Oriole™ Fluorescent Gel Stain: A Novel Fluorescent Stain for a Simpler 2-D PAGE to MALDI Workflow

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Introduction

Proteomics research involves the parallel identification and quantitation of proteins in complex biological samples. The most commonly employed workflow consists of extraction of the proteins from the biological source, fractionation or separation of the material, and digestion of the proteins followed by mass spectrometric analysis of the resultant peptides. Protein identification is performed by automated comparison of the peptide masses to a known database of predicted peptide masses generated from known protein sequences. Selected peptides may be subjected to fragmentation and a second dimension of mass spectrometry for further confirmation of protein identity. A common variant of this procedure involves separating the proteins by 2-D gel electrophoresis followed by staining, imaging, and excision of the proteins from the gel. The gel plugs are digested with trypsin and the eluted peptide mixture is analyzed directly by MALDI mass spectrometry. This method takes advantage of the extraordinary resolving power of 2-D electrophoresis. Proteins are substantially separated from one another prior to digestion and the resultant reduction in the complexity of the proteome prior to mass spectrometry (MS) analysis gives a higher chance of detecting posttranslational modifications. Relative protein quantitation can also be performed by analysis of the stained gel image. This procedure does not require a high-end mass spectrometer and is relatively accessible to the nonexpert user.

In the 2-D/MALDI workflow, only proteins detected on gel after staining are subjected to mass analysis. Various staining methods, with differences in time to result, sensitivity, and dynamic range can be used. The most commonly used technique is Coomassie blue staining. It typically allows detection of tens of nanograms of protein (Neuhoff et al. 1988) over two orders of magnitude. The more sensitive silver staining method can detect spots of 0.1 ng of protein, but is experimentally more demanding and has a dynamic range of only one order of magnitude. In addition silver-stained proteins are more difficult to identify with MS methods (Lin et al. 2008, White et al. 2004). In contrast, fluorescent stains combine the ease-of-use of Coomassie blue with the sensitivity of silver staining and display a linear response over three to four orders of magnitude. Furthermore, fluorescent staining is easily coupled with MS. The disadvantages of fluorescent staining methods are the need for an expensive fluorescent scanner, long staining procedures, and the need to counterstain with Coomassie blue for gel spot cutting.

Recently, Bio-Rad introduced a new stain, the Oriole stain (Berkelman and Walker 2009), which has the sensitivity typical of fluorescent stains and is imaged using simple and relatively inexpensive UV imagers. The staining protocol is compatible with MS methods. Here we compare the use of available fluorescent stains with the Oriole stain in a 2-D/MALDI proteomic workflow (Ball and Karuso 2007). We focused on evaluating workflow advantages in terms of saving time and reducing manual steps, while preserving sensitivity and a large linear dynamic range.

Materials and Methods

Materials

All materials were from Bio-Rad Laboratories, Inc. unless otherwise noted. Krypton protein stain was obtained from Thermo Scientific. Deep Purple total protein stain was obtained from GE Healthcare; sodium borate, ACS grade, was obtained from Spectrum Biosciences; ethanol was purchased from J.T. Baker; and acetonitrile (ACN) was purchased from Sigma-Aldrich. Acetic acid, glacial, was obtained from EMD Biosciences.

Isoelectric Focusing and Gel Electrophoresis

2-D SDS-PAGE standards were diluted at 15 µl/ml into an isoelectric focusing buffer containing 2 M thiourea, 7 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% w/v Bio-Lyte® 3/10 ampholyte, and 0.002% bromophenol blue. This sample was used to rehydrate twenty-one 11-cm ReadyStrip™ IPG strips, pH 3–10 NL (200 µl/strip). Samples were passively rehydrated on a PROTEAN® IEF cell, and then focused for a total of 35,000 V-hr.

The strips were then treated with ReadyPrep™ protein extraction kit equilibration buffer I (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 2% DTT) for 20 min at room temperature, and then alkylated for 20 min using ReadyPrep protein extraction kit equilibration buffer II (containing iodoacetamide). The strips were assembled onto the tops of 8–16% linear gradient Criterion™ precast Tris-HCl gels. Strips were secured

using PROTEAN Plus proteomics grade agarose (0.75% agarose), containing 0.003% bromophenol blue. The gel cassettes were loaded into a Criterion™ Dodeca™ cell, and electrophoresis was performed in 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS buffer for 55 min at a constant 200 V. Gel cassettes were then randomized and disassembled, and the gels were subjected to the various staining procedures.

Gel Staining

Gel staining was performed at room temperature following each manufacturer's guidelines. These procedures are briefly described below. Full protocols are available with each product from the manufacturer. Colloidal Coomassie staining was performed according to the method of Neuhoff (Neuhoff et al. 1988). Silver staining was performed using a modified Shevchenko method (Shevchenko et al. 1996). Gels stained with Deep Purple total protein stain were fixed overnight in a 15% solution of ethanol containing 1% citric acid, stained for 60 min then washed for 30 min in 15% ethanol. Gels were then acidified by treatment with 15% ethanol containing 1% citric acid for 30 min, and rinsed with 15% ethanol for 5 min prior to imaging. For Krypton, SYPRO Ruby, and Flamingo™ stains, 40% ethanol with 10% acetic acid was used for the fixation step. Gels stained with Krypton stain were fixed two times for 30 min each, washed with water, and then stained overnight. Gels were briefly destained using 5% acetic acid and washed with water for another 2 x 15 min prior to imaging. SYPRO Ruby stain gels were fixed two times for 30 min and stained overnight. Destaining was performed using 10% methanol with 7% acetic acid for a total of 60 min. Flamingo stain gels were fixed for 120 min prior to overnight staining and were not destained in this study.

Oriole stain gels were transferred, following electrophoresis, directly from the gel cassette to the stain solution. Staining took place for 90 min.

All gels were stored post-staining according to the manufacturers' suggestions, and rinsed briefly with water or aqueous ethanol (for Deep Purple total protein stain only) prior to any imaging.

Imaging

Gels were imaged either on a GS-800™ calibrated densitometer or a VersaDoc™ MP 4000 system. Imaging was conducted using the software-supplied parameters for each stain. Settings for SYPRO Ruby stain were also used for the Oriole stain samples, and included a 10 sec exposure. Settings for Flamingo stain were also used for the Krypton stain samples. Deep Purple, Flamingo, and Krypton stain gels all required 180 sec exposures. The resultant images were cropped and sized for consistency, and then subjected to analysis using PDQuest™ advanced 2-D analysis software. Automatic spot counting was performed, and results confirmed through manual spot counting of the SDS-PAGE standard sample. Standard spot numbers (SSP) were assigned to the matched spots common to all gels analyzed to produce a master gel. Spot volumes and variances are from the average of three gels. The volumes were summed and combined for each protein area. The combined volumes are expressed relative to the whole.

MALDI Analysis

Following imaging, the fluorescently stained gels were counterstained using Bio-Safe™ Coomassie stain following the manufacturer's protocol. The gels were then prepared for automatic spot excision using an EXQuest™ spot cutter. The counterstained gels were placed on the cutting surface and imaged using a white light source. The images were then compared to the master gel to confirm the alignment, and spots from the cut list were excised. In parallel, an Oriole stain gel was placed directly on the EXQuest spot cutter, with samples being excised following UV epi-illumination. The image was compared to the master gel image to confirm the placement of the cuts.

Samples were automatically distributed into 96-well protein purification plates for processing on a Tecan Freedom EVO100 liquid handling robot. Following centrifugation, the plugs were treated with 50 µl of ACN/100 mM ammonium bicarbonate (ABC) (1:1) three times, for 20 min each, to destain. This method is applicable to Coomassie brilliant blue (CBB), and all of the fluorescent stains presented in this report. The plugs were then dehydrated with 50 µl of ACN/water (9:1) for 30 min. The solvent was removed by centrifugation, and the plugs allowed to air-dry. Five µl of trypsin (20 ng/µl in 25 mM ABC) were added to each plug at 0°C, followed by a 30 min incubation. The excess solution was centrifuged and 5 µl of 25 mM ABC was added to each plug. The plates were then placed in a controlled humidity environment at 20°C for 15–18 hr. At this time, the digest solution was centrifuged into clean microtiter collection plates, and the plugs further treated with 5 µl of 0.5% v/v trifluoroacetic acid (TFA) for 30 min. This was filtered, and combined with the original filtrate to give approximately 10 µl of sample solution at pH ~2.

The acidified digest samples were prepared for MALDI-TOF analysis by the dried droplet method using α-cyano-4-hydroxycinnamic acid (CHCA). Briefly, 0.5 µl of sample was applied to the plate followed by 0.5 µl of 5 mg/ml CHCA in ACN/0.1% TFA (2:1). Calibration sites on the plate were prepared in a similar fashion using Bruker peptide calibration standards dissolved in 0.1% TFA. Upon drying, the sample plate was introduced into the mass spectrometer.

MALDI mass spectrometry was performed on a 4800 Plus MALDI TOF/TOF analyzer (AB Sciex) in MS-MS/MS mode using the 4000 Series Explorer control software (v3.5.3, AB Sciex). Samples were initially irradiated at 355 nm with a Nd:YAG laser. A total of 400 shots from 16 subregions per sample were acquired in positive, reflector mode over the 750–3,500 Da range for MS processing. The five most intense signal peaks from each sample not associated with matrix or trypsin autolysis were then subjected to MS/MS analysis using a 2 kV collision energy with the CID gas set to off. Fifty

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shots from each of twenty subregions were totaled, and these data were combined with the parent MS data to be subjected to database searches.

Peaks were filtered to the 750–3,500 Da mass range. Peak lists were created, and submitted to the MASCOT database search program (Matrix Sciences) using either ProteinPilot (v3.0, AB Sciex) or GPS Explorer software (v3.6, AB Sciex). Spectra were subjected to three subgroup searches of SwissProt v57.9 ("other mammalia", "bony vertebrates", and "green plants") to cover the different species present in the sample. Only results with a probability of randomness of less than 0.05 ($P < 0.05$) were considered significant, and included in this evaluation. Results are presented in regard to peptides matched and protein sequence coverage achieved.

Results and Discussion

Staining

The fluorescent stains used in this study can achieve sensitivities of protein detection on the order of hundreds of picograms, and possess linear dynamic ranges of three to four orders of magnitude (Berkelman 2008, Berkelman and Walker 2009, Harris et al. 2007). Additionally, most of the staining processes are designed to give clean gel images with limited background staining. Each stain is compatible with MS analysis, but results have been shown to vary among stains (Ball and Karuso 2007, Lanne and Panfilov 2005).

Table 1 summarizes the processing steps necessary for each stain following electrophoresis, and prior to image collection. Most methods include a fixation step, staining, and various washing and destaining steps for optimal imaging. All four stains used in this study (other than Oriole stain), require an overnight procedure for optimal performance. Deep Purple stain employs an overnight fix while Flamingo, Krypton, and SYPRO Ruby stains utilize overnight staining. The recommended methods often call for several solution changes over the course of the protocol. Typical waste streams can be greater than 700 ml/gel. In contrast, staining with Oriole stain occurs immediately following electrophoresis in a single treatment without the need for fixation. Additionally, destaining is not required. Staining with Oriole stain is complete within 90 min, allowing imaging and further processing to occur the same day as electrophoresis.

Image Analysis

The simple 2-D SDS-PAGE standard used in this study contains seven proteins across a wide range of pIs and molecular weights. These proteins contain a number of isoforms producing a multispot pattern on the gel. The amount of each protein in the sample was set above the limit of detection of CBB in order to facilitate subsequent sample processing. Spot counting was performed in an automated fashion using PDQuest software, and confirmed by manual inspection of the assigned locations. The average of the three experiments is reported. Representative gel images from each stain are shown in Figure 1. Staining using the visible reagents (CBB or silver) produced densitometer images that detected all of the proteins. Staining with CBB gave an average of 37 spots while silver staining (data not included) showed an average of 28 spots. All of the fluorescent stains revealed more spots than silver staining. Deep Purple stain averaged 35 spots/gel and Krypton stain showed 39 spots. Flamingo, SYPRO Ruby, and Oriole stains averaged 40, 41, and 41 spots, respectively. These results concur with earlier work (Ball and Karuso 2007).

Marked image similarities allowed for interrogation of preferential staining by the various fluorescent stains. Using the known pI/MW values for each protein, the stained isoform regions were assigned to one of each of the proteins. Combining the spot volumes from the individual locations into protein groups provided information about stain distribution (Table 2). The variation of protein staining intensity for each

Table 1. Comparison of gel staining procedure using different fluorescent stains.

	Stain Processing Times				
	Krypton	**Deep Purple**	**Flamingo**	**SYPRO Ruby**	**Oriole**
Fixing	65 min (3 solution changes)	overnight	1 × 120 min	2 × 30 min	none
Staining	overnight	60 min	overnight	overnight	90 min
Destaining/washing	35 min (3 solution changes)	2 x 30 min	10 min	60 min	none
Total time	100 min + overnight stain	120 min + overnight fix	130 min + overnight stain	120 min + overnight stain	90 min
Number of steps	7	4	3	4	1
Volume of all reagents used per gel, ml	700	400-500	300	400	100

Table 2. 2-D gel protein spot volumes.

	Protein Stain									
Protein	**Krypton**		**Deep Purple**		**Flamingo**		**SYPRO Ruby**		**Oriole**	
	Percent Total	**%CV**	**Percent Total**	**%CV**	**Percent Total**	**%CV**	**Percent Total**	**%CV**	**Percent Total**	**%CV**
Trypsin Inhibitor	13.2	20.9	7.1	7.6	4.4	19.4	11.8	9.8	7.6	20.5
Actin	7.5	12.3	3.9	43.5	6.7	20.5	8.1	23.9	7.6	21.3
BSA	7.6	25.4	7.5	35.5	10.6	15.0	8.5	22.7	13.4	13.1
Carbonic Anhydrase	7.7	15.4	6.9	32.8	6.0	36.4	8.5	13.2	9.1	15.3
Ovotransferrin	22.0	6.0	25.4	14.6	18.3	0.5	22.4	11.3	24.1	6.2
GAPDH	25.3	10.1	26.3	11.1	23.9	18.8	25.8	3.4	25.1	10.3
Myoglobin	16.7	16.3	22.8	11.5	30.1	15.0	14.8	9.3	13.2	5.3
Average CV %		**15.2**		**22.4**		**17.9**		**13.4**		**13.1**

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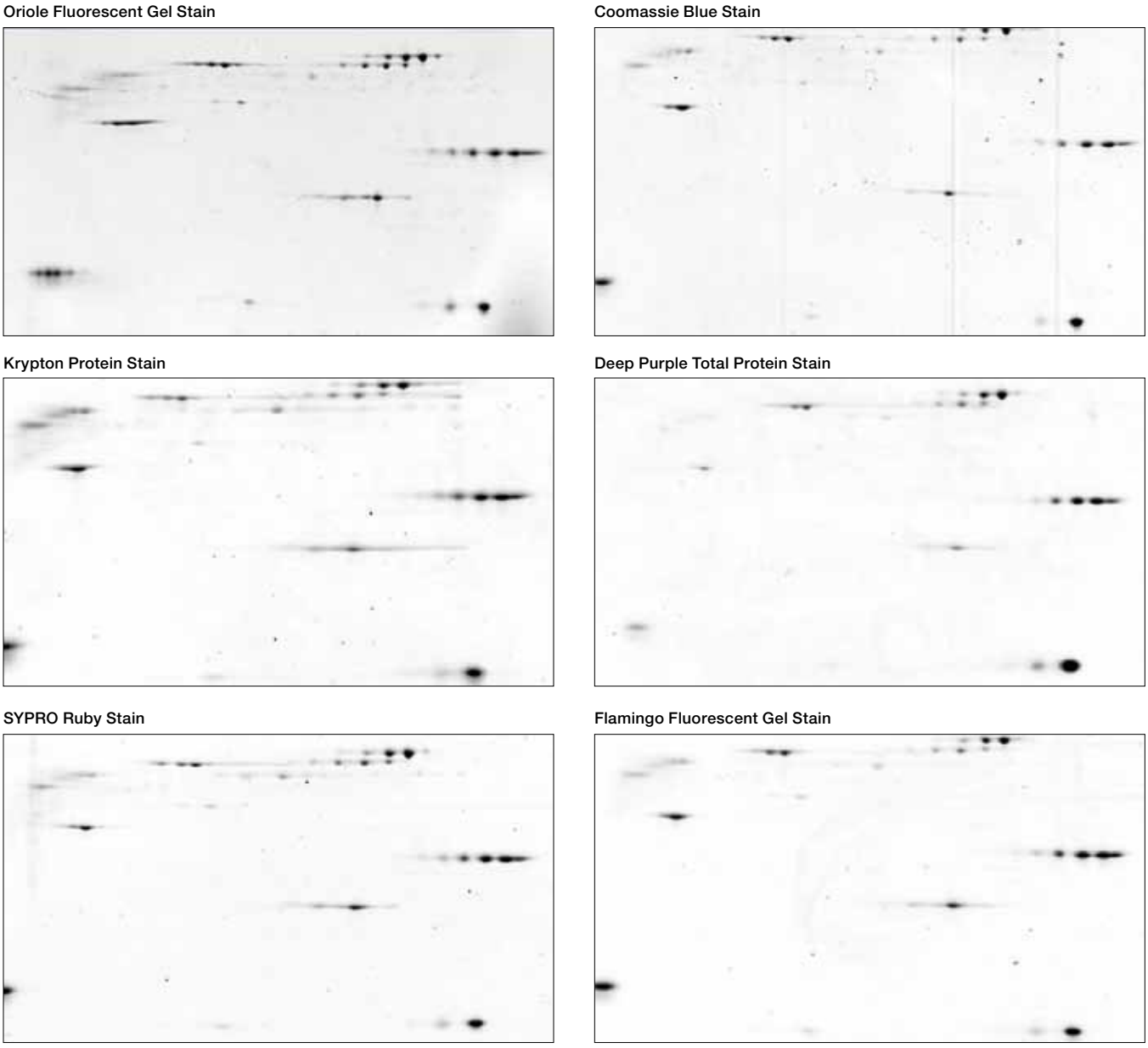

Fig. 1. Comparison of protein gel staining techniques. 2-D SDS-PAGE standards were separated by 2-D gel electrophoresis and stained using various commercially available reagents.

fluorescent stain ranged from 13–22%. Most stains had a strong preference for binding to ovotransferrin and GAPDH, which contributed to about 50% of the total gel fluorescence. Deep Purple, Krypton, SYPRO Ruby, and Oriole stains had similar distributions, however, Oriole stain stained BSA preferentially to the trypsin inhibitor. As seen in Figure 1, Deep Purple stain tended to have a preference for the more basic proteins within the sample set. Like Flamingo stain, Deep Purple stain exhibited a higher staining intensity with myoglobin than the other stains. The sample lacks the required concentration breadth to investigate dynamic ranges, however, this information has been previously reported for these stains by the manufacturers.

Spot Cutting and MALDI Analysis

Earlier work on fluorescent stains and their compatibility with MS provided excellent insight into stain differences using the same sample as our current study (Ball and Karuso 2007). The protein levels used were above the limit of detection for CBB staining in order to facilitate spot cutting from counterstained gels. The images obtained above were used to create a common set of identifiers for the spots on each gel. This matchset was used to determine which spots would be excised for MALDI analysis. A set of 24 spots common to all gels was selected and one gel from each fluorescent staining method was processed for MS. From these 24 spots, 16 produced at least one positive database hit on at least one

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of the gels. These 16 spots were then the basis for MS analysis on a second gel from each group. Mass spectral data are reported as an average of the two experiments where applicable.

In order to locate the spots of interest for cutting, gels were washed in water for 5 min and then treated with CBB for 1 hr. Destaining in water proceeded for another 30 min before the gels were positioned for re-imaging and spot excision. This method can be applied to all of the fluorescently stained gels in this study. Alternately, by taking advantage of the UV epi-illumination of the EXQuest spot cutter it is possible to proceed to spot cutting of gel stained with Oriole stain without counterstaing with CBB. Figure 2A shows a typical Oriole stain gel image obtained on a VersaDoc MP 4000 imager following a 20 sec exposure. Exposures of 30–60 sec on the EXQuest spot cutter are sufficient to produce similar images in terms of contrast and resolution (Figure 2B). This approach of UV imaging is feasible as photobleaching is minimal with Oriole stain. When exposed to UV irradiation for 20 min, more than 70% of the peak fluorescence was retained (data not shown). The higher-contrast image produced through UV illumination also makes the spot alignment procedure prior to spot excision straightforward. Ultimately, lowering the number of processing steps decreases the chance for sample depletion or alteration prior to MS analysis.

Table 3 shows the results of the MASCOT database search. The data are presented as the number of unique peptides matched for the Protein ID given, and the sequence coverage represented by those matched peptides. MS/MS data (not shown) was used to confirm the protein assignments. All of the fluorescent stains were able to identify ($P < 0.05$) at least half of the isolated spots, with Krypton stain matching only eight submissions (no matches to the myoglobin spots). Deep Purple stain was able to ID a second form of carbonic anhydrase (SSP 4101, Figure 2B), as was Oriole stain, but was unable to ID myoglobin like Krypton stain. The strong staining preference for myoglobin shown by Deep Purple stain, combined with the lack of an MS response, was not expected. SYPRO Ruby and Oriole stains led to the correct identification of 13 and 14 spots, respectively. Whereas SYPRO Ruby stain permitted the ID of a fourth GAPDH isoform (SSP 8201), and a second sample from myoglobin

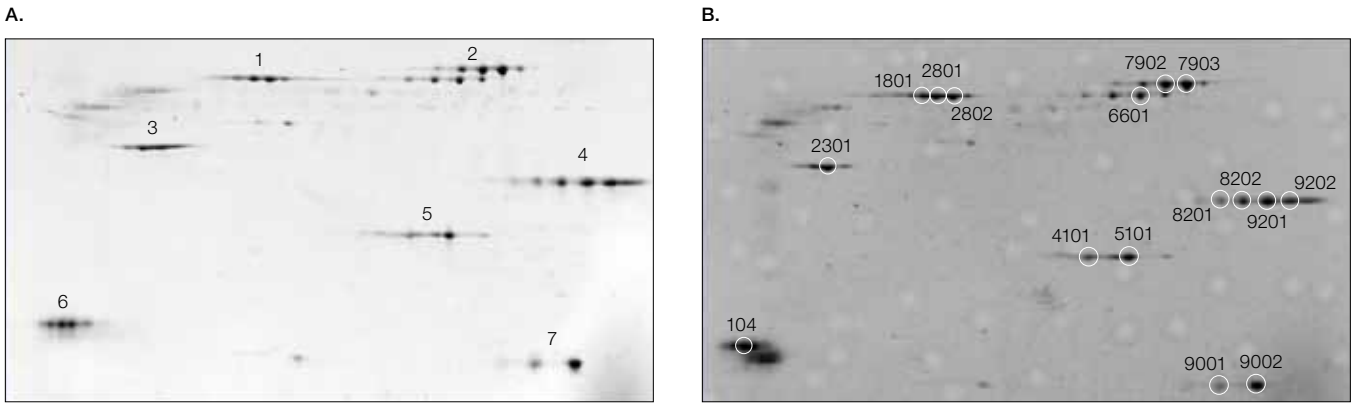

Fig. 2. Imaging of gel stained with Oriole stain. A, Gel imaged on a VersaDoc MP 4000 imager using a 10 sec exposure; 1, BSA; 2, ovotransferrin; 3, actin; 4, GAPDH; 5, carbonic anhydrase; 6, trypsin inhibitor; 7, myoglobin; **B**, gel stained with Oriole stain imaged on the EXQuest spot cutter using UV illumination for 60s. Numbers are the SSP identifiers of excised spot.

Table 3. Results of MASCOT database search.

		Protein Stain									
		Krypton		Deep Purple		Flamingo		SYPRO Ruby		Oriole	
SSP	Protein ID*	%Coverage	Peptides	%Coverage	Peptides	%Coverage	Peptides	%Coverage	Peptides	%Coverage	Peptides
104	ITRA_SOYBN	35	12	35	13	35	11	21	7	35	13
2301	ACTA_BOVIN	45	18	29	14	38	19	35	16	36	15
1801	ALBU_BOVIN	–**	–	–	–	–	–	–	–	4	6
2801	ALBU_BOVIN	–	–	–	–	15	10	20	10	37	19
2802	ALBU_BOVIN	11	8	14	7	20	14	20	13	57	39
4101	CAH2_BOVIN	–	–	31	7	–	–	–	–	28	10
5101	CAH2_BOVIN	43	12	45	13	30	6	53	17	48	21
6601	TRFE_CHICK	–	–	–	–	–	–	–	–	27	18
7902	TRFE_CHICK	–	–	36	22	–	–	20	12	45	37
7903	TRFE_CHICK	28	17	28	16	14	13	26	14	52	43
8201	G3P_RABIT	–	–	–	–	–	–	15	7	–	–
8202	G3P_RABIT	18	10	–	–	26	9	17	9	18	14
9202	G3P_RABIT	23	10	24	13	17	11	29	15	31	17
9201	G3P_RABIT	24	12	17	10	19	12	32	15	28	18
9001	MYG_HORSE	–	–	–	–	–	–	15	7	–	–
9002	MYG_HORSE	–	–	–	–	50	8	54	12	62	15

* Swiss-Prot/UniProt KB protein ID

** Protein not identified

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(SSP 9001), Oriole stain was unique in identifying a third form of ovotransferrin (SSP 6601) and BSA (SSP 1801). Most spectra were peak-rich and free of interfering contaminants. In general, the sequence coverage of the proteins was similar amongst the stains. The dissimilarities included the sequence coverage, and number of identified ovotransferrin and BSA peptides derived from gels stained with Oriole stain, which surpassed those from the other stained gels. This was surprising given the equivalent loading of proteins on all gels, and randomization prior to staining. The ability to clearly and efficiently excise spots of interest may have an effect in this regard. If a spot is harvested only partially, the amount of protein for analysis will predictably drop. At some point, the limit of detection of the MALDI method may be reached. Inteference with MS analysis due to the staining process is also still debatable. SYPRO Ruby stain has been reported to give MS results that can lack a significant amount of cysteine-containing peptides in the spectra (Lanne and Panfilov 2005). Results obtained from the analysis of BSA (SSP 2801) from SYPRO Ruby and Oriole stains samples were compared. When the spectra and peaklists from the two sample sets were evaluated it was found that eight of the ten peptides identified from the SYPRO Ruby stain gels, one of which contained a cysteine residue, were common with those from gels stained with Oriole stain. The other 11 peptides unique to the Oriole stain sample had 9 peptides that contained at least one cysteine residue. That suggests that Oriole stain does not cause peptide modification in the same manner reported for SYPRO Ruby stain (Lanne and Panfilov 2005). This increased sequence coverage allows for more confident protein identifications and gel mapping.

Conclusion

These results, and those from earlier studies (Berkelman and Walker 2009), show that Oriole stain contains all of the positive aspects of other fluorescent protein stains: sensitivity down to nanogram levels, linearity of response over a wide range, and compatibility with UV-based gel imaging systems and downstream MS analyses. The advantage of Oriole stain is realized in the actual processing of samples. Following SDS-PAGE, gels are placed directly into staining solution without the need for fixation. Staining is optimal in 90 min. Imaging of these gels may then take place directly without the need for extensive washing. In contrast, the other fluorescent gel stains examined in this study require a fixation step as part of their processing prior to staining, and rely on an overnight processing step for best results. These other fluorescent stains generally also require destaining and washing protocols prior to imaging. If CBB counterstaining is employed, this leads to the use of even more time and resources. Oriole stain provides multiple advantages in the 2-D gel MALDI proteomic workflow. In practical terms this means that while the gels stained with Oriole stain have been imaged and are ready for spot cutting, gels stained with other reagents need another day until they are at the same stage.

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