User Guide

for the streamlined, less biased, but still manual registration of fluorescent tracks to the Allen mouse brain Reference Atlas

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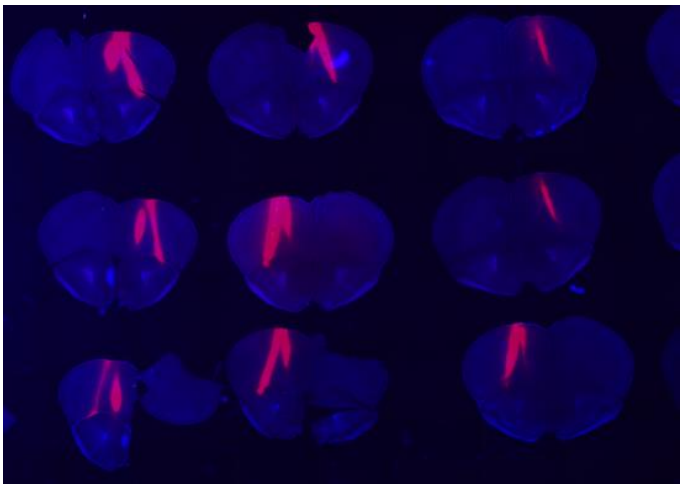
1. Data & code to have on hand

- Imaged slides of brain slices with fluorescent track(s)



- Microns per pixel of these images

- Prior knowledge of the number, entry point, and insertion depths of all probes is highly recommended

- MATLAB (R2017 on a Windows computer used for testing)

- A (computer) mouse with a scroll wheel

- Get the code at github.com/cortex-lab/allenCCF and add this folder with subfolders to your MATLAB Path

- Additionally, download the reference atlas data at http://data.cortexlab.net/allenCCF/ and suporting code at https://github.com/kwikteam/npy-matlab

2. Pre-processing

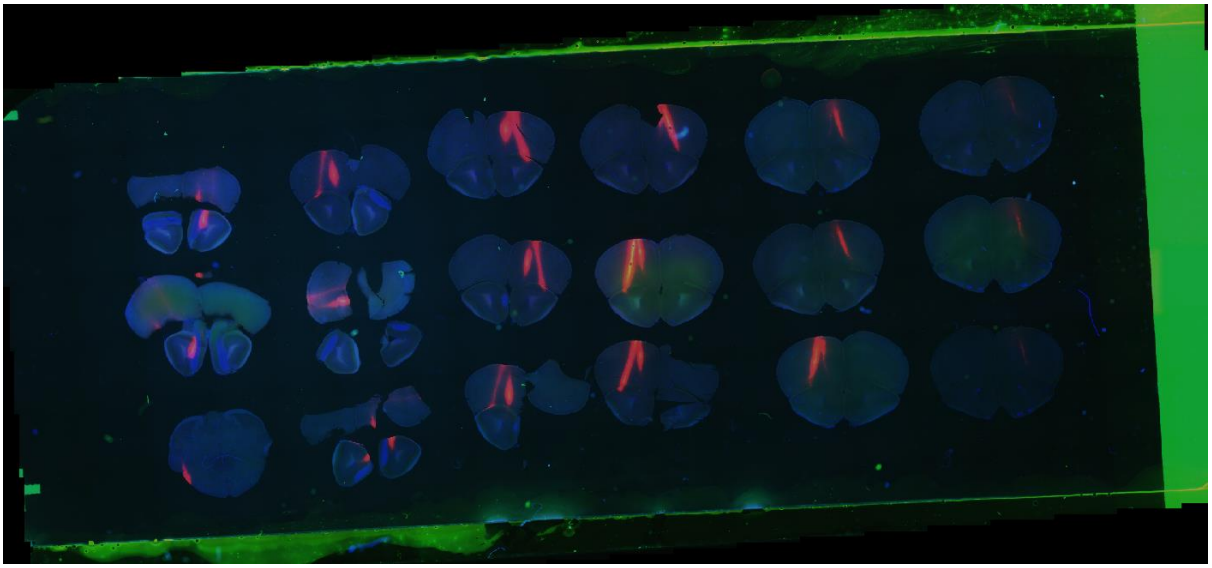
Go from an unprocessed image of many brain slices, to a folder of nice-looking, properly oriented, and ordered brain slice images (skip if you already have this)

- Open *ProcessHistology.m*
- Enter the folder containing the histology slide images, the folder the processed slice images should be saved to, the name of the histology slide images in anatomical order, and a few other parameters
    - If your images are downsampled to the correct size (10 microns per pixel), set use_already_downsampled_image = true

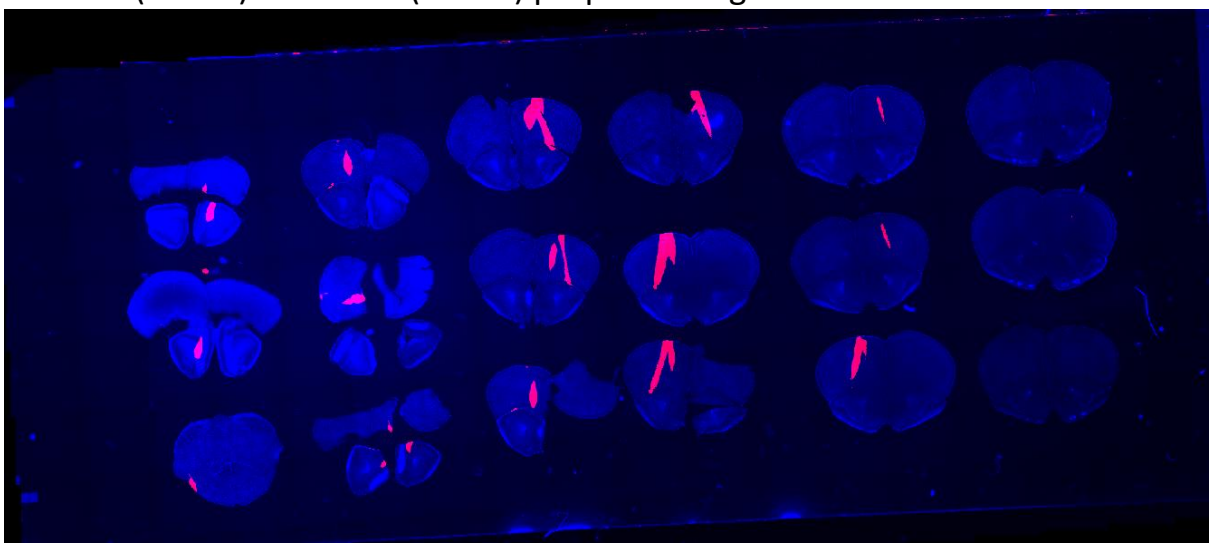
```
%%  SET FILE AND PARAMETERS

% directory of histology
image_folder = 'C:\\zubjects\\Subjects\\Richards\\Histology\\';
save_folder = 'P:\brain volumes\slices\Richards\';


% name of images, in order anterior to posterior or vice versa
image_file_names = {'Large Image 1.tif','Large Image 2.tif','Large Image 13.tif','Large Image 12.tif',...
                    'Large Image 11.tif', 'Large Image 9.tif','Large Image 8.tif','Large Image 7.tif','Large Image 6.tif',...
                    'Large Image 10.tif','Large Image 5.tif','Large Image 4.tif'};
                   % note that image 14/15/16/17, which fit inbetween 2 and 12, have no dye visible

% name to save cropped slices as
save_file_name = 'Richards_';

% parameters
microns_per_pixel = 3.233; %1.62;
microns_per_pixel_after_downsampling = 10;
```

- Run this cell to initialize parameters
- Run the following *cell* to downsample and adjust the large histology images (downsampling very large images may take a minute); Instructions should appear on adjusting the image
    - Scrolling shifts the max or min pixel intensity saturation points; space bar shifts between control of the max vs. the min; ‘c’ shifts to the next color channel; arrow keys save the image move to the next one

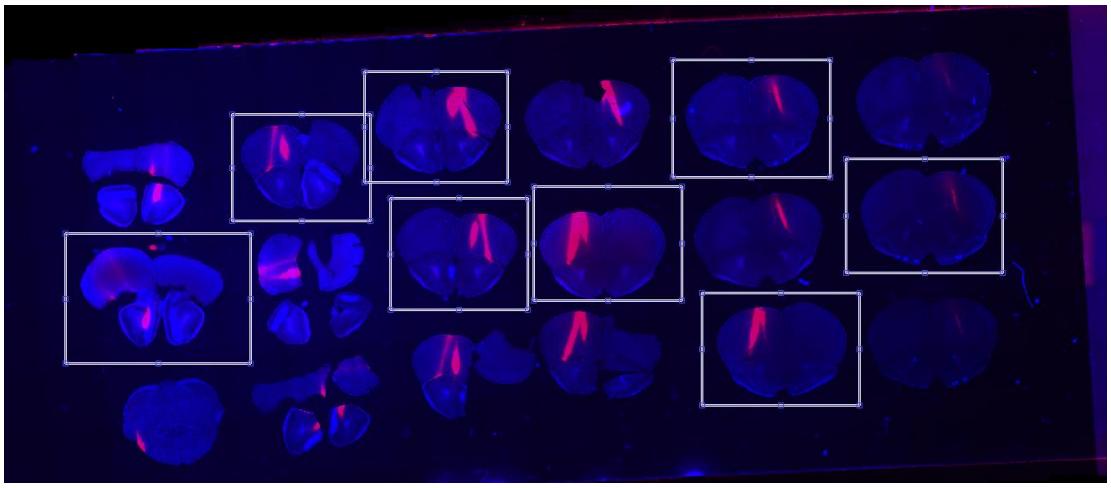
```
Controls:

scroll: adjust contrast
space: switch btwn adjusting upper and lower saturation points
e: view original version
any other key: return to modified version
r: reset to original
c: move to next channel
s: save image
left/right arrow: move to next slide image
```



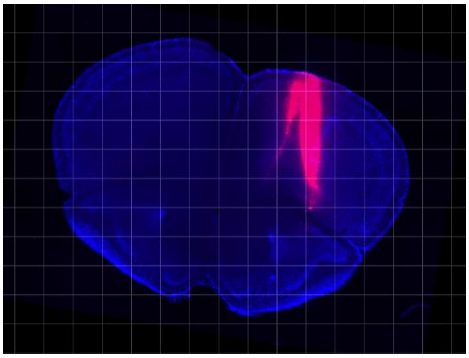
Before (above) and After (below) preprocessing



- When done with all slides, close the figure, and run the following cell ('CROP AND SAVE SLICES'). More instructions will appear in the console.
    - Select a slice image by clicking and dragging around it; try to go in order, either anterior to posterior or posterior to anterior
    - The slices needn't be perfect; they will be after the next, and last, pre-processing step. Cropping too big is ok.
    - Do so for each desired slice on the slide – for now, the most important is to crop the entire slice (and press 'space' to move to the next histology slide)
    - Every cropped slice will automatically be saved in the save_folder/processed folder

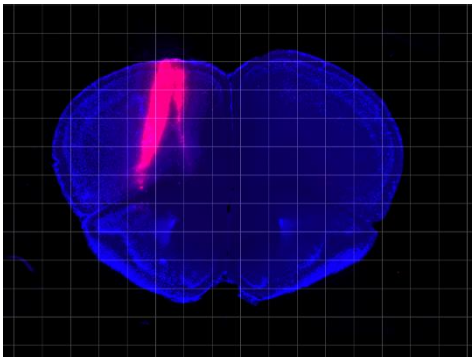


- Finally, run the last cell in *ProcessHistology.m*
    - Instructions appear. Scroll to get the slice oriented straight, press ‘f’ to reflect the slice horizontally (so all slices are in the same orientation – necessary for the next steps), press ‘c’ to crop the image further (highly recommended if the image is larger than 800 x 1140), and left/right arrows to save and navigate between slices.

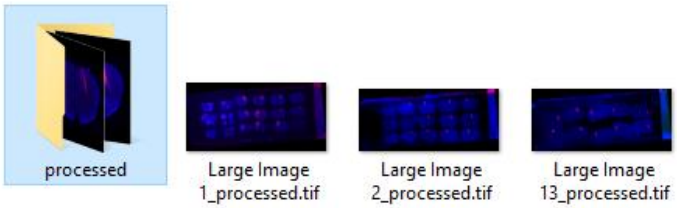


Before (left)

After (right)



- Done! Now you should have the large images saved in your designated ‘save_folder’, as well as a subfolder in there called ‘processed’ containing the processed slices, in order.

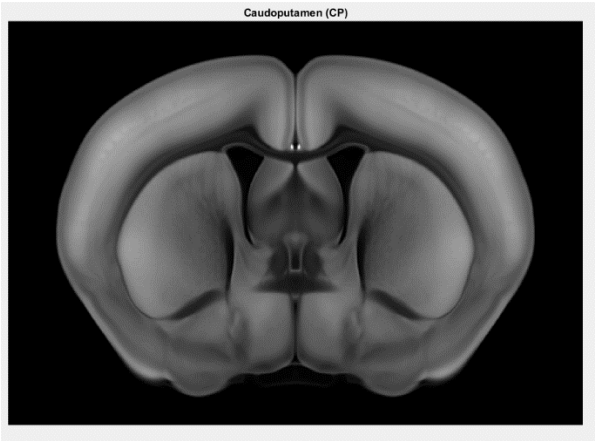


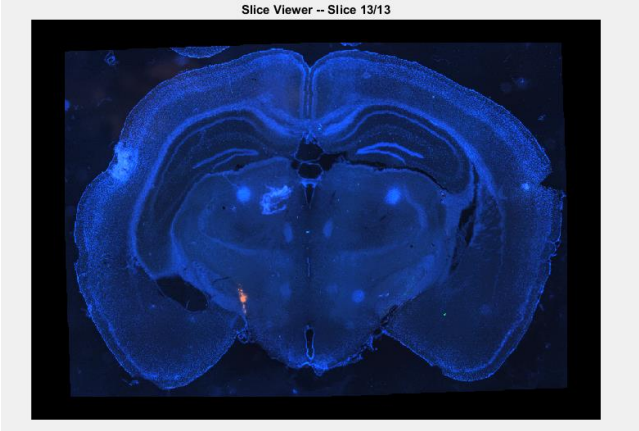
3. Navigate to the appropriate slice in the Allen reference atlas

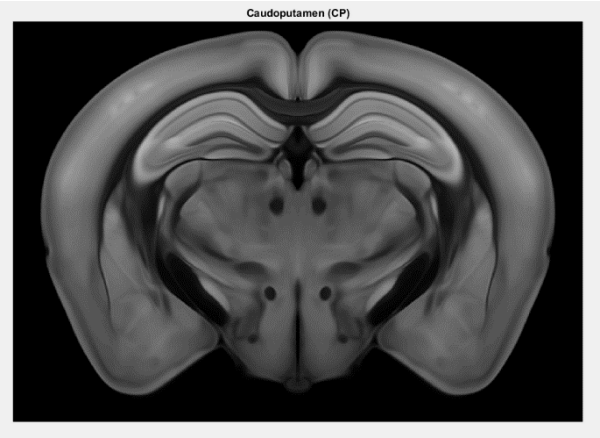
With the folder of processed images, find each corresponding slice in the reference atlas; or search the atlas without the slice images in a simpler GUI

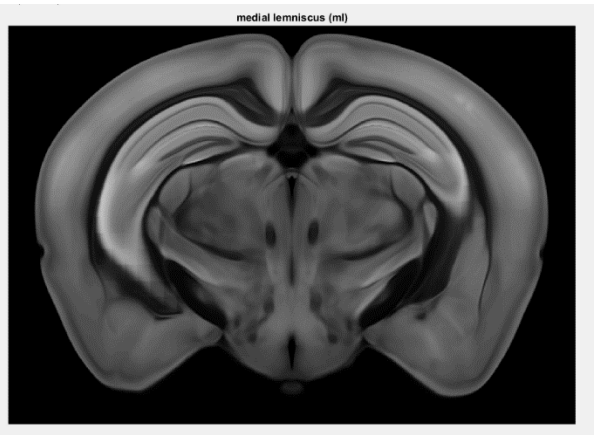
- Open the file *AlignHistologyToProbe.m* and enter the folder with the ordered, processed slices.
    - if you did not process the images through the above process, you may still need a similar naming convention (*Name of Animal_slide number_slice number.tif*) – e.g. 'Richards_2_12.tif'
    - You will need to first download the reference atlas structural volume, annotation volume, and annotation data. This can be done on http://data.cortexlab.net/allenCCF/
        - Download the structure tree ending in '2017.csv'
    - You must also download and add to your path the following repository: https://github.com/kwikteam/npy-matlab
    - Enter the file paths of the annotation volume in the line starting 'av = …'; of the structural volume in the line starting 'tv = …'; and of the annotation data in the line starting 'st = …'
    - Run the file. The first time, it will take a little while to load the reference brain data.
- The simpler function *allenAtlasBroswer(…)*, which is commented out on the last two lines of this script, can be used to find slices in the reference atlas, without any accompanying GUI that uses the histology images (Slice Viewer). It is not compatible, however, with sections 4 -6.

- Basic instructions appear.
    - On the Slice Viewer, press ‘left’ or ‘right’ to switch slices.
    - On the Atlas Viewer, scroll to move through coronal slices. Press ‘up’ to make scrolling now adjust the angle of the slice, along the dorsal-ventral axis or press ‘right’ to make scrolling now adjust the angle of the slice, along the medial-lateral axis. Using these features, navigate to the approximately correct slice as seen in the Slice Viewer





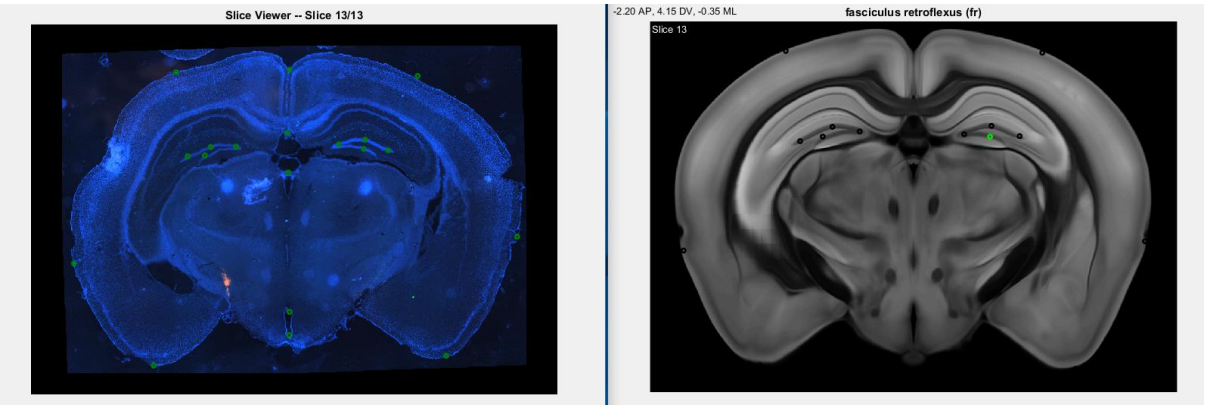




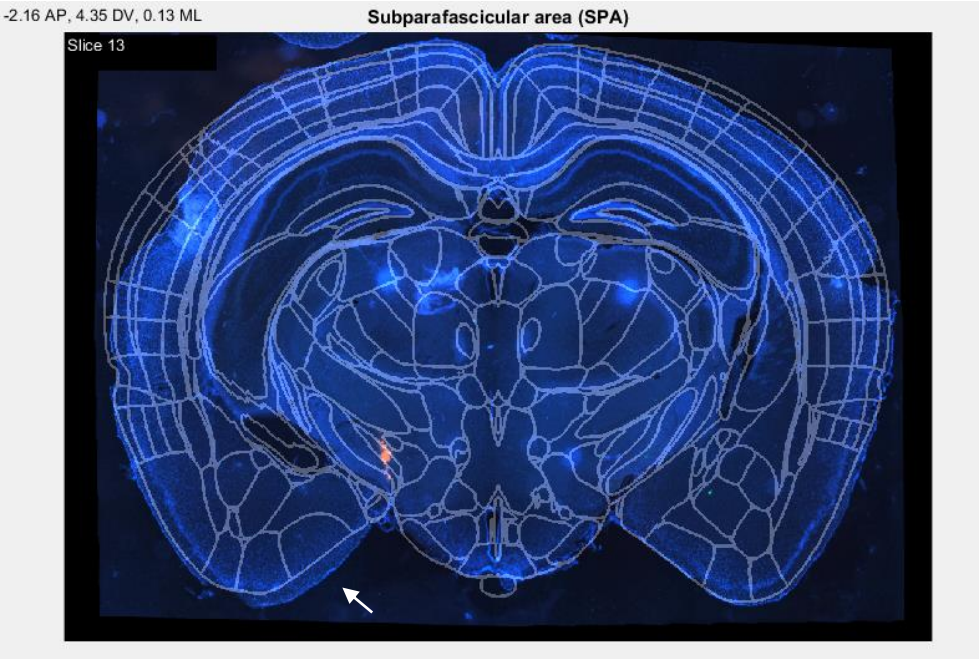
4. Transform and overlay histology to match reference

We've found a similar slice in the reference atlas, but room for error remains – transform and overlay the slice with the reference to get even more exact

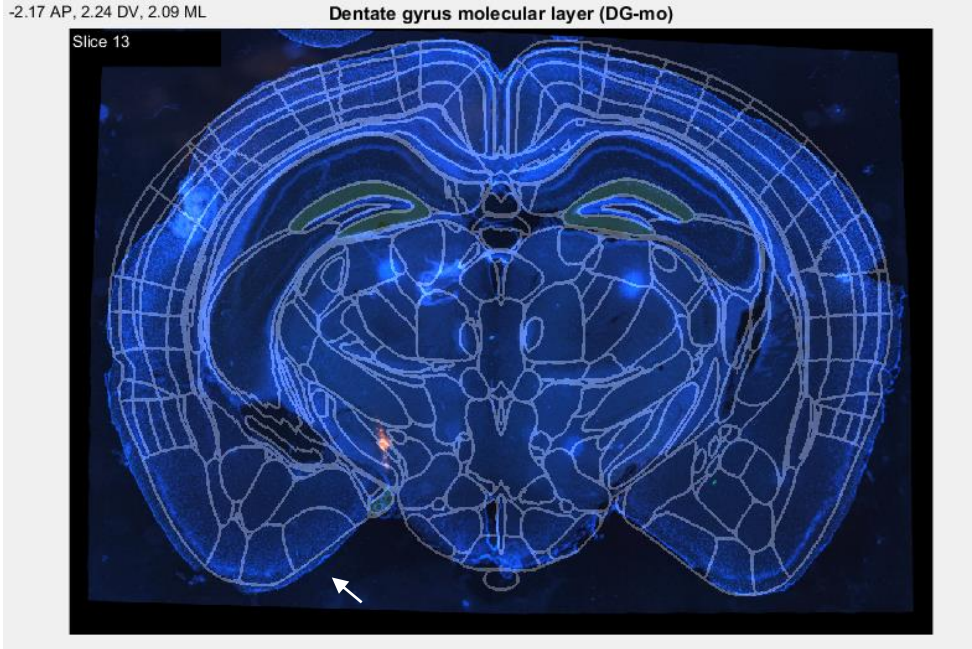
- When you're done exploring the brain regions on this slice with the mouse pointer, or by pressing 'o' to highlight regions (press 'o' again to turn that off), press 't' on the Atlas Viewer to initialize a transform
- Also press 't' with the Slice Viewer figure selected, so that both figures are in transform mode.
- Select salient points on one figure, and then the corresponding point on the other. 10-20 points should do, depending on the slice.
    - You can always add more points later
    - Press 'd' to erase the current transform and start over



- Press ‘h’ to transform and overlay the histology, and thereafter switch between 3 viewing modes. This also saves the transform.
    - Press ‘a’ to toggle region annotation outlines. Now you can slightly alter the reference outlines position (using the scroll bar and arrow keys as before) and/or alter the transformation by clicking more points in both figures and pressing ‘h’ again
        - Press ‘h’ 3 times total to return to present view
        - If you change just the reference atlas position, you can also press ‘x’ to save that change



After pressing ‘h’ and ‘a’; *Arrow shows a minor improvement made by adding 2 points to the transform and modifying the angle of the slice in the reference atlas*



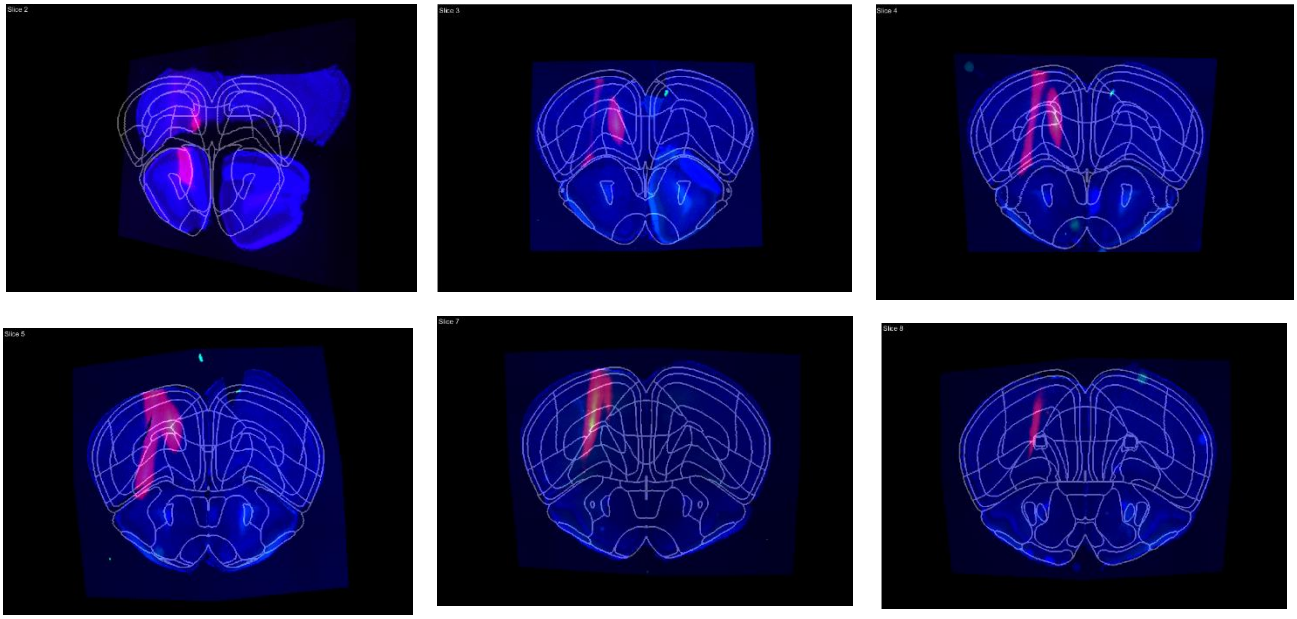
After improving the fit

- To move on to the next slice, use the left/right arrows, with the Slice Viewer selected
    - Then, you can press ‘h’ to switch to reference mode, scroll to the slice in the atlas corresponding to your new histology image, and repeat the process.
    - to bring up a particular transform that has been saved, navigate to that slice in the Slice Viewer and press ‘l’
- However, there is a more streamlined way to move between slices on the Atlas Viewer: press ‘left’, and then you can slowly scroll between the slices you’ve transformed! If a slice is not yet transformed, it will take you to the reference view so that you can do so.
    - It is not necessary to transform every single slice, if for example two slices show redundant information about the probe

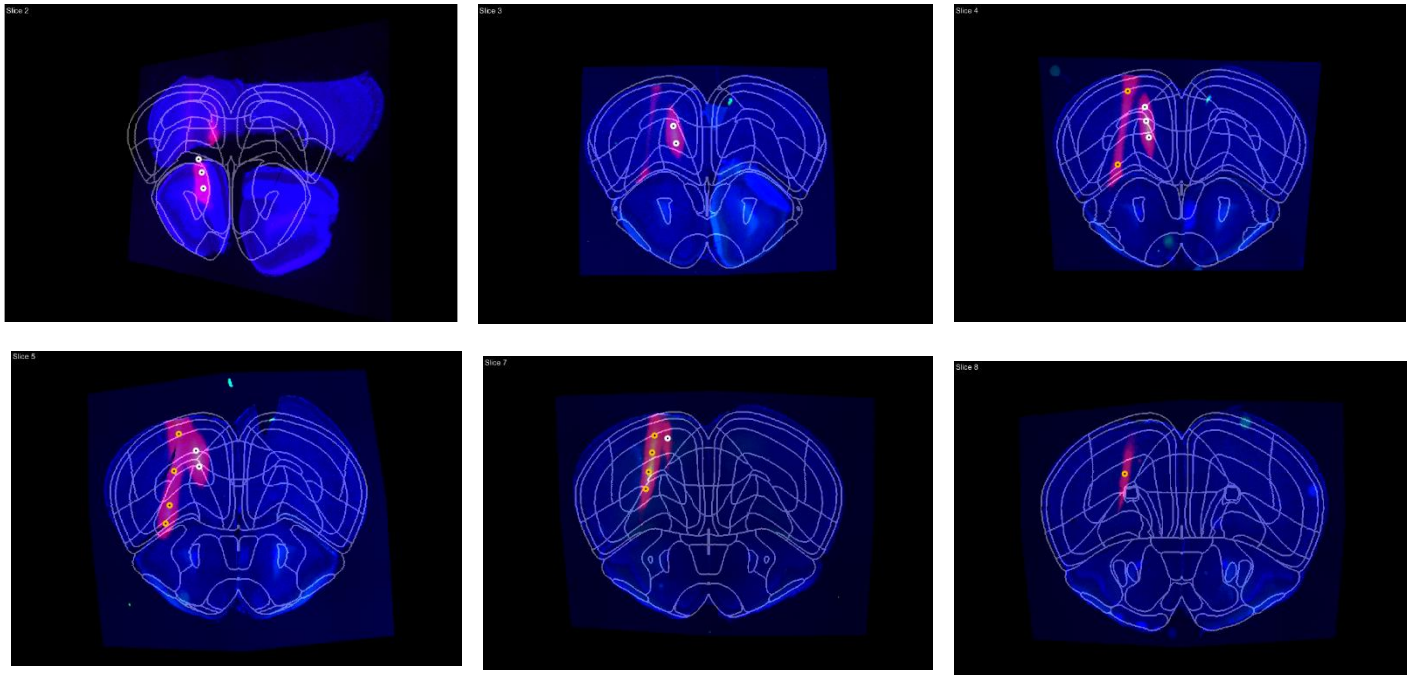
5. Select dye track locations

Localize the dye track(s) in the histology

- Navigate through transformed slices on the Atlas Viewer by pressing ‘left’ and then scrolling. Press ‘a’ to see the region boundaries



- Press ‘p’ to toggle probe point mode. Now you can click points on the figure corresponding to the probe track.
- Press ‘n’ to add a probe (up to 11 probes), and press ‘p’ to toggle between them (check the console to see which probe has been selected)
- Press ‘d’ to delete the most recent probe point from the currently active probe.
- **Press ‘s’ to save the probe points for probe visualization**
- Press ‘l’ *twice* (once loads a transform) to load saved probe points (or ‘w’ to launch probe view mode – see below)

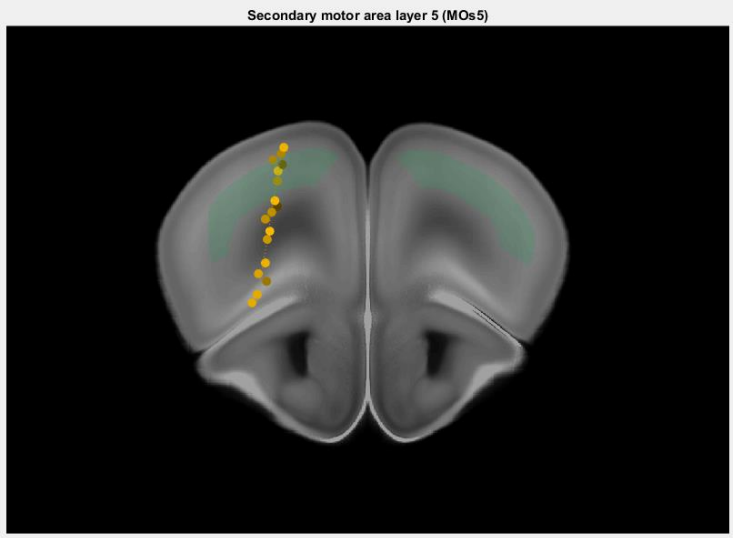


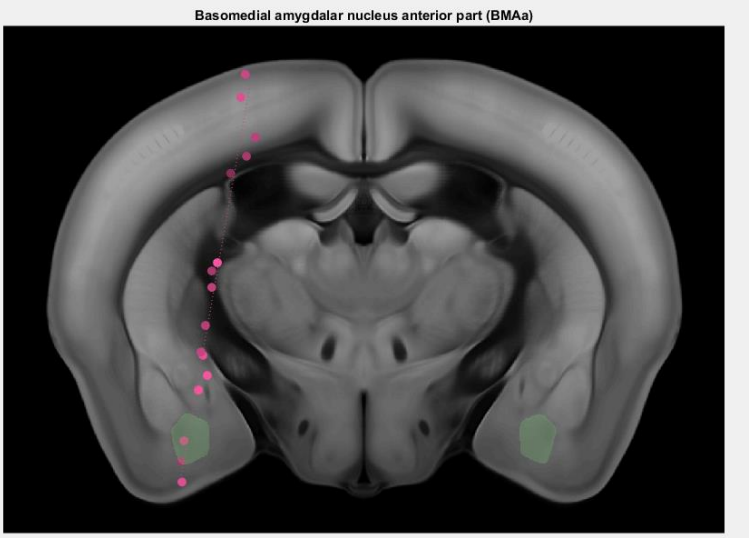
- Pressing ‘p’ will also bring up a third window: the Transformed Slice & Probe Point Viewer. When this figure is selected, pressing left and right will move through the slice images (transformed, if applicable)
    - This allows you to see surrounding slices with their probe points, to help determine which probe a particular fluorescent track belongs to

6. Visualize Probe Track

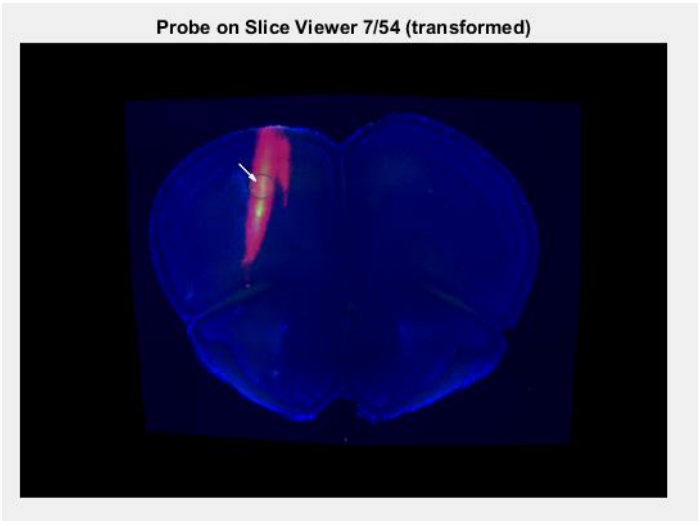
See the fruits of your labour

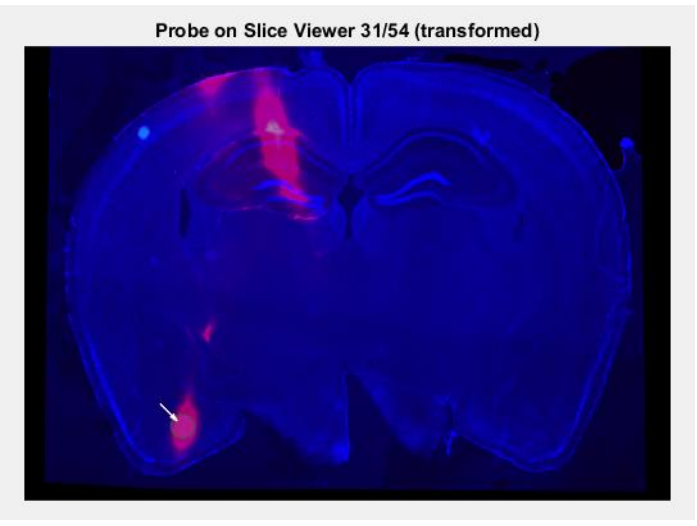
- Load saved probe points by pressing ‘l’ twice, and toggle the selected probe (as stated in the console) by pressing ‘p’
- Press ‘w’ to toggle probe viewer mode for the currently selected probe (will load probe 1 if none is selected or loaded).
    - This will show a best fit line, the slice that this line lies along, all the clicked probe points (with proximity from the best fit indicated by brightness)
    - Feel free to press ‘o’ to see the probe intersects, or to scroll around and explore nearby regions





    - Clicking on or around the probe track will find the point in the *histology images* closest to the clicked point, and display it in the Transformed Slice & Probe Point Viewer window





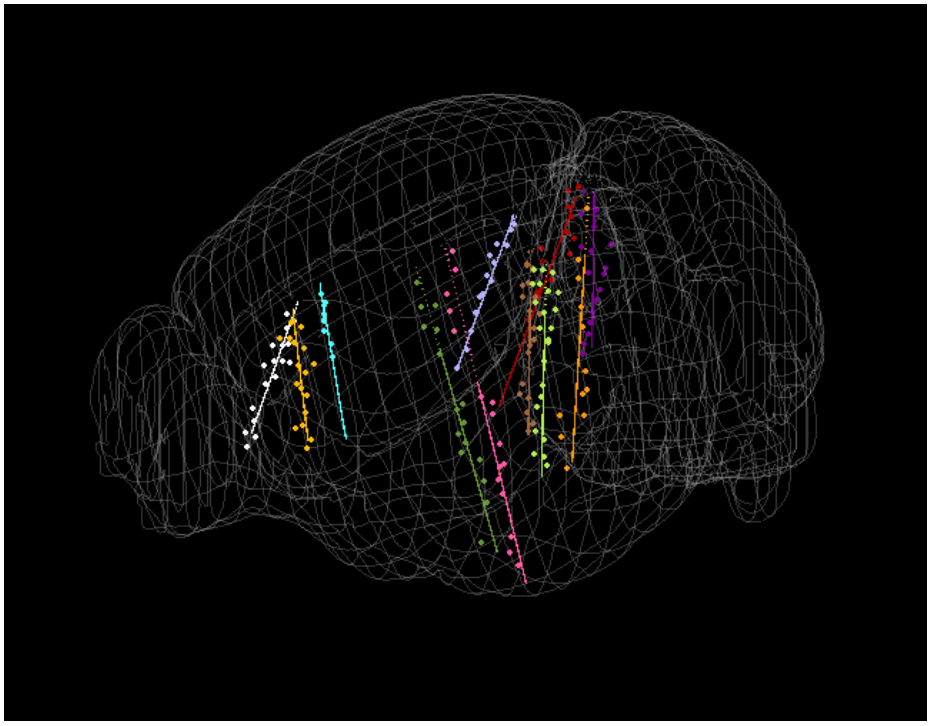
- Open *displayProbeTrack.m* and enter some parameters
    - *processed_images_folder* and *probe_save_name_suffix* should be the same is those used in AlignHistologyToProbe
    - *probes_to_analyze*: a list of the probes you would like to analyze found in the file set by the two aforementioned variables. 'all' will analyze all saved probes; [1, 5] will analyze the 1st and 5th probes
    - *probe_lengths* is a list of the known insertion depths of all probes saved in this file (if you are not analyzing some probes, you can set their depths to 0). Setting it as one number sets all probes to that depth.
    - *active_probe_lengths* is the distance from the bottom tip of the electrode that contains recording sites
    - *probe_radius* is the range in microns around the best fit line corresponding to the probe that will be queried to estimate a confidence metric (distance from best fit line to nearest different brain region – see plot below)
    - *show_parent_category* will also include a confidence metric for 2nd-order parent regions (e.g. prelimbic area vs. prelimbic area layer 6a) – this feature is slow.
    - *distance_past_tip_to_plot:* how far past the estimated tip of the probe should be plotted? (see plot below)
    - *scaling_factor*: set the scaling between the probe length that you know from the insertion depth and the depth in the reference atlas brain. 1.0-1.2 is a reasonable range. Aligning the electrophysiology to known landmarks (e.g. white matter) can also help to estimate this value. Set this variable to 'false' to use the most ventral clicked point as the expected electrode tip, and to then derive the scaling factor automatically.
    - *show_region_table:* output and show a *borders_table* with data on the regions displayed in the plot (see below): their upper and lower borders in microns from the surface, acronym, name, area index as represented in the downloaded annotation volume
        - see all abbreviations and hierarchical region structure is the downloaded csv file 'structure_tree_safe_2017.csv'

```
%% ENTER PARAMETERS AND FILE LOCATION


% file location
processed_images_folder = 'P:\brain volumes\slices\Richards\processed\\';
probe_save_name_suffix = '';

probes_to_analyze = 'all';  % either set to 'all' or e.g. [2,3]


% probe parameters
probe_lengths = [3.0, 2.5, 3.8, 5.5, 6.5, 3.8, 5.2,5.2, 5, 4.2, 4]; % in mm -- how far into the brain did you go, for all probes or just one num.
active_probe_length = 3.84; % in mm
probe_radius = 100; % in um -- error range queried for confidence metric
show_parent_category = false; %true; % overlay in gray distance between parent regions (takes a while)

probage_past_tip_to_plot = .3; % in mm -- plot this far or to the bottom of the brain, whichever is shorter

scaling_factor = 1.1; % set scaling e.g. based on lining up with ephys, or set to *false* to get scaling automatically from histology

show_region_table = true;
```

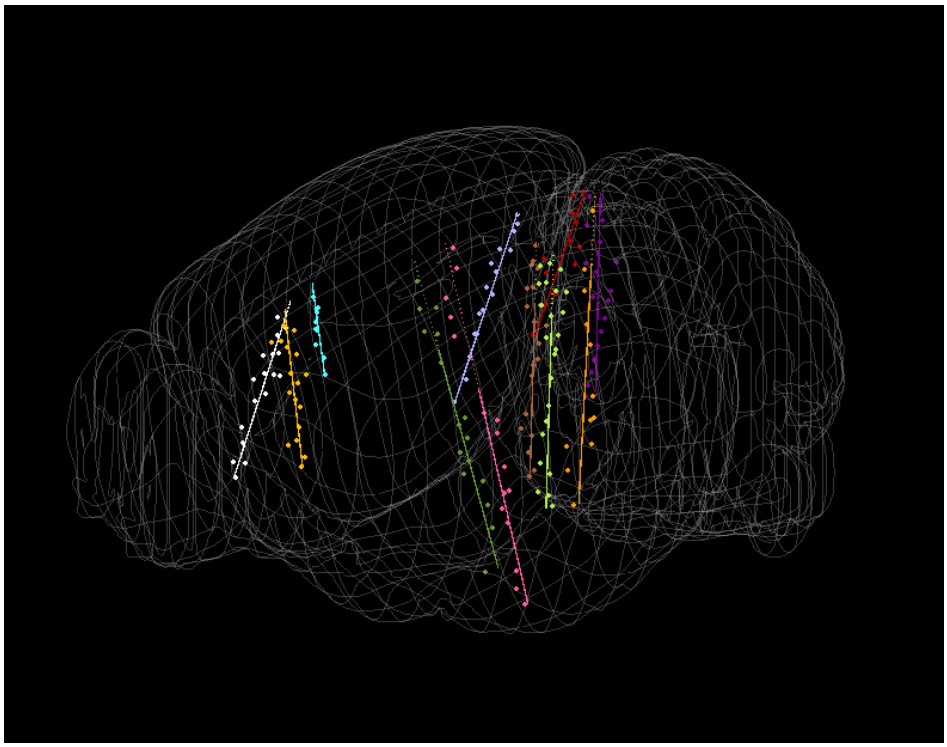
example parameters for a brain with 11 probe tracks

```
probe scaling of 1.1 determined by user input

borders_table =

  21×5 table

    upperBorder    lowerBorder      acronym                    name                                    avIndex
    ___________    ___________    ___________    ____________________________________________________    _______

       0              140          'MOp1'         'Primary motor area Layer 1'                             20
     140              160          'MOp2/3'       'Primary motor area Layer 2/3'                           21
     160              170          'SSp-ll2/3'    'Primary somatosensory area lower limb layer 2/3'        68
     170              190          'MOp2/3'       'Primary motor area Layer 2/3'                           21
     190              540          'SSp-ll2/3'    'Primary somatosensory area lower limb layer 2/3'        68
     540              720          'SSp-ll4'      'Primary somatosensory area lower limb layer 4'          69
     720              890          'SSp-ll5'      'Primary somatosensory area lower limb layer 5'          70
     890             1130          'SSp-ul5'      'Primary somatosensory area upper limb layer 5'          84
    1130             1620          'SSp-ul6a'     'Primary somatosensory area upper limb layer 6a'         85
    1620             1650          'SSp-ul6b'     'Primary somatosensory area upper limb layer 6b'         86
    1650             1740          'scwm'         'supra-callosal cerebral white matter'                 1190
    1740             1800          'ccb'          'corpus callosum body'                                 1199
    1800             1830          'ccb'          'corpus callosum body'                                 1199
    1830             5100          'CP'           'Caudoputamen'                                          574
    5100             5280          'CEAc'         'Central amygdalar nucleus capsular part'               597
    5280             5350          'ec'           'external capsule'                                     1194
    5350             5570          'BLAa'         'Basolateral amygdalar nucleus anterior part'           564
    5570             5700          'CTXsp'        'Cortical subplate'                                     556
    5700             6020          'EPv'          'Endopiriform nucleus ventral part'                     561
    6020             6100          'EPv'          'Endopiriform nucleus ventral part'                     561
    6100             6320          'PIR'          'Piriform area'                                         417
```

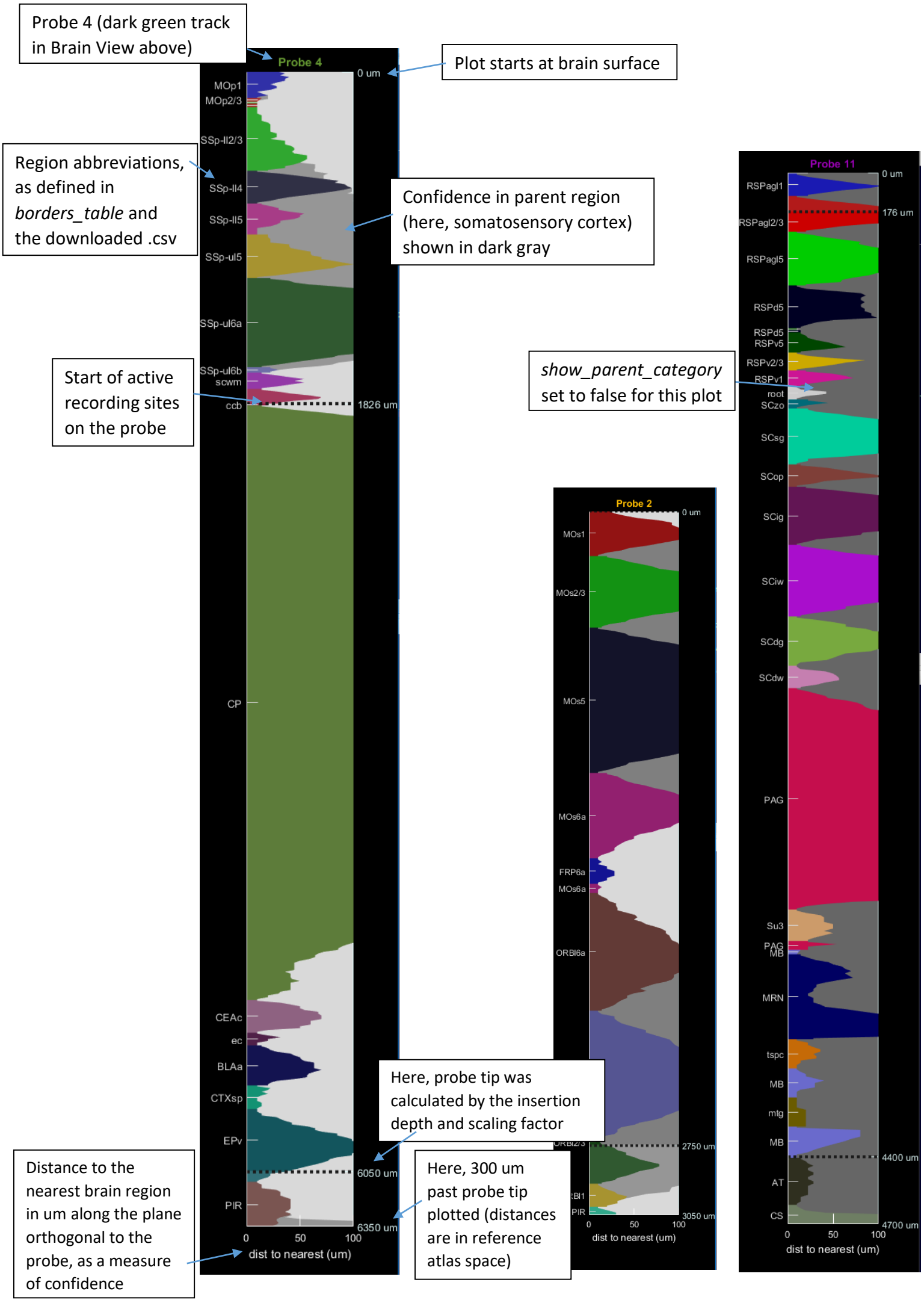
Example table output from probe 4 (the dark green probe below)



All probes overview output (Brain View), using scaling_factor = 1.1



Same data, using scaling_factor = false (get probe scaling from histology)


Probe 4 (dark green track in Brain View above)
Plot starts at brain surface
Region abbreviations, as defined in *borders_table* and the downloaded .csv
Confidence in parent region (here, somatosensory cortex) shown in dark gray
Start of active recording sites on the probe
show_parent_category set to false for this plot
Here, probe tip was calculated by the insertion depth and scaling factor
Here, 300 um past probe tip plotted (distances are in reference atlas space)
Distance to the nearest brain region in um along the plane orthogonal to the probe, as a measure of confidence
Probe 4
0 um
MOp1
MOp2/3
SSp-ll2/3
SSp-ll4
SSp-ll5
SSp-ul5
SSp-ul6a
SSp-ul6b
scwm
ccb
1826 um
CP
CEAc
ec
BLAa
CTXsp
EPv
PIR
6050 um
6350 um
0
50
100
dist to nearest (um)
Probe 2
0 um
MOs1
MOs2/3
MOs5
MOs6a
FRP6a
MOs6a
ORBl6a
2750 um
PIR
3050 um
0
50
100
dist to nearest (um)
Probe 11
0 um
RSPagl1
176 um
RSPagl2/3
RSPagl5
RSPd5
RSPd5
RSPv5
RSPv2/3
RSPv1
root
SCzo
SCsg
SCop
SCig
SCiw
SCdg
SCdw
PAG
Su3
PAG
MB
MRN
tspc
MB
mtg
MB
4400 um
AT
CS
4700 um
0
50
100
dist to nearest (um)

7. FAQ

Frequently asked questions

- There's a bug or error – what should I do?
    - Close all the figures and re-start the script

- That doesn't work – what should I do?
    - Feel free to raise an issue at github.com/philshams/allenCCF

- Is there a tool to know how I should orient my probe, before I do the recording?
    - Andrew Peters crafted one called *allen_atlas_probe.m*, using the same reference data as above (arrays called *tv, av,* and *st*). This file can be found in the repository.