**Chrysalis**

This script is for analyzing static images like those acquired on confocal or epifluorescent microscopes as well as 2-photon movies.

**Prior to running this script:**

The CFI analysis workstation has bfmatlab and DIPimage installed, however if you are running this program on a different computer or trying to install a new version of either software then follow the below instructions:

Install bfmatlab and save it in C:\Program Files as bfmatlab.

<http://www.openmicroscopy.org/site/support/bio-formats5.2/users/matlab/>

Go to downloads and select matlab toolbox. When downloaded, unzip and copy bfmatlab folder to an accessible location like C:\Program Files. Make sure line 693 in the matlab code for the Chrysalis.m file reflects the location of the bfmatlab folder [addpath (‘C:\Program Files\bfmatlab’);].

**Running this script:**

To analyze images with this software, save images as leica files in the .lif format. Put all the leica files to be analyzed into one folder. If the images will be spectrally unmixed then use the leica software (Leica Application Suite) to generate a compensation matrix by using Leica Dye Separation, which is found in the Process tab of the LAS software, on single color control images.

Start the script by opening Chrysalis.m which is found in the Chrysalis folder on the desktop of the CFI analysis workstation. Once Matlab opens, click the editor tab and then click the run button which will make the Chrysalis window appear. In the Chrysalis window, click the “Input folder” button and select the folder containing your files then click on the “output folder” button and select the folder into which the analyzed files will be saved. If spectrally unmixing the images, then click on the the “Compensation Matrix” button and select the .sdm file that contains the compensation matrix for the images that will be analyzed. Next, select the file type to be analyzed by selecting either movie or static image. Then, select the type of image processing that will be done on the files by selecting any of the options found under image processing (each option is described in detail in the features section below). Once all of the settings for the image analysis are chosen, click the run button in Chrysalis window (not the matlab window) to start the analysis.

**Features:**

Spectral Unmixing: Applies compensation to the analyzed images based on the values in the.sdm file selected as the compensation matrix.

New Channel: Generates a new channel that consists of only voxels that are above the threshold of the channels selected in the include menu and that are below the threshold of the channels selected in the exclude menu. The signal intensity for each of these voxels is based of the signal intensity for the selected base channel in each of those voxels. For example, this feature can be used to create a new channel that only contains voxels for DCs by including channels for CD11c and MHCII while excluding channels for B220, F4/80, and CD3 and using CD11c as the base channel.

When entering values in the “Number of New Channels” and the “Number of Channels” boxes the value must be a whole number typed as an integer rather than spelled out (for example, enter “1” rather than “one”). Multiple options can be selected in the listbox menu of the include and exclude channel menus by clicking control + left mouse button. Multiple new channels can be generated by entering the number of new channels you would like to generate in the number of new channels box and then selecting between the new channels with the selected new channel menu. Each different new channel can have unique settings but the number of channels in the image needs to stay the same for all of the new channels.

Rescale Data: Rescaling the data is recommended to improve how the image appears in Imaris. Rescaling will change the intensity values for each channel to utilize the entirety of the dynamic range. The changes in intensity values after rescaling make it difficult to compare images quantitatively in Flowjo, therefore the rescale factor for each image is exported alongside the image when this feature is selected. This rescale factor file can be used by the XTExportStatWtihRescaleOffset Batch Xtension in Imaris to export normalized statistics for each image that factors in the rescale factor that was initially applied during processing thereby providing accurate image to image quantitative comparisons in Flowjo.

When analyzing movies:

Save movie as AVI file: This feature saves the movie as an AVI file. When this option is selected a window appears that allows for color selection for each channel. This option is great for quickly looking over movies to determine which movies have healthy tissue and worth analyzing further.

Save movie as BigDataViewer file: This feature saves the analyzed movies as a BigDataViewer file which can be directly opened up Imaris. If you do not select this option then any spectral unmixing and new channel generation that was done on the analyzed movies will not be saved.

When analyzing static images:

Merge all images in each file: If the file contains multiple images then selecting this feature will merge all of the images in the Z axis so they are stacked one after the other. This is a great option if a file contains multiple images from one tissue sample. Combining all of the images into one large image allows for the same analysis to be applied to all of the images from one tissue sample and expedites the analysis.

**After Running the Matlab code:**

Open the processed files in Imaris by selecting the h5 file for the image when using Imaris 9 or either the XML or h5 file for the image when using Imaris 8. Alternatively, use the Imaris fileconverter to convert the BigDataViewer files (XML + h5 file) to a .ims files (Imaris format). It can take a while to open a BigDataViewer file directly by Imaris, so it can be nice to convert the BigDataViewer files to .ims files before analyzing them in Imaris if analyzing multiple files.

Upon opening the file in Imaris, the color of each channel can be changed and each channel can be labelled by clicking “image properties” under the “edit” tab.

Processing the images with Chrysalis will change the voxel size of the image which will provide inaccurate distance measurements and surface generation. Therefore, while in “image properties” change the voxel size for X, Y, and Z to the images original voxel size (voxel size settings around found in image properties under the geometry tab). If you are not sure about the voxel size of your original image, the voxel size can be found by opening up the original leica file in the leica LAS software then right clicking the leica file and selecting properties but this does not work on merged files so if your file is merged then the non-merged version needs to be selected. When you close the image properties window you won’t see your images because they have moved weirdly. Just hit “Fit” and “reset” in the bottom right hand corner of the Imaris window and your image will appear in front of you.

**If Matlab has a memory error in the middle of processing images:**

1. Making sure that you have allocated all possible memory to Matlab.

2. Close matlab and restart the computer. Log back in and move any processed files out of the input folder for the Matlab script. Run the matlab script again to continue processing files left in the input folder. Just closing and opening matlab doesn’t free up the memory.