## **Two-photon imaging analysis pipeline for optogenetic experiments**

***Before processing, make sure all raw imaging data are properly organised and named***:

The file organization example:

*D:\2PhoData\210518\M1T3i-4m-ZT-IN1\_GCa,25F11A24D12D\_Chr,St,FO,LP20G20,10mA1s,RA*

## **A. Processing raw data**

***Step1: Z-projection***

***Run “Step1\_BatchZProjectionMaker.m” by changing the list\_of\_directories to the directory of the data folder. Set the parameters as below.***

ProjectionType=2; %1-MaxProjection, 2-MeanProjection

WriteMultiPageTiff=0; % Set this to 0 if you want to write separate tif images

WriteSeparateTiff=1; % Set this to 0 if you want to write multipage tif images

list\_of\_directories = {...

'D:\2PhoData\230702'...

% Put the path of the folders to be processed here

% 'D:\2PhoData\210929'...

% 'D:\2PhoData\210930'...

% 'G:\2PhoData\210915'...

};

**Explanation:** This code will make the averaged z-axis projection of all captured z-stacks and save them in a new folder, “ZProjected”, under the data folder.

***Step2: Image registration***

***Run “Step2\_BatchRegistration.m” by changing the list\_of\_directories to the directory of the data folder. Set the parameters as below:***  
  
GetFixedFrameBeforeStart=1;

ChooseFixedFrame=0;

DoAutoFixedFrame=1;

AutoFixedFrameNo=345; %Arbitrarily chosen fixed frame number

RunRegistration=1;

UseChoosedFixedFrame=0;

UseAutoFixedFrame=1;

UseAdjustedFixedImgForReg=0;

xmlname='Experiment.xml';

ProjFolderName='ZProjected';

TransformType='rigid'; % Change if needed! Either use translation or rigid.

list\_of\_directories = {...

'F:\2PhoData\240119'...

'F:\2PhoData\240121'...

'F:\2PhoData\240122'...

% 'D:\2PhoData\231210'...

};

**Explanation**: This code will register all the Z-projected images inside the “ZProjected” folder and save the registered images in a new folder called “Reg\_Matlab\_translation” or “Reg\_Matlab\_rigid” inside the “ZProjected” folder.

**Important:** After this step finishes, manually check the registration quality of the registered images! If the registration quality is too bad, run this code again by changing “TransformType='rigid'” to “TransformType='translation’” or the other way around. If the quality is still bad, use TurboReg (a FIJI plugin, website: <https://bigwww.epfl.ch/thevenaz/turboreg/>) to register the z-projected image stack. If TurboReg can produce a good registration result, make a new folder named “TurboReged” inside the “ZProjected” folder, and save the registered image stack as a .tif image sequence in that “TurboReged” folder. If TurboReg also cannot produce a good registration result, either discard this data or manually register every z-projected frame of the stack using the TrakEM2 function in FIJI (In FIJI, File>>New>>TrakEM2 (blank), drag the Z projected folder into the TrakEM2 window, unlock all frames, set one frame as red and another frame as green, and manually edit the XY coordinates of each frame. When finished, export the whole image sequence to .tif flat images).

***Step3: Extraction of Raw fluorescent data.***

***Run “Step3\_BatchExtractionMultiROIVer.m” by changing the list\_of\_directories to the directory of the data folder.***

*Set the parameters in the code as below:*  
  
xmlname='Experiment.xml'; ( this is the Thor image file for imaging parameters)

UseMeanProjOrMaxProj='mean';% Input 'mean' for mean t-projection, 'max' for max t-projection

ThresholdRatio=0.5;

list\_of\_directories = {...

'D:\2PhoData\240119'...

'D:\2PhoData\240121'...

'D:\2PhoData\240122'...

};

This code will first request the user to draw the ROI(s) and background ROI.

Once the parameters are set, it will extract the average grey value within each ROI from every frame of the Z projected image stack and store it into an Excel file “RegFluoDatL.xlsx

If the code has run successfully, it will save two copies of the Excel data file: one inside the registered image folder and the other in the RawFluo folder. There should be a time series column for each ROI and a column for the background.

***Step4: Extract the optogenetic stimulation time series for optogenetic trials and add the stimulus ON column***

**Before running the code:** For optogenetic stimulation, change the “ThorSync001” folder names in the Date Folder to “ThorSync[TrialName].” The Thorsync file name and the 2Pdata names must match for the code to work.

Run “**Step4\_BatchPlotting\_MultiROI.m**” after changing the list\_of\_directories to the directory of the data folder. Set the parameters as below:

**BackGroundSubtractionOn1OrOff0=1**;%Set this to 1 if you want background subtraction, set this to 0 if you don’t want background subtraction

FineTuningLightOnFrame=0;(not being used)

RawFluoFileName='RegFluoDatL.xlsx';

xmlname='Experiment.xml';

ThorSyncFileName='Episode001.h5';

ThorSyncSettingFileName='ThorRealTimeDataSettings.xml';

SetColorSpec={[0,0,1];[0,1,0];[0.9290, 0.6940, 0.5250];[1, 0.3, 1];[0.5, 0.70, 0.7410];[0.940, 0.840, 0.6560];[0.5, 0.5, 0.75];...

[0.660, 0.8740, 0.480];[0, 0.5, 0.16];[0.5010, 0.7450, 0.9330];[0.6350, 0.0780, 0.2840];[0.6350, 0.0780, 0.55];[0.6350, 0.0780, 0.3];[0.6350, 0.2, 0.55];[0.6350, 0.2780, 0.65];[0.9350, 0.0780, 0.55];[0.8350, 0.780, 0.55];[0.8500, 0.6250, 0.280];[0.6, 0.6250, 0.980];[0.16, 0.1620, 0.980];[0.26, 0.26, 0.9];[0.36, 0.36250, 0.2];[0.46, 0.50, 0.90];[0.1, 0.5, 0.16];[0.2, 0.15, 0.16];[0.3, 0.25, 0.16];[0.4, 0.5, 0.16];[0.5, 0.5, 0.16];[0.6, 0.5, 0.16];[0.7, 0.5, 0.16];[0.8, 0.5, 0.16];[0.9, 0.5, 0.16];[0.4, 0.25, 0.16]};

**BasalFrameTime=10;**

**BalsalFEndTime=3;%This means be basal frame is set to "from the 13 secs before first stim to 3 secs before first stim"**

CutTailFrameNo=0;

LeastStimInterval=100;%In frame

BGIndThre=0.55;

AutoDetectTrialTime=1;% Set it to 1 ->this code will read the 7th character of your Trial Folder's name and determine the trial time. Set it to 0 to manually input the trial time below

**ImageTimeMin=8; %Change here!! Set to 8 for 8 minutes trials, 4 for 4 minute trials.**

ImageTimeSec=0;%Change here!!

PulseOn=0;

PesudoFirstStimTime=31;%This is the time of the pseudo-first stimulation used for basal F time window selection when no optogenetic stimulation exists in the trial.

list\_of\_directories = {...

'D:\2PhoData\240119'...

'D:\2PhoData\240121'...

'D:\2PhoData\240122'...

};

**Explanation:** This code will read the 'RegFluoDatL.xlsx', and compute and plot the dF/F of the whole trial for each trial in the data folders enlisted in list\_of\_directories. This code will also read the optogenetic stimulation pattern data stored in “Episode001.h5” file from ThorSync, find and write the onset and offset time of each optogenetic stimulation, and write them into a new column in its output.

**Important**: The dF/F and plots generated in this step are only a quick preview of the data, and they are NOT used for any figure in the study.

**Note:** After this step, manually check the optogenetic stimulation onset and offset frame number by comparing them to the actual frame number in which the background elevation appears in the data stack. This correction is needed because of issues in ThorSynch.

## **B. Calculating and plotting dF/F0**

***Step 5. Put all raw fluo data (“RegFluoDatL-xxxx.xlsx”) of the same data group into the same folder. In the following text, that folder will be referred to as “Folder A.”***

Run “**Step5\_Segmentation\_df\_f\_calculation\_Opto.m**” with changing the list\_of\_directories to the directory of the folder A.  
  
For 1-second stimulation trials, use these parameters:

**stimtime**=1; %In Second, Change here if needed!!! Usually 1s or 10s

**TimeStartBeforeStim**=7;%in seconds. Change here!!,usually 7 for 1s stim trials, or 10 for 10s stim trials

**TimeStopAfterStim**=7;%in seconds. Change here!!,usually7 for 1s stim trials, or 20 for 10s stim trials

TimeResolution=0.25;% in second,usually 0.25

dFFOutlierThreshold=10;%Change If need, usually 10

ImageTimeMin=4;%Change here!!

ImageTimeSec=0;%Change here!!

For 10-second stimulation trials (used in Fig.5e), use these parameters:  
**stimtime**=10; %In Second, Change here if need!!! Usually 1s or 10s

**TimeStartBeforeStim**=10;%in seconds. Change here!!,usually7 for 1s stim trials, or 10 for 10s stim trials

**TimeStopAfterStim**=10;%in seconds. Change here!!,usually7 for 1s stim trials, or 20 for 10s stim trials

TimeResolution=0.25;% in second,usually 0.25

dFFOutlierThreshold=10;%Change If need, usually 10

ImageTimeMin=4;%Change here!!

ImageTimeSec=0;%Change here!!

Importantly, for both 1-second and 10-second stimulation trials, set **BasalFTimeLength=5;BalsalFEndTime=1;**(the basal F time window will be set to -6 to -1 second before stimulation onset)

**Explanation**: This code will generate the binned dF/F and interpolated dFF of each stimulation segment and save them into a new folder named “BindFFSeg” or “IntdFFSeg” inside the folder A, respectively.

**Step6.** Run “**Step6\_Plotting\_Opto.m**” with changing the list\_of\_directories to the directory of the folder A.

For 1-second stimulation trials, set   
**stimtime**=1; %In second, Change here if need!!!

**XLimSetting**=[-7 8];

**TimeStartBeforeStim**=7;%in seconds. Change here!! for 1s stim, set to 7, for 10s, set to 10

**TimeStopAfterStim**=7;%in seconds. Change here!! for 1s stim, set to 7, for 10s, set to 20  
  
For 10-second stimulation trials, set   
**stimtime**=10; %In second, Change here if need!!!

**XLimSetting**=[-10 20];

**TimeStartBeforeStim**=10;%in seconds. Change here!! for 1s stim, set to 7, for 10s, set to 10

**TimeStopAfterStim**=10;%in seconds. Change here!! for 1s stim, set to 7, for 10s, set to 20

For both 1-second and 10-second stimulation trials, set:

TimeAxisResolution=0.25;

YStep=0.5;

YLimSetting=[-0.5 2.5];

UseBinnedData=1;

AllFontSize=15;

TitleFontSize=18;

XFontSize=20;

YFontSize=20;

AutoLineColor=0;

SetColorSpec={[0.5, 0.6470, 0.7410];[0, 0, 1];[0.8500, 0.6250, 0.980];[0.9290, 0.6940, 0.5250];[0.4940, 0.6840, 0.5560];[0.75, 0.5, 0.75];...

[0.4660, 0.9740, 0.8880];[0, 0.5, 0];[0.5010, 0.7450, 0.9330];[0.6350, 0.0780, 0.2840];[0.6350, 0.0780, 0.55];[0.6350, 0.0780, 0.3];[0.6350, 0.2, 0.55];[0.6350, 0.2780, 0.65];[0.9350, 0.0780, 0.55];[0.8350, 0.780, 0.55];[0.8500, 0.6250, 0.280];[0.6, 0.6250, 0.980]};

**Step7.** This code calculates absolute peak dF/F in Fig. 1h and Fig. 4b (can be positive or negative) dF/F in 0 to 4 seconds after optogenetic stimulation onset.

Run the MATLAB code “**Step7\_Calculate\_peak\_df\_f\_Opto.m**” using the following parameters:

(Parameter used for Figure 1h).

StimStartRow=31;

TimeAxisResolution=0.25;%in seconds.

**CompareRangeStartInTime=0;%in sec. Relative to Stim start time. If it equal to stim start time, this parameter should be 0.**

**CompareRangeEndInTime=4;%in sec. Usually set to 4**

UseBinnedData=1;

list\_of\_directories = {...

'G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\Wiso,St(p\1s'...

'G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\Gr64f(P'...

'G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\Gr66a(P'...

'G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\ppk28,St(p'...

'G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\TMC(p'...

};

OutputPath='G:\My Drive\Worki\AvgdFFResponsePlot,Chec-Current\IN1 imaging\Starved\GutLines\Stats';

XlsxTitle={'Wiso','Gr64f','Gr66a','Ppk28','TMC'};%Fig 1, Make sure this sequence is in accordance with the input sequence of folders.