

# AR INSIGHTS

## Quantitative Analysis of Histological Staining and Fluorescence Using ImageJ

### BACKGROUND

An important objective for scientists is to statistically compare staining intensity (Fig. 1) or fluorescence (Fig. 2) for a particular marker between treatments or groups. Simply “eyeballing” an image and stating that a particular treatment or group is more densely stained or brightly fluorescent than another treatment or group is insufficient for scientific publications. Systems are available for image analysis in immunohistochemistry. However, many of these systems require expensive software and hardware attachments for acquisition, analysis, and storage of images. Therefore, an inexpensive and reliable alternative for image analysis is desirable.

The cost effective answer for quantitative immunohistochemical analysis is ImageJ, developed by Wayne Rasband (<http://imagej.nih.gov/ij/docs/index.html>). ImageJ is a Java image processing and analysis program based on NIH Image for the Macintosh. ImageJ is available in the public domain (i.e., free). ImageJ runs on any computer that is a Java 1.5 or later virtual machine. Java runtime environments are available for free from Sun Microsystems or bundled with platform-specific installations of ImageJ ([rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)). Downloadable copies are available for Windows, Linux, and Mac OSX. For the evaluation of immunohistochemistry slides using ImageJ, images are captured onto the hard drive of the workstation computer. Thereafter, captured images are opened in NIH Image/ImageJ for evaluating indices of positivity on immunohistochemistry slides, as well as fluorescence images.

### PLUGINS FOR IMAGEJ

ImageJ's strength lies in the extensive number of plugins that extend its functionality. Hundreds of plugins are available for free downloading to ImageJ (Collins, 2007). ImageJ add-ons are available from multiple sources (see p 2 of the ImageJ user guide for suggestions). Various projects address the difficult task of using ImageJ beyond the basics. Biologists who are unsure of which distribution to choose should opt for Fiji. Another option is MBF ImageJ bundle or ImageJ for Microscopy (formerly WCIF-ImageJ), featuring a collection of plugins and macros, collected and organized by Tony Collins at the MacBiophotonics Facility, McMaster University.

### USING IMAGEJ TO ANALYZE IMMUNOHISTOCHEMISTRY

ImageJ opens the following file formats: TIFF (default format), JPEG, PNG, GIF, BMP, DICOM, PGM, and FITS. Other formats can be opened with the aid of plugins.

### Intensity Measurements

If the researcher wishes to count stained or fluoresced cell profiles, these can be counted directly from the screen by placing marks of different colors onto positive and negative nuclei by clicking the mouse. ImageJ will then automatically generate the immunohistochemistry index.

Staining can be assessed by setting a “threshold” using the thresholding tool (Fig. 3). The following information is based on a guide provided by the University of Auckland ([http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis\\_resources.aspx](http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis_resources.aspx)). The image needs to

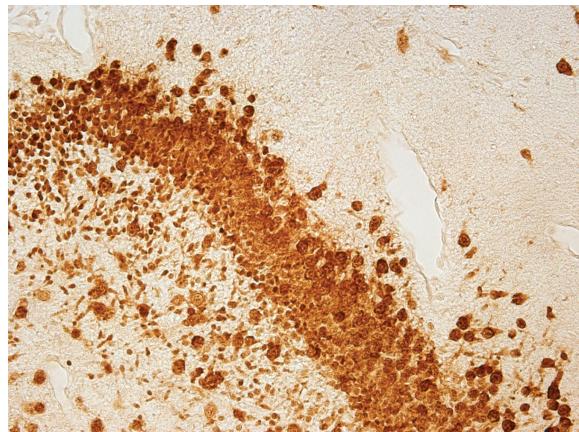


Fig. 1. A 6- $\mu$ m paraffin embedded section stained with NeuN and visualized using DAB. The image was taken using a Zeiss Axioscope microscope equipped with a Zeiss HRC microscope camera at 2.5 $\times$ . The image shows the dentate gyrus in the hippocampus of a 0.7 gestation fetal sheep after 25 min of umbilical cord occlusion. In this example, the researcher may statistically determine the number of stained cells, etc, to compare the results with other treatment or control groups. The image was kindly provided by Dr. Robert Barrett, PhD, from the University of Auckland.



be opened and then the following steps applied. (1) Select “Image–Adjust–Threshold.” The auto setting can be selected or the sliders can be manually moved until all the stained areas are selected. A histogram is displayed to provide assistance. (2) Choose “Dark background” for fluorescence. (3) Click on “Set” to set the threshold of the image. (4) Select “Analyze–Set

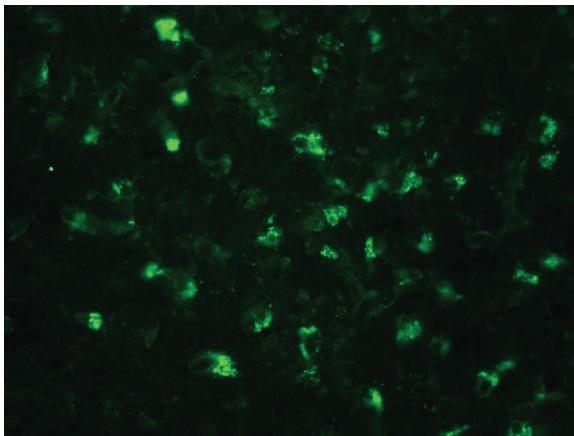


Fig. 2. A 6- $\mu\text{m}$  paraffin embedded section stained with IB4 and Alexa Fluor 468-conjugated antibody (green). The 40 $\times$  image was taken on a Nikon i90 microscope with a motorized stage. The image shows gyral white matter of a 0.7 gestation fetal sheep 3 days after a 25-min umbilical cord occlusion. In this example of a fluorescent image, the researcher may statistically determine the amount of fluorescence and compare the results with a control group. The image was kindly provided by Dr. Robert Barrett, PhD, from the University of Auckland.

Measurements” and choose the parameters to be measured. Ensure that all the gray level measurements are selected. The “Limit to Threshold” option should also be selected, because otherwise the entire image will be measured, rather than the selected area. (5) Select “Analyze–Measure.” The results table will appear, which can then be saved. “Analyze–Analyze Particles” can be used to measure individual feature profiles. Intensity measurements can also be performed within regions of interest (ROIs; see below).

To compare multiple specimens, staining and image acquisition should be performed in parallel for the entire set. Identical reagents and processing should be used, with identical image acquisition settings and exposure times. Thresholding tool settings that successfully quantify staining in a positive control specimen should then be duplicated in every image to be compared. When staining intensity is being quantified, a uniform background is required in all areas of the image. One method to correct for uneven illumination in fluorescence images has been previously described (Model and Burkhardt, 2001). This is particularly recommended when intensity is measured for an entire image or when differing portions of separate images are to be compared.

## ROI

A specific ROI can be analyzed rather than the whole image. A useful feature of ImageJ is the ROI Manager that allows selection of specific areas for evaluation, or deselecting areas of unwanted elements (Fig. 4) (Girish and Vijayalakshmi, 2004). These steps are achieved by

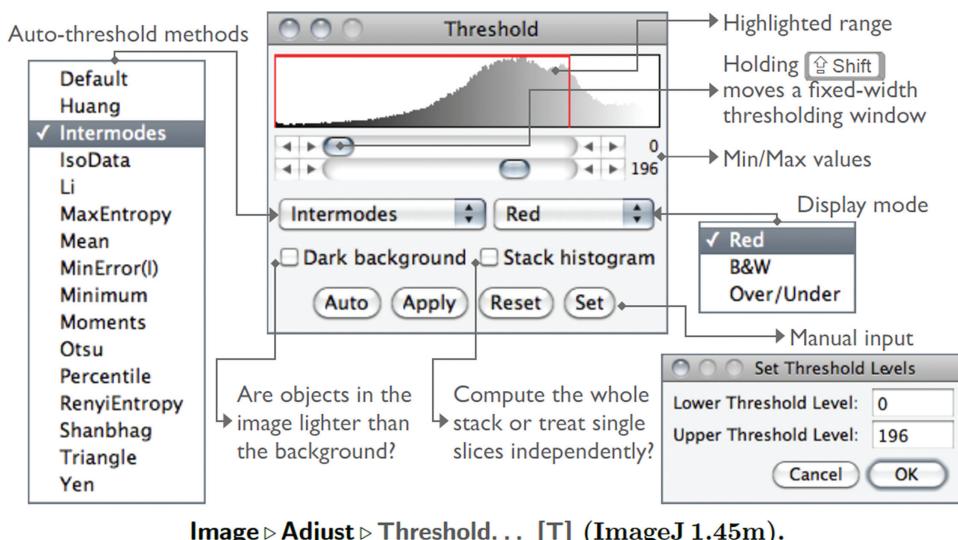
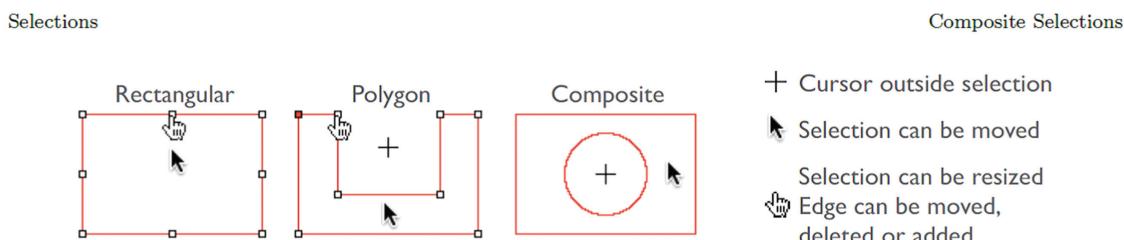


Fig. 3. Snapshot of ImageJ user guide showing the threshold feature (p 79).

drawing the ROI, using one of the drawing tools (e.g., freehand or ellipse tool) (Fig. 5). Subsequent steps are: (1) open the image and draw the ROI; (2) select “Analyze–ROI Manager”; (3) select “Analyze–Set

Measurements” to select the parameters to measure; and (4) click on “Measure” in the ROI Manager window. The results will appear in a Results window as Excel files. If “Analyze–Measure” is selected instead of using



**Three types of area selections In ImageJ.** Notice the cursor changes: to an *arrow* when it is within the selection, to a *cross-hair* when outside the selection, to a *hand* when over a selection vertex or ‘handler’. Notice also the filled handler in the polygon selection and the absence of point handlers in Composite Selections. Overlays, i.e., non-active selections displayed in the non-destructive image overlay, are also displayed without handlers.

Fig. 4. Snapshot of ImageJ user guide showing types of area selections of an ROI (p 18).

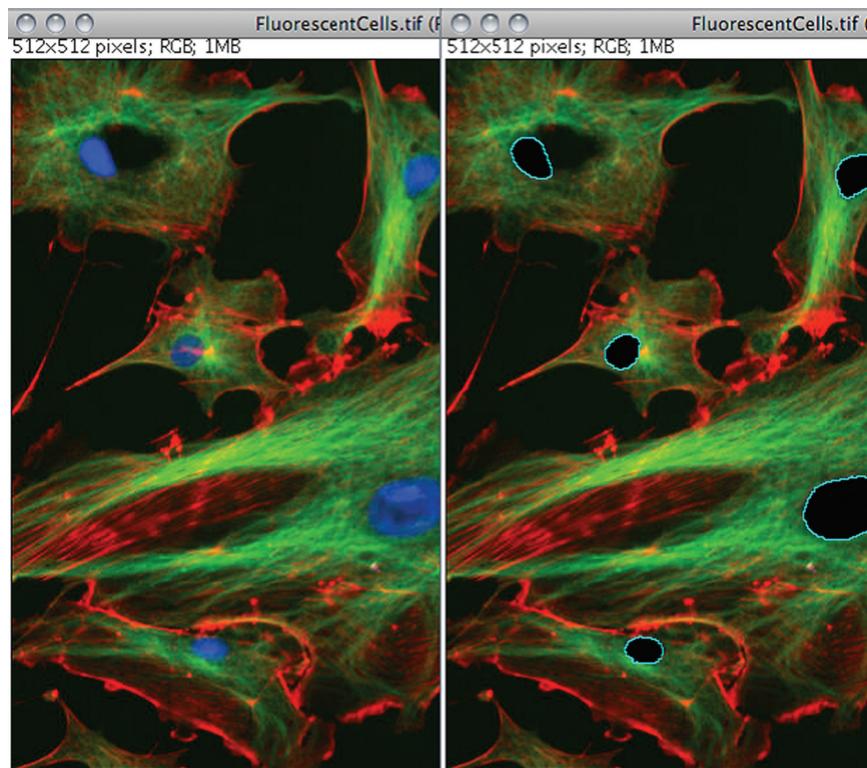


Fig. 5. Snapshot of ImageJ user guide showing selected ROIs drawn on an image (p 80).

the ROI Manager, then results for the entire image will be obtained instead of only the ROI.

### Issues of Selection Bias

For examining a particular organ, tissue, or cell type, microscopic sections should be selected to provide a representative sample of the whole sample (Hsia et al., 2010). Selecting specific samples or sections fails to provide equal opportunity for analysis and misses the effect of regional heterogeneity. This leads to biased conclusions. A sampling scheme should be used that includes all regions to avoid selection bias.

A reference space in which the label is counted is required (e.g., number of cells per unit volume or area, total cells, etc.). A reference space should be defined at each level of magnification (Hsia et al., 2010).

### ONLINE SITES AND HELP FOR IMAGEJ

Help resources that complement the ImageJ guide are listed in the guide (p 6). If a user is having problems with ImageJ, various mailing lists are provided in the guide. Below is also a small list of websites that describe how to measure histological staining/fluorescence using Image J.

- The ImageJ homepage: <http://imagej.nih.gov/ij/>
- The User Guide for ImageJ is available at: <http://imagej.nih.gov/ij/docs/index.html>
- Overall guide covering intensity analysis: [http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis\\_resources.aspx](http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis_resources.aspx)
- For measuring cell fluorescence: <http://scientechnetblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej/>
- <http://lab.methodmint.com/methods/37/measuring-cell-fluorescence-using-imagej/>
- ImageJ mailing lists: <http://imagej.nih.gov/ij/list.html>  
<http://imagejdev.org/mailing-lists>

For additional information on the ROI Manager, see the following websites.

- [http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis\\_resources.aspx](http://www.fmhs.auckland.ac.nz/sms/biru/facilities/analysis_resources.aspx)
- <http://scientechnetblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej/>
- <http://lab.methodmint.com/methods/37/measuring-cell-fluorescence-using-imagej/>

### ACKNOWLEDGEMENT

The author thanks Dr. Robert Barrett, PhD, for providing images, and Damon Shutt for advice on ImageJ.

Ellen C. Jensen\*  
*The Anatomical Record*

### LITERATURE CITED

- Collins TJ. 2007. ImageJ for microscopy. *BioTechniques* 43:S25–S30.  
Girish V, Vijayalakshmi A. 2004. Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer* 41:47.  
Hsia CCW, Hyde DM, Ochs M, Weibel ER on behalf of the ATS/ERS Joint Task Force on the Quantitative Assessment of Lung Structure. 2010. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med* 181:394–418.  
Model M, Burkhardt J. 2001. A standard for calibration and shading correction of a fluorescence microscope. *Cytometry* 44:309–316.

\*Correspondence to: Ellen C. Jensen, PhD, 35 Southern Cross Road, Kohimarama, Auckland 1071, New Zealand. E-mail: ellen\_knapp2004@yahoo.com.au

Received 20 July 2012; Accepted 5 November 2012.

DOI 10.1002/ar.22641

Published online 4 February 2013 in Wiley Online Library (wileyonlinelibrary.com).