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Comparison of SYPRO Ruby and Flamingo fluorescent stains for application in proteomic research

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ABSTRACT

Fluorescent dyes are widely used for the detection and quantitation of proteins separated by polyacrylamide gel electrophoresis. SYPRO Ruby is one such fluorescent dye widely used for this purpose. More recently, another fluorescent dye, Flamingo, is available for expression proteomic research. Using a standard ultraviolet (UV) transilluminator and a charge-coupled device (CCD)-based imaging system, the relative sensitivity of these two different fluorescent stains with regard to detection of protein spots separated by two-dimensional gel electrophoresis (2D–GE) and identification by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) were compared. Using mouse kidney and liver homogenates as well as *Escherichia coli* extract, we detected a greater number of protein spots using Flamingo compared with SYPRO Ruby. In addition, when we compared the number of matched peptides and the percentage of amino acid residues identified for 22 different protein spots of mouse kidney proteome, we observed a higher number of matched peptides and a higher percentage of amino acid residues for the majority of the proteins using Flamingo compared with SYPRO Ruby. Also, we were able to characterize a protein spot that can be detected by Flamingo only. Therefore, we recommend Flamingo over SYPRO Ruby to be used for studies on expression proteomics.

Expression proteomics is becoming increasingly important in the postgenomic era, and fluorescent dyes are widely used for gel-based quantitative proteomic research. Although fluorescent dyes can be relatively expensive and require a gel documentation system, there are several major advantages compared with traditional dye-binding methods such as Coomassie brilliant blue (CBB)[1] or other methods such as silver staining and radiolabeling [1]. Fluorescent dyes can be widely used in life science research such as qualitative and quantitative analysis of nucleic acids and proteins. Fluorescent dyes are more sensitive, have a broad linear dynamic range, and are safe to use compared with traditional CBB, silver stain, and autoradiography. In addition, fluorescent stains are compatible with mass spectrometers. As a result, fluorescent stains are being widely used compared with colorimetric and radiometric detection of proteins on polyacrylamide gels and membranes. In addition to their application in expression proteomics, fluorescent stains are used to study posttranslational modifications, including phosphorylation, glycosylation, and stress-related protein modification such as carbonylation/nitrosylation/ADP ribosylation [2–5]. Differential phosphorylation and glycosylation are known to play important roles in the pathogenesis of various diseases such as cancer [6,7]. The identification of posttranslationally modified proteins that are also expressed differentially in control and diseased samples can provide valuable information for the identification of drug targets and/or diagnostic tests for various diseases. In the current study, we used a standard ultraviolet (UV) transilluminator and a charge-coupled device (CCD)-based imaging system [8] for the detection of fluorescent dye-stained proteins separated by two-dimensional gel electrophoresis (2D–GE). This type of electronic imaging system is less expensive compared with a laser-based/photomultiplier system. In addition, it is possible to have rapid multiplex analysis of proteins (exposure time typically is milliseconds to seconds) due to multiple fluorescent images captured as a result of single UV excitation and multiple emission spectra of the different dyes used to stain a single gel. On the other hand, laser scanners can be used for only those fluorophores whose excitation/emission spectra will match with the output of the available laser sources. The current study is an extension of our previous study that compared the staining efficiency and equivalency for liquid chromatography–tandem mass spectrometry (LC–MS/MS) identification of two different

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[1] *Abbreviations used:* CBB, Coomassie brilliant blue; UV, ultraviolet; CCD, charge-coupled device; 2D–GE, two-dimensional gel electrophoresis; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectrometry; ESI, electrospray ionization; IEF, isoelectric focusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OD, optical density; NCBI, National Center for Biotechnology Information; MALDI, matrix-assisted laser desorption/ionization.

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fluorophores: SYPRO Ruby and Deep Purple. Although the above two fluorophores showed comparable mass spectrometry (MS) compatibility, SYPRO Ruby was more sensitive with regard to identification of protein spots separated by 2D–GE while using UV transillumination and CCD-based imaging. Hence, we recommended the use of SYPRO Ruby for studies on expression proteomics [1]. More recently, a fluorescent stain, Flamingo, has been recommended by Bio-Rad as more sensitive than SYPRO Ruby, a dye that is widely used in expression proteomic studies (http://www.bio-rad.com/cmc_upload/Literature/195737/Bulletin_5346.pdf). However, Harris and coworkers [9] reported comparable staining efficiency of the two stains.

In the current investigation, we compared the staining efficiency of the most commonly used fluorescent dye, SYPRO Ruby (absorption peaks ~280 and 470 nm, emission peak ~610 nm), with that of Flamingo (absorption peak ~512 nm, emission peak ~535 nm) for proteins, followed by their identification by LC–electrospray ionization (ESI)–MS/MS. We used a standard UV transilluminator and a CCD-based imaging system, which is less expensive compared with a laser scanner, to compare the staining efficiency of the two stains. Later, we compared the MS compatibility for the identification of the protein spots detected by these two different fluorophores using LC–ESI–MS/MS.

Materials and methods

The details of the reagents, preparation of samples for 2D–GE, isoelectric focusing (IEF), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), gel scanning and spot detection, in-gel trypsin digestion and LC–ESI–MS/MS of tryptic peptides, database searching, and data processing have been described by Chakravarti and coworkers [1,10,11]. Briefly, proteins samples for 2D–GE were prepared from the kidneys or livers of C57BL/6 male mice (~5 months old, Harlan Sprague–Dawley) as described previously [11]. *Escherichia coli* protein extract was obtained commercially from Bio-Rad. IEF was carried out using an IPGphor system (GE Healthcare) and an 11-cm pH 3–11 NL ReadyStrip IPG strip. Following second-dimension gel electrophoresis (8–16%, w/v, acrylamide) using a Criterion gel apparatus (Bio-Rad), the gels were stained with either SYPRO Ruby or Flamingo according to the manufacturer's instructions. For excision of the protein spots, the gels were subsequently stained with Bio-Safe Coomassie (Bio-Rad) to visualize the protein spots. Protein markers (molecular weight 14,400–97,000 Da) of known concentrations (GE Healthcare) were separated by one-dimensional SDS–PAGE and used to compare the limit of detection and linear dynamic range of SYPRO Ruby and Flamingo.

Gel scanning and spot detection were carried out using a CCD-based bioimaging system (UVP) containing a UV transilluminator (300–340 nm) and the emission filter (570–640 nm for SYPRO Ruby-stained gels and 515–570 nm for Flamingo-stained gels). Computer-assisted two-dimensional gel image analysis was performed using the Image Master Platinum 2D software package (version 5.0, GE Healthcare) for the detection and manual editing of the protein spots. Quantitation of the images of different proteins separated by one-dimensional SDS–PAGE was carried out by measuring the total optical density (OD, arbitrary units) of the stained bands of each lane of the gel using VisionWorksLS (UVP).

Tryptic digestion, LC–ESI–MS/MS identification of proteins using an LCQ Deca XP ProteomeX System (Thermo Electron), and the search criteria used for the identification of proteins have been described in detail by Chakravarti and coworkers [1,10,11]. Briefly, each trypsin-digested protein was analyzed three times consecutively for identification by LC–ESI–MS/MS. All MS/MS spectra were searched with the SEQUEST algorithm-based Bioworks 3.3 (Thermo Finnigan) against a database created by extracting mouse entries from the National Center for Biotechnology Information (NCBI) ftp site. As described earlier, the following stringent SEQUEST criteria were used to calculate the number of peptides for the identification of any particular protein: (i) Delta Cn score $\geqslant 0.1$, (ii) Rsp score $\leqslant 3$, (iii) Xcorr $\geqslant 1.5$ for +1 charged peptides, (iv) Xcorr $\geqslant 2.0$ for +2 charged peptides, (v) Xcorr $\geqslant 2.5$ for +3 charged peptides, and (vi) protein probability values (representing the values for the best peptide match, which is the peptide with the lowest score, i.e., the highest probability of a match between the actual sequence and the observed spectrum) $\leqslant 0.001$. Spectra for all of the hits were further verified manually. The following criteria were taken into account during the manual verification stage: (i) continuity of the *b* and *y* ion series and (ii) good quality of the MS/MS spectrum (i.e., the fragment ions should be clearly above the baseline). Unless otherwise mentioned, proteins with three or more unique spectra (at least with one of the fluorophores) were accepted as positive identification.

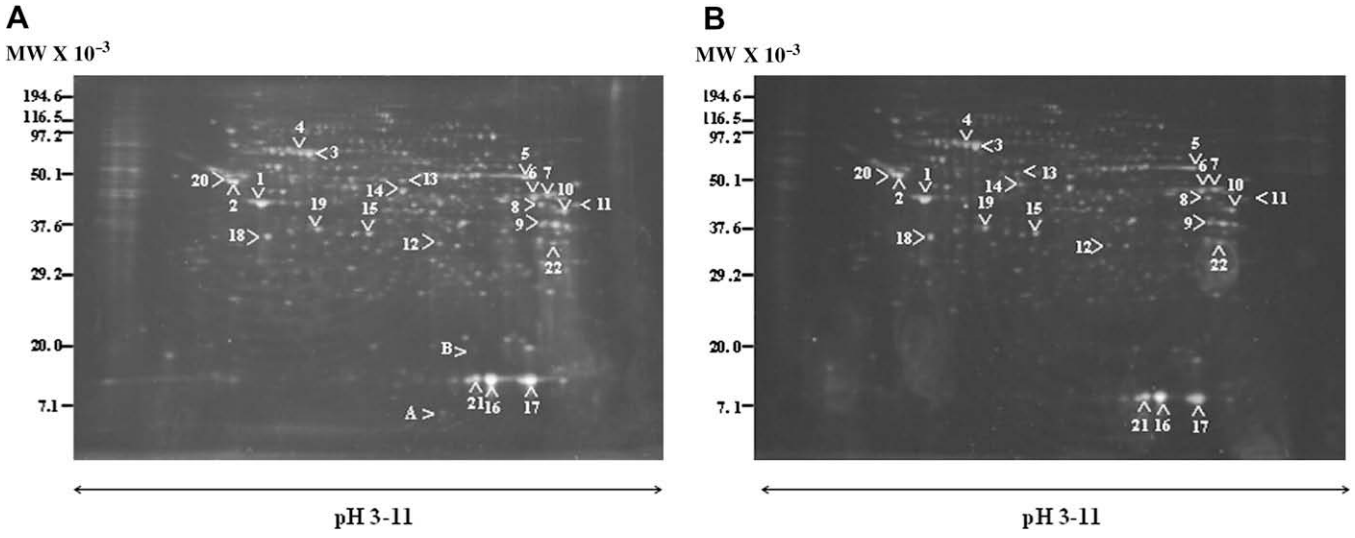

Fig. 1. Detection of proteins using Flamingo (A) and SYPRO Ruby (B) fluorescent stains. Mouse kidney proteome (20 μg) was analyzed by 2D–GE. Five different gels were stained with either SYPRO Ruby or Flamingo. A higher number of spots was consistently detected by staining with Flamingo. The results are summarized in Table 1. MW, molecular weight.