NOTE: Habituating the mouse to head restraint often results in clearer intrinsic imaging results. It is recommended that you habituate the mouse over 2-3 days by placing it in head restraint for progressively longer durations, beginning with 10-20 minutes and progressing to a full hour before conducting the imaging.

NOTE: Many of these settings will vary depending on the particular experiment. The parameters described here are specific to intrinsic autofluorescence imaging of V1 and the higher visual areas. C57 also have autofluo!

Hardware Set Up

- Turn on camera
  - Power button located on top
  - Sends out information about image collection
- Turn on Q Imaging trigger box nope
  - Linked to Lab Jack
  - Converts pulses from camera
- Turn on MPC-200
  - Linked to ROE Coordinate controller
- Turn on X-Cite light source only for WF2
- Turn on display monitor
  - Displays visual stimuli
- Check to make sure the emission filter set in place is the Longpass 500/LP
  520 for GFP

Software Set Up

NOTE: Left computer password = hullglick
Right computer password = hullglick

- On left computer, open 2 programs:
  - First click "LoadVisStimRet.command" on desktop - this batch file will set up the MWorks variables for VisStimRet
  - MW Server
  - MW Client
    - Start connection
    - For Experiment, choose "VisStimRet"
    - For Variable set, choose "Ret_4_Pos"
      - Open up variables
        - Speed Counters keep track of pulses - should start at 0 and be aligned with images collected
        - GratingExpt Structure
          - Subject # = Mouse Identification #
          - # Trials = 88
          - Boolean describes types of stimuli
            - 0 = false, 1 = true
            - RetStim = 1

        - StimSpecs = stimulus properties
            - nScans = # of frames for on/off stimulus presentation = 20 frames on, 20 frames off
            - Azimuth = x-coordinate: + right, - left
            - Elevation = y-coordinate: + up, - down
- On right computer, open Micro Manager
    - NOTE: Micro Manager will only open properly if the camera is already turned on!
    - On the opening welcome screen, click OK
    - Exposure describes # ms that the camera is collecting light for a given image
        - Most common exposures for II fall between 300-450 ms
        - NOTE: For II you will be collecting frames every 500 ms, so do not set the exposure to more than 450 ms. set 450 exposure
    - Binning describes # of pixels
        - Set binning to 4
    - The histogram displays the image's pixel intensities
        - You want as few pixels saturated as possible
    - Open "Multi-D Acq."
        - Enter the total # of frames to collect
            - Based on (trial length) x (# of trials)
            - For Ret_4_pos, collect 3520 frames
        - Enter the interval for frame collection
            - For Ret_4_pos, set to 500 ms, or 2 Hz
        - Choose the storage path for the imaging data at the bottom of the screen
            - Make sure "Save Images" is checked
            - Select your desired Directory Root
            - Include name, date, and animal # for the Name Prefix
            - Make sure "stack" is selected so that images are saved as a stack and not individual image files

<u>Mouse Set Up</u>

- Place mouse on the rig wheel with its nose pointed back and to the right, so that its right eye has an angled view of the display monitor. The top of its head should be directly below the microscope
    - Use a screwdriver to tighten the mouse's headpost into the restraints
        - NOTE: Tighten the perpendicular arm of the headpost first, followed by the angled arm
    - Once the mouse is in place and secure, turn up the light source by a notch or two and carefully move the rig until the light is shining directly on the cranial window. low limit!
        - Once content with the position of the rig, turn down the light source again
        - NOTE: Try and minimize light exposure. Turn light off when not imaging, and use lower light levels with higher camera exposure time if possible.
- Use a cotton-tipped applicator and DI water to clear the cranial window of any debris or smudges.

- Attach the black light shield to the mouse's 3D-printed cap by snapping it in place and then velcroing the shield around the objective to block out all light.

Imaging

NOTE: At this point, turn off the lights in the imaging area and close the curtains surrounding the imaging area to block out light.

- On the Micro Manager main screen (not Multi-D Acquisition) click "Live" to switch to live mode.
- Turn up the power on the light source by one or two notches and adjust the exposure time accordingly until an image of the brain comes into view.
- Adjust the X, Y, and Z coordinates until you see a sharp image of the brain focused on the lateral-posterior portion of the cranial window (V1 and the higher areas).
    - Once at the sharpest possible image, move the Z coordinate ever so slightly lower until the image is slightly blurry - this should place the focus below the surface of the brain.
- Once content with the position and focus of the microscope, go to Micro Manager and exit Live mode.
    - NOTE: It is essential that you exit Live mode before beginning imaging or else MWorks will start sending pulses before you hit "Acquire".
- In MW Client, double check the variables to make sure they are correct, and check to make sure your speed counters are set to zero. Once confident that the visual stimulus is set, click the green "Play" button, hit "Stop" immediately, then hit "Play" again.
    - NOTE: For some reason MWorks requires you to hit Play, Stop, Play in order to save your stimulus settings, so don't forget this step.
- In Micro Manager on the Multi-dim. Acquisition screen, double check the total frame #, interval, and directory root for the data. Then hit "Acquire".
    - Once you hit "Acquire", a new screen should open up in Micro Manager displaying the brain images being collected.
        - The top of this screen should display the current number of the collected image. Check to make sure this number is in line with the speed counters in MWorks.
    - The first 20 pulses (or 10 seconds) should show a blank screen on the display monitor , and then the next 20 pulses should trigger the visual stimulus.
- The 3520 images taken every 500 ms should take approximately 30 minutes to complete. You can leave the mouse alone to view the stimuli during this time and return at the end of the collection period.
    - NOTE: Before leaving the imaging area, check on the mouse to make sure it is calm and its eyes are open, viewing the stimulus.
        - If the mouse does not appear calm, you may want to spend more time habituating it to head restraint before imaging.
        - If the mouse is closing its eyes or it looks like it is falling asleep, you may want to try imaging at another time when the mouse will be more alert.

        - Be on the lookout for a gooey white substance that sometimes collects around the mouse's eyes. This is a sign of stress and can interfere with the mouse's vision.
            - If you observe this eye goop, try removing it with cotton-tipped applicators and DI water.
            - If the goop persists even after removal, you may want to habituate the mouse more or try imaging at another time.
- After the 30 minutes is up and all 3520 images have been collected, quit MW Client and MW Server and then reopen the programs.
    - This step resets the stimulus for the next collection.
    - Repeat the MW Client setup with all the same variables, and again check that the speed counters are set to zero.
- In Micro Manager, leave all the settings as they are, except provide an updated file name for the next image stack.
    - I often leave the file name exactly the same, except add "Session 2".
- In MW Client, hit "Play", "Stop", then "Play" again to prepare the stimulus for the next image collection.
- In Micro Manager Multi-dim. Acquisition, hit "Acquire" to begin the next image collection.
    - This second round of images is going to be averaged with the first set, so the settings should be identical for both collections.
        - Be careful not to adjust the mouse's position, microscope coordinates, exposure time, stimulus variables, binning, etc. in between runs.

Post-Imaging

- After the second round of image collection is complete, turn the light source down to the lowest setting so that there is no longer any light shining on the brain.
    - NOTE: Exposing the brain to light can over time lead to photobleaching, so it is important to only have the light source turned up when necessary.
- Now that the image collection is complete, feel free to turn on the lights and open the curtains to make cleanup easier.
- Un-velcro and remove the light shield from the mouse's cap.
- Use a screwdriver to carefully loosen the head restraints holding the mouse's headpost in place. Return the mouse to its cage.
- Use paper towels and a cleaning solution to clear the wheel and rig of any mouse excrement.
- If you and others in the lab are finished imaging for the day, close out of MW Client, MW Works, and Micro Manager.
    - Power down the light source, display monitor, camera, Q Imaging trigger box, and MPC-200.

Data Analysis

- Use Remote Desktop Connection to log on to Nuke
- From Windows Explorer, select "Map Network Drive" to connect to Crash (file server).

- In the save directory you specified in Micro Manager, locate the two image stacks and copy them to your Crash.
- In Nuke, open the program ImageJ.
    - Open the two image stacks in ImageJ.
- Take the following steps in ImageJ to process your raw images:
    - For EACH image stack, click “Image”, “Type”, and select “32 bit” to increase the range of pixel values
        - The image should appear slightly brighter after completing this step
    - Click “Plugins”, “Stacks” and select “Concatenate” to make the two image stacks into a single file.
        - Choose the proper stacks for Image 1 and Image 2, and select “None” for Image 3.
        - Select “Keep Original Images” if you don’t want to lose the raw data.
        - You should now have one concatenated master stack. The rest of the processing will be directed to the concatenated stack.
    - Click “Plugins”, “Macros” at the bottom, and select “Trial Avg AK2”.
        - Align Frames = 0 (No)
        - # Frames per Trial = 160 (40 frames each for 4 positions)
        - Frames per Condition = 40 (20 on, 20 off)
        - This should result in two stacks
            - tavg is the average images for the stimulus presentations
                - Discard this stack
            - tavgn normalizes the change in the fluorescence from the mean
                - Direct the rest of the processing to this stack
    - Click “Plugins”, “Stacks”, and select “Grouped ZProjector”
        - Group size = 20 frames
        - Projection Type: Average Intensity
        - Direct the rest of the processing to this stack
    - Click “Plugins”, “Hypervolumes”, and select “Hypervolume Shuffler”
        - Select Unshuffle
        - Volume Depth = 2
        - This groups together the frames associated with “off” (no visual stimuli) vs. “on”
        - Direct the rest of the processing to this stack.
    - Click “Plugins”, “Stacks”, and select “Substack Maker Plus”
        - Slices = 5-8
        - This isolates only the frames associated with “on”
        - Direct the rest of the processing to this stack
    - Click “Process”, and select “Enhance Contrast”
        - Check the “Normalize” and “Process all 4 Slices” boxes; leave the other two blank
    - Rotate 90 degrees left to orient mouse posterior at bottom and anterior at top
    - Save this final stack

    - Additionally, generate a FOV image by taking one of the raw data images and duplicating the first frame, then rotate 90 degrees left and save
- Congratulations! You should now have a map of your mouse's visual cortex.
    - Each of the four slices in the final stack corresponds to one of the stimulus positions.
    - Colorize and overlay:
        - Duplicate each of the 3/4 slices as individual images
        - Select Image/Color/Merge Channels, then choose the slices for RGB channels and select *none* for the remaining channels
        - Check Create composite, Keep source images
        - Will create an overlay, you can adjust the color contrast/brightness by selecting Image/Adjust/BrightnessContrast, and use the slider at the bottom of the composite to shift between color channels
    - If done correctly, you should be able to observe retinotopy in the images: as the visual stimulus position moves in the mouse's field of view, the portion of the visual areas being stimulated should move correspondingly.
        - Example: As the stimulus moves from a lateral to a more medial position, the part of V1 being activated should move from a medial to a more lateral portion.
    - You should be able to utilize Wang & Burkhalter's area map to identify the higher areas in your map.
        - The areas most commonly seen using this approach are V1, LM, AL, RL, AM, and PM