

Semi-quantitative Determination of Protein Expression Using Immunohistochemistry Staining and Analysis

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[Abstract] Semi-quantitative immunohistochemistry (IHC) is a powerful method for investigating protein expression and localization within tissues that involves using software, such as the freely available Fiji (ImageJ), to conduct deconvolution and downstream analysis. Currently, there is lack of an integrated protocol that includes a detailed procedure on how to measure or compare protein expression. Publications that use semi-quantitative methods to ascertain protein expression often don't provide enough details in their methods section, which makes it difficult for the reader to reproduce their data. The current protocol provides an example and detailed steps of conducting semi-quantitative analysis of IHC images using Fiji software.

Keywords: Immunohistochemistry, Semi-quantification, Fiji ImageJ, DAB staining, Hematoxylin

[Background] Semi-quantitative immunohistochemistry (IHC) has been widely used for investigating protein expression and localization within tissues (Matkowskyj et al., 2000; Cregger et al., 2006; Taylor and Levenson, 2006; Braun et al., 2013; Fedchenko and Reifenrath, 2014; Bauman et al., 2016; Pike et al., 2017; Lu et al., 2018; Crowe et al., 2019), and various software and methods have been used in semi-quantitative IHC to conduct deconvolution and downstream analysis. Some methods require advanced coding/mathematical experience or the use of subscription-based software that may not be feasible for every scientist (Matkowskyj et al., 2000; Shu et al., 2016; Guirado et al., 2018). Fiji (ImageJ) is a freely available software (version 1.2; WS Rasband, National Institute of Health, Bethesda, MD). Publications using ImageJ for image quantification are often directed towards the creation of specific plugins for ImageJ or lack a step-by-step protocol (Varghese et al., 2014; Chen Y, 2017; Guirado et al., 2018). However, there is no integrated protocol that includes a detailed but simple procedure of conducting such steps, including the use of ImageJ software. There are many open online forums where users post their questions about using Fiji ImageJ for image quantification purposes. This is often due to publications not providing sufficiently detailed methods sections, which makes it difficult for the reader to reproduce the data. It is also time-consuming for researchers who are not initially familiar with ImageJ software to figure out how to use this software. The current protocol provides an example for conducting semi-quantitative analysis of IHC images using ImageJ.

Equipment

Computer Specifications:

1. A 64-bit operating system that has Windows 7 or greater, Mac OS X 10.11 or greater, or Linux with kernel supporting GLIBC 2.14 and GLIBCXX 3.4.15 (typically kernels 2.6.39)
2. NVIDIA graphics card (GeForce, Quadro, or Tesla) with CUDA capabilities 2.0 or greater, see <https://developer.nvidia.com/cuda-gpus> for more details
3. Need up-to-date NVIDIA drivers (minimum version of 369)
4. Any computer with Java-based operating system and Excel available

Software

1. Free ImageJ Fiji software (Johannes Schindelin, Albert Cardona, Mark Longair, Benjamin Schmid, and others, <https://imagej.net/Fiji/Downloads>), version 1.2 (no specific plugin was used)

Procedure

A. Staining of tissue using immunohistochemistry procedure

1. Reference to [the](#) procedure used for the staining of cells for the IHC protocol can be found in a previously published manuscript (Crowe *et al.*, 2019).
2. Brief protocol for immunohistochemistry staining:
 - a. Cut FFPE tissue blocks into 4 μm sections and mount on positively charged slides.
 - b. Deparaffinize and rehydrate tissue.
 - c. Retrieve antigen in buffer of choice (citrate buffer at pH 6 [was](#) used for this protocol).
 - d. Block antigen with normal goat serum and incubate with primary antibodies (*i.e.*, anti-OATP1B1 for this protocol).
 - e. Incubate with horseradish peroxidase (HRP)-conjugated secondary antibody and visualize by 3,3'-diaminobenzidine (DAB) to detect the protein of interest (OATP1B1 in the current protocol).
 - f. Stain nuclei with hematoxylin.
 - g. Hematoxylin and eosin (H&E) staining is not necessary for this protocol, but is useful when looking at the pathology of the tissue.

B. Image exporting and saving

The microscope should be set up correctly (*i.e.*, white balanced) before acquiring new data for the ImageJ default values to work. Export and save the raw immunohistochemistry (IHC) immunohistochemistry images [as](#) a .tiff file format. Tiff format for images is preferred to prevent the loss of raw data and associated metadata.

- C. **Using Fiji for deconvolution** of the IHC image.
1. Download and open Fiji software.
 2. Click the “File” option and click “Open”. The IHC image will open up on the computer screen.
 3. Click on the IHC image to make the image active.
 4. Click the “Image” option and select “Color” > “Color Deconvolution.”
 5. A new pop-up Color Deconvolution window will show up. For IHC images stained with 3,3'-diaminobenzidine (DAB) and hematoxylin (H), select the “H DAB” vector option. Leave “Show Matrices” and “Hide Legend” unchecked and click “Okay.” (Figure 1).

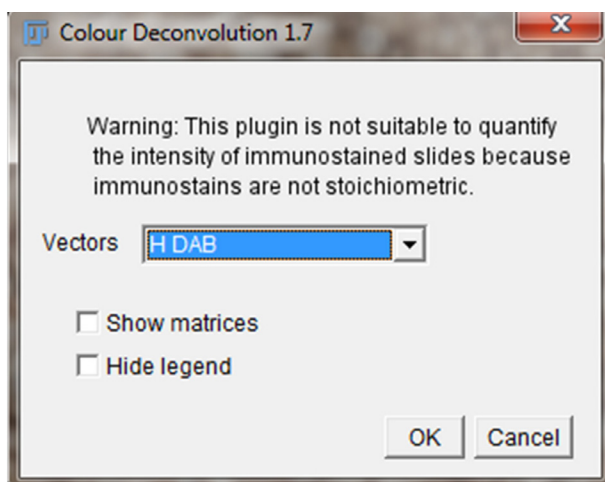


Figure 1. Color Deconvolution Window. The Color Deconvolution Window will be used to separate the staining of the IHC image. The H DAB vector separates the IHC image into DAB staining (brown staining) for the protein of interest and Hematoxylin (H) staining for the nucleus.

6. After selecting the H DAB option, three different images will pop up on the computer screen. Color 1 window represents only the Hematoxylin staining (blue/purple) and Color 2 window represents only the DAB staining (brown) (Figure 2).

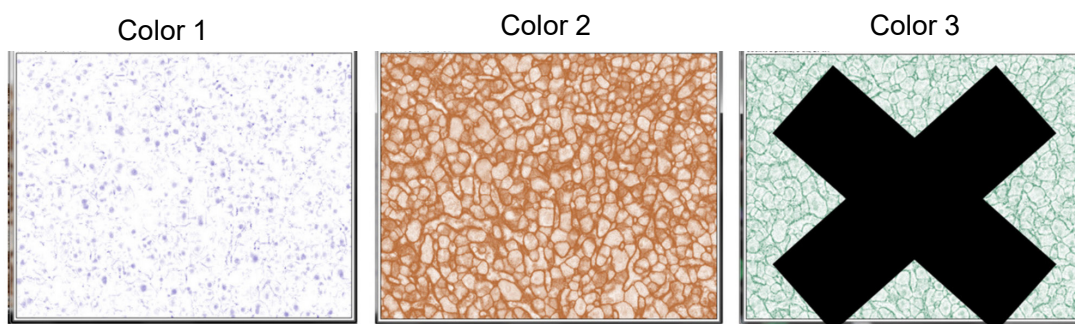


Figure 2. Deconvolution of IHC image. Separation of the IHC image into hematoxylin staining for the **nuclei** (Color 1) and DAB staining for OATP1B1 protein expression (Color 2) in hepatocytes **within** human liver tissue. Color 3 panel is for another staining, if applicable. For DAB and hematoxylin staining **alone**, the Color 3 panel can be **eliminated**. In DAB and

hematoxylin staining, as in current studies, a warning “X” is shown so that users understand that the amount of antibody staining (*i.e.*, DAB staining) in this case cannot be mathematically quantified, as it is not stoichiometric.

- Exit out of the Color 3 window, as this will not be needed for the image analysis.

D. Thresholding the DAB-stained IHC image

- Click on the DAB Color 2 image to activate it. DAB staining represents your primary antibody of interest. In this case, liver tissue was incubated with an OATP1B1 primary antibody, and the OATP1B1 expression is detected by DAB.

- Go to “Image” and select “Adjust” and “Threshold”. After selecting the threshold, the brown image is converted into a black and white binary mask image, where all the values above the threshold are converted and represented in white. The background is black.

Note: A shortcut to threshold the image is achieved by pressing “Ctrl” + “Shift” + “T”.

- A new threshold window will pop up. The top bar indicates your minimum threshold value and the bottom bar indicates your maximum threshold value (Figure 3).

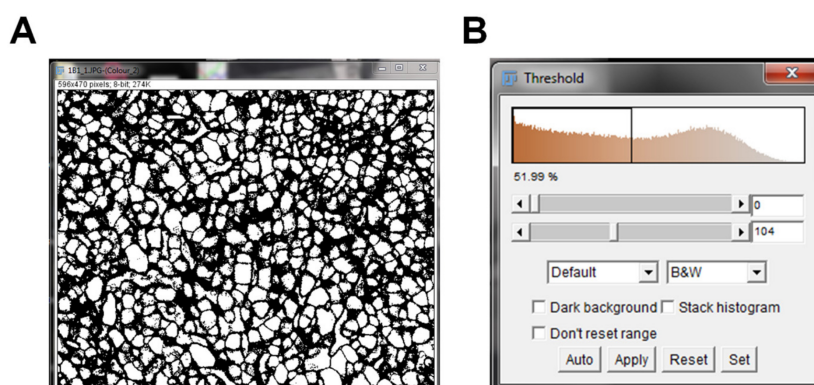


Figure 3. IHC image pre-threshold. IHC image converted to black and white pixels prior to thresholding the image (A). The threshold pop up window pre-threshold indicates the baseline threshold values (B).

- Leave the minimum threshold value set at zero.
- Adjust the maximum threshold value so that the background signal is removed, without removing the true DAB signal (Figure 4A). This is an arbitrary value, since it is set by the user. The maximum threshold value should be tested for at least five images to obtain an average maximum threshold value. Once the maximum threshold value is chosen, this will be set for all future IHC images. The measured threshold will only remain relevant provided that the microscope acquisition settings (color balance, exposure time, magnification, light source, camera vendor, etc.) remain constant throughout future experiments.
- Once the maximum threshold image is set, click “Apply” on the threshold window. After clicking

apply, the minimum and maximum threshold values will be 255 (Figure 4B).

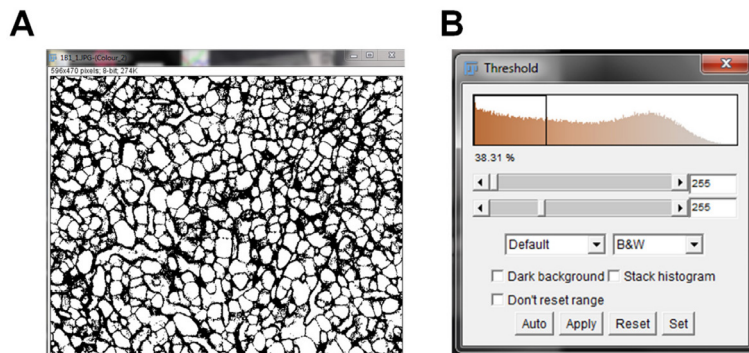


Figure 4. IHC image post-threshold. The background for the DAB staining in the IHC image was removed by adjusting the maximum threshold value (A). The minimum and maximum threshold values were set and applied (B).

E. Quantifying the DAB signal area in the IHC image

1. Go to “Analyze” and select “Set Measurements”.
2. A “Set Measurement” pop up window will open. Select the “Area”, “Mean grey value”, and “Display Label” boxes, and leave all other boxes unchecked. “Area” will give the size of the IHC image. “Mean grey value” represents the quantified signal, and “Display Label” gives the information on the image name being quantified (Figure 5A).
3. Select “Okay” in the Set Measurement window. These options only need to be set once for the first image, and will be remembered for all other future images measured.
4. Go to “Analyze” and select “Measure”.

Note: A shortcut for measuring the signal area is “CTL + M”.

5. A “Results” window will pop up giving the name of the image (Label), size of the image (Area), and the average pixel intensity of the thresholded image as an indirect way to measure staining area (Mean) (Figure 5B).
6. Export results for later by clicking File>Save, as to avoid copy/paste errors.
7. Exit out of the Color 2 DAB-stained image.

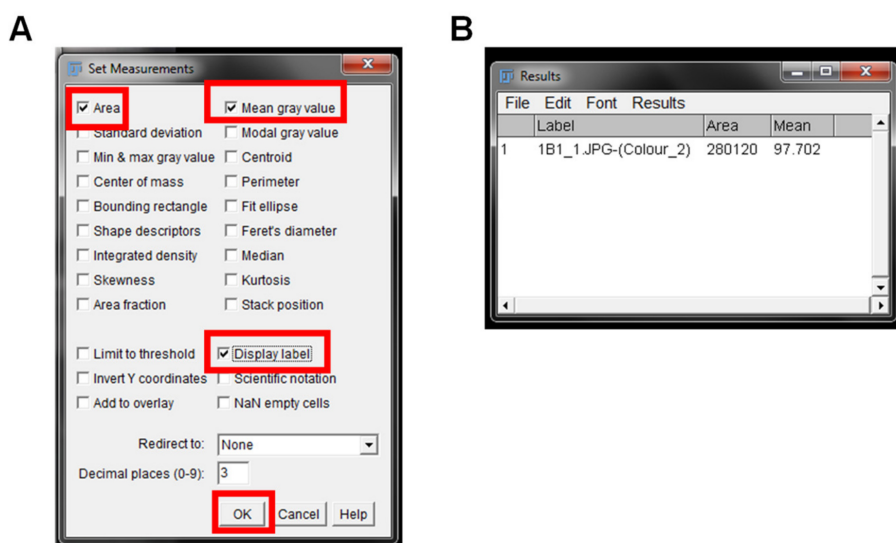


Figure 5. Measurement of DAB staining. The Set Measurements window to choose the options for output of each IHC image is shown. The suggested options for output are highlighted with red boxes (A). The Results output window is shown in (B), and contains the name of the image (Label), area of the image (Area), and mean grey value intensity (Mean).

F. Measuring the size of the nucleus

1. Click on the Hematoxylin Color 1 image to activate it.
2. Select the "Straight line" tool on the Fiji panel.
3. Measure the length of a nucleus by drawing a line across the nucleus with the Straight line tool (Figure 6A).
4. Go to "Analyze" and select "Measure".
5. A Results window will pop up with the diameter of the nucleus ("Length") (Figure 6B).
6. Measure ~10 different nuclei, using Steps F1–F5 in a representative IHC image, and take the average of these lengths to determine the average size of the nuclei. This serves as the average size of the nuclei for all IHC images from hereon. It should be done across multiple images if images are significantly different and represent multiple conditions.

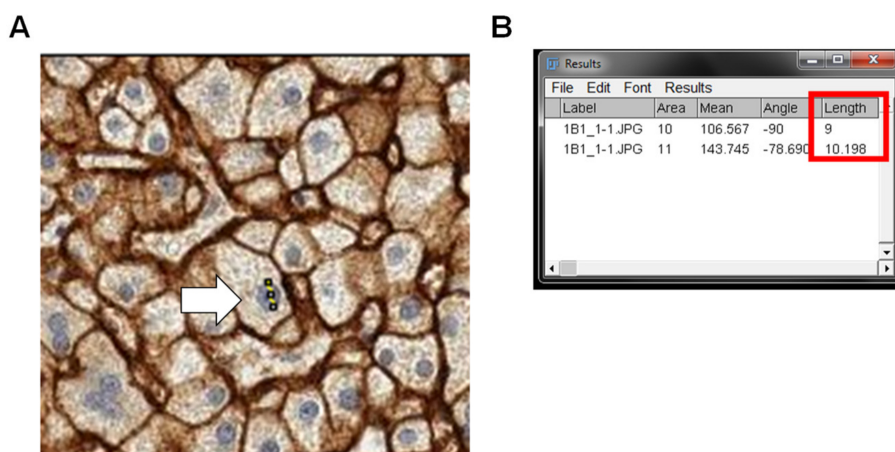


Figure 6. Measurement of nuclei size. The raw IHC image stained with DAB for OATP1B1 staining and hematoxylin for nuclei staining is used to measure the size of the nuclei. Using the straight-line tool, a line is drawn over the nucleus (A, white arrow pointing to yellow line). The distance of the line is measured by going to Analyze > Measure in the ImageJ Fiji toolbox panel. The Length (highlighted in red box) represents the diameter of the nucleus (B).

G. **Thresholding the hematoxylin-stained IHC image**

1. Click on the Hematoxylin Color 1 image to activate it.
2. Go to “Image”, and select “Adjust” and “Threshold”. After selecting threshold, the blue/purple image is converted to a black and white image.
3. Set the minimum threshold value to zero.
4. Adjust the maximum threshold value so that the background signal is removed, without removing the true hematoxylin/nucleus signal. This is an arbitrary value, since the user sets it. The maximum threshold value should be tested for at least five images to get an average maximum threshold value. Once the maximum threshold value is chosen, this will be set for all future IHC images.
5. Once the minimum and maximum threshold values for the image are set, click “Apply” on the threshold window. After clicking apply, the minimum and maximum threshold values will be 255. **Of note**, automatic thresholds **are** preferred, **since** manual thresholds must be adequately tested and **kept** constant for **all** other images in the experiment.

H. **Quantifying the hematoxylin/nucleus signal in the IHC image**

1. Select the Color 1 Hematoxylin image to activate it again.
2. Go to “Process” and select “Binary” > “Watershed”. This action will split the nuclei that are joined together into multiple nuclei.
3. Go to “Analyze” and select “Analyze Particles”.
4. An “Analyze Particles” window will pop up giving multiple options (Figure 7A).
 - a. Size (pixel²): Set the size of the nuclei to the average nuclei size measured in Step F6 to infinity (*i.e.*, 6–Infinity).

- b. Circularity: Leave it set at 0.00–1.00.
 - c. Show: Leave it set to “Nothing”.
 - d. Select “Summarize” and “Exclude on Edges” in the window. Summarize will give a summary of the particle’s measurements. Exclude on the edges means that nuclei on the outer edge of the image will not be included in the measurement.
5. Select “Okay” on the Analyze Particles window, and a Summary window with the output will pop up (Figure 7B). The output data includes:
 - a. Count indicates the number of nuclei in the IHC image.
 - b. Total area indicates the total area of the nuclei in the image.
 - c. Average area indicates the average size of the nuclei in the IHC image.
 6. Copy the results in the Summary window to the same excel file for later analysis.
 7. Exit out of the Color 1 Hematoxylin/Nuclei stained image.

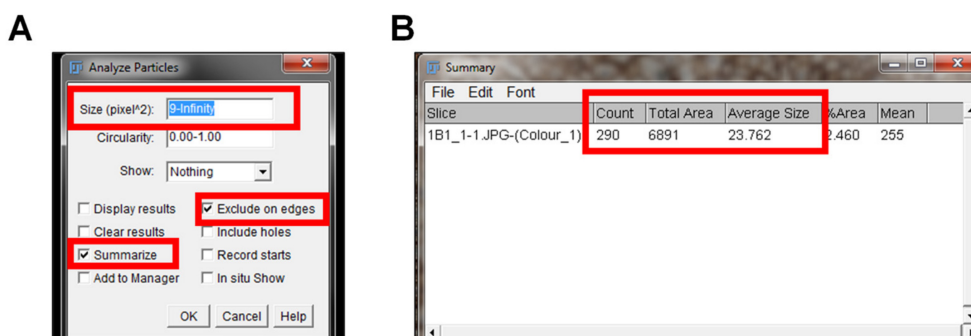


Figure 7. Quantification of nuclei in IHC image. To measure the number of nuclei in each IHC image, the Analyze Particle window pops up after selecting Analyze > Analyze Particles (A). The size of the particle measured is set to the average diameter of nuclei to infinity. The options to summarize and exclude on the edges are selected prior to clicking okay. The summarize option leads to the summarized output of the count, total area, and the average size of the nuclei particles in the IHC image (B). Exclude on the edges indicates that no nuclei particles will be included in the analysis.

- I. Semi-quantitative analysis of the IHC image
 1. Open the excel file containing the DAB and hematoxylin results outputted from the Fiji software.
 2. For each image, divide the Mean grey intensity value from Step E5 by the number of nuclei measured in Step H5. This value represents the area of DAB stain normalized to the number of nuclei.
 3. Take the average normalized area of DAB of for all IHC images for each sample/treatment, etc. to obtain an average and standard deviation values.

Data analysis

Expression of OATP1B1 and OATP1B3 were compared in genotyped human liver tissue stained with OATP1B1 or OATP1B3 DAB staining and hematoxylin. Using 79 genotyped human liver IHC samples, there was no significant difference between the genotypes for OATP1B1 (c.521 TC) polymorphism using a Student's *t*-test (Crowe *et al.*, 2019).

Application of this protocol to other studies should be **done** with caution, as it may not work for others if their microscope or staining protocols differ slightly, even if they were to **imagestain** the same protein with the same antibody. In addition, this protocol is not intended to replace the ImageJ manual, which is a great resource for **users**, and can be found at https://www.researchgate.net/publication/260261544_Analyzing_fluorescence_microscopy_images_with_ImageJ or <https://petebankhead.gitbooks.io/imagej-intro/content/chapters/rois/rois.html>.

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Competing interests

No competing financial interests for this study.

Ethics

Use of human tissues was approved by the Institutional Review Board at the University of Oklahoma Health Sciences Center. A total of 79 formalin-fixed, paraffin-embedded (FFPE) archived human liver (42 from surgical resection and 37 from liver biopsy) and normal kidney tissue blocks were obtained from OUHSC Stephenson Cancer Center Biospecimen Acquisition Core and the Bank from the Department of Pathology at the University of Oklahoma Health Sciences Center.

The authors have noticed some errors in the original protocol **that were now** amended and clarified in the current protocol. The authors apologize for any inconvenience caused.

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