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RESEARCH ARTICLE

A comprehensive evaluation of imidazole-zinc reverse stain for current proteomic researches

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In this paper, we comprehensively evaluated the capability of imidazole-zinc reverse stain (ZN) in comparative proteomics. Three commonly used protein gel staining methods, including silver (SN), SYPRO Ruby (SR), and CB stain were investigated alongside for comparison purpose. A transparency scanning procedure, which may deliver more even and contrasting gel images, was found best for documenting ZN stained gels. Our results showed that ZN was more sensitive than SN, SR, and CB. It may reveal as few as 1.8 ng of proteins in a gel. Moreover, ZN was found to provide a linear dynamic range of staining for revealing proteins up to 140 ng, and show an insignificant staining preference. To analyze a ZN stained 2-D gel image that generally comprises an apparent but even background, the Melanie 4 software was found more suitable than others. Furthermore, ZN demonstrated an equivalent or better MS compatibility than the other three staining methods. Intense and comprehensive MS profiles were frequently observed for ZN stained gel spots. Approximate two-third of ZN stained gel spots were successfully identified for protein identities. Taken together, our results suggest that the prompt, cost effective and versatile ZN is well suited for current proteomic researches.

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1 Introduction

Proteomics is a fast growing subject that analyzes the expression of whole gene products within an organism. Several disciplines, including global proteomics, comparative proteomics, and functional proteomics, subdivide this new field into different aspects [1]. Among all disciplines, comparative proteomics captures most interest because it directly reveals the identities of differentially expressed proteins for each biological event. The identified protein targets may serve as biomarkers for clinical diagnosis, or landmarks for brand new biological pathways.

In current comparative proteomics, 2-DE is one of the most frequently employed techniques [2]. It separates thousands of proteins simultaneously according to their p*I* and

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Abbreviations: GP, glycogen phosphorylase; **LAC,** lactalbumin; **LSD,** least significant difference; **SN,** silver stain; **SR,** SYPRO Ruby; **ZN,** imidazole-zinc reverse stain

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molecular weight in one simple electrophoretic procedure. The differentially expressed protein spots can be excised from the gels and further analyzed by MS for annotating protein identities. The combination of 2-DE and MS has become the most utilized workflow in tasks of protein target discovery. To accomplish a 2-DE experiment of quality, one key issue is to develop the separation result by a sensitive protein gel staining method, which may reveal as many protein spots as possible on a 2-D gel.

To assess a protein gel staining method for comparative proteomic researches, several evaluation criteria should be considered. For example, such staining method should provide a sufficient sensitivity of staining for revealing minor proteins in gels. Moreover, it should also comprise a broad dynamic range for linearly staining proteins. Furthermore, a less significant staining preference to different protein species is preferable. On the other hand, such staining method should be very compatible to the downstream experiments, such as the protein annotation by MS. For a long time, protein gel staining methods, such as CBB stain (CB) [3–6] and silver stain (SN) [6–8], have been commonly used in proteomics. Recently, the-state-of-art fluorescent staining method, SYPRO Ruby (SR) stain [9–11], has been becoming the most utilized staining methods in comparative proteomics. In this study, it was found that imidazole-zinc reverse stain (ZN), another sensitive but less frequently used protein gel staining method, was remarkably ideal for proteomic researches as well.

ZN that utilizes zinc and imidazolate ions for protein visualization on poly-acrylamide gels was originally introduced in the 1990s [12]. This staining method is based on the selective precipitation of imidazolate-zinc complex in the gel except the zones [13, 14] where proteins or other macromolecules, such as DNA [15, 16] or lipopolysaccharides [17, 18] are present. It has been reported that ZN delivers an equivalent or better sensitivity of staining than some versions of SN, which have been considered one of the most sensitive protein gel staining methods [19, 20]. Notably, the most appealing feature of ZN is that it can be accomplished within only 10–15 min [20]. Thereafter, this prompt protein gel staining method should be a good alternative to other protein gel staining methods and increase the throughput of proteomic researches. Intriguingly, even with such advantages, limited numbers of the ZN stained 1- or 2-D gel images have ever been published, especially in the field of comparative proteomics. Presumably, it is not easy to obtain a ZN stained gel image with quality. For a long time, many laboratories have utilized cameras to photograph the ZN stained gels against a black or dark background. However, the corresponding gel images are not even and less contrasting, and thus less suitable for subsequent quantitative analysis (see Section 3.1).

To overcome this dilemma, we proposed to use the transparency scanning procedure for documenting the ZN stained gels. With the aid of the proposed setup, the visualization of the ZN stained gel images was significantly improved. The capabilities of ZN in comparative proteomics, including the staining effects, the compatibility to the software-based gel image analysis, and MS analysis, were then comprehensively evaluated and quantitatively compared with three commonly used protein gel staining methods, including SN, SR, and CB. Our results demonstrated that the prompt and cost effective ZN is well suited in many aspects for current proteomic researches.

2 Materials and methods

2.1 Gel electrophoresis

The 1-DE (15% SDS-PAGE) was performed based on Lameili's protocol [21] for separating a two-fold serially diluted LMW standard protein mixture (GE Healthcare, Piscataway, NJ) containing rabbit muscle glycogen phosphorylase (GP), BSA, chicken egg ovalbumin (OVA), bovine erythrocyte carbonic anhydrase (CA), soybean trypsin inhibitor (TI), and bovine milk lactalbumin (LAC) from 8 μg to 15.6 ng. The actual protein content of individual protein band in a 1-D gel was described in Supporting Information Table 1. The 2-DE was performed as described previously [22] with minor modification. Briefly, TCA precipitated proteins from human hepatocytoma cells (HepG2) were dissolved into standard 2-DE rehydration buffers containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, and 18 mM DTT. Protein samples (100 μm) were applied to IPG strip (18 cm, pH 3-10 L, GE Healthcare) using a rehydration loading method. After 12 h of rehydration on the IPGphor II system (GE Healthcare), IEF then was performed as follows: 500 V for 1 h, linear; 1000 V for 1 h, linear; linear ramping to 8000 V for 1 h, and finally 8000 V for 3.5 h. The second dimension was performed using 12.5% SDS-PAGE in the Daltsix electrophoresis system (GE Healthcare) at 40 mA constant current *per* gel after equilibration.

2.2 Protein gel staining methods

All 2-D gels were revealed by protein gel staining methods as described below. ZN was performed by using a commercial kit, VisPro 5 min protein stain kit (Visual Protein, Taipei, Taiwan), in a black staining box as following procedure. After electrophoresis, gels were washed briefly with distilled water. Sensitization solution was then added and incubated for 5 min. After briefly washed with distilled water, gels were developed with development solution. Within 20 s, the background of gels turned white while protein spots left opaque. This developing reaction was stopped by immersing the gel into large quantity of distilled water. Developed gels were stored in distilled water prior to image acquisition.

SN was performed by using a photochemical version of commercial SN kit, PlusOne™ Silver Staining Kit (GE Healthcare) according to manufacturer's instructions. The mass compatible, noncross-linker version of SN (MS-SN)

was performed as previously described [23]. SR was performed by using a commercial SR gel stain kit (Invitrogen, Grand Island, NY) according to manufacturer's instructions. CB was performed by using the colloidal CBB stain as previously described [4].

2.3 Gel image documentation and analysis

All developed gels except the SR stained gels were scanned at 200 dpi resolutions in TiFF format by using an optical flatbed scanner with transparency unit (Xlite, Avegene, Taipei, Taiwan). The ZN stained gel images were reversely documented for positive images. The SR stained gels were documented by using a xenon arc lamp-based CCD camera system (ProX-PRESS, Perkin Elmer, Waltham, MA, USA) with excitation/emission wavelength at 488 and 610 nm, respectively. For gel image analysis, 1-D gel images were analyzed by TotalLab 120 software (Nonlinear, Durham, NC, USA), and 2-D gel images were analyzed by TotalLab 100 software (colony function), Phoretix 2D Elite software (Nonlinear) and Melanie 4 software (Genebio, Geneva) with a low, medium and high spot detection stringency as described in Fig. 3.

2.4 MS sample preparation

Selected protein targets on 2-D gels were manually excised at approximately 1 mm in diameter. Spots excised from the SR, ZN, and CB stained gels were processed according to the standard MS sample preparation protocol [24]. Spots excised from the MS-SN stained gel were processed according to Gharahdaghi's protocol [25]. In-gel digestion of proteins was carried out using MS-grade Trypsin Gold (Promega, Madison, WI, USA) overnight at 37°C. Tryptic digests were extracted using 10 μL Milli-Q water initially, followed by two times extraction with a total of 20 μL 0.1% TFA. The combined extracts were dried in a vacuum concentrator at room temperature, and then dissolved in 1 μL of 5% ACN with 0.5% TFA. The prepared MS samples were mixed with equal volume of the CHCA matrix solution (10 mg/mL) before spotted on to the target plate of MALDI-TOF MS analyzer.

2.5 MALDI-TOF MS analysis and protein identification

The mass spectrometer utilized for protein analysis was a Bruker Biflex IV MADLI-TOF MS (Bruker Daltonics, Billerica, MA, USA). The accelerating voltage was 20 kV. For unbiased collection of MS signals, each spot was randomly ionized by laser with 25% of the maximal intensity for 40 trials, in which encompassed 100 laser shots. For PMF, all collected MS signals were integrated into a summary spectrum and processed by using FlexAnalysis and Biotools software (Brukers Daltonics). The processed data were analyzed by using the MASCOT searching engine (www.matrixscience.com). The search parameters were defined as follows: Database, NCBInr; Taxonomy, Homo sapiens; enzyme, trypsin; fixed modification, carbamidomethylation; peptide MS tolerance, 0.2 Da; and allowance of one missed cleavage.

2.6 Miscellaneous

All utilized electrophoretic reagents and standard protein mixture were purchased from GE Healthcare. Reagents used for cell culture and MS sample preparation experiments were purchased from Invitrogen and Sigma (St. Louis, MO, USA), respectively. Analysis of variance (ANOVA) and least significant difference (LSD) tests were performed by using the SAS software (Version 9.13, Cary, NC)

3 Results and discussion

3.1 Optimization of the image documentation procedure for the ZN stained gels

Although introduced for over fifteen years, the prompt and sensitive ZN has not been widely used in proteomic experiments yet. ZN delivers a negative gel image with transparent bands or spots and a white background. In general, researches may place a dark or black background beneath the ZN stained gel to facilitate the observation. Such method provides merely acceptable visualization of the ZN stained gels. Recently, we have developed a backlit light plate setup that significantly enhances the visualization of the ZN stained gels. Utilizing Snell's law, this backlit light plate setup allows researchers to see as few as 1 ng of proteins on a ZN stained gel [26]. However, documentation of the ZN stained gel images is still troublesome. The traditional method that photographs a ZN stained gel against a black or dark background generally delivers a gel image with poor contrast. In addition, there is sometimes a bright area seen in the photographed ZN stained gel image (Supporting Information Fig. 1), which might be due from the reflection of illumination light [10, 20, 27]. Such less contrasting and uneven ZN stained gel images seem not suitable for the quantitative analysis.

In this study, we proposed to use the optical flatbed gel scanner equipped with a transparency unit to document the ZN stained gels. This kind of scanner has a light source and a detector located at opposite sides of the scanned objects. Consequently, there is no interference of the reflection of illumination light. When performing the transparency scanning procedure with the image reverse mode turned on, the obtained ZN stained gel image was not only even but also a positive gel image (Fig. 1G). Such ZN stained gel image was much contrasting than the one documented by the traditional photography (data not shown) or by using the reflective scanning procedure (Fig. 1C). Coincidentally, more minor protein bands could also be detected on the CB stained gel image documented by using the transparency scanning procedure (Fig. 1H) than by using the reflective scanning procedure (Fig. 1D). Therefore, this transparency

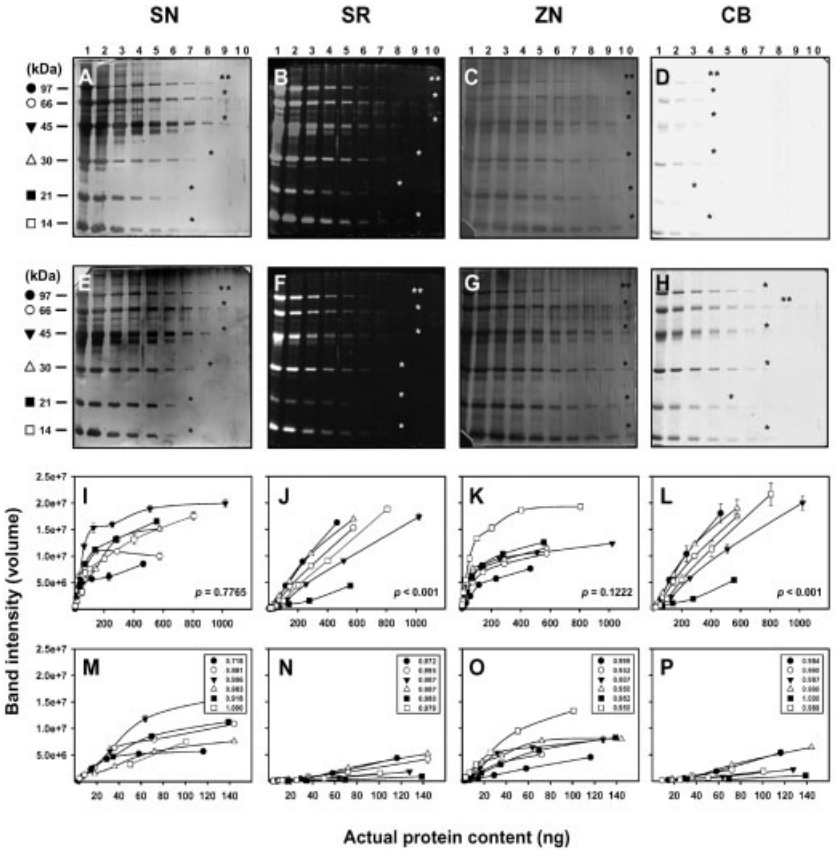

Figure 1. Staining effects on 1-D gels. The images of 1-D gels stained by (A) MS-SN (scanned by the transparency mode), (B) SR (10 s exposure), (C) ZN (scanned by the reflection mode), (D) CB (scanned by the reflection mode), (E) SN (scanned by the transparency mode), (F) SR (2 s exposure), (G) ZN (scanned by the transparency mode), and (H) CB (scanned by the transparency mode). Single and double asterisks indicate the position of least visible band of each standard protein and of all standard proteins on a 1-D gel image, respectively. M_r of each standard protein was labeled alongside the represented symbol. The gel images in Figs. 1E–H was analyzed by TotalLab 120 software for quantitating the image intensity of all protein bands. The band intensity (volume) was plotted against the corresponding protein content (ng) for six standard proteins from 0 to 2000 ng (I–L) or from 0 to 140 ng (M–P). In Figs. 1I–L, the heterogeneity of the first order coefficients of six protein curves (linear algorithm) was analyzed by ANOVA. The corresponding *p* values was shown in the lower right corners. In Figs. 1M–P, the linear coefficient determinants of six protein curves are shown in the upper right boxes.

scanning procedure should be well suited for documenting gels developed by most colorimetric protein gel staining methods. It was also observed that if employing the transparency scanning procedure to documented a ZN stained 2-D gel, more protein spots could be detected by 2-D gel image software, in comparison with using other image documentation procedures (data not shown).

3.2 Evaluation of the staining effect using 1-D gels

In order to evaluate the applicability of ZN in comparative proteomics, we initially compared the staining effect of ZN with three commonly used protein gel staining methods, SN, SR, and CB. SDS-PAGE 1-D gels (15%) that separated a serially diluted standard protein mixture from 8 μg to 15.6 ng were used as the evaluation materials. The SN, ZN, and CB stained gels were documented by using the above transparency scanning procedure. The SR stained gels were documented by using a laser gel image acquisition system. Employing a prolonged exposure procedure to document the SR stained gels was found to bring about the image saturation (Fig. 1B, 10 s exposure), which is not suitable for the quantitative analysis. For the utilized image acquisition system, a 2 s exposure procedure was found optimal to document the SR stained gel (Fig. 1F). When analyzing the four 1-D gel images (Figs. 1E–H) by using TotalLab 120 software, the image intensity of any detected protein band was found at least three-fold higher than the one of the background region.

3.2.1 Comparison of the sensitivity of staining

Our results demonstrated that ZN had a highest sensitivity of staining among the four staining methods. It allowed the detection of all kinds of standard proteins at highest dilution (Fig. 1G, lane 10). In terms of the LOD, the least visible protein bands in the SN, SR, ZN, and CB stained 1-D gel image contains 3.6 ng of GP, 3.6 ng of GP, 1.8 ng of GP, and 9 ng of BSA, respectively (indicated by double asterisks). On average, ZN was found much more sensitive than CB, and two- to four-fold more sensitive than SN and SR. The above result is inconsistent with some reports that SN and SR had an equivalent or much better sensitive of staining than ZN [2, 10, 27, 28]. Two possible reasons may explain the above discrepancy. Firstly, the utilized transparency scanning procedure improved the quality of the documented ZN stained gel images and hence increased the performance of ZN. Secondly, different versions of ZN kits or protocol that were utilized by other laboratories or us provided distinct staining effects. In brief, the sensitivity of staining was observed for the following order: ZN>SR>SN>CB. However, it has to be mentioned that many other versions of SN have been devel-

oped [29–31], and some of them might provide a better sensitivity of staining. The utilized mass compatible version of SN (MS-SN, Fig. 1A) delivered an inferior sensitivity of staining to ZN.

It was also observed that ZN was more capable of revealing low M_r protein bands than other high sensitivity staining methods. For example, standard proteins smaller than 30 kDa, such as CA, TI, and LAC, were more effectively revealed by ZN than by SN and SR. Possibly, the prompt staining procedure of ZN reduces the likelihood of diffusion of low M_r proteins from gels. In contrast, these proteins might be more easily eluted from gels during the lengthy staining procedures used in SN and SR. Thereafter, more protein molecules might be retained in the ZN stained gels than the SN or SR stained gels. If it were true, more intense MS profiles will be seen for the bands or spots excised from the ZN stained gels (See Section 3.4).

3.2.2 Comparison of the dynamic range of staining

Another evaluation criterion for a ideal protein gel staining method is the dynamic range of staining, which stands for the linear relationship between the image intensity and actual protein content of a revealed band or spot. In comparative proteomics, a protein gel staining method with a broader dynamic range of staining should be used because either major or minor protein signals in experimental gel pairs will be compared for the ratio of differential expression.

The dynamic range of staining of SN, SR, ZN, and CB were assessed by analyzing the 1-D images in Figs. 1E–H using TotalLab 120 software. For six tested standard proteins, the image intensity (volume) of each visible protein band was plotted against the corresponding protein content (ng). Six protein curves were obtained for a staining method. It was found that SR delivered an exceptional dynamic range of staining for revealing proteins from nanogram to microgram level (Figs. 1J and N), which is in agreement with the previous reports [10, 11, 32]. In contrast, SN showed a poorer dynamic range of staining (Fig. 1I), which was merely acceptable only when revealing proteins less than 40 ng (Fig. 1M). Alternatively, ZN delivered a better dynamic range of staining than SN but worse than SR (Fig. 1K). As determined by the linear coefficient of determination of individual protein curve, ZN showed an acceptable dynamic range of staining for revealing most tested proteins less than 140 ng (Fig. 1O). On the other hand, even CB was the least sensitive staining method tested; it showed a good dynamic range of staining from sub nanogram to microgram level (Figs. 1L and P). In brief, SR encompassed a best dynamic range of staining for revealing either high or low abundant proteins in gels. CB and ZN comprised an ideal dynamic range of staining for revealing high and low abundant proteins in gels, respectively. In contrast, SN had a linear dynamic range of staining only for revealing very low abundant proteins in gels.

3.2.3 Comparison of the staining preference

A good protein staining gel method should also have an insignificant staining preference of revealing specific protein species. However, it was found that all four kinds of staining methods might demonstrate a staining preference to specific standard proteins, as six protein curves in Figs. 1I–L do not overlap. Additionally, each kind of staining methods demonstrated a distinct staining preference. For examples, SN more effectively stained OVA (Fig. 1I, closed triangle) but less effectively stained GP (Fig. 1I, closed circle) than other proteins; ZN more effectively revealed LAC (Fig. 1K, opened square) than other five proteins; CB more effectively stained GP and CA (Fig. 1L, closed circle and opened triangle) but less effectively stained TI (Fig. 1L, closed square). Intriguingly, inconsistent with previous reports that SR had an insignificant staining preference [10, 11], an apparent staining preference was found for SR (Fig. 1J). It was found that SR and CB shared a very similar staining preference to six tested proteins. Presumably, the Ruthenium complex and the Coomassie dye involve a similar binding mechanism to proteins [32].

However, when using ANOVA to examine the heterogeneity of the first order coefficients of six protein curves under linear algorithm, an insignificant heterogeneity was found for ZN ($p = 0.1222$), while a significant heterogeneity of was observed for SR and CB ($p<0.001$). Surprisingly, although the six protein curves in Fig. 1I looked very discrepant, an insignificant heterogeneity was also observed for SN ($p = 0.7765$). Similar conclusion was made if utilizing the quadratic algorithm to examine the heterogeneity of the first and second order coefficients of six protein curves (data not shown). It indicates that ZN has a less evident staining preference, at least than SR and CB. Theoretically, the negative staining nature of ZN does not rely on the interaction between the dye molecule and protein and should not contribute a staining preference of revealing specific proteins.

3.3 Evaluation of the staining effects using 2-D gels

Since 2-D gels are the most utilized separation strategy in current proteomics, we further compared the staining effects for the four staining methods by using 2-D gels. A large format 2-D gel resolving 100 μg of human hepatocytoma cellular proteome was used for evaluation. As examining the 2-D gel images in Fig. 2A by the eye, a comparable number of protein spots can be seen on the SN-stained and ZN stained 2-D gel images; fewer protein spots are seen on the SR stained 2-D gel image while fewest protein spots are seen on the CB stained 2-D gel image.

3.3.1 Comparison of the image quality

As compared the enlarged image sections of the four 2-D gel images (image section S1 and S2 series in Figs. 2B and D, respectively), distinct staining effects were clearly observed

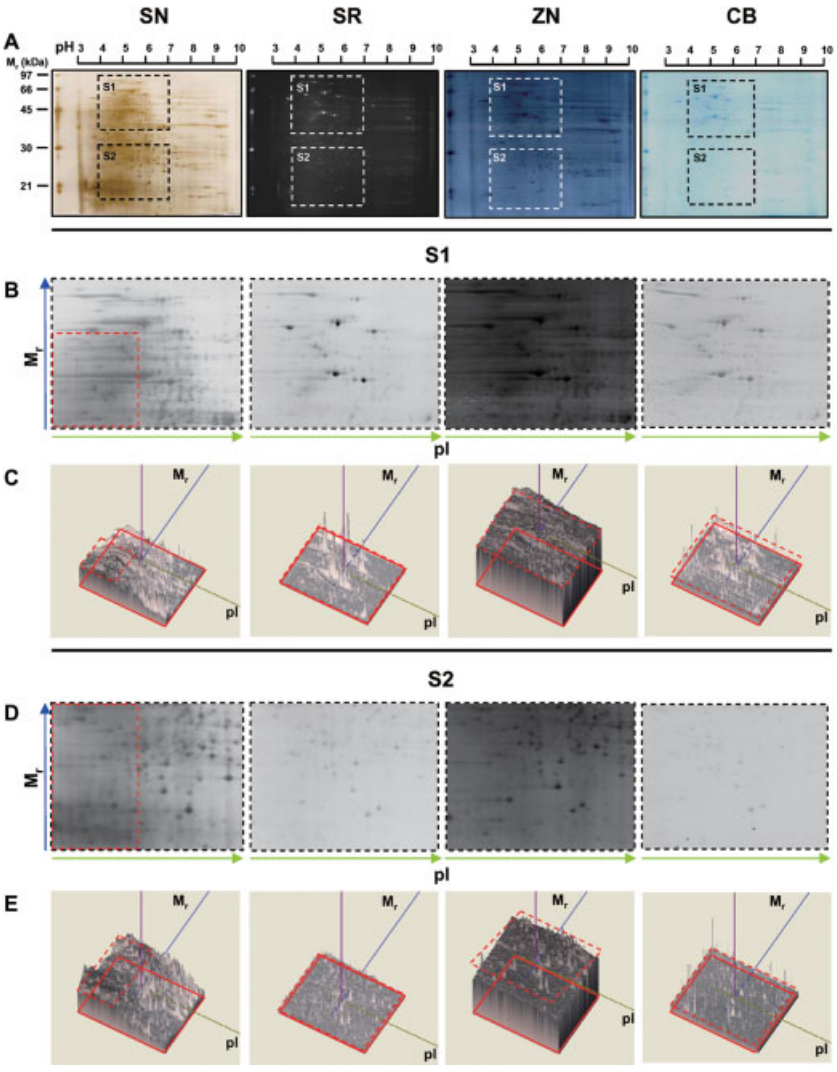

Figure 2. Staining effects on 2-D gels. (A) The documented 2-D gel images developed by the four staining methods. Two images sections (S1 and S2) on each 2-D gel image were selected for further analysis by Melanie 4 software. The four-fold enlarged grayscale images of S1 and S2 are shown in (B) and (D), and the corresponding 3-D histograms were shown in (C) and (E). Red-dashed squares indicate the high background area seen in a gel image or a 3-D image histogram. The X, Y, and Z axes used in the 3-D histogram represents the distribution of p*I* (green), M_r (blue), and image intensity (purple), respectively.

for the four staining methods, For instance, SR delivered a lowest background in a gel among the four staining methods. CB demonstrated a moderate background in a gel, if proper de-staining procedure had been performed. Alternatively, ZN introduced an overall high background in a gel while SN brought in an uneven background especially in the acidic area of a 2-D gel. We further utilized the 2-D gel image analysis software, Melanie, to investigate the above image sections. The mentioned background features were more clearly present by examining the 3-D histograms of gel images (Figs. 2C and E). In brief, the SR and CB stained gel images showed overall flattened 3-D histograms while the ZN stained gel images showed towering 3-D histograms. On the other hand, gibbous areas were observed in the 3-D histograms of the SN stained 2-D gel images. The above observations raise a question whether a 2-D gel image with a high or uneven background is compatible to the software-based gel image analysis in comparative proteomic researches.

3.3.2 Comparison of the compatibility to the software-based gel image analysis

In comparative proteomics, most documented 2-D gel images will be analyzed by 2-D gel image software for calculating the expressions, in terms of image intensity, of all revealed protein spots. Thereafter, a comprehensive and accurate detection of all revealed protein spots on a 2-D gel image is the most critical step when performing the software-based gel image analysis. To investigate the compatibility of the high background 2-D gel image to the software-based gel image analysis, we compared three packages of 2-D gel image software, including TotalLab 100 (colony function), Phoretix 2D Elite, and Melanie 4, for their performance of detecting protein spots on the S1 and S2 image sections for ZN. The 2-D gel images stained by SN, SR, and CB were evaluated in parallel. Three different detection stringencies were tested for each software package. Theoretically, more

spots could be detected by software under lower detection stringency conditions.

Under a low or medium detection stringency condition, TotalLab 100 and Phoretix 2D Elite were able to detect more spots from the SR or CB than the SN or ZN stained 2-D gel images (Figs. 3A and B, white and hatched bars). Possibly, both TotalLab 100 and Phoretix 2D Elite are developed by the same manufacturer, Nonlinear, and thus share a similar spot detection algorithm that preferably detects spots from the low background images. However, it has to be noted that the average intensity of detected spots were lower on the SR and CB stained 2-D gel images (Figs. 3D and E, white and hatched bars). It implies that many of the detected spots on the SR and CB stained 2-D gel images are false positive signals, which may be dye deposits or dirt on gels. Under a high detection stringency condition, similar numbers of spots can be detected from all four kinds of image sections for both S1 and S2 series (Figs. 3A and B, black bars). Nevertheless, it is found that the above two software packages always detect fewer spots from the ZN stained 2-D gel images. Presumably, TotalLab 100 and Phoretix 2D Elite are not suitable for analyzing the high background 2-D gel images.

In contrast, Melanie 4 seems more suitable for analyzing the ZN stained 2-D gel images. Under a medium or high detection stringency condition, comparable number spots can be detected by Melanie 4 from all four kinds of image sections for both S1 and S2 series (Fig. 3C, hatched and black bars). Notably, the average intensity of detected spots was similar on the SR, ZN, and CB stained gel images (Fig. 3F, hatched and black bars). Possibly, Melanie 4 comprises a spot detection algorithm that is less affected by the background in a 2-D gel image. Indeed, as examining the spot detection profiles, TotalLab 100 and Phoretix 2D Elite were much capable of identifying spots from gel images with a low (SR and CB) or moderate background (SN), but failed to locate some eye visible spots from gel image with a high background (ZN) (Figs. 4A and B). On the contrary, Melanie 4 delivered a similar spot detection profile for all four kinds of 2-D gel images, regardless the background interference (Fig. 4C).

3.3.3 Comparison of the visualization of 2-D gels by direct observation

Under the circumstance where interested protein spots need to be manually excised from a 2-D gel, direct observation of the 2-DE result would become an issue of importance. To achieve the above purpose, a compatible protein gel staining method should provide a comprehensive visualization of the stained 2-D gels that allows researchers to see as many protein spots as possible. To evaluate the visualization of the four 2-D gels in Fig. 2A by direct observation, ten volunteers who have performed at least twenty 2-DE experiments were enrolled for the following test. They were asked to observe and estimate the number of eye visible protein spots in each evaluated 2-D gel. Illumination apparatuses, such as the white light table for SN and CB, the backlit light plate was used for ZN [26], and the backlit blue light plate for SR (direct visualization of fluorescent signals in protein gels using a backlit blue light plate, Wu *et al.*, to be published in Proteomics), were utilized to facilitate the observation processes. For the number of eye visible protein spots on a 2-D gel, eight participants considered the order ZN>SR>SN>CB. The

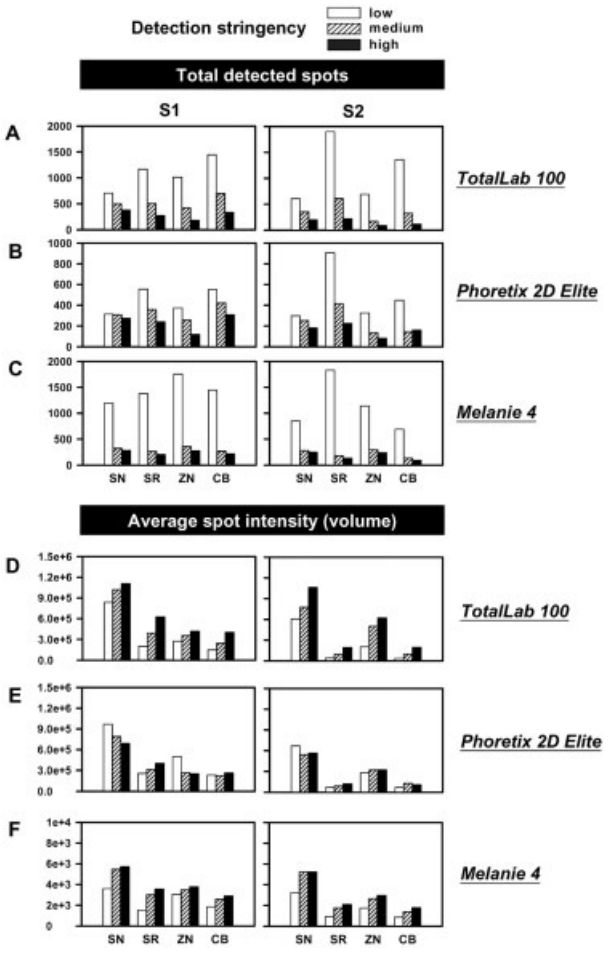

Figure 3. Results of the software-based 2-D gel image analysis. S1 and S2 image sections were analyzed by using TotalLab 100 (colony function), Phoretix 2D Elite, and Melanie 4 software. (A–C) The number of total detected spots and (D–F) the average spot intensity were in individual image section were assess under a low (white bars), medium (hatched bars), or high (black bars) detection stringency condition. Three applied detection stringencies of each software packages were as follows. TotalLab 100 (colony function, operator size 25): low stringency: sensitivity 9900, noise factor 3, background 1; medium stringency: sensitivity 9700, noise factor 5, background 2; high stringency: sensitivity 9500, noise factor 7, background 4. Phoretix 2D Elite (operator size 25): low stringency: sensitivity 9900, noise factor 1, background 1; medium stringency: sensitivity 9700, noise factor 3, background 2; high stringency: sensitivity 9500, noise factor 5, background 4. Melaine 4: low stringency: smooth 2, min area 5, saliency 10; medium stringency: smooth 5, min area 10, saliency 50; high stringency: medium stringency: smooth 5, min area 10, saliency 100.

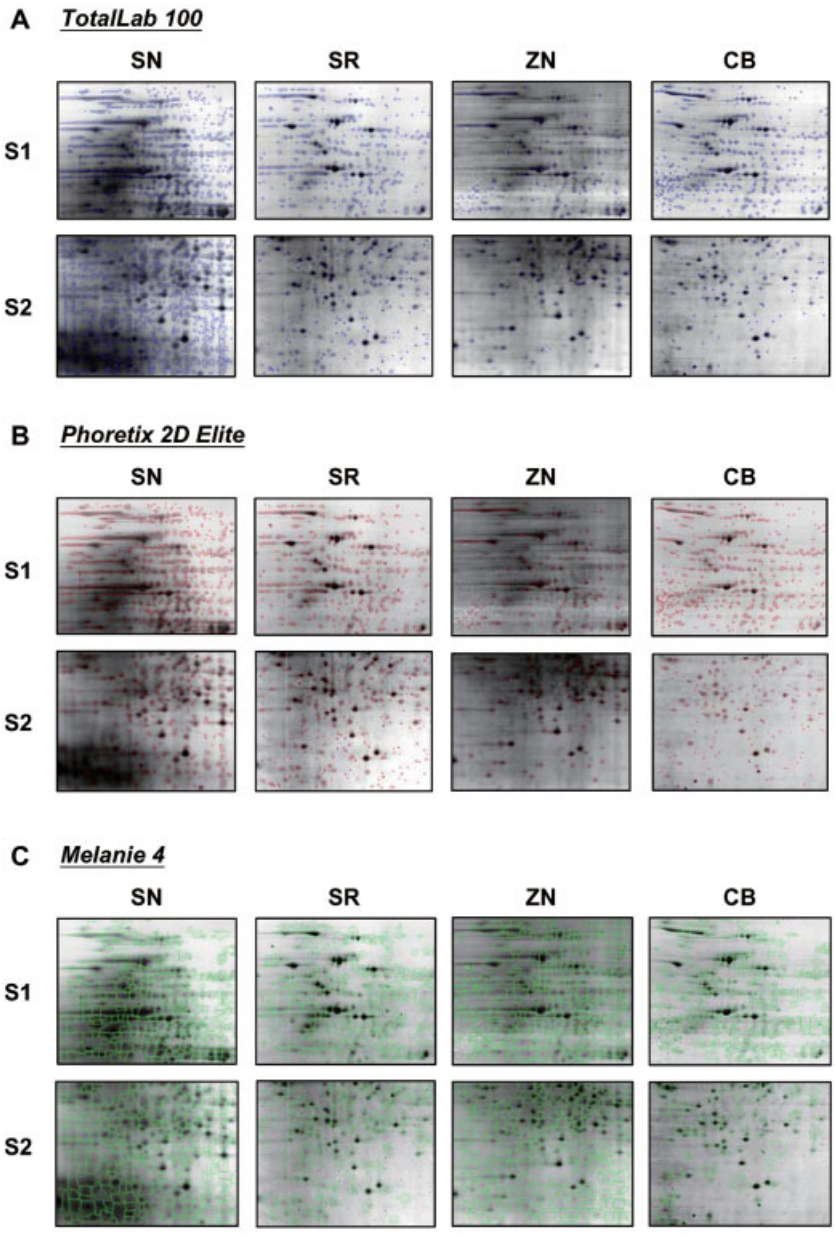

Figure 4. Spot detection profiles of the evaluated 2-D gel images. The outlines of spots detected on S1 and S2 image sections by using (A) TotalLab 100 (colony function), (B) Phoretix 2D Elite, and (C) Melanie 4 were shown in blue, red and green, respectively. The high detection stringency was applied for each package of software. The grayscale distribution of the input gel image sections have been automatically adjusted by all three packages of software. The shown output gel image sections provided a better visualization than the corresponding ones in Fig. 2A.

other two participants considered the order ZN>SN>SR>CB. Thereafter, if direct observation of the protein signals in 2-D gels is required, ZN may provide a better visualization of 2-D gels than the other three staining methods, especially with the aid of the backlit light plate setup.

3.4 Evaluation of the MS compatibility

Excising the interested protein spots from the stained 2-D gel and then annotating the protein identity by MS is probably the most utilized workflow in proteomics. Thereafter, an ideal protein gel staining method in proteomics should not only deliver a good staining effect but also be compatible with MS analysis. Recently, the MS compatibility of SR and other protein gel staining methods have been investigated [33–36]. However, although some laboratories have been identified proteins from the ZN stained gels [37–39], the MS compatibility issue of ZN has not been fully addressed yet. In this study, we evaluated the MS compatibility for ZN, and compared the results with MS-SN, SR, and CB. The MALDI-TOF MS, which generally has a higher requirement of the quality of the prepared samples, was used as the evaluation platform. Twenty-four identical protein spots that were all visible on the SR, ZN, CB, and the additionally prepared MS-SN stained 2-D gels were manually excised and processed for MS analysis. The locations of individual spots on the 2-D gel

are shown in Supporting Information Fig. 2. According to the apparent M_r, the twenty-four selected protein targets can be evenly sub-divided into three groups as follows, group 1: larger than 45 kDa; group 2: between 30 and 45 kDa; group 3: smaller than 30 kDa. By PMF, the protein identification results are summarized in Supporting Information Table 2. Out of twenty-four protein targets, only five of them were systematically identified in all four kinds of 2-D gels, including chaperonin (spot 1), prolyl 4-hydroxylase, β subunit precursor (spot 5), ER 60 protease (spot 6), ATP synthase H^+ transporter, β subunit precursor (spot 7), and ACTB protein (spot 9). Overall, the rate of identification was 50% for SR and MS-SN, 63% for ZN, and 71% for CB (Table 1). For MS-SN and CB, the rate of identification is similar to a recent report [35]. For SR, the rate of identification is lower than the previous observations [35, 36]. Nevertheless, considering the higher rate of identification the better MS compatibility of a protein gel staining method, the MS compatibility should follow the order: CB>ZN>MS-SN = SR. Therefore, in agreement with some previous opinions [2, 28], ZN should be well compatible to MS analysis.

Only five out of twenty-four protein targets (21%) were systematically identified from all four kinds of 2-D gels, suggesting that the populations of spots successfully amenable to MS analysis differed according to the nature of the protein gel staining methods. In a MS spectrum, both the intensity and comprehensiveness of MS signals may influence the result of subsequent protein annotations. To obtain a more comprehensive understanding of the MS compatibility in different staining methods, we then investigated the above two features of the MS signals for the four staining methods.

Table 1. Comparison of the number of input MS signals and the subsequent identification results for the four staining methods

	MS-SN	SR	ZN	CB
Group 1 (M_r>45 kDa)				
Input MS signals	30.1/25.3	40.3/15.2	56.6/19.7	48.8/11.2
Identified protein targets	**6**	**7**	**7**	**7**
Matched peptide	16.2/10.4	12.3/3.9	20.9/5.2	18.4/3.5
MOWSE score	161.1/97.7	117.6/34.5	203.9/48.4	165.6/34.4
Sequence coverage (%)	45.0/22.0	34.6/5.3	56.4/9.3	47.2/9.6
Group 2 (M_r 30–45 KDa)				
Input MS signals	24.5/27.1	27.9/13.6	39.3/31.3	49.1/26.1
Identified protein targets	**3**	**3**	**5**	**5**
Matched peptide	9.0/2.8	5.0/2.83	12.0/1.0	13.3/3.5
MOWSE score	105.5/48.8	71.5/10.61	128.7/43.1	119.0/56.6
Sequence coverage (%)	30.6/11.0	38.5/21.4	51.3/0.6	47.5/12.9
Group 3 (M_r<30 kDa)				
Input MS signals	13.6/13.3	15.1/15.0	27.5/20.8	33.9/21.6
Identified protein targets	**3**	**2**	**3**	**5**
Matched peptide	7.1/1.0	5.0/1.4	6.0/1.0	7.6/2.6
MOWSE score	98.3/42.2	68.5/6.4	80.0/4.2	91.0/26.9
Sequence coverage (%)	52.8/5.1	19.3/13.3	52.3/3.2	59.8/9.3
Overall summary				
Input MS signals	22.8/22.9	27.8/17.5	41.1/26.4	43.9/21.0
Rate of successful identification	***0.50***	***0.50***	***0.63***	***0.71***
Matched peptide	10.7/8.7	7.4/4.9	13.0/7.2	13.1/5.8
MOWSE score	121.7/79.7	85.9/36.1	137.5/65.2	125.2/48.7
Sequence coverage (%)	42.8/17.9	30.7/11.2	53.3/7.4	51.5/11.2

Twenty-four protein targets were grouped according to the apparent M_r as follows, group 1: larger than 45 kDa; group 2: between 30 and 45 kDa; group 3: smaller than 30 kDa. Eight protein targets were categorized into a group. After screening off the noise in raw spectra by the FlexAnalysis software, the number of input MS signals in a processed spectrum was statistically analyzed (mean/SD) for eight targets in a group or for all twenty-four protein targets (overall summary). After analyzing by PMF, the number of identified proteins (in bold), along with the number of matched peptides, MOWSE score and the sequence coverage of an identified protein were further analyzed. In the overall summary, the rate of identification (in bold and italic), instead of the number of identified proteins, was displayed.

3.4.1 Comparison of the intensity of MS signals

When using software to process a MS spectrum, only stronger MS signals will pass the screening threshold, S/N (ratio), and be used as input MS signals for subsequent protein annotations. Weaker MS signals will not pass the screening threshold and be eliminated as noises. Presumably, a more intense MS profile provides more input MS signals and brings about a better identification result. In the MS spectrum pairs of the five systematically identified proteins (Fig. 5), more intense MS profiles were seen for MS-SN, ZN, and CB. More input MS signals in a processed spectrum were seen for ZN (spots 1, 5, and 6) and CB (spots 7 and 9). Highest MOWSE scores were observed for MS-SN (spots 5 and 6) and ZN (spots 1, 7, and 9). For SR, the corresponding five spectra demonstrated relatively weak MS profiles and the identification results comprised lower MOWSE scores.

Similar phenomenon was observed when examining the identification results for the total twenty-four protein targets. For instance, more intense MS profiles were generally seen for CB and ZN (data not shown). Besides, the number of input MS signals in a processed spectrum follows the order, CB (43.9)>ZN (41.1)>SR (27.8)>SN (22.9). Furthermore, the above order is associated with the order for the rate of identification, and the number of matched peptides, MOWSE score and the sequence coverage of an identified protein (Table 1, summary). Taken together, the above results imply that by providing more intense MS profile and therefore more input MS signals in a spectrum; ZN should be well compatible to MS analysis. In contrast, SR seems less compatible to MS analysis than the other three staining methods.

Notably, for annotating low M_r proteins (Table 1, group 2 and 3), ZN and MS-SN demonstrated a lower rate of identification than CB. For ZN, it is possible that the extensive gel spot washing procedure in the employed standard MS sample preparation protocol eluted considerable amounts of the smaller proteins from the unfixed gel spots. Prior to washing the unfixed gel spots, performing a brief acidic gel fixation procedure might prevent the elution of proteins and thus increase MS compatibility for ZN. For MS-SN, it is likely that the potassium ferricyanide/sodium thiosulfate reaction and the additional washing procedure unavoidably elute some proteins as well as the bound silver ions from the gel spots.

3.4.2 Comparison of the comprehensiveness of MS signals

As examining the sequences of matched peptide for proteins systematically identified in all four kinds of 2-D gels (Supporting Information Table 3), distinct inclinations of MS peptide presentation were found for the four staining methods. In general, a more comprehensive match of peptides were seen for MS-SN, ZN, and CB, but not for SR. Considering the corresponding MOWSE scores, the comprehensiveness of MS signals in a spectrum definitely affects the protein identification result.

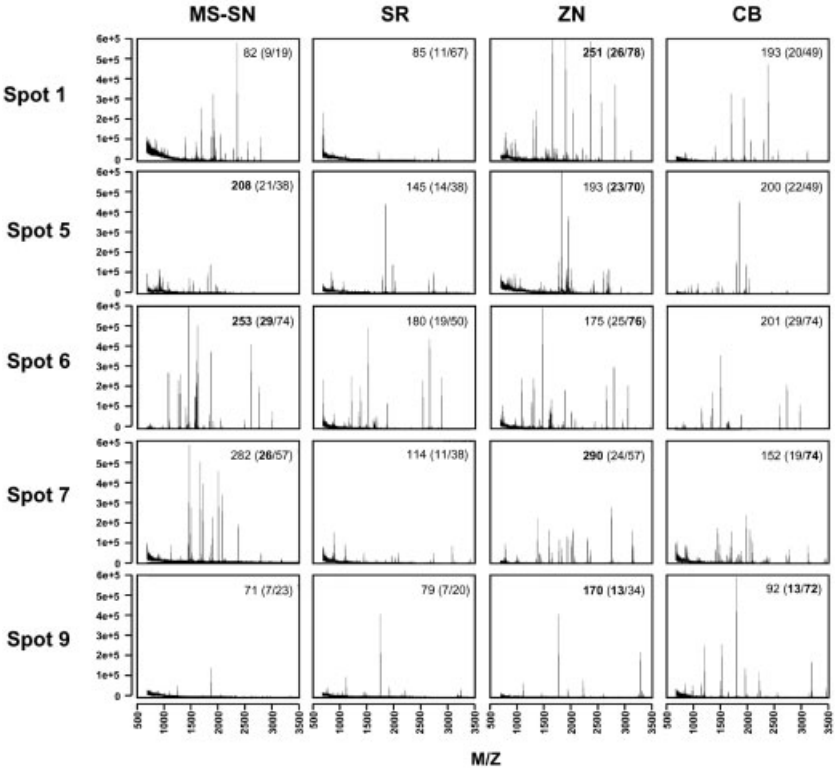

Figure 5. MS spectrum pairs of the five systematically identified protein targets. spot 1, chaperonin (gi|31542947); spot 5, prolyl 4-hydroxylase, beta subunit precursor (gi|20070125); spot 6, ER 60 protease (gi|1208427); spot 7, ATP synthase H^+ transporter, beta subunit precursor (gi|32189394); spot 9, ACTB protein (gi|15277503). For each spectrum, the absolute MS counts (Y axis) of m/z value (X axis) from 650 to 3,500 are shown. The corresponding MOWSE score, the number of matched peptides and input MS signals are shown in the upper right corners for each spectrum as following order: MOWSE score (matched peptide/input MS signals). For the above three aspects, the highest values among the four MS spectrum pairs are shown in bold.

To clearly discriminate the inclinations of MS peptide presentation for the four staining methods, the sequence features of the matched peptides in identified protein targets were investigated (Table 2). Statistical analysis (ANOVA) revealed that all four staining methods did not show significant difference on the MS presentation of basic residue-containing peptides (C-terminal K and C-terminal R), basic residue-rich peptides (missed cleavage = K + R/2K/2R), acidic residue-containing peptides (D and E), acidic residue-rich peptides (D + E/2D/2E), or aromatic residue-containing peptides (F, Y, and W). However, there was a significant discrepancy on the MS presentation of Cys- (C, $p = 0.0460$), and Met- (M, $p = 0.0355$) containing peptides, and a possible discrepancy on the MS presentation of His-containing peptide (H, $p = 0.0916$). Specifically, in comparison to the other three staining methods, SR seems to have an apparent declination of presenting Cys- (0.9 peptides/protein target) and Met- (1.1 peptides/protein target) containing peptide, while ZN had an inclination of presenting His-containing peptides (8.9 peptides/protein target). The above phenomenon can also be observed when examining the sequence of matched peptides for the five systematically identified proteins (Supporting Information Table 3).

LSD test was further used to categorize the inclinations of MS peptide presentation for the four staining methods. For basic residue-containing (C-terminal K and C-terminal R), basic residue-rich or aromatic residue-containing peptides, all four staining methods demonstrated a similar inclination of MS presentation. For His-containing, acidic residue-containing or acidic residue-rich peptides, the inclination of MS presentation follows the order, ZN $\geq$ CB = MS-SN>SR. For Cys-containing peptides, the inclination of MS presentation follows the order, ZN $\geq$ CB>MS-SN = SR. For Met-containing peptides, the inclination of MS presentation follows the order, ZN = CB $\geq$ MS-SN>SR. The above results imply that ZN may have a more comprehensive presentation of MS signals than the other three staining methods. On the other hand, consistent with previous reports [34–36], we observed that SR less effectively revealed the MS signals for the Cys- and/or Met-containing peptides in a protein target.

Table 2. Sequence features of the detected peptides in identified protein targets

Contain at least one	Matched peptides in an identified protein					Inclination of MS peptide presentation			
	Mean/standard error				ANOVA *p*	LSD			
	MS-SN	SR	ZN	CB		MS-SN	SR	ZN	CB
Basic amino acid									
C-terminal K	5.09/1.54	4.27/1.18	8.33/1.81	6.80/1.13	0.2172	A	A	A	A
C-terminal R	6.82/1.43	5.36/0.84	7.50/1.18	7.07/0.97	0.5911	A	A	A	A
Missed cleavage (K + R/2K/2R)	5.82/1.54	4.45/0.71	6.75/1.28	6.07/0.62	0.5127	A	A	A	A
H	3.64/1.05	2.45/0.63	5.50/8.68	4.33/0.71	**0.0916**	AB	B	A	AB
Acidic amino acid									
D	8.45/1.65	6.36/1.19	10.17/1.34	9.27/0.89	0.2046	AB	B	A	AB
E	8.82/2.21	6.55/1.28	12.50/1.80	10.13/1.32	0.1101	AB	B	A	AB
D + E/2D/2E	8.45/2.03	6.55/1.45	11.75/1.55	9.73/1.20	0.1420	AB	B	A	AB
Aromatic amino acid									
F	6.27/1.34	5.64/1.36	8.92/1.52	7.67/1.10	0.3316	A	A	A	A
W	1.36/0.52	0.45/0.25	1.42/0.51	1.53/0.46	0.3535	A	A	A	A
Y	5.55/1.56	4.27/1.14	7.50/1.30	6.87/1.02	0.2976	A	A	A	A
Sulfur containing amino acid									
C	1.15/0.29	1.00/0.42	2.25/0.41	1.87/0.32	**0.0460**	B	B	A	AB
M	3.18/1.30	0.73/0.33	4.58/1.05	3.27/0.63	**0.0355**	AB	B	A	A

For individual staining method, the number of matched peptides in an identified protein of being basic residue-containing peptides (C-terminal K, C-terminal R, and H), basic residue-rich peptides (missed cleavage = K + R/2K/2R), acidic residue-containing peptides (D or E), acidic residue-rich peptides (D + E/2D/2E), aromatic residue-containing peptides (F, Y, or W), and sulfur amino acid residue-containing peptides (C or M) was calculated (mean/standard error), and statistically analyzed for discrepancy of the inclination of MS peptide presentation by ANOVA. A possible and significant discrepancy was considered when $p<0.1$ (in bold) and $p<0.05$ (in bold underlined), respectively. For the four staining methods, the inclinations of a specific MS peptide presentation were further categorized by LSD. For instance, A is similar to AB but dissimilar to B.

4 Concluding remarks

Currently, 2-DE is still the most powerful and convenient technique for the simultaneous analysis of interested cellular proteomes. Due to the loading capacity limitation in an electrophoresis system, when researchers want to detect more minor protein targets in a 2-D gel, employing a protein gel staining method with a high sensitivity of staining becomes mandatory. Several high sensitive protein gel staining methods, such as the well documented SN or the-state-of-art fluorescent SR, have been thoroughly investigated for their usefulness and limitation in current proteomic researches [2, 3, 6–8]. However, ZN, the other high sensitive protein gel staining method early introduced in 1990s [12], has drawn limited attention. In this study, through a comprehensive evaluation in many aspects, this prompt, high sensitive and cost effective protein gel staining method should be very applicable in comparative proteomics.

The evaluation results are summarized in Table 3. Firstly, as reported elsewhere [20, 40], ZN may demonstrate an equal to or better sensitivity of staining than SR or some versions of SN. The utilized ZN kit can reveal electrophoretic bands or spots containing as few as 1.8 ng of protein. Secondly, subordinate to SR but superior to SN, ZN provides a linear dynamic range of staining for revealing proteins up to 140 ng. This dynamic range should be sufficient for accurately quantitating the expression of most revealed protein spots in a 2-D gel. Thirdly, ZN shows an insignificant staining preference of revealing specific protein species, and more effectively reveals smaller proteins than SN, SR, or CB. Fourthly, an apparent but even background is generally observed in a ZN stained gel image. To properly detect protein spots in a

Table 3. A summary of the capabilities and application features for the four staining methods

	SN[a)]	SR	ZN	CB
Staining effect				
LOD (ng)	~3.6	~3.6	~1.8	~9
Dynamic range (ng)	3.6–40	3.6–1000	1.8–140	9–1000
Staining preference[b)]	No	Yes	No	Yes
Quality of gel image				
Background	Medium	Very low	High	Low
Evenness	Uneven	Even	Even	Even
Compatibility to the software-based gel image analysis[c)]				
TotalLab 100 (colony function)	Good	Good	Poor	Good
Phoretix 2D Elite	Good	Good	Poor	Good
Melanie 4	Good	Good	Good	Good
Compatibility to MS				
Intensity of signals in a MS spectrum	Moderate ~ high	Moderate	High	High
Numbers of input MS signals in a processed spectrum	Moderate	Moderate	More	More
Inclination of MS peptide presentation	–	Fewer C, M[d)]	More H[e)]	–
Rate of identification (0%: 0, 100%: 4)	2	2	3	3
Other features				
Cost	Moderate	High	Low	Low
Duration of operation [f)]	~16 h	~18 h	15 min	~18 h
Direct observation apparatus	White light table	UV transilluminator Blue light trans-illuminator Backlit blue light plate	Backlit light plate	White light table

a) The MS compatible SN (MS-SN) is used for comparison of the compatibility to MS.
b) The staining preference was based on the result of ANOVA.
c) A poor compatibility to the analysis by 2-D image software is considered when significant amount of visible spots was not detected by software.
d) SR presents fewer Cys- or Met-containing peptides in a spectrum than the other three staining methods.
e) ZN presents more His-containing peptides in a spectrum than the other three staining methods.
f) The duration of operation calculated according to the protocols in individual staining kits. The thickness of gel was considered as 1.5 mm. For SN, an overnight acidic fixation procedure is considered. For SR and CB, an overnight staining procedure is considered.

ZN stained 2-D gel image, 2-D gel image software such as Melanie 4 is recommended. Fifthly, ZN may provide a comparable MS compatibility to other protein gel staining methods. An intense MS profile with comprehensive peptide signals, which may bring about a better protein identification result, is generally observed for a ZN stained gel spot.

Therefore, considering the above features along with the prompt and economic character, we suggest ZN a new allocation in current proteomics as follows. ZN can be used when an immediate examination of the 2-DE result is needed. For those unsatisfactory 2-DE results, the decision of reperforming another 2-DE experiment can be made within 10–15 min if ZN is utilized. For the quantitative analysis of a ZN stained 2-D gel image, the reverse stained gel is suggested to be documented by using the transparency scanning procedure, and analyzed by using certain 2-D gel image software like Melanie 4. To manually excise the interested protein spots from a ZN stained 2-D gel, the backlit light plate setup may be employed to facilitate the visualization of very minor protein signals. For MS analysis, a ZN stained gel spot can be processed by using the standard MS sample preparation protocol. A previously reported ZN specialized MS sample preparation protocol might provide better MS compatibility for ZN [40].

Alternatively, a ZN stained 2-D gel can be re-stained by other staining methods with a broader dynamic range of staining, such as SR, to obtain a gel image for subsequent quantitative analysis. On the other hand, for laboratories those do not equip with spot pickers but want to recover minor protein targets stained by the fluorescent dyes such as SR, Cy3 or Cy5, they may utilize ZN to restain those fluorescently stained 2-D gels and manually excise the interested protein targets on a backlit light plate.

In sum, this study demonstrates that ZN should be revitalized and recognized as another feasible protein gel staining method in proteomics.

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