicarbonate, pH 8.1, for 15 min each while shaking on an orbital rotator set at 200 rpm at room temperature. This solution was removed and replaced with enough digestion buffer, 25 mM ammonium bicarbonate, and modified trypsin solution (Promega, Madison, WI) at a final concentration of 0.03 mg/mL, to entirely cover the gel pieces in the bottom of the tubes. The gel pieces were allowed to incubate in this solution at 37°C overnight for at least 16 h. Upon completion of digestion, the peptide fragments were extracted by adding 70% acetonitrile to the tube containing the gel pieces. The gel pieces were allowed to incubate in this solution for 1 h while shaking on an orbital rotator set at 200 rpm at room temperature. This wash was collected and placed into a new 1.5 mL siliconized microcentrifuge tube. The gel pieces were then incubated in deionized water for 30 min while shaking. This wash was removed and pooled with the wash acquired from the previous step. The gel pieces were then subjected to an additional incubation in 70% acetonitrile for 1 h while shaking. This wash was pooled with the previous two

washes. The samples were then dried to completion using a vacuum centrifuge. The digests were resuspended using 20 μL of 0.1% trifluoroacetic acid followed by vigorous vortexing. The samples were purified using C18 reversed-phase microcolumns (ZipTip; Millipore, Bedford, MA), followed by analysis using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Mass spectra were obtained on a Voyager DE-STR time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). A 0.25 μL aliquot of sample was deposited onto the sample stage and mixed with 1 μL of matrix solution. The matrix solution used was 12 mg/mL α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) dissolved into acetonitrile:water:trifluoroacetic acid, 50:50:0.1. For each spectrum, 100 scans were averaged. The spectra were internally calibrated using trypsin autolysis peaks. Peptide mass mapping analysis of the tryptic digests was achieved using the software tool PAWS (Version 8.5, ProteoMetrics, LLC, New York, NY).

3 Results

3.1 Optimization of staining and detection conditions

Fluorescence excitation/emission spectra of SYPRO Tangerine stain demonstrate that the dye is optimally excited with 300 or 490 nm light (Fig. 1). This excitation profile is ideally suited for visualization of the stain using a 302 nm UV-B transilluminator, 473 nm second harmonic generation (SHG) laser or 488 nm argon-ion laser. Like SYPRO Orange dye, SYPRO Tangerine dye can also be visualized on a blue light box (Dark Reader; Clare Research, Boulder, CO). The emission maximum of SYPRO Tangerine dye is observed at 640 nm, and is thus spectrally well matched to Texas Red emission band-pass filters. For comparison, the excitation/emission profile of SYPRO Orange dye is excitation = 300 nm, 470 nm/emission = 569 nm and of SYPRO Red dye excitation = 300 nm, 547 mm/emission = 631 nm [10].

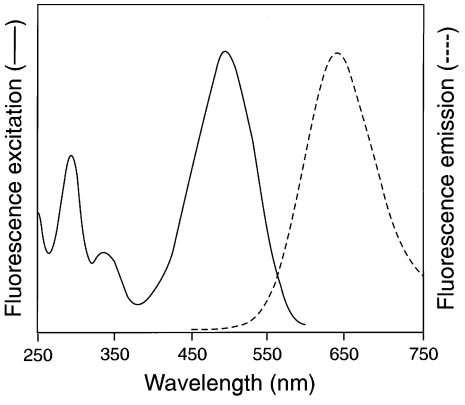


Figure 1. Fluorescence excitation/emission spectra of SYPRO Tangerine stain. Spectra were generated in 7% acetic acid, 2% SDS. Identical spectra were obtained in phosphate-buffered saline, 2% SDS (data not shown). The left trace (solid line) demonstrates excitation maxima at 300 and 490 nm with emission set at 640 nm. The right trace (dotted line) demonstrates emission maximum at 640 nm with excitation set at 440 nm.

SYPRO Tangerine dye does not effectively stain proteins in water alone or in common buffers such as 50 mM citrate, formate, or acetate (data not shown). Salt is required for effective staining. While 500 mM–1 M NaCl produces bright background staining, 150–250 mM NaCl is effective for protein staining with low background. SYPRO Tangerine dye effectively stains proteins in a wide range of buffers if 100 mM NaCl is included as well. Acceptable buffers include 50–100 mM formate buffer, pH 4.0, sodium citrate, pH 4.5, sodium acetate, pH 5.0, MES, pH 6.0, imidazole, pH 7.0, HEPES, pH 7.5, Tris-acetate, pH 8.0, Tris-HCl, pH 8.5, Tris-borate, pH 9.0, and sodium bicarbonate, pH 10. SYPRO Tangerine dye staining can also be performed in 12.5% trichloroacetic acid. SYPRO Orange dye only faintly stains proteins a blue color in this solvent. In 12.5% trichloroacetic acid SYPRO Tangerine dye stains proteins brightly, but also with bright background fluorescence. A 20 min wash in water removes the background and sensitive staining is obtained. Overnight exposure of the SYPRO Tangerine dye to 12.5% trichloroacetic acid destroys the dye and prevents detection of proteins.

Though the optimal salt concentration for SYPRO Orange and SYPRO Red dye staining of proteins in the absence of acetic acid is roughly 30 mM NaCl, the fluorescence intensity obtained in this solution is only about 25% of that obtained in 7% acetic acid. Significant protein-to-protein variability is readily apparent, especially with SYPRO Orange dye in this buffer system. Soybean trypsin inhibitor, bovine serum albumin and ovalbumin stain with much lower intensity than the other molecular mass markers. Carbonic anhydrase shows slightly diminished staining as well. SYPRO Tangerine dye shows slightly diminished staining of lysozyme but all other proteins stain intensely in 150 mM NaCl.

A comparison of SYPRO Orange and SYPRO Tangerine dye staining of broad-range molecular mass protein standards after SDS-polyacrylamide gel electrophoresis is shown in Fig. 2. Twofold dilution series of proteins were prepared in standard SDS sample buffer and the amount of protein loaded ranged from 2 μg per band to 1 ng per band. Both dyes effectively stain the protein standards in 7% acetic acid, though background fluorescence is slightly higher for SYPRO Tangerine dye stained gels

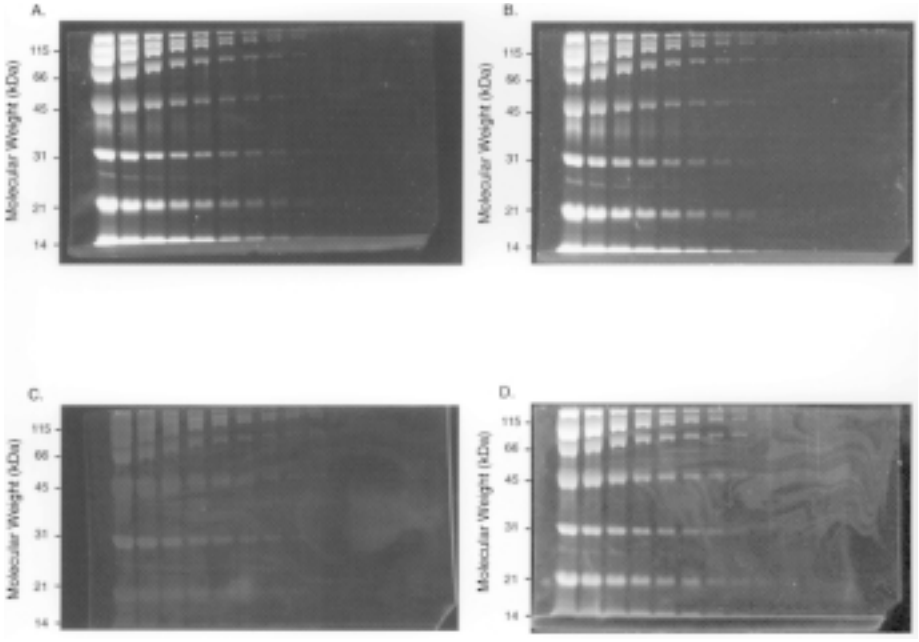


Figure 2. Staining of broad-range molecular mass protein standards with SYPRO Orange and SYPRO Tangerine dye. (A) SYPRO Orange dye staining in 7% acetic acid solution. (B) SYPRO Tangerine dye staining in 7% acetic acid solution. (C) SYPRO Orange dye staining in phosphate-buffered saline solution. (D) SYPRO Tangerine dye staining in phosphate-buffered saline solution. Twofold dilution series of proteins were prepared in standard SDS sample buffer and the amount of protein loaded ranged from 2 μg per band (1st lane) to 1 ng per band (12th lane).

(Fig. 2 A, B). SYPRO Orange dye does not effectively stain proteins using a phosphate-buffered saline solution in place of 7% acetic acid (Fig. 2C). SYPRO Tangerine dye effectively stains proteins in phosphate-buffered saline with staining sensitivity that is comparable to the 7% acetic acid solution (Fig. 2D). The pictured gel was imaged directly after staining, without any intervening washing steps. This leads to a small amount of fluorescence from the stain solution itself that is apparent in the right-hand portion of Fig. 2D. A brief rinse in deionized water minimizes this background, but extensive washing removes the protein-bound dye as well. Using the CCD camera-based Roche Lumi-Imager, Bio-Rad Fluor-S or Genomic Solutions BioImage systems, 4–10 ng of protein stained with SYPRO Tangerine dye is readily visualized, with linearity extending to 2 μg ($r = 0.9911$). Similar detection sensitivity is obtained using a Polaroid camera system in combination with a 302 nm UV transilluminator. This detection sensitivity matches SYPRO Orange and Red stains. For perspective, colloidal CBB-G detects about 8–16 ng while silver stain detects 2–4 ng of protein under similar electrophoretic conditions (data not shown). With a photomultiplier tube-based detector, such as the one in the Fuji FLA-3000G Fluorescent Image Analyzer, 4–10 ng of SYPRO Tangerine dye-stained protein is also detectable using the 488 nm argon ion laser source to excite the dye.

3.2 Mechanism of staining

The interaction of SYPRO Tangerine dye with various amino acid side chains was examined using a series of amino acid homopolymers and random copolymers. For comparison, the interaction of CBB-R, colloidal CBB-G, silver, SYPRO Red dye and SYPRO Orange dye were also evaluated with the same battery of polymers (Table 1). In the table, intense staining is indicated in boldface and moderate staining is indicated in standard font. Weakly staining polymers are not listed. Intense staining was typically 2–3 times as strong as moderate staining. Among the stains evaluated, colloidal CBB G, SYPRO Red, SYPRO Orange and SYPRO Tangerine dyes bind to the widest range of amino acid polymers. The stains interact strongly with basic amino acids, as well as some hydrophobic amino acids. CBB-R primarily stains lysine- and arginine-containing polymers while the acidic/silver nitrate procedure used in this study primarily stains lysine-containing polymers. The three fluorescent dyes show subtle preferences in amino acid staining with respect to one another in the standard 7% acetic acid staining solution. SYPRO Orange dye binds to tyrosine residues less well than SYPRO Red dye and SYPRO Tangerine dye. The poorer performance of SYPRO Orange dye in the heteropolymer component of the study is undoubtedly due to this reduced affinity for tyrosine residues (Table 1). SYPRO Red dye binds histidine residues to a lesser extent than the other two fluorescent dyes while SYPRO Tangerine dye binds tryptophan residues to a lesser extent.

The suggested mechanism of protein staining by the SYPRO dyes is by association with the SDS micelle [10, 11]. Thus, the staining properties of SYPRO Orange dye, SYPRO Red dye, and SYPRO Tangerine dye were further evaluated with respect to this anionic detergent.

Table 1: Semi-quantitative analysis of dye interaction with amino acid homo- and heteropolymers.

Silver	SYPRO Orange dye	SYPRO Red dye	SYPRO Tangerine dye	Colloidal Coomassie Blue-G	Coomassie Blue-R
Lys	**Lys**	**Lys**	**Lys**	**Lys**	**Lys**
Ile	Ile	Ile	Ile	Ile	Ile
Leu	Leu	Leu	Leu	Leu	Leu
	Arg	**Arg**	**Arg**	**Arg**	**Arg**
	His	His	**His**	**His**	His
	Tyr	**Tyr**	**Tyr**	**Tyr**	Tyr
	Trp	**Trp**	Trp	Trp	Trp
	Gly	Gly	Gly		
Lys:Tyr	**Lys:Tyr**	**Lys:Tyr**	**Lys:Tyr**	**Lys:Tyr**	**Lys:Tyr**
Glu:Lys:Tyr	**Glu:Lys:Tyr**	**Glu:Lys:Tyr**	**Glu:Lys:Tyr**	**Glu:Lys:Tyr**	**Glu:Lys:Tyr**
	Arg:Tyr	**Arg:Tyr**	**Arg:Tyr**	**Arg:Tyr**	**Arg:Tyr**
	Glu-Tyr	**Glu-Tyr**	**Glu-Tyr**	**Glu-Tyr**	
	Glu:Ala:Tyr	**Glu:Ala:Tyr**	**Glu:Ala:Tyr**	**Glu:Ala:Tyr**	

Strong reactions are noted in bold face, 12-point font. Moderate reactions are noted in standard 10-point font. Polymers that did not stain with any of the dyes are absent from the table.

None of the basic amino acid polymers stain in the absence of SDS. Dye binding is limited to the poly-L-tyrosine and poly-L-tryptophan polymers under these conditions. However, staining of these bulky hydrophobic side chains is reduced 2- to 3-fold in the absence of SDS. The staining profile of SYPRO Tangerine dye in phosphate-buffered saline is similar to the profile obtained in 7% acetic acid. Prominent staining of tyrosine, arginine, lysine, and histidine homopolymers is observed. Among the heteropolymers, poly Arg:Tyr and poly Lys:Tyr provide the strongest signals. As expected, SYPRO Red and SYPRO Orange dye staining is reduced substantially in phosphate-buffered saline. The primary reason these dyes perform poorly in phosphate-buffered saline appears to be due to a lack of staining of the basic amino acid residues. While silver stain detects proteins with a slightly higher sensitivity than the SYPRO dyes described in this study, the amino acid polymer studies suggest that staining with SYPRO dyes yields more accurate estimates of protein amounts as silver stain primarily visualizes lysine residues.

Some dyes are known to stain both nucleic acids and proteins. For example, the cationic carbocyanine dye "Stains-All" (1-ethyl-2-[3-(3-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl]-naphtho[1,2d]thiazolium bromide) stains RNA, DNA, phosphoproteins and calcium-binding proteins blue while unphosphorylated proteins and hyaluronan are stained red [21–25]. SYPRO Tangerine dye was compared with a conventional fluorescent nucleic acid stain, SYBR Gold dye, for the detection of DNA in denaturing, polyacrylamide gels (Fig. 3). As demonstrated by the figure, SYPRO Tangerine dye interacts specifically with proteins and not nucleic acids. SYPRO Orange and SYPRO Red dyes also selectively stain proteins [10, 11].

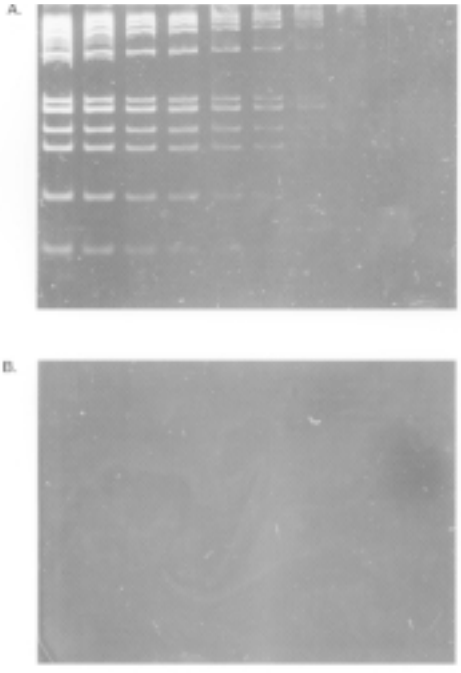


Figure 3. Staining of DNA by SYBR Gold dye and SYPRO Tangerine dye. (A) SYBR Gold dye staining of ϕX174 RF DNA in a 5%T, 5%C urea/polyacrylamide gel. (B) SYPRO Tangerine staining of a duplicate gel. SYPRO Tangerine dye does not stain DNA.

3.3 Performance advantages of SYPRO Tangerine dye

SYPRO Tangerine dye is unusual in its ability to detect proteins in nonfixative, neutral pH buffers. Two main advantages of such a solution environment are that proteins are more easily eluted from the polyacrylamide gel matrix after electrophoresis and enzyme activity is more likely to be recovered. The nonfixative nature of the staining solution is illustrated by evaluating passive elution of proteins from a polyacrylamide matrix. Passive diffusion of proteins was evaluated by staining two-dimensional gels in 7% acetic acid or in phosphate-buffered saline using SYPRO Tangerine dye. As demonstrated in Fig. 4A and B, the 7% acetic acid solution acts as an effective fixative for the majority of proteins resolved by two-dimensional gel electrophoresis. Proteins remain stained in the gels, even after a 24 h incubation period. Staining in phosphate-buffered saline for 2 h already reveals some diffusion of lower molecular mass proteins compared with 7% acetic acid stain (Fig. 4C). Further diffusion of proteins is observed after a 24 h incubation in the phosphate-buffered saline formulation of the stain (Fig. 4D). Extending this experiment to 48 h demonstrated that certain proteins, such as tubulin, are fairly resistant to passive elution from a polyacrylamide gel matrix. Thus, tubulin was selected to examine the transfer of protein to membranes by electroblotting. As demonstrated in Fig. 5, tubulin was readily transferred from SYPRO Tangerine-stained polyacrylamide gels using the phosphate-buffered saline staining formulation. Tubulin was subsequently visualized by using alkaline phosphatase-conjugated secondary antibody with similar detection sensitivity as unstained control gels. The transfer of tubulin from gels stained with SYPRO Orange dye in 7% acetic acid was not possible despite a 20 min incubation in transfer buffer containing 0.1% SDS prior to blotting.

The feasibility of staining proteins with SYPRO Tangerine dye followed by in-gel detection of enzyme activity was evaluated using rabbit liver esterase and *Escherichia coli* β-D-glucuronidase zymography. SYPRO Tangerine dye was found to require SDS for binding to proteins in polyacrylamide gels. SYPRO Tangerine dye does not stain proteins separated in the presence of alternative detergents such as Triton X-100 or benzyldimethyl-*n*-hexadecylammonium chloride. On the other hand, esterase activity was not detectable in the presence of 2% SDS. Enzymatic activity could readily be detected in gels, however, if washing steps were included to lower the SDS

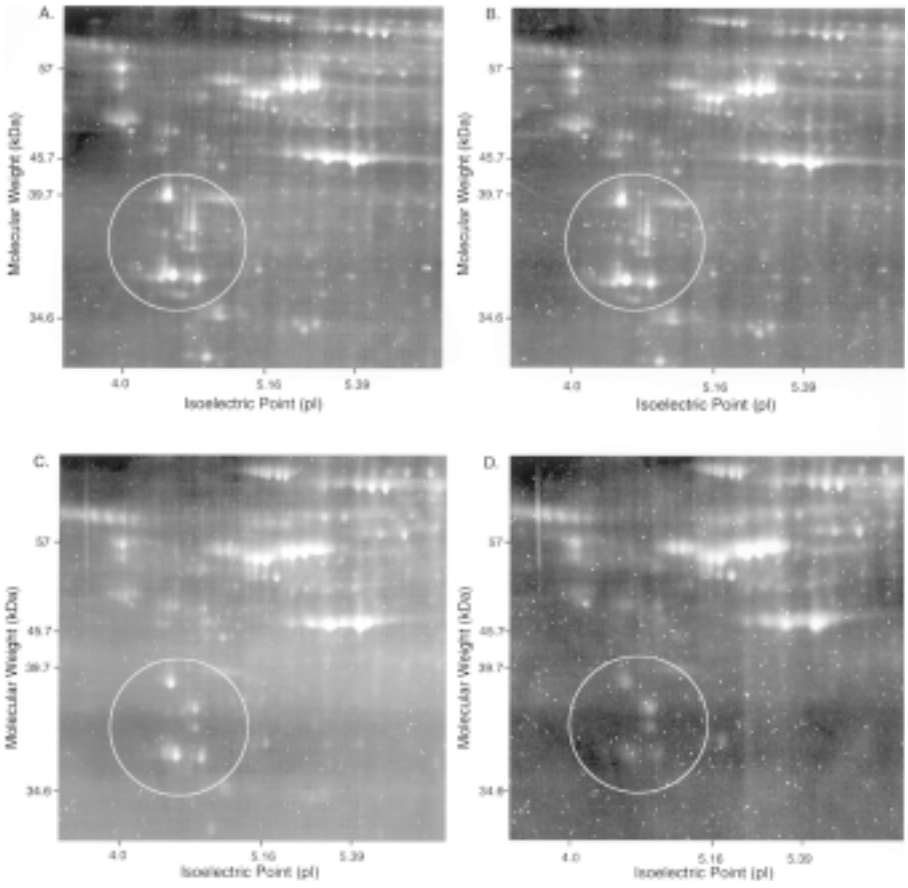


Figure 4. Demonstration of the nonfixative nature of SYPRO Tangerine dye staining by 2-D gel electrophoresis. (A) Region of a 2-D gel stained with SYPRO Tangerine dye in 7% acetic acid for 2 h. (B) Same region 24 h later. (C) Region of a 2-D gel after staining with SYPRO Tangerine dye in phosphate-buffered saline for 2 h. (C) Same region 24 h later. Diffusion of proteins from the polyacrylamide gel matrix is especially apparent when viewing the tropomyosin region of the pattern (circled area). The acidic end of the gels is displayed on the left, and the basic end on the right. Higher molecular mass proteins are near the top, while lower molecular mass proteins are towards the bottom of each gel.

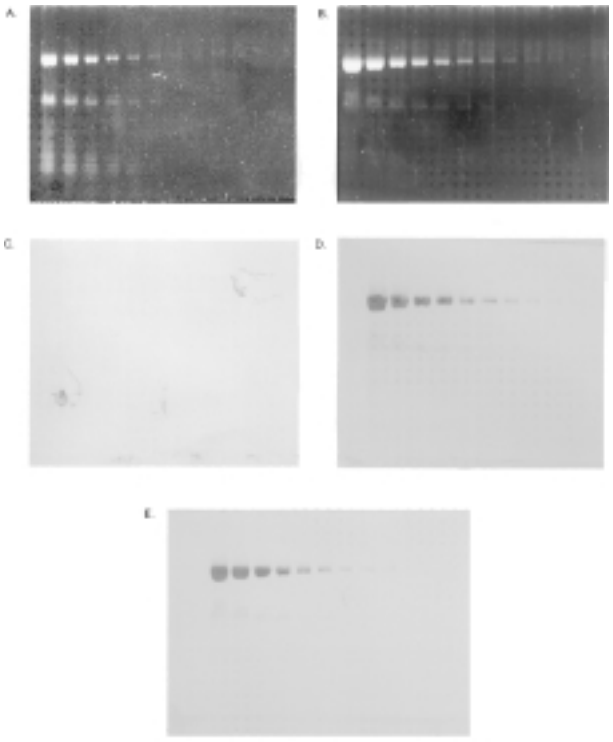


Figure 5. Electroblotting tubulin from SYPRO Orange and SYPRO Tangerine dye-stained gels. Tubulin was selected as a model protein since it is relatively difficult to elute from gels by simple passive diffusion. (A) SYPRO Orange dye-stained dilution series of bovine tubulin. (B) Same dilution series of tubulin stained with SYPRO Tangerine dye. (C) Immunostained electroblot membrane after transferring tubulin from the SYPRO Orange-stained polyacrylamide gel. (D) Immunostained electroblot membrane after transferring tubulin from the SYPRO Tangerine dye-stained polyacrylamide gel. (E) Immunostained electroblot after transferring tubulin from an unstained polyacrylamide gel. A bromochloroindoyl phosphate/nitroblue tetrazolium detection system was used to visualize antitubulin antibody in C–E.

concentration. Gels were run using a standard SDS-polyacrylamide gel protocol except that protein samples were not boiled in sample buffer [13]. Then, gels were stained with SYPRO Tangerine dye in phosphate-buffered saline. Afterwards, fluorescent total protein profiles were recorded, gels were incubated in two 10 min changes of 0.1% Triton X-100 in phosphate-buffered saline and enzyme activity was detected utilizing either α-naphthyl acetate combined with Fast Blue BB for esterase activity measurement or ELF-97 β-D-glucuronide for β-glucuronidase activity as described in Section 2.8. The protocol allowed sensitive detection of both enzyme activities as discrete, sharp bands (Fig. 6B, C).

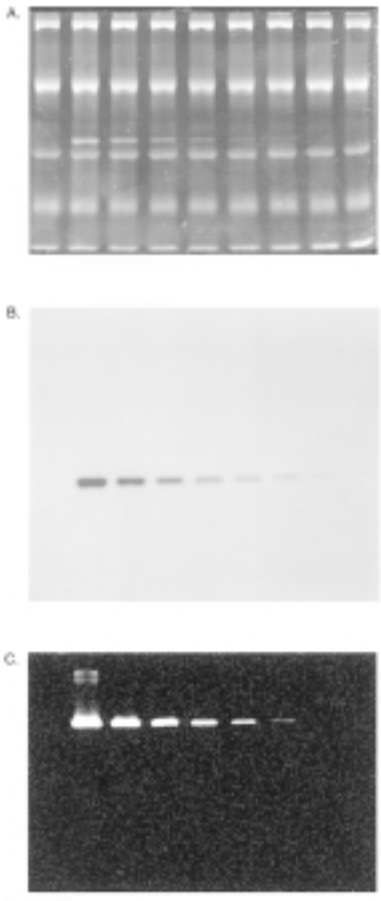
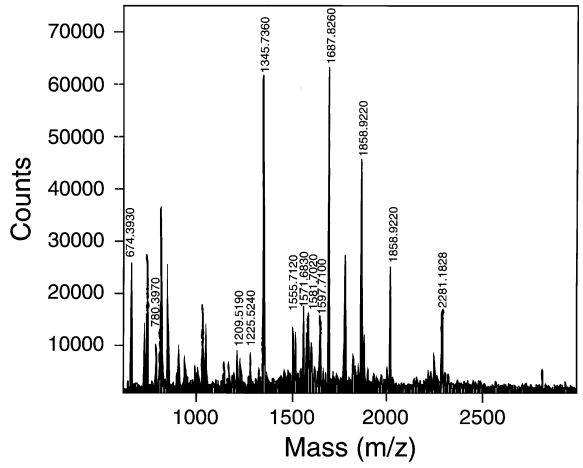


Figure 6. Zymographic detection of enzyme activity after visualization of total protein patterns using SYPRO Tangerine dye. (A) Detection of the total protein profile using SYPRO Tangerine stain. (B) Selective staining of rabbit liver esterase using a mixture of α/β-naphthyl acetate with Fast Blue BB for detection. (C) Selective staining of *Escherichia coli* β-glucuronidase activity using ELF-97-β-D-glucuronide.

3.4 Compatibility with MALDI-MS

To evaluate compatibility with MS, *in situ* tryptic digestion was carried out on proteins stained with SYPRO Tangerine dye. Peptide digests were subjected to MALDI-MS as described in Section 2.10. High quality peptide mass fingerprint spectra were obtained without incorporating additional steps to remove the dye from the protein samples (Fig. 7A). The only artifactual modification of the peptides observed was oxidation of some of the methionine residues. This is likely to occur from protein interaction with residual gel polymerization catalysts during electrophoresis or as a result of the lyophilization of samples during their preparation for MS and has frequently been observed with silver and CBB-R staining as well. A database search of the resolved peptide fragments correctly identified the protein as ovalbumin (Fig. 7B). Similar results

A.



B. **Ovalbumin**

m/z submitted	MH+ matched Ions	Delta (ppm)	Peptide Sequence	Modifications
647.3930	647.3881	7.6133	VYLPR	
780.3970	780.3892	10.0034	LYAEER	
1209.5190	1209.5210	-1.6638	DEDTQAMPFR	
1225.5240	1225.5159	6.5874	DEDTQAMPFR	Met-ox
1345.7360	1345.7381	-1.5455	HIATNAVLFFGR	
1555.7120	1555.7215	-6.1085	AFKDEDTQAMPFR	
1571.6830	1571.7164	-21.2620	AFKDEDTQAMPFR	Met-ox
1581.7020	1581.7219	-12.5794	LTEWTSSNVMEER	
1597.7100	1597.7168	-4.2635	LTEWTSSNVMEER	Met-ox
1687.8260	1687.8404	-8.5155	GGLEPINFQTAADQAR	
1773.8660	1773.8996	-18.9521	ISQAVHAAHAEINEAGR	
1858.9220	1858.9663	-23.8313	ELINSWVESQTNGIIR	
2281.1700	2281.1828	-5.6271	DILNQITKPNDVYSFSLASR	

Figure 7. Suitability of SYPRO Tangerine Protein Gel Stain for protein identification by MALDI-MS. (A) Peptide mass profile of ovalbumin obtained after SDS-polyacrylamide gel electrophoresis and visualization with SYPRO Tangerine dye. (B) Protein identification based upon the peptide mass profile.

were obtained with another test protein: lysozyme (data not shown). Therefore, SYPRO Tangerine stain is fully compatible with current protocols for protein identification by mass profiling using MALDI-MS.

4 Discussion

The primary objective of this study was to develop a sensitive fluorescent staining procedure that avoids the need for handling potentially harmful acids and organic solvents such as methanol, acetic acid and trichloroacetic acid. On a total mass basis, methanol is one of the top five chemicals appearing in the United States Environmental Protection Agency's (EPA's) Toxic Chemical Release Inventory database that is discharged by industrial facilities in 32 states ([26] http://www.epa.gov/opptintr/tri/). Typical symptoms of methanol toxicity include fatigue, headache, vomiting, visual impairment and death [27]. While the toxicity profile of haloacetic acids is incomplete, trichloroacetic acid is known to be teratogenic [28]. Acetic acid is a very weak tumor promoter in the multistage mouse skin model for chemical carcinogenesis but is a potent agent in the tumor progression phase of the model [29]. A solution of 7% acetic acid, as typically used in SYPRO Red and SYPRO Orange stains, is comparable to vinegar and thus should not be considered hazardous. Some laboratory personnel do find acetic acid vapors a nuisance, however.

Few environmentally benign staining methods for protein detection in SDS-polyacrylamide gels appear in the literature. A method for destaining CBB-R stained gels using 0.5 M NaCl was reported but staining of gels still required methanol, acetic acid, and trichloroacetic acid [30]. An environmentally benign staining procedure using CBB-G involves washing gels twice in 5 mM HCl for 2 h, staining gels in 1 mM HCl containing CBB-G for 16 h, and destaining in 1 mM HCl [31]. Though staining in dilute HCl is a reasonable alternative to the harsher solvents normally employed, the extended duration of the protocol is inconvenient. For quantitative measurements in gel electrophoresis it is important to avoid any destaining procedure as dye is invariably removed from the protein band as well as the background [32]. In addition, CBB stains are relatively insensitive, detecting 30–50 ng of protein per band [1, 2]. Recently, Bio-Rad Laboratories introduced a colloidal Coomassie blue formulation referred to as Bio-Safe Coomassie stain. The composition of this stain is a trade secret but, based upon the Materials Safety Data Sheet (MSDS), consists of 2.5–5% phosphoric acid, 0.1–1% methanol and possibly other chemicals not disclosed to the end user. In contrast, SYPRO Tangerine dye provides a flexible and sensitive approach to staining proteins under a wide range of solution conditions. Lack of a destaining step makes SYPRO Tangerine stain particularly suitable for protein quantitation. The staining procedure requires only saline, and the dye itself is easily recovered by adsorption to charcoal prior to solvent disposal.

SDS appears to bind along an unfolded polypeptide chain as a series of 6.2 nm spherical micelles [33, 34]. The necklace-like structure of the SDS/protein complex is characterized by the polypeptide backbone and polar side chains interacting with the sulfate head groups on the surface of the micelle while the hydrocarbon chain of the SDS molecules and the hydrophobic side chains of the protein are buried in the micelle's interior. Studies using amino acid homopolymers suggest that SYPRO Tangerine dye binds to proteins *via* interactions with the basic amino acids lysine, arginine and histidine, as well as through weaker interactions with hydrophobic amino acids such as tyrosine (Table 1). Omission of SDS completely prevents binding to the basic amino acid polymers and staining of hydrophobic polymers is greatly

reduced. This demonstrates that the fluorescent dye binds indirectly with proteins through interaction with SDS. Though synthetic amino acid homopolymers only serve as approximations of real proteins, our results obtained with CBB dye are consistent with previously published dye binding studies (Table 1; [35]). Due to the tremendous variety of silver staining methods practiced, it is impossible to assign a single staining mechanism or amino acid preference to the group as a whole. Aspartic acid, glutamic acid, histidine, cysteine, methionine, and lysine have all been proposed as candidate residues for silver ion binding [36]. The acidic silver nitrate staining method utilized in this study does appear to interact strongly with lysine residues, however. Significantly, the staining of defined amino acid heteropolymers corroborates results obtained from the homopolymer studies for all stains evaluated in this investigation.

Studies with sulfonated dyes suggest that the nucleation sites for SDS micelles along a polypeptide chain are likely to be basic amino acid residues. The primary binding mechanism of the fluorophore, 1-anilino-8-naphthalene sulfonate (ANS), is through ion pair formation with lysine, arginine and histidine residues [37]. Similar behavior has been observed with other organic sulfonate and sulfate ligands, including detergent sulfates such as SDS [38, 39]. Collectively, these observations suggest that basic amino acids play a fundamental role in binding anionic detergent and are therefore crucial to SDS-mediated interactions of hydrophobic fluorophores such as SYPRO Tangerine dye. This study further establishes that the binding of SYPRO Tangerine dye to the SDS/protein complex requires 150–250 mM NaCl. SDS binding to proteins is known to increase from roughly 1.0 to 2.2 g/g protein as salt concentration is increased from 10 to 220 mM [40, 41]. It is likely that a similar phenomenon promotes binding of the hydrophobic SYPRO Tangerine dye to SDS micelles. It is reasonable to speculate that the salt facilitates binding by reducing electrostatic repulsion between charged groups on both the detergent and the fluorescent dye.

Besides addressing legitimate environmental, health and safety concerns, a gentle staining procedure for protein detection may have benefits with respect to electroelution of proteins from polyacrylamide gels and the identification of gel-separated proteins by MS peptide mapping. Successful recovery of a protein by electroelution requires prior electrophoretic separation from contaminating species and identification of the band of interest. Staining gels with dyes such as CBB allows localization and excision of the target protein, but fixing and staining decreases protein recovery during the subsequent electroelution procedure [42]. Proteins processed in this manner often require resolubilization in 0.1 M NaOH, 0.1% SDS for 30 min prior to further equilibration in electroelution buffer [42]. In addition, the aspartate-proline bond is susceptible to hydrolysis in acidic staining and destaining solutions, resulting in cleavage of some proteins [43]. To circumvent these problems, the location of a protein in an unstained gel is often determined from bands in a corresponding strip that has been stained [42]. This interpolation method is often inaccurate as gel dimensions change during fixing, staining and destaining procedures and protein diffusion can occur in the unstained gel while the reference gel strip is being processed. SYPRO Tangerine dye staining permits convenient, direct protein visualization for electroelution without an intervening resolubilizing step.

Several artifactual protein modifications readily occur during staining of SDS-polyacrylamide gels, and undoubtedly other modifications are yet to be discovered. Trichloroacetic acid, present in many Coomassie blue formulations, often leads to esterification of glutamic acid residues while formaldehyde, present in silver staining recipes, may lead to alkylation of α- and ε-amino groups [3, 8, 44]. Such modifications hinder identification of proteins using MS by complicating interpretation of spectra. While peptide databases are often constructed to account for artifactual protein modifications, the modified species can potentially overlap other peaks that would otherwise provide useful sequence information. The only protein modification observed with SYPRO Tangerine dye in this study was partial oxidation of methionine residues to methionine sulfoxide in some peptides (Fig. 7). This modification is commonly observed in gel-separated proteins and may arise from residual persulfate in the gel due to the polymerization reaction or exposure of samples to air during lyophilization prior to analysis by MS [44]. We find that SYPRO Tangerine dye-stained protein spots generate spectra of comparable quality to CBB or SYPRO Orange dye stained spots. No artifactual peaks attributable to the dye itself or to dye-peptide complexes could be identified in the profiles. Furthermore, no polymer background signals were observed such as those reported for colloidal gold or silver stain [8, 45].

The use of SYPRO Tangerine dye in saline should make it superb for applications such as zymography, electroelution, gel staining prior to electroblotting and other downstream microchemical applications where high yield protein recovery is critical (*e.g.*, Edman-based sequencing, MS). Since proteins are not fixed, their recovery from gel matrices is superior to currently used staining methods. This improvement in protein recovery is readily demonstrated by comparing the transfer of tubulin from SYPRO Orange dye *versus* SYPRO Tangerine dye-stained gels

(Fig. 5), but is true for comparisons with silver staining and CBB-R staining as well (data not shown). Staining gels in saline should also be attractive in educational settings, especially in combination with a harmless blue light source such as the Dark Reader (Clare Chemical Research). The fluorescent dye is environmentally friendly and less of a nuisance to laboratory personnel due to the absence of noxious solvent fumes. SYPRO Tangerine stain allows sensitive detection of proteins under gentle staining conditions that minimize artifactual modification of proteins.

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5 References

[1] Brush, M., *The Scientist* 1998, *12*, 16–22.

[2] Wirth, P., Romano, A., *J. Chromatogr. A* 1995, *698*, 123–143.

[3] Haebel, S., Albrecht, T., Sparbier, K., Walden, P., Korner, R., Steup, M., *Electrophoresis* 1998, *19*, 679–686.

[4] Rabilloud, T., *Electrophoresis* 1992, *13*, 429–439.

[5] Christiansen, J., Houer, G., *Electrophoresis* 1992, *13*, 179–183.

[6] Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., Mann, M., *Nature* 1996, *379*, 466–469.

[7] Shevchenko, A., Jensen, O., Podtelejnikov, A., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., Mann, M., *Proc. Natl. Acad. Sci. USA* 1996, *93*, 14440–14445.

[8] Scheler, C., Lamer, S., Pan, Z., Li, X., Salnikow, J., Jungblut, P., *Electrophoresis* 1998, *19*, 918–927.

[9] Singer, V. L., Steinberg, T. H., Jones, L. J., Malekzadeh, N., Haugland, R. P., *J. NIH Res.* 1995, *7*, 82.

[10] Steinberg, T. H., Jones, L. J., Haugland, R. P., Singer, V. L., *Anal. Biochem.* 1996, *239*, 223–227.

[11] Steinberg, T. H., Haugland, R. P., Singer, V. L., *Anal. Biochem.* 1996, *239*, 238–245.

[12] Steinberg, T. H., White, H. M., Singer, V. L., *Anal. Biochem.* 1997, *248*, 168–172.

[13] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[14] Patton, W., Lopez, W., Barry, P., Skea, W., *BioTechniques* 1992, *12*, 580–585.

[15] Patton, W., Pluskal, M., Skea, W., Buecker, J., Lopez, M., Zimmerman, R., Belanger, L., Hatch, P., *BioTechniques* 1990, *8*, 518–527.

[16] Patton, W., *J. Chromatogr. A* 1995, *698*, 55–87.

[17] Tuma, R., Beaudet, M., Jin, X., Jones, L., Cheung, C., Yue, S., Singer, V., *Anal. Biochem.* 1999, *268*, 278–288.

[18] Towbin, H., Staehlin, T., Gordon, J., *Proc. Natl. Acad. Sci. USA* 1979, *76*, 4350–4354.

[19] Zhou, M., Upson, R., Diwu, Z., Haugland, R., *J. Biochem. Biophys. Methods* 1996, *33*, 197–205.

[20] Lauber, W., Carroll, J., Duffin, K., in: Savage, L. (Ed.), *Proteomics: Integrating Protein Based Tools and Applications for Drug Discovery*, International Business Communications, Southborough, MA 1998, pp. 385–400.

[21] Dahlberg, A., Dingman, C., Peacock, A., *J. Mol. Biol.* 1969, *41*, 139–147.

[22] Green, M., Pastewka, J., Peacock, A., *Anal. Biochem.* 1977, *56*, 43–51.

[23] Campbell, K., MacLennan, D., Jorgensen, A., *J. Biol. Chem.* 1983, *258*, 11267–11273.

[24] Wade, M., O'Conner, J., *BioTechniques* 1992, *12*, 794–796.

[25] Gee, H., Cowman, M., *Anal. Biochem.* 1994, *219*, 278–287.

[26] Neumann, C., Forman, D., Rothlein, J., *Environ. Health Perspect.* 1998, *106*, 217–226.

[27] Pamies, R., Sugar, D., Rives, L., Herold, A., *Postgrad. Med.* 1993, *93*, 183–184.

[28] Hunter, E., Rogers, E., Schmid, J., Richard, A., *Teratology* 1996, *54*, 57–64.

[29] Rotstein, J., Slaga, T., *Cancer Lett.* 1988, *42*, 87–90.

[30] Sreeramulu, G., Singh, N., *Electrophoresis* 1995, *16*, 362–365.

[31] Nivinskas, H., Cole, K., *BioTechniques* 1996, *20*, 380–385.

[32] Neuhoff, V., Stamm, R., Pardowitz, I., Arold, N., Ehrhardt, W., Taube, D., *Electrophoresis* 1990, *11*, 101–117.

[33] Samso, M., Daban, J., Hansen, S., Jones, G., *Eur. J. Biochem.* 1995, *232*, 818–824.

[34] Shirahama, K., Tsujii, K., Takagi, T., *J. Biochem. (Tokyo)* 1974, *75*, 309–319.

[35] Tal, M., Silberstein, A., Nusser, E., *J. Biol. Chem.* 1985, *260*, 9976–9980.

[36] Rabilloud, T., *Electrophoresis* 1990, *11*, 785–794.

[37] Matulis, D., Lovrien, R., *Biophys. J.* 1998, *74*, 422–429.

[38] Matulis, D., Richardson, T., Lovrien, R., *J. Mol. Recognit.* 1996, *9*, 433–443.

[39] Conroy, M., Lovrien, R., *J. Cryst. Growth* 1992, *122*, 213–222.

[40] Allen, G., *Biochem. J.* 1974, *137*, 575–578.

[41] Shinagawa, S., Kameyama, K., Takagi, T., *Biochim. Biophys. Acta* 1993, *1161*, 79–84.

[42] Shoji, M., Kato, M., Hashizume, S., *J. Chromatogr. A* 1995, *698*, 145–162.

[43] Hunkapillar, M., Lujan, E., Ostrander, F., Hood, L., *Methods. Enzymol.* 1983, *91*, 227–236.

[44] Patterson, S., Aebersold, R., *Electrophoresis* 1995, *16*, 1791–1814.

[45] Loo, R., Dales, N., Andrews, P., *Prot. Sci.* 1994, *11*, 1975–1983.