

2). ExVivo_InVivo_Registration Pipeline

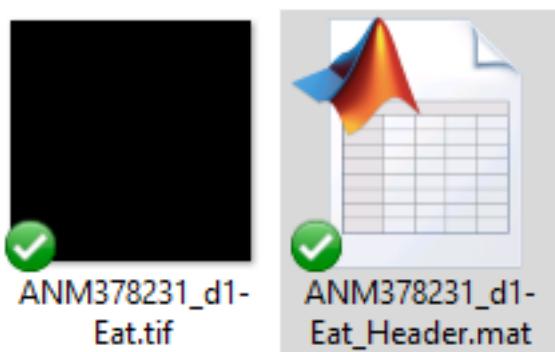
1. About the example data

The example data in **ExVivo_InVivo_Reg\ZStack_XDays** subfolder are the slow zstack images aquired in meta trials. These slow zstack images are used for **ex vivo to in vivo registration** and **image registration across days**. The organization of these images are shown below.

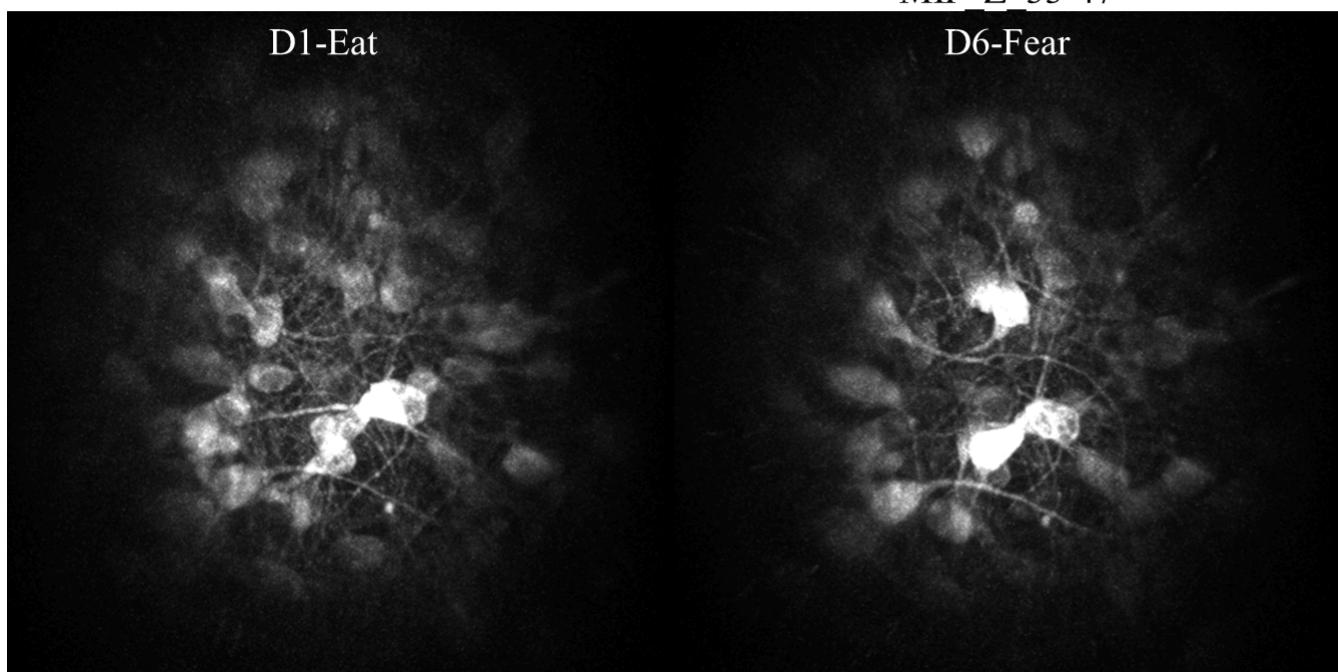
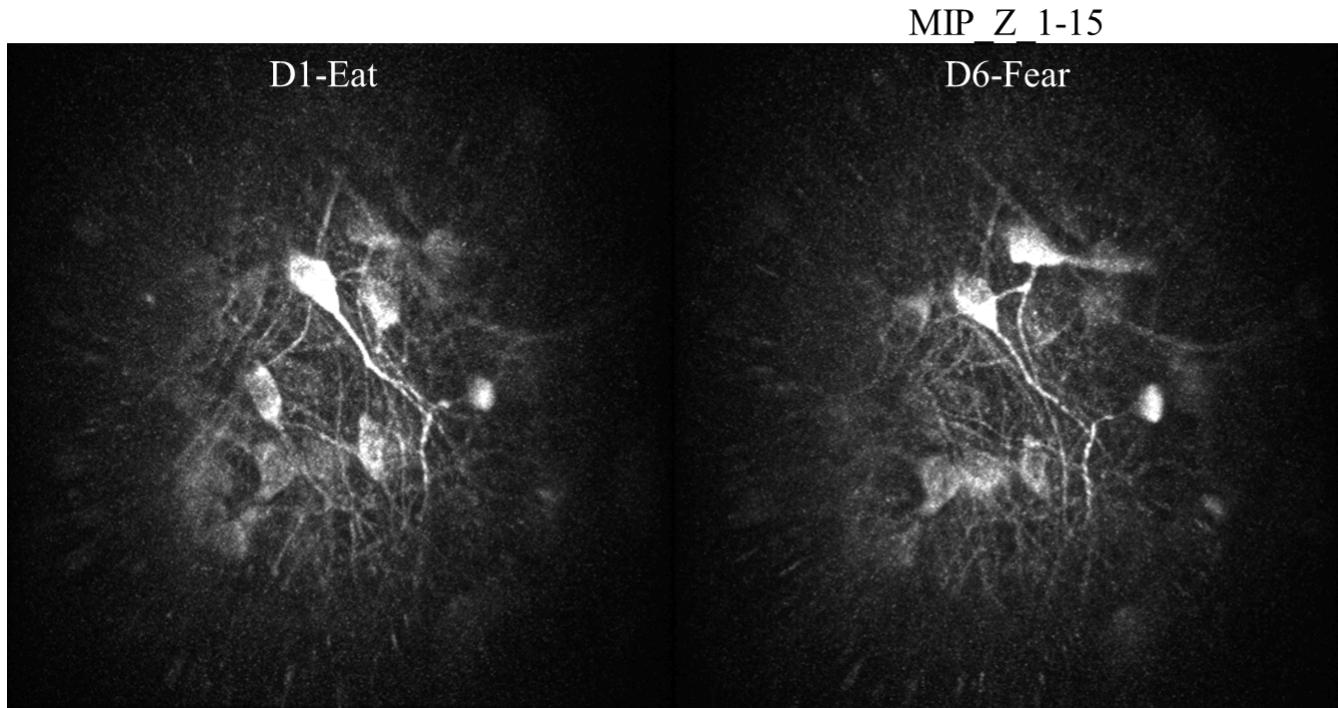
Name
d1-Eat
d2-Drink
d4-Training
d6-Fear
d8-Ghrelin
d9-Saline
d10-Leptin
d10-Perfusion

Except for **d10-Perfusion**, the subfolder names are in **dn-xxxx** format , where **n** indicates the **nth day** of the whole imaging experiments and **xxxx** is the imaging experiment related behavior. **ANM378231_d10-Perfusion.tif** in **d10-Perfusion** folder is the image zstack acquired through GRIN lens **after perfusion**. Except for **\d6-Fear\ANM378231_d6-Fear_Full.tif** and **\d10-Perfusion\ANM378231_d10-Perfusion.tif**, other images are acquired in the same imaging depth ranges as those in fast-z images. **\d6-Fear\ANM378231_d6-Fear_Full.tif** and **\d10-Perfusion\ANM378231_d10-Perfusion.tif** are acquired in larger imaging depth range.

Note1: For each image file, there is a corresponding .mat file as shown in gray below. The .mat file provides the original Scanimage meta information of the image zstacks, which is useful for the reconstruction of 2p image 3D volume in next section.



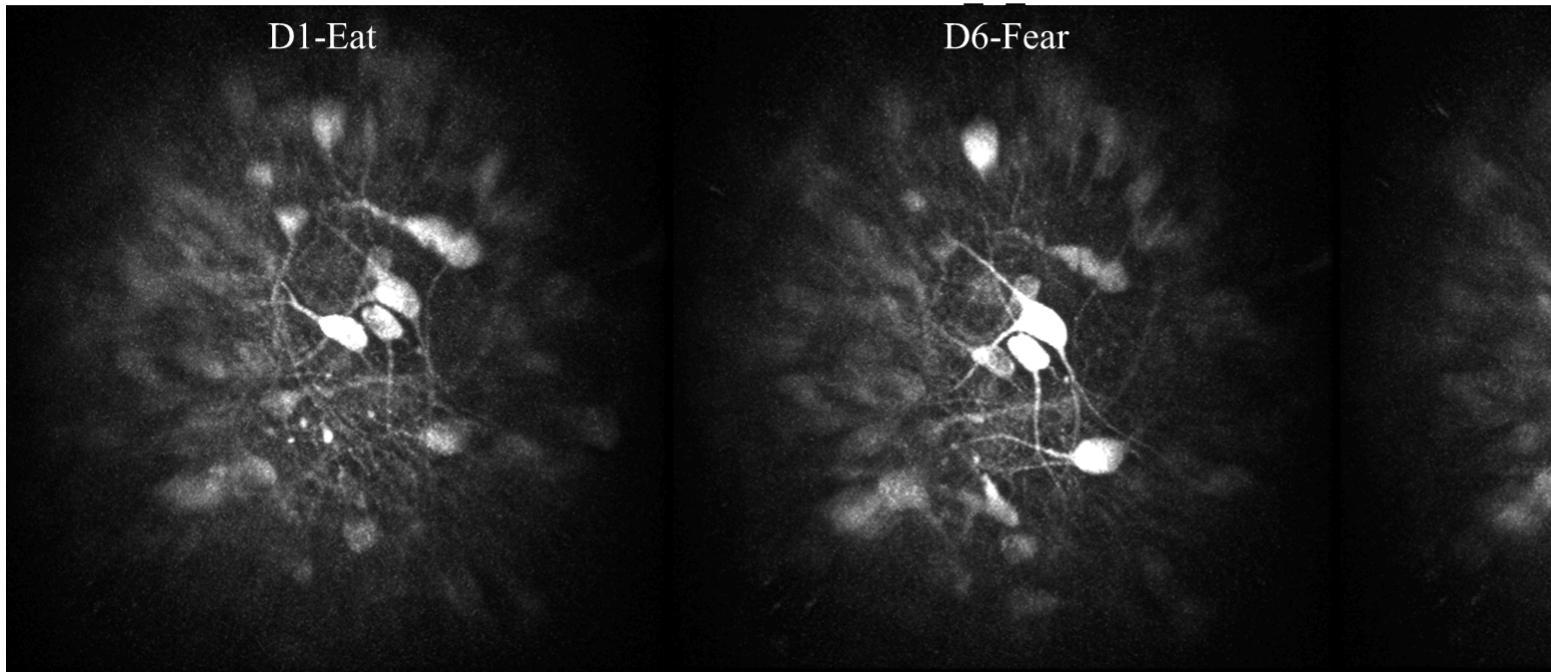
Note2: Following MIP_Z_nn-mm images are the maximum intensity projection of images on day 1, 6 and 10, where *nn* and *mm* indicate the starting and end z depth of the projection volume. MIP_Z_1-15, MIP_Z_33-47 and MIP_Z_60-74 compare the neuronal structure in superficial, middle and deep layer of the imaging volume on different days respectively. Comparison of these slow zstack images showed that the FOV of imaging brain region was quite stable during the whole imaging experiments.



MIP Z 60-74

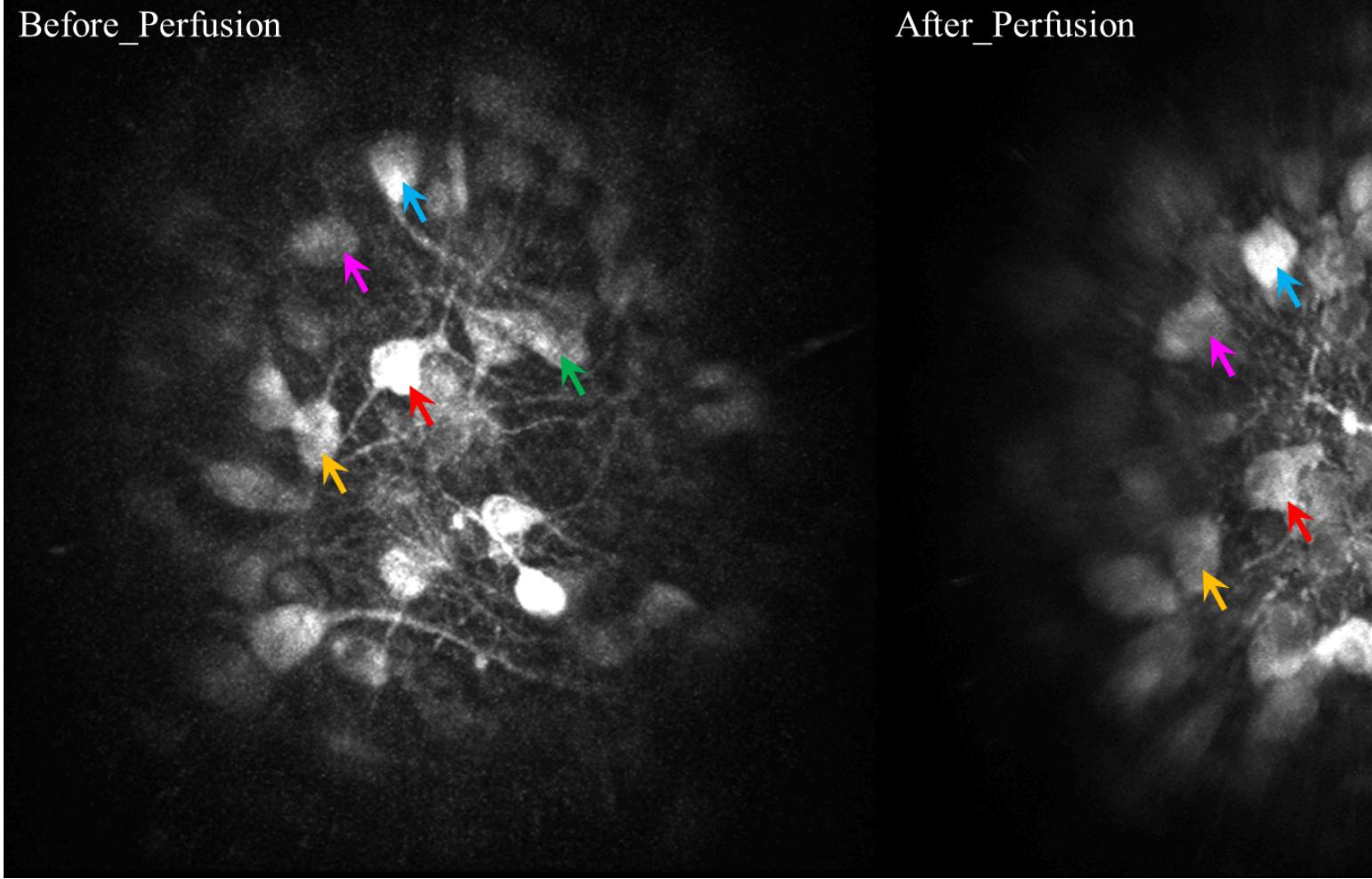
D1-Eat

D6-Fear



Note3: The neuronal image of the brain region under GRIN lens largely changed after perfusion. As shown below, after perfusion, although we can find many corresponding neurons, as marked with the same color arrows, the imaging FOV shifted down and the fluorescence intensities of some neurons, such as those pointed by the red and green arrows, dramatically changed. The image zstack after perfusion provides a crucial intermediate guidance between the ex vivo images and in vivo 2p images acquired in behavioral experiments.

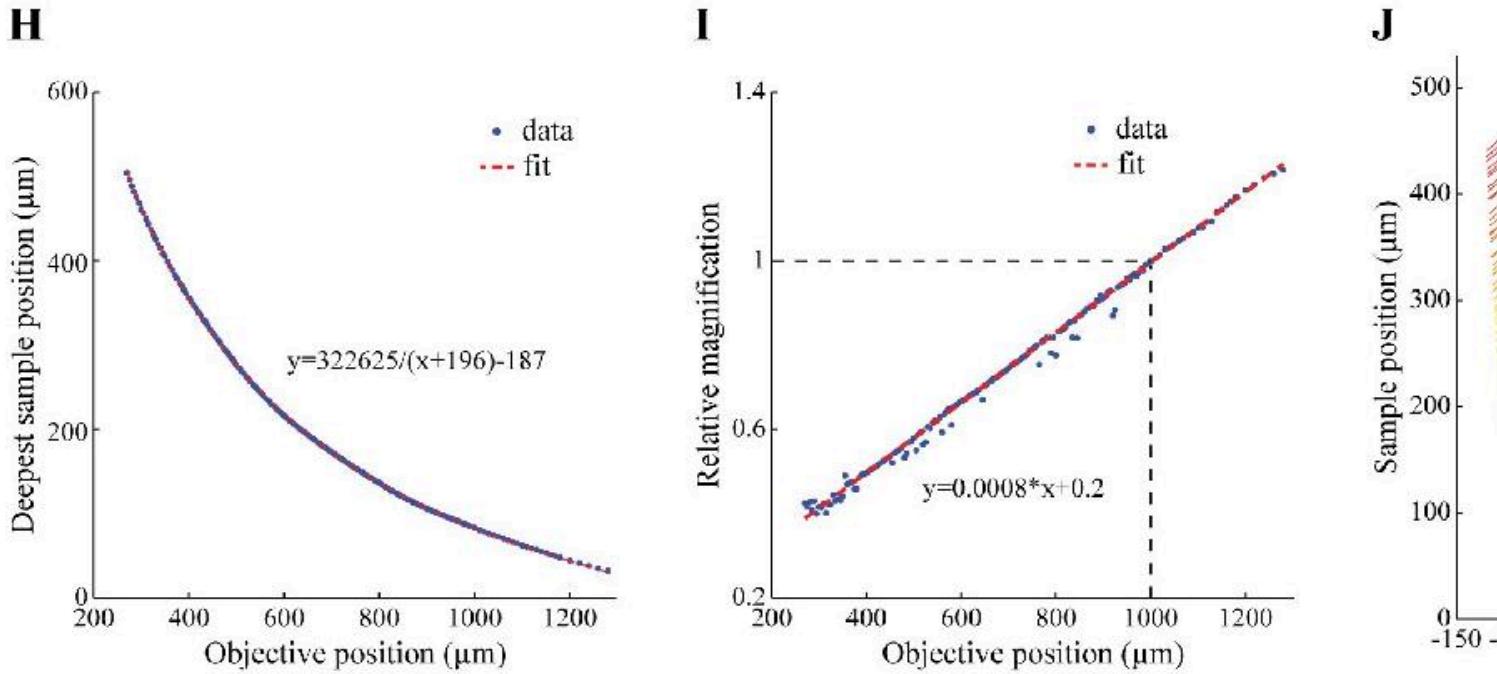
Before_Perfusion



After_Perfusion

2. Reconstruct the 2p image 3D volume by correcting the FOV curvature, remapping the objective-sample and objective-magnification relationships

As shown below (from the figS.3 in our paper 1), the two-photon-GRIN-lens imaging system has nonlinear objective-sample relationship (**H**), objective-position-dependent magnification (**I**) and FOV curvature (**J**). To find the same neurons in *in vivo* imaging volume and *ex vivo* stack, we need to reconstruct the 2p image 3D volume by correcting the FOV curvature, remapping the objective-sample and objective-magnification relationships using the calibrated optical properties of the two-photon-GRIN-lens imaging system.



The optical properties measured with 2p excitable GRID target are stored in `\ExVivo_InVivo_Reg\FOVs.mat`. There are 5 variables in the file: ***GRID_SPACE***, ***vtPosZ***, ***matCt_Md***, ***cLFits*** and ***cLFOVs***. Details about these variables are in `\ExVivo_InVivo_Reg\FOVs_readme.txt`.

- The following script uses the calibrated optical properties to reconstruct the 2p image 3D volumes acquired on day 6 (the middle of the whole imaging experiments) and after perfusion on day 10. These reconstructed 2p imaging volumes will be for the *ex vivo* to *in vivo* registration in next sections.

Batch_ReconstructImage3D

- This script generated reconstructed images, which were suffixed with `_Reconstructed.tif` as shown in gray below, in ***d6-Fear*** and ***d10-Perfusion*** subfolders. The script also plotted objective-position-dependent magnification, nonlinear objective-sample relationships.

Name

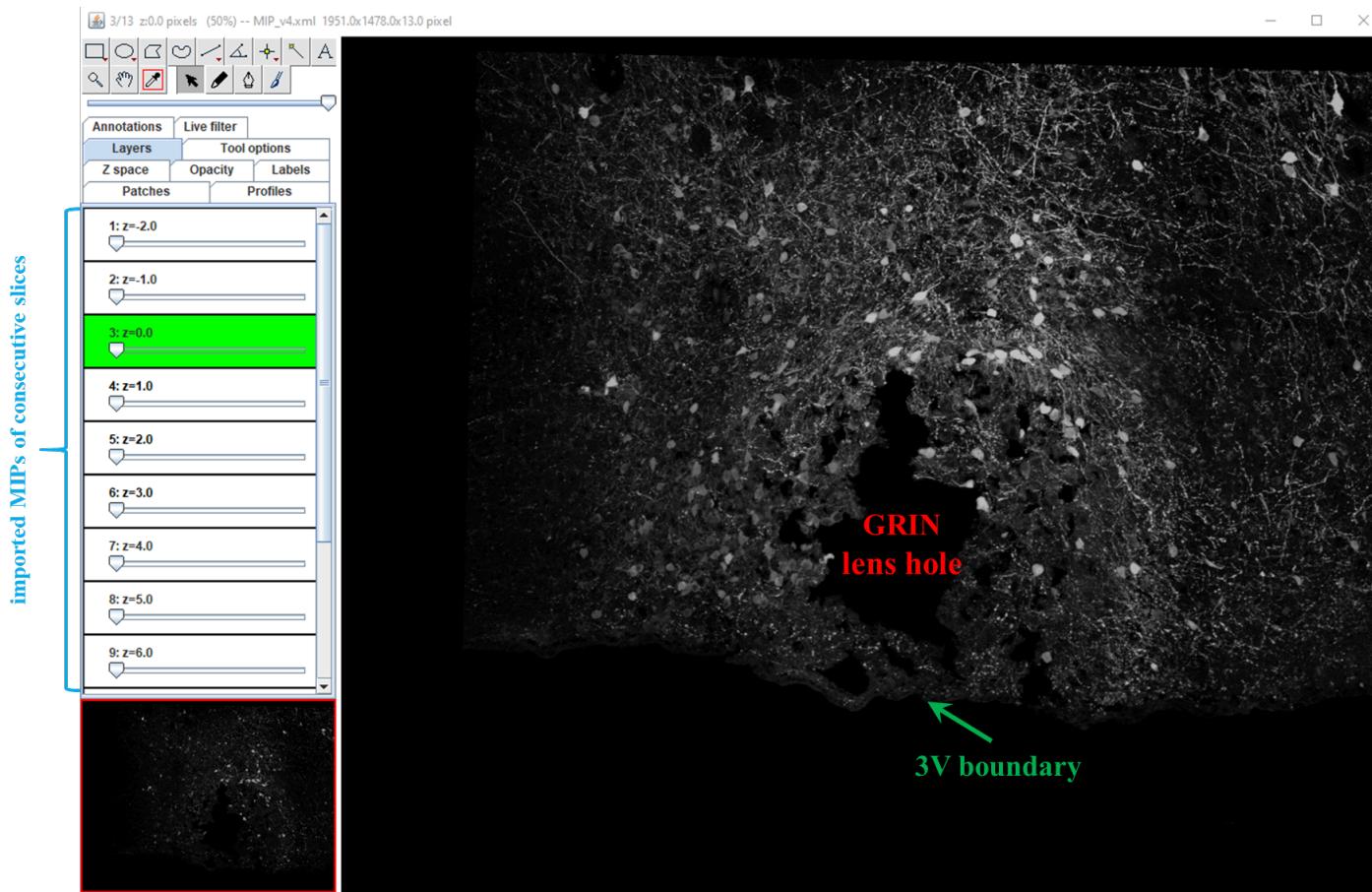
-  ANM378231_d6-Fear.tif
-  ANM378231_d6-Fear_Full.tif
-  ANM378231_d6-Fear_Full_Header.mat
-  ANM378231_d6-Fear_Full_Magnification.fig
-  ANM378231_d6-Fear_Full_Optical-Prop.fig
-  ANM378231_d6-Fear_Full_Reconstructed.tif
-  ANM378231_d6-Fear_Header.mat
-  ANM378231_d6-Fear_Magnification.fig
-  ANM378231_d6-Fear_Optical-Prop.fig
-  ANM378231_d6-Fear_Reconstructed.tif

Note1: I left these reconstructed images in the example data folder so that you can examine the correction changes.

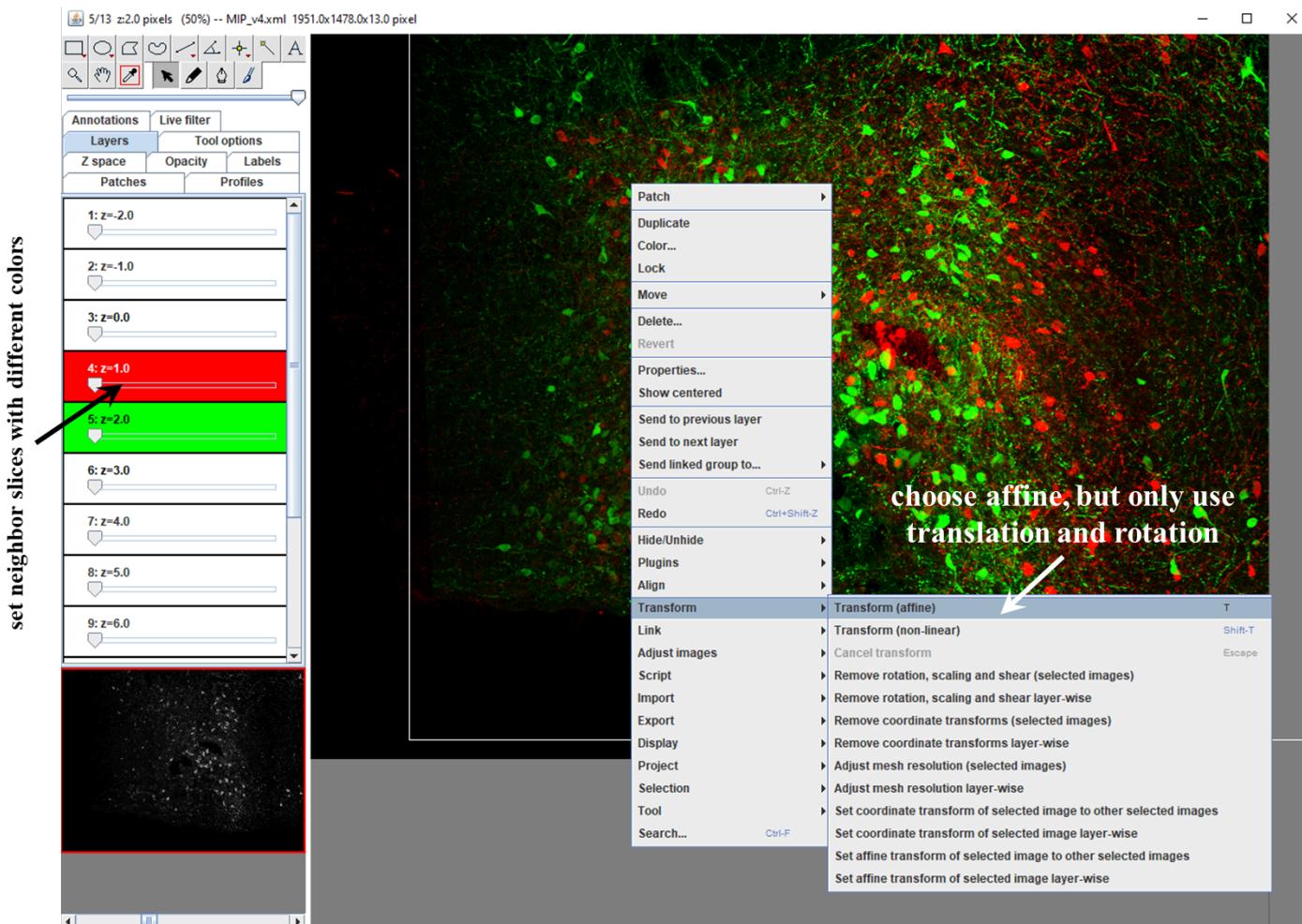
3. Stitch image stacks of *ex vivo* brain slices using the anatomy landmarks and under the guidance of the reconstructed 2p image 3D volume

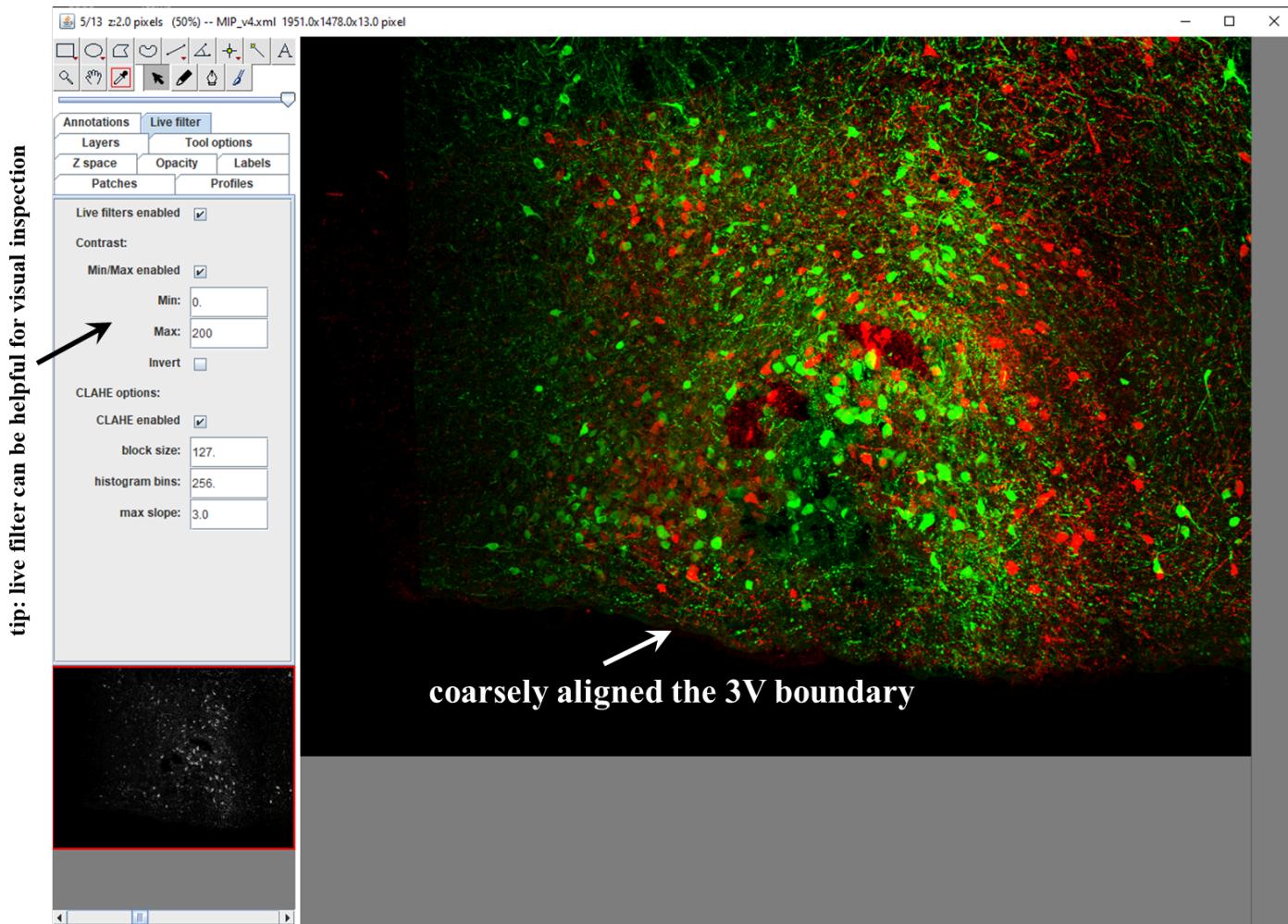
1. Coarsely stitch consecutive brain sections in [TrakEM2](#) using the GRIN lens hole and anatomy landmarks, such as 3V.

a). Import MIP images of consecutive brain sections into TrakEM2 in Fiji as shown below



b). Manually transform (translate and rotate) the neighbor images so that they are well aligned with anatomy landmarks

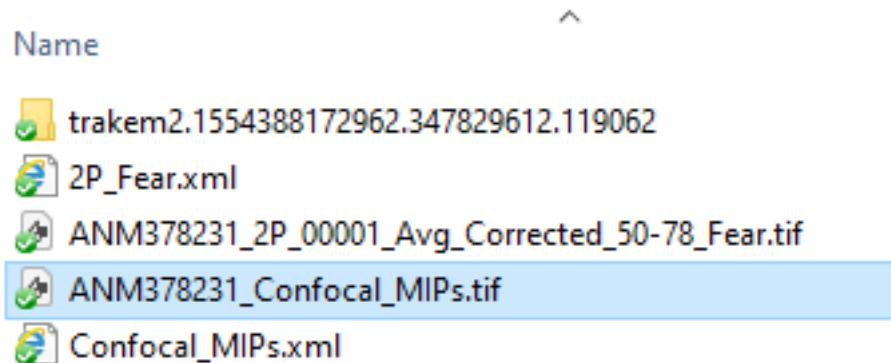




Note: the live filter as shown above can be helpful in the coarse alignment processing.

2. Perform fine stitching under the guidance of the reconstructed 2p image 3D volume.

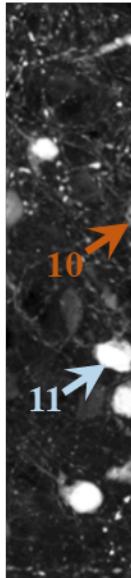
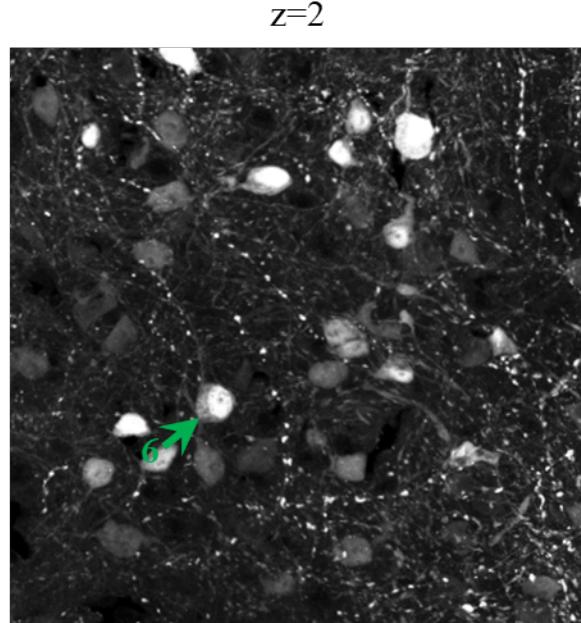
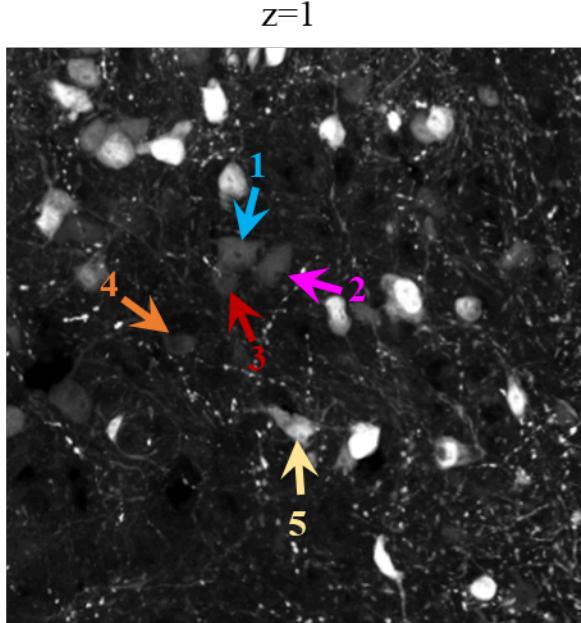
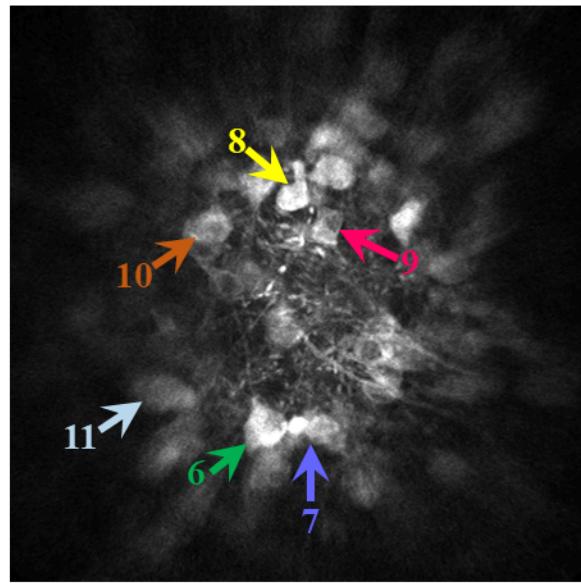
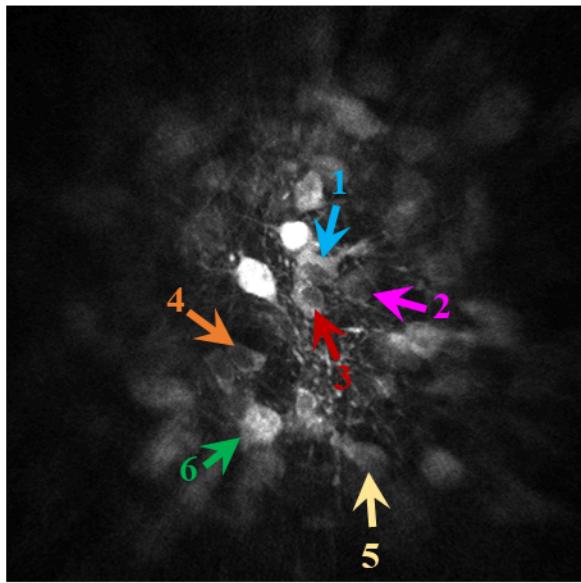
ANM378231_Confocal_MIPs.tif in \ExVivo_InVivo_Reg\Reg\Reg_Demo subfolder contains coarsely aligned MIPs of 3 consecutive sections.



a). Find neurons with distinct features in the reconstructed 2p image volume and ex vivo images of brain sections

As shown below, 11 neurons with distinct features could be easily identified in ANM378231_d10-Perfusion_Reconstructed.tif and ANM378231_Confocal_MIPs.tif. These neurons will be used as fiducial markers in next steps.

Confocal_MIPs.tif

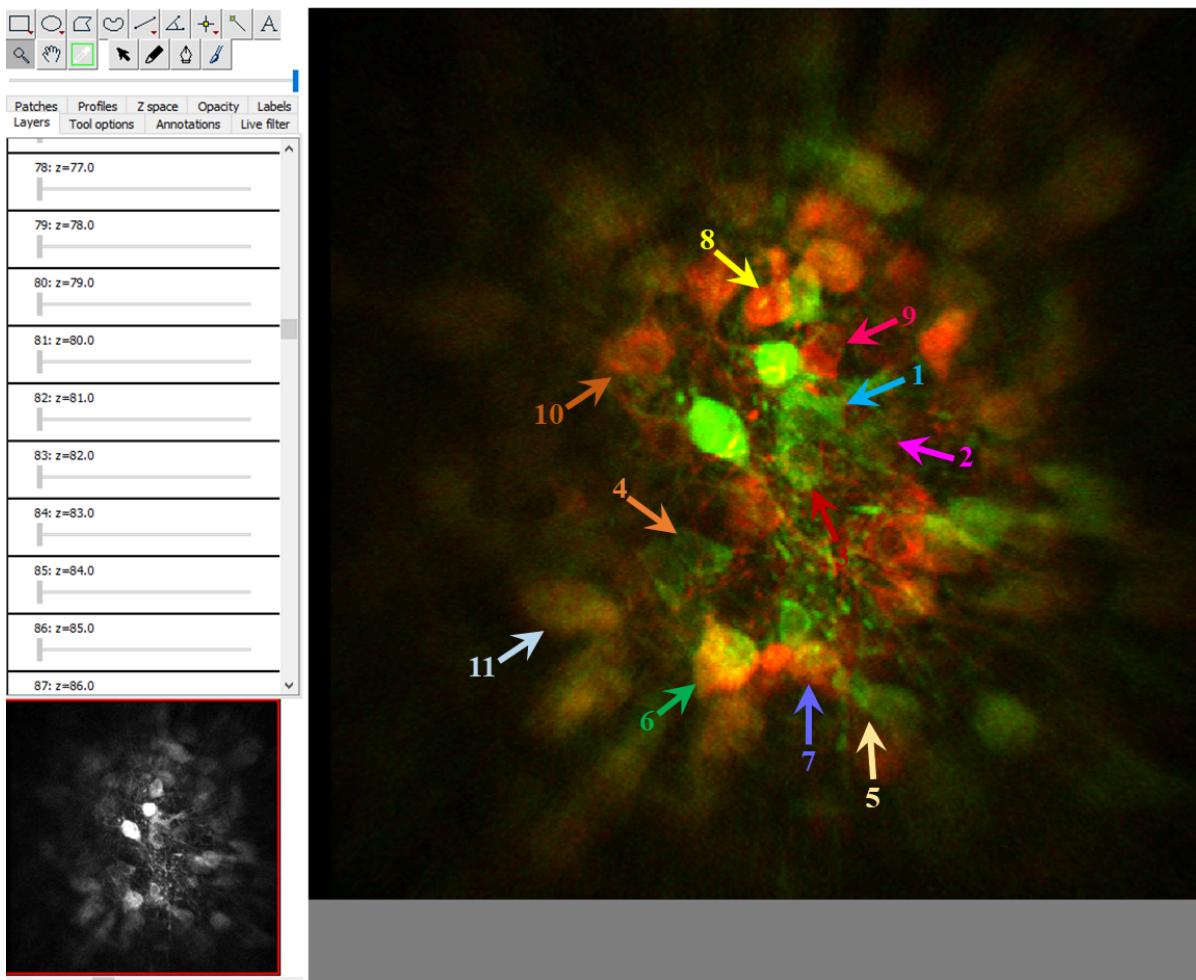


Note: the images from Confocal_MIPs.tif above were zoomed-in subregion of ANM378231_Confocal_MIPs.tif.

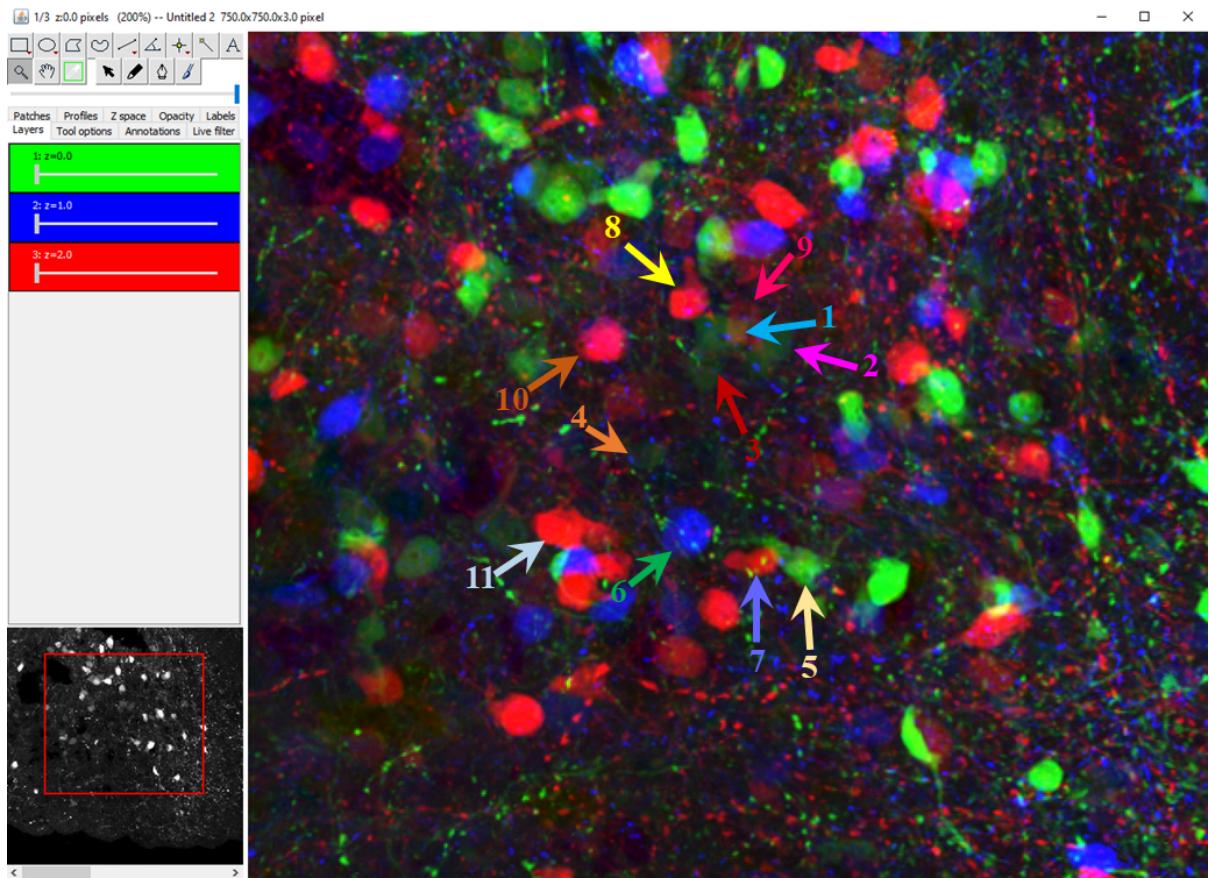
b). Manually adjust the neighbor images under the guidance of the reconstructed 2p image 3D volume so that the stitching precision < 10 um (about 2/3 neuron size)

Below shows the relative locations of those 11 fiducial neurons in corrected 2p image volume. Image at Z=73 is green and image at Z=89 is red.

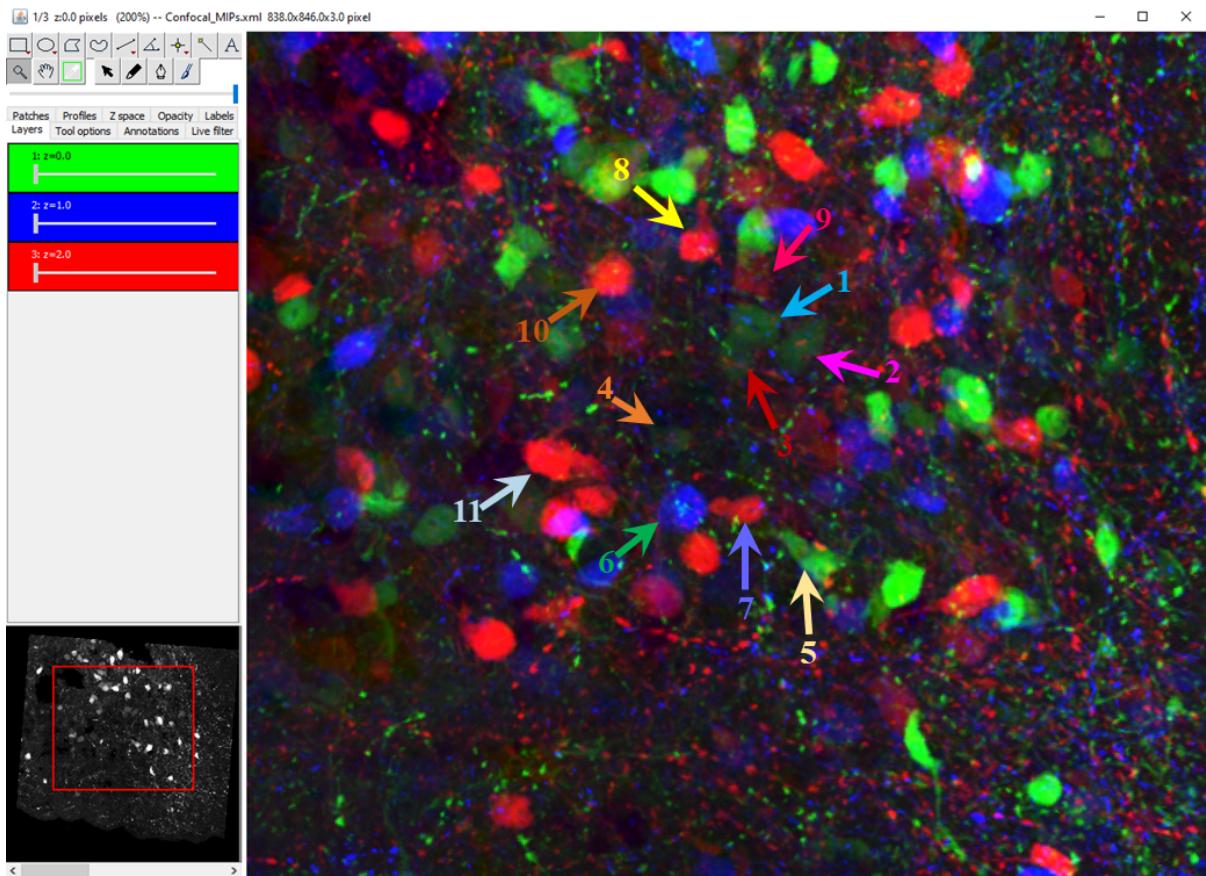
73/247 z:72.0 pixels (134.0%) -- Untitled 0 601.0x601.0x247.0 pixel



As shown below, the coarsely aligned images **ANM378231_Confocal_MIPs.tif** provides largely right location of those neurons. However, the relative positions of those fiducial neurons were mildly messed up compared with those in reconstructed 2p image volume. For example, the relative locations among neuron 1, 8 and 9, relative locations among neuron 5, 6 and 7 are different from those in intact 2p volume.



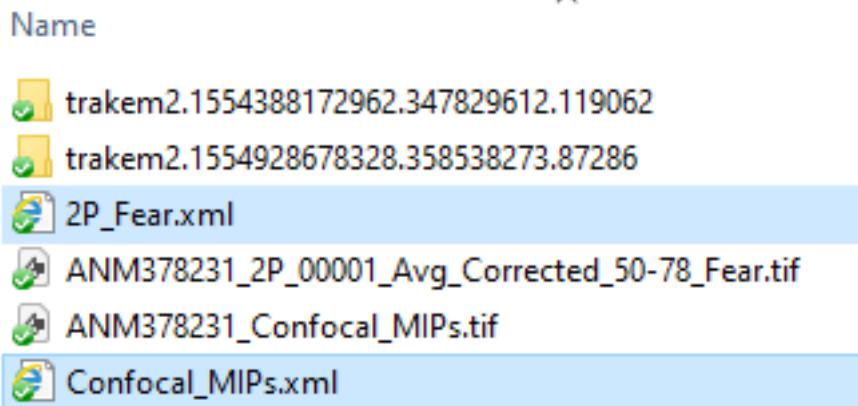
With the guidance of the reconstructed image volume, we can manually adjust (translate and rotate) the neighbor images so that the relative positions of those fiducial neurons are almost the same as those in reconstructed 2p image volume. Below shows the well stitched images of ANM378231_Confocal_MIPs.tif.



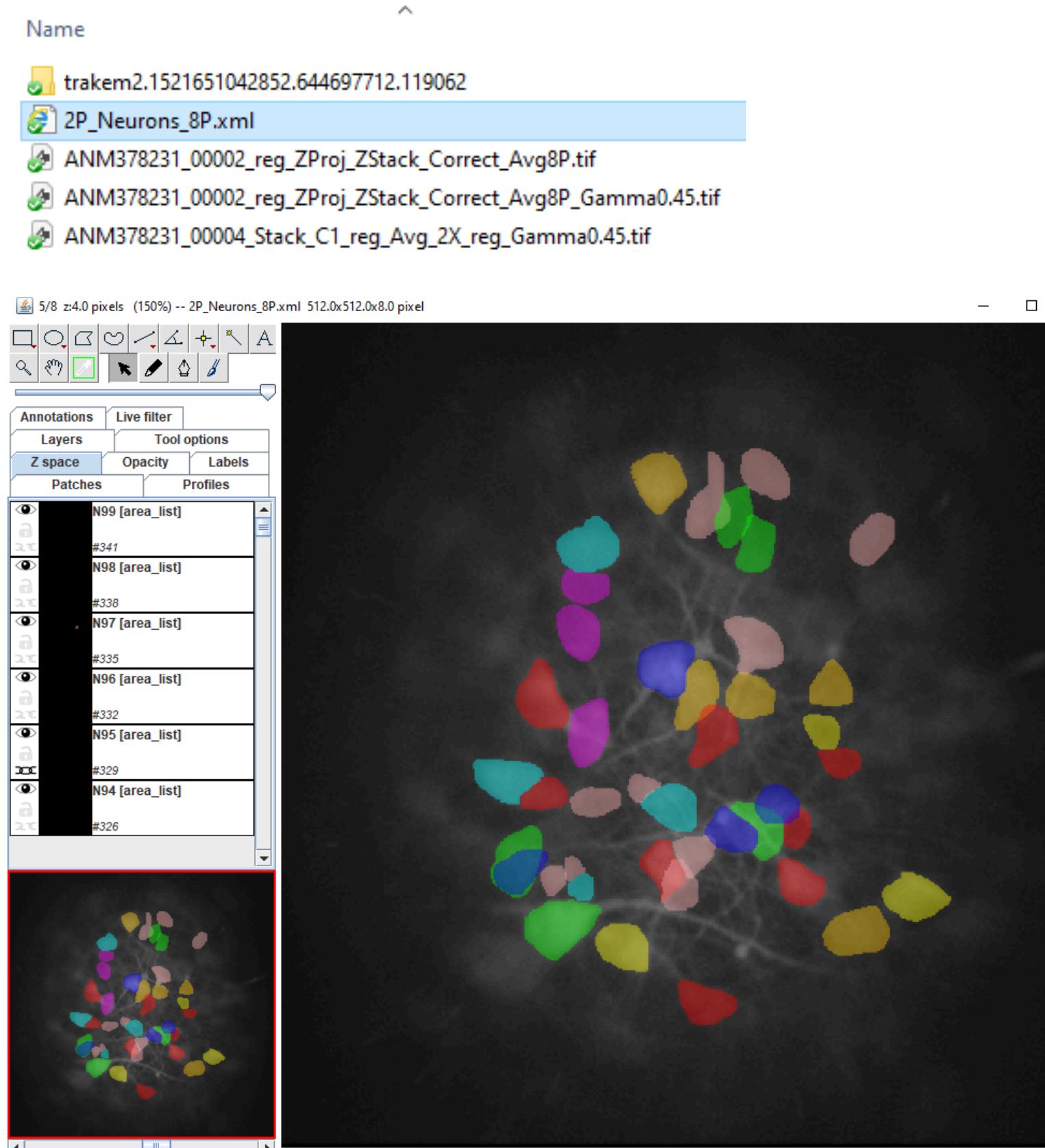
4. Find the corresponding neurons in *in vivo* 2p image volume and ex vivo confocal image stack using TrakEM2

After stitching ex vivo images, we used these identified fiducial neurons as starting points to find their neighbor neurons matched between the corrected ZStack images in fear retrieval experiment (middle of the entire imaging experiments) and ex vivo images.

2P_Fear.xml and **Confocal_MIPs.xml** in **\ExVivo_InVivo_Reg\Reg\Reg_Demo** subfolder are TrakEM2 projects demonstrating the ex vivo to *in vivo* registration with 40 matched neurons in a subset of the 2p imaging volume.



2P_Neurons_8P.xml in **\ExVivo_InVivo_Reg\Reg\Reg_All_Planes** is a TrakEM2 project with 99 neurons identified in ex vivo images in all 8 imaging planes.



Note1: to facilitate observing the dark neurons, the gamma of the images imported into **2P_Neurons_8P.xml** was adjusted to 0.45.

Note2: because of the relative elongated axial resolution (~10 µm) of the two-photon-GRIN-lens imaging system, the images of some neurons may be spatially overlapped as shown above.

5. Export ROIs of individual neurons from TrakEM2

Because the images of individual neurons can be spatially overlapped and across multiple imaging planes, each neuron was exported as a 3D ROI mask.

Save_AreaList_As_ROIs.py in **\Analysis_Pipeline\Local\ExVivo_InVivo_Reg** subfolder was used to export ROIs of individual neurons from TrakEM2. To run this Jython script in Fiji, you need to add Fiji-Legacy to your update sites and initiate Jython interpreter in Fiji plugin as shown below.

```
Save_AreaList_As_ROIs.py %in Fiji
```

Manage update sites

A...	Name	URL	Host	Directory on Host
<input checked="" type="checkbox"/>	ImageJ	https://update.imagej.net/		
<input checked="" type="checkbox"/>	Fiji	https://update.fiji.sc/		
<input checked="" type="checkbox"/>	Fiji-Legacy	https://sites.imagej.net/Fiji-Legacy/		
<input checked="" type="checkbox"/>	Java-8	https://sites.imagej.net/Java-8/		
<input type="checkbox"/>	2015-Conference	https://sites.imagej.net/2015-Conference/		
<input checked="" type="checkbox"/>	3D ImageJ Suite	https://sites.imagej.net/Tboudier/		
<input type="checkbox"/>	3Dscript	https://romulus.oice.uni-erlangen.de/updatesite/		
<input type="checkbox"/>	ActogramJ	https://romulus.oice.uni-erlangen.de/imagej/upd...		
<input type="checkbox"/>	AIC Janelia - Course	https://sites.imagej.net/AICjanelia-course/		
<input type="checkbox"/>	Angiogenesis	https://sites.imagej.net/Angiogenesis/		
<input type="checkbox"/>	AngioTool	https://sites.imagej.net/AngioTool/		
<input type="checkbox"/>	Archipelago	https://sites.imagej.net/Lindsey/		
<input type="checkbox"/>	AxoNet	https://sites.imagej.net/AxoNet/		
<input type="checkbox"/>	BACMMAN	https://sites.imagej.net/Ljp/		
<input type="checkbox"/>	BAR	https://sites.imagej.net/Tiago/		
<input type="checkbox"/>	BaSiC	https://sites.imagej.net/BaSiC/		
<input type="checkbox"/>	BIG-EPFL	https://sites.imagej.net/BIG-EPFL/		
<input type="checkbox"/>	BigStitcher	https://sites.imagej.net/BigStitcher/		
<input type="checkbox"/>	BigVolumeViewer Demo	https://sites.imagej.net/BigVolumeViewer/		
<input checked="" type="checkbox"/>	Bio-Formats	https://sites.imagej.net/Bio-Formats/		
<input type="checkbox"/>	Biomat	https://sites.imagej.net/Biomat/		
<input type="checkbox"/>	Biomedgroup	https://sites.imagej.net/Biomedgroup/		
<input type="checkbox"/>	BioVoxel	https://sites.imagej.net/BioVoxel/		
<input type="checkbox"/>	Blind Analysis Tools	https://sites.imagej.net/Actual/		

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Scripting ▾

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- Stitching ▾
- Time Lapse ▾
- Tracking ▾
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- Utilities ▾
- Volume Viewer

BeanShell Interpreter

Refresh BSH Scripts

Macro Interpreter

Refresh Macros

Clojure Interpreter

Refresh Clojure Scripts

JRuby Interpreter

Refresh JRuby Scripts

Javascript Interpreter

Refresh Javascript Scripts

Jython Interpreter

Two variables `savDir` and `Label_Max` need to be set. Please read the details about them in the script. The script generates a Tiff stack for each ROI. The file name of the Tiff stack is in **N*iii*** format as shown below, where *iii* is the ROI ID in TrakEM2.

Name

-  N99.tif
-  N98.tif
-  N97.tif
-  N96.tif
-  N95.tif
-  N94.tif
-  N93.tif
-  N92.tif

6. Register images from fastZ to slow zstack

During fastZ volumetric imaging, the image size was set to 256x256 to increase the temporal resolution. However, in slow zstack imaging, to increase the spatial resolution, the image size was set to 512x512. Beside the difference in image size, we found during fastZ imaging, there are slight and consistent mechanical vibrations of the objective lens during the fast movement.

-  ANM378231_00002_reg_ZProj_ZStack_Correct_Avg8P
-  ANM378231_00004_Stack_C1_reg_Avg
-  ANM378231_00004_Stack_C1_reg_Avg_2X
-  ANM378231_00002_reg_ZProj_ZStack_Correct_Avg8P.tif
-  ANM378231_00004_Stack_C1_reg_Avg.tif
-  ANM378231_00004_Stack_C1_reg_Avg_2X.tif

In subfolder **\ExVivo_InVivo_Reg\Reg\ZStack_FastZ_Reg**, ANM378231_00004_Stack_C1_reg_Avg_2X.tif is the 2x template images in individual fastZ imaging planes and ANM378231_00002_reg_ZProj_ZStack_Correct_Avg8P is the average images of slow zstack in the same imaging ranges as those during fastZ imaging. Comparing these two image stacks, you can find the vibrations in fastZ image (ANM378231_00004_Stack_C1_reg_Avg_2X.tif) and the stability in slow zstack (ANM378231_00002_reg_ZProj_ZStack_Correct_Avg8P). Because of the stability in slow zstack, we use the slow zstack to do the ex vivo to *in vivo* registration. To generate the ROIs for fastZ imaging, we need to register the fastZ image stack to slow zstack.

The script below was run in **Janelia Computer Cluster** to split the image stacks into images of individual imaging planes and register the images in fastZ to their corresponding images in slow zstack.

```
MatlabCmd_Reg_StackZ_FastZ.m
```

For the example data, the script finally generates the registered a fastZ image stack and saves the transformation information in subfolder **\ExVivo_InVivo_Reg\Reg\ZStack_FastZ_Reg\ANM378231_00004_Stack_C1_reg_Avg_2X\imgSeq** as show below.

-  ANM378231_00002_reg_ZProj_ZStack_Correct_Avg8P.tif
 -  ANM378231_00004_Stack_C1_reg_Avg.tif
 -  ANM378231_00004_Stack_C1_reg_Avg_2X.tif
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_reg.tif
-
-  ANM378231_00004_Stack_C1_reg_Avg_2X_00081Warp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00081InverseWarp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00080GenericAffine.mat
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00071Warp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00071InverseWarp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00070GenericAffine.mat
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00061Warp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00061InverseWarp.nii.gz
 -  ANM378231_00004_Stack_C1_reg_Avg_2X_00060GenericAffine.mat

7. Transform ROIs to fast-z images

Using the script below, the ROIs, which were identified during *ex vivo* <-> *in vivo* registration, were transformed to fastZ imaging planes with the transformations above. The script was also implemented to run in **Janelia Computer Cluster**.

```
Batch_MatlabCmd_ApplyTrans_RegROIs_Inverse_AllNeurons
```

The transformed ROIs are in **\Fear_Imaging_Exp\2P_Imaging\Avg3\ROIs_Reg** and can be used to extract calcium dynamic responses from images as shown in **TwoPhoton_Imaging_Processing_Pipeline mlx**.

Name

-  N99_reg.tif
-  N98_reg.tif
-  N97_reg.tif
-  N96_reg.tif
-  N95_reg.tif
-  N94_reg.tif
-  N93_reg.tif
-  N92_reg.tif
-  N91_reg.tif
-  N90_reg.tif