Step-by-step Guide to Compensating Bleed-through in Imaris

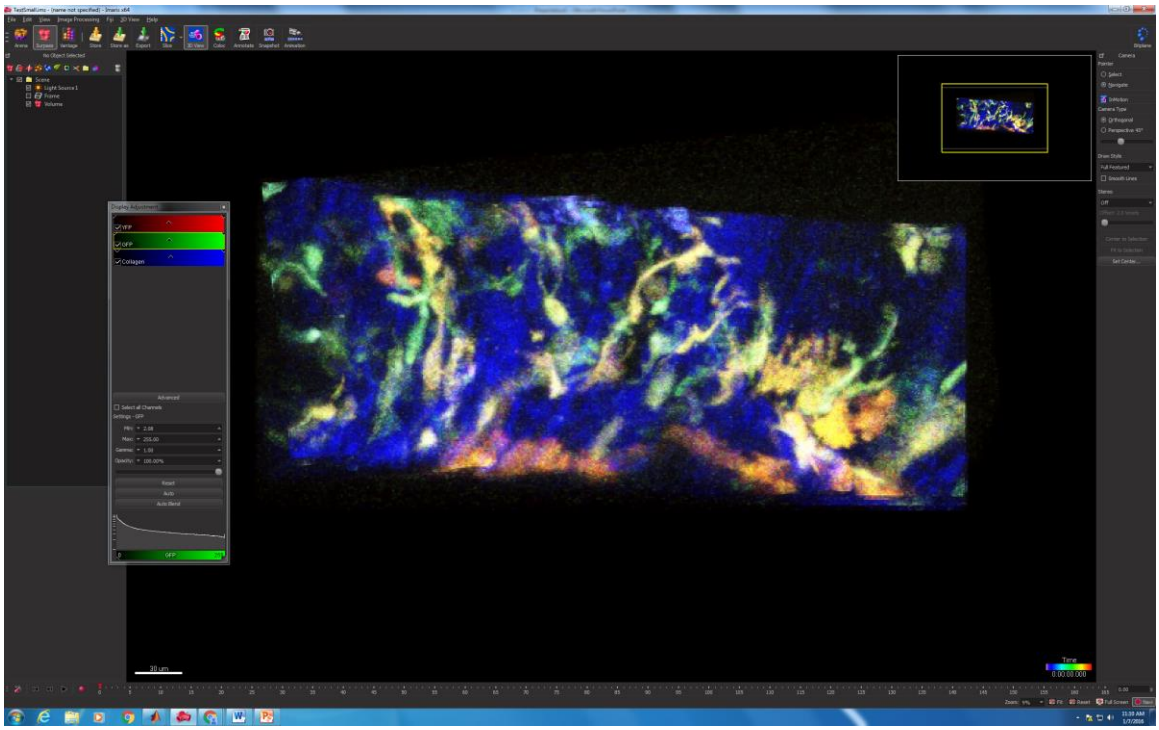
Compensation_SmallData

This program corrects for bleed-through between channels in microscopy data. It is based on the same math as compensation in FlowJo. Compared to flow cytometry, microscopy images typically have higher signal-to-noise ratios, more background signal from tissue autofluorescence, and more variation in relative signal intensities. This makes bleed-through correction difficult. To use this program, first segment the cells apart from background so that only cellular fluorescence is used. Intensity values within the cell volumes are averaged to minimize the effect of noise. Use these values to approximate a bleed-through matrix. Then, this matrix is applied to the entire data set on a pixel-by-pixel basis. The new compensated channels can be used for further analysis.

Written by Sara McArdle of the La Jolla Institute Microscopy Core, 2018. For questions, please contact smcardle@lji.org

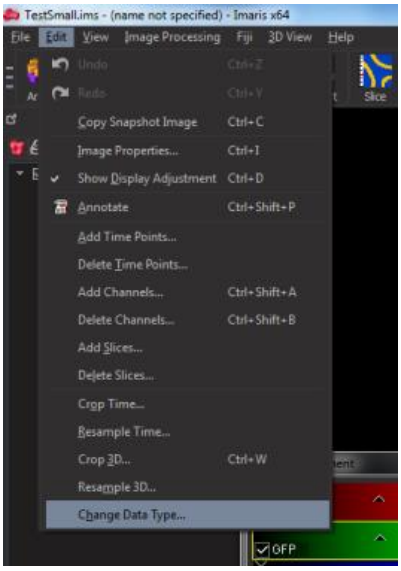
1. **Combine the Channels**

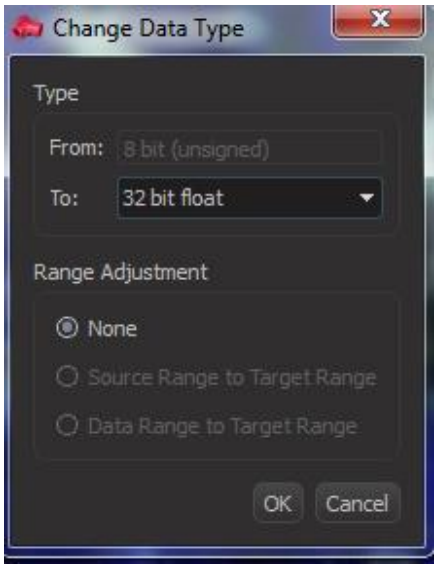
   There can be any number of colors in your dataset, but currently the program can only correct for bleed-through between 2 channels. In the example, we have GFP and YFP cells, which bleed into each other, and collagen, which is spectrally far from both.



The data must be first converted from 8 bit (integers 0-255) to 32 bit float (which allows for negative numbers, decimal points, and large numbers). If your data set is large, this can take a long time.

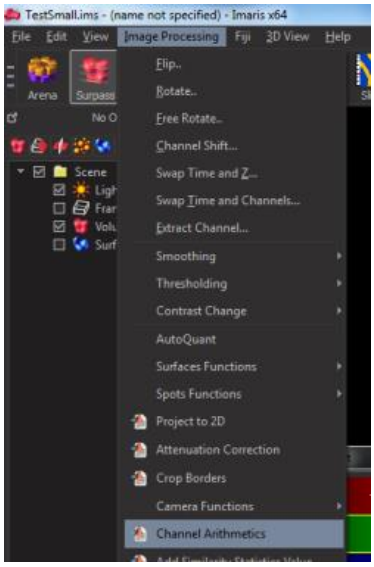
Go to Edit > Change Data Type; select 32 bit float

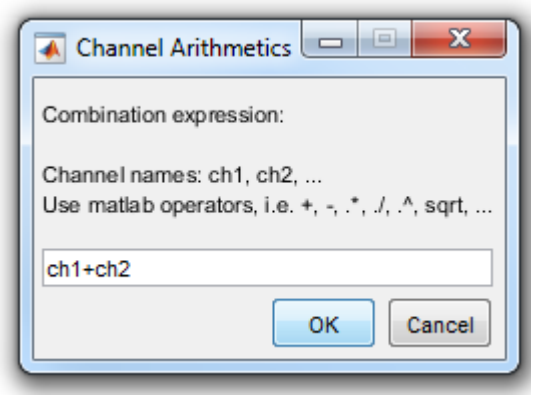




Then, to be able to make surfaces that include all of the cells of both colors, combine the 2 colors into 1 channel.

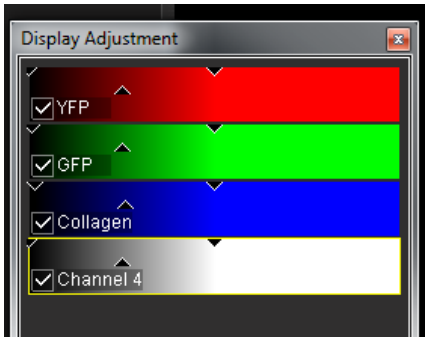
Go to Image Processing > Channel Arithmetics; add the 2 relevant channels

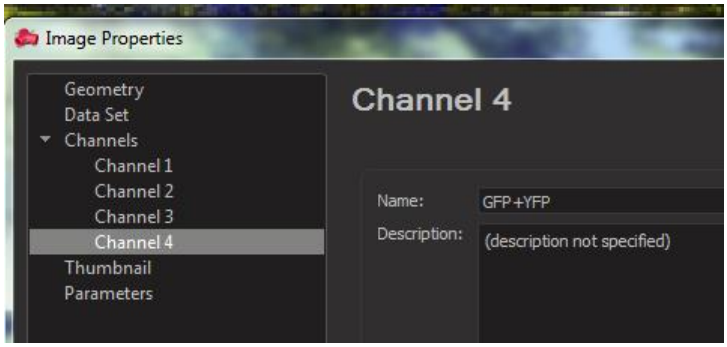




Use your channel numbers (found by counting on the display adjustment).

Click on the new channel name to give it a more descriptive name

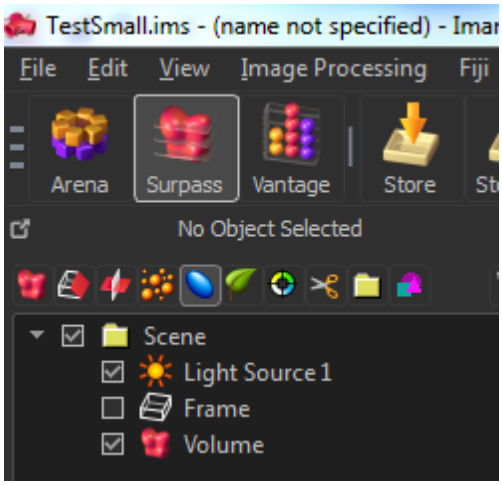




Hit “Auto” in display adjustment to fix the way the new channel appears. Now is a good time to save your file

2. Make Cell Surfaces

Create surfaces with the new, combined channel using the Surfaces wizard.

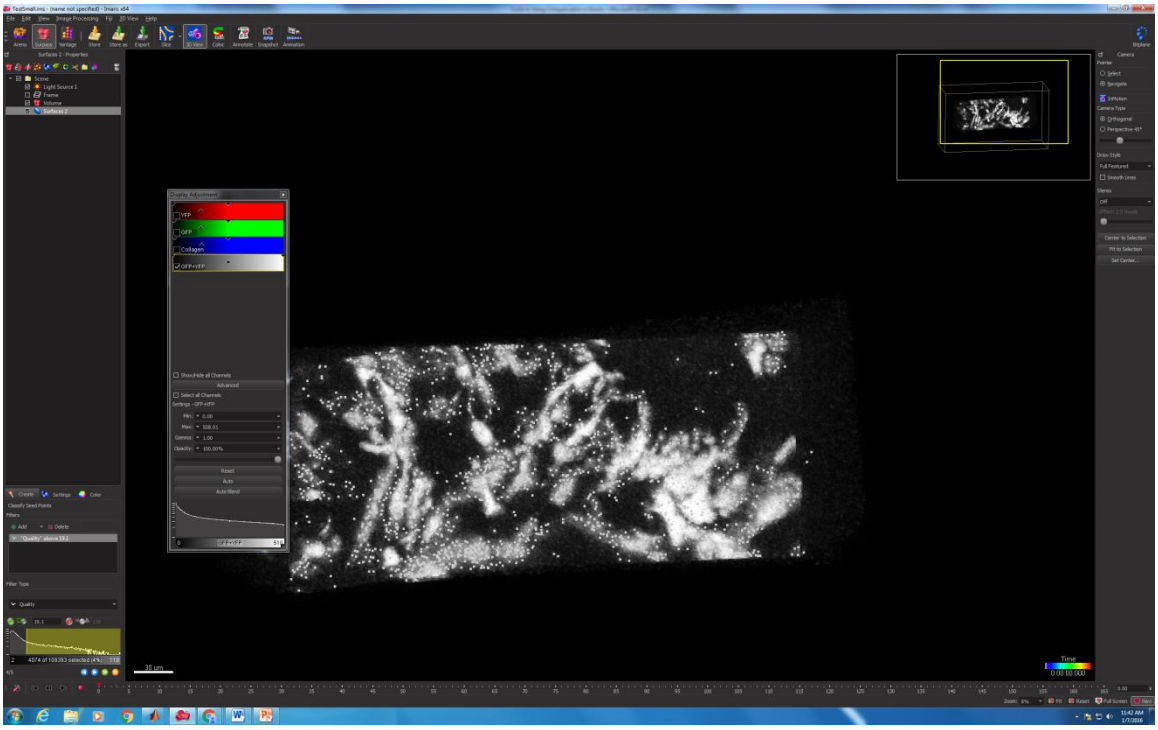


- The best results are achieved by processing the entire data set, but a region of interest can be used if the data set is too large. Do not track surfaces over time.

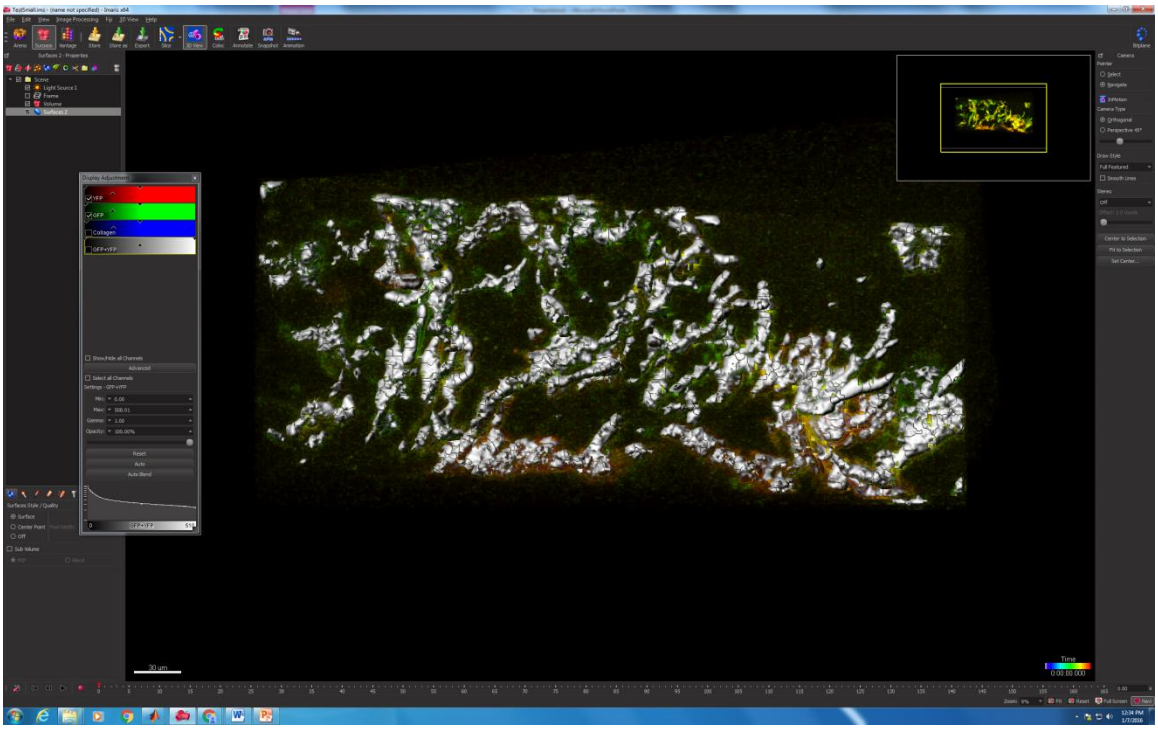
-Use smoothing, but use a small detail level (1 pixel width). Capturing dim cells is important, so typically thresholding with background subtraction is best, but use whichever method best captures the cells.

-It is more important to capture dim cells than to remove all background, so use a generous (low) threshold. If you have cells of different colors than are in contact with each other, **enable split touching objects**. It is very important to make sure that touching cells are broken apart along their borders, but it is perfectly fine and even useful to have a single cell broken into multiple pieces. Therefore, choose a very small seed point diameter (less than half the actual diameter of the cells)

-For the same reason as above, choose a low quality threshold



-Choose a voxel threshold that removes most non-cellular surfaces, and finish surface creation:

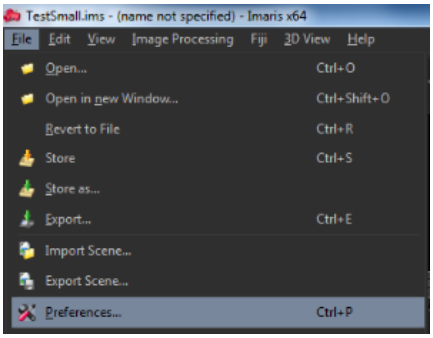


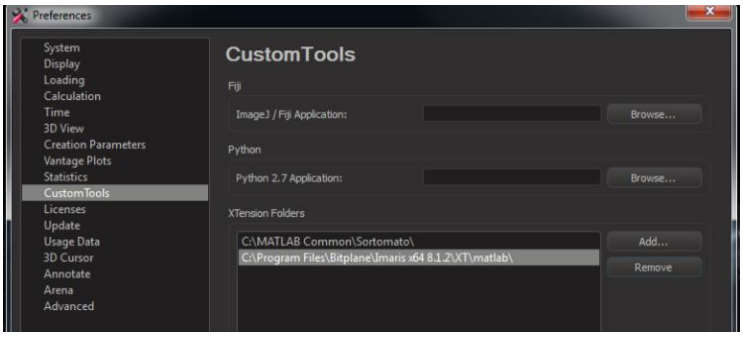
Save your file again here.

3. Add Compensation Program to Imaris (first time only)

Make sure the 2 files (Compensation.m and Compensation.fig) are in the folder C:\MATLAB Common\Compensation. Enable Imaris to read that folder.

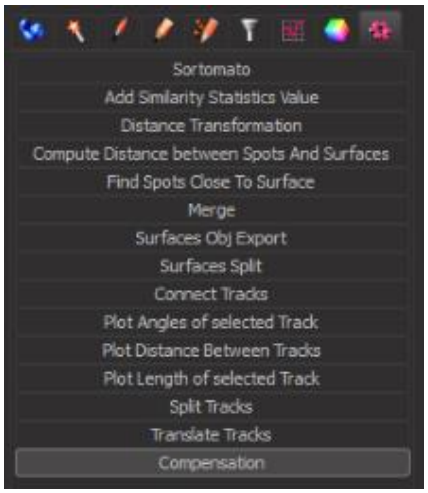
Go to File> Preferences; Click Custom Tools; Click Add; Select Compensation folder



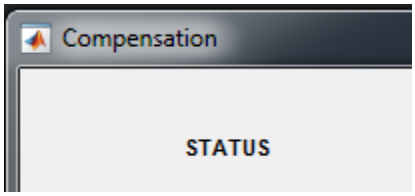


4. Run the program

Click on the new surface, then click Tools (Gear Symbol) > Compensation

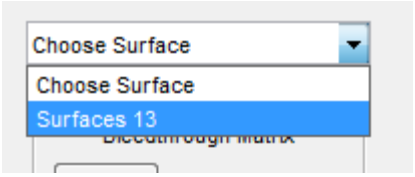


A screen will pop-up, with a status indicator at the top. This will give instructions or tell you that calculations are occurring.

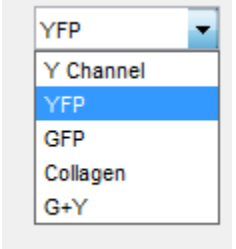


Press Start

Choose the surface you just made from the pop-up menu



Choose the channels with the *raw* data you want to correct to go on the X and Y axes of the figure



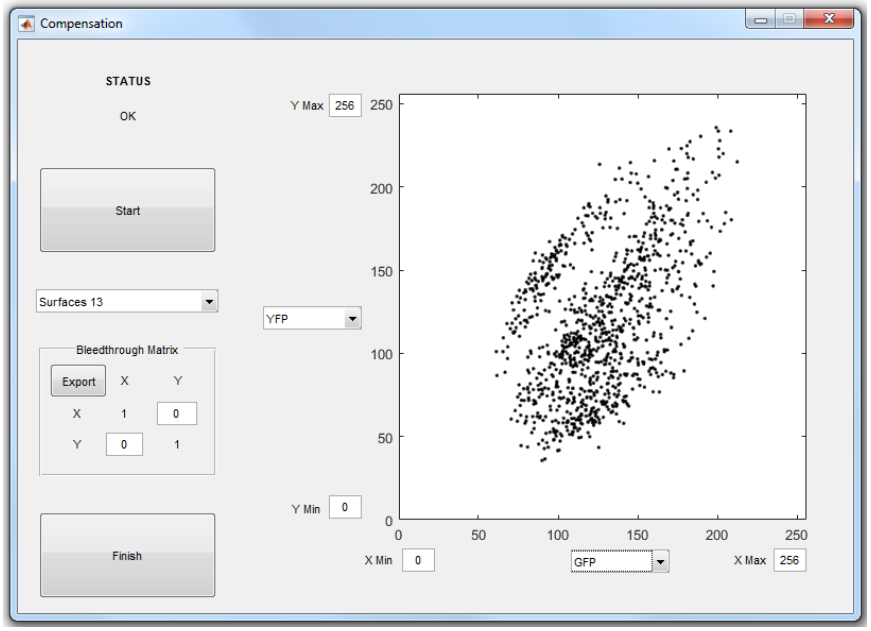
Data will populate the figure. Each point shows the median intensities of the two chosen channels for an individual cell or cell fragment.

Adjust Bleed-through Matrix

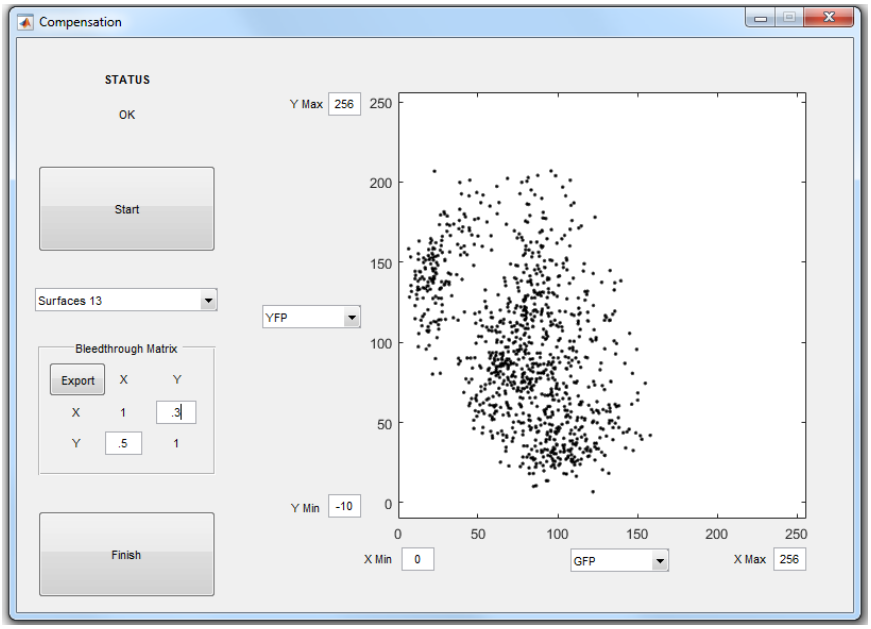
This part should feel familiar to FlowJo users who have manually corrected its automatically-generated compensation matrix. The matrix gives how much of the signal from 1 color (row header) appears in the detector designated for the other color (column header). Higher numbers mean more bleed-through occurs, and 1 = 100%.

For the reasons given at the beginning, it is difficult to calculate an objectively correct compensation matrix and threshold cutoffs defining positive vs negative populations. Single color controls can be helpful in determining approximate bleed-through values, but tissue composition, depth of imaging, and noise can alter the bleed-through percentages. For this step, it is useful to have a measure (typically from flow cytometry) of how large each single positive and the double positive populations should be. Change the numbers in the bleed-through matrix until the channels are separated to your satisfaction. Remember, due to autofluorescence from the cells and the tissue matrix, cells that are negative for a color may still have values above 0.

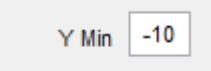
Before:



After:

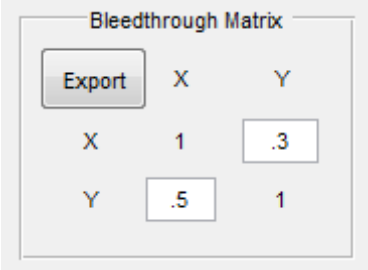


You can adjust the range of both axes to better visualize your data

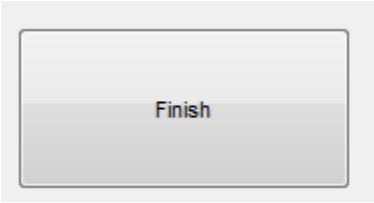


When you are satisfied with the compensation, you can export the matrix as an excel sheet.

Click Export; Choose a file name and location



Click Finish

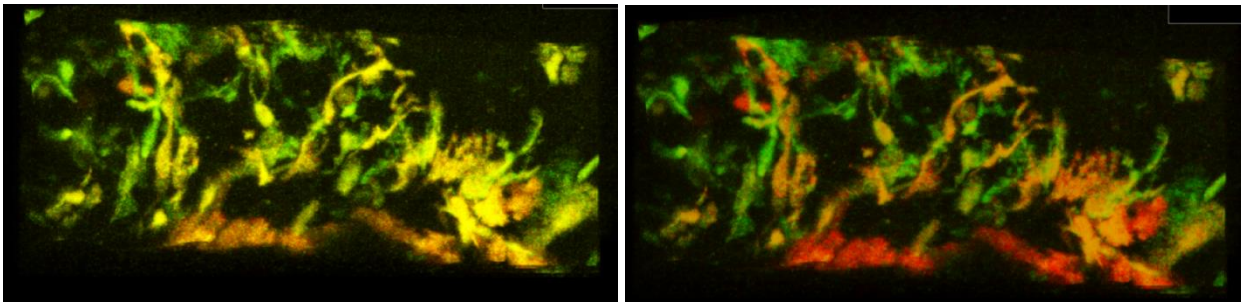


2 new channels with compensated data will be added to the Imaris file. This will take a long time, and the status box will turn green when it's done.

5. Final Results

On the Display Adjustments, hit "Auto" or manually adjust the min and max values to optimize the look up table. Typically, the minimum displayed value should be at least 0.

Before **After**



YFP cells are clearly red and GFP cells are clearly green, instead of both being shades of orange.