Manual thresholding method for quantitation of DAB-labeled structures in whole-slide images of cervix

Purpose

Measure number and area fraction of DAB-positive cells in large areas of tissue stained for nerves with DAB and counterstained with hematoxylin.

Setup

Images

This protocol is designed for TIFF images.

Software

1. Use the Fiji package pre-loaded with plugins here (for Windows). Or, if you have downloaded regular Fiji, activate the **PTBIOP** update site (instructions). Run Fiji by double-clicking **`ImageJ-win64.exe`**.
2. Copy the **`1_cervix_preprocess_tiff_revised.ijm`** script, and the **`1_DAB_preprocess_tiff_batch.ijm`** script, available here, to your computer.

Pre-processing

Batch processing (easier):

1. Place all of your input TIFs into a folder.
2. Create an output folder to hold the split colors. This cannot be inside the input folder.
3. Open the **`1_DAB_preprocess_tiff_batch.ijm`** script in Fiji and click Run. Follow the prompts to choose input and output folders, and indicate which objective was used. It will take some time to process all the images in the folder.

Single image processing

1. In Fiji, open one TIF image.
2. Crop out empty space: If the image is very large, make a rectangular ROI to just cover the tissue of interest. *Image* > *Duplicate*.
3. When the new window appears, close the original image window to preserve memory.
4. Open the **`1_cervix_preprocess_tiff_revised.ijm`** script (drag and drop to ImageJ toolbar) and click **Run**.
    - **Input image:** Click Browse and select the original TIFF file.
    - **Output directory:** Click Browse and select where you want the output images to be saved.

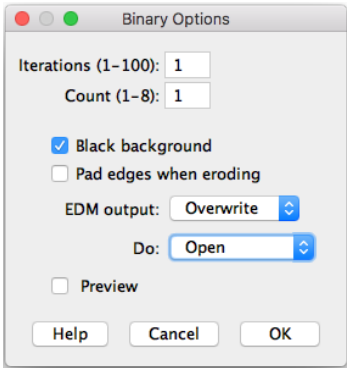
    The script will do the following for you:

- Subtract background to correct some tile artifacts
- Correct color balance
- Correct spatial calibration so area measurements will be accurate.
- Split colors and save original, hematoxylin, and DAB images in the output directory.

Detect and analyze DAB-positive areas

1. Set up measurements: *Analyze* > *Set Measurements* > check **Area**, **Limit To Threshold**, **Display Label**.
2. Set background color to white: Double-click the eyedropper tool  and click in the bottom section to set **black foreground, white background.**

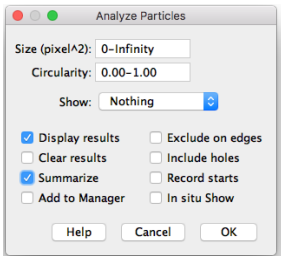
3. Open the DAB image saved in the pre-processing step.
4. If there is any nonspecifically stained area you want to exclude, draw an ROI around it and press Delete or Backspace to clear it to white.
5. *Image* > *Adjust* > *Threshold.* Uncheck Dark Background. Adjust the slider to include the positive DAB stain as much as possible. Use the + and - keys to zoom the image in and out.
6. To save a copy of the image showing the deleted areas and the thresholded areas, *Image* > *Overlay* > *Flatten* and save the new window under a different name.
7. At this point you can either measure the thresholded area directly (typical for nerve bundles) or do some additional processing (typical for single cells).
    a. For direct measurement: After setting the threshold, **proceed to step 8**.
    b. For additional processing:
        i. After setting the threshold, click **Apply** in the Threshold window.
        ii. *Process* > *Binary* > *Options*; set as below and click OK. This will remove stray pixels and clean up cell edges.



        iii. *Image* > *Adjust* > *Threshold.* Click Auto so the white areas turn red. Proceed to step 8.
8. *Edit* > *Selection* > *Select None* to get rid of any region of interest on the image.

9. *Analyze* > *Measure* (or press M). The Results window will show the **DAB-positive** area in μm^2. If you get 0 for area, make sure you don't have an ROI active from the previous step. Do *Edit* > *Selection* > *Select None* to get rid of it.

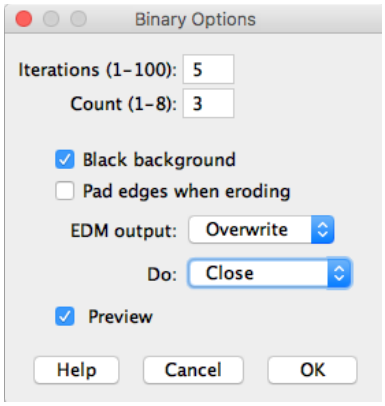
Optional: Count DAB-positive cells



1. Follow the steps for "additional processing" above. Click on the the binary (black and white) image.
2. *Analyze* > *Analyze Particles*; set as shown at right. If needed, set size limits to exclude large artifacts or small areas that are not cells. Click OK. You'll get a Summary window with the total count, and a Results window with data on each detected particle.

Measure total tissue area

1. Set background color to **black**: Double-click the eyedropper tool  and click in the bottom section to set **white foreground, black background.**
2. Open the H (hematoxylin, purple) channel saved in the pre-processing phase.
3. *Image* > *Adjust* > *Threshold.* Adjust the slider to include the tissue as much as possible. It's ok if there are tiny holes. Click **Apply** to produce a black and white image.
4. Click on the black and white image. *Process* > *Binary* > *Options*. Set parameters as shown in the screenshot. Check Preview to see the effect. Tiny holes should be mostly closed up, and large holes should persist. If you don't like the result, adjust the parameters, or re-open the H image and set a different threshold.
    - When ready, click **OK**.



5. Use the magic wand tool  to click in the tissue to select it. If it's not all outlined, hold down Shift and click on other areas to add them to the selection.
6. *Edit* > *Clear Outside.* This removes background artifacts.
7. *Edit* > *Selection* > *Select None.*
8. *Image* > *Adjust* > *Threshold.* Check **Dark Background**. Adjust the slider to include the tissue.
9. *Analyze* > *Measure* (or press M). The Results window will show the area of the **whole tissue** in μm^2.
10. To save a copy of the image showing the processed, thresholded mask, *Image* > *Overlay* > *Flatten* and save the new window under a different name.

Calculate area fraction

1. Copy the data in the Results window and paste into a spreadsheet.
2. Calculate area fraction = DAB area divided by tissue area.