Light Sheet Software Guide

by Dawnis Chow, Xinyue Cui, and Eileen Troconis

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Introduction

This is a guide to the software for use in light sheet data acquisition and processing. This document explains both the procedures involved in using the programs and details of what each program does and how it works.

The light sheet pipeline can be broken down into several stages. There are a number of software programs (primarily in Matlab) that have been developed for each step.

Important software should be already accessible within Matlab by name (having previously been stored on the Matlab path, i.e. type the name or part of the name into the command line and press *tab*). However, backups exist for all software at http://github.com/fetcholab. Source files may either be downloaded directly or copied (from Github's interface or Raw format on the site).

A walkthrough for the data_explorer_gui has been written in a Word (located in the demo data folder K:/DATA_EXPLORATION_GUI_DEMO/walkthrough.docx) along with example data for the GUI.

Stages of the Experiment and Analysis Pipeline

I.) Running the Experiment

II.) Conversion of stacks to fluorescence time series

- *** Compression**
- *** Registration**
- *** Segmentation**

III.) Validation

IV.) Analytic Approaches

Software Description.

I. Running the Experiment

Programs to allow stimulation and physiological recording during light sheet experiments was created using Labview. The software was designed to run using the Behavior Laptop connected to the NI USB-6221 board with appropriate inputs, depending on desired experimental configuration.

For piezo-electric stimulation experiments, I recommend using the LSExpt_Variable_Timing_Pulse_GO+RAND LabView file. (It is located under Dropbox/Labview/LightSheet). This program relies on the generation of a stimulus amplitude and timing text file

from a Matlab script (GenerateRandomStimulusFile.m). The subsequent stimulus file is saved under Dropbox\Light Sheet\StimulusFile\GoRandomStimulus.txt. Previous stimulus files that have the same name are moved to a new file that have the creation date of that file appended to prevent the loss of information of previously used stimulation patterns.

Another useful LabView program is the BuzzPiezoelectricDevice program. This allows testing of the piezo stimulation device to see if everything is wired correctly before the experiment.

Experiment Procedure

To perform the experiment, in the LSExpt Lab View program, the user must specify the frames per stack and the amount of time to acquire signal data (for physiological experiments). The program automatically reads the stimulus file called GoRandomStimulus.txt. The LabView program is started **before** acquisition is started on the light sheet computer. Once acquisition starts on the computer, the current frame count should increment if things are working correctly.

II. Conversion of Stacks to Useable Data

1. Data Compression and Registration

Note: for processing speed, the data should be located on the MAIN hard drive on the light sheet computer!

After the collection of data with the light sheet microscope, image stacks are written to a folder containing the '.stack' files along with meta-data including 'ch0.xml', 'stack_dimensions.txt', and 'stack_frequency.txt'. The primary goal of this stage of analysis is to convert the .stack files into either the more compressed '.klb' format (10x smaller) or '.tif' format. At the same time, the conversion software allows cropping of regions that do not contain data, and also simultaneously performs a rough 2D (in X and Y) rigid registration of stack volumes over time.

The getConversionParameters.m helper script in Matlab saves user-defined parameters into each folder to allow batch-conversion of multiple experiments at once. Once all user settings have defined per experiment, specify the appropriate folders under *list_of_directories* in the script Batch_parLightSheet_Convert_Stacks.m and run.

For co-extraction of red channel: If there is red (or other color) channel structural information associated with your stack, there is an additional step which involves copying the conversionParameters.mat file generated by the process into the folders containing the red stack, detailed below.

Note: There the possibility of performing a mutual-information based registration which takes much longer. Anecdotally, I've seen that this can reduce the amount of jitter in time series over just using the 2D method. Whether or not to use this should be decided a priori for each paper's worth of experiments.

Procedure:

On the light sheet computer do the following:

- Open MATLAB

- Open script: **getConversionParameters.m,** set the user parameters as (or whatever is desired):
    - Outputformat = '.klb'
    - registerConcurrently = true
    - Crop_and_mask_image = true
    - computeTimeSeriesMaxProjection = true
    - computeTimeSeriesMeanProjection = true
- Run
- A new window will open. Find and select the folder that contains the stacks
- A new window called StackCropperGUI will open
    - Fit the fish inside the red square by using the arrow keys and the commands written on the window. The amount that the square moves with each click can be set in the %size scaling box.
    - Move the bar under the histogram to reduce the black background as much as possible. Check the 'show masking' box to preview.
    - **double click on stacks on right to check with them. Check beginning and see end at least...**make sure still in the red box across all stacks! Accept
- A new window will open. Select the same folder as before to save all output. ??? No folder selection option????
- A new window called 'Stack2DRegistrationGUI' will open.
    - In Flattening Method, select 'Mean of selected frames' if you want to average
    - Choose a slice in box below flattening method and then click in stack number on left then press enter (or choose a new stack number on left and put it in and press return) . Image on left should update to the selected slice. Pick a slice that contains components of varying brightness... middle of stack ish. The program will use this slice to compare across stacks by assigning green and red spots to the corresponding slices of the two stacks being compared at a time. If the slices from the two stacks are perfectly aligned, the overlay image will appear black and white (no green or red spots).
    - By clicking on arrow keys a yellow square will appear on the slice. Move it to an area that contains components of varying brightness. The dimensions of the square may be modified, but enlarging it increases processing time. Default size is good!
    - Fixed stack # should be the middle stack. Compare a few stacks to middle stack by typing in Stack # box.
    - Preview Registration. If fixed and moving look really different try reentering the stack numbers...

    - If everything looks acceptable, click Register! This does NOT cause registration!!!! It only saves the parameters... you need to go to the batch processing below to have the registration happen!!!
    - **Co-registration note:** If you are co-registering a red channel, copy the conversionParameters.mat file that is created for each trial into the folder containing the red stacks. I recommend that a structural stack is taken as closely as possible in time before and after each experimental trial and to take GCaMP stacks in quick succession when doing structural stacks (i.e. take 5 red stacks, switch filters/lasers then take 5 green stacks to insure you have a green/red set that is perfectly aligned). In the BATCH_parLightSheet_Convert_Stacks.m script, you will specify which are experimental and structural folders.

- Follow the prompts to add experimental stacks. You will have to navigate to the appropriate folders (being inside the folder one level is also OK) followed by structural stacks (CO-LABEL DIRECTORIES). You will also be prompted on whether or not you want the machine to shut down after it is done.

Press CONTINUE if you are finished adding directories or wish to leave one of the fields empty.

- Files are generated in the previously selected folder. Copy these into a drive and take them to the IMARIS computer: tS_maxproj and tS_meanProj (in timeProj folder), Background_0, ch0, conversionLog, Stack dimensions, Stack_frequency.
- The co-label directories generate cropped (un-registered) .tif files under \processed. Copy just one of these files to add as another channel in Imaris.
- **Note**: the script 'timeseries_viewer' allows visualization of slices across time for closer visual inspection

*Possibly consider re-moving stacks where there are significant movement artifacts before computing max projections.

2. Cell Segmentation in Imaris

The goal of this part of the pipeline is to generate spots in Imaris for each nuclear-labelled cell in the time series. To do this, I would recommend only moving files that are absolutely necessary between computers. This includes the max/mean projection from the previous step (.tif files) and the meta-data files (ch0.xml, stack_frequency.txt, and stack_dimensions.txt).

The segmentation is done in three steps. In the first step, software from the Ahrens lab (from Kawashima et al., 2016) finds candidate nuclei in each plane. These candidates are communicated to Imaris to generate spots. In the second step, filtering operations are applied in Imaris to keep user-specified spots. Lastly, the final segmentation is exported into a file format that can be read by software on the light sheet computer to extract fluorescence time series for each cell.

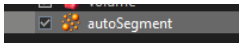
Note: The program currently checks within each slice for overlapping neurons, but doesn't check for this in adjacent slices at the moment.

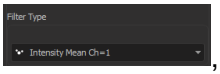
There is an Imaris XTension under IMAGE PROCESSING to change spot diameters in place by some fixed amount in X and Y.

Procedure:

- Move time projection files and meta-data to the Imaris computer
- Open IMARIS (make sure that only one window is open using Task Monitor)
- In IMARIS, open tS_meanProj (or maxProj, whichever one you think best reflects your cell distribution)
- Make sure that on IMARIS, go into 3D view and also: Edit -> Image Properties -> Geometry(or use ctrl+I) -> Voxel size -> x = 0.41, y = 0.41, z = 5
- Open the MATLAB script '**segment_HuCH2BGCamP_ims.m**'(File location(at 01Jun2018): C:\Users\Joe\Dropbox\CellAnalysisUtilities\LightSheet\segmentation)
    - Adjust brightness threshold(br_threshold), contrast threshold (cont_threshold) and cell radius (cell_rad) for cell detection. Usually I use br_threshold=100, cont_threshold=7, cellrad=3. Please adjust according to your needs
    - Run
    - Please enter segmentation channel: 1
    - If the program starts counting numbers -> good sign! Each number is a slice where the program is finding cells

NOTE: to find right segmentation parameters you can 3D crop the Imaris image to a slice or a few and more quickly see how different settings do! But if you crop 3D do to edit menu image properties and make the initial x, y and z zero!!!

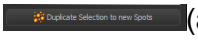
- In IMARIS
    - Select 'autosegment' 

in the scene window on left below volume line
    - Filter icon  -> Add, In Filter Type 

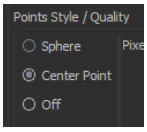
, select in popmenu:

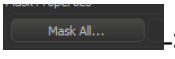
If the below are not in the filter list go to preferences in Imaris and statistics then spots and check the boxes for the missing statistics.

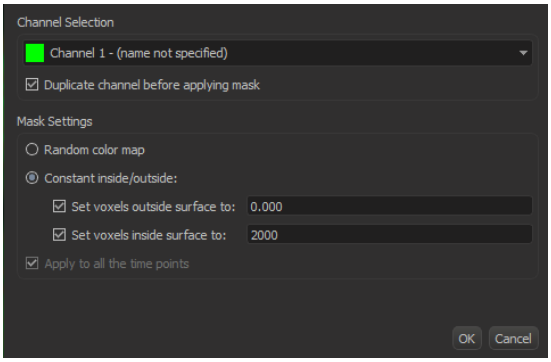
- Intensity Mean Ch=1 -> 500 (recommended by Dawnis(Cui sometimes use 100 to get largest coverage of neurons))
- Number of voxels -> 50 to 80 (recommended by Dawnis)????? No such selection
- Position Z -> get rid of most superficial layer. This is to get rid of reflections outside the fish usually at the top of the s stack... they may not exist in your data!

    - Duplicate selection to new Spots using this 

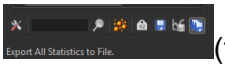
(at bottom left)

- Use ‘Center Point’ 

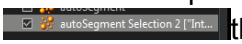
- Check the cell detection quality: (Cui comment: Usually I will just add two clipping plane to get a slice of brain (using this ) and check the distribution of center points in that slice, but there are other ways to do that…)
  - Pencil icon  -> Mask all 

-> Set outside to 0 and inside to 2000 

  - This gives a new channel in Imaris - change its color on display adjustment and window. Click on channel name to get colors. Change them in this way or as in the next step.
  - Edit -> Image Properties -> make Channel 1 (max projection data) green and Channel 2 (cells you just masked) magenta, check if they match. If not, you can discard current segment spots, and adjust brightness threshold (br_threshold), contrast threshold (cont_threshold) and cell radius (cell_rad) in the ‘segment_HuCH2BGCamP_ims.m’, run that program again until satisfied. Or adjust your Imaris fluo mean threshold or etc.
- File ->Export(or directly press ctrl+E) -> save .ims file in Imaris K drive

- Then we have two ways to extract cell position info:
  - Way1: Click on the ‘autoSegmentation Selection[“Position…..’ spots, click Statistics icon (), then click 

(the rightest one) export All Statistics to file. Then open the saved statistics xls file, use save as… to convert it to xlsx. file. Copy that xlsx to your portable hard drive.
  - Way2: In Matlab, first make sure you’re in the right directory which you want to put the position .mat file in, then open script ‘imSegmentation2matfile’ (File location(at 01Jun2018): C:\Users\Joe\Dropbox\CellAnalysisUtilities\LightSheet)
    - backTrackData = false

- Run
- Please type the name of the spots objection to use: (Note: type in the name of the spots object you want to extract position from, which usually should the name of be 

this one. Press F2 and then Cltr+C to copy the name of this spots object and past it here to tell the matlab program)
- Creates file called 'imsSegmentationData.mat' in whatever directory MATLAB is. Move file along with 'autoSegmentData.mat' file to drive.

A note on co-labeled neurons: If you have a red channel, you can use the filter function in Imaris to specify which of your previously segmented neurons are co-labeled. To do this, load the one of the .tif files generated with the stack conversion script above into Imaris as an additional channel. Check to see that your two channels are properly registered together. Then you can use the spots filter function to generate a new subset of spots that have a threshold intensity on your red channel. Later, this label may be applied to your extracted dataset using the data_explorer_gui.

3. Obtaining Fluorescence Time Series (FTS)

Matlab files on light sheet are on C: in custom_matlab_scripts folder

In this step, the cell segmentation information is transferred back to the light sheet computer to extract a fluorescence time series for each segmented neuron.

Procedure: On the light sheet computer

- In MATLAB
  - Script: 'extract_lightsheet_time_series_to_file'
    - useExcelFile = true (if you used Way1, which uses excel file)
      useExcelFile = false (if you used Way2, which didn't use excel)
    - Run
    - A new window will open. Select 'processed' folder (this is where you select the images folder with all the brain images)
    - Enter 1 to select '.klb'
    - Enter
    - A new window will open. Select 'imsSegmentationData' in drive
      - Note: Or, if useExcelFile=true, you'll need to choose ch0.xml file and the .xlsx file (the one just generated from Imaris) in this step. There will be a warning could not start excel server.... IGNORE It. There will be a delay before it continues and then it takes a bit to process..will say starting parallel pool when it begins.

- This takes some time…
- The saved fluorescence time series file will appear in the .klb directory. This is the file needed to perform analysis on data.

THIS is the final data file called: ls_fluorescence_time_series.mat.

- Note: voxels are only considered a part of the volume if the center of the voxel is within the ellipse defined by the spot.

IV. Validation Approaches: Older Approaches (independent of data_explorer_gui)

The main visually-based validation approaches are to use timeseriesviewerGUI or to draw ROIs onto slice movies using FIJI. Both methods need to use timeseriesviwerGUI first to generate the time slice movies.

On the light sheet computer:

- Generate of tsView files:
  - In MATLAB run 'timeseries_viewer_GUI.m' (Should be at Custom_Matlab_Scripts\timeseries_viewer_GUI), add to path if asked.
  - Click 'Load', check if '.klb' or '.tiff' is selected, load the corresponding file folder that contains .klb or .tiff data(usually should be the 'processed' folder).
  - Click Generate All Slice Movies, wait. This could take ~10s/timeframe. After the program is done, we'll have our time series of each plane in a newly created folder called 'tsView'.
  - You can transfer any/all of the planes you want to check onto external storage.

Drawing ROIs in timeseries movies

The information for spot positions/diameters is in the fluorescence_time_series.mat file included in the variables spas and spRaddiiXYZ. You also need the relevant tsView (.tif) files. This procedure generates ROIs in FIJI for each specified neuron passed into the command line argument of genFIJI_ROIs (a MATLAB function).

Procedure: On the Imaris computer

- Code: genFIJI_ROIs, drawRois.py. Copy these in same folder as everything else that you need.
- In MATLAB, load your fluroescence_time_series data extracted from raw image. (use load('filepath/filename');)
- In MATLAB, run: genFIJI_ROIs(spPos, [1:size(spPos,1)]', spRadiiXYZ)
  - Substitute highlighted with indices of cells of interest. This will be the index of the cells within both spPos and spRadiiXYZ. Best way to do this is to create spPos2 and spRadiiXYZ2 with cells of interest and then make index be 1:9 or wtv, but the entire set.
- This generates a folder called spotROIs
- Open ImageJ
- Open movie for the slice of interest, which should be a .klb file located in processed/ tsView folder
- Open 'drawRois.py' by dragging that .py file onto FIJI menu.
- Run
- Choose the position file (in folder spotROIs) of your plane of interest

- In ROI manager, make sure that it's empty initially and tick show all
- Saves ROI data as a zip
- Make sure ROI manager is clear before you load the ROIs for another plane.

data_exploration_gui

NOTE: A walkthrough has been added to the GUI folder (see data_exploration_gui/DOCUMENTATION) along with demo data.

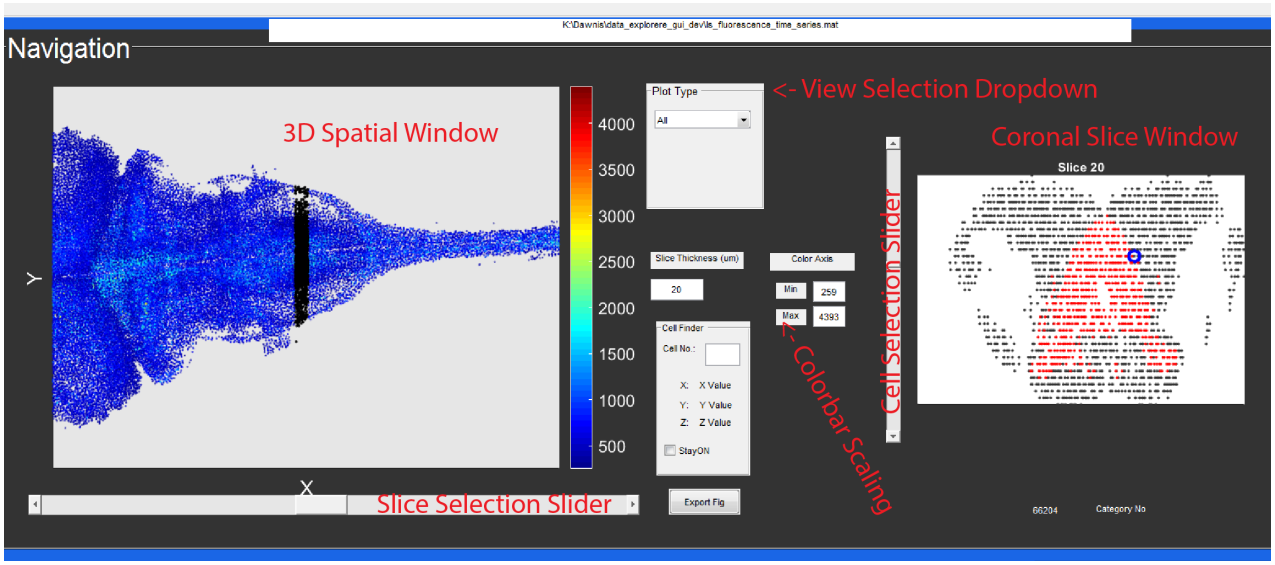
This GUI allows visualization of the spatial and time series information associated with extracted fluorescence time series, and provides a number of options manipulating sets of cells and interfacing with Imaris. **Additionally, it allows simultaneous visualization of calcium time series and slice movies for validation**.

On installed systems, this GUI will be located in the folder data_explorer_gui (for example, on Imaris 2 it is located in ~\Dropbox\data_explorer_gui\GUI) and is also on the path. The current version of the GUI is data_explorer_gui.m and its associated .fig file. If the path in MATLAB is correctly configured, typically the guy can be run simply by typing data_explorer_gui in the command window.

A Brief Tour

Navigation Window
Here, spatial data associated with your data set appears. The left window plots the whole dataset in 3D, while the right window plots coronal slices, highlighting neurons of interest in your selected ROI. Plot type has three modes: Exploration, ROI, and Validation. The colors of the dots in the 3D spatial window correspond to a map defined by the *metric* object in the metric listbox (by default this is the max. fluorescence exhibited by a time series. However, correlational analysis will generate a *metric* object mapped to correlation coefficient). The default mode, ‘all’, allows general exploration of the data via coronal slices. Neurons in this slice window are plowable via mouse or slider selection. In ROI mode, the background neurons may be displayed somewhat more sparsely, and all highlighted ROI groups will be plotted (selected under All ROIs listbox). In validation mode, the user can explore time slice movies by loading slice movies (see button under Tools) and loading a .klb Z-Stack.

(Cui added: If user want to find the cell no of one specific dots in the 3D spatial window or in the coronal slice window, they can choose the Data Cursor (showing at right panel) in the tool bar -> right click on the plot ->choose "Choose Call Back Function" -> choose "111CXYDataTipNewCallback051518.m" in the "... \Data_Explorer_Dawnis&Cui_20180910" folder -> Open. Then whenever the user use the "Data Cursor" tool to click on a data point in 3D plot, the X Y Z information and Cell No will be shown in the data tip that pops up.) (Note: This function may not be usable in new versions of MATLAB. Probably needs upgrade.)



Navigation Panel

(Note2: That is the reason I made "AllPosition" the only global variable at line 96 of this gui)

Time Series Window
In general, calcium time series appear in the top window here. The second window is currently unused, but can be adopted for displaying averaged results or stimuli. The plot types changes whether raw or delta f/f is displayed. (Delta f/f must be calculated using the Compute dFF button if it is not included in your fluorescence time series .mat file). If multiple members of an ROI are selected, they can be plotted simultaneously using the PlotFTS button.

ROI Window
Here, groups of neurons can be managed. The default group added upon load contains all neurons in the data set. A new group containing no neurons can be created by pressing the ‘a’ key with the ROI Master (left listbox) selected. ROIs can also be imported from analysis code or from Imaris (see Imaris menu).

ROIs can be imported or exported to file using the Export or Load button, or to FIJI ROIs using the Gen. Fiji ROIs button.

Tools Window
A variety of custom tools appear here.
Generate Time Slice Movies: Generates tsView.tif files for viewing time slice movies from a .klb stack directory.
*Load Slice Movie: loads one of the tsView files for viewing in validation mode.

*Compute dFF: Uses a dFF converter written by Dawnis that provides a number of options for filtering and windowing.

Correlation Window
After user finished the "All to All"/"ROI to ROI"/"Series to All_Corr"/"Series to All_CrossCorr" calculation, the correlation result will be presented here. See below "Correlation Analysis" section for details.

Imaris Menu
This allows interface with a data set in Imaris. Use the connect menu item to connect to an Imaris instance. Imaris visibility toggles the visibility of the Imaris window and is useful to know whether or not the connection was successful. The main function of connecting to Imaris is to import and export sets of spots. These must match positions in the data set precisely to be recognized as valid. (Meaning that the positions must be on a proper scale of 0.41 microns per pixel in X and Y and 5 microns per Z).

Loading Data and Manipulating Plots
Upon opening the GUI, the first task is to load a fluorescence_time_series.mat file into memory. This is accomplished by going to the *DataSet* menu item and selecting *Load Dataset*. If all goes well, neuronal locations will be marked with points colored by maximum intensity in the Navigation window. The slider can be moved to change the coronal slice in the slice window. The previous two windows are plotted in 3D, and the view can be changed using the rotation tool in the toolbar underneath the menu.
(Cui's Note: You can load the file "Joes_Sample_ls_fluorescence_time_series_Elb_012717Fish01Trial02_90Hz.mat" for test purpose. It should be right at the "Data_Explorer_Dawnis&Cui_20180910" folder. It contains a simplified light sheet fluorescence time series data, which consists of 7107 cells' fluo traces (matrix size: 7107x1280). But DFF WAS NOT CALCULATED IN THIS SAMPLE FILE!
After sample data gets loaded, the coronal slice window might be empty, in that case, just slide the slice selection slider of the 3D Spatial View window, and everything should be ready.)

Toolbar: hand (moves figure), data_tip (can select data points and display information), rotation for 3D figures.

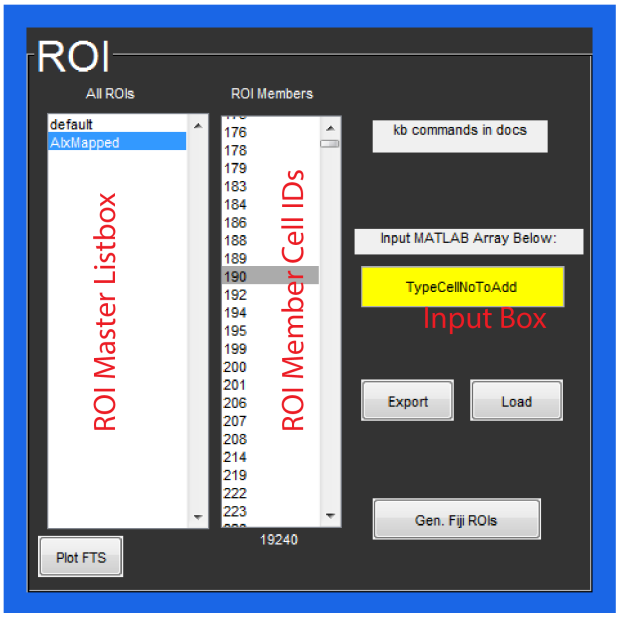
In general, the toolbar buttons can be used on any axis with a plot.

Manipulating ROIs

Create a new ROI by pressing a in the ROI Master listbox (left). Neurons can be added manually by updating the circled neuron in the Navigation slice window, clicking on the ROI members box, and pressing ‘a’. In addition, valid array expressions can be passed in through the textbox, (for example [5:5:1000] will select every fifth neuron between 5 and 1000) in the input box in the ROI window (highlighted yellow).

ROI Master Keyboard Commands
‘a’: Create an empty ROI set.
‘delete’: delete highlighted ROI set.
’r’: rename highlighted ROI set.
‘u’: take the logical union of all highlighted ROI sets
‘i’: take the logical intersection of all highlighted ROI sets.

ROIListbox Keyboard Commands
‘a’: add currently selected cell to ROI
‘delete’: delete currently selected cell(s).
‘k’: keep currently selected cell(s) and delete everything else.
‘p’/‘q’: plot currently selected members.
‘f’: find the selected ROI member(s) in space and plot



Metric Listbox
The metric listbox (underneath the ‘Plot Type’ dropdown in the Navigation Pane) controls what mapping is used for the color of dots in Exploration and ROI plot mode. The metric mapping in the list may also represent the outputs of analysis (for example, correlation to a particular bait sequence). Metrics are saved with the data when using the ‘Save Dataset’ option under the ‘Dataset’ menu.

Similar to the ROI Master Listbox, ‘delete’ and ‘r’ can be used to rename and delete metrics objects respectively.

Validation Mode
To use the validation mode view, first load a slice movie (and remember which one it is!) using the button under Tools. The Matlab command window will let you know when it is done loading (typically 30 to 90 seconds). Then, switch to Validation mode under Plot Type. Use the Load Z Stack button to visualize neurons in each slice. Any neurons that are selected under ROI members will be circled in the left Navigation window. Select the appropriate slice in your data set using the slider. Then, hit throw ROIs to highlight the appropriate members of each ROI for visualization on the movie. (Note, this will note update until the slider is moved).

Note that the color axis scaling of the top windows can be controlled by inputing appropriate min. and max. under the color axis prompt.

The Tools Panel

Note to future developers: *The code for the GUI (data_explore_gui.m and .fig) should only contain things related to display and data handling. DO NOT CODE ANALYSES*

INSIDE THE GUI. The tools panel exists as a connector to your fancy analysis, which should exist in a separate GUI or function. This is so that if your analysis happens to need a tweak, you do not screw up the GUI. Please see the 'Remove Overlaps' button for an example of how to pass data to and from the explorer GUI.

Generate Time Slice Movies
This button will generate a time varying slice file for each stack in the selected folder (stacks can either be in the .tif or .klb format, the program will select whichever type has the largest number in that directory). The generated files will be stored in a folder called tsView in the selected stack directory.

Compute dFF
This function uses a sliding window to compute dFF for each neuronal time series in your data. It has an option to apply a low-pass filter to smooth your data (default on). The low pass filter will allow frequencies lower than (1/5)*stack frequency through (that is, it is a low pass Butterworth filter with cutoff at 20% stack frequeny of order 3*stackfrequency).

The input required is the acquisition frequency of your data, the size of the baseline window desired (in seconds) and what percentile you want to treat as baseline (default 50% for median).

The 'Load' button and 'Save' button allow you to load the dFF from a previously saved file, or save it to file. Compute performs the computation.

(Cui added at 101618) Also, it is common to need to subtract background when convert raw fts into dFF. In that case, the "Subtract Background" checkbox should be checked, and background .csv file (can be exported from ImageJ) should be loaded by clicking "Load Back Ground Time Series" button. After the background .csv file get successfully loaded, the file name of that background .csv file will be presented below the "Subtract Background" checkbox.

Also, if you want to simply use lowest 5% as basal fluo, you can set moving window size to 99999(make sure it exceed the total time length), set basal percentile to 5%, uncheck Low-Pass Filter, load background fluo .csv file if you like, remember to check the Subtract Backgroud if needed, then Compute. (This paragraph is also presented in the pop-up window that will show up if you click on the "Help" button))

When the window is closed, the dFF information is transferred back to the main GUI.

(Cui's Comment: Users can also use the "Batch_dFF_Computer.m" in "... \Data_Explorer_Dawnis&Cui_20180910\IndependentScripts" folder to perform batch dFF calculation. You just need to make sure all to-be-calculated fts .mat files and background .csv files are in one same folder, and make sure each pair of fts .mat file and backgroun .csv file have the exactly same name (to prevent the program from

reading them with wrong sequence), and then set the directory, stack_frequency, window_size, basepercent, lowpass_on, and then run the program.)

Remove Overlaps Button
This tool can be used to remove neighbors within a certain distance either in 3D space or on adjacent slices only (excluding the each neuron's own slice) to help alleviate double counting. Currently, for each pair that is within the threshold distance, the one that has the highest maximum fluorescence is kept.

The tool asks for a threshold distance (default 5.4 microns) and whether to only check adjacent slices. If adjacent slices is not checked, the tool will also remove neighbors on the same slice that are too close to each other.

The tool creates two new ROIs, 'toKeep' and 'toRemove' which can be used to save the updated dataset and to examine the performance of the overlap removal algorithm. To save the filtered data, use the Export ROI button and select yes when asking to 'Export All ROI subsets'. The ROI 'toKeep' and all associated cell data will be saved in the ***toKeep.mat Matlab binary and can be loaded back into the GUI via 'Open Dataset'.

Correlation Analysis
Currently, correlational of an ROI (including the All ROI) to a single bait sequence is supported. Bait sequences (located beneath the time series plot window) can be loaded by either navigating the data set or using the 'Make Bait' button, which will average all highlighted items in the ROI members window. The correlation function can be found in the 'Correlational Analysis' menu, and can be performed with either $\Delta F/F$ or raw fluorescence depending on the radio selection on the GUI.

The results of the analysis generates a new addition to the 'Metrics' window below 'Plot Type'. It is possible to visualize the values of the correlation by selecting the newly generated item.

Selecting a data subset by Threshold
This feature has been added under the Dataset menu item and brings up its own sub GUI. Upon thresholding by the highlighted metrics item (selected by user), it returns an ROI with cells meeting the threshold criteria.

Possible Future Features and Work
-Event triggered analysis (button in Tool panel)