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Quantitation in two-dimensional fluorescence difference gel electrophoresis: Effect of protein fixation

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Abstract

Analyzing a large number of unfixed gels in a 2-D fluorescence difference gel electrophoresis (2-DIGE) experiment presents a challenge of avoiding variable protein diffusion within and across the comparison groups. The characteristics of protein detection and quantitation in a 2-D differential in gel fluorescence experiment were compared for gels with and without protein fixation. The current study tests and concludes that when dealing with a large sample size with variable protein diffusion across the 2-D gel over a period of 2–4 days, it is a preferred choice to fix the gel without affecting the protein quantitation.

Keywords

Cocaine; Difference gel electrophoresis; Diffusion; Protein fixation

Since the development of 2-DE by O'Farrell, 2-D fluorescence difference gel electrophoresis (2-DIGE) has been a fundamental improvement in gel-based separation [1, 2]. The DIGE method is based upon covalently labeling the lysine residues of proteins with cyanine (Cy)-based fluorophores. A major advantage of 2-D DIGE over 2-DE is its capability to normalize the control and the experiment sample (Cy3 and Cy5 labels) to the combined/pool sample (internal standard) labeled with distinct dye (Cy2) run in the same polyacrylamide gel. The internal standard theoretically contains all the spots present across all the samples making the intergel spot matching and quantitation very simple. It is also made expedient as the spot maps are superimposed and compared unequivocally. This also provides the ability to measure protein spot in a biological sample to be measured as a ratio to its matching spot in the internal standard. This significantly augments the confidence with which a protein concentration variation can be quantified accurately, separating the inherent experimental variation in a 2-D gel from the biological deviation. This fluorescence-based technique also has a dynamic range of 10^4 in addition to having a good linearity and sensitivity making it possible to quantify the low-abundance proteins [3].

Fluorescence-labeled proteins in 2-D DIGE are scanned by Typhoon variable mode imager. As all the three images generated by the Cy 2, 3, and 5 dyes are to be optimized to a similar maximum pixel intensity it can take variable time from 1 to 3 h to scan one gel depending upon the sample variability. We have employed 2-D DIGE for protein expression profiling of postmortem human brain tissue from cocaine overdose (COD) patients as compared with control. For this we routinely use a sample size of 10–12 for the analysis which requires 2–4

days for scanning the gels. In 2-DE where the proteins are stained with traditional stains (Coomassie, Silver-nitrate, *etc.*), the proteins are fixed in the gel to prevent their diffusion from the gel matrix in subsequent steps of staining and imaging [4–10]. Even though a standard step in the staining protocol for all the traditional staining methods, protein fixation is not included in the contemporary protocol for DIGE. The protein diffusion in the gels scanned without protein fixation, especially the gels which are scanned later on days 2–4, is a concern. This raises the probability of variable protein diffusion and eventual inaccurate quantitation when the gels are scanned at differing time points. On the other hand, if the proteins are fixed in a 2-D gel in order to prevent their movement in the gel matrix and out of the gel they get precipitated from the soluble form. Once the proteins are precipitated, the attached fluorescent dye could have a different response to the laser than what it would have had on the soluble form of protein. This in turn may lead to the inaccurate quantitation. The current study was therefore designed to compare the effect of protein fixation on the quantitation of proteins in a DIGE experiment as compared to traditional nonfixing protocol.

The nucleus accumbens from five control and four COD human subjects was used for this study. The cytosolic protein fraction was obtained as described previously [11]. The cytosolic protein fraction was precipitated and purified using the 2-D Cleanup kit from GE Healthcare (Piscataway, NJ). The pellet from each sample was resuspended in 50 μ L of sample buffer (30 mM Tris Cl, 2 M thiourea, 7 M urea, and 4% CHAPS, pH 8.5). The protein concentration was determined at this stage by the 2D-Quant kit (GE Healthcare). Normalization (pooled sample) was prepared by combining 50 μ g from each sample. We have optimized the labeling of 50 μ g of protein sample (each sample) by labeling with 400 pmol of appropriate dye (suspended in >99.5% pure dimethylformamide) [12]. This allows us to attain optimum labeling of the comparatively less abundant protein spots while still maintaining the most abundant protein spots in the dynamic linear range for quantitative image analyses. The pooled sample was labeled with Cy2 while the COD/control samples were labeled with Cy5 and the labeling reaction stopped by 1 μ L of 10 mM lysine. For each gel the labeled pool sample and the labeled COD/control sample were mixed and an equal amount of rehydration buffer (2 M thiourea, 7 M urea, 2% dithiotrietol, 4% CHAPS, and 2% Pharmalyte (GE HealthCare)) was added and the total volume was brought up to 450 μ L by Destreak rehydration buffer (GE HealthCare) [13].

The IEF was done on Immobiline DryStrips (240 mm $\times$ 3 $\times$ 0.5 mm, linear 4–7 pH, GE Healthcare) using the GE Healthcare IPGphor apparatus [13, 14]. Sequential IEF was done by step and hold at 0 V for 10 h, 100 V for 100 V·h, 500 V for 500 V·h, 1000 V for 1000 h, 8000 V for 13 500 V·h (gradient), and 8000 V for 60 000 V·h. A maximum current of 50 μ A was applied *per* strip, maintaining the platform temperature at 20°C. After IEF, the IPG strips were equilibrated to reduce the disulfide bonds with gentle rocking for 10 min in 3 mL of equilibrating solution *per* strip (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% v/v glycerol, 2% w/v SDS, and 2% w/v DTT). To alkylate the SH groups of the proteins, the IPG strips were rocked for 10 min in 3 mL of solution *per* strip containing 6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% v/v glycerol, 2% w/v SDS, and 2.5% w/v iodoacetamide [15]. The proteins were further separated on the basis of their molecular weight on 12% SDS-PAGE (2400 mm $\times$ 2000 mm $\times$ 1 mm) at a constant 150 V for 360 min using the Ettan Dalt II System (GE Healthcare) [1].

All gels were scanned at 100 μ m resolution using the Typhoon 9400™ scanner (GE Healthcare). The blue laser (488 nm) for excitation and 520 band pass (BP) emission filter was used for scanning the gel image with Cy2 label and the red laser (633 nm) for excitation and 670 BP emission filter was used for scanning Cy5-labeled sample. The photomultiplier tube was set to ensure maximum pixel intensity of 80 000–90 000 for both the images in all the gels. The images of the scanned gels were cropped using the ImageQuant™ V5.2 to

remove extraneous areas to the gel image. After scanning all the gels, the gels were fixed for 2 h in 500 mL of fixation solution (30% v/v methanol, 7.5% v/v acetic acid in distilled water). This was followed by washing the gels twice with distilled water (500 mL) for 10 min *per* wash. The gels were scanned once again after the protein fixation. The image analysis was performed by DeCyder 5.01. The protein spot detection and the normalization of COD and control gel images with respect to the pooled sample gel image were carried out in the DIA mode of DeCyder. After spot detection, the abundance changes are represented by the normalized volume ratio, represented by ratio of COD or the control sample (Cy5) with respect to the pooled sample (Cy2). The DeCyder DIA normalization method assumes that most proteins are unchanged, having unit volume ratio. The spot filtering was done on all the gels using the following parameters; slope >1.0, area <350, peak height <350, and volume <100 000. The spots were authenticated manually for all the gels. Spot maps from all the gels were first matched by manual landmarks and then in automatic mode by DeCyder BVA. The protein spot matches were confirmed manually for all the gels [16].

A comparison of the spot detection and protein spot quantitation reproducibility of the protein fixation with respect to nonfixing protocol was conducted. The number of spots detected for the control ($n = 5$) and COD ($n = 4$) gels are tabulated in Table 1A. The number of spots detected irrespective of the protein fixation were comparable in both the sample groups with the difference in the CV (%) being 9.2% for the control group and 0.8% for the COD group. A *t*-test failed to show any statistically significant difference in the number of detected spots in both the study groups notwithstanding protein fixation. Overall the numbers of spots detected were comparable in all the gels.

A thorough comparison of the effect of protein fixation on the protein quantitation was done after the initial comparison of the spot detection. The study was designed to compare the protein quantitation within replicate gels in both the groups as well as across the two groups. The five gels loaded with control samples were run through the protein detection DeCyder DIA software before and after fixing the gels. All the gel images generated by the DIA were matched later in the DeCyder BVA software. Four hundred and eighty four spots which were present in at least three out of the five replicate gels, before and after gel fixation, comprising different molecular weights, pIs, low and high abundance were included in the comparison. The *t*-test was applied to check if there was any statistically significant difference in the protein quantitation after gel fixation as compared to the traditional nonfixing protocol for DIGE. Out of the 484 protein spots analyzed only two protein spots showed a statistically significant difference ($P < 0.05$) in protein quantitation. If there is no difference in the protein quantitation between the two protocols, the average ratio, (protein quantity before gel fixation)/(protein quantity after gel fixation), should be ideally equal to 1. The average ratio for the spots with statistically significant difference was 1.27 and -1.33, instead. A similar comparison was done on a set of four gels loaded with COD samples. Out of the 346 protein spots, which were present in at least three out of the four gels, analyzed only one protein spot showed a statistically significant difference ($P < 0.05$) in protein quantitation with an average ratio of 1.49 instead of 1. The remaining spots did not have a statistically significant difference between the protein quantitation. Thus, the protein quantitation remained unaltered for 99.6 and 99.7% of the gel spots after fixation of COD and control sample gels, respectively.

Finally the gels were subjected to map the average ratios between the COD and control samples. The replicate gels from COD samples were matched with the replicate gels of control samples. The average ratios of COD/control were calculated for 56 spots with distinct spot boundaries encompassing all the different molecular weights, pIs as well as low and high abundance (Figs. 2A–D). The *t*-test revealed that there was no significant statistical difference in the average ratios of the selected spots. The above analysis was performed

initially on the set of gel images without gel fixation (Figs. 2A and C) and later with gel images with fixed proteins (Figs. 2B and D). The comparison of the average ratios generated using the gel fixation and nonfixation protocol is summarized in Fig. 1 and Table 1B. Briefly, 63% of the spot average ratios from the protein fixed gels showed less than 10% deviation as compared with the average ratio of protein spots from gels which were not fixed. Also, approximately 83% protein spots deviated less than 25% in their original average ratio calculation. Spots representative of the above subset with their spot volume displays have been shown in Fig. 2; spots a, b, c, d, e, h, i, j, k, and l. The spot labels in Figs. 1, 2 correlate as: Spot 28 = a, 41 = b, 49 = c, 13 = d, 45 = e, 14 = f, 55 = g, 30 = h, 19 = i, 24 = j, 42 = k, and 22 = l. Of these the spots a, b, c, d, e, h, and i have volume ratio of 1, showing 100% correlation between the fixed and non-fixed proteins. On the other hand, the spots j and k showed a volume ratio of 1.01 and 1.02, respectively. Thus, the spots j and k also showed 1 and 2% statistically nonsignificant deviations from the original spot volume. Of the 17% protein spots which showed more than 25% deviation from the original average ratio (Fig. 2; spots f and g), 10% protein spots were deviated due to: (1) variable spot boundary due to the variable second-dimensional protein separation in the gel and (2) variable spot detection during image analysis (*e.g.*, Fig. 2; spot g). Thus, the average ratios of approximately 93% protein spots remained in comparable range irrespective of whether the proteins were fixed or not prior to scanning of the gels. Overall, the protein quantitation is similar for the gels with or without protein fixation in the gel.

Ideally, if the protein fixation does not affect the protein quantitation, there should be no change in the number of spots detected and the spot intensities. Since identical analyses were performed the differences observed are due to the variable at question or due to errors inherent to the analysis system. These differences are reflected as unmatched spots and spots with differential protein amounts without a significant statistical correlation. It appears from the present study that the attached fluorescent dye on the proteins precipitated after fixing the gel does not differ at all or differs by an insignificant manner in their response to the laser as compared to the attached fluorescent dye on the soluble form of protein. Both the scanning techniques show comparable quantitation and consistent reliability regardless of the molecular mass, *pI*, and low and high abundance of the proteins. The two techniques have equal sensitivity, ease of use, and cost, and both are highly recommended for quantitative analysis of 2-D gels. Most of the differential proteomic studies in recent times persevere to identify the differentially expressed proteins between the proteomes under consideration by various MS techniques. The variable diffusion of proteins caused by nonfixing technique of DIGE also opens up the possibility of decreasing the amount of protein available for downstream MS. In complex regions of the gels where there is abundance of protein spots packed together there is also a possibility of proteins diffusing into neighboring spots confounding the MS correlation with the protein spot quantitation. This would not be relevant to most of the studies which employ the concept of running a separate gel (pick gel) loaded with large amount of starting amount of cell lysate to increase the amount of protein available for MS and separating the quantitative gels from the MS gel. However, there are always situations where the available sample is limited, *e.g.*, samples from clinical studies employing cerebrospinal fluid and small but functionally important regions of brain such as pituitary, especially from small animals such as rats. In these studies where the low sample amount precludes the running of "pick gel," it becomes very crucial to prevent the loss of protein from the protein spot in the gel as occurs in the nonfixing protocol of DIGE. In conclusion, when dealing with a large sample size where there is variable protein diffusion across the 2-D gel over a period of 2–4 days due to the nonfixation of proteins in the gels, it will be a preferred choice to fix the gel without affecting the protein quantitation. This will prevent the protein quantitation as well as the generation of MS data from getting skewed.

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Abbreviations

COD	cocaine overdose
Cy	cyanine
2-DIGE	two-dimensional fluorescence difference gel electrophoresis

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Figure 1.

Comparison of average ratio of COD/control samples, before and after fixation of proteins in 2-D DIGE. Protein spot numbers are depicted on the *x*-axis, while the *y*-axis shows the comparative standardized log abundance (before and after gel fixation).

Figure 2.

2-DIGE of control (A and A') and COD (C and C') samples without gel fixation. Same gels control (B and B') and COD (D and D'), after protein fixation. Proteins labeled with Cy2 and Cy5 were resolved in 4–7 linear pH gradient (Immobi-line DryStrips; $240 \times 3 \times 0.5$) and 12% SDS-PAGE ($2400 \text{ mm} \times 2000 \text{ mm} \times 1 \text{ mm}$). A representative of protein spots with good (a, b, c, d, e, h, i, j, k, and l) and poor (f and g) correlations are shown on the gel images (A, B, C, and D) with their corresponding volumes in fixed and unfixed states.

Table 1

(A) Reproducibility of the total number of spots detected in control ($n = 5$) and COD ($n = 4$) in 2-D DIGE and (B) percentage deviation of the average ratios of COD/control samples after protein fixation (Fig. 1; comparing statistically nonsignificant average ratios)

(A)

Sample group	Before fix			After fix		
	Mean	SD	CV (%)	Mean	SD	CV (%)
Control	828	53	6.4	786	122	15.6
COD	778	35	4.5	737	27	3.7

(B)

Deviation from original average ratio (%)	Number of spots
<10%	35
10–25%	11
25–50%	3
50–100%	2
>100%	4