Snapshots for intra- and inter-ocular differences at retinal proteins levels

Research Article

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Abstract

We attempted to study the intra- and inter-animal variations of protein expressions in chick retina using two-dimensional electrophoresis (2-DE). The posterior retinas of 10 day-old chicks were harvested. The proteins were extracted and 2-DE protein profiles between two eyes of the same individual and among different animals were compared and analysed using Melanie gel analysis software. Computerised Melanie gel analysis showed a high similarity of 83.1 ± 2.1% (mean ± SEM, n=3) between the protein profiles of right and left retinas from the same animal. However, this percentage dropped to 64.1 ± 3.1% (Mean ± SEM, n=3) when comparing between different animal samples. We have shown that there were good similarities in intra-animal comparison but poor in inter-animal comparison. We have documented the caveat in comparing the protein profiles between different animals where apparent differentially display protein could actually be a false positive signal. The finding highlighted the potential usefulness of the proteomic approach in basic eye research where comparison between control and experimental tissues could be carried out in the same animal, rather than using different animals.

Keywords: Proteomics; 2De; Retina, Chicks.

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Introduction

Chick (Gallus gallus) is a popular non-mammalian vertebrate model which has been used for studying many biological systems. In myopia research, chick has become the most widely used model for studying the pathophysiology of myopia [1,2]. Different approaches have been applied to study experimental myopia. A number of growth-related candidate pathways have been suggested to play important roles in myopic growth. For example, proteoglycan [3], ZENK [4], basic fibroblast growth factor and transforming growth factor beta have been implicated in the myopia development [5]. Although a growing body of knowledge on the myopic development has been accumulated with time, the putative signalling pathway or mechanism involved in the myopic development is yet to be determined. Animal studies over the past 30 years have revealed an active emmetropization mechanism localized in ocular system without the control from higher centre [6,7]. Besides, eye elongation takes place where the corresponding part of the visual field was occluded [8]. Biochemical signals from retina to sclera are generally accepted as bidirectional regulations in controlling compensated eye growth. However, clinical and animal studies underscore that myopia is a very complex disease which is not likely to be explained by a single target approach. Proteins are the cellular building blocks and the key players for executing nearly all cellular functions. Understanding how proteins are regulated and modified in downstream myopic tissues is pivotal for developing targeted drug therapies.

Proteomics presents a fundamental paradigm shift in biological research in the post–genomic era. Proteomic approach can typically profile cellular proteins in a high-throughput manner as well as to investigate the temporal physiologic states of tissues by studying their differential protein expressions. It is particularly useful in comparing the protein expression profiles between normal and diseased tissues or drug effects on cellular tissues. Although the technique has been widely applied in many basic and clinical studies such as in cancer research [9,10], Alzheimer's disease study [11] and Severe Acute Respiratory Syndrome disease (SARS) study [12], traditional proteomics employing two-dimensional gel electrophoresis (2-DE) [13,14] provides molecular "snap-shots" in which thousands of cellular proteins can be profiled, catalogued and compared efficiently [15]. This approach has been applied recently to study several of ocular diseases such as in diabetic retinopathy [16,17], cataract [18] and retinal disorders [19,20]. In myopia research, biological signals involving in ocular growth in chicks, mouse and tilapia retina [21,22,23], scleral tissue in tree shrew sclera [24] were also attempted using 2-DE. Modern advances in proteomic methods have enhanced our knowledge in many diseases for both diagnostic and therapeutic purposes. By comparing changes between normal and diseased tissues, key biochemical pathways may be revealed. However, proteome expressions are known to be dynamic at timely manner. Also, a tissue specific protocol is important to profile the proteome of a cellular system in any proteomic research [25].

Although different proteomic platforms have been employed for the studying a number of ocular diseases and conditions, the proteomics application in eye research and particularly in myopia research is yet to be popularised. Our long term interest is to study protein signals by comparing animal tissues between the myopic and normal control eyes. It is thus important to look at the potential intra- and inter-animal protein profiles difference detectable by two-dimensional gel electrophoresis. This fundamental but critical issue is commonly overlooked in many proteomic eye studies. In the present study, we outlined a workable procedure and attempted to monitor the 2-DE retinal protein profiles between the eyes of the same animal and among different animals.

Materials and Methods

Newly hatched white leghorn chicks were kept in temperature-controlled brooders on a 12/12 hour light/dark cycle, and fed food and water ad libitum. Their normal growth was monitored by body weights. Ten day-old white leghorn chicks were randomly selected in the same batch and sacrificed by CO_2 overdose. The eyes were immediately enucleated. The extraocular tissues were removed and the eyes were hemisected at the equator. The retina was isolated from the posterior hemisphere, frozen in liquid nitrogen and stored at $-80^{\circ}C$ until further processing. Three pairs (6 eyes) of 10-day old normal chick retinas were harvested and their proteins were extracted by a lysis buffer containing 7 M urea, 2 M thiourea, 40 mM Tris, 0.2% (w/v) Biolyte, 1%(w/v) DTT, 2% (w/v) CHAPS, 1% (v/v) tetradecanoylamido-propyl-dimethylammonio-propane-sulfonate (ASB14) and 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich, USA). Each tissue was homogenized in a liquid nitrogen cooled Teflon freezer mill (Mikrodismembrator; Braun Biotech, Germany) at the highest speed (1600 rpm) with 100 µl of the lysis buffer for 4 mins. The dismembrator homogenized the tissues to powder form at a low temperature. Another 150 µl buffer was added to the tissue powder and the tissue was further homogenized for another minute. Finally, 50 µl buffer was added to help recovering all homogenate. Samples were collected, vortexed briefly and then centrifuged at 16.1 x 1000 G for 20 mins at 4°C. The supernatants were collected and the pellets were discarded. In order to perform reagent compatible protein quantification, the amount of soluble protein recovered was measured by a 2-D Quant Kit (GE Healthcare Life Science, US).

The soluble retinal proteins were separated by iso-electric focusing (IEF) in the first dimension of 2-DE. Ready Strip™ Immobilized pH gradient (IPG) strips (11 cm) of pH 5-8 were loaded with 300 µg of protein and focused for a total of 36,000Vh at constant temperature (20°C) under linear voltage ramp after an active IPG rehydration at 50 V in a PROTEAN IEF Cell (Bio-Rad, US). Following IEF, strips were incubated in an equilibration buffer (6M urea, 30% glycerol, 50 mM Tris, 2% SDS) containing 2% DTT for 15 mins. The strips were then incubated for an additional 15 mins in the equilibration buffer supplemented with 2.5% iodoacetamide. They were rinsed briefly with Milli-Q H_2O and sealed with 0.5% agarose. The strip(s) was placed on top of a SDS-PAGE gel (1 mm thick, 25 x 20 cm). Separation of proteins in the second dimension was achieved by using 12% continuous gels and running at 30 mA/gel overnight until completion. The two IPG strips for paired right and left eyes were run side-by-side on a single SDS-PAGE gel and all three gels were run in the Bio-Rad Dodeca tank system simultaneously to minimise any experimental variations in the running conditions. All gels after 2D-E were also stained together in the same container. Protein spots were visualised with standard Coomassie Brilliant Blue R250 after overnight gel fixation.

Stained gels were scanned as uncompressed "TIFF" images at 300 dpi resolution with a flatbed scanner "ScanMaker 4600" (Microtek, Taiwan). The images were exported to the Melanie 2D gel analysis software package (ver 4. Geneva Bioinformatics, Switzerland) for protein spots matching and quantification without prior manual editing. Twenty spots were randomly selected as references for gel alignment, automatic spot detection and spots matching.

Intra- and inter-animal protein profiles were matched and compared between the paired retinal samples. In terms of the quantitation of spots intensity, the program first calculated the optical density (OD) of the spot. It was based on the highest calibrated pixel intensities of the spot from which the background was subtracted. The background was defined as the minimum pixel value in the neighbourhood of the spot. The spot volume was calculated as the total pixel volume above the spot border which was virtually equivalent to the protein amount of the spot.

Results and Discussion

We investigated the variation of retinal protein profiles of the same and different animals. Figure 1a showed the protein profiles of three pairs of normal retinas. Using the computerised Melanie gel analysis software, there were 215 ± 7 (Mean ± SEM, n=6 gels) spots detected in each gel. The overall matching percentages of spots between the right and left retinae of the same animal ranged from 80 to 87%, averaged at 83.1 ± 2.1% (Mean ± SEM, n=3 pairs). Of these matched spots, the spot volume (represents the protein content per spot) correlated extremely well between the eyes of the same animal (correlation coefficient=0.98). Comparing the protein profiles between the eyes of different animals, lower percentages of spot matching were observed in all three comparisons: #1 vs #2, #1 vs #3 and #2 vs #3, and the values were 69.1%, 58.3% and 64.8% respectively, averaged at 64.1 ± 3.1% (Mean ± SEM). The difference in the overall matching percentages between the intra- and inter-animal comparisons (83.1% and 64.1% respectively) was statistically significant (P<0.05) by paired t-test. However, of those matched spots between different animals, the spot volume correlations were also good, with a typical value of 0.96. This was only slightly less than that in the same animal comparison (0.98).

It was also noted that in the automatic spot matching, the spot mismatch between the eyes from different animal (inter-animal) was more than that of the same animal (intra-animal). These mismatches were confirmed by a detailed spot-to-spot manual checking. Therefore, the reported successful matching rate in the inter-animal comparison was actually overestimated.

To further illustrate the differences in intra-animal and inter-animal comparison, we also studied the scattered correlation plots of the spot OD between the eyes of the same animal (RE and LE of chick #2) and also between animals (RE of chick #2 & #3) in Figure 1b as an example. Again, the number of proteins matched was higher in the same animal than between different animals (167 pairs vs.156 pairs). Most strikingly, the OD value, which relates to the protein amount, differed by 5.1 unit between different animals whereas a very slight difference of 0.49 unit was seen in the same animal (Figure 1b, the regression lines). Enlarged gel sections from both eyes of chick #2 and #3 were shown in figure 2 for further discussion.

Figure 1a. Protein profiles of three pairs of normal retinae separated on pH 5-8 IPG strips and 12% SDS-PAGE. The gels were stained with Coomassie blue R250. (IEF: isoelectric focusing; Da: Dalton; RE: right eye; LE: left eye.) (Colour hue enhanced for display).
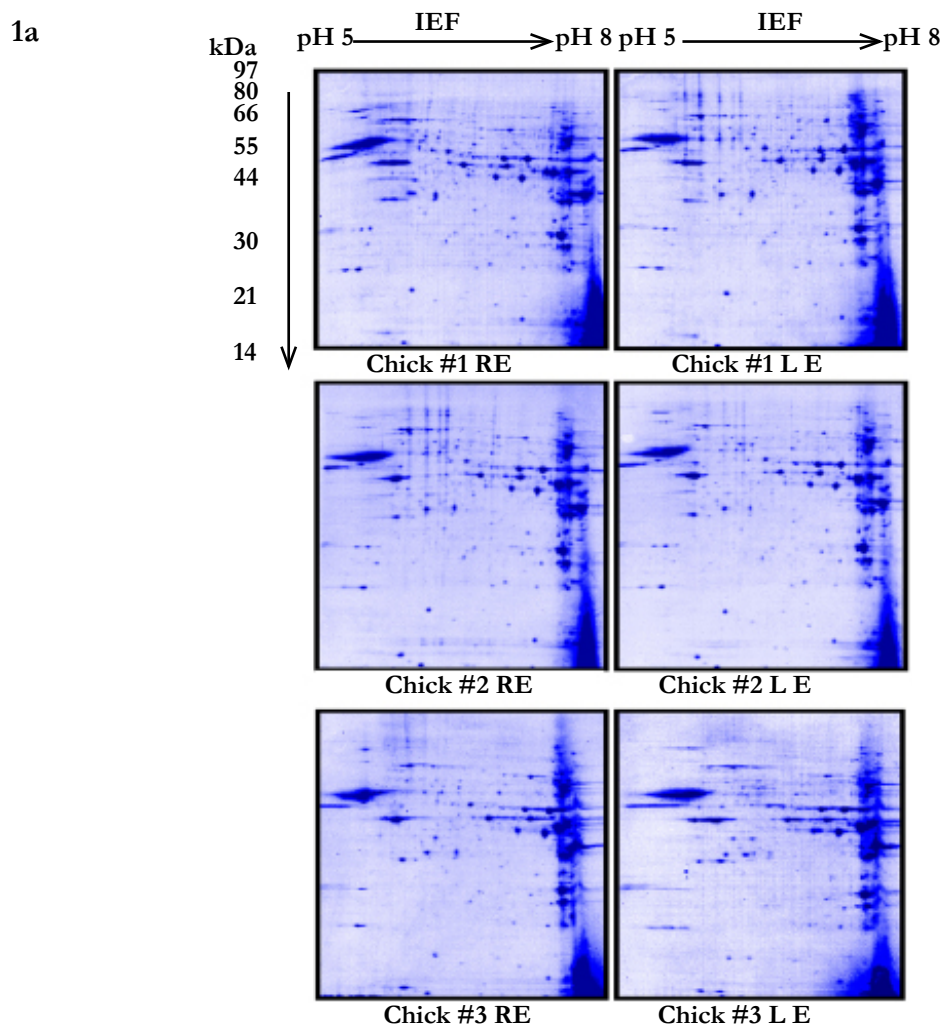
The proteomic approach aims at identifying the differential protein display by comparing the protein profiles between the control and experiment proteomes. In proteomic eye research, for example, the differences in protein expressions between the control and diseased eyes of animals are usually profiled for comparison. These differentially expressed protein changes could reflect underlying physiological changes and shed important light on the pathogenesis of various ocular diseases.

Different from many diseases in other parts of our body, one unique experimental consideration for eye research is that we have binocular system whereby the treatment and control conditions could be manipulated in the same body with identical genetic makeup. For a meaningful comparison in proteomic study, the protein profiles of the samples must be sufficiently repeatable and consistent. However, it is well known that protein expressions are very dynamic and they could vary rapidly in response to external stimuli. Typical proteomic studies used to compare proteomes between experimental/treated samples to control/untreated samples while different individuals with age-matched samples are employed. Despite rapid development in proteomic eye research in recent years, there is still lack of investigation of how dynamic displays of ocular proteomes are reflected in the same animal and among different age-matched animals. Hence, we tr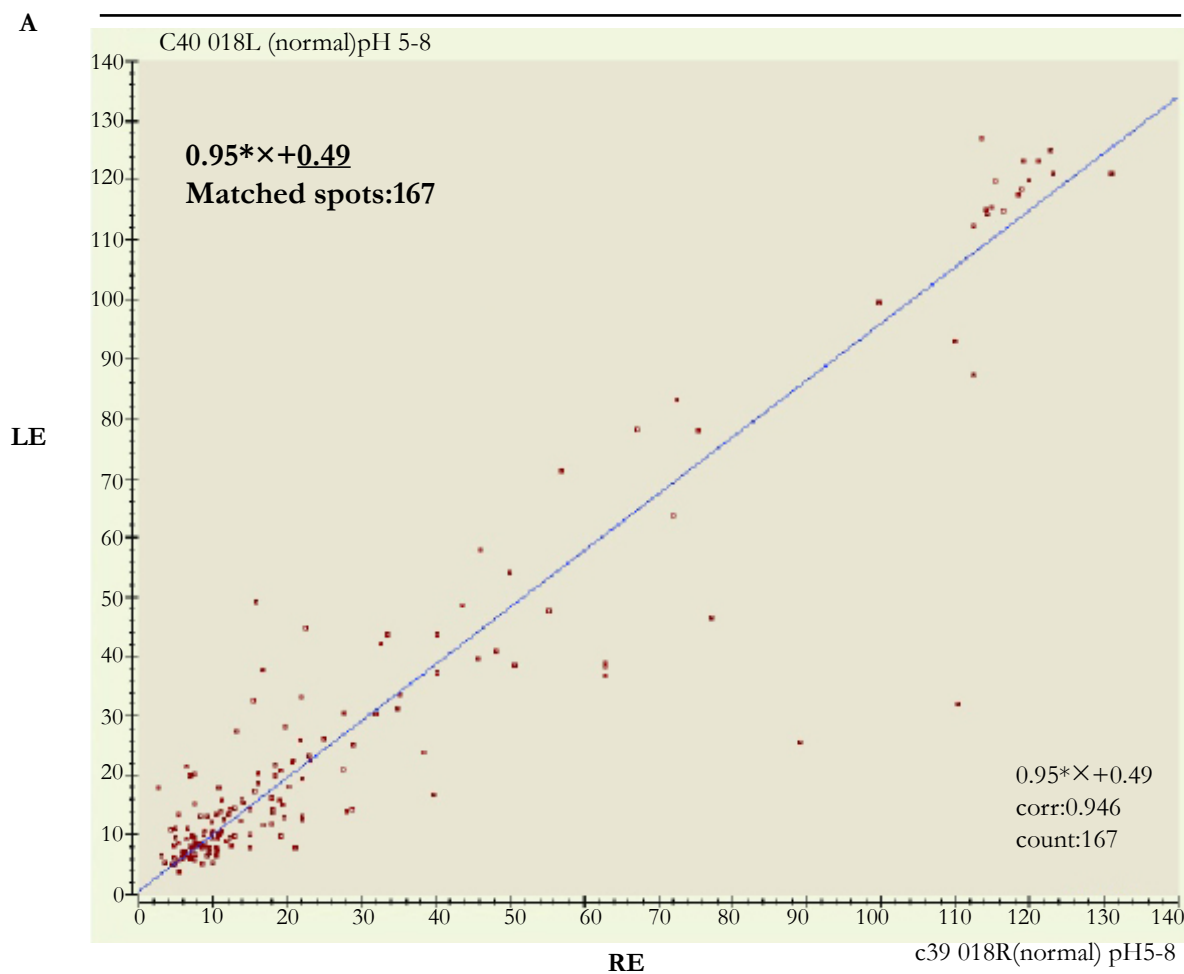ied to address this fundamental and yet important question by studying the protein 2-DE profiles between the right and left eyes of the same animal (intra-animal comparison) as well as the eyes between different animals of the same age (inter-animal comparison). Coomassie blue was used because it gives better linearity in quantifying the protein amount when compared to silver stain. Narrow IPG pH range (5-8) was used as most of the soluble retinal proteins were located within this range and a better spot resolution could be obtained on 11 cm IPG strips according to our pilot study.

Our results have shown clearly that under the same experimental conditions, the inter-animal variation was significantly ($p < 0.05$) higher than the intra-animal variation in terms of protein spots matching. On the other hand, based solely on the OD values (Figure 1b, the regression lines) generated by the gel matching software, in which the numeric abundance values are not altered by manual image enhancement for display, one might have concluded that there were protein changes between the apparently similar age-matched retinal samples. It highlighted the risk of interpreting changes in the protein displays solely by using samples from different animals. This phenomena may be minimised, but could not be eliminated even by increasing the sample numbers or using large pooled sample numbers once comparisons are made upon different animals.

Figure 1b. Scatter plots comparing the matched spots according to the OD (optical density). A.: A typical intra-animal protein profile comparison between the right and left retinas of chick #2.

1b   **#2 RE and LE (intra-animal match)**

A

C40 018L (normal)pH 5-8

LE

0.95*×+0.49
Matched spots:167

0.95*×+0.49
corr:0.946
count:167

RE   c39 018R(normal) pH5-8

One of the possible reasons for the big variation between animals is that since very young and fast growing chicks were used in the experiment, subtle differences in the development or growth rates between the chicks could produce very different protein 2-D maps. Different from genomic counterpart, protein expressions are known to be very 'dynamic' and responsive to external stimuli in relatively shorter time. Early studies have pointed out there is a poor correlation between mRNA levels and protein expressions [26,27,28]. Expressed retinal proteins could be different between individual animals due to external variations such as visual stimuli or / and different retinal maturation rate. This argument is consistent with the observation in the 2-D protein patterns from the right eye (RE) and left eye (LE) of two animals in Figure 2. Figure 2A showed the comparison of the spot OD at two different gel areas between chick #2 and #3 (inter-animal comparison). The spots being tested (t) seemed to be "up-regulated" in both eyes of chick #3 by about 28.9% (SD ± 4.0, n=2) in area 1 and 52.6% (SD ± 8.5, n=2) in area 2. However, it was obvious that the spots (t) between two eyes (RE vs. LE) of the same animal were similar (intra-animal comparison) in both cases. Therefore, a false "up-regulation" of protein (t) in the experimental eyes #3 might have

been concluded if we took either eye of another individual #2 as a control. Similarly in Figure 2B, a differential protein display between different animals might have been suggested, but those apparently different spots were equally present in both eyes of animal #3 (as indicated) while they were missing in animal #2 altogether.

These differences between animals were very repeatable in all the above cases and they cannot be attributed to staining variations among the different gels as all gels were processed together throughout the experiment. Most importantly, it was clearly shown that the intensity of reference spots (ref), which were near to the test spots (t), remained fairly unchanged in all cases as shown in Figure 2A. This indicated that the changes of intensity of the test spots being compared were not due to the overall gel staining variation as the intensity of the reference spots remained fairly consistent

Although not completely unexpected, it is important to note that even under the same experimental conditions and gel software analysis settings; the computerized protein expression data indi-

Figure 1b. Scatter plots comparing the matched spots according to the OD (optical density).B.: A typical inter-animal protein profile comparison between the right retinas of chick #2 and chick #3. (Higher false up-regulation as indicated).

1b

#2 and #3 (inter-animal match)
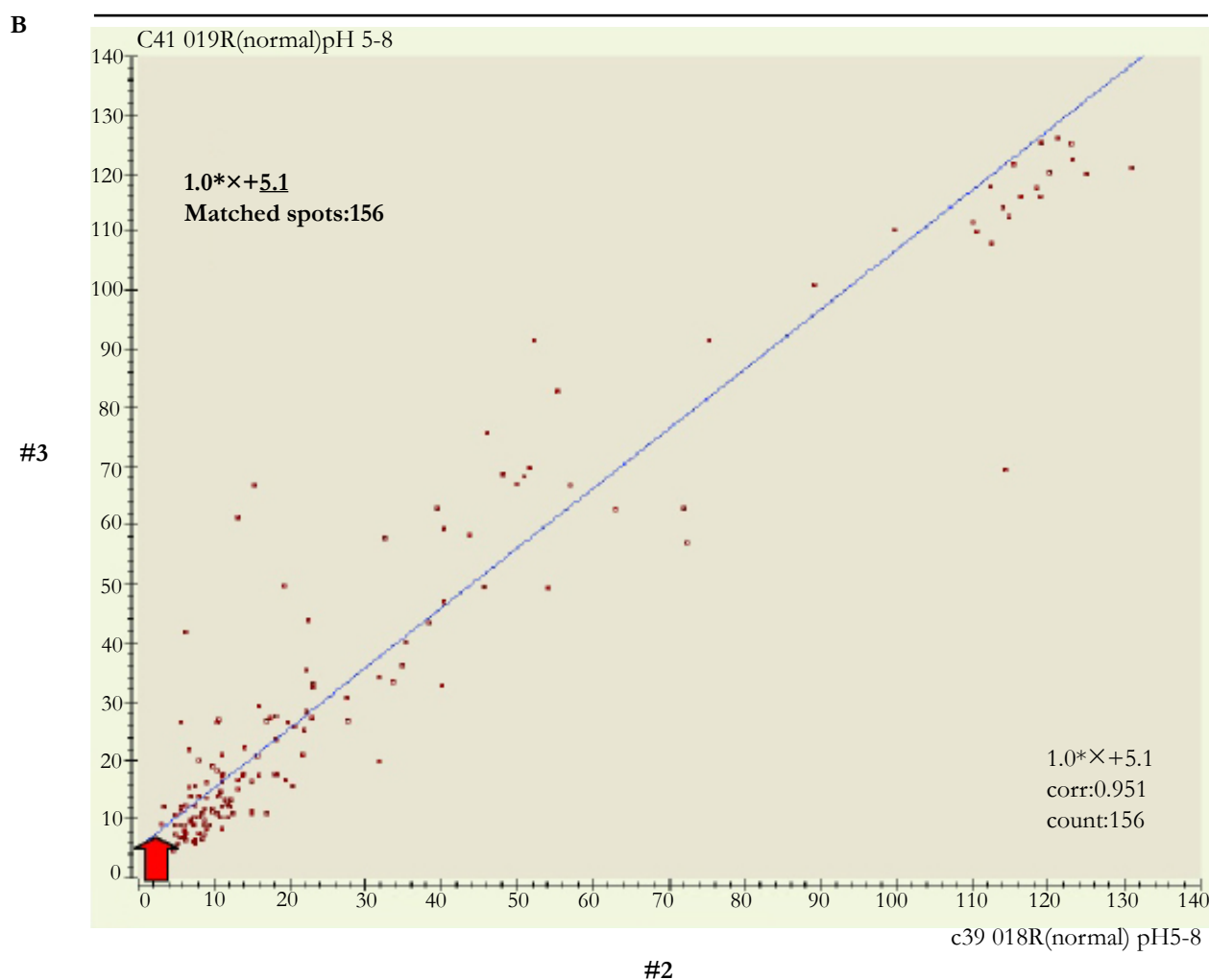
cated significant variations among different animals but it varied far less in the eyes from the same animal, at least in those higher abundant proteins detectable in our study. Our findings highlight the caveat in comparing the protein profiles of tissues from different animals. Most importantly, it accentuates the advantages of comparing the eyes of a single animal in experimentation. It is particularly applicable in the proteomic study where the eyes of the animal can be manipulated separately and compared, such as in the animal myopia research or other ocular diseases when one eye is being affected. Given that the background variation of protein within an animal is small, the subtle differential changes in proteins can be identified with more confidence. Although cell lines generally offer the possibility of repeating an experiment in order to confirm the findings and enhance their significance, this approach will be very difficult to apply to certain ocular problems such as in myopia which is accepted as a multifactorial disease contributed by both genetic factors and visual experience in higher order animals.

It is worth noted that non-gel based proteomic approach, or liquid chromatography–mass spectrometry is getting dominant in many large scale proteomic discovery studies because of its large throughput and better detection sensitivity. The present study highlighted the intrinsic problems when differential protein quantifications are being studied using two physically different samples, in regardless of whether gel-based or non-gel based proteomic platform is used. In fact, applying the traditional 2-DE gel based experiment with visualisation of the quantitative protein changes in the current study demonstrated the intra- and inter-animal variations which could not be shown in the short-gun gel-free approach.

We have demonstrated the variations in the protein samples within the same animal as well as between different animals in retinal tissues. We anticipate that similar findings could be observed in other parts of ocular tissues if samples from the same animal and different animals were compared. Although we should not completely ignore the fact that these variations could be the reflection of individual phenotypic plasticity, we aimed to point out the potential false positive result of 2-DE analysis using pool animal eyes from different individuals or comparing the individual eye of different animals as this improper way of proteomics comparison is not uncommonly seen nowadays. In other words, the contralat-

Figure 2. Enlarged panels showing intra- and inter- animal differences in the 2-D gels of chick #2 and #3. Gel spots were converted to greyscale and the spot boundaries were detected by Melanie 4 gel analysis software. A: The spot intensities (OD) were represented as numeric values according to the automatic gel analysis data. (t: test spot, ref: reference spot). B: Two-dimensional and corresponding three-dimensional spot patterns comparison for both eyes of chick #2 and #3.
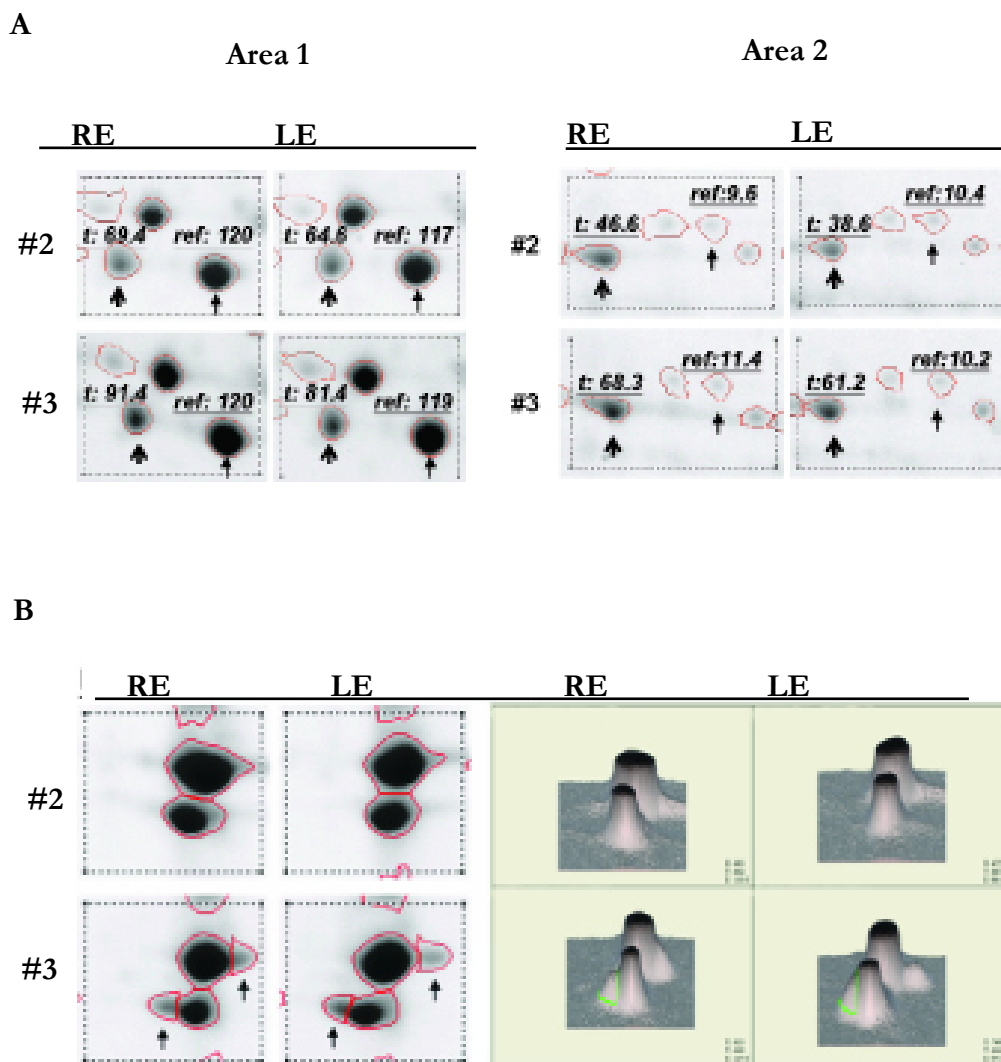
eral eye of the same animal could act as a much powerful control in the proteomics eye study. Considering the smaller intra-animal variation in the protein patterns between eyes, the proteomic analysis may reveal subtle changes in the protein expressions between the two eyes of the same animal. We believe that eye research can be greatly benefited from the proteomic technology since the differential protein expression from different experimental manipulations on the two eyes of the same animal, with the same genetic makeup, can be readily studied.

Acknowledgements

Conflicts of Interest

The authors have no conflicts of interest to be disclosed

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