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mAO-MPFM Operation and Image Acquisition

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We use this protocol and it's working

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Abstract

A streamlined method to running a multimodal Adaptive Optics Multiphoton Fluorescence Microscope (mAO-MPFM) built at the University of Kansas with software and pathway components from 3i. This protocol is intended to show in depth with multiple checkpoints how to run the microscope from start to finish as well as troubleshoot multiple modes within the system.

Guidelines

All guidelines and troubleshooting can be found within the protocol.

Materials

Laser: Chameleon Discovery Classic (NX), tunable: 680-1300 nm, fixed: 1040 nm, pulse duration: 100 fs, repetition rate: 80 MHz

Microscope and Software: 3i/Zeiss Upright Two-Photon, Slidebook (software, 2024, current version 2.0.45035), Chameleon Laser GUI

Safety warnings

 This system uses a class four laser.

Before start

Notes: Best to work in moderate light when initially setting up. Lock "garage door" when in use. Should hear a click near the right door lock by the laser when closing the door (this is the safety shutter opening). Select the current version of Slidebook (2024 currently). Will need to use the laser GUI (blue cloud, Chameleon Discovery icon) to monitor power of the laser at the source.

Vocabulary

- 1
 - 1- AO – adaptive optics
 - 2- Pol – polarization
 - 3- GUI – Guided user interface
 - 4- PMT – photomultiplier tube (detector)
 - 5- GFP – green fluorescent protein
 - 6- Gain – numerical value associated with PMTs, corresponds to the PMT's ability to receive signal from the sample
 - 7- Galvos – components within RS+ unit that control the laser steering when scanning and acquiring an image
 - 8- Dual scanning – galvos are operating independently of one another; one controls x steering/scanning and the other controls y steering/scanning
 - 9- Resonant scanning – both galvos are operating together at a resonant frequency to each other to steer the laser
 - 10- Offset – used in resonant scanning, this aligns the galvos for a better image; when scanning there will be some errant lines in the scan this offset will align those errant lines from the scan
 - 11- Branding – burning a pattern into the surface of your sample, can be spirals/circles or a variation of lines

Computer Set up

- 2 Log into the computer and open current version Slidebook and laser GUI.
 - 2.1 When turned on, the laser GUI will guide the laser to tune to the last wavelength used.
 - 2.2 It should also open with both shutters closed; make sure these are greyed out (shutters are closed).
- 3 Make sure the stage in microscope is cleared, and the garage door is shut.

System Set up

- 4 On the control stack, locate the large white switch that controls the power to the laser. Switch it on.



- 5 Above the chiller is a black control box; flip the key to "Laser Enable". This box controls the shutters.
- 6 Turn on the chiller located below the laser enable key switch box.

- 7 Make sure the Pockels cell control box (just above laser box) says 005.

Note

The knob should be touched only if the number does not match the value from the alignment session. The alignment value for this laser is 005.

- 8 All other control boxes should be on.
- 9 Double check the safety shutter box; the shutter should be lit up (orange) to signify that the safety shutter is open.

Note

When the system is turned on, it should click initially; if it does not, turn the key to the off position.

- 9.1 Wait 10 seconds, then turn the key to the "on" position. A click should be heard and the "open" light should be lit.

10

Wait a minimum of 10 minutes before using the laser for imaging.

Note

- a. As the system warms up in the laser GUI, both tunable and fixed lines will widely fluctuate in power measurements. The fluctuations may be over 100 m, but this is normal during warmup time.
- b. The laser has sufficiently warmed up when the fluctuations become much smaller, roughly 5-8 mW ranges for the tunable and 1-2 mW for the fixed line. If the tunable is still moving a little too much (jumping around by 5 instead of increments of 1-2), then wait another 5-10 minutes.
- c. These values should match the ranges seen on the performance pages from Coherent (depends on wavelength).

Laser GUI

- 11 Looking at the GUI, the shutters for both emissions will be closed (grayed out and say "closed" underneath the button).

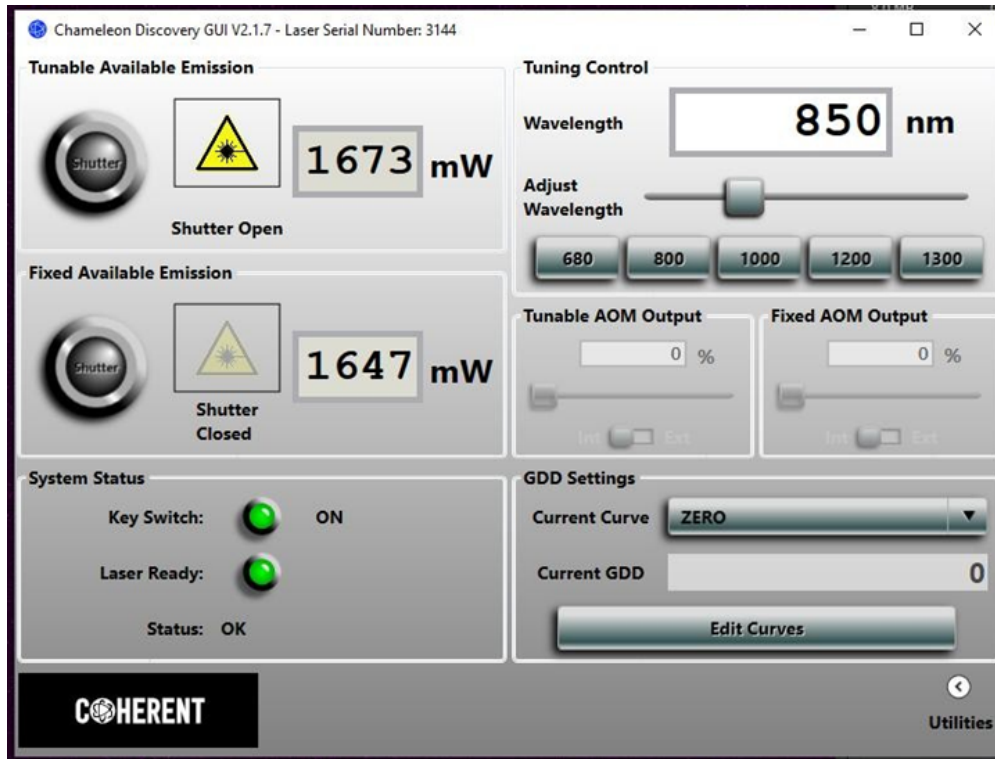


Figure 1. Laser GUI from Coherent for the Chameleon laser system.

- 12 To set the desired starting wavelength, either type it in, then hit "enter," or use the slider beneath the numerical box.
- 13 The laser will tune to this new wavelength. As it does so, the second light indicator on the GUI, found below the fixed shutter, will blink red; beneath the light the word "tuning" will appear.
- 14 Once tuned, the indicator light will become solid green and be ready for use (after warmup).
- 15 The first indicator light on the GUI should remain green; if the laser has been tripped, the light will turn red.
- 15.1 To correct this, all PMTs must be turned off, and then shutter the laser through the GUI by clicking the button.

- 16 Control the shutter as described below.
- 16.1 To turn shutter **on**, click the button. The laser will click loudly, and there will be a delay from off to on in the GUI. The laser is **not** engaged in the system. The button is now greyed out.
- 16.2 To turn Shutter **off**, click and hold the button until the laser clicks loudly. There will be a slight delay from on to off in the GUI. The laser **IS** engaged and running through the system. The button is no longer greyed out (has yellow caution triangle) and the garage door should be closed. Failure to adequately click and hold the button will result in the system's signaling "wait" in the GUI underneath the button. Click and hold again.

Slidebook

- 17 Open the most current version of Slidebook.
- 18 Select the configuration on startup that meets your need. All Slidebook configurations will operate the same but will have different PMT options and other added (or subtracted) hardware.

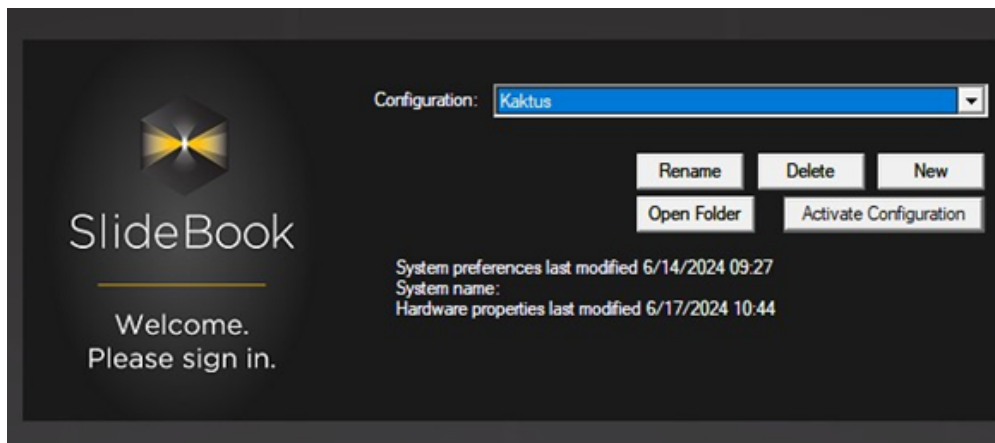


Figure 2. Slidebook startup dialog, selection for hardware made here.

- 18.1 Kaktus will have the 4 PMT options and AO capabilities.
- 18.2 Nosepiece will have 2 PMT options and AO capabilities.



- 18.3 FLIM will have the filter wheel activated (use Pol 1) and AO capabilities.
- 18.4 Kaktus (no AO) will have 4 PMT options without AO capabilities.
- 19 Once the laser is turned on and tuned, but **shuttered**, open the garage door and place sample in the stand.
- 20 Adjust the height of the stage to the objective so they are close enough to place a drop of distilled water that will touch the objective lens to "connect" the objective and the sample. The proper distance will create a bubble effect; once the bubble is created, another drop of two of distilled water can be added.
- 21 On Slidebook, save the file before beginning to scan.
- 22 Open the focus window and click on mu button (nextliti-photon system to capture).
- 22.1 Click multi-photon; the delay after clicking is to be expected.
- 23 Once loaded, 3 windows will open the focus window, the scanning console, and the visualization window. One small black window will need to be enlarged in order to see the sample.
- 24 On the **Focus** window, use the **Filter Set** drop down menu to **Widefield**.

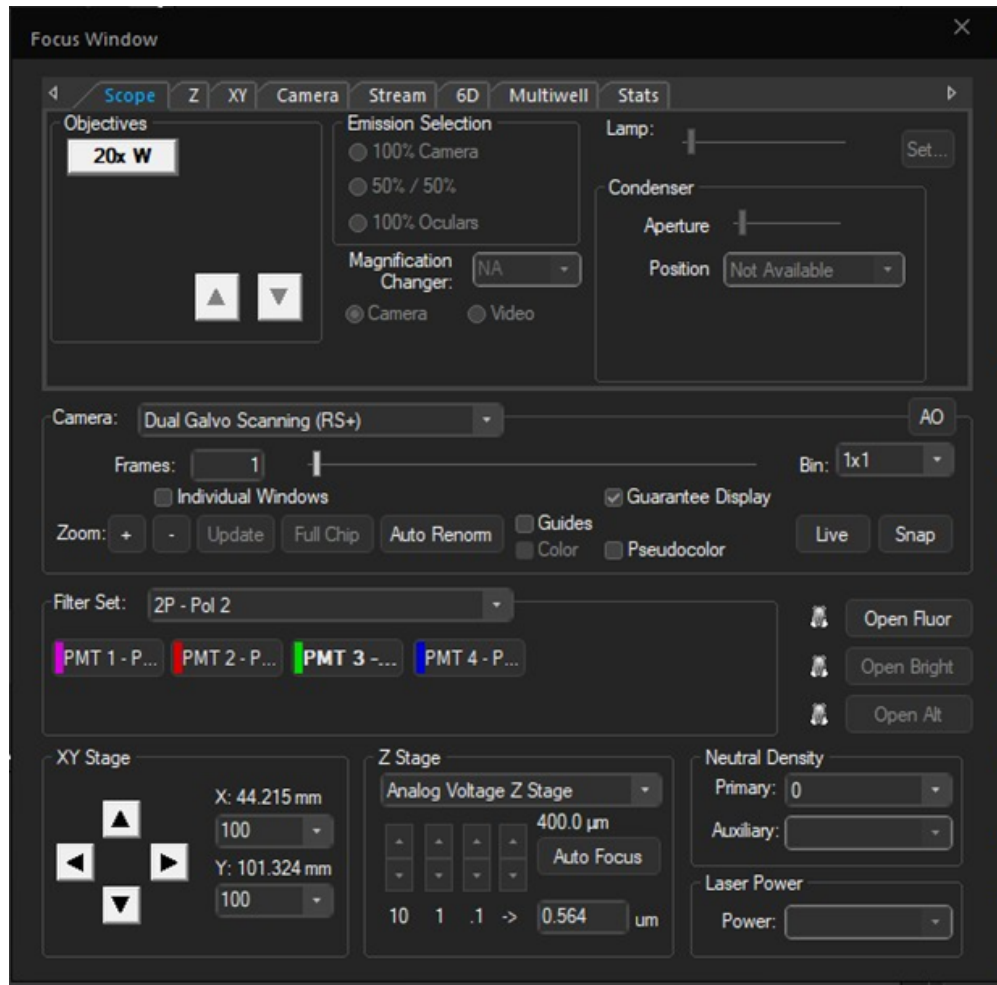


Figure 3. Focus window. It can be used to change between scanning modes, establish distances for z stacks (via z tab), move the sample in X and Y directions, and change the filter set.

- 24.1 Two filter buttons are found under the dropdown.
- 24.2 One is brightfield and one is GFP.
- 24.3 Click on the GFP; you will hear a click.
- 24.4 Click on "Open Fluor" to see sample through the eyepieces.



- 24.5 Use the joystick to align in XY. Use the course/fine focus knob on the microscope base to align in Z. Do not "break" the water bubble; the objective must be in the water.
- 25 A silver plunger is located on the right side immediately behind the eyepieces. Push the silver plunger into the side of the microscope. This will move the light into the eyepieces so placement of the specimen can be observed.
- 26 Once aligned, pull the plunger away from the microscope to "close" the eyepieces and direct light to the PMTs.
- 27 Close the "garage" door; listen for a click, then lock the door.
- 28 On the **Focus** window, click "Close Fluor".
- 29 On the **Focus** window, use the Filter Set drop down menu. Select Pol 1 if using nosepiece for FLIM. Select Pol 2 for everything else.

Note

In the VectorRS+box, Pol 1 places a half wave plate in the light path; Pol 2 takes it out of the light path.

- 30 Use the **scanning window** to establish controls for capturing images.

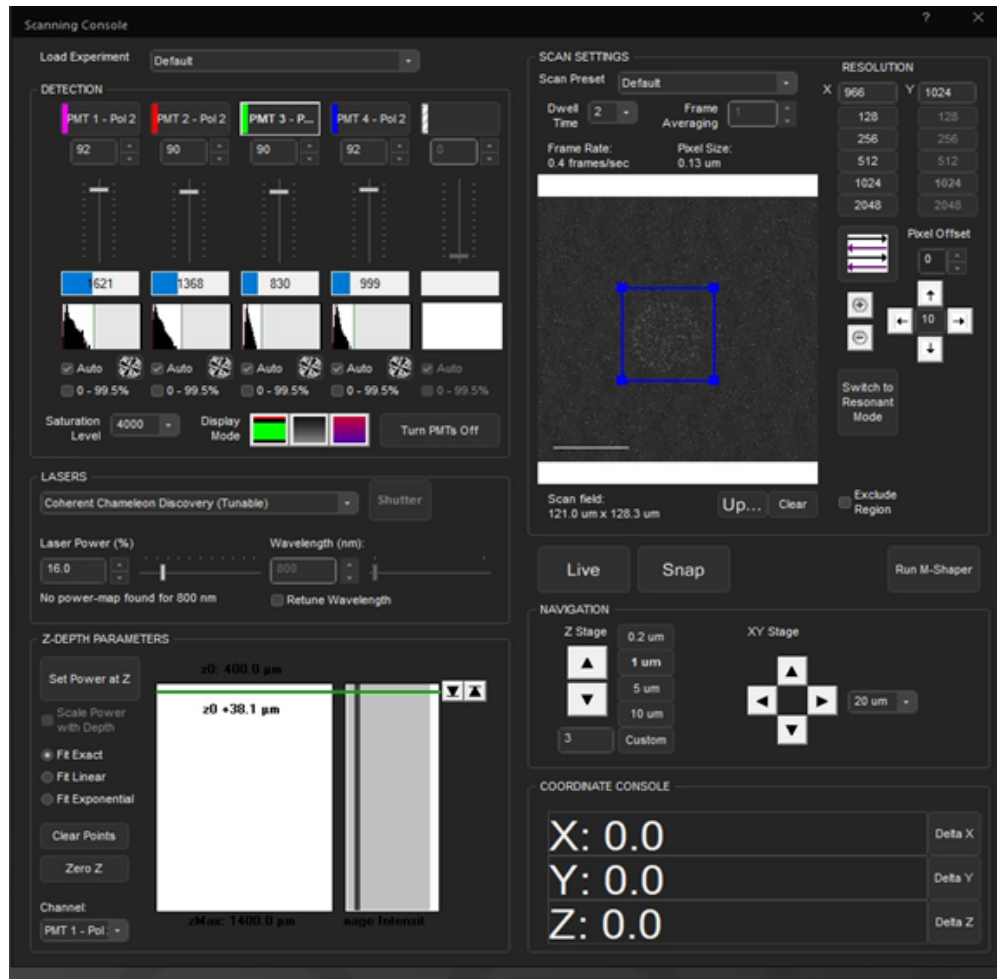


Figure 4. Scanning Console window. Use this window to adjust final imaging resolution, adjust gain on the PMTs, select the PMT for live scanning, switch between dual and galvo scanning, adjust saturation levels, adjust laser power, change the scanning pattern (raster or single direction), and adjust the offset.

- 30.1 Engage the laser by turning off the shutter in the laser GUI.
- 30.2 Click "Turn on PMTs".
- 30.3 Click "Live" in scanning window.
- 30.4
 - i. The laser will hit the sample and return to PMTs.
 - ii. The visualization window will be black or fuzzy
 - iii. Choose one or more than one PMT to see the sample.
- 30.5 Click on choice of PMT



- i. If more than one color is wanted for scanning observation, hold the “ctrl” key on the keyboard and click on additional PMTs.
- ii. Now signal is being received to the computer from the PMTs, but the live window will still be black or fuzzy until the following steps.

30.6 Increase the power of the laser from 0.

- i. Use the up and down arrows or the slider.
- ii. Start at or below 10% power before adjusting.

30.7 Now adjust the gain on the PMTs.

- i. Move the slider up from 0 under the PMT(s) you are using. This needs to be done one PMT at a time.
- ii. Start around 70-90

Note

Adjust the gain and power until best combination is found for sample.

- a. If the signal is weak at 100 gain on the PMT, increase the power from the laser.
- b. If the signal is strong and red spots are being created, decrease the gain on the PMT. If the gain is at 100, laser power might need to be increased to compensate for the full potential of the PMT being open.
- c. End goal should be low power and low gain values (relative).

- iii. If the sample is not immediately visible, see step iv below
 - a. Maintain 256 pixels upon initial investigation/scanning

30.8 Once a power and gain are set, a signal should be seen.

- i. If the visualization window is still fuzzy, adjust the focus on the external coarse/fine focus module.
- ii. A region of the sample should be seen, depending on label/wavelength/PMT choice.

30.9 If nothing is seen using focusing (Z adjustment), adjust the power and gains.

- i. Start with slight power increases (1-3%) then move to gain adjustment.
- ii. If necessary, use one PMT to make this easier.

30.10 Once the PMTs pick up the sample, adjust the focus slightly for more clarity

- i. Once the sample is found, readjust PMT gains if necessary.

- 30.11 Depending on the sample and imaging technique you can switch between Resonant Scanning or Dual Galvo (in **Camera** drop down menu on **Focus** window or using the button on the **Scanning** window).
- 30.12 In either mode some edges of the sample might seem mismatched; you will need to adjust the offset (the issue with edges is most common with resonant scanning).
- The offset adjustment is an up and down arrow set in the scanning console.
 - The system starts at 0 and can be adjusted in the positive or negative direction depending on the movement of the visual mismatch.
 - If using dual galvo mode, raster scanning might need to be switched from bidirectional to single direction; to make the switch, use the big button with 4 rows of arrows located next to offset.
- 31 Once the image looks acceptable in the live mode, click "Stop" and open the capture window.
- 31.1 Select the PMTs desired to image.
- Make sure to use the correct polarization.
- 31.2 The number of frames used to average per PMT per image can be adjusted (1 is a good place to start).
- 31.3 For 3D volume images (z-stack), that can be engaged here as well.
- The scanning depth (distance) can be set up in the **focus** window under the Z tab or 3D tab.
 - If the Z tab is greyed out, scroll in Z to observe the sample and gauge distance to be scanned.

Note

- Enable 3D in **Capture** window.
- Input the number of microns to be scanned. The remaining boxes will be populated with their respective values once the microns has been filed in.
- If gauging the distance yourself, I would keep the sample in the middle of the distance you want to scan (find what you believe is the middle while in "Live" scanning).
- Enable the "Range around current" option.
- Enable the "Return to current position after capture" option.

- 31.4 Timelapse can be engaged here.



- i. Set the number of frames needed per scan as well as the number of scans and intervals needed for a set amount of time.

Use of AO Properties

- 32 Use dual scanning mode; AO cannot be used in resonant scanning mode.



Note

Unless the dual scanning mode is engaged, images will not be captured using the AO deformable mirror. Light will continue to pass through the entire system without adjustment.

- 32.1 Initial calibration of the AO unit can be run in 256 or 512 pixel resolution.

- 33 To open the AO console, first click on the slide containing the images.

- 33.1 Now use the drop-down menu on the **Special Capture** option in the acquire ribbon.

- 33.2 Scroll down to AO testbed.

- 34 To turn on the mirror, run a preliminary acquisition.

AO settings

New Mix 2

Bypass

DM

Setup...

SAC-DM Calibration

Compute

A

5.32647

B

-0.7353

Plot...

Index 1 for Fit

0

Index 2 for Fit

0

Fit...

Acquisition

Zernike Modes List (ex: 3-6,7,9)

12,3,5,8

1

Zernike Amplitudes

Start

-2

End

2

Increment

0.3

Iterations

Exposure time (ms):

1

Filter:

PMT 3 - Pol 2

☒ Lock SAC Defocus

Name:

1

Status: Complete

Acquire

Remote Focusing Range

Get Range

GO AO

Analysis

Polynomial Fit Degree

5

☒ Iterative Optimization

☐ Limit to ROI No.

1

Plot

Merit Function

☒ Resolution
 ☐ Intensity
 ☐ Contrast

☐ Use Mean Intensity

Analyze

Analyze data file

DM Ratios: Intensity

0.511714

HF

0.988764

Contrast

0.822057

Use DM

Reset DM

Amplitude (μm)

2.857

-12.000

12.800

-2.500

12

3

5

8

Zernike Modes

Mode

8

Amplitude

-2.5

Set

Spherical-Z Depth Fit

☒ Rolling SAC-DM

S = A + B * Z

Z1 μm

0

Sph 1

0

☒ Use AO pattern

A

0

B

0.015733

Z2 μm

75

Sph 2

1.18

Calc S

Close

Figure 5. AO testbed window. All adjustments to the DM will be done through this window including pulling up saved calibrations, recalibrating for new sessions, and finding numerical value of the Zernike modes.

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- 34.1 First, choose the Zernike modes needed. Find the full list of the mode values by clicking the "setup" button found at top left corner.
- 34.2 Most common modes will be 12 (spherical), 3 (astigmatism in x), 5 (astigmatism in y).
- i. Coma in Y and X (7 and 8 respectively) can be included.
 - ii. List the modes in the order the aberrations are to be corrected; for example, if using 12, list it first in the Zernike modes box in the **Acquisition** section.
- 34.3 Initially, set the amplitudes to -5 to 5, located below the Zernike modes box.
- 34.4 To begin, the increment can be kept at 0.5.
- i. Exposure time should be kept at 1.
 - ii. Choose the PMT and polarization that are the easiest to observe the corrections on the sample.

Note

1. The PMT chosen should clearly illustrate the aberrations of the sample needing correction.
2. AO calibration is not needed for each PMT. Once calibrated the corrections will be applied globally to all PMTs.

- 34.5 In the **Analysis** box, choose the **Merit** function, Resolution, Intensity, Contrast, for which the AO calibration is needed .
- i. Keep the "**Lock SAC Defocus**" box checked.
 - ii. Click "**Go AO**" outside of the acquisition section.
- 35 After the initial calibration, check the plot to determine if the Zernike plots are level or erratic. To open the plots, click the **Plot** button in the **Analysis** box.
- 35.1 In the blue plot, a clear point or apex should be seen; in the red plot a flat or linear line should be seen.
- 35.2 If neither plot in 35.1 is accomplished, run the AO again, but adjust the amplitudes and possibly the increment.



- 35.3 Another means of checking Zernike plots is to visually track improvements by comparing the AO test image to the "Flat comparison" image.
- 35.4 If the calibration needs to be run again, click the "**Reset DM**" then the "**Use DM**" buttons to return the DM to a zeroed position before running another calibration.
- 36 This iteration process can be repeated multiple times until the right values are found for the sample.
- 37 Once the DM has been set, the calibration can be run under the **SAC-DM** section.
- 37.1 Set the A value to 0. 
- 37.2 Click "**calibrate**".
- 37.3 This will need to be run only once.
- 38 Now calibrate the **Spherical Z-Depth Fit** box.
- 38.1 Set the A value to 0. 
- 38.2 Keep the **Sph 1** and **Z1** values at 0.
- 38.3 Z2 can be a value based on the current sample, a previously scanned depth, or the depth required for 3D acquisition.
- 38.4 **Sph2** can be any value but it should be compared to the value on the slider in the **Setup** menu.
- 39 Once all sections are calibrated and set, save calibrations to a file name using the **Save As** in the **AO Settings** drop-down menu at the top of the testbed window. 



- 40 Once saved, make sure the **Rolling SAC-DM** and **Use AO pattern** boxes are checked before returning to the capture window to take an image.
- 41 If running an imaging session with a sample that was previously calibrated, pull up the saved calibration to use from the **AO settings** drop-down menu.
 - 41.1 The system would need to run a quick initial calibration (-2 to 2 in the amplitudes) to use the saved values as well.
 - 41.2 To change the histogram values (in the **Analysis** box) to the values from a previously saved session, click on the Zernike histogram.
 - i. To the right of the histogram, make sure the Zernike mode shows up in the **mode** box.
 - ii. Click the **Amplitude** box and input the value from the saved session.
 - iii. Repeat until all Zernike values are updated.
 - 41.3 Make sure to check the **Rolling SAC-DM** and **Use AO pattern**.
 - 41.4 Take AO images.

Branding

42

Note

Branding can be done at any point during imaging. Make sure the AO unit is not engaged and use dual scanning mode. Only one pattern can be done per timelapse. Both 256 and 512 pixels resolution can be used, but 256 pixels are best.

- 43 Click the **"Live"** button.

- 44 Bring the sample into focus. .

- 45 Determine the area of interest to brand.





- 46 In the region section, choose either the line or spiral function.
- 47 While still in live scanning, trace the area to brand with the line, or place the arrowhead for the spirals at the center of where the spiral will be branded.
- 48 Once the patterns are made, click "**Stop**".
- 49 Click the **Photomanipulation** option in the acquire ribbon, and in the drop down arrow of that option click "**Specify 2P Photostimulation.**"
- 50 Open the **2P Photostimulation** window. Choices in this window depend on whether lines or spirals were drawn.

2p Photostimulation

☐ Insert stimulation sequence during timelapse

☐ Turn off gain on PMTs before stimulation

☐ Stimulate with 1p laser

☐ Change 2p wavelength during photostimulation

☐ Use spiral patterns with radius of microns

Region: Plane (4D only):

Before time point: Through time point:

Number of pulses: Pulse width (ms):

Interval (ms): Wavelength (nm):

Power:

Description:

Region	Before Ti...	Through ...	Plane	Pulse Wi...	Channel	Pow
--------	--------------	-------------	-------	-------------	---------	-----

Figure 6. 2P Photostimulation window. Both line and spiral branding are "coded" here to be carried out as a timelapse in the capture window. Pattern adjustments to width, time points, and regions as well as protective measures can be made here.

50.1 For both lines and spirals,

- a) "Insert stimulation sequence during timelapse"
- and
- b) "Turn off gain on PMTs before stimulation"

MUST be checked.

50.2 For lines:





i. **Region:** Instruct the program what line(s) are to be drawn. Multiple lines can be designated by writing 1-3, 1-2, etc., or individual lines can be designated by listing a single value.

ii. **Before time point and through timepoint** determine when the line will be branded. Both should contain the same number.

Note

If programming lines separately (region only lists one value), then separate the time values (i.e., region 1 will be before and through timepoint 10; region 2 will be before and through timepoint 11).

iii. Keep the number of pulses as is.

iv. **Pulse width (ms):** this refers to how wide/dark the line can be.

Note

When burned, there is a visual difference between 500 ms pulse width and 1000 ms pulse width.

v. **Interval (ms):** this refers to roughly how long the laser will remain on this region

Note

1. Start at 1000 ms.
2. Leave Wavelength as is.

vi. **Power:** this refers to how much power will be used to create the pattern.

Note

1. Depending on the depth of the sample in the medium or mounting medium, the higher the power the better.
2. If the sample is too deep in the mounting medium, then the sample will not brand regardless of power or wavelength of the laser.

vii. Click "**Add**".

50.3 For spirals:

i. Click "Use spiral patterns with radius of ____ microns".

ii. In the box for spiral pattern selection, input the numeric value of the desired size of the spiral.

iii. **Region:** line(s) can be controlled here. Multiple lines can be branded by entering 1-3, 1-2, etc.; entering a single value will provide individual control of individual lines.

iv. Keep plane as it is.



v. **Before time point and through timepoint:** these are when in the timelapse the spiral will be branded. Both should contain the same number.

Note

If programming lines separately ("region" lists only one value), separate the time values (i.e., region 1 will be before and through timepoint 10; region 2 will be before and through timepoint 11).

vi. Keep number of pulses as is.

vii. **Pulse width (ms):** this refers to how dark the spiral will be branded.

Note

When burned, there is a visual difference between 500 ms pulse width and 1000 ms pulse width.

viii. **Interval (ms):** this refers to roughly how long the laser will remain on this region.

Note

1. 1000 ms is a good starting point.
2. Wavelength will remain as is.

ix. **Power:** this refers to how much power will be used to create the pattern.

Note

1. Depending on the depth of the sample in the medium or mounting medium, the higher the power the better.
2. If the sample is too deep in the mounting medium, then the sample will not brand regardless of power or wavelength of the laser.

x. Click "**Add**".

50.4 Once the patterns are added to the list, the timepoint listed last must be remembered; close the 2P photostimulation window.

50.5 Open the **Capture** window.

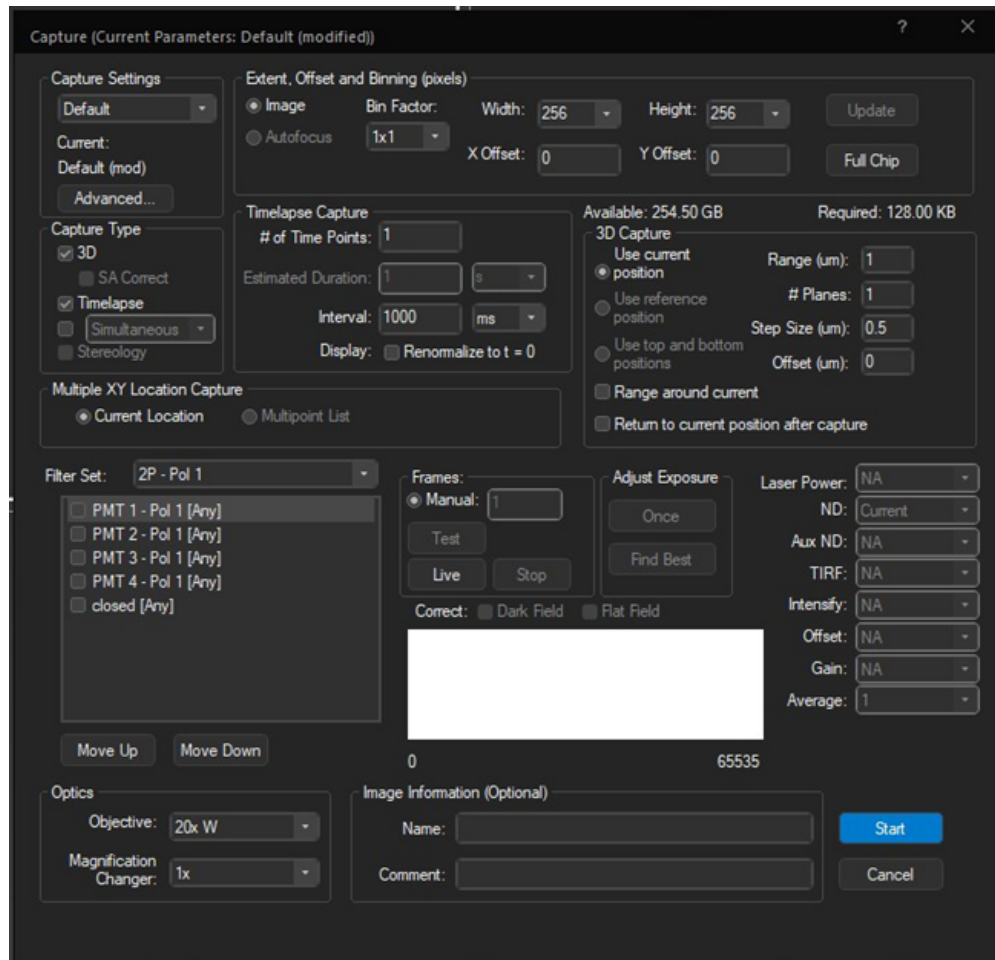


Figure 7. Capture window controls all settings for the image or stack to be captured. The timelapse and 3D options are controlled from this window for acquisition purposes.

- 51 Enable the timelapse capture type.
- 52 In the "**# of Time Points**" enter one time point higher than was listed in the photostimulation window list; for example, if the last region was set at time point 12, then the capture should be 13).
- 53 In the "**Interval**" box change it from 1000 ms to 0 ms.
- 54 Select only one PMT channel to make the scan faster.
- 55 Capture the timelapse.



- 56 Once timelapse is complete, check the last timepoint to determine if the brand worked or take a single 2D image without changing any settings (besides removing timelapse capturing).
- 56.1 If the brand does not show, return to the **2P Photostimulation** window; and try the brand regions again, but adjust the power, pulse width, OR interval. Do only one at a time.
- 56.2 For further issues, check the troubleshooting section.

System Shut Down (end of imaging session)

57

Note

If taking a short break (under 1.5 hours), then shutter the laser, turn off the PMTs and lock the computer. If closing for the day follow the steps below.

Shutter the laser.

- 58 Turn off the PMTs.
- 59 Save file for the last time.
- 60 Open the garage door.
- 61 Move the objective all the way up (off the sample).
- 62 Remove sample from sample stand or holder.
- 63 Use a lens wipe to blot excess water on the objective.





Note

IMPORTANT: Do NOT wipe across the objective lens; only blot. Wiping will scratch the lens. Gently place and remove the lens wipe on the objective until water is removed.

- 64 If needed, use a kimwipe to clean excess water from sample,
- 65 Dispose of cleaning materials, unused water, and disposable pipette.
- 66 Close and lock the garage door.
- 66.1 When closing the door, listen for the safety shutter click.
- 67 Close Slidebook.
- 68 Close Laser GUI.
- 69 Go to the control tower.
- 70 Flip the laser key switch to "Standby".
- 71 Flip the large white switch to off.
- 72 If it is the last imaging session on this instrument for the week (i.e., it is Friday) or for more than 2 days, all boxes in the control tower (white boxes and Pockels cell box) can be turned off.
- 73 Chiller will remain on 24/7.





Troubleshooting: Laser Errors

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Note

The major steps (whole number) in the troubleshooting sections occur in no particular order. The sub-steps (75.1, 75.2, etc.) should be followed in order.

75 If the laser GUI shows an R232 fault, follow 75.1-75.6.



75.1 Shutter the laser.

75.2 Turn off the PMTs.

75.3 Flip the key on the laser control box to standby.

75.4 Keep the key switch there for 10 seconds.

75.5 Flip the key switch back to laser enable.

75.6 Fault should be cleared.

76 If the laser is tripped or emergency shuttered (in the GUI), follow steps 76.1-76.5.



76.1 Laser GUI indicator will be solid red

76.2 Shut off all PMTs and click "stop" (in the scanning window).



- 76.3 Find the key switch on the control tower; flip it to "standby" for a few seconds; then switch to "laser enabled."
- 76.4 After cycling the laser, check the GUI to make sure laser power is running around 1.5-2 W, depending on the wavelength being used.
- i. Laser power will change dependent on wavelength used. Consult Coherent provided pages in blue binder if not sure.
- 76.5 Make sure garage door is shut and locked properly.
- i. This will deter the safety shutter from being engaged.

Troubleshooting: Slidebook/Sample Errors

- 77 If a PMT is tripped, the bar beneath the gain sliders will turn bright red.  
- 77.1 Shutter the laser in the laser GUI.
- 77.2 Turn off all PMTs in the scanning console window in Slidebook.
- 77.3 The tripped PMT (red bar beneath gain slider) means something could have sparked, there was overload in the receiving signal, the laser was too strong, or the gain was not proper for that signal.
- 77.4 Increase the gain slightly and decrease the laser power (1-2%).
- 77.5 Turn the PMTs on and unshutter the laser.
- 77.6 If the PMTs trips almost immediately, move to a new region of interest, the previous region shown might have been burned by over imaging.
- 78 If no sample appears on the visualization window and "snow"/grey "fuzz" is seen:  
- 78.1 Turn off PMTs



78.2 Shutter the laser

78.3 Check the focus window to make sure the correct polarization (Pol 1/Pol 2) is chosen.

78.4 Check live button to determine if it says "stop" or "live,"

i. If it says "stop," the laser was active

ii. If says "live," the laser was not actively engaged in scanning the sample.

Note

1. If option i is the case, click stop and open the garage door. Look at the plunger position and make sure it is pulled out (this guides the light to the PMTs in this position). If plunger is out, move to step 78.5; if plunger is pushed in, pull it out and reset to "look at the live image."

2. If option ii is the case open the laser shutter, turn on the PMTs and click live (should be able to see something now)

78.5 If the above does not correct the problem, adjust the focus, the laser power or the gain.

78.6 If sample is seen in the GFP filter through the eyepieces and the above steps are not correcting the problem after initial focus/power/gain adjustments, the wavelength of the laser may need to be adjusted.

i. To adjust wavelength, make sure the laser is shuttered and the garage door is closed (plunger is pulled out).

ii. Adjust the wavelength within the laser GUI, wait for the laser to tune, then open the shutter and begin the focus adjustments again.

78.7 If the problem persists, the label/stain/fluorophore might not be suitable for 2P.

79 If the sample signal looks considerably weaker in the live window after multiple images taken, or some time has passed (over 30 mins):

79.1 Turn off PMTs.





79.2 Shutter the laser.

79.3 Click stop on the scanning window.

- i. Turn “Fluor on”; switch the filter to GFP in the Widefield drop down selection in the Focus window.
- ii. Or use cell phone flashlight as an external light source.

79.4 Open garage door.

- i. Make sure safety switch has engaged (click sound).

79.5 Check water on slide.

- i. Add more water if bubble is gone, or if all water has evaporated.

79.6 If water level is fine, move objective up (disconnect from water). An air bubble in the water might have been caught between the objective and the sample.

- i. Use lens paper to wipe water from objective.
- ii. Remove excess water from slide using a corner or part of a kimwipe to absorb the water.
- iii. Place fresh drop of **distilled** water onto slide.
- iv. Reconnect objective to water drop; add one or two more drops to “immerse” the objective.

79.7 If the water was not the problem, check Pockels cell value in the control tower.

- 79.8
- i. If the value has not changed, check Pockels cell position on the table.
 - ii. Shutter laser, turn off PMTs, stop the live window.
 - iii. In laser GUI, change the laser to alignment mode, keep shuttered once it is ready.
 - iv. Remove the back panel, locate the Pockels cell.

Note

1. The bundled cords on the Pockels cell should be pointing straight up.
2. If the bundle is leaning towards one side where the cords are strapped down, loosen the cords a little and reorient the bundle to face straight up and down.
3. The bundle should be up and down as alignment of this unit is crucial. Pockels cell works with voltage across the glass in the unit to adjust the laser in the path. If the cell is rotated, it changes the bias application of the voltage and can disrupt the alignment.



v. Open the shutter (in the GUI) to the tunable laser line; now the laser is in alignment mode.

vi. Check the path to make sure the laser is entering the center of the Pockels cell and entering the beam splitting cube at the center.

80 If a crosshatch pattern is seen in any PMT channel during live scan and continues to be seen in a captured image:



80.1 Realign the system.

80.2 Turn off PMTs.

80.3 Shutter the laser.

80.4 Stop the live scanning.

80.5 Switch the laser in the GUI to the tunable alignment mode.

80.6 Open Scanning Diagnostics window.

i. Find Pockels Power

ii. Start with 0.5 V (The working range is from 0-2.0 V).

iii. Click set.

iv. Set Galvos to 0.

80.7 Connect the safety shutter magnets. If the magnets cannot be removed from the garage door, turn the key to the "off" position on the control tower.

80.8 Open laser shutter.

80.9 If no laser comes out at the objective, remove the filter cover above the objective.

80.10 Use a laser safety card to examine the back aperture of objective.

i. If no red light is showing check:

Note

1. Safety shutter,
2. Laser shutter,
3. Pockels Power in Scanning Diagnostics window.
4. If 1-3 are fine, move to next step

80.11 Remove safety panel below Vector RS+

- i. Keep laser unshuttered.
- ii. Make sure laser is hitting corner mirror (being turned into M-shaper).
- iii. Check alignment of beam entering the M-shaper (make sure it is centered). If not:

Note

1. Adjust corner mirror in small increments and use the laser safety card to check that the laser is centered.
2. If the laser is entering the m-shaper properly or was realigned through this unit, use the laser card to again check the back aperture of the objective.
3. If still no laser is seen at the back aperture, realign through m-shaper.
 - a. Remove side panel.
 - b. Remove screws to panel of m-shaper.
 - c. Check that the laser is hitting center of DM.
 - i. Need to rotate aligning adjustments between the laser entering from the bottom and exiting at the top of the DM
 - ii. If laser not centered, center it.
 - iii. Use the entrance mirror knobs (below the m-shaper) to adjust the laser on the DM
 - d. Again, using the laser safety card, check the back aperture of the objective

80.12 If there is still no laser, realign through Vector RS+.

- i. In the Scanning Diagnostics window, make sure galvos are set to 0,0 for X and Y locations.
- ii. With a laser card, look at the entrance to the Vector RS+.

**Note**

1. If no light is at the entrance, check the base of the AO unit.
2. If there is light at the base of the AO unit, adjust the corner mirror screws (across from the AO unit) to move the light out the top of the AO unit into the Vector RS+.
3. If light is entering Vector RS+ but not exiting, adjust within that unit.

81 If branding pattern does not show up:



81.1 In the stimulation window, make sure the timepoints "before" and "through" are matching values.

81.2 In the capture window, make sure the capture time is longer than the stimulation time was in the last region (if stimulation was 12, capture should be 13).

81.3 Make sure the PMTs are turned off during stimulation capture.

- i. Make sure the PMTs are **ON** during timelapse capture.
- ii. Make sure the laser is unshuttered during capture.

81.4 Ensure the PMT used to see the live image is the same channel used to capture timelapse (also ensure the use of the same polarization, "pol 1" vs "pol 2").

81.5 If branding pattern does not appear once the above steps are followed, repeat steps in the Branding section.

- i. If repeating the steps does not work, try a different wavelength.
- ii. Use the laser GUI to change the wavelength.

Note

1. 800, 850, 900, 920 nm have been tested for branding and work well for lines and spirals.
2. Make sure the laser is shuttered before changing the wavelength

81.6 If repeating steps does not work for spirals, try changing the radius before the wavelength. Begin with smaller values (1 micron vs 10 microns).

81.7 If all the above does not work for lines or spirals, or both, the area of interest is too deep in the mounting medium.



i. Adjust microscope in z to move closer to the top of the sample and find a different area.

Protocol references

Simar, L., & Rosa-Molinar, E. (2025) "Benchmarking Assessment and Implementation of an Imaging Phantom in Nonlinear Optical Microscopy. Unpublished manuscript. *Current Protocols*.

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Acknowledgements

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