Quantification of Immunohistochemistry on Adjacent Sections Comparing Fluorescent and DAB Markers

Baun, J., Zurhellen, C., York, T., Tipton, B., Switzer III, R.C.

NeuroScience Associates, 10915 Lake Ridge Drive, Knoxville, TN 37934

ABSTRACT

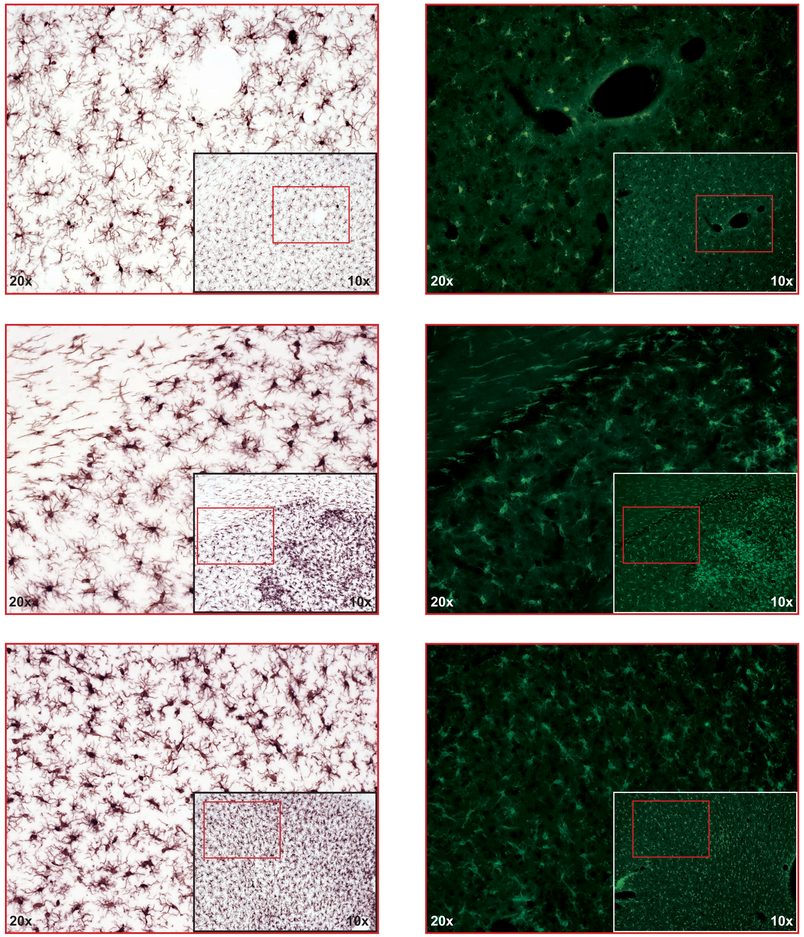
The location of antibody binding sites in brain tissue sections is commonly detected with fluorescent markers or with colored reaction products such as diaminobenzidine. We sought to answer the question: Do both types of detection schemes reveal the antibody binding sites to the same extent as judged by density of staining?

Adjacent sections of tissue were stained with several antibodies, including GFAP for astrocytes and Iba1 for microglia from rat brains that were unilaterally rendered ischemic by middle cerebral artery occlusion. Sets of adjacent sections were immunohistochemically stained free-floating using standard IHC methods. For one set, the antibody binding site was detected using a secondary antibody conjugated with a fluorescent molecule. The second set was stained using the sequence of a secondary antibody conjugated with a biotin molecule, followed by an avidin-HRP complex and then reacting with diaminobenzidine and H_2O_2.

Two areas of interest were digitally captured for each detection scheme/stain: An ischemic area in one hemisphere and an unaffected area in the other hemisphere. The same region of interest for each stain was analyzed on adjacent sections to rule out any random differences in brain areas. Images of both the DAB and fluorescent stains were converted to 8-bit grayscale. The 8-bit grayscale images of fluorescent-stained tissue were inverted to mimic the 8-bit grayscale images of the DAB images. All 8-bit grayscale images were converted to a binary image for densitometric analysis. Densitometry (measured as the percent area occupied by signal) was performed to determine any differences between the DAB and fluorescent images. The percent area in both the ischemic and normal areas of interest were analyzed in both DAB and fluorescent images for each stain.

There were dramatic differences observed between the two staining methods. In both the GFAP and Iba1 stains there were obvious variations in staining intensity in both the affected and normal brain regions. This is not surprising as there is considerably greater amplification of the antibody presence using the DAB multiple stage sequence vs. the “single” stage fluorophore-secondary antibody sequence. Nonetheless, the degree of hypertrophied astrocytes and microglia seen with the fluorescent method is meager compared to the images using the DAB sequence.

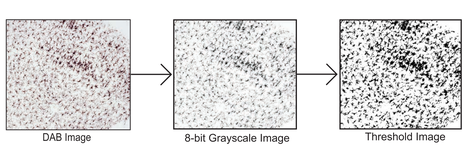
Iba1

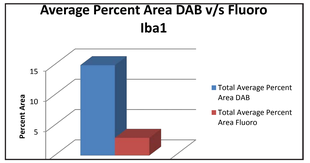


Shown above are exemplary DAB and fluorescent Iba-1 images that were processed for densitometric analysis. Adjacent sections were stained (using either DAB or fluorescent protocols) and corresponding regions were imaged from those adjacent sections for DAB/fluorescent comparison.

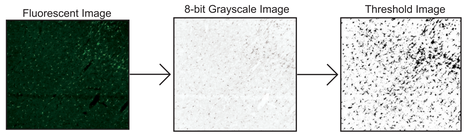
Methods

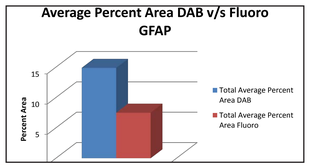
Images were captured at 10x for both DAB and fluorescent stained adjacent sections. Those images were then converted to 8-bit grayscale. The 8-bit grayscale images of fluorescent-stained tissue were inverted to mimic the 8-bit grayscale images of the DAB images. All 8-bit grayscale images were converted to a binary image for densitometric analysis. A percent area was then calculated for each image.





Original Images



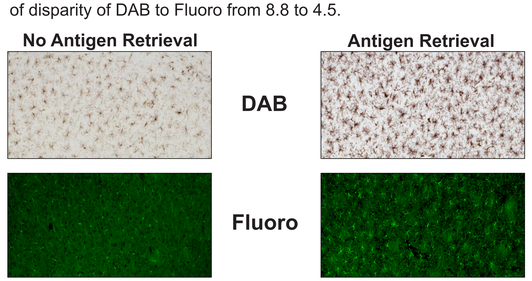


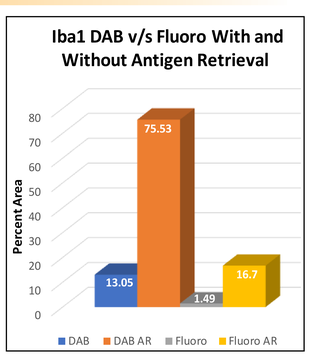
Conclusion

There was a significant percent area difference observed between the fluorescent and DAB images, analysis of these images provided further evidence of a staining trend. In the Iba1 stain, percent area occupied with DAB staining was 14.9% while the fluorescent staining was 2.9%, nearly 80% difference. A similar trend was observed in the GFAP staining, DAB resulted in 14.8% while fluorescent staining resulted in 7.5%, nearly 50% difference. Fluorescent staining is necessary in some instances such as when colocalization is known or expected, however if total percent area is to be measured in an experiment, the difference between traditional DAB and fluorescent staining should be considered.

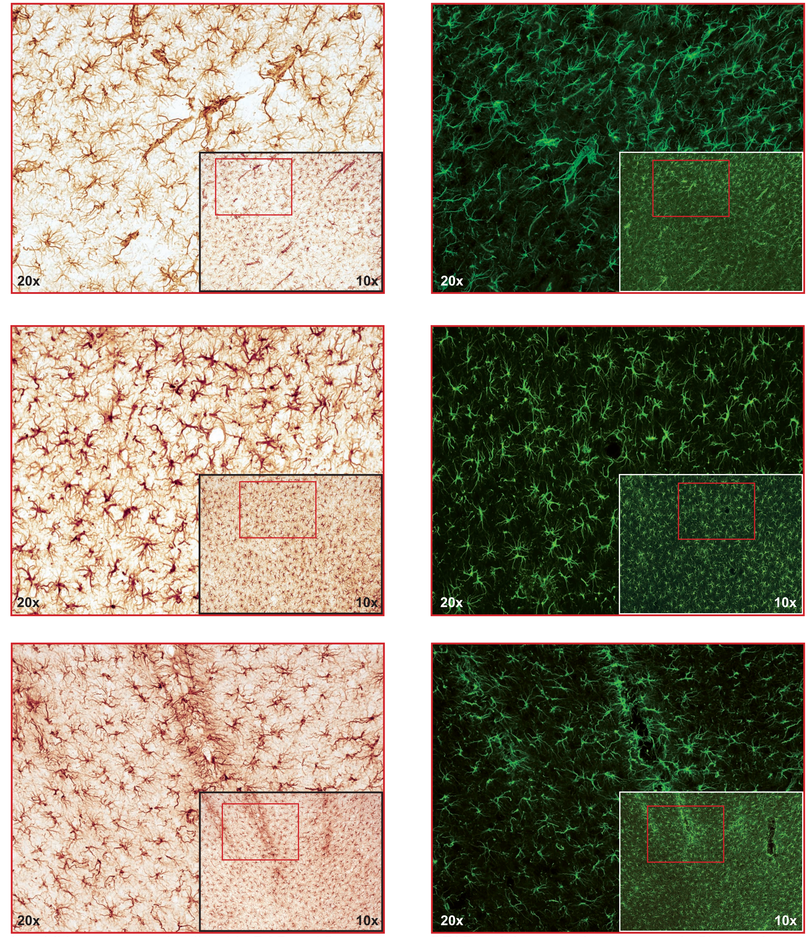
• SUPPLEMENT •

The striking difference between resultant staining with DAB vs Fluoro tags may be due to differences in amplification. Possible enhancement using Antigen Retrieval methods was explored and changed the ratios of disparity of DAB to Fluoro from 8.8 to 4.5.





GFAP



In addition to comparing Iba-1 in DAB and fluorescence, GFAP staining was also measured. Shown above are exemplary DAB and fluorescent GFAP images that were processed for densitometric analysis. Adjacent sections were stained (using either DAB or fluorescent protocols) and corresponding regions were imaged from those sections for DAB/fluorescent comparison.