OBTAINING THE FLUORESCEIN PIXEL INTENSITY PROFILE FROM PENTERO VIDEO SCREENSHOTS USING PHOTOSHOP

1. Open image file (*.tif, *.jpg, *.gif, *.png) in Photoshop. The higher the resolution the better.
2. Select your background layer in the layers navigator. In the Adjustments palette, choose Channel Mixer (click on the little Channels icon).
3. With that Channel Mixer sublayer selected, go to the Properties palette.
    - Check Monochrome.
    - Set RED -100, GREEN +200, BLUE -100.
4. Save as a *.psd with the same base name.

SIZING & CHOOSING THE ROIs:

I. Make a new layer, name it "FIELD", get on it.
- Use the rectangle marquee selection tool to choose the illuminated surgical field. Include the surgical field; it is acceptable to be approximate. Don't include the video play bar at the bottom (either crop this from the file or just don't select it).
- Use the bucket fill tool to fill it. I always use orange (#FFAA00).
- Deselect the rectangle.
- Hide the layer.

II. Make a new layer, name it "B", get on it.
- Use the elliptical marquee selection tool to draw a CIRCLE that encompasses the biopsy (the diameter of the selection should equal the longest axis of the fluorescence in the biopsy).
- Use the bucket fill tool to fill it. I always use green (#00FF00).
- DO NOT deselect the circle.
- Hide the layer.

III. Make a new layer, name it "C", get on it.
- In the Select menu, choose "Transform Selection". Once you're in transform mode, in the bar at the top of the window, make sure height and width are linked, then type in "200%". Your selection will double in diameter.
- Use the bucket fill tool to fill it. I always use blue (#0000FF).
- DO NOT deselect the circle.
- Hide the layer.

IV. Make a new layer, name it "A", get on it.
- In the Select menu, choose "Transform Selection". Once you're in transform mode, in the bar at the top of the window, make sure height and width are linked, then type in "25%". Your selection will be 1/4xC diameter, which is 1/2xB diameter. Arrange this small circle over the brightest spot on the biopsy.
- Use the bucket fill tool to fill it. I always use cyan (#00FFFF).
- Deselect the circle.
- Hide the layer.

EXTRACTING PIXEL HISTOGRAMS FROM ROIs

I. Make the FIELD layer visible, get on it.
- Use the Magic Wand selection tool inside the bucket-filled (orange) region to select that ROI.
- Hide the FIELD layer, get on the Channel Mixer layer.

- In the Window menu, check Histogram. Make sure that "Entire Image" is selected, and channel = green.
- In the Window menu, check Measurement Log. There is a "Record Measurements" button in the Measurement Log window. Hit it – a measurement should appear.

II. Make the A layer visible, get on it.
- Use the Magic Wand selection tool inside the bucket-filled (cyan) region to select that ROI.
- Hide the A layer, get on the Channel Mixer layer.
- Hit "Record Measurements". For the biopsy ROIs, the Mean Gray value is what will be used as the Raw Fluorescent Intensity in downstream analysis.

III. Make the B layer visible, get on it.
- Use the Magic Wand selection tool inside the bucket-filled (green) region to select that ROI.
- Hide the B layer, get on the Channel Mixer layer.
- Hit "Record Measurements".

IV. Make the C layer visible, get on it.
- Use the Magic Wand selection tool inside the bucket-filled (blue) region to select that ROI.
- Hide the C layer, get on the Channel Mixer layer.
- Hit "Record Measurements".

You should have four lines of Measurements recorded.

EXPORTING PIXEL HISTOGRAM DATA TO FILES

1. Select all lines by Shift+click.
2. Click the little menu icon in the top right corner of the Measurement Log window.
3. Choose "Export Selected." Save the directory by the base name of the sample; it will contain the actual pixel histograms of each ROI and a text file with the exported statistics (as they appeared in the lines of the Measurement Log) will go wherever you choose to save them.
4. Knowing the order of the Measurements as you took them, rename the *.csv files in the directory according to their ROI (i.e., A, B, C, FIELD).

NOTES ON USAGE OF ROIs:

- The FIELD ROI is meant to be the largest, most rough estimation of the illuminated surgical field, and does not change much throughout the surgery. Biopsies from the same patient therefore generally have comparably drawn FIELD ROIs.
- We have compared the values that result from A, B, C, and a finely-handdrawn outline of the biopsy, D, as rendered by someone who was present for the surgery. The smallest ROI, A, which has a diameter ~1/2 that of the longest axis of the biopsy, consistently provided the smallest difference between the median and mean for the ROI, and agreed best with the subjective intensity call.
- We use the mean intensity of the ROI pixels, appearing in the first column of the biopsy data sheet.