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Research Article

COMPUTATIONAL METHOD FOR SEMI-QUANTITATIVE ANALYSIS OF IMMUNOBLOTS OF MODIFIED PROTEINS USING IMAGEJ

Aditya Arya[1], Anamika Gangwar[1], Narendra Kumar Sharma[2], Niroj Kumar Sethy[1] and Kalpana Bhargava[1]

[1]*Peptide and Proteomics Division, Defence Institute of Physiology and Allied Sciences, Defence Research and Development Organization, Lucknow Road, Timarpur, Delhi -110054, India.*
[2]*Division of Infectious Diseases, Hospital São Paulo, Escola Paulista de Medicina Universidade Federal de São Paulo- 04024-002, Brazil.*

Abstract: Oxidative stress is associated with the generation of reactive oxygen/nitrogen species (RNOS) which non-enzymatically modify active functional groups in proteins mostly turning them into protein carbonyls or nitrosyls. These changes render the protein molecules non-functional and drive them to degradation or formation of cross linking aggregates. Limited methods are available for detections of protein modifications. Enzyme linked immunoassays as quantitative method and Immunoblotting based qualitative methods are most common. Visual examination of Immunoblots containing a characteristic pattern is difficult for a fair comparison if the differences are trifling or the modifications are of varying degree across the complete range. This necessitates the use of image processing tools for a fair comparison. We, report here a computational approach using ImageJ, to process and obtain significant inter group comparison. Also, this method provides, software developers and programmers an opportunity to augment the gel processing tools with such plugins and features.

Keywords: Protein nitrosylation; Protein Carbonylation; Relative Quantification; ImageJ

Introduction

Reactive oxygen species include both radical and non-radical molecules, which rapidly react with various biomolecules in the living systems. Proteins, contain chemically reactive amino and carboxyl group in their side chains and terminals, which on reacting with the reactive oxygen species undergo oxidation and form protein carbonyls (Dalle-Donne *et al.*, 2003b; Curtis *et al.*, 2012a; Curtis *et al.*, 2012b). Reactive nitrogen species such as peroxynitrite and nitric oxide radicals also react irreversibly with cysteine and tyrosine amino acids to form nitrosylated derivatives in proteins (Gaston *et al.*, 2003; Schonhoff *et al.*, 2003). These modifications irreversibly alter the protein topology and therefore cause loss of their function. Degradation of these protein by 20s proteasome or accumulation of cross linking aggregates is implicated to several pathological conditions (Squier, 2001). Protein oxidation is directly involved in age-related disorders such as Alzheimer's diseases (Hensley *et al.*, 1995), Parkinson's disease (Alam *et al.*, 1997), Amyloid lateral sclerosis (ALS) (Beal *et al.*, 1997; Ferrante *et al.*, 1997) and Huntington's disease (Ross *et al.*, 2014).

Protein modifications, primarily carbonylation and nitrosylation are often analyzed quantitatively with the help of ELISA, however the qualitative techniques based on

Corresponding Author: **Kalpana Bhargava**
E-mail: kalpanab2006@gmail.com
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immunoblotting have also been used. These methods are of interest when we need to identify the specific protein with modification. Immunoblotting techniques for detection of protein carbonylation are based on derivitization of proteins with dinitrophenylhydrazine (DNPH) followed by western transfer of proteins and probing with anti-DNPH antibodies (Wehr and Levine, 2013). Similarly, for nitrosylation blots are probed with anti-nitrotyrosine antibodies and developed with luminescent substrate (Weber *et al.*, 2012). ELISA based methods are usually easy to perform and provide absolute quantification of proteins carbonyl or nitrosylation, yet a degree of false positive and also the complexity with biological samples render them less preferred methods over immunoblotting (Matthijssens *et al.*, 2007). Semiquantitative analysis of the immunoblots can provide a much stratified estimation of protein modifications on the basis of their molecular weight. Immunoblots of protein carbonylation and nitrosylation are usually examined visually to report the change; this limits the sensitivity of the assay and poses a difficulty when differences are minimal. At present no computational method is available for analysis of such immunoblots that is available in the public domain. Some tools available for similar analysis include Un-Scan-it from Silk Software and those from BioRad and GE Healthcare. However, the latter tools are proprietary and cost-intensive and more importantly, the algorithms are defined mainly for the analysis of routine gels with regularly spaced bands and do not provide users flexibility to analyze the irregularly spaced bands (as in the case of carbonylation and nitrosylation blots). We therefore, report here a statistically tested method for the semi quantitative analysis of Immunoblots of Nitrosylated or Carbonylated proteins using ImageJ, an image analysis software developed by National Institute of health (NIH, Maryland, USA). This method can also be extrapolated to phosphorylation, acetylation, sulphehydration and thionylation.

Materials and Methods

Materials

All materials except kits and those specified in the methods separately were purchased from Sigma Aldrich (USA), anti nitrotyrosine antibody (cat # sc-32757) was purchased from Santa Cruz, HRP conjugated anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz (USA). Oxyblot assay kit (cat # 7150) was obtained from Millipore (USA). H_2O_2 (cat #107298 was purchased from Merk, and NO donor sodium nitroprussite (cat #228710) was purchased from Sigma Aldrich (USA).

Protein Sample preparation

Blood was isolated from male sparague Dawley rats of 6 weeks (~200 g) in heparinized tubes and centrifuged at 3500 g for 15 minutes. Plasma was collected as supernatant and added with 2 µl protease inhibitor cocktail to prevent protein degradation and stored at -80°C for further use. Samples were then grouped into two groups one was labelled control (n=15) and second as oxidized or nitrosylated (n=15) The plasma was oxidized by incubating in 50 µM H_2O_2 for 30 minutes and another group was nitrosylated by incubating in 100 µM NO donor sodium nitroprussite for 30 minutes in non-reducing environments. All the animal care and experimental protocols were approved by the Institutional Ethical Committee of Defence Institute of Physiology and Allied Sciences.

Oxyblot Assay

Oxyblot protein oxidation assay was performed using Oxyblot™ protein oxidation detection kit (cat# 7150, Millipore, USA) as per the manufacturer's instructions. Briefly, proteins were quantified and aliquots with equal concentrations were prepared. 5 µl protein sample was added with 12% SDS upto a final concentration of 6%. Immediately, 10 µl dinitrophenylhydrazine was added to each sample and incubated at room temperature for 15 minutes. Samples were then added with 2 µl 5% Mercaptoethanol and finally reaction was stopped with neutralization solution. Samples were then, loaded on 10% Sodium dodesyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a nitrocellulose membrane. Membrane was blocked overnight in 5% skimmed milk prepared in Phosphate Buffer Saline with tween-20 (PBST).

Further, the membrane was incubated in anti DNPH antibody at 1:150 dilution on rotary platform for 2 hours, washed three times with PBST and then incubated in secondary antibody at 1:300 dilution for 1.5 hours. Finally, the blots were developed using chemiluminescent substrate (Sigma cat# CPS1300-1KT) and imprints were acquired on X-ray films. Films were then scanned using a high resolution scanner at 300 dpi resolution.

Protein carbonylation assay

Protein carbonylation assay was performed using crude protein extracts using the Protein carbonylation colorimetric assay kit (Cayman, cat# 10002050) as per the manufacturer's instruction. Briefly, Samples were quantified for protein content and equal volume of protein was dispensed and added with 800 µl DNPH, incubated at room temperature for one hour and then added with 1 ml 20% Trichloroacetic acid (TCA). Tubes were then centrifuged at 10,000 g for 10 minutes. Supernatant was discarded and pellet was resuspended in 10% TCA and incubated on ice for 10 minutes. Tubes were again centrifuged at 10,000 g for 10 minutes and pellet was washed with 1:1 ethanol: ethyl acetate mixture. Further, pellet was resuspended in 500 µl of grandly hydrochloride and centrifuged at 10,000 g for 10 minutes to remove left over debris. Finally 220 µl sample was dispensed in duplicate in 96 well ELISA plate and optical density was measured at 370 nm using spectrophotometer.

Nitrotyrosine immunoblot

Proteins samples were prepared by adding equal volumes of crude protein with 2X Laemmeli buffer and boiled for 15 minutes and immediately kept in ice to prevent renaturation. Proteins were then loaded and separated on 10% SDS-PAGE. Separated proteins were then, transferred to nitrocellulose membrane and blocked overnight with 5% skimmed milk prepared in PBST. Further, membrane was incubated in anti-nitrotyrosine antibody 1:500 dilution for 2 hours on rotary shaker, washed 3 times in PBST and again incubated in secondary antibody for 1.5 hours. Finally, membrane was developed using chemilumiscent substrate and finally imprints were acquired on X-ray films. Images were then scanned with a high resolution image scanner at 300 dpi resolution.

Protein nitrosylation assay

Protein nitrosylation in the sample was measured using OxiSelect™ Nitrotyrosine ELISA Kit (Cell Biolabs inc., USA, cat# STA 305) as per the manufacturer's instructions. Briefly, 50 µl nitrated BSA or samples were added to each well of 96 well plates and incubated with anti-nitrotyrosine antibody at 1: 300 dilution for 1 hour on orbital shaker. Wells were then washed three times with wash buffer and added with 100 µl secondary antibody and incubated at room temperature for 1 hour. Further, wells were washed three times and 100 µl substrate solution was added to each well. Finally after the colour development the reaction was stopped using the stopping solution and optical density was measured at 450 nm. Nitrosylation was calculated using standard curve and plotted as nM/mg of protein.

Image analysis procedure

Image J is an open source image analysis tool developed by NIH. We used this tool for analysis of immunoblots (Abramoff *et al.*, 2004; Schneider *et al.*, 2012; Rasband, 2014). High resolution image of scanned immunoblots, both from protein carbonylation and nitrosylation were imported in ImageJ and converted to 8-bit format using the type 8-bit function in image menu, and rotated 90 degrees anticlockwise in order to match the lane selection function of Image J. As the image J analysis in 8-bit measures the gray values therefore it was imperative to invert the image colours using the function in edit menu or using shortcut ctrl+shift+I. The individual lanes were selected as shown in the Figure 1 using the rectangular selection tool, the dimensions of the selection area were chosen in such a way that largest lane is selected completely. Finally the profiles of the plot were obtained from plot profile function under analyse menu. Curve and data points were then exported to GraphPad Prism™ v5.0 for further analysis. Mean pixel density and total area under the curve were measured and values were used for further analysis. The poorest quality of the image that can be analysed by this

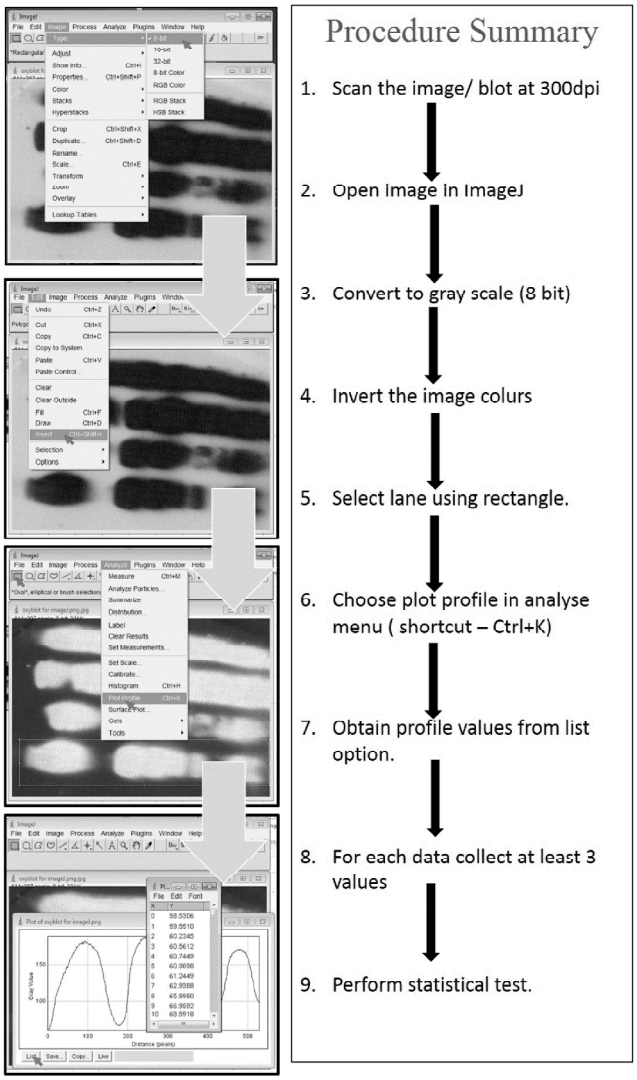

Figure 1: **Standard procedure developed for the image analysis and obtaining pixel values. A. screen shots and B. sequence of steps.**

method include an 8-bit image with minimum resolution of 120 dpi provided the images does not have background noise below a grey value of 80 dpi.

Statistical analysis

Image analysis was performed in triplicates with same image and student's t-test was performed to obtain the significance. Further the relationship between the image analysis and ELISA based methods was assessed using one way ANNOVA followed by Bonferoni's Post hoc analysis. A confidence level of 95% was considered significant.

Results

Analysis of protein oxidation

Protein oxidation by reactive oxygen species mainly leads to the formation of carbonyls. These carbonyls can be derviatised into hydrazones, a coloured product obtained after reaction of protein carbonyls with dinitriphenolhydrazine (DNPH). Two common methods are practiced, one ELISA based absolute estimation and second, immunoblotting based oxyblot method (Matthijssens *et al.*, 2007). In this study we first calculated the total protein carbonyls using ELISA method in order to correlate the results with computational image analysis. We obtained 1.67 nM, 1.04 nM, 2.08 nM, and 2.2 nM of protein carbonyls per mg of protein in four different samples (Figure 2A). Further, we separated the proteins on SDS-PAGE and developed an immunoblot as discussed in method section. Representative SDS-PAGE with same loading was stained with Coomasie stain for internal loading reference (Figure 2B). The image analysis was performed based on the mean intensity of the data and also the total number of pixels in curve area. Representative plot profiles were developed in GraphPad Prism and a statistical analysis was performed to check the normal distribution of the data. The mean intensity of the blot area was calculated in GraphPad Prism and plotted as a bar graph (Figure 2C). Further, in order to visualize the heterogeneity in the band thickness 3 dimetional plot profile was also plotted (Figure 2D). The 3D plots are to provide enhance visualization of density of immunoblots, which would include the differences in the density of specific band in terms of the grey values on the 8-bit scale of 0-255. These plots could also differentiate the bands appearing similar on visual examination.

Analysis of protein nitrosylation

Peroxynitrites produced from reaction of peroxides and nitric oxide are high reactive towards proteins especially the tyrosine residues. This deforms the structure and impacts the function of protein. Again as a measure of oxidative damage to protein we also measured protein nitrosylation in 4 different types of samples first by using ELISA based nitrosylation

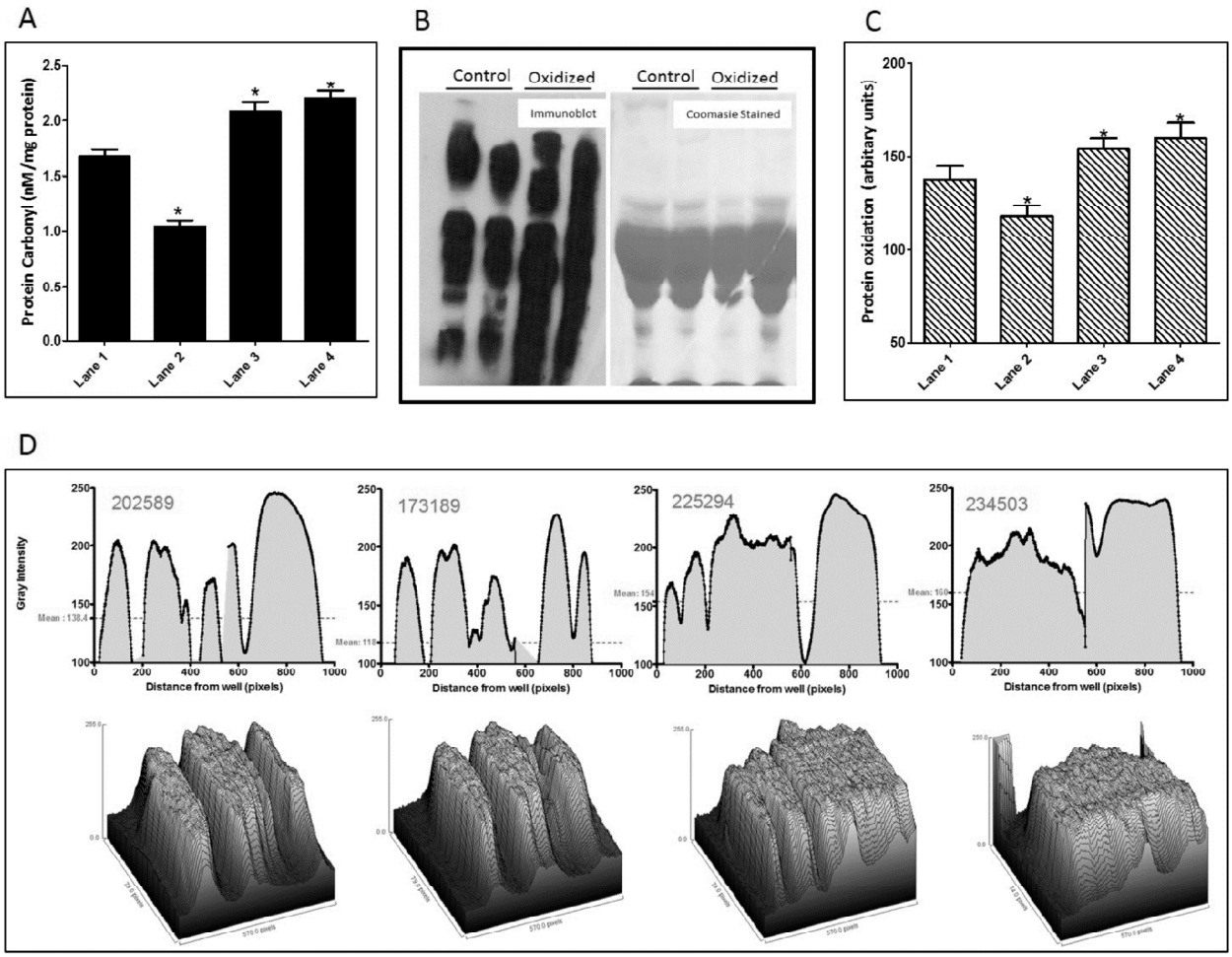

Figure 2: **Immunoblot image analysis for protein carbonylation in four different samples. A. Estimation of protein carbonyl content (oxidized protein) using ELISA based kit. B. Representative Immunoblots and corresponding coomasie stained gel. C. profile plot and 3D plots of each lane corresponding to immunoblot each graph contains mean intensity (dotted red line) and total number of pixels (Bold red, numeric value) computed using GraphPad Prism. (* p<0.01)**

quantification method and then by ImageJ based immunoblot image analysis. In ELISA we obtained 1.76 nM, 1.87 nM, 3.6 nM and 3.9 nM of nitrotsylated adduct per mg of protein in the sample (Figure 3A). Further, we performed an immunoblot using anti-nitrotyrosine antibody and analysed the blot image using ImageJ, representative gels with same loading were stained with coommasie blue (Figure 3B). The mean intensity of the blot area was calculated in graph pad prism and plotted as a bar graph (Figure 3C). Plot profile and 3 dimensional wireframe profiles were also developed using the pixel values obtained during the ImageJ analysis (Figure 3D).

Statistical correlation analysis

All the pixel values obtained from ImageJ analysis were reproduced using slight change in the selection area and statistical significance and correlation was measured using standard statistical tests. In order to establish a relationship between the results obtained from the ELISA based assays and image J based assays (both mean based and total area based) were compared and it was observed that the fold change in protein carbonylation and nitrosylation obtained by ELISA was very similar to fold change pattern obtained by ImageJ based analysis (Figure 4A, 4B). Finally we performed extensive correlation analysis of fold changes obtained by ELISA and image analysis using several sample images with different levels of carbonylation or nitrosylation. Pearson coefficient of 0.9821 and 0.9865 were achieved for the protein carbonylation and nitrosylation assays respectively (Figure 4C, 4D), showing a significant correlation. Detail of

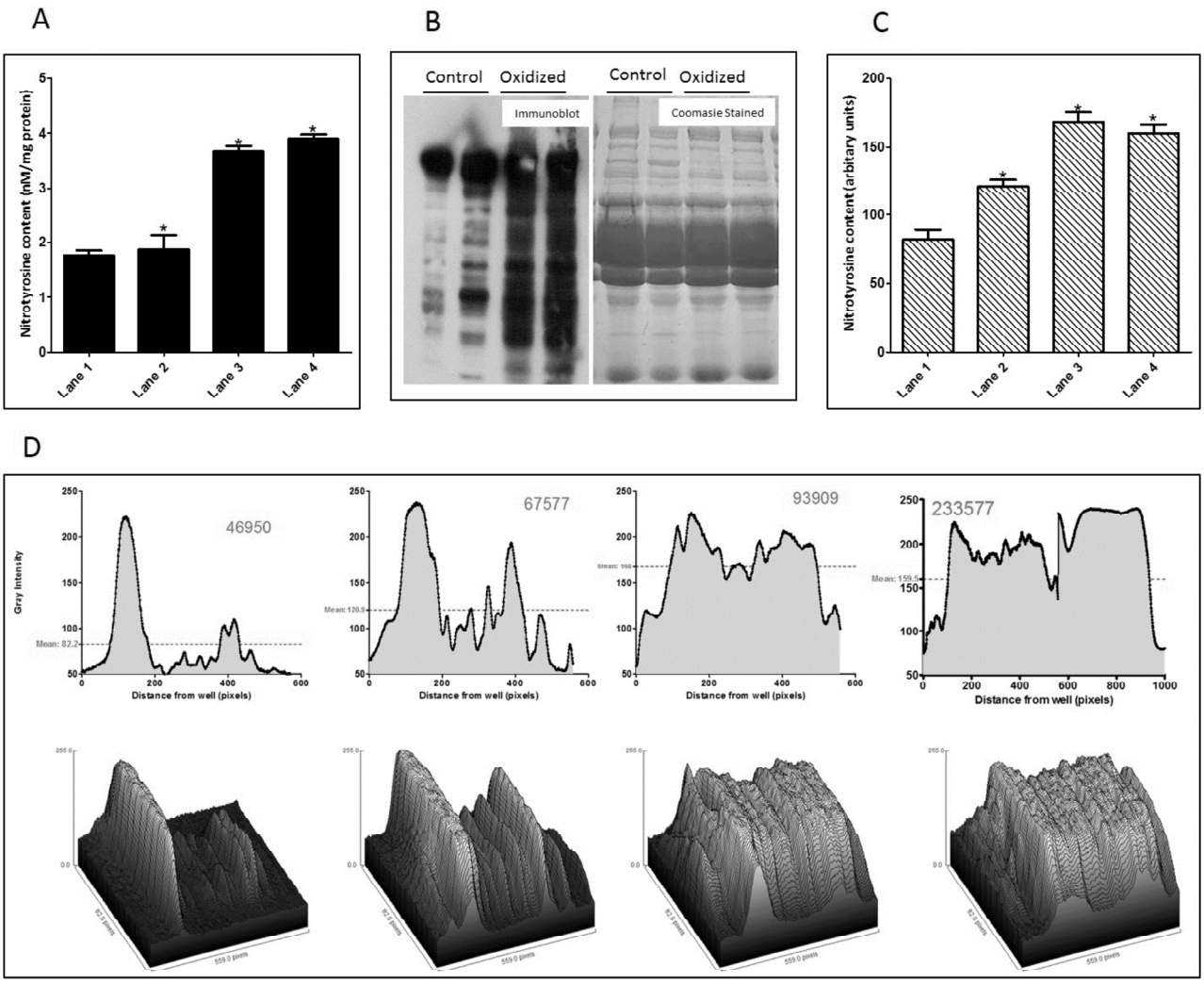

Figure 3: **Immunoblot image analysis for protein nitrosylation in four different samples. A. Estimation of protein nitrosylation (at tyrosine residues) using ELISA based kit. B. Representative Immunoblots and corresponding coomasie stained gel. C. profile plot and 3D plots of each lane corresponding to Immunoblots each graph contains mean intensity (dotted red line) and total number of pixels (Bold red, numeric value) computed using GraphPad Prism. (* p<0.01)**

correlation analysis performed using GraphPad Prism is shown in Table 1. However, with this method we were not able to perform absolute quantification in contrast to ELISA based assays.

Discussion

Protein modifications during adverse conditions especially due to impaired redox balance are the emerging domain in redox biology (Hensley *et al.*, 1995; Dalle-Donne *et al.*, 2003a; Dalle-Donne *et al.*, 2003b; Rabek *et al.*, 2003; Nystrom, 2005; Biswas *et al.*, 2006). Two commonly used techniques for the estimation of modified proteins are ELISA and Immunoblots. Both the techniques have their own set of pros and cons. ELISA on one hand is quantitative technique and could be used for the estimation of absolute amount of modified proteins in a given sample, on the other hand, immunoblots cannot estimate absolute quantity of modified protein. Despite of this advantage ELISA cannot predict the distribution of protein modifications over the span of varying protein size. Nevertheless, ELISA suffers from a technical drawback of false positives, which is seldom observed in Immunoblots; for this reason researchers looking for identification of modified proteins or understanding the pattern of protein modifications prefer to use Immunoblots.

Estimations of ELISA can be performed using spectrophotometry, but the analysis of Immunoblots is mainly dependent on visual observation. Conventional methods of

densitometry of gel do not work well here due to poor symmetry in the bands. This difficulty in assessing the closely related modifications necessitated the computational analysis of Immunoblots (Goto *et al.*, 1999). Although the ImageJ provides an inbuilt program for the analysis of gel by plotting the curves, this method was not suitable for the analysis of the carbonylation or nitrosylation blots due to irregular plots and inability to quantitate each data point. In the present study, we report and effective method to perform semiquantitative analysis of the blot and reach the similar conclusions as obtained by ELISA based methods. This method was routinely used and continually tested at the author's laboratory for its robustness and significance. This method however be challenged if the image quality is poor and blots have been scanned with poor background lighting. During the robustness testing (routine use by various labs) showed that background grey pixels drastically affect the measurement efficiency and results may be erroneous. So one should be careful about the use of this method if image quality is compromised.

The inability to determine the absolute quantification still remains a major disadvantage and for the same we will need to depend on the ELISA based techniques. Still, in most of the experimental designs, where absolute quantification is not the prime concern, this method is much more effective than visual examination of the blots. Rigorous parametric and non-parametric statistic evaluation of the method demonstrate the significance of the inter group comparisons attained by the analysis.

It is evident that the method outlined here is not for absolute quantification but then none of the immunoblotting techniques are either. The primary objective of the study was to emphasize that the relative quantification easily achievable with this technique is enough to understand the changes occurring in protein modifications as one would like to know this information in comparison to suitable controls which may also have some level of protein modification that might be clinically insignificant. Further, we are proposing here a new application of the ImageJ tool, which is an open source and thus easily

Table 1
Linear regression analysis for fold change using ImageJ based method and ELSIA based method.

Best-fit values for Protein Carbonylation	
Slope	0.5674 ± 0.02425
Y-intercept when X=0.0	-0.05560 ± 0.05429
X-intercept when Y=0.0	0.09799
1/slope	1.762
95% Confidence Intervals	
Slope	0.5134 to 0.6215
Y-intercept when X=0.0	-0.1766 to 0.06535
X-intercept when Y=0.0	-0.1253 to 0.2887
Goodness of Fit	
r^2	0.9821
Sy.x	0.1028
Is slope significantly non-zero?	
F	547.6
DFn, DFd	1.000, 10.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	12
Maximum number of Y replicates	1
Total number of values	12
Number of missing values	0
Best-fit values for Protein Nitrosylation	
Slope	0.9331 ± 0.03147
Y-intercept when X=0.0	-0.2228 ± 0.08672
X-intercept when Y=0.0	0.2387
1/slope	1.072
95% Confidence Intervals	
Slope	0.8645 to 1.002
Y-intercept when X=0.0	-0.4117 to -0.03380
X-intercept when Y=0.0	0.03850 to 0.4174
Goodness of Fit	
r^2	0.9865
Sy.x	0.1919
Is slope significantly non-zero?	
F	878.9
DFn, DFd	1.000, 12.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	14
Maximum number of Y replicates	1
Total number of values	14
Number of missing values	0

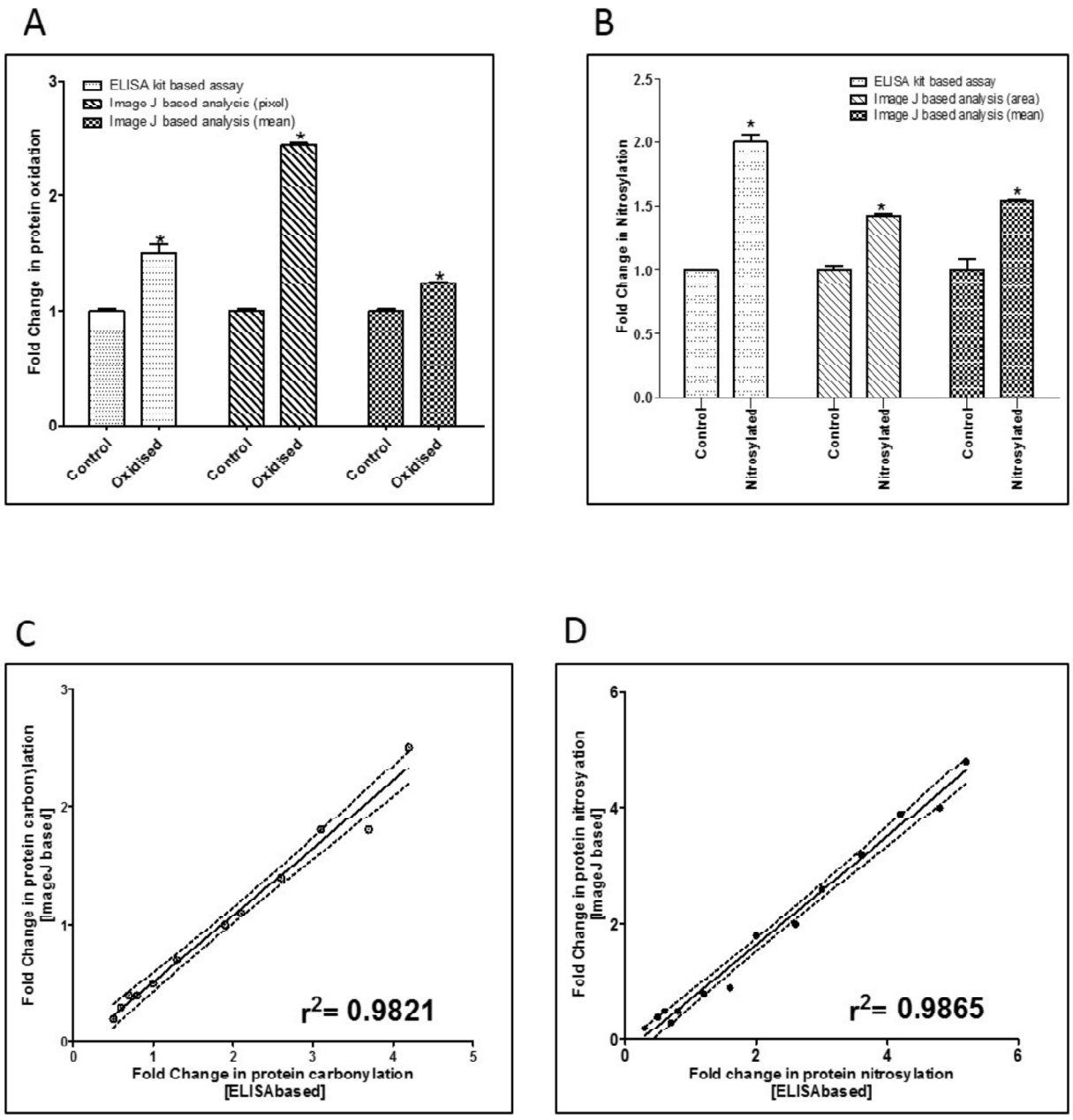

Figure 4: **Correlation analysis of image processing method with conventional ELISA based technique. A. Bar graph showing the fold change (log2 values) of carbonyl content in each of the four samples calculated using ELISA based method, ImageJ based analysis using mean values and using total number of pixels under the plot area. B. Bar graph showing the fold change (log2 values) of nitrosylation content in each of the four samples calculated using ELISA based method, ImageJ based analysis using mean values and using total number of pixels under the plot area. C. Correlation analysis of ELISA vs ImageJ based method for fold change in protein carbonylation using statistical package of GraphPad Prism reveals the squared correlation value of 0.9821. D. Similar corelation analysis for protein nitrosylation revealed a squared corelation coefficient of 0.9865. (* $p<0.01$).**

accessible. The idea was also to inspire computer programmers to develop suitable plugins to enhance the applicability vis-à-vis providing the researchers with a user friendly method which reduces the errors caused due to visual examination.

Another word of caution is the fact that some of the blots used in the investigation for comparisons might look overexposed or saturated. One must realize that the blots shown here are not ordinary blots for single protein rather they represent a number of modified

proteins on the same blot and therefore appear to be saturated (as we have obtained this after determining the optimal time of exposure) and most of the blots for such protein modification appeared similar in various reports. Also, it is not possible to use housekeeping gene product to normalize, as the housekeeping genes would also suffer from variable protein modification during the stress conditions. However we relied on accurate protein concentration measurements by alternate methods to ensure equal loading (aplty reflected in Coomasie stained gel) and this is the standard mode of representation.

Conclusion

In conclusion, present computational approach to semiquantitate the immunoblots of protein modifications carbonylation and oxidation provides a user friendly and enhanced comparative analysis. This method can also be recommended for similar images such as protein phosphorylation, lipid and carbohydrate modifications. Software developers and programmers may develop a new plugin or tools based on these algorithms and may further simplify the process. Further, as the analysis displays output in the form of distance from the wells, therefore a parallel analysis of molecular weight ladder can be useful in predicting the degree of protein modification with respect to each molecular weight.

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Abbreviations

RNOS, Reactive Nitrogen and Oxygen Species; ELISA, Enzyme linked immunosorbent assay; DNPH, Dinitrophenylhydrazine; NO, nitric oxide, PBS, Phosphate buffer saline, SDS-PAGE, Sodiumdodesylsulphate polyacrylamide gel electrophoresis; TCA, Trichloroacetic acid; µl- microliter.

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