Using ImageJ to analyze RNAscope® and BaseScope™ data

Introduction

This Technical Note provides guidelines for performing semi-quantitative image analysis with Fiji ImageJ on *in situ*-hybridized (ISH) slides that have been processed using the RNAscope® or BaseScope™ assays. You can use Fiji ImageJ to analyze single-plex, duplex, and fluorescent multiplex images. Duplex or multiplex image analysis requires some changes in the procedure.

Download the Fiji image processing package distribution of ImageJ using the following link: **https://fiji.sc**. The latest version of Fiji is bundled with all of the plugins used here. If you are using an older version of Image J, the Color Deconvolution and Weka Classifiers will need to be downloaded as separate plugins before performing the analysis.

Note: ImageJ has limited capability of handling large, high-resolution images. We recommend using a field of view limited to a maximum number of two gigapixels in (x,y) to count cells and punctate dots within cell boundaries.

Workflow

Below is an example of an image stained with one probe. We will use this image to demonstrate image analysis using FiJi ImageJ.

Figure 1. Single-plex image example
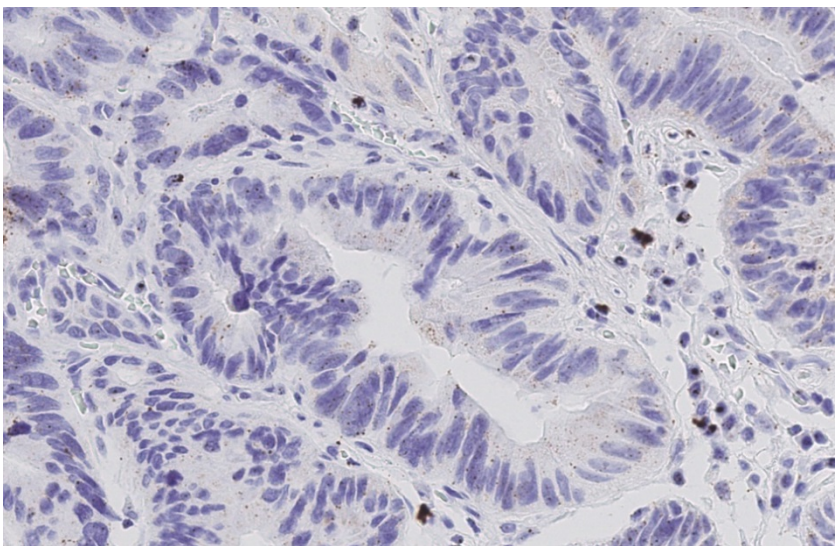
## Part 1: Counting the number of cells

Brightfield images (chromogenic assays) require a color separation step before analysis. Follow **Part1a** to separate your marker signals into different color channels, and then follow the steps in **Part1b** to perform analysis.

For **fluorescent image analysis**, skip **Part1a** and begin directly with **Part1b**.

Part 1a: Color Deconvolution

Follow the steps to use color deconvolution to separate the cells from other marker signals.

1. From the ImageJ drop-down menu, select **File > Open**.
2. Select the appropriate image and click **Open**.
3. Find a region in the image to select a distinct nuclear stain and a marker stain.
4. Zoom into the region of choice using the magnifying glass tool.
5. Select the rectangle tool. From the ImageJ drop-down menu, select **Image > Color > Color Deconvolution**.
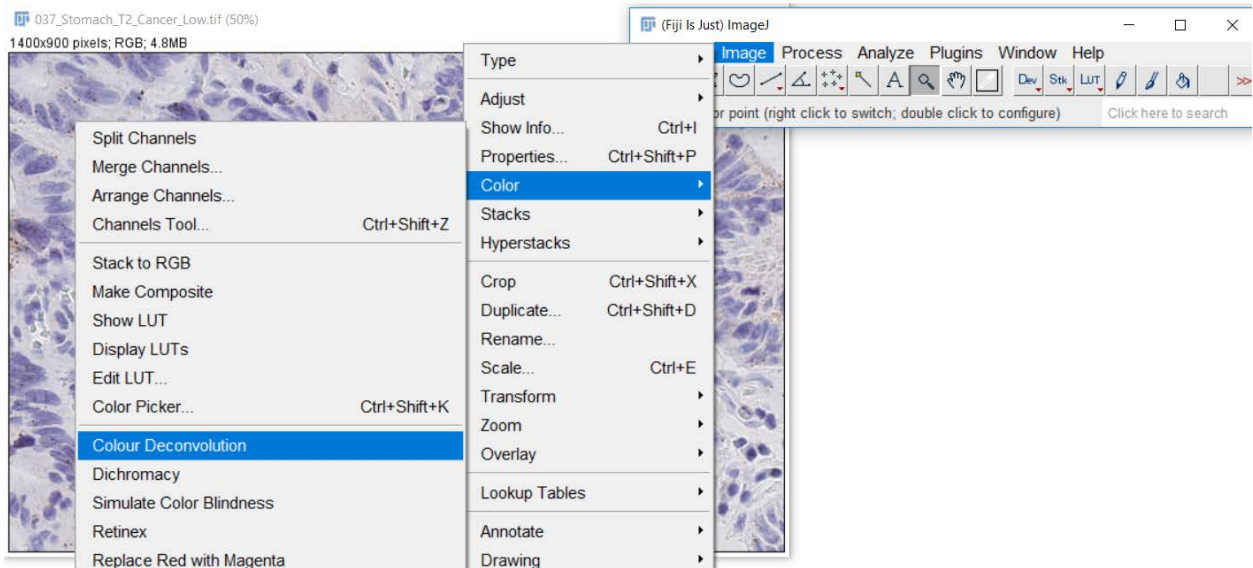
6. From the Options menu, select **From ROI**.
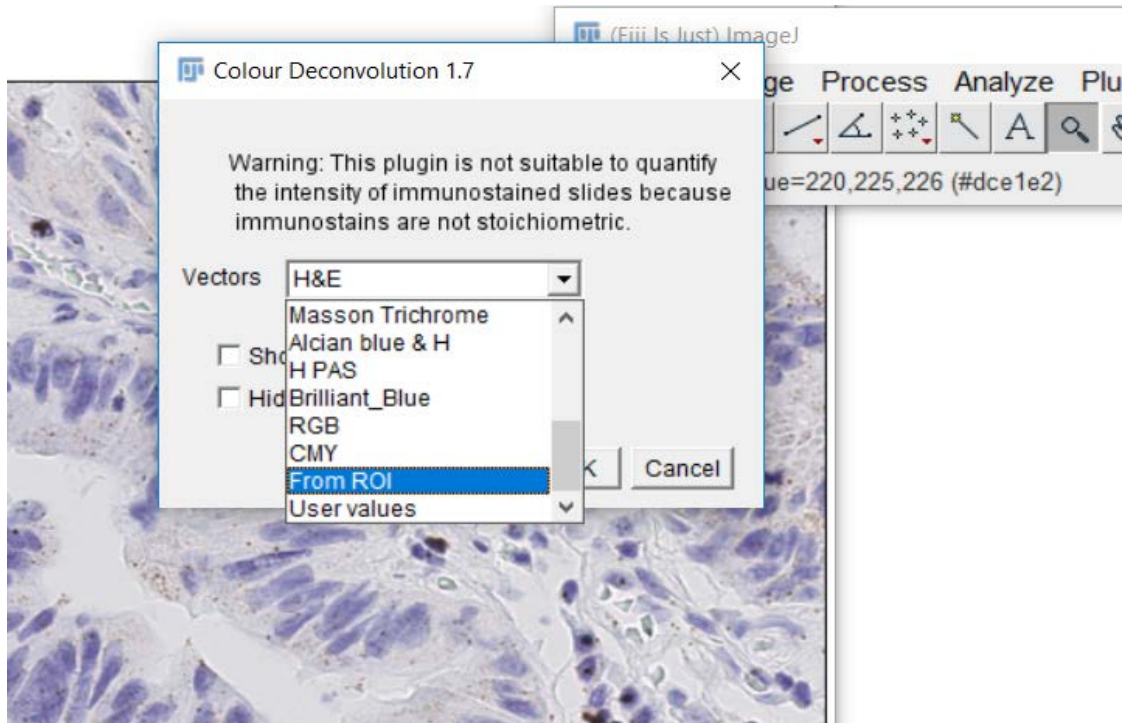
7. Using the rectangle tool, do the following in the image:
   a. Select an example of a nucleus stain for color 1. Right-click to move onto the next step.
   b. Select an example of a probe stain for color 2. Right-click to move onto the next step.
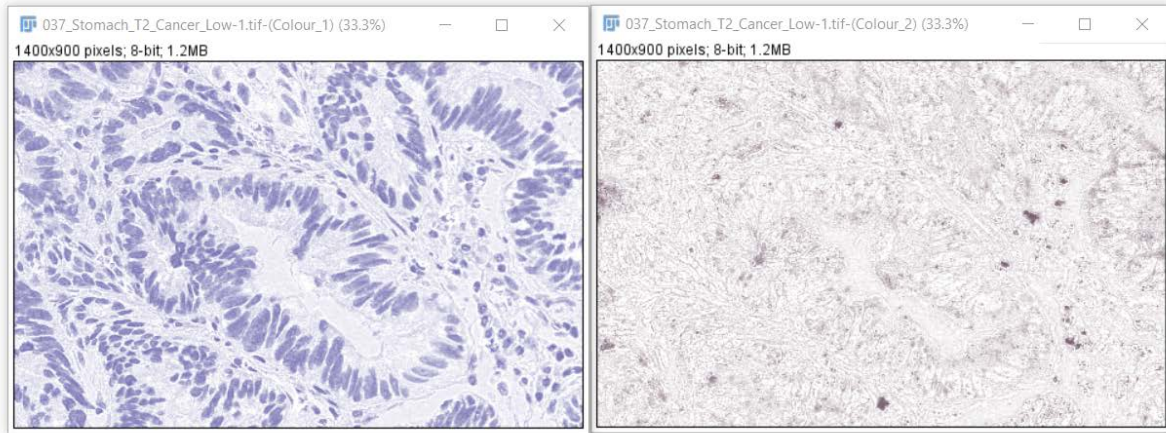   c. Select an example of a background stain for color 3. Right-click to move onto the next step.
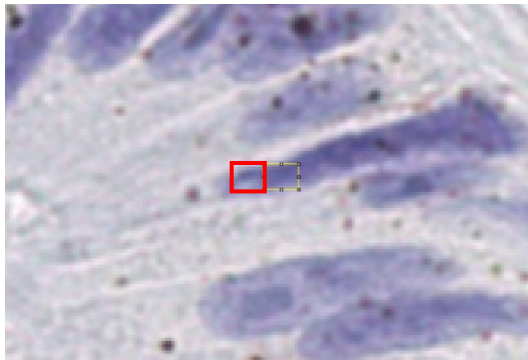
Selected nucleus stain example
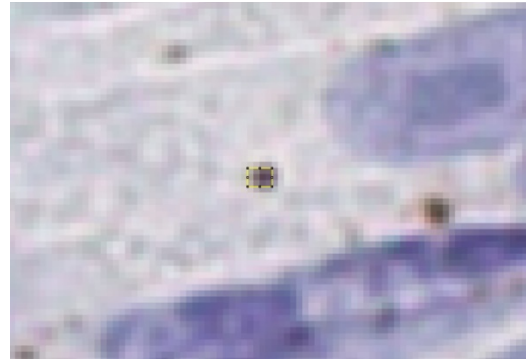
Selected probe stain example

8. Run the color deconvolution.

   You should see an output of three images; one for each channel. The first channel will contain the nuclei.


Channel 1 (nuclei)

9. Save the image of the nuclei as a separate image.

Part 1b: Image analysis

Begin multiplex fluorescent image analysis here using the nuclear image. Otsu thresholding, Watershed segmentation, or both can be performed as described. ImageJ allows other image processing methods as well. Your fluorescent image (white foreground on a black background) can be inverted (putting black objects on a white background using Ctrl+Shift+I). Inverting the image may aid in the ease of subsequent image analysis.

1. To automatically select a threshold based on the two-pixel populations in the image (nuclei and background), select **Image > Adjust > Threshold** and choose **Otsu** from the threshold options.
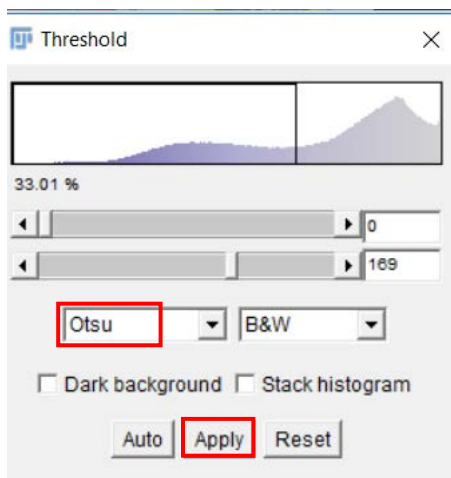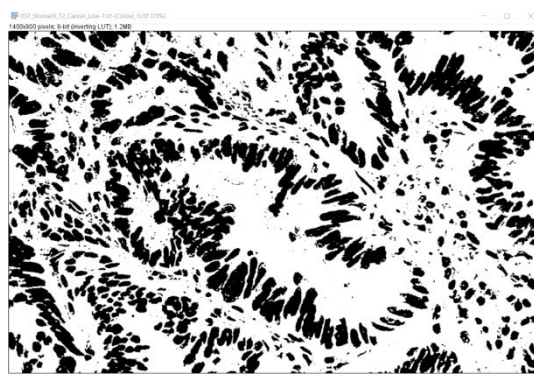
2. Click **Apply**. See the following images for results (nuclei are shown in black):
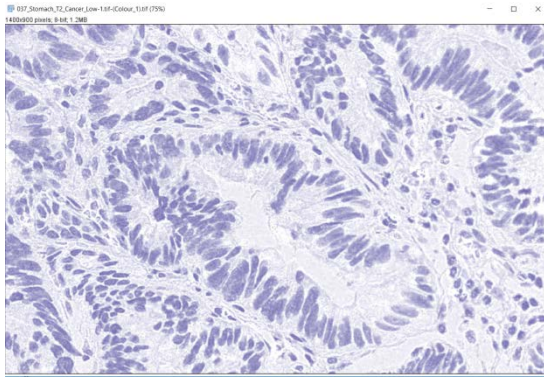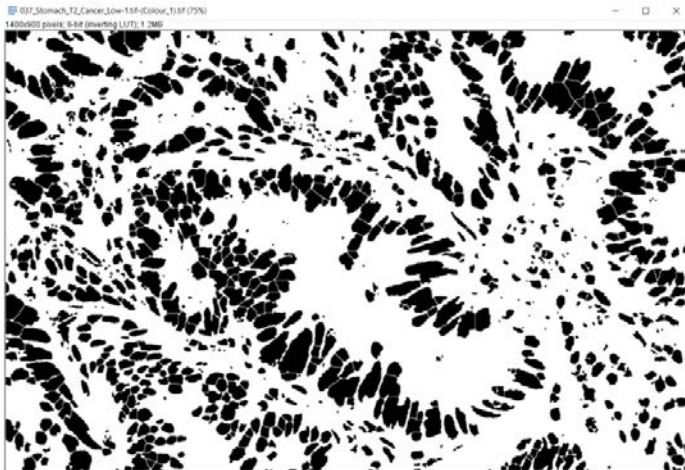

Original image	Converted image

3. If needed, adjust the image to make the nuclei smoother and remove background noise. Use the following morphological operations:

- **Process** > **Binary** > **Close**
- **Process** > **Binary** > **Open**

4. To split overlapping nuclei, use **Process > Binary > Watershed**. See the following image for results:

5. To count the cells, use **Analyze** > **Analyze Particles**.

6. Adjust the size and circularity options to avoid counting fragments and to count cells only. The number of detected cells in the image example is 485.

|	Area	Mean	StdDev	Mode	Min	Max	IntDen	Median
477	544	255	0	255	255	255	138720	255
478	458	255	0	255	255	255	116790	255
479	639	255	0	255	255	255	162945	255
480	447	255	0	255	255	255	113985	255
481	688	255	0	255	255	255	175440	255
482	383	255	0	255	255	255	97665	255
483	302	255	0	255	255	255	77010	255
484	306	255	0	255	255	255	78030	255
485	343	255	0	255	255	255	87465	255

Part 2: Quantifying the probe signal

Brightfield images (chromogenic assays) require using Weka classifiers/ segmentation. Follow **Part2a** to analyze the probe signal, and then follow the steps in **Part2b** to perform analysis. For fluorescent image analysis, skip **Part2a** and begin with **Part2b** to analyze each probe image separately.

Part 2a: Weka Classifier

To analyze the probe signal, use Weka classifiers.

1. Open the original image and select **Plugins > Segmentation > Trainable Weka Segmentation**.
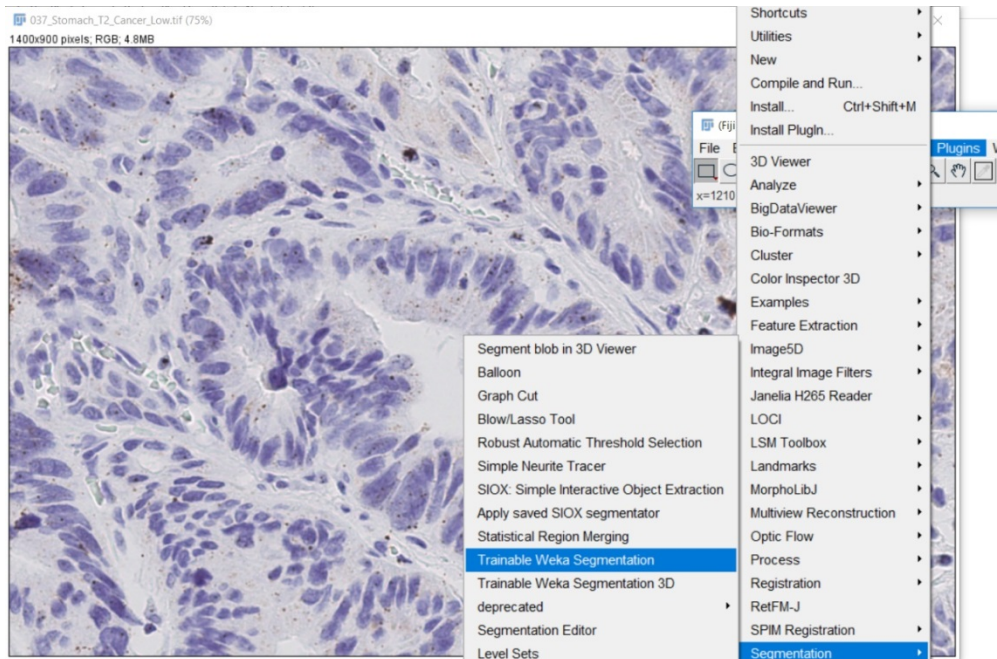
The default number of classes is two.

2. Depending on the number of probes you have, add more classifiers by selecting **Create new class**.
    The example image has three classifiers: background, nuclei, and probe. For a duplex image, add a fourth classifier for the second probe.
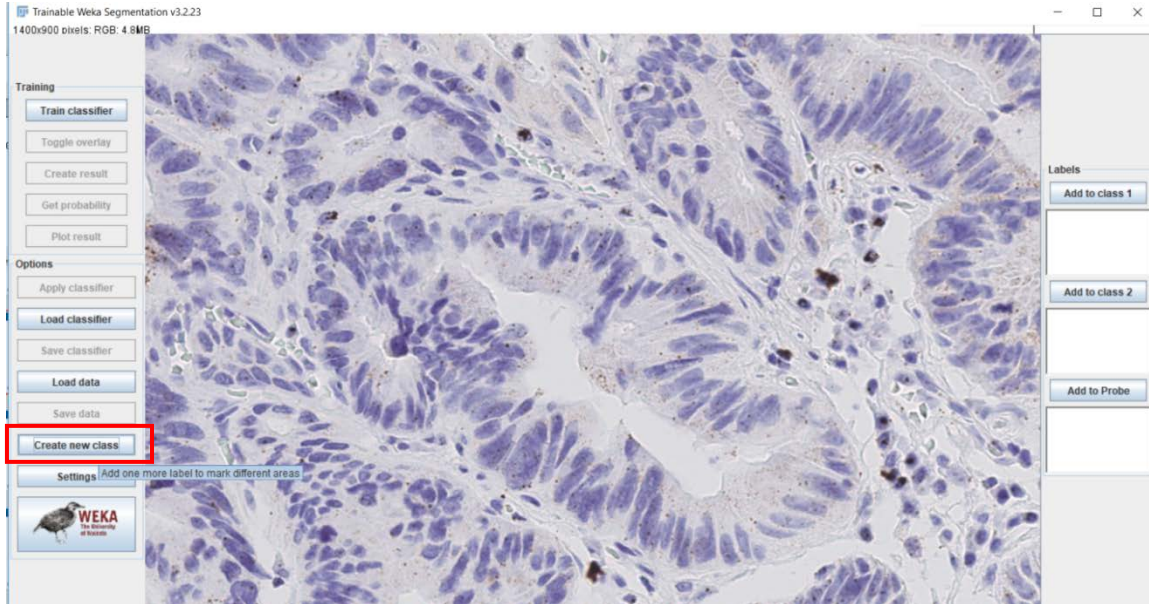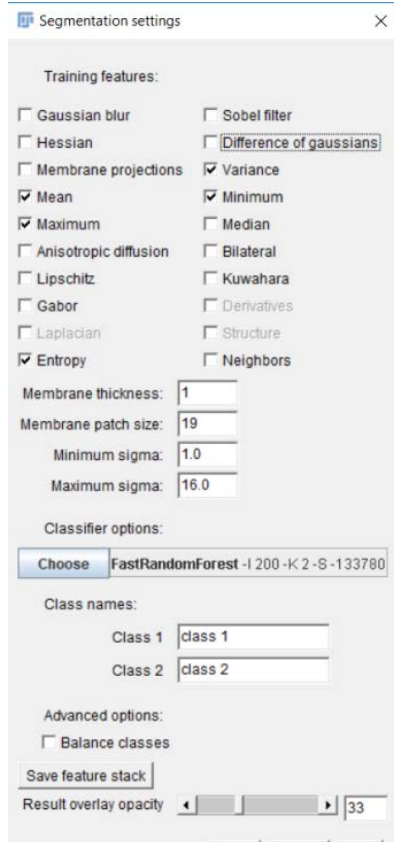
3. Using the freehand selection tool, select 2–4 examples for each class as shown.
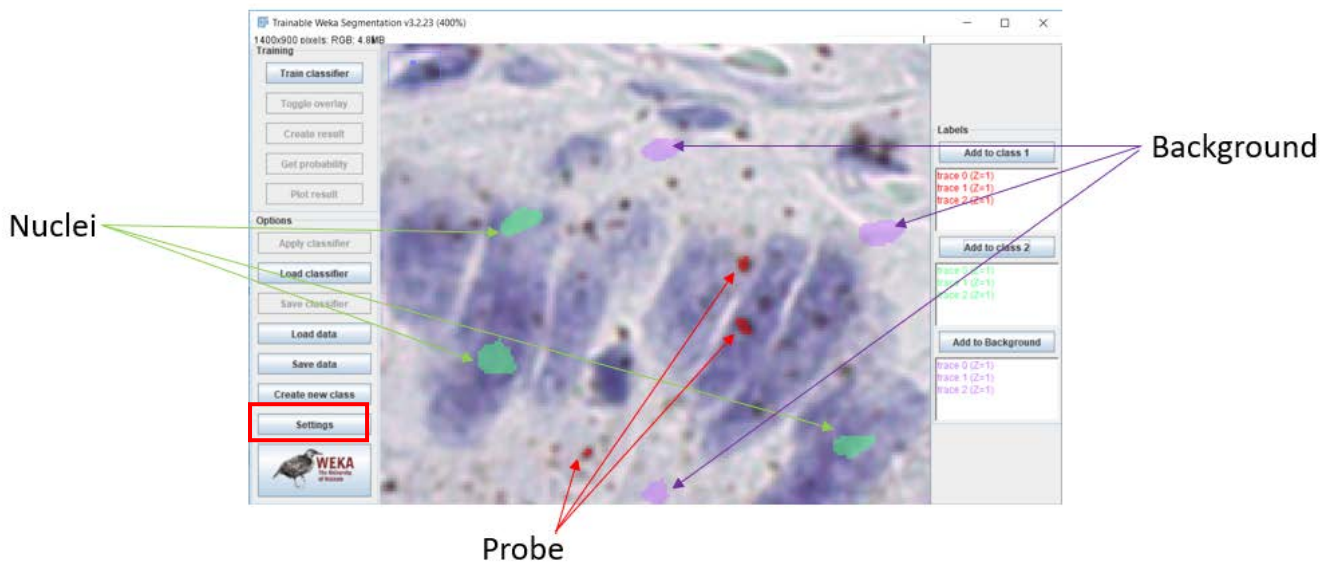
4. Click **Settings**.
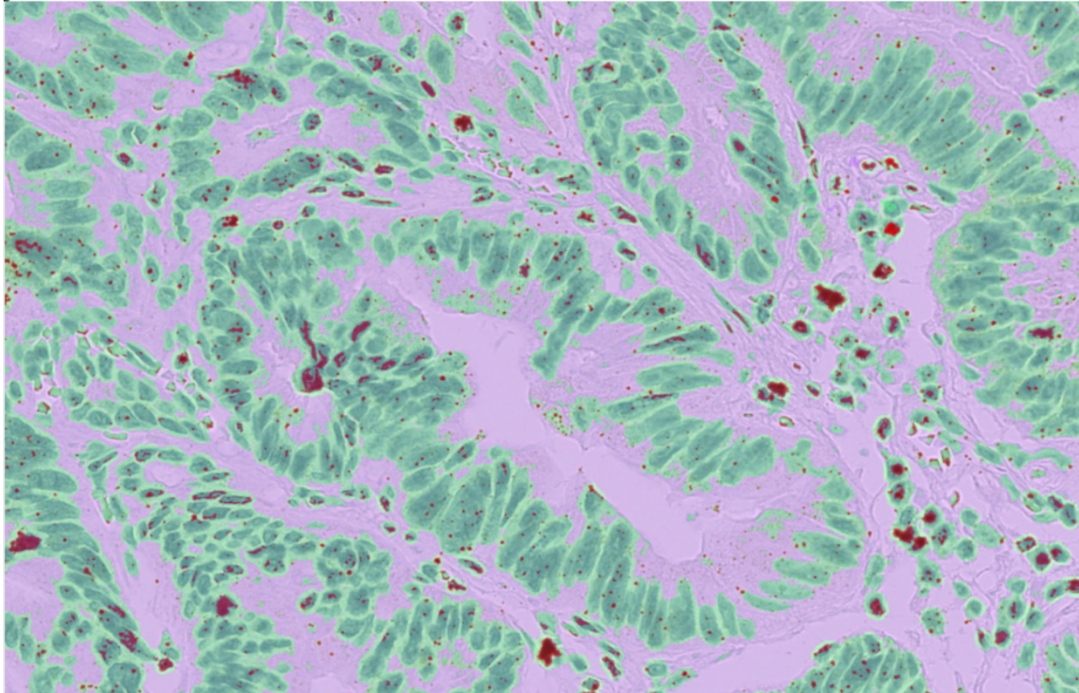5. Select the following features and click **OK**.

6. Click **Train Classifier** on the main screen, and check the results.

Note: This step may take a few minutes depending on the size of your image and computer specifications.

7. If the results need refining, select more training regions and repeat steps 4–6.
   You should see a result similar to the following image:

8. Click **Create result**.
9. Separate the probe class from the other classes:
    a. Select **Image** > **Adjust** > **Threshold**.
    b. Change the range until only the probe class is visible in the image.
    c. Click **Apply**, and close the window.
10. If you have a duplex image, repeat step 9 for the second probe class.

Part 2b: Analyze Probes

For multiplex fluorescent analysis, you can process each of your probe images using Otsu thresholding, Watershed segmentation, or both as described in **Part 1: Counting the number of cells** prior to continuing with this step. ImageJ also allows other image processing methods. Your fluorescent image (white foreground on a black background) can be inverted (putting black objects on a white background using Ctrl+Shift+I). Inverting the image may aid in the ease of subsequent image analysis.

1. To count the probe spots:
    a. Select **Analyze** > **Analyze Particles**.
    b. Adjust the circularity and size parameters so that only the probe clusters are detected.

    The area for each detected particle is displayed in a table.
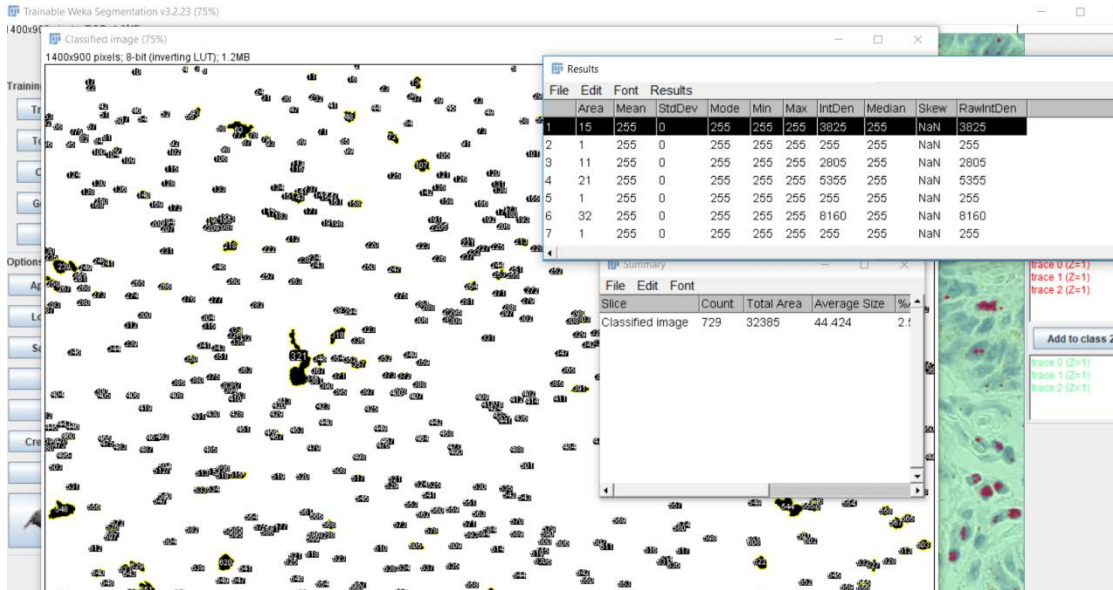
Note: To obtain the total probe count within each probe cluster, determine the area of a single probe using the image and accompanying results table, then divide the area of the larger clusters by the single probe area.

2. Visually inspect all of the detected particles and exclude the particles that do not look like a real signal.
3. Click Next to proceed. Ignore any pop-ups that may appear on the screen.
4. To calculate the number of dots per cell, divide the total number of probe count obtained in Part 2: Quantifying the probe signal by the number of cells obtained in Part 1: Counting the number of cells.
5. If you have a duplex or three- or four-plex image, repeat steps 1–4 for the second, third, and fourth probe.