U.S. Army Medical Research
Institute of Chemical Defense

USAMRICD-TR-08-02

An Unbiased Quantitative Method for Assessing Alterations in Microtubule-Associated Protein 2 (MAP-2) Immunostaining

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February 2008

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
February 2008	Technical Report	May 2007 to November 2007

4. TITLE AND SUBTITLE
An Unbiased Quantitative Method for Assessing Alterations in Microtubule-Associated Protein 2 (MAP-2) Immunostaining

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kan, RK, Tompkins, CP, Kniffin, DM and Hamilton, TA

5d. PROJECT NUMBER

5e. TASK NUMBER
6.1

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
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Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-08-02

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ-P
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The present study evaluated the use of computer-assisted image analysis for investigating the temporal alterations in microtubule-associated protein 2 (MAP-2) immunoreactivity in the piriform cortex following acute soman intoxication. Brain samples harvested at various time points after the onset of soman-induced seizures were immunostained with MAP-2 antibody. Color digital images captured with a color digital camera were converted to grayscale images and enhanced to identify the areas of MAP-2 immunoreactivity from the background. Segmentation was then performed by thresholding to highlight pixels occupied by the MAP-2 immunostain. The average total area of MAP-2 immunostaining in the piriform cortex was significantly decreased by 20% ($P<0.001$) 1 hr after soman-induced seizure onset as compared to control counterparts. Differences in the mean total area of MAP-2 immunostaining at 3 and 6 hr after seizure onset were not significantly different from the average total value obtained from 1 hr. At 12 hr, loss of MAP-2 immunostaining was visually obvious in layers II and III, and the total area occupied by MAP-2 immunostaining was significantly reduced by 65%. At 24 hr, only 17% of the total area was occupied by MAP-2 immunostaining. Using this technique, alterations in MAP-2 immunoreactivity were consistently quantified and reproducible.

15. SUBJECT TERMS
imaging analysis, immunohistochemistry, MAP-2, nerve agent, soman

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Robert K. Kan
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	30	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-6502

Acknowledgement

This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

Abstract

Immunohistochemical stains are typically interpreted qualitatively by pathologists and researchers as either absent or present. This method of interpretation is subjective and does not accurately provide information regarding the extent of changes in the immunohistochemical staining pattern, especially when the difference in immunohistochemical staining between control and experimental tissue sections is subtle and indiscernible by the human eye. The present study evaluated the use of computer-assisted image analysis for investigating the temporal alterations in microtubule-associated protein 2 (MAP-2) immunoreactivity in the piriform cortex of the rat following acute soman intoxication (1.6 LD_{50} or 180 ug/kg). Brain samples harvested at various time points (1, 3, 6, 12 and 24 hr) after the onset of soman-induced seizures were immunostained with mouse monoclonal MAP-2 antibody using the avidin-biotin-peroxidase complex (ABC) method. Brightfield images of MAP-2 immunostaining were captured with a color digital camera. Color digital images were converted to grayscale images and enhanced to clearly identify the areas of MAP-2 immunoreactivity from the background using Adobe® Photoshop 7.0. Segmentation was then performed by thresholding to automatically highlight pixels that are occupied by the MAP-2 immunostain, using ImagePro Plus (version 6.0). The average total area of MAP-2 immunostaining in the piriform cortex (n = 6) was found to be significantly decreased by 20% ($P<0.001$) as early as 1 hr after soman-induced seizure onset as compared to control counterparts (n = 6). Although there were differences in the mean total area of MAP-2 immunostaining at 3 and 6 hr after the onset of seizures, they were not significantly different from the average total value obtained from 1 hr. At 12 hr after the onset of seizures, loss of MAP-2 immunostaining was visually obvious in layers II and III, and the total area occupied by MAP-2 immunostaining was significantly reduced by 65%. At 24 hr, only 17% of the total area was occupied by MAP-2 immunostaining. These findings were consistent with the results obtained by two other operators who have no experience conducting computer-assisted image analysis. Using this technique, alterations in MAP-2 immunoreactivity were consistently quantified and reproducible. In addition, since there were no significant differences in the total area of MAP-2 immunoreactivity between 1, 3 and 6 hr after seizure onset, a therapeutic window of up to 6 hr may exist for administering neuroprotective compounds to halt brain injury progression. Moreover, although this unbiased quantitative immunohistochemistry was used to quantify the amount of MAP-2 immunostaining, it could be employed to quantitatively measure the changes in the immunoreactivity of any proteins following injury.

Introduction

Immunohistochemical (IHC) staining methods have been extensively used in both clinical and basic research settings. In clinical practice, IHC has been used as a diagnostic tool to establish malignancy of tissue biopsies. As a research tool, IHC has been used in a number of laboratories for evaluating brain injury induced by ischemia (Matesic and Lin, 1994; Kitagawa et al., 1989) and trauma (Folkerts et al., 1998; Posmantur et al., 1996), skin lesions such as junctional epidermolysis bullosa (Vodegel et al., 2003; Kirtschig et al., 1998) and pulmonary injury induced by endotoxins such as lipopolysaccharides (Janardhan et al., 2006; Mitsuhashi et al., 1999). In our laboratory, IHC is extensively used to investigate temporal progression of skin injury produced by the vesicating agent sulfur mustard (Kan et al., 2003; Petrali and Oglesby-Megee, 1997) and neurodegeneration induced by chemical nerve agents (Kan et al., 2005; Ballough et al., 1995).

IHC is typically interpreted qualitatively using a binary present-absent or increased-decreased end point. This interpretation method was generally accepted before the advent of digital processing and the development of image analysis software. A major criticism of this scoring method is its lack of objectivity; additionally, it is not accurate when both control and experimental tissue samples display immunohistochemical staining. Today, quantitative IHC has become a main-stream method for measuring changes in immunoreactivity of proteins. Most funding agencies and prestigious journals require quantitative analysis of immunostaining for submission of grant proposals and for consideration of publication.

Given the advancement in computer hardware and imaging analysis software, there is no reason not to incorporate these technical dimensions to obtain quantitative pathological measures. The present study employed Adobe® Photoshop 7.0 and ImagePro Plus 6.0 to quantify the changes in MAP-2 immunoreactivity in the piriform cortex following soman-induced seizures. MAP-2 is the most abundant neuron-specific cytoskeletal protein in the brain, localized mostly in dendritic processes (Caceres et al., 1984; De Camilli et al., 1984). Loss of MAP-2 immunoreactivity has been shown to be a sensitive marker for brain damage induced by soman toxicity (Ballough et al., 1995), cerebral ischemia (Kitagawa et al., 1989; Matesic and Lin, 1994) and traumatic brain injury (Folkerts et al., 1998; Posmantur et al., 1996). This study employed Adobe® Photoshop 7.0 (Adobe System Incorporated, San Jose, CA) and ImagePro Plus software 6.1 (Media Cybernetics, Silver Spring, MD) to quantitate MAP-2 immunostaining, using image analysis, in the rat piriform cortex following exposure to a lethal dose of soman. The piriform cortex was used to study the progression of injury since it is a brain region that consistently showed damage in the rat exposed to soman.

Materials and Methods

Adult male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 200-250 g, were used in the study. Animals were pretreated with the oxime HI-6 (125 mg/kg, ip) 30 min prior to soman challenge (1.6 LD_{50} or 180 μg/kg, sc). One minute after soman injection, animals were treated with atropine methyl nitrate (AMN) (2.0 mg/kg, im). HI-6 and AMN were used to decrease the mortality of soman-exposed animals (Shih et al., 1991). The concentration of soman was

selected to produce seizures in 100% of the animals (Shih et al., 1991). Vehicle control animals received an equivalent volume of vehicle, HI-6 and AMN. All experiments were conducted in compliance with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

At 1 hr, 3 hr, 6 hr, 12 hr or 24 hr after soman-induced seizure onset, animals were anesthetized with an injection of sodium pentobarbital (65 mg/kg, ip). Animals were perfusion-fixed with 10% phosphate buffered formalin (PBF). Brains removed immediately after perfusion from the skull were submersion fixed in 10% PBF at 4°C for 18-24 hr, coronally cut into 3-mm thick slices using a rat brain matrix (ASI Instrument, Warren, MI) and processed in paraffin. Sections cut at 5 μm were immunostained with mouse anti-MAP-2 monoclonal antibody. Only sections between bregma -3.00 mm and -3.60 mm as described by Paxinos and Watson (1998) were used; these coordinates encompassed all brain regions of interest, including the hippocampal formation, piriform cortex, thalamus, amygdala and sensory and motor cortices. Since the piriform cortex consistently showed damage following soman-induced seizures, it is used to quantify the magnitude of brain injury.

Microwave-Assisted MAP-2 Immunohistochemistry

Brain sections were dewaxed in xylene and hydrated in decreasing percentage of ethanol. Following washing in distilled water, sections were treated with 5% hydrogen peroxide at room temperature for 20 min to suppress endogenous peroxidase activity. Following thorough washing in running tap water (5 min), sections were rinsed in dH_2O and subjected to microwave antigen retrieval (MAR) procedures. Briefly, sections were boiled 2 times (5 min each time) in 10 mM citric acid (Sigma-Aldrich, St. Louis, MO; Lot 30H-0627) in a microwave (BioGenex EZRetriever Microwave; BioGenex, CA) and cooled at room temperature for 20 min prior to immunohistochemical staining (Kan et al., 2005; Pleva et al., 2005).

Indirect immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu and co-workers (Hsu et al., 1981). Following microwave antigen retrieval, brain sections were incubated in 5% horse serum at 4°C for 30 min to block tissue immunoglobulins that could react with secondary antibody. Sections were then incubated in anti-MAP-2 monoclonal antibody (1:100; Clone AP-18; NeoMarkers, Fremont, CA) for 18 hr at 4°C. After being thoroughly rinsed in PBS, sections were incubated in biotinylated secondary antibody for 1 hr at room temperature (Vector, Burlingame, CA) and ABC (avidin-biotin-peroxidase complex) solution for 30 min at room temperature (Vector, Burlingame, CA). MAP-2 immunoreactivity was then visualized by incubating sections in diaminobenzidine (DAB) for 5 min at room temperature (Sigma-Aldrich, St. Louis, MO). Negative control sections were simultaneously processed without either MAR or primary antibody to ensure specificity of MAP-2 immunostaining.

Computer-Assisted Image Analysis

Quantification of immunohistochemical of MAP-2 was performed as diagrammed in Figure 1.

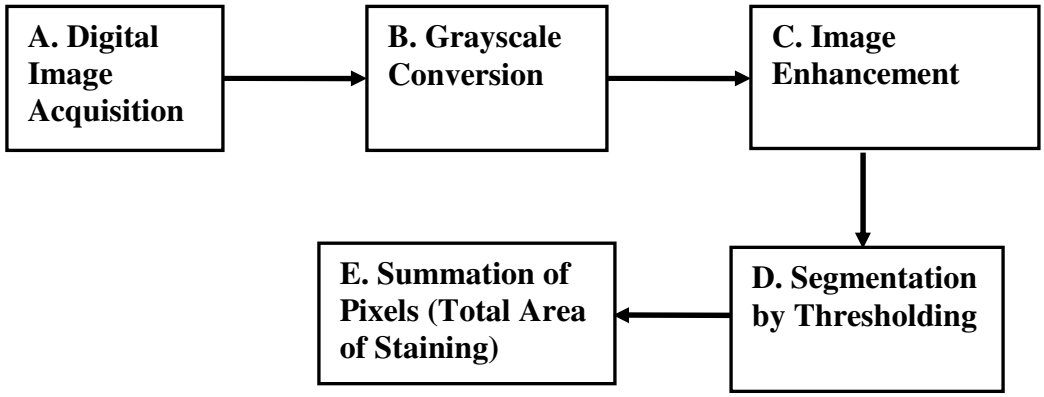

Figure 1. A schematic diagram showing processes for quantification of immunohistochemical staining (i.e. MAP-2 immunoreactivity). A. Digital image acquisition is a process in which brightfield images of area of interest are digitalized. Digital images are intrinsically made up of a collection of small boxes, which are known as pixels. Each pixel has a specific pixel value that represents the color or shade of gray of that pixel. B. Grayscale conversion is performed to convert color digital images to 8-bit grayscale images with each pixel given a pixel value between 0 (white) and 255 (black); pixel values between 0 and 255 represent different shades of gray. C. Image enhancement is performed to produce better contrast between area of staining and background. D. Segmentation by thresholding is performed to automatically highlight pixels with the pixel values below the threshold value. E. Summation of pixels is performed to calculate the total area occupied by immunostaining. Note: the size of each pixel and the number of pixels in each digital image are directly proportional to the magnification of the image and the resolution of the image, respectively. Therefore, it is important to keep all images at the same magnification and resolution.

Digital Image Acquisition

Quantitative immunohistochemistry begins with the translation of stained cellular components on tissue sections into raw data in the form of digital images. The digital image is intrinsically divided into a rectangular grid of pixels representing the color spread of the image. Each pixel is given a pixel value, ranging from 0 to 255, that represents the color (in the case of a color image) or shades of gray (in the case of a black and white image) of that pixel.

Acquisition of digital images from brightfield images was performed on a BX61 light/fluorescent microscope mounted with a DP-70 digital color camera (Olympus America Inc., Woodbury, NY) (Appendix A). Brightfield images of MAP-2 immunostaining of control and soman-exposed animals were captured after the white-balanced function was performed. All images were stored in the computer as TIFF files at a magnification of 200X and resolution of 1039 X 1063 pixels. Each pixel was calibrated at 0.1024 μm^2. The exposure, which determines the brightness of the digital image, was set at "Auto" to remove operator bias. The importance of the brightness level

in the consistency and reproducibility of quantitative immunohistochemistry is discussed in the Discussion section.

Color Image Conversion

Color digital images were converted to 8-bit grayscale images using Adobe® Photoshop 7.0 (Adobe System Incorporated, San Jose, CA) (Appendix B). A grayscale image (black and white) contains 256 shades of gray with 0 representing black and 255 representing white. Values in between denote different shades of gray. Using the pixel value information unique to the areas of immunostaining, quantitative image analysis of immunohistochemical stains is the summation of all the values of the pixels that reflect the areas of immunostaining on the image.

Image Enhancement

Grayscale images were enhanced, using Adobe® Photoshop 7.0 (Adobe System Incorporated, San Jose, CA), to render the areas of MAP-2 immunostaining clearly distinguishable by the human eye from the background (Appendix C). In all cases, enhancement of the grayscale images was done using the auto function to eliminate operator bias. The enhanced grayscale images were named to indicate that they had been modified and then saved in the computer.

Segmentation by Thresholding

Segmentation by thresholding is a process in which the areas of immunostaining are separated from the background. This process is done by setting the threshold value using ImagePro Plus software (version 6.1; Media Cybernetics, Silver Spring, MD) (Appendix D). The threshold value is where pixels with grayscale value below the threshold are included in the measurement of areas of immunostaining and those above the threshold are excluded in the measurement. After the segmentation by thresholding step is performed, all pixel values that are below the threshold value are automatically highlighted by the software.

Summation of Pixels

Summation of pixels is a process in which all pixels that are highlighted during segmentation by thresholding are summated. The total number of pixels represents the total area that contains the MAP-2 immunostaining (Appendix E).

Statistical Analysis

The average total area of MAP-2 immunoreactivity (Mean area ± SEM) was calculated by the summation of the total area that displayed MAP-2 immunostaining divided by the number of samples per group (n=6). The differences in the mean total area of MAP-2 immunoreactivity between control and experimental groups were compared by one-way analysis of variance (ANOVA) followed by multiple comparisons using a Tukey's test. In all analysis, p-values less than or equal to 0.05 were interpreted as being statistically significant.

Results

Figure 1 shows MAP-2 immunoreactivity of control and experimental (1 hr, 3 hr, 6 hr, 12 hr and 24 hr) groups. In control animals, MAP-2 staining was localized mostly in dendrites (Figure 1A). As early as 1 hr after the onset of seizures, loss of MAP-2 was evident as indicated by the reduction of dendritic staining pattern (Figure 1B). At 3 and 6 hr after seizure onset, loss of dendritic MAP-2 immunoreactivity was concomitant with an increase in neuronal MAP-2 immunostaining (1C and 1D). By 12 hr, MAP-2 immunoreactivity in layers II and III was markedly decreased (1E). At 24 hr after the onset of seizures, layers II and III were completely devoid of MAP-2 immunostaining (1F). In addition, MAP-2 loss was evident in layer I of the piriform cortex.

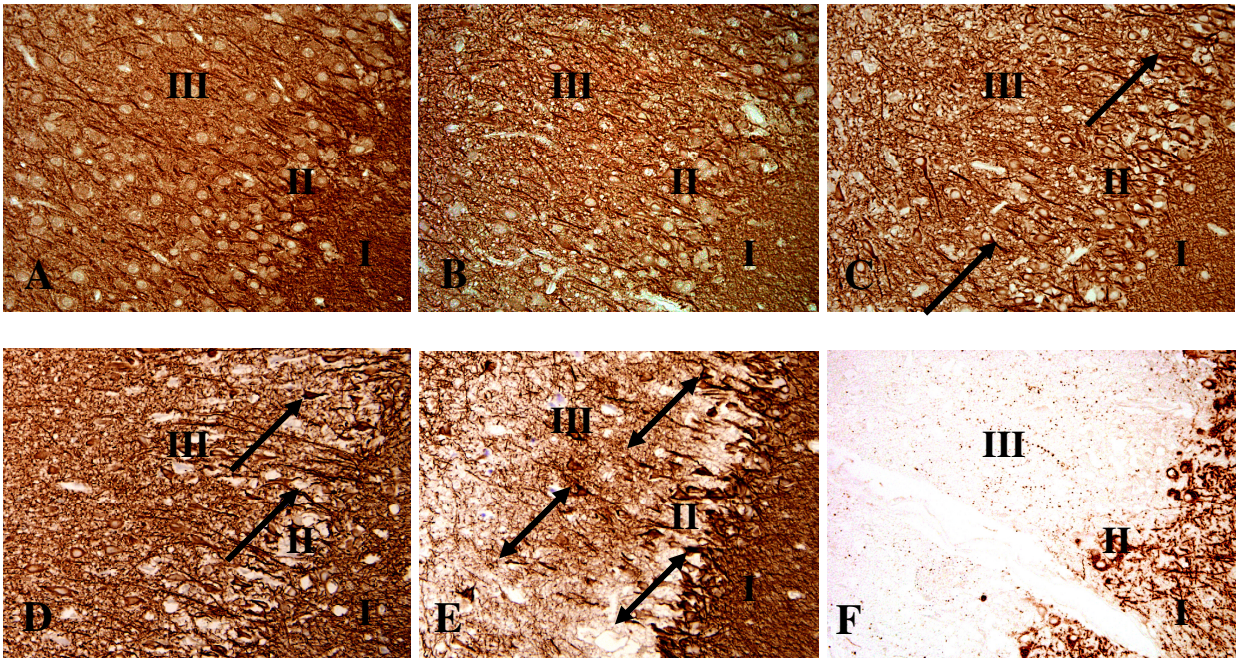

Figure 1. Time-course MAP-2 immuoreactivity in piriform cortex of vehicle control (A) and at 1 hr (B), 3 hr (C), 6 hr (D), 12 hr (E) and 24 hr (F) after soman-induced seizure onset. Vehicle control (A) sections show a typical pattern of MAP-2, predominantly in neuronal dendritic processes. At 1 hr after onset of seizures (B), loss of MAP-2 immunoreactivity in layers II and III was observed. At 3 (C) and 6 hr (D), MAP-2 loss in dendritic processes and increase in MAP-2 staining in morphologically altered neurons (arrows) were detected. At 12 hr (E), MAP-2 loss in layers II and III is pronounced and degenerated neurons (double arrows) are intensely labeled with MAP-2. By 24 hr (F), only scattered punctate MAP-2 immunostaining and a few MAP-2 positive neurons remained in layer III and II, respectively. In addition, loss of MAP-2 immunoreactivity has spread to layer I.

The extent of MAP-2 immunostaining loss in the piriform cortex from control and soman-exposed animals was measured. The results show a temporal reduction of MAP-2 immunoreactivity when compared to control counterparts (Figure 2). A statistically significant decrease (-20%) in the average total area of MAP-2 immunostaining in the piriform cortex was detected as early as 1 hr after the onset of seizures induced by soman ($P<0.001$). At 3 hr and 6 hr, the average

percentage of MAP-2 immunostaining was increased from -20% at 1 hr to -14% and -16%, respectively. However, the differences in the mean percentage of MAP-2 immunostaining at 1 hr, 3 hr and 6 hr after seizure onset was not statistically significant (P>0.05). At 12 hr, MAP-2 loss continued and it was significantly reduced by 65% as compared to the control value (P<0.001). At 24 hr after seizure onset, there was an 83% reduction in MAP-2 immunostaining.

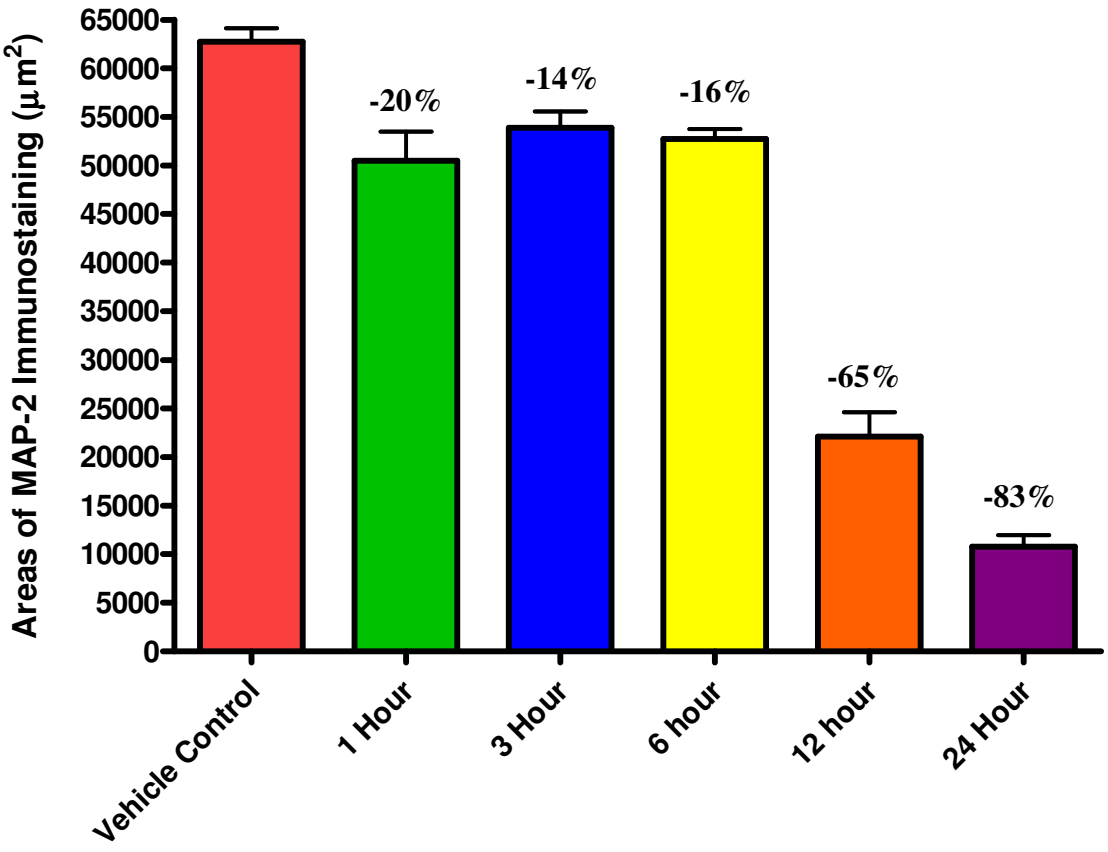

Figure 2. MAP-2 immunoreactivity in piriform cortex following soman-induced seizure. A significant reduction (20%) in MAP-2 immunoreactivity was observed as early as 1 hr after seizure onset (p>0.01). No further significant decrease in MAP-2 immunostaining was detected at 3 and 6 hr (P>0.05). The most significant decrease (65%) in MAP-2 immunoreactivity commenced at 12 hr (p<0.001). At 24 hr, MAP-2 immunoreactivity continued to decline (83%) (p<0.001).

Discussion

Histopathological methods such as H&E remain the "gold standard" for pathologists and most researchers for diagnostic and therapeutic decisions in autopsy or necropsy pathology. However, the interpretation of H&E sections is an inherently subjective process based on primarily on the cellular up-take of the stain and morphological features. In contrast, immunohistochemical evaluation is based on the levels of protein content in the cell or tissue and thus, it is a better tool for examining the functional status of a cell or tissue following an insult. Therefore, it is not surprising that immunohistochemistry has been used widely in the past to assist pathologists and researchers to complement morphological information.

When immunohistochemistry was first introduced, it was largely used as a qualitative method to assess tissue samples as either positive or negative. This type of qualitative interpretation is useful for characterizing cell types, but not for studying the levels of cellular expression of proteins, such as MAP-2. Additionally, with the explosion of gene expression microarray technology in recent years, thousands of genes can be screened for identification of specific pathways that could be operating in the pathogenesis of injury. Since proteins are products of genes, it is important to confirm expression microarray results by detecting protein expression levels using Western blot, mass spectrometry or immunohistochemistry. Of these validation methods, only immunohistochemistry can provide precise anatomical and cellular location of proteins of interest. For these reasons, quantitative immunohistochemistry is a method required for manuscripts and grant proposals if immunohistochemistry is used in the study.

The present study used Adobe Photoshop and ImagePro Plus image analysis software to quantitate the extent of MAP-2 loss as an approach to measure the severity of brain injury after the onset of soman-induced seizures. The results showed that brain injury is progressive, as indicated by an increase in the reduction of MAP-2 immunoreactivity in the piriform cortex during the first 24 hr after soman intoxication. As early as 1 hr after the onset of seizures, MAP-2 immunostaining was reduced by 20%. At 3 and 6 hr, changes in MAP-2 immunoreactivity as compared to 1 hr were not statistically significant. This finding could be explained by the observation that MAP-2 immunoreactivity was strongly increased in both morphologically altered and healthy neurons at 3 and 6 hr. Though there was loss of dendritic MAP-2 immunostaining, the increase in neuronal cytoplasmic MAP-2 immunoreactivity keeps the total area of MAP-2 immunostaining unchanged. Our quantitative results indicate that the degree of brain injury induced by soman remains unchanged up to 6 hr, suggesting that the therapeutic window for neuroprotection is at least 6 hr following soman intoxication. In addition, it is unlikely that neuroprotective compounds could have any therapeutic benefits beyond this time point due to the severity of brain injury.

Image analysis is a quantitative technique that primarily deals with image information such as intensity in the case of color images or shades of gray in the case of black and whites (or grayscale) images. The most important factors that produce high variability of image information are tissue preparation, reproducible immunohistochemistry and the brightness of the captured images. It is impossible to process and stain all the tissues to be used for image analysis at the same time. To eliminate batch-to-batch processing and immunochemical staining variation, it is critical to perform all procedures according to standard operating procedures.

The brightness of the captured images can affect the final image information. The brightness of the digital image is the function of the exposure time and determines the grayscale value of the pixels within the image. During the segmentation process by thresholding, separation of objects (areas of immunostaining) from the background staining is based on the pixel value. Changes in pixel value due to increase or decrease in brightness of the digital images can increase or decrease the threshold value, and therefore, the amount of immunostaining in the image is overestimated or underestimated. For this reason, it is critical to eliminate operator bias of the brightness of the image. One common practice in our laboratory is to set the exposure time at auto exposure to eliminate brightness bias of the operator.

This technical report is written to provide a technical method for quantification of immunohistochemical stains and to illustrate the value of quantitative immunohistochemistry to differentiate subtle changes of proteins of interest over time. Possible issues in generating reproducible and reliable quantitative immunohistochemical results are also discussed. "A picture may be worth a thousand words," but quantitative data are worth a thousand pictures. We hope that scientists and technical staff at the institute who are conducting immunohistochemical studies will consider using quantitative image analysis to expand the database on tissue pathology induced by chemical warfare agents and other toxic chemicals.

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APPENDIX A
DIGITAL IMAGE ACQUISITION

1. Turn on the computer and microscope.
2. Left click on the DP Controller icon to open the image acquisition window (Figure 1).
3. Left click on the Blue Eye icon (Arrow) to interface the camera with the microscope.

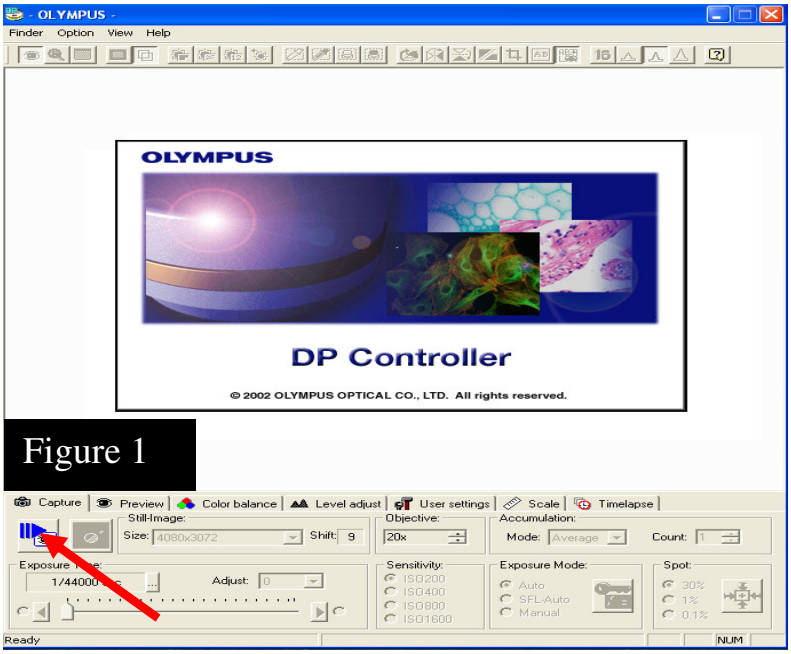


Figure 1

4. Place a slide on the stage and find the area of interest (for example the piriform cortex). The brightfield image of the piriform cortex will automatically appear in the DP Controller window (Figure 2). If the image is not in focus, use the fine focus of the microscope to adjust the focus.

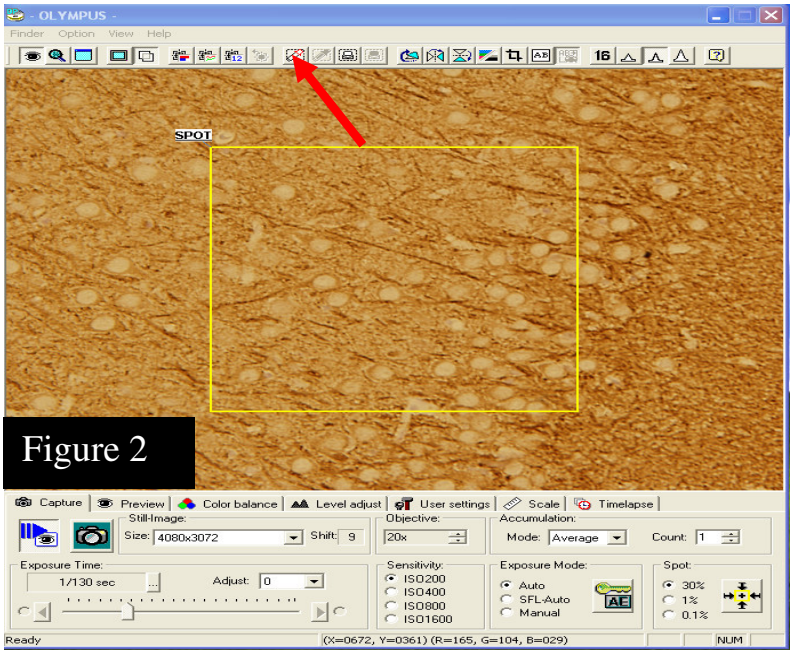


Figure 2

5. Left click on the White Balance icon (arrow) to set the background as shown in Figure 2. Place the cursor and then left click on an all white portion of the slide. The background of your image will appear white (Figure 3).

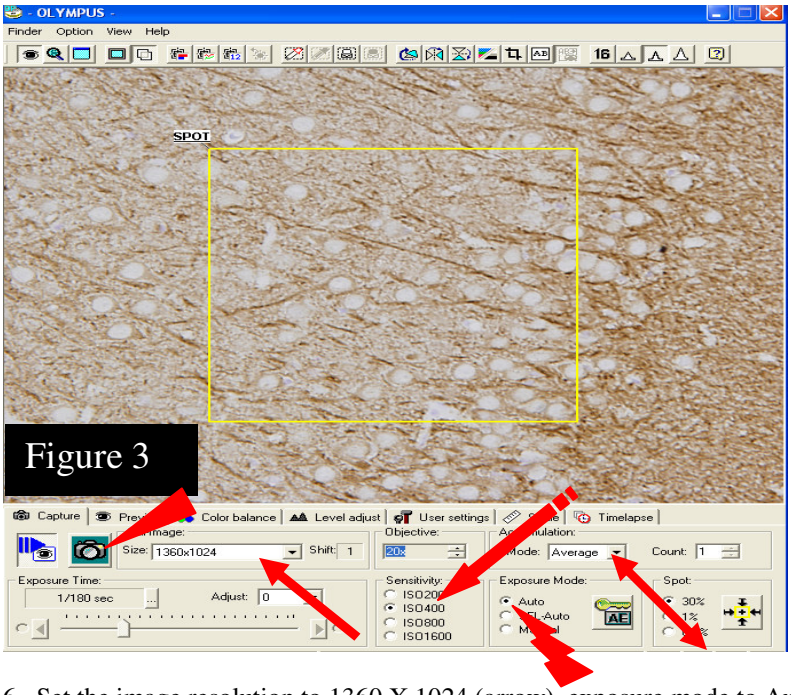


Figure 3

6. Set the image resolution to 1360 X 1024 (arrow), exposure mode to Auto (double arrow), sensitivity to ISO400 (lightening bold) and objective to 20X (broken arrow). These parameters should be the same throughout the study.
7. Make sure the brightfield image is in focus. Use the fine focus of the microscope to adjust the focus. When ready, left click on the Green Camera icon (arrow head) to capture the brightfield image.
8. The DP Manager window with your image that you just captured will appear (Figure 4)

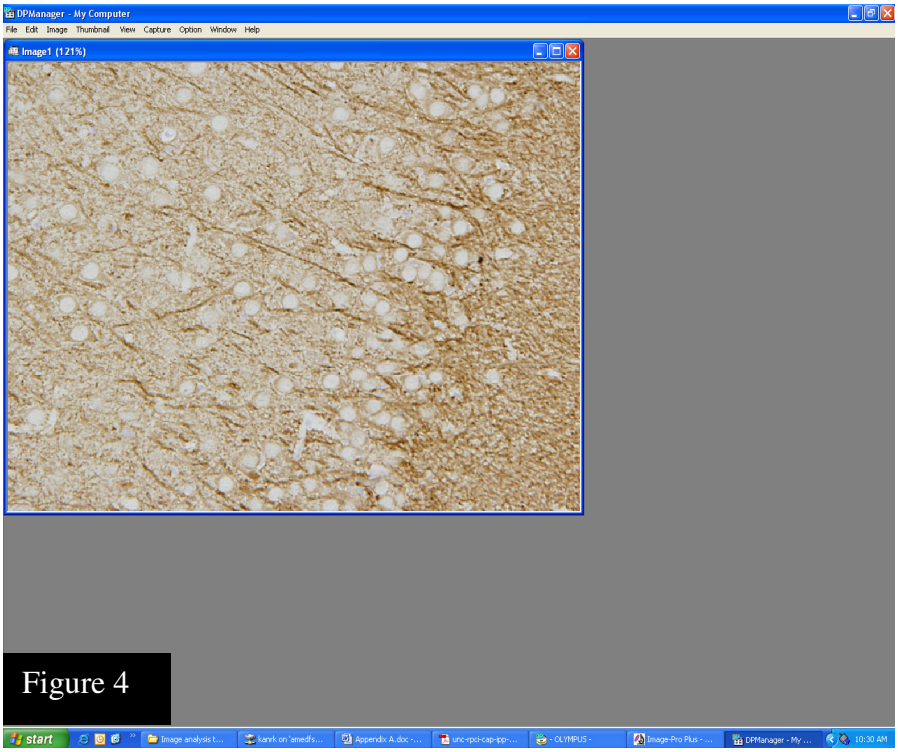
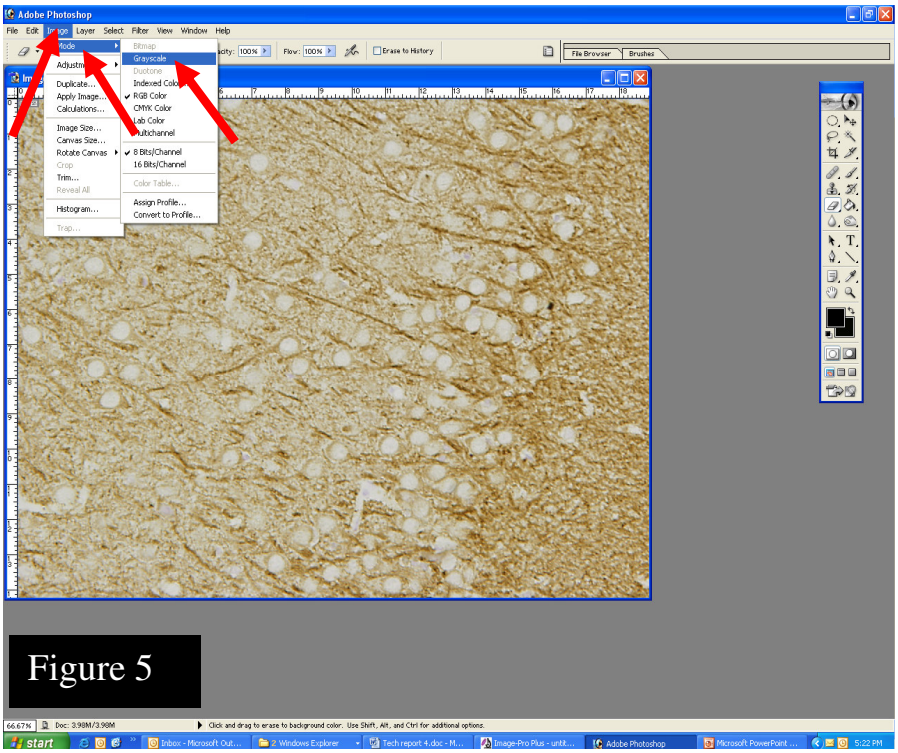


Figure 4

9. Left click File/Save as and name the image. Make sure to indicate the objective size when naming the image file. You will need this information when determining the area of the image that occupied by the stain.

APPENDIX B
GRAYSCALE CONVERSION

1. To convert the color brightfield image that you just captured, open the color images in Photoshop.
2. Left click Image/Mode/Grayscale (arrows) as shown in Figure 5.



3. A submenu Discard color information? will appear. Click OK and Figure 6 showing a grayscale image will appear. Left click File/Save as and give a name to the image. Make note of where you save the image.

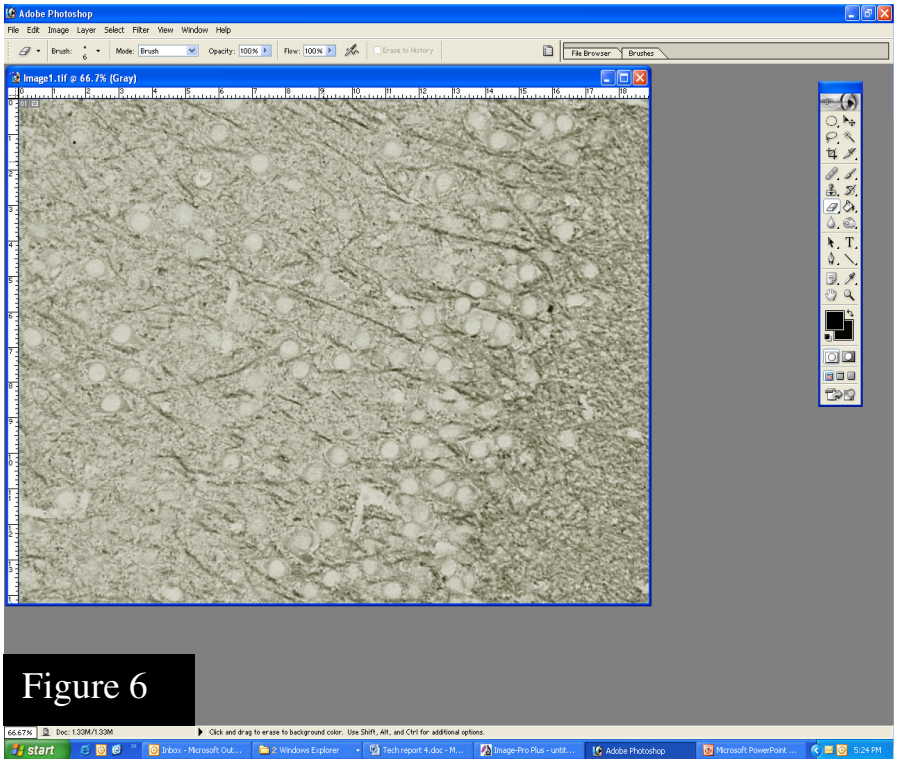

Adobe Photoshop
File Edit Image Layer Select Filter View Window Help
Image1.tif @ 66.7% (Gray)
start

Figure 6

APPENDIX C
IMAGE ENHANCEMENT

1. Open grayscale images in Photoshop as shown in Figure 7.

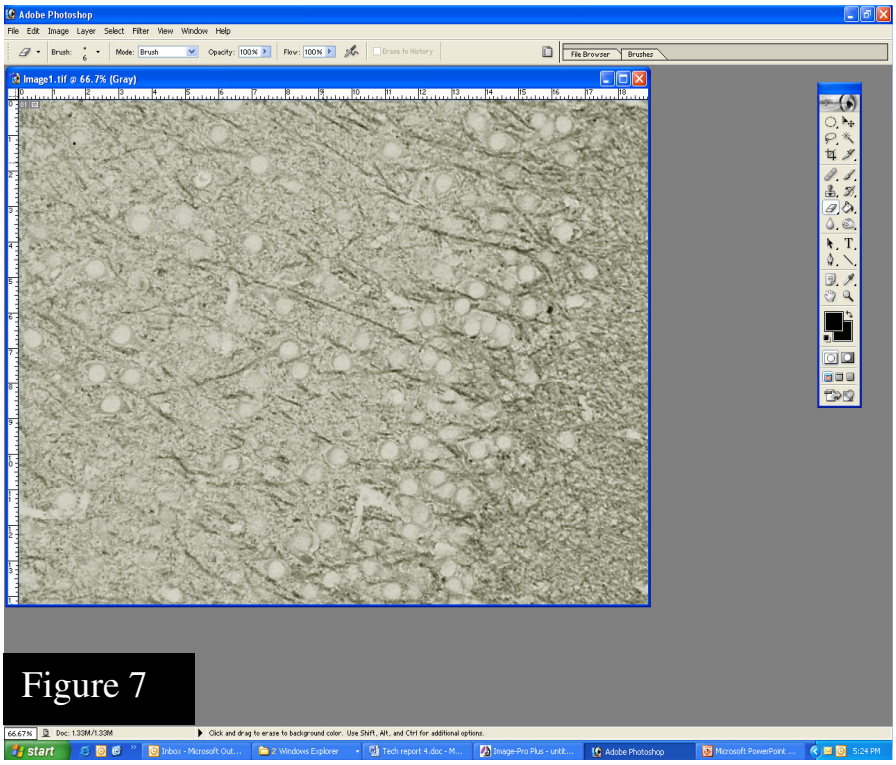


Figure 7

2. Left click Image/Adjustment/Levels (arrows) as shown in Figure 8. Figure 9 will appear.

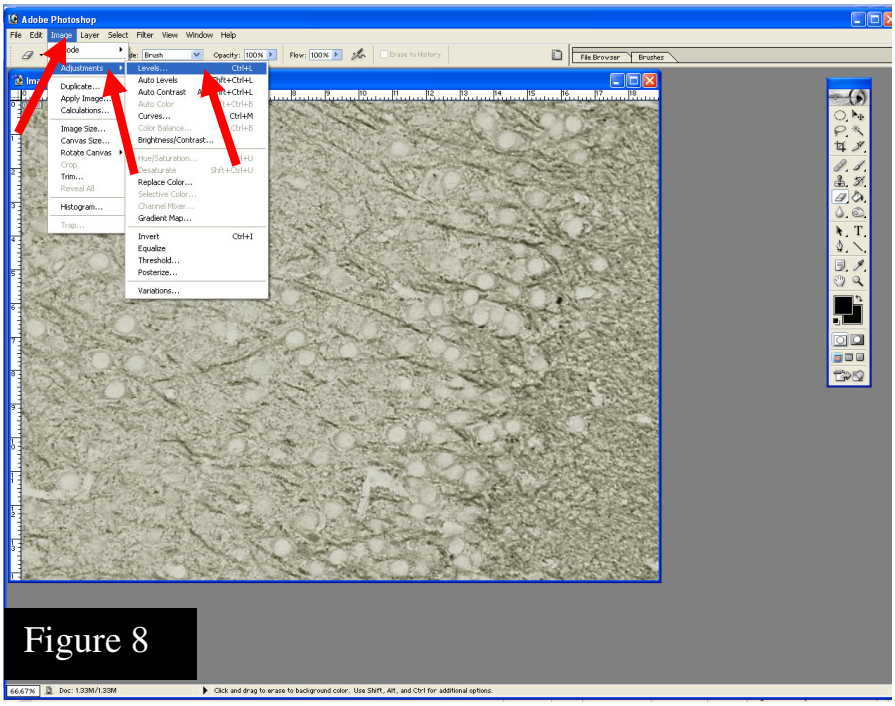


Figure 8

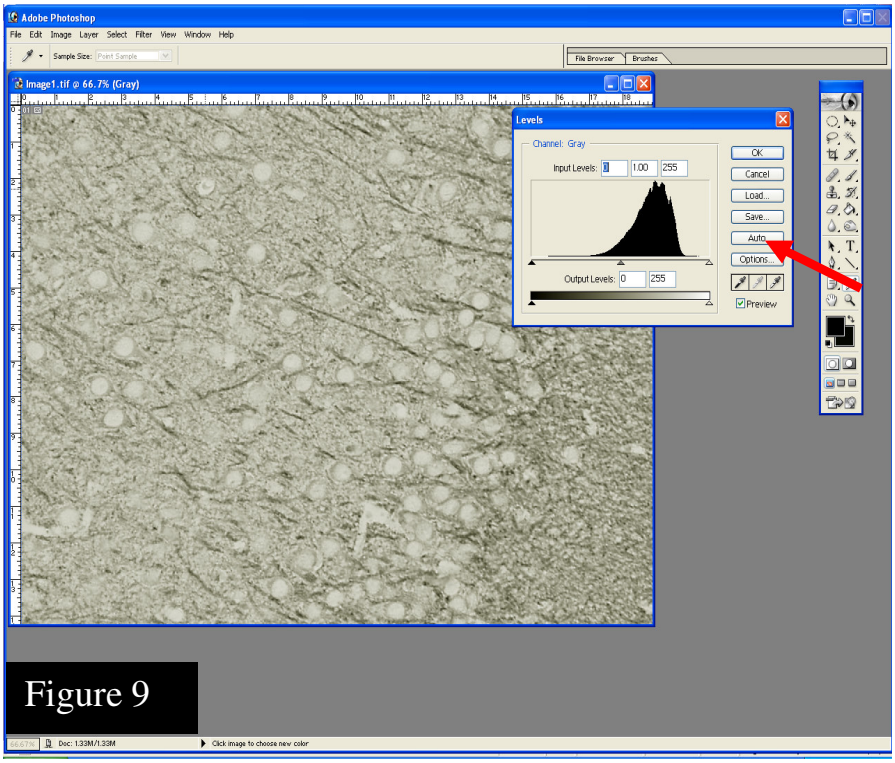


Figure 9

3. Click Auto (arrow) to enhance the grayscale image as shown in Figure 9. Click OK. Figure 10 will appear.

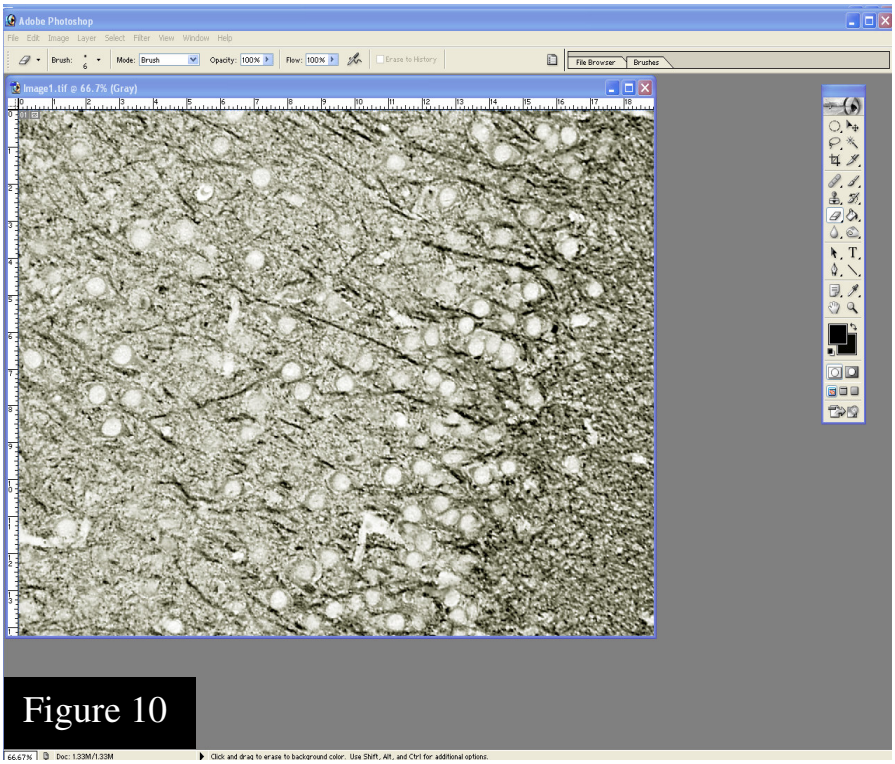
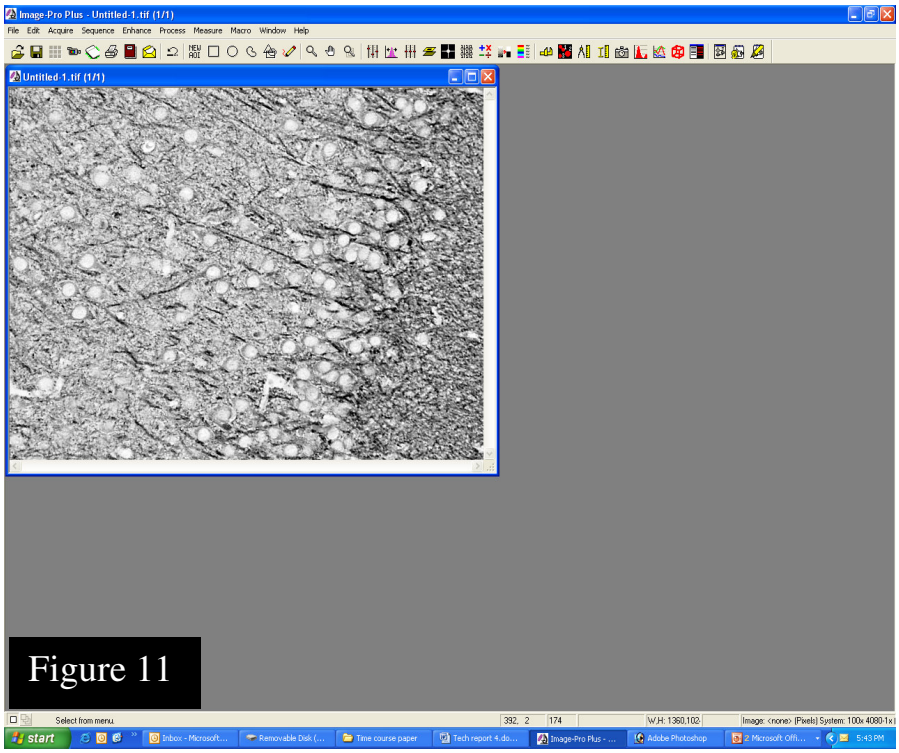


Figure 10

4. Click File/Save as and give a name to the grayscale image. Make note of where you save the image.

APPENDIX D
SEGMENTATION AND THRESHOLDING

1. Open enhanced grayscale images in ImagePro Plus 6.0 as shown in Figure 11.



2. Click Measure/CountSize (arrows) as shown in Figure 12. Figure 13 will appear.

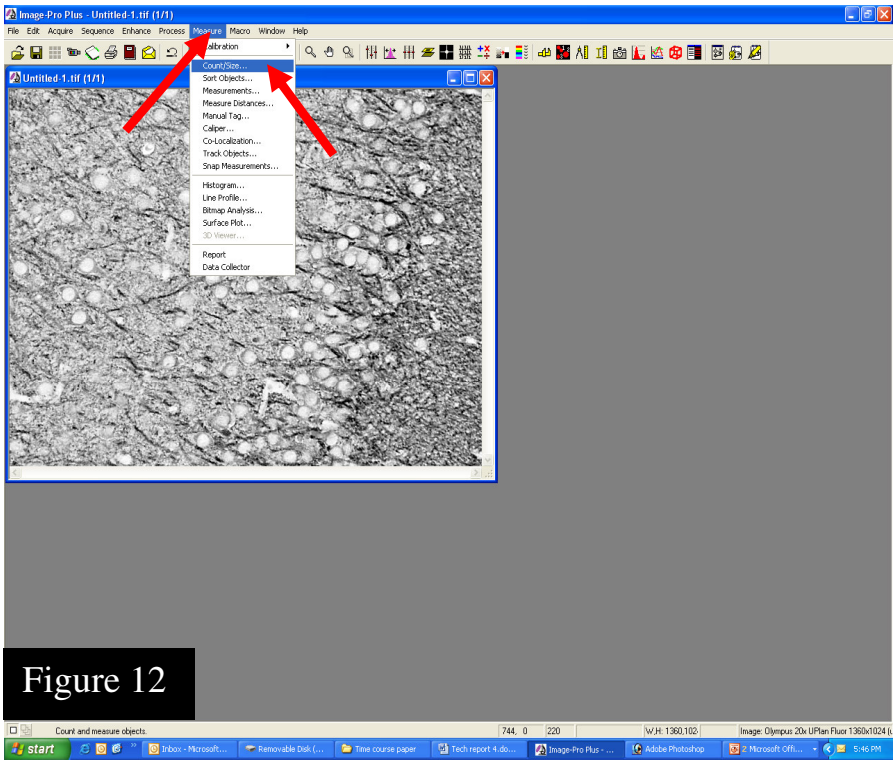


Figure 12

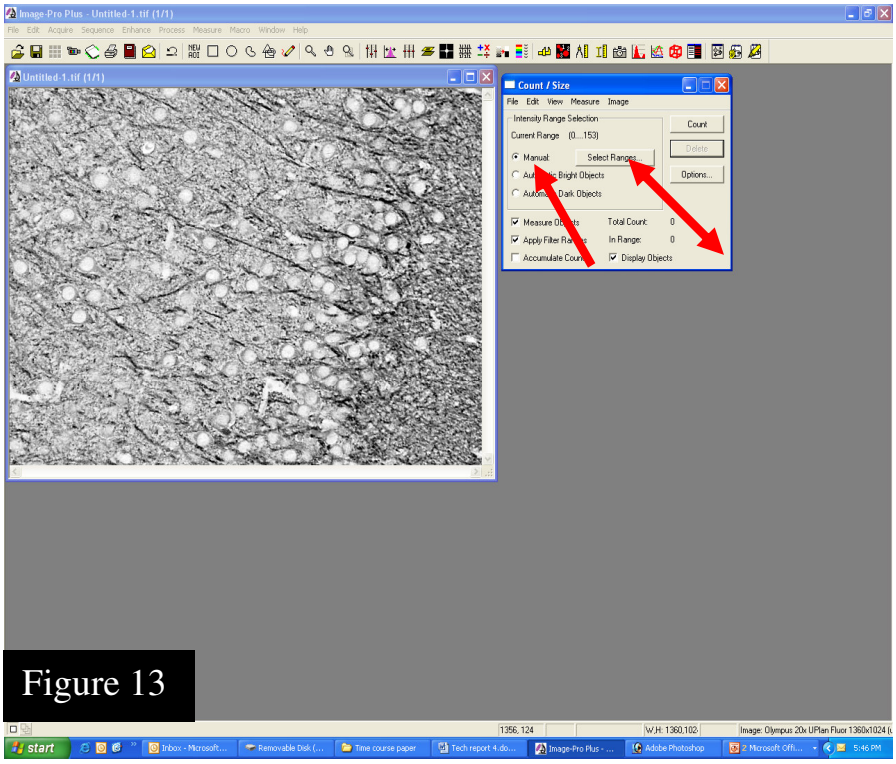


Figure 13

3. Select Manual (Arrow) from the Count/Size window as shown in Figure 13.
4. Left click Select Ranges (double arrow) from the Count/Size window as shown in Figure 13. The areas containing immunolabeling will be automatically highlighted to generate a binary image as shown in Figure 14.

The number in the box indicated by the double arrow represents the threshold value. Pixel values below this threshold value are highlighted.

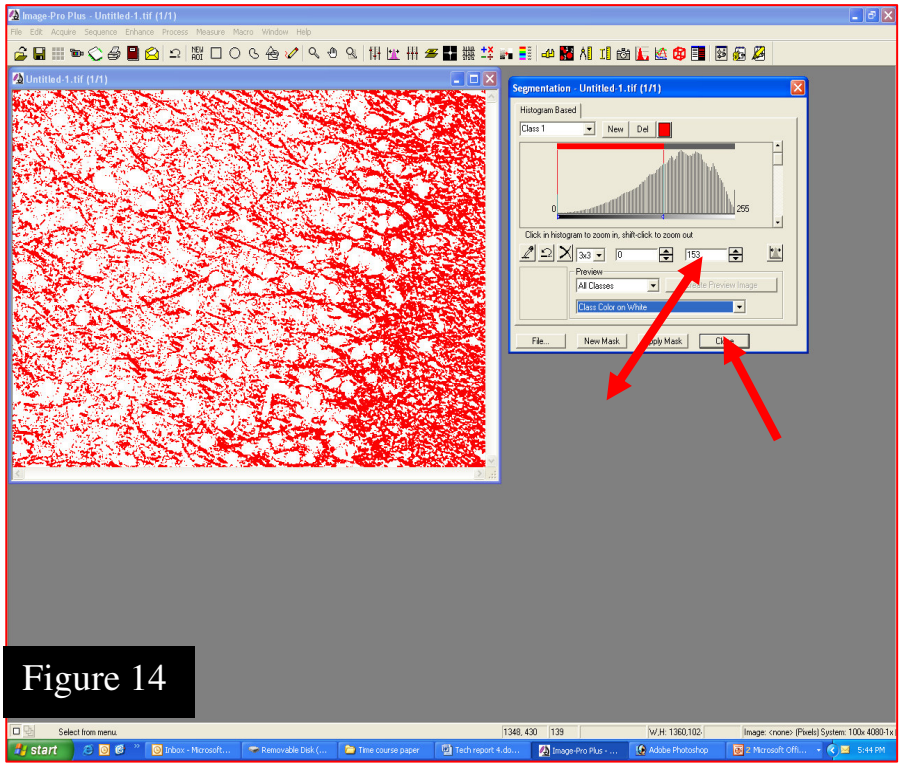


Figure 14

5. Left click Closed (arrow) as shown in Figure 14. Figure 15 will appear.

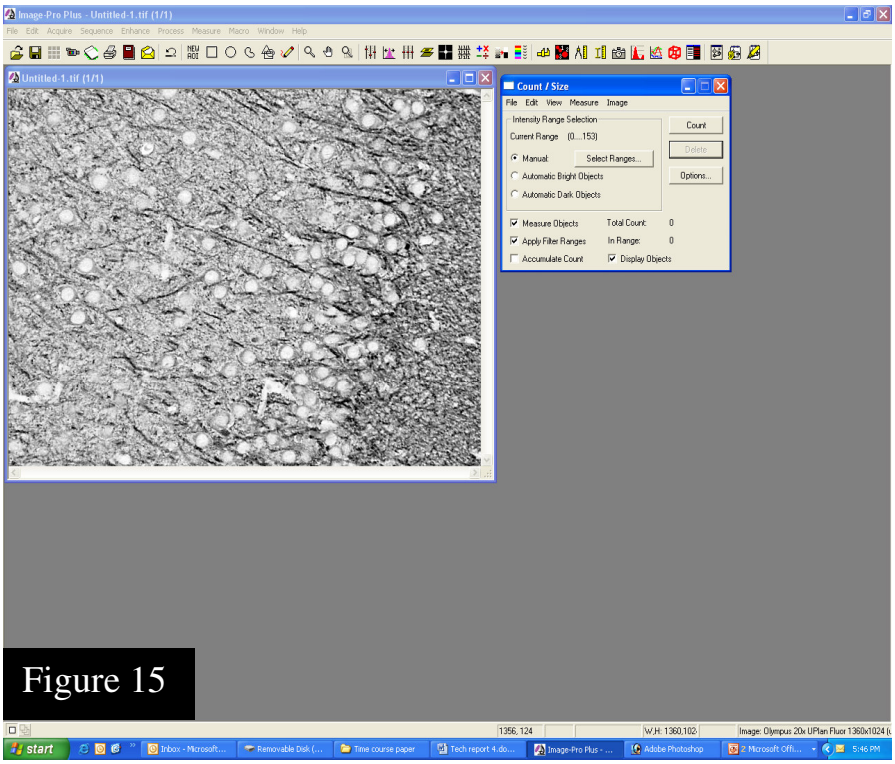


Figure 15

6. Do not close the image. Go to Appendix E.

APPENDIX E
COUNTING IMMUNOLABELED AREAS

1. Click Measure/Calibration/Select Spatial Calibration (arrows) as shown in Figure 16. Figure 17 will appear.

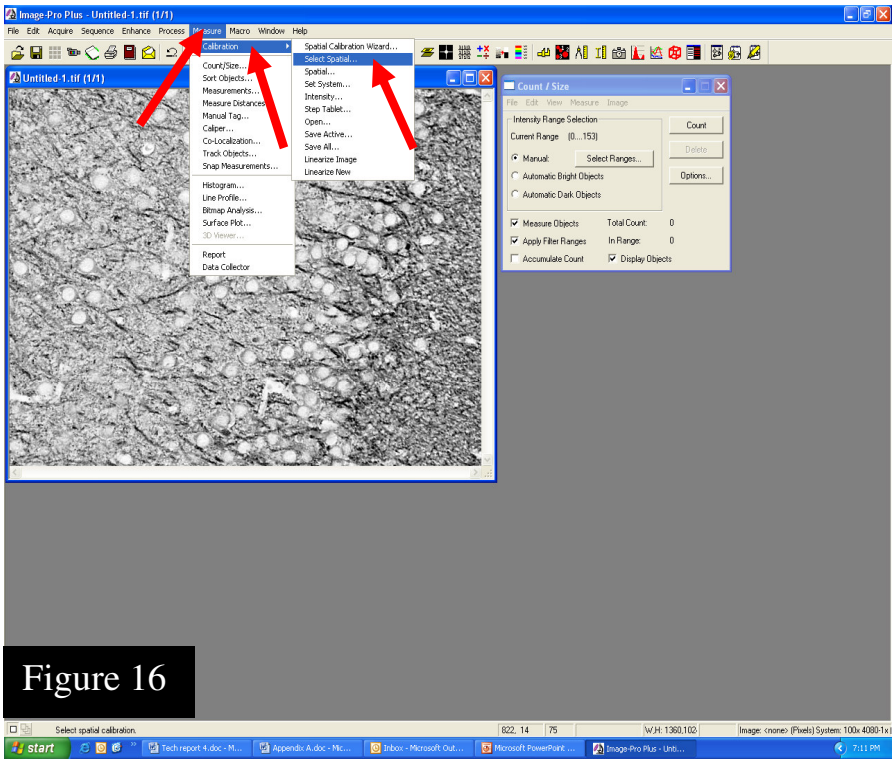


Figure 16

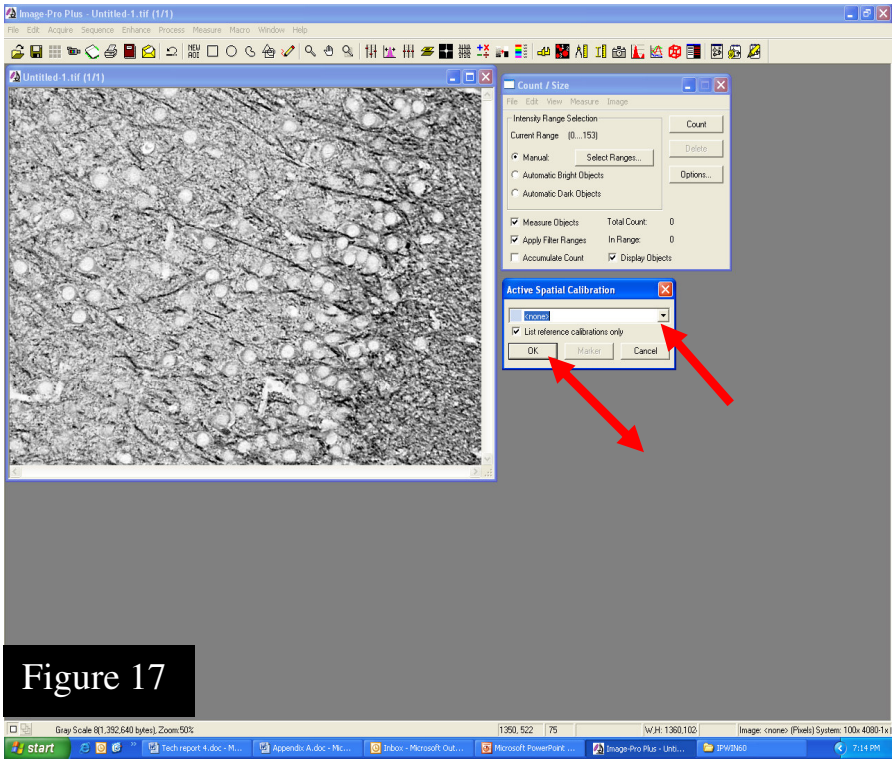


Figure 17

2. Left click the drop down menu (arrow) in the Active Spatial Calibration window as shown in Figure 17 to select the appropriate magnification of the objective and resolution of the image that you used to acquire your images.
3. Left click OK (double arrow) to close the Active Spatial Calibration window as shown in Figure 17.

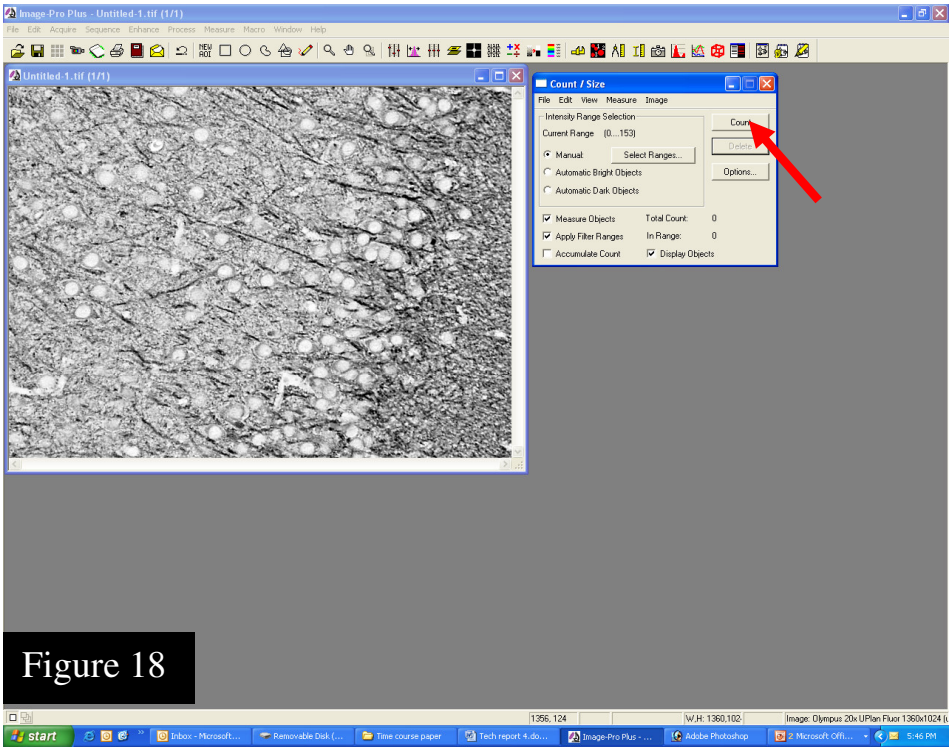


Figure 18

4. Left click Count (arrow) in the Count/Size window as shown in Figure 18. Figure 19 shows that highlighted immunolabeling will appear. The highlighted pixels represent the total area occupied by immunolabeling.

If the highlighted image does not look like Figure 19, then left click Option in the Count/Size window. The display options, Outline Style, Label Style and Label Color should be set at Filled, None and Red, respectively. Left Click OK to close the Count/Size Option window.

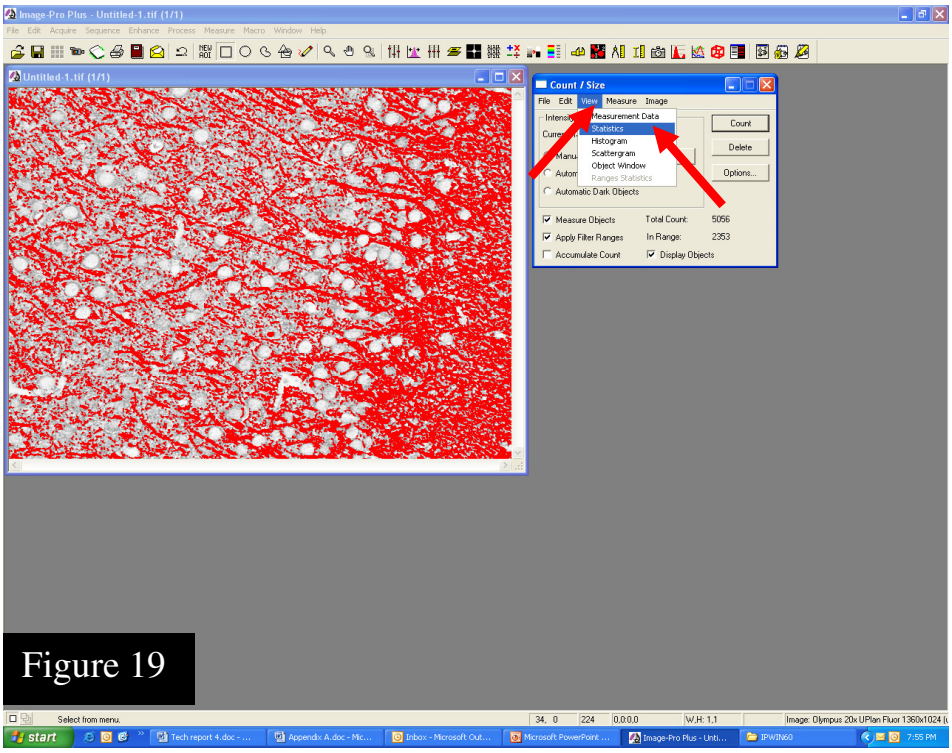


Figure 19

5. To obtain a numerical value of the total immunolabeled area, left click View/Statistics (arrows) in the Count/Size box as shown in Figure 19. Figure 20 shows that the Statistics window will appear.

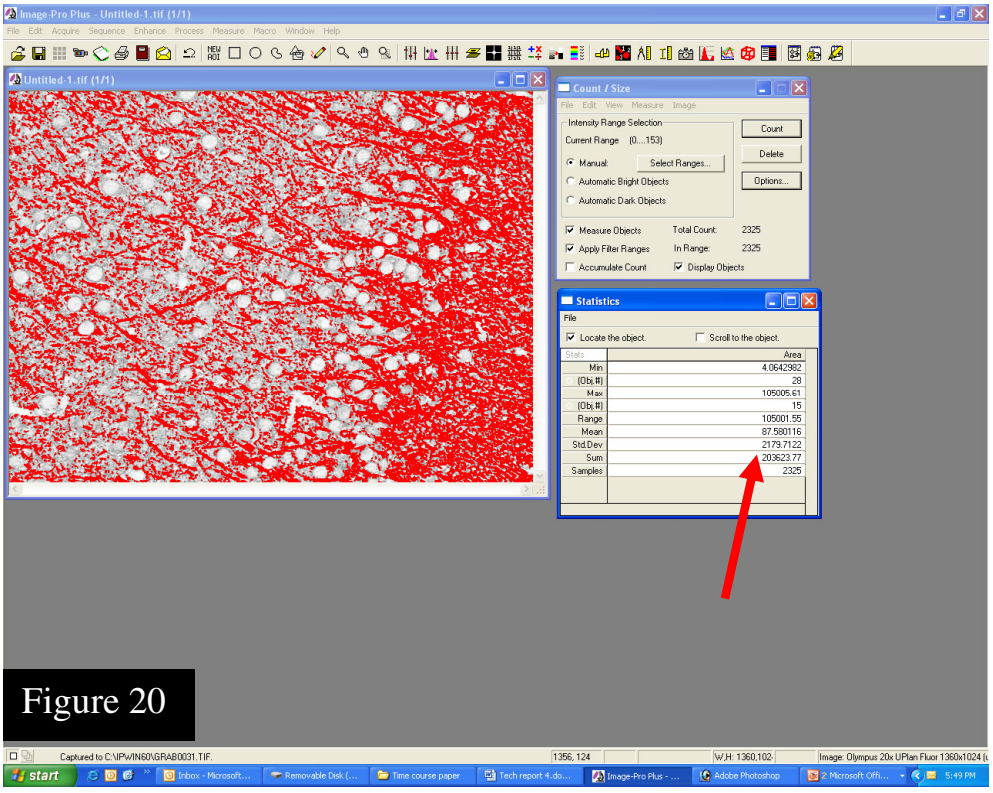


Figure 20

6. The numerical value of the total area occupied by immunostaining is showing in Row 8 (arrow) in the Statistics window.

7. Left click File/Export data (arrow) in the Statistics window as shown in Figure 21 to export the statistical data into Excel .

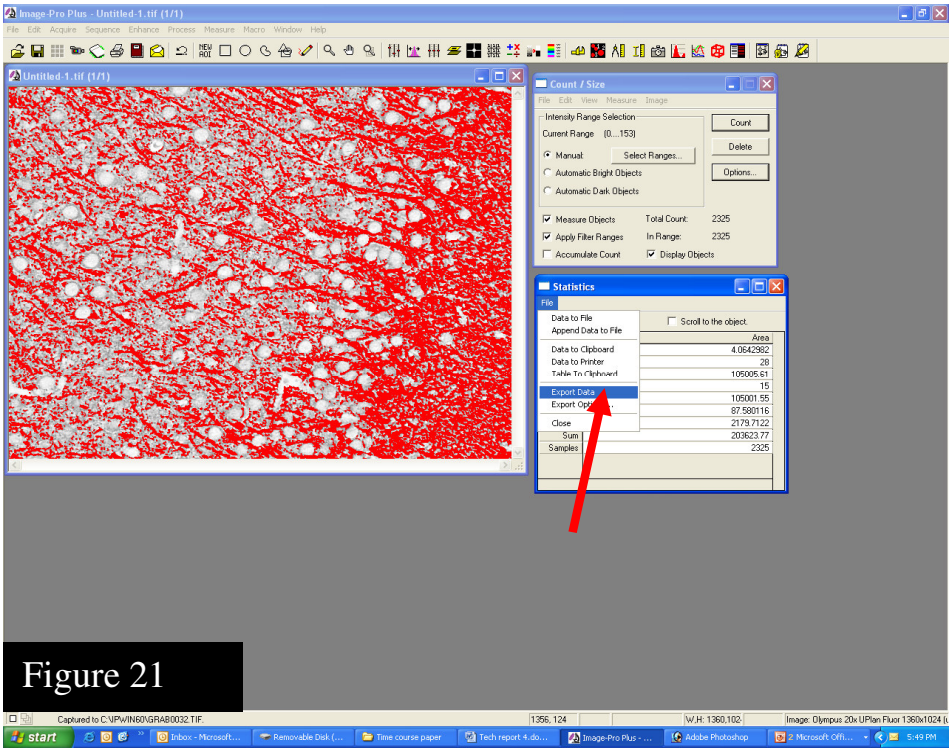


Figure 21