ImageJ segmentation guidelines

nPAsym identifies objects of interest as arrays of black foreground pixels. Nuclei segmentation is an important early step of the procedure and its accuracy can affect output quantities. Segmentation is robust when nuclei are silhouetted sharply against the background. It is often not the case. To make segmentation efficient, the staining methods should avoid dye combinations producing absorption overlaps. It is also desirable to ensure high intensity of nuclear staining.

There is more than one way to segment an image in ImageJ, each having its own advantages and limitations. Here, we mention only one used in the test study. Raw 24-bit RGB images were thresholded using HSB (Hue-Saturation-Brightness) routine detailed below. If you are going to try another one, be sure it does not involve a look-up table (LUT) inversion. To secure proper nPAsym operation it is critical to have 0 for black foreground. Segmentation is performed as follows:

1. In ImageJ, open a raw image of interest.

2. Go to **Process** → **Filters** → **Gaussian Blur** and select **Sigma (Radius)** = 2. This step depends on the quality of a raw image and can be skipped.

3. Choose **Image** → **Adjust** → **Color Threshold**. In the lower part of the Color Threshold box select thresholding method (Default), threshold color (B&W, i.e., black and white) and color space (HSB) (Fig. 1).

4. By dragging sliders that alter values of **Hue** parameter select a blue-violet part of the histogram. Those colors dominate in the images of nuclei. The upper and lower sliders adjust, respectively, the minimum and maximum threshold values.

5. Adjust **Saturation** level by putting the upper slider somewhere in the rosy pink part of the bar. Leave the lower slider at 255.

6. Place the upper slider of the **Brightness** parameter to the left from the histogram. Leave the lower slider at 255.

7. Go to **Image** → **Type** and tick in 8-bit box.

8. Then, choose **Edit** →**Invert**. Now your image is properly segmented into nuclear masks and a background being ready for nPAsym operation.

Run nPAsym from the **Plugins** submenu.

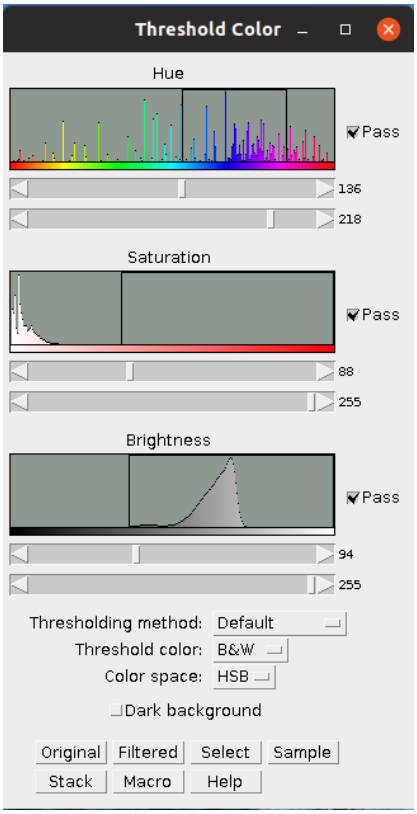

Fig. 1 Example selections made in the Color Threshold box of ImageJ while performing a nuclei segmentation routine.