Using ImageJ to analyze RNAscope® and BaseScope™ data

Introduction

This Technical Note provides guidelines for performing semi-quantitative image analysis using Fiji ImageJ on *in situ*-hybridized slides using the RNAscope® or BaseScope™ assays. Download the Fiji image processing package distribution of ImageJ using the following link: **https://fiji.sc**.

Note: ImageJ has limited capability with 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 present 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
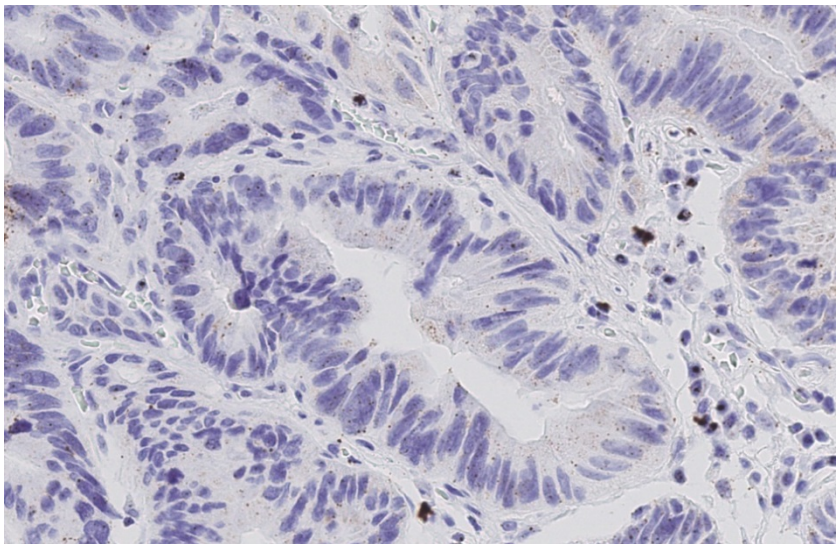

Part 1: Counting the number of cells

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

1. From the ImageJ tabs, select **File > Open**.
2. Select the image you want and click **Open**.
3. Find a region in the image where you can select a nucleus stain and a marker stain.
4. Zoom into your region of choice using the magnifying glass tool.

5. Select the rectangle tool.
6. From the ImageJ tabs, select **Image > Color > Color Deconvolution**.
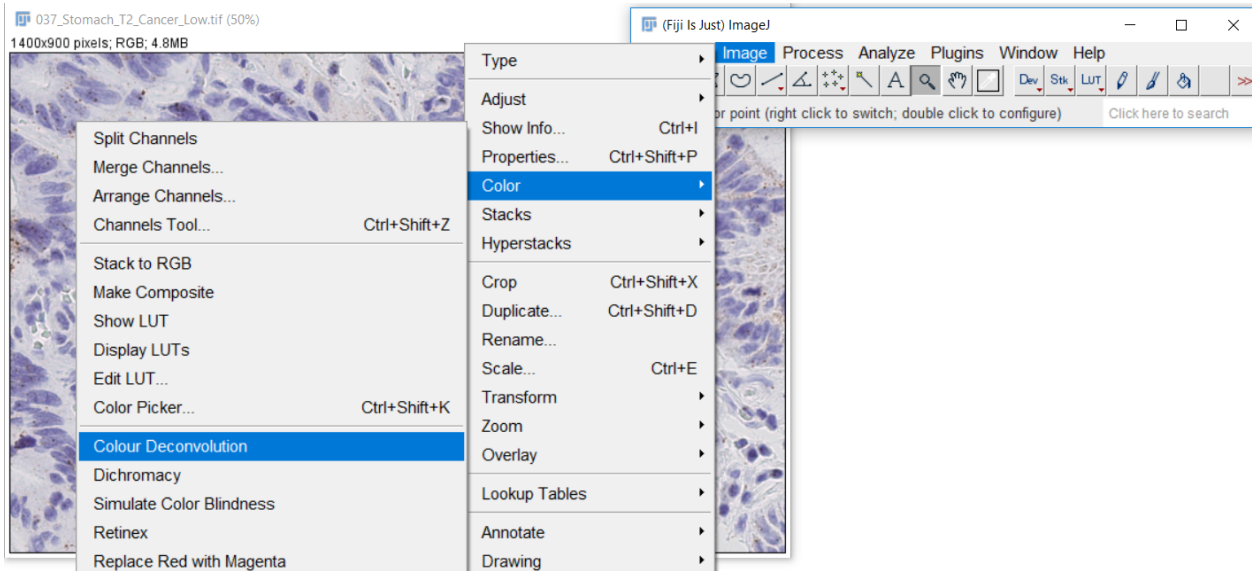
7. From the Options menu, select **From ROI**.
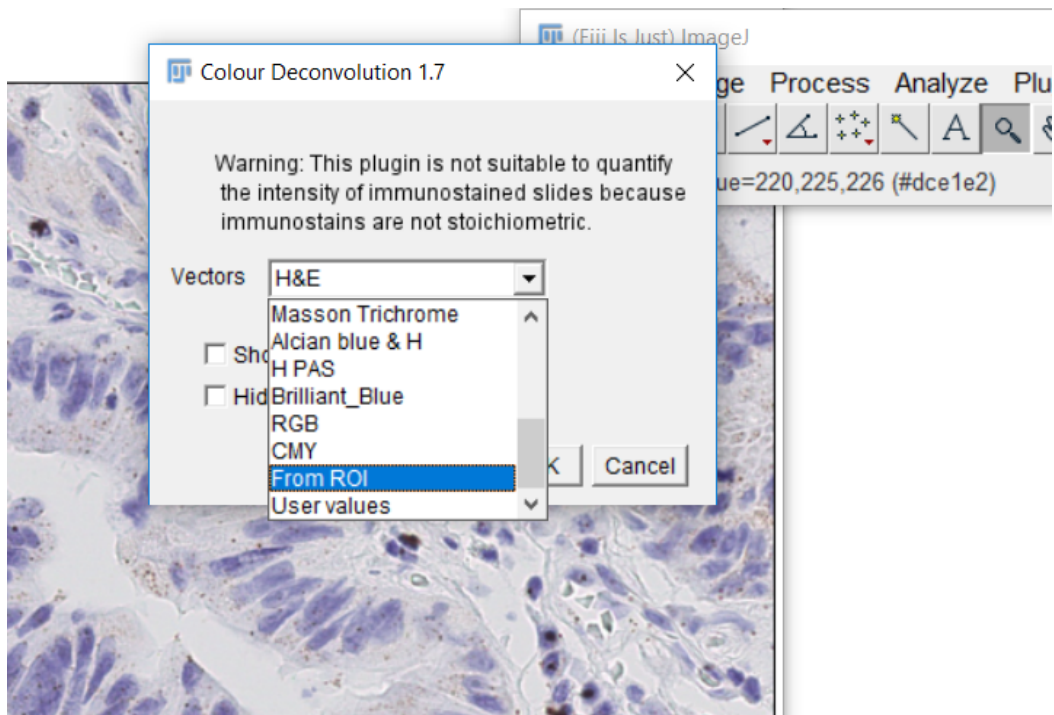
8. Using the rectangle tool, do the following in the image:
   a. Select an example of a nucleus stain for color 1. Right-click to move onto the next step.
   b. Select an example of a probe stain for color 2. Right-click to move onto the next step.
   c. Select an example of a background stain for color 3. Right-click to move onto the next step.
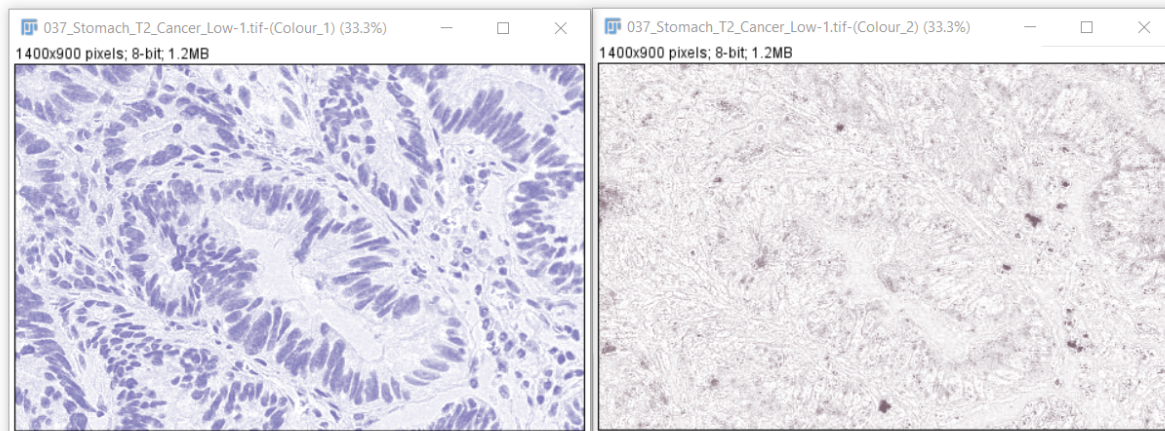

Selected nucleus stain example
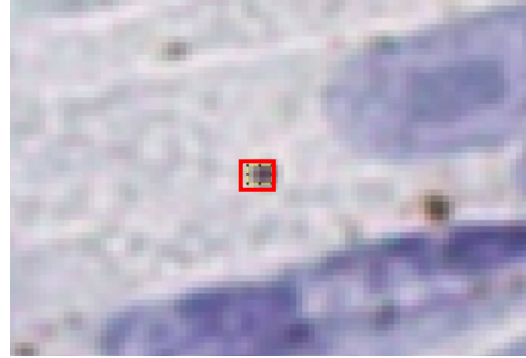

Selected probe stain example

9. Run the color deconvolution.

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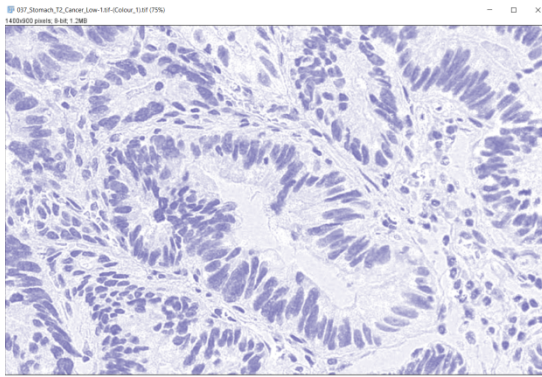

Channel 1 (nuclei)

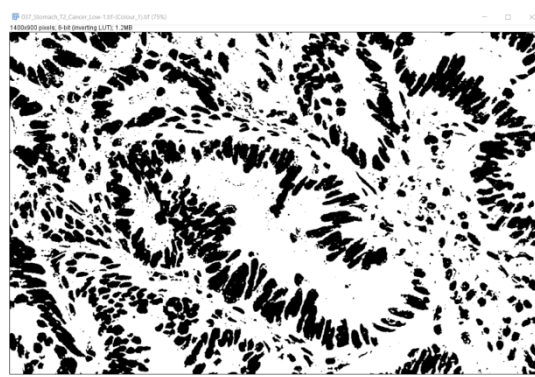10. If desired, save the image of the nuclei as a separate image.

11. 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.
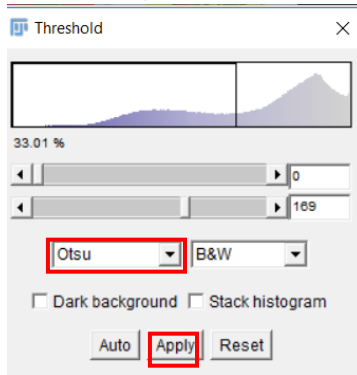
12. Click **Apply**. See the following images for results (nuclei are shown in black):
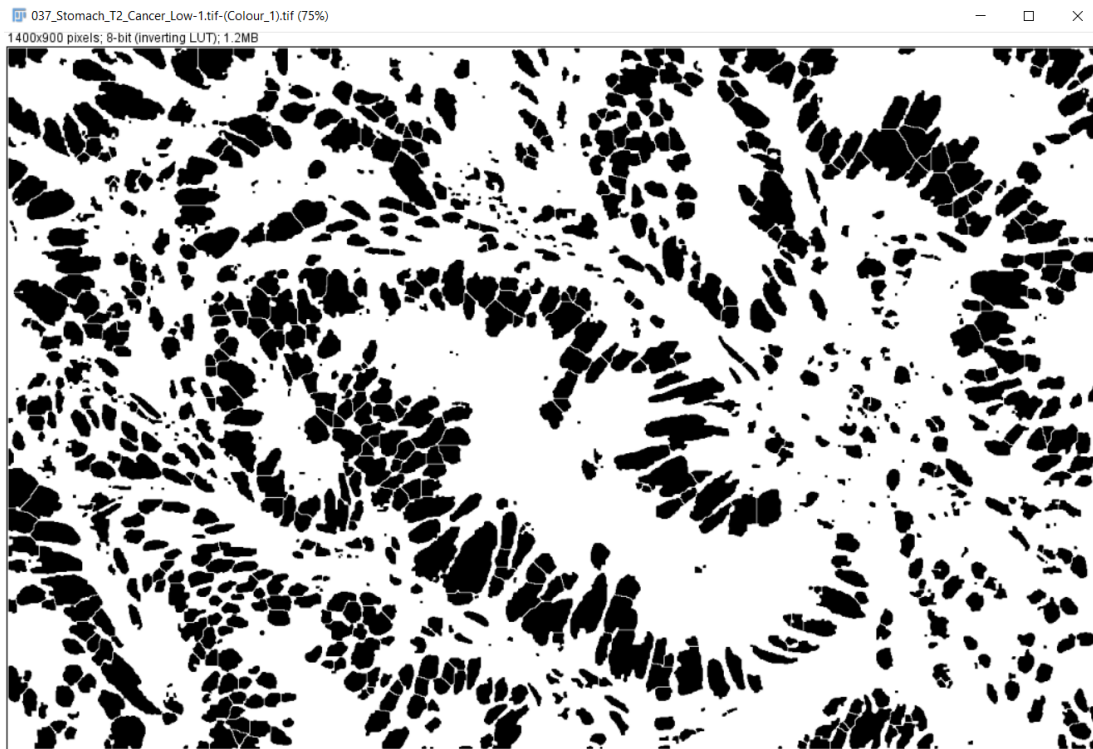

Original nuclei image   Converted image

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

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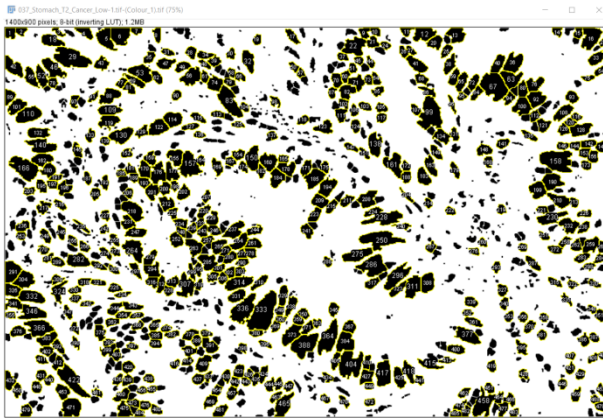- **Process** > **Binary** > **Open**

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

15. To count the cells, use **Analyze** > **Analyze Particles**.
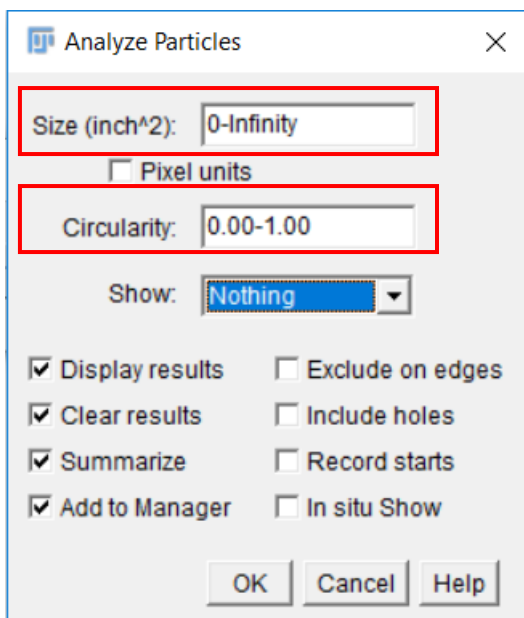
16. Adjust the size and circularity options to avoid counting fragments and to count cells only. The number of detected cells in the image example is 485.
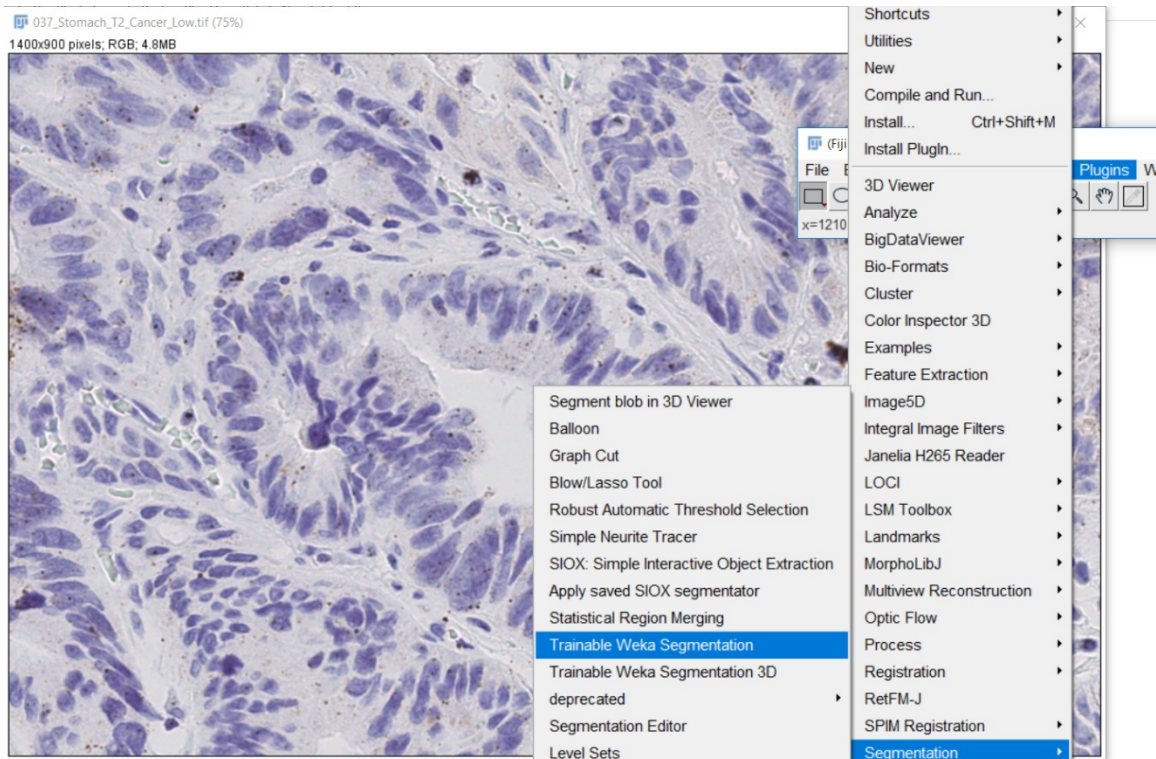

## Part 2: Quantifying the probe signal

To analyze the probe signal, use Weka classifiers.

1. Open the original image, and select **Plugins > Segmentation > Trainable Weka Segmentation**.
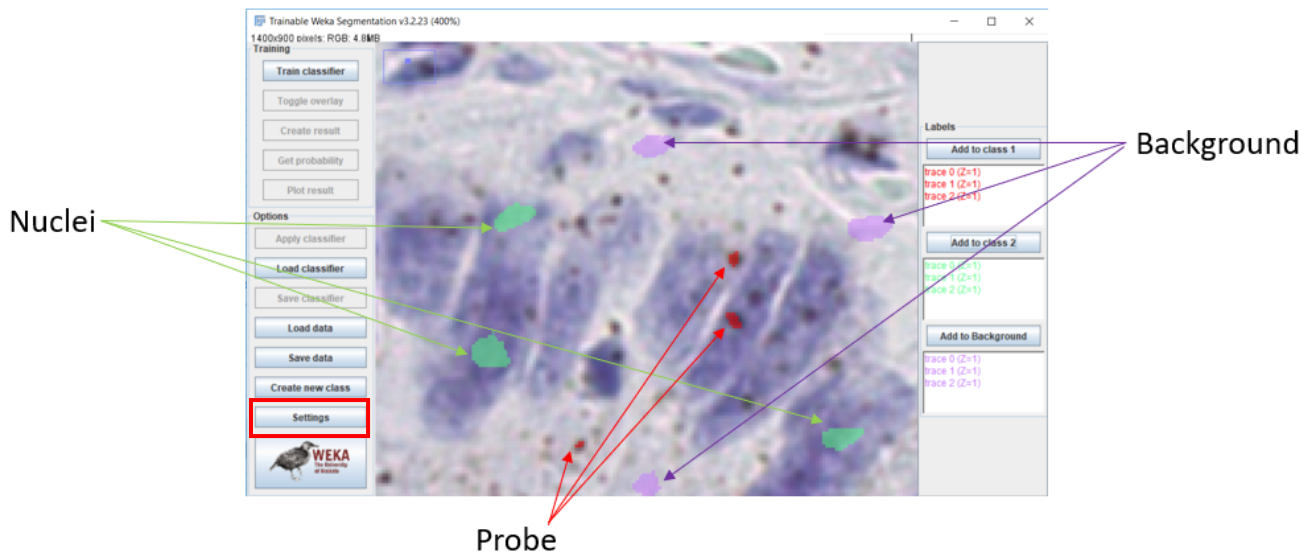

The default number of classes is two.

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.
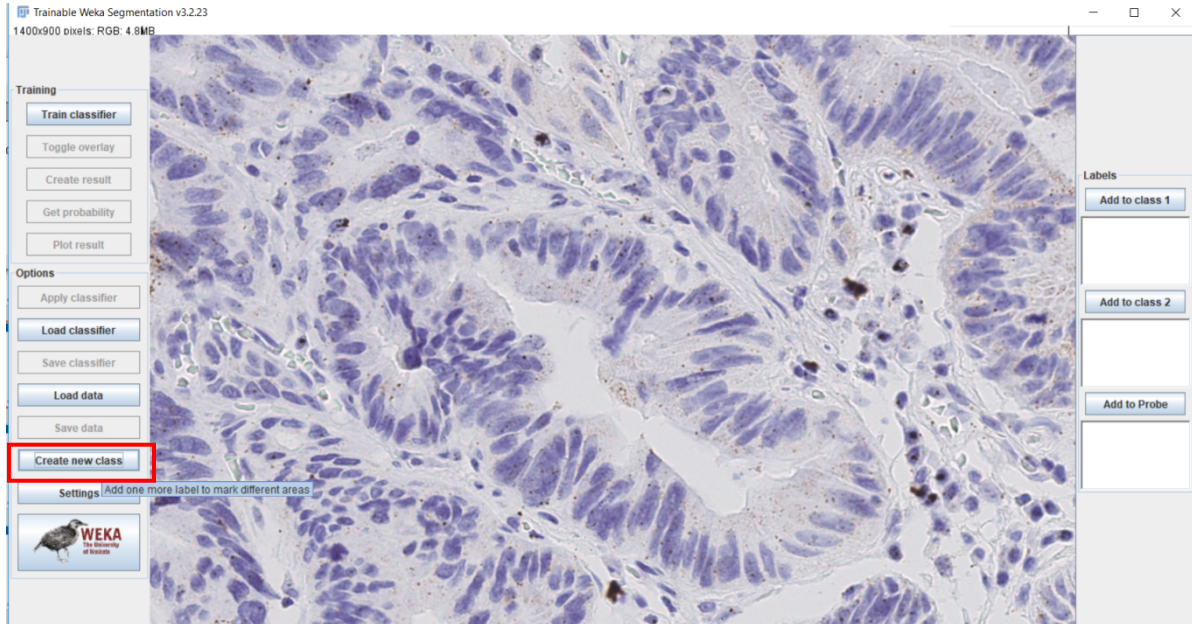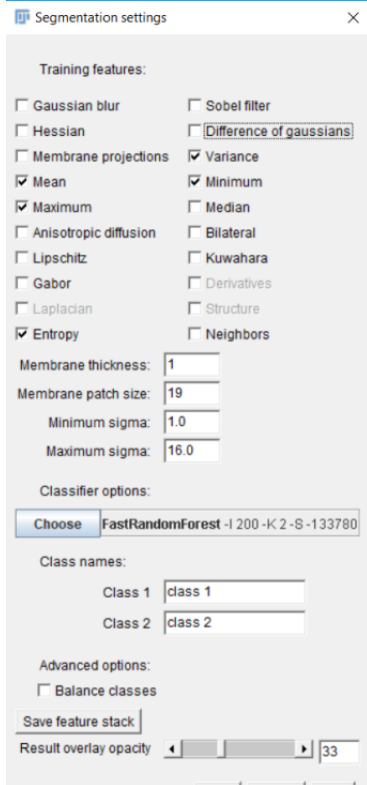

3. Using the freehand selection tool, select 2–4 examples for each class as shown.
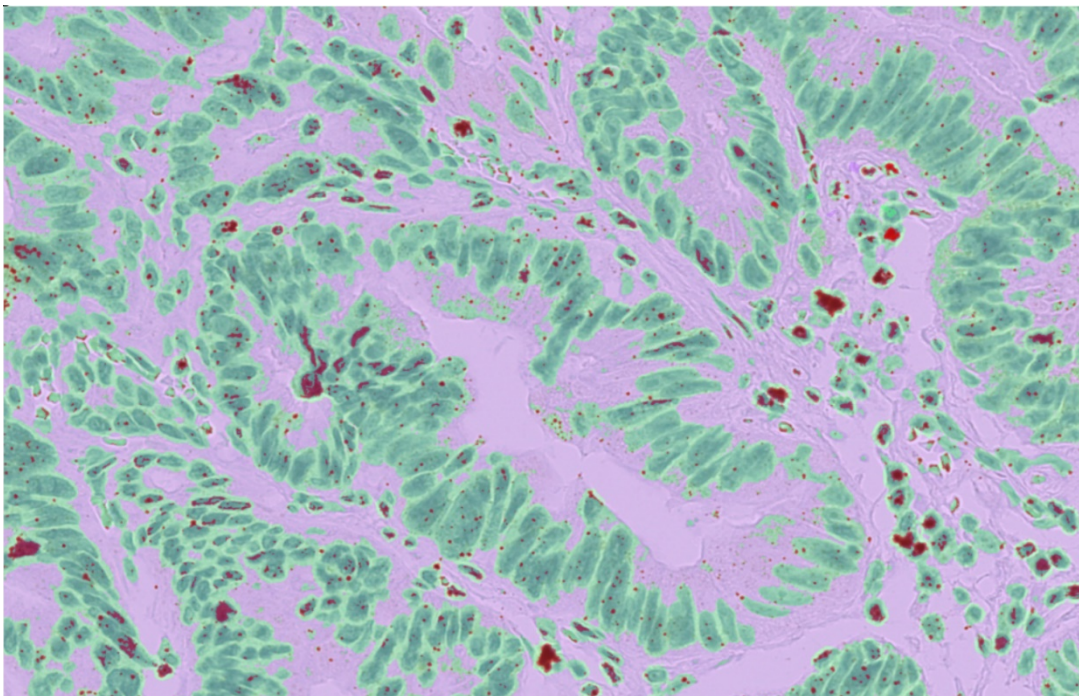

4. Click **Settings**.

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 specification.

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. 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.
11. Visually inspect all of the detected particles, and exclude the particles that do not look like a real signal.
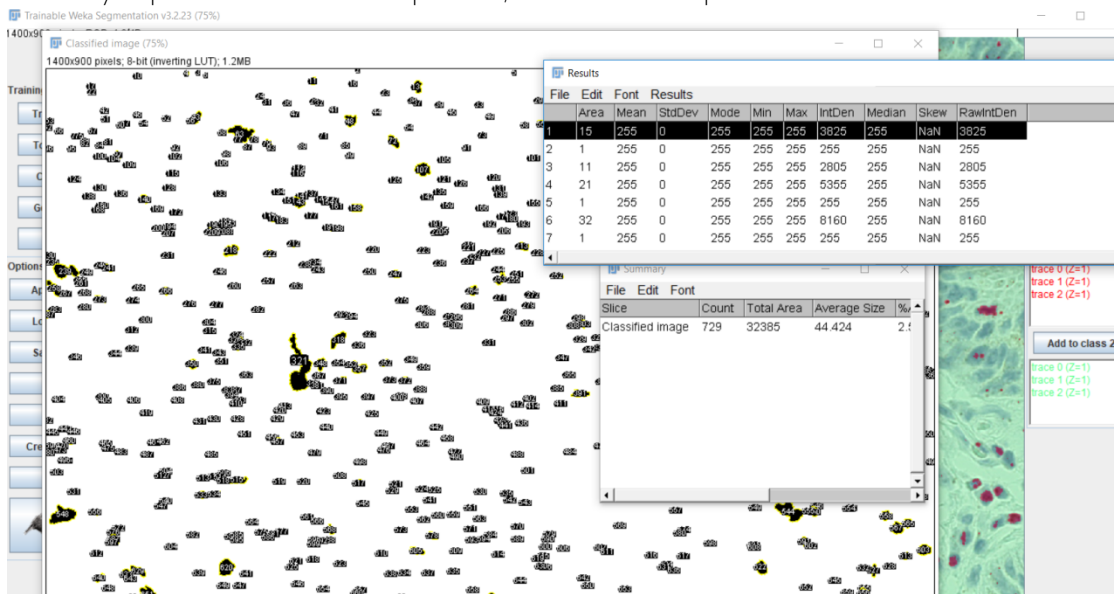
Note: To obtain the total probe count within each probe cluster, determine the area of a single probe using the image and accompanying results table, then divide the area of the larger clusters by the single probe area.

12. Click **Next** to proceed. Ignore any pop-ups that may appear on the screen.
13. To calculate the number of dots per cell, divide the total number of probe count obtained in Part 2 by the number of cells obtained in Part 1.