**Generate Compensation Matrix**

This script is run through FIJI (FIJI is just ImageJ) and is used to generate a compensation matrix from single color control images saved as a .sdm file and a csv file. Additionally, a plot of each dyes/single color control images of the spectras is saved as a .png file.

**Prior to Running GenerateCompensationMatrix:**

This script has only been tested on images saved in Leica’s .lif format, however it supports a wide range of other formats like Nikon’s .ND2 format. Every format on this list (<https://docs.openmicroscopy.org/bio-formats/5.5.3/supported-formats.html>) that can have multiple images per file can be used with this script.

1. One file, like a .lif file, must contain all of the single color control images that are in order based on their channel. For example, if BV421 is primarily detected in channel 1 then the BV421 single color control image should be the first image in the .lif file.
2. Each image must only represent one color and not have any contaminants, like Ultracomp ebeads stained with other colors. If necessary, crop the images.
3. There must be a single color control image for every channel that is present in the image of the tissue that will be spectrally unmixed. For example, if the image of the tissue has 10 channels then there should be 10 single color images in the file.
4. FIJI (Fiji is just ImageJ) needs to be installed (<http://fiji.sc/#download>) to run this script.
5. FIJI/Bioformats might need to be updated to run this script. Update by using the Update command from the Help menu. Then go to Manage Update sites, tick Bio-Formats, click Close, apply changes and restart Fiji.

**Running GenerateCompensationMatrix:**

1. Start FIJI.

2. In FIJI, open GenerateCompensationMatrix.py

3. Select Run in the Window that appears.

4. In the new GenerateCompensationMatrix window, select the file with the single color control images in the compensation file box.

5. Select the features of interest, like “Save as SDM” and “Save the plot as PNG”.

6. Select run.

7. The script will open each series (single color control image) and calculate a threshold from the maximum intensity projection using the 'triangle' algorithm. It then uses the threshold to calculate the mean intensity above the threshold.

8. The generated files are saved in the same folder that has the file with the single color control images.