**1P AOSLO System Startup Procedure**

Patterson Lab – G.3193

Last Updated: 3/18/2025

Before working with the system.

1. Take off any reflective jewelry (rings, watches, bracelets)
2. Switch the laser light outside G.3193 on (light switch to the left of door)
3. If imaging an animal, put the experiment sign on the outside of the door.

After working with the system.

1. Double check lasers are off
2. Switch laser light outside room off
3. Remove experiment sign from outside of door, put back on inside of door

General rules of thumb.

1. Turn the lights off before using the visible PMT
2. Avoid bumping mirrors when reaching in the system – tell someone if you do.
3. Don’t touch lenses/mirrors/filters directly, only use the edges (with gloves if unmounted)

Turning the system on. Going in clockwise order, beginning facing the desk

1. Turn on the shutter controller on the desk. Follow instructions taped to the top to adjust the settings and enable the shutter.
2. Rack to the left of the desk:
   1. Turn on the inverting amplifier (top front), laser signal box (middle front), and the scanner sync box (back top). Each has a switch on the back of the box.
3. Optical table above the rack has the Toptica, the reflectance imaging source and the deformable mirror controller stacked on top of each other.
   1. Turn on the Toptica (if needed). turn the key counter-clockwise. Check that the cable is in the appropriate wavelength and mode (analog/digital). The laser will usually be warmed up (number display shows “0”) by the time you are finished turning everything else on, then follow the instructions in the Laser SOP.
   2. Turn on the reflectance source. Turn the key clockwise, press “Select” to switch the mode from “Low” to “High”. Press “On/Off” once so that the red light is off. Press “On/Off” again to emit – the green light will turn on.
   3. Turn on the deformable mirror (DM) controller by pressing the button. The blue light should turn on and the orange LED labeled “Activity” will start flashing. When the LED flashing and turns off, the DM is warmed up.
4. Bottom optical table, left side.
   1. Switch on the galvo scanner box (switch on back towards desk). The green light on the front of the box (far side from the desk) should turn on.
   2. If using the marmoset relay, place magnetic mounted beam block on the stage.
5. Under the optical table, left side.
   1. Turn on the Velmex stage motors if using macaque model eye. ***Switch them on at the same time.*** Doing them separately can cause a mechanical issue – if this happens, they will start making a whirring noise, just turn them off and try again.
6. Top optical table, left side.
   1. Turn on LED controllers, if using Maxwellian View. If using the LEDs, turn the dial on each LED controller box up to “Limit”. Check the switches on each box – for alignment, they should be set to “CW”, for stimuli controlled by the software, they should be set to “MOD”.
7. Top optical table, far side between M8 and the last flat of the macaque system.
   1. Check the magnetic-mounted pellicle beamsplitter. If using the Maxwellian View, it should be placed on the magnetic mount closest to M8 (giant mirror). If using marmoset relay, it should be removed. Put in engineering room cabinet.
   2. Check the magnetic-mounted marmoset flat mirror. If imaging a macaque, make sure the marmoset mirror is removed from the magnetic mount. It’s stored in the engineering room cabinet when not in use. If imaging a marmoset, place the mirror so that it reflects light from M8 into the marmoset relay. Be careful when placing to not bump the last flat mirror before the eye.
8. Top optical table, far side, center.
   1. Uncap the DM and place face-down on table so dust does not collect.
   2. Check filters in front of visible PMT. If using model eye, remove filters. If imaging a macaque, make sure you have placed the right filter.
9. Under the optical table, right side of the desk.
   1. Switch the resonant scanner box on. Switch is on the side closest to the desk, light should illuminate on the far side of the box.
   2. Turn on 488 nm Mustang laser, if needed. Turn the key clockwise and press the “Reset” button. Check behind the black tape to make sure the white light is on.
   3. Turn on the PMT control box. Switch is on the far side of the box from the desk at the back (long reach). The lights the box should turn on.
10. Bottom optical table, under the PMT array shelves.
    1. Turn on the wavefront sensing source (847 nm). Switch is on the back of the box (left side).
    2. Press the “On/Off” once and the red light labeled SLD will turn off. Press again to start emitting – a green light will turn on.
    3. Press “Select” until the display reads “SLD Current”. This is what you will adjust to increase the laser’s output. Usually it will be <100 mA for the model eye and >100 mA for a real eye. You can make initial adjustments by using the screwdriver in front of the light source to turn the key under the light labeled SLD or wait until you have the AO software on.
11. Left-most PC (“Analysis PC”).
    1. Open the Toptica software, if needed. It’s the red circle icon. Follow instructions in the Laser SOP to connect to the Toptica and set the laser intensity.
    2. Open the ThorLabs Kinesis software, if not already open. Close warning box that appears behind the loading image. If there are less than 7 boxes, click “Connect” to search.
12. Center PC (“SLO PC”).
    1. Open the SLO software in the “software” folder on the desktop, “real-time” subfolder, choose “Tracking-Sara”. Or click on the light blue PC icon on the taskbar.
    2. Move the Channel 2 window over to see Channel 1 and make sure to adjust the width of both windows to show the full display.
    3. On the “Imaging Configuration” tab of the SLO software, check desinusoid and click “Camera Start”.
13. Right-most PC (“AO PC”).
    1. Open Tsunami Wave (Python icon on the taskbar). If the orange light on the DM is still blinking after turning on, wait until it stops to open Tsunami Wave.
    2. Go to the AO tab, click “load AO settings” and click the one beginning with “ready-2-go”.

**1P AOSLO Macaque System Optimization**

Patterson Lab – G.3193

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Aligning the macaque model eye.

1. Attach the model eye to the Velmex stage with the two ¼-20 screws (should be in place). Once in place, make sure it is screwed in all the way by lifting the stage up slightly and tightening the screws
2. Turn on one of the visible light sources and open the shutter to use for alignment. Check “Calibration” next to the target laser (Mustang or Toptica) and turn up the intensity to ~20% for Mustang, 100% for Toptica assuming the Toptica software has the laser emitting and is set relatively low, ~5-10% depending on the NDFs.
3. Check the “visible shutter” checkbox above the laser controls on the SLO software.
4. Check where the beam is relative to the aperture of the model eye (center cage on breadboard) using a card or a piece of paper. If one isn’t nearby, there are more in the green “Cards” bin in the 1P sideroom.
5. Center the model eye. Use the controller to the right of the Velmex stage to move the model eye into place. ***Don’t press the buttons on top***, just use the joystick.
   1. Start by using the visible laser to get close to the center of the model eye’s aperture, using the card as needed to see where the beam is located. When nearly centered, you should see the laser coming through the model eye’s lens onto the slide.
   2. Then close the visible light shutter and use the wavefront sensing on Tsunami Wave (SHWS tab, click “WS Live”) to fine-tune.
   3. If the pellicle is in, make sure you are centering to the beam coming from the flat mirror, not the light coming through the pellicle (there should be a magnetic beam block in the way, but sometimes it isn’t there if the Maxwellian view hasn’t been used recently).
6. Once you have the model eye centered, adjust the wavefront sensing source with the screwdriver until the max value under the wavefront sensing image is slightly less than 255 (or by eye to decrease the size of the spots). You might need to rotate the screen for the AO PC slightly to see it while adjusting.
7. Turn on AO. Turn “WS Live” off, go to the AO tab and click “AO Live”

Imaging the model eye (macaque).

1. Center the model eye as above.
2. Check “desinusoid” on the Calibration tab of the SLO software and press “Camera Start”.
3. Make sure the reflectance source is on and emitting.
4. Turn on reflectance PMT. Go to the last tab and check the “Reflectance PMT gain” box, then gradually turn the PMT gain up with the slider to get an image (usually ~0.3 in the model eye).
   1. Keep the mean value in the Ch1 window <100, a conservative value for protecting the PMT (damage is usually around 150 mean pixel value, but over 100 the image is too saturated to be useful).
   2. If too much light is shown to the PMT relative to the gain, it has an internal shutter that closes to protect the sensor. You will hear a click and the image will disappear. Set the PMT gain down to zero, uncheck the “Reflectance PMT gain” box, re-check it and try again.
5. Turn on a visible light source. In the calibration tab, turn up the laser (20% on Mustang, 100% on Toptica in SLO software and 5-10% on Toptica software is sufficient). Open the visible shutter if closed.
6. Turn on visible PMT. ***Turn off the room lights while visible PMT is on and close curtains on right and back of system.*** Check the “fluorescence PMT gain” box. Turn the gain up slowly (click above and below slider to change value instead of dragging slider) until you can see the model eye paper. Stop when the image is clear and keep it under 100.
7. Set up experiment folder, if needed. If you are optimizing before an experiment, create the experiment folder in the “Data” folder (pinned to quick access). Name it “monkeyID\_date” (e.g., MC00851\_20220415). Inside, create 5 subfolders: “Ref”, “Vis”, “Pretest”, “Analysis” and “Stimulus files”. The last one is only needed if using stimuli in the experiment.
8. Set the save folders (bottom of first tab in SLO software) for CH1 and CH2 to the “Pretest” folder if optimizing before an experiment.

Optimizing the reflectance PMT position.

1. Open ThorLabs APT software and MATLAB on the SLO PC and the ThorLabs Kinesis software on the Analysis PC.
   1. If the values for any of the actuators you plan to use are set to zero and there is an orange light next to the text “Not Homed” on the Kinesis software or the “Homed” light is not bright green on the APT software, you will need to first home the motors. See Troubleshooting docs.
2. Set the FOV in the imaging software to the largest you plan to use in your experiment.
3. Change the default step size of the reflectance X and Y PMT controllers to 0.002 if you already have a decent image of the model eye paper. You may need a larger step size if you are far away, but eventually you want to work your way back down to 0.002
   1. To change the step size in the APT software, click Settings on the lower left and change the value for “Step Distance”.
   2. To change the step size in the Kinesis software, click the settings for the “Jog” arrows
   3. To jump to a value, click the number display on the APT software,
4. Move the X and Y position up and down while watching the mean value and stop where it is highest. This doesn’t need to be absolutely perfect – the next step will fine-tune.
5. Close the ThorLabs APT software, then run “APTGUI\_Channel1” (should be open in the MATLAB editor, if not run “APTGUI\_Channel1” from the command line).
   1. The ThorLabs software must be closed before starting the optimization app because only one program can communicate with the actuator control boxes at a time. If you forget to close it, just cancel out of all the error boxes that show up and try again after closing it.
6. Turn the PMT gain down until the mean value is ~30-40. Set the search window to 0.03 or 0.02 if you have a decent image, then run the XY optimization. This will iterate through X and Y PMT positions and find the combination that results in the highest mean pixel value.
   1. If the PMT gain started too high and the optimized position exceeds ~150, the PMT will shut down and the search will fail. You have to close out of MATLAB and reopen
   2. Same if the search window is too big and the XY position get into a region with 0 mean pixel value (no info for Nelder Mead algorithm).
7. Copy the values into the Source and PMT Position Excel sheet (pinned)
8. We don’t touch the reflectance PMT cage so the source position and PMT Z position don’t require adjustment between sessions. They get set during the alignment process so the cone mosaic image provides a consistent reference for the other channels. If Z and/or source position does need to be adjusted, follow the steps for the visible PMT and lasers below.

Optimizing the visible PMT and source position for different lasers­.

1. ***Turn off the room lights before turning on the visible PMT!***
2. Set the source position and PMT Z position to the model eye values from the previous experiment in the Excel sheet.
3. Turn on the visible laser to be optimized (on the first tab of the imaging software, check calibration, increase intensity to ~20% for the Mustang and 100% for the Toptica, assuming the software on the analysis PC is ~10%). Open the shutter. I usually start with Toptica 561 nm as it’s the clearest.
4. Increase the visible PMT gain as described above until you see an image. Unless the PMTs have been reset or the system has been changed, the last experiment’s values should get you pretty close.
5. Optimize X and Y PMT positions as above with the APT software.
6. Next optimize with the MATLAB program (run the Channel 2 version). Before beginning, remember to decrease the PMT gain until the mean pixel value is between 20-40. I use a search window of 0.03 unless you are not very confident in the current positions.
   1. You can run this even before the Z PMT position and source position are perfect, as long as you have enough signal and some sort of image – the heatmap produced will show a broader sensitivity, but the center should be close. Having the X and Y PMT in place is useful for the next steps.
7. Adjust the Z PMT to optimize the mean pixel value.
   1. Watch out for hysteresis on the Z PMT and source actuators (e.g., when you move in one direction for awhile and then see a huge drop in mean pixel value for a small movement in the opposite direction). Once you reach a good place, go in the opposite direction, then back to the optimal value
8. Adjust the source position, focusing on image clarity. I usually turn up the PMT gain some for this to have a better image. If the source position is way off, look at mean pixel value, then switch to image clarity once it’s close. I usually move in largish steps (0.1 or so) until I identify where the image definitely begins to go out of focus for both directions, then work my way towards the best focus in the middle with smaller step sizes (~0.01, no need to go down to 0.002).
9. I usually return to the Z PMT after optimizing source position to make final adjustments at ~0.002 step sizes. If you weren’t close to begin with, you might need to iterate between the Z PMT and source position.
10. If you optimized XY with the MATLAB program before fine-tuning Z and source position, rerun.
11. Copy the values into the Excel spreadsheet.
12. Add the name of the laser to the file prefix for CH2 (e.g., CH2\_Mustang). Take a 10 second video of the model eye.
    1. If “Manual Ctrl” isn’t checked on the “Calibration” tab, the shutter will close afterwards. Check the visible shutter checkbox to open it back up.
13. Repeat with any other lasers you plan to use in the next experiment. Remember to turn the visible PMT gain down and uncheck before switching lasers to avoid sending too much light to the PMT. Once the new laser is emitting and the shutter is open, recheck and increase the gain slowly.

Imaging the Maxwellian View Position

1. Do this step only if using the Maxwellian View in your experiment.
2. Turn up the dials on all three LEDs all the way. Switch from “MOD” to “CW”. You should see the LEDs lit up.
3. On the imaging software, turn down all visible lasers, uncheck “Calibration”, and close the visible shutter.
4. Turn the visible PMT up. To detect the LEDs, the gain often needs to be 0.8-0.9. You should at least see some speckle where the LEDs are hitting.
5. Adjust the CH2 prefix to be “CH2\_LEDs” and take a 10 second video (increase duration if needed after the next steps – may be necessary if you have NDFs in the Maxwellian View).
6. Open the CH2 video in ImageJ and create a Z-projection. Choose “Standard Deviation”. You should now see where the LEDs are hitting – they should be off to the left or right side so as not to interfere with the calcium imaging side.

Setup the system for the experiment.

1. Change the Mustang and Toptica (if needed) PMT Z and source positions to the biased values provided in the Excel spreadsheet.
   1. For example, if you’re imaging the ganglion cell layer with the Mustang and presenting spatial stimuli with the 561 nm line of the Toptica, you would set the Mustang source position and PMT Z position to the ganglion cell layer biased values and the Toptica source position to the photoreceptor layer biased values.
2. Put filter on the visible PMT. The 525-40 filter for GCaMP6 is in the 1P side-room cabinet, the others are usually laying around the optical table.
3. Put your stimulus files into the “Stimulus files” folder. If using new spatial stimulus videos, put them into the stimulus videos folder. Run one of your stimuli in the physiology tab to make sure it doesn’t throw an error and check your layout (todo: docs).
4. Leave the model eye in place for morning power measurements before the experiment.

**1P AOSLO Troubleshooting**

Patterson Lab – G.3193

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Homing the actuators. When the connection between the motor controls and the software is disrupted (like when the USB cables are disconnected and then reconnected or the power supply is disrupted or unplugged), the software no longer knows what position the actuators are in. To fix, you need to rehome them. Until they are homed, whatever position they were last in is now 0.

1. Take a note of the mean value and current PMT gain. This is where things should be once you have homed the PMT and returned it to the correct position. Beware of increasing the PMT gain further, because it could risk showing too much light to the PMT once you find the right position.
2. Press the “Home” button on one of the actuator UI boxes (I usually start with X, then Y) and watch the numbers as they change. The PMT will reverse to the end of its range, then return to a midpoint. The point of reversal (where the numbers stop decreasing and start increasing) is often close to the value you will have once optimized (but positive instead of negative).
3. Start by typing in values for the position, incrementing in steps of 1 or 2. Watch the image as the actuator moves. If you see the model eye image, you know it’s lower than your final value. Then decrease the value in smaller steps until you see the image again. Iterate until you have a decent image, then you can follow the steps above for optimization.
4. If you are really having a hard time finding the right place (often occurs when multiple actuators were away from the optimal model eye positions before the reset), start increasing the PMT gain conservatively to get more information about the correct direction of movement from the mean value. Drop the PMT gain any time you seem to be approaching a mean value of 100 (conservatively 80), decrease the PMT gain some along with the step size.
5. Repeat with the next actuator.

If it’s the morning of an experiment and there isn’t time to fix this, you can go ahead with a few caveats:

1. The Thorlabs APT GUI on the SLO PC won’t allow you to directly input negative values. If your biased value is negative, you have to set the value to zero and the step size to your target value, then press the down arrow to step down.
2. The MATLAB optimization software needs to set the value directly so it won’t work if the X and Y positions are zero because the program needs to test a range of values around that point, including the negative ones. If you are short on time, prioritize homing the X and Y PMT position for the visible PMT so that you can optimize in vivo (placing the filter in front of the visible PMT after imaging the model eye moves things slightly so the in vivo optimization can have a big impact on image quality).