

Notes on Using the Upright Zeiss LSM 710 at the University of Chicago

<http://pondside.uchicago.edu/oba/Cal/booked/Web/?>

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Important updates in this version:

- The Main Switch on the remote power control must be **turned off** at the end of each session.
 - The reason for previous policy of turning off the sub-switches but leaving the Main Switch on was to allow the fans continue to cool the lasers; preventing condensation which shortens the laser's shelf life. However, the downside of leaving the lasers off but still connected to the circuit is the exposure to electrical power surge. This new policy reflects the perception of a greater risk of power surge than the risk of condensation.
- The troubleshooting section is on the last three pages of these notes.
- The confocal calendar has been moved to a new URL.

BEGIN



Step1: Turn ON the PC (you might have to wait a few mins for the blue HP screen to disappear). Click Ctrl+alt+del and login to your account (DO NOT LAUNCH THE ZEN2012 yet!!!)

Step2: on remote power control **a.** MAIN switch ON(wait 6-8secs) (Check the big black LASOS box. Both the interlock and power red lights should be ON!! If not, you have to switch off and back ON the switch below the big silver plug on the box), **b.** SYSTEM/PC ON (wait for a few secs until everything is loaded on the little screen by the microscope), **c.** COMPONENTS/SWITCHES ON (the key for the lasers on the remote control should always be at the horizontal "ON" position)

Step 3: Turn on X-Cite for epi-fluorescence.

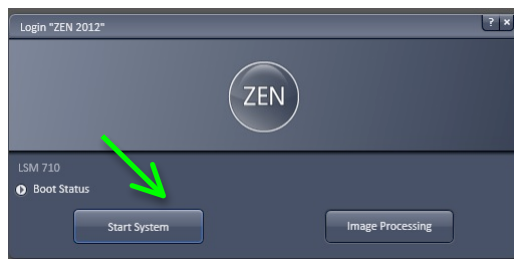
Step 4: If you will be using argon (Ar) laser, which is for 458nm (CFP), 488nm (GFP), or 514nm (YFP) excitation:

Make sure the red toggle on a panel (located left of HP PC, and it says Lasos RMC . . .) is on "idle" and *not* on "run".

Turn on Lasos argon laser by turning the turn key from vertical "0" position to horizontal "1" (ON) position.

Wait for a few seconds before you flip the toggle from "idle" to "run". Depends on the room conditions, after several minutes, the green LED should come on to indicate that power is stable.

Step 5: Launch the ZEN2012 software and start your experiments (click Start System)



If the process is stuck for quite some time, then reset the real-time PC (the big black Zeiss box), which is located behind and perpendicular to the HP PC. Open the cover, press and release the bigger button.

→ See more tips on the Troubleshooting pages at the end pages of these notes.

LOCATE

Choose a stage insert: standard slide holder, long cover slip holder, 35mm Petri dish holder, 60mm Petri dish holder, or welled-plate holder (to be used with stage heater).

On the touchscreen monitor to the right of the microscope, select the **Home button** on the upper left corner, then select **Load Position** in the right column, the stage will be lowered, put the stage insert in place by pushing against a set of loading springs that are in the bottom left corners.

Place your sample on the stage insert. Either manually turn the focusing wheel to raise the stage, or press the upper left triangle button to raise the stage back to its original “working” position. The automatic movement can be stopped by pressing stop button, when it is necessary as the objective lens is getting too close to the sample.

Click on the Locate tab.

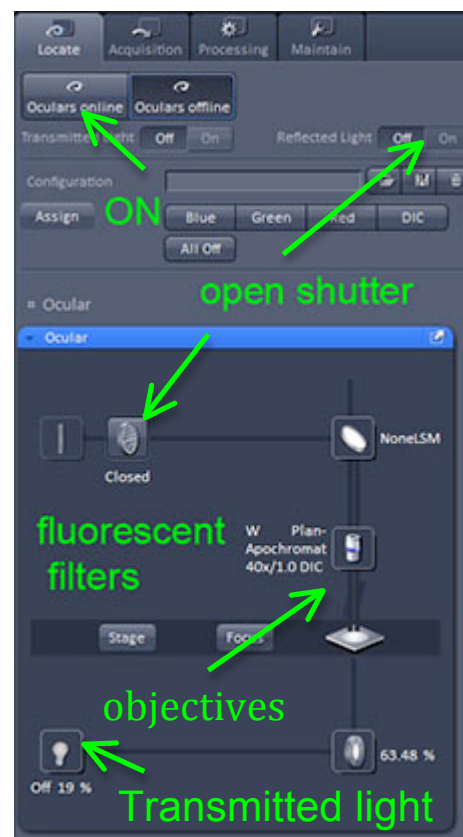
Choose an objective: 10x dry, 10x dipping, 20x dry, 40x dipping, or 40x water immersion (with a long working distance), or 100x oil immersion. Other objectives available but not in the rotation: 5x dry, 63x water immersion (usually on the LSM 510).

Alternatively, to choose an objective using the touchscreen, select the Microscope button in the left column, then select the objective.

On the computer screen, click "Oculars Online" and To locate a region of interest on your sample through the eyepieces, choose a type of illumination: transmitted light (bright field) or fluorescence.

For transmitted light, click the icon for transmitted light, and drag the scale bar to 15% or more (at source; and with 60+% transmission) for transmitted light illumination.

For fluorescence, click icon to choose from blue, green, or red fluorescent filters. Be sure that the shutter is opened in order to view under the fluorescence mode.



Alternatively, using the touchscreen, on the right column under the Home menu, you can turn on/off the RL (reflector light) Illumination and the TL (transmitted light) Illumination

X-Y axes: Use the joystick to adjust the automated stage. Toggle the button on top to switch between fine (slow) and coarse (fast) adjustment. Z axis: Focus by using one of the three focusing wheels. **Fine focus** by using the smaller knob or by finger-turning the dimpled wheel. Focus speed is fastest while using the 10x dry lens, and the speed is slower with an objective lens of a higher magnification.

After you have found the region of interest, turn off the illumination, and click "Offline".

Cf. Select Online to enable viewing sample through the eyepieces (or the oculars); select Offline to send emission signal to the signal detector and thus the computer.

OPTIONAL ADJUSTMENTS

Adjustment of the Condenser's Z Position

Adjustment of the condenser is necessary when you are switching between the stage inserts. Notice that the slide holder is indented, but the cover slip and Petri dish stage inserts are flat.

- Stage insert in place, but without the sample.
- Switch to the 10x objective.
- Turn on transmitted light.
- Minimize the condenser iris by sliding the bar from left to right (pass 0.3).
- Lower the F focus by pressing the lower button (immediately behind the dimpled focus wheel on the right side of the microscope stand).
- Adjust the height of the condenser until the edge of the iris is in focus.
- Raise the F focus by pressing the upper button; expand the condenser iris by sliding bar right to left.

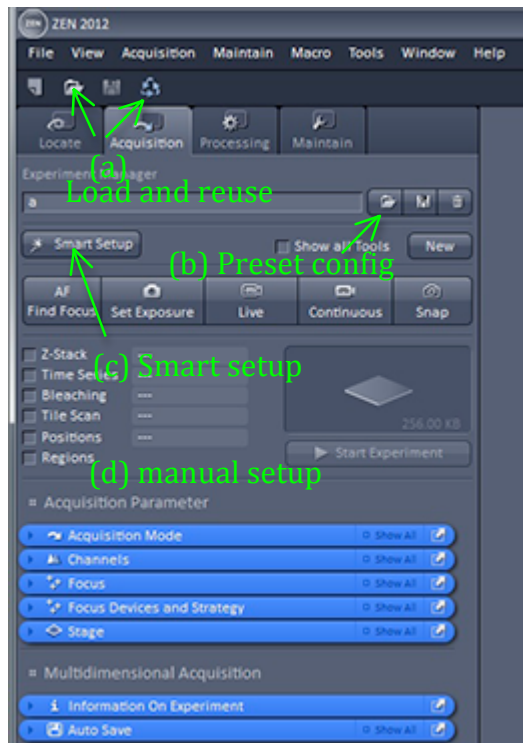
Adjustment for DIC Imaging

- Polarizer is at the bottom of the stage, set the polarizer pin on 0. Always leave polarizer in position.
- Condenser prism: if using low power objective use prism II (NA 0.4 or lower, e.g. the 10x dipping), if using higher power objective use prism III (for NA 0.5 or higher, i.e. most of the lenses).
- Objective prism above each objective is specific to that objective lens. Twist the knob to adjust DIC.
- Put in #4 filter “Analyzer module DIC ACR”: select either on the computer or on the touch-screen in the reflector menu.

ACQUISITION

Click on the Acquisition tab.

CONFIGURATION



Set up the configuration in 4 ways: (a) load a *.czi file and reuse its configuration, (b) choose from a preset configuration, (c) use "Smart Setup", or (d) Set up the configuration in 4 ways: (a) load a *.czi file and reuse its configuration, (b) choose from a preset configuration, (c) use "Smart Setup", or (d) manual setup.

(a) Load and reuse

Click on the first icon to load the *.lsm or *.czi image with old configurations, once the image has been loaded, click on the third icon to reuse the configurations

Caution: Before load and reuse old configurations, make sure that the option to reuse objective lens has been *unchecked*. To do so, go to the Maintain tab, under Options, the LSM Options panel, the Re-use tab, and uncheck the box.

(b) Preset configuration

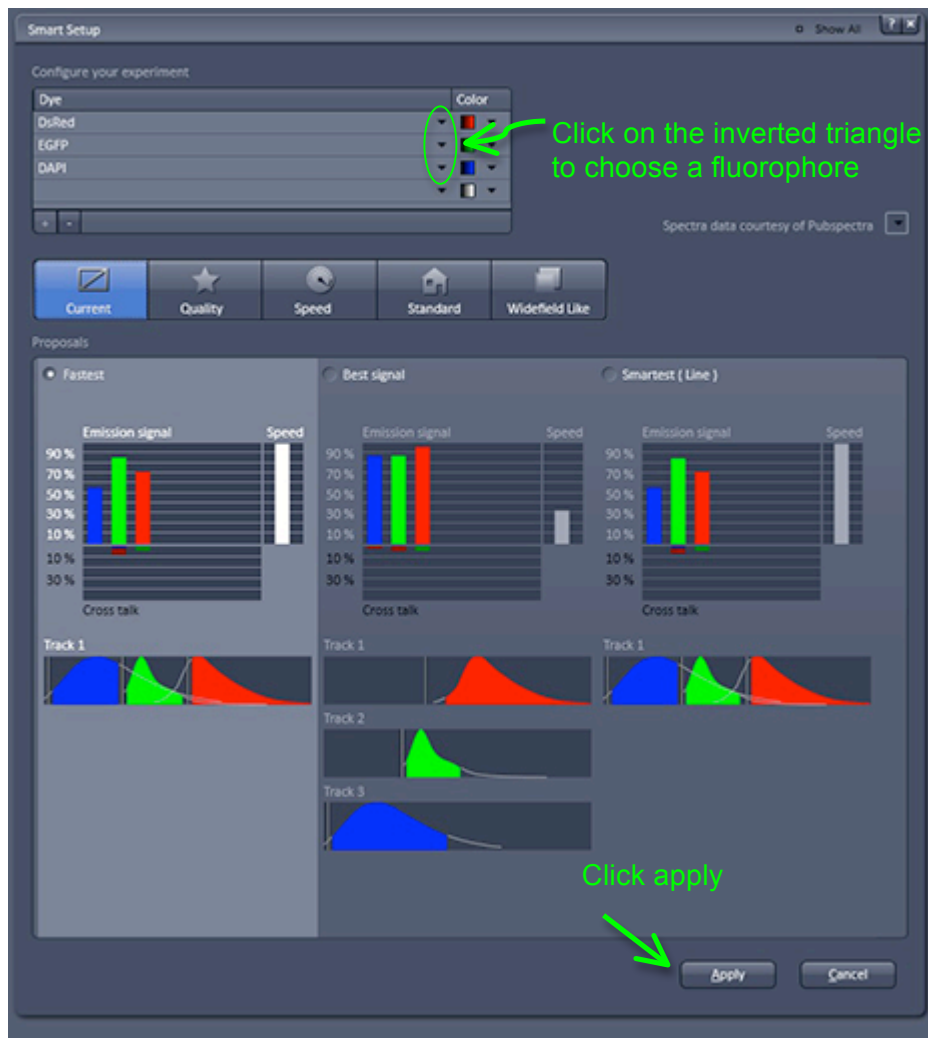
To use one of the preset configurations, click the open folder button, choose from one of the configurations by clicking on it to apply.

(c) Smart Setup

If using the guided "smart setup", click on the inverted triangle. Choose a fluorophore from the menu. Repeat this step for all types of the fluorophores present.

Then, choose from one of the four options. These four options are different ways of detecting the emission signals by varying between switching every track or switching every line as illustrated by each of the diagram.

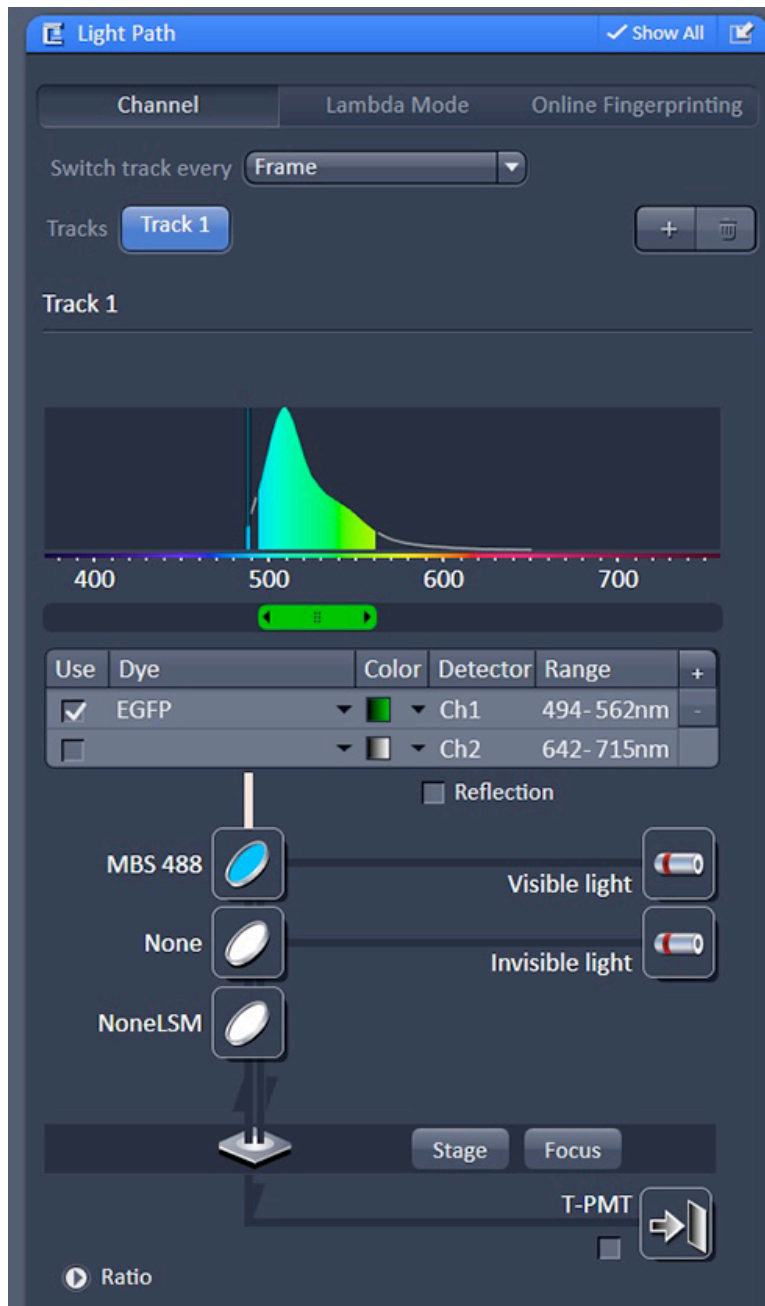
When decided on the options, click apply.



FASTEST: For simultaneous imaging. Favors speed over cross talk and uses FRAME wise switching

BEST SIGNAL: For sequential imaging, slow speed. Attempts to eliminate cross-talk and uses FRAME wise switching.

SMARTTEST: Attempts to combine spectrally distinct channels into the same track in order to optimize speed and minimize cross-talk.



(d) Manual Setup

If manually set up the configurations, check both the **Light Path** panel and the **Channels tool**, change track, excitation lasers, spectral range (or emission band), dichroic, etc.

In the Light Path panel, the dichroic (Main Beam Splitter, or MBS) should match the excitation lasers in the 405 and visible light paths.

Single track: use one track for simultaneous imaging of up to 10 channels theoretically for each track.

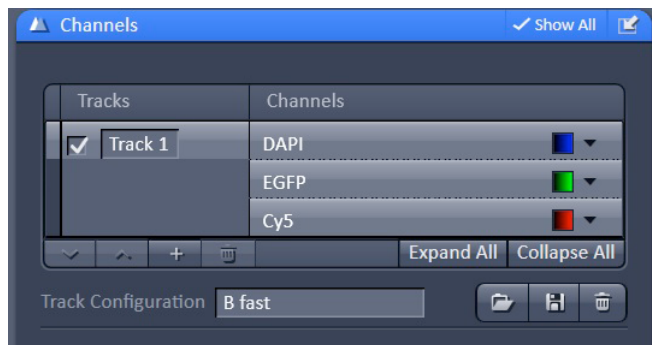
Multi-track: Use two or more track for sequential imaging.

Switch track every line (or line switching) is only suitable when *no* physical parts are changing between the tracks that means switching between photomultiplier tubes (or PMTs); however, any electronic changes in the control of laser excitation, or detection gain is fine.

Switch track every frame (or frame switching) should be used if any physical part moves when switching between the tracks, such as the dichroics, pinhole sizes, spectral ranges, etc.

T-PMT is for transmitted light (or bright field) picture that can be added independent of or attached to one of the tracks. Ch1 and Ch2 are standard alkali PMTs, but ChSx is an array of 4x8 high-sensitivity PMTs.

There can be up to 8 ChSx channels in each track. Thus, overall an overall of 40 channels (4 x (8 ChSx + Ch1 + Ch2)). Detector gain for all of the ChSx is the same, but digital gain for each of the ChSx can be adjusted independently. You may save the configuration clicking the diskette button in the middle, name it and click OK.



Setup for Simultaneous Acquisition

For simultaneous acquisition using four or more channels, click on the Light Path panel, click on the "+" button in the upper right corner, and additional "ChSx" channel appears.

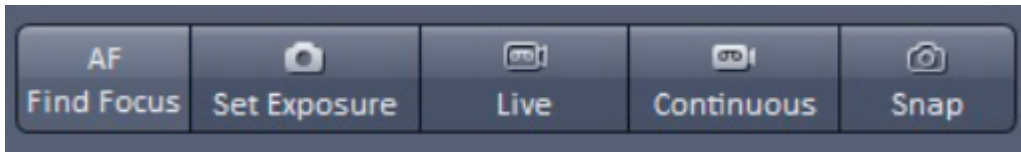
Click on the inverted triangle in the Dye column to specify the fluorophore, and similarly on the inverted triangle in Color column to specify the false color desired for the particular channel.

Notice the slider for the spectral range for the ChSx appears as a small slider; adjust each of the sliders to set the spectral range for each channel

The theoretical limit is a maximum of eight "ChSx" channels, thus 10 channels for each track. Overall, the theoretical limit is 40 channels per image with scan switch track every frame (in the Imaging Setup panel).

LINE switching: with that method only the channels and lasers can change between tracks. Line method is preferred unless it is necessary to change something else between tracks like pinhole or MBS. In any other case you need the FRAME switching method.





Modes of Acquisition: Set Exposure, Live, Continuous, or Snap

Set Exposure:

Set Exposure changes the detector gain and digital offset to detect the signal to form an image.

Live : For a Fast continuous scan

Live (formerly Fast XY) is fast, continuous scan of 512x512 pixels at one frame per second, providing a quick feedback, which is useful when adjusting the scanning parameters. Live image is usually a dimmer image because of its fast scan speed, when compared to Continuous.

Continuous : for a high quality, slow, continuous scan

Continuous is continuously scanning at the parameters that you have specified.

Snap: to take an image

Snap is a single scan at the parameters that you have specified so what you see is what you get from those scanning parameters, including averaging, scan speed, etc.

Zoom / Crop

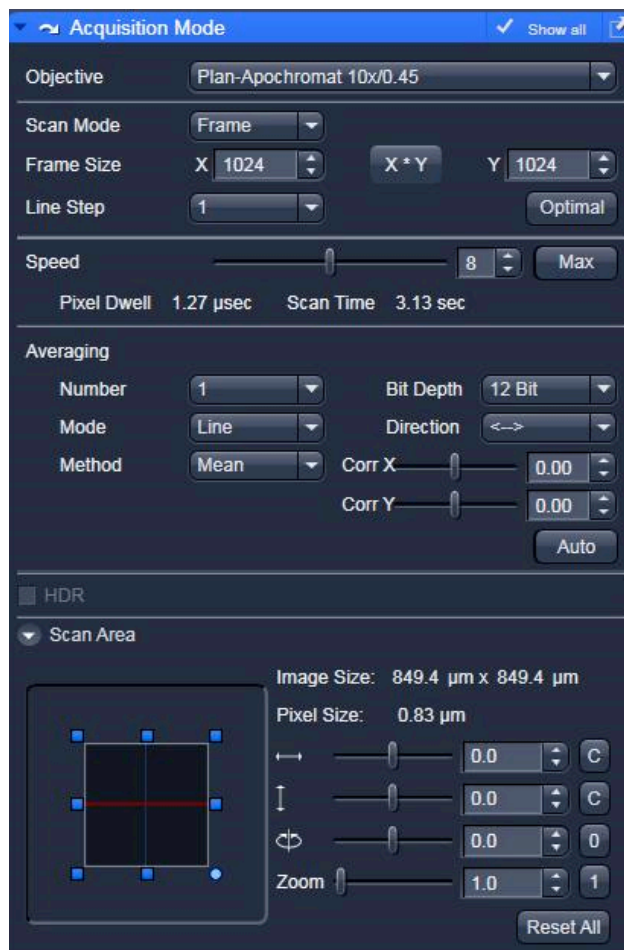
Look for **the zoom function** in the Scan Area of the Acquisition Mode panel. You can zoom out as much as 0.6x.

Real optical Zoom, increases resolution and decreases image size.

Alternatively, you **can crop the image**. Look for the crop button (between the Reuse and Positions button) at the bottom of the image window.

Scan Speed and Averaging

A recommended scan speed is 6, 7, or 8. Slower scan speed improves quality of the image because the detector has more time to detect the emission signals. But of course, the slower the scan speed the longer it takes to finish a stack of images (multiply scan time with the number of slices); also, a slower scan speed may increase the risk of photo-bleaching. Typical settings for averaging are: Number 4, Mode Line, and Method Mean.



Averaging also improves the quality of the image because the repeated acquisition (line mode or frame mode) averages out the random noise. However, this is not an option for fast live imaging, e.g. with calcium green.

Consider this: a scan speed of 7 with line averaging twice could result in better images over a scan speed of 6 with no line averaging, in same amount of time and other factors being equal.

Frame Size

You may choose the number of pixels in the image. To choose from default sizes (512x512, 1024x1024, etc.), click on the X * Y button.

The **Optimal button** calculates the **optimal number of pixels for this objective, at the excitation laser's wavelength, and the level of zooming**.

Bit Depth

8-bit ($2^8=256$, which means 256 levels of grey) is fine, but 12-bit ($2^{12}=4096$, which means 4096 levels of grey) is better, especially for quantifying analysis.

Direction

Choose bi-directional imaging for faster imaging. Choose uni-directional imaging to minimize defects in non-alignment.

NOTES on resolution:

Collect 2-3 pixels over the smallest object you want to resolve

Do not collect less than the minimum pixel size

Use higher mag/NA lens if needed

To get smaller pixels but a larger field you can collect 1024x1024 (frame size) or use tiling

Maximum Zoom (at 512x512) and minimum pixel size

10X Zoom 5 = pixel size ~ 0.3um (300nm)

40X Zoom 6.3 = pixel size ~ 0.08um (80nm)

63X Zoom 4 = pixel size ~ 0.07um (70nm)

Ingredients of confocal imaging = Pinhole size + Percent of laser excitation + Gain + . . .

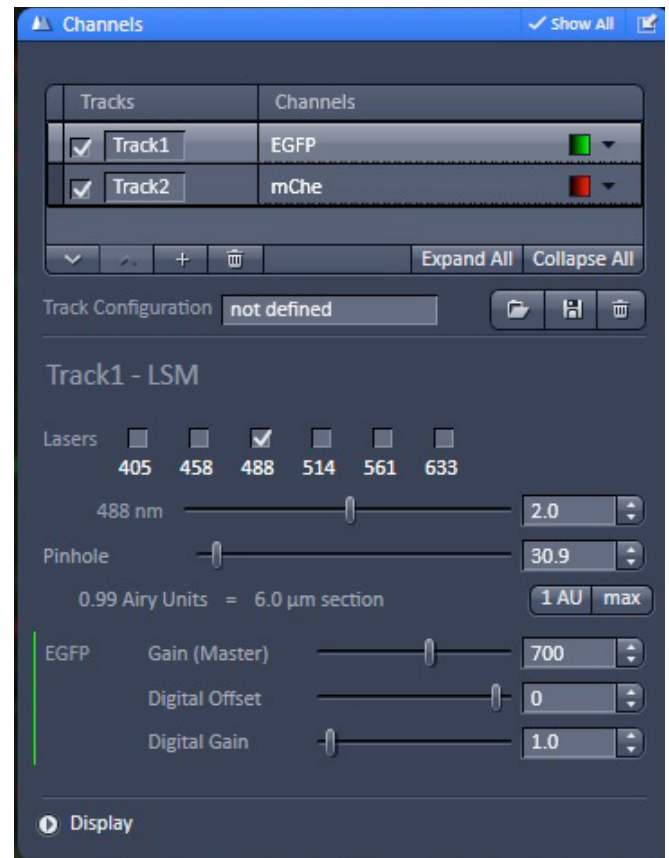
Pinhole

The larger the pinhole, the thicker and brighter the optical slices. The smaller the pinhole, the thinner the slice, but the images tend to be dimmer. Notice that there is a trade-off between the thickness and the brightness of the optical slices.

The recommended pinhole size is (at minimal) 1 Airy unit, but no smaller than that. So start with 1 Airy unit, and then increase the pinhole to get a good image. 1AU ensures that you are taking a confocal slice (optimal section thickness)

Power of laser excitation

On this LSM 710, only the argon (Ar) laser which its power can be adjusted at source. For all lasers (including the Ar laser), the power of laser excitation can be adjusted as percent of transmission.



The power of laser excitation can be adjusted as percent of transmission. In the Channels panel, look for the active lasers in each track.

Always start with a low excitation ($\leq 1\%$) and then increase the percent of transmission because this is a strategy to minimize photo-bleaching of the fluorophore.

Gain and Digital Offset

The twin functions of gain and offset allow you to adjust the sensitivity of the detector (photomultiplier tube or PMT), for each channels.

On this LSM 710, the recommended starting values are the Gain (Master) are 500-700, and the Digital Offset at an integer below zero.

It's useful to adjust the gain and offset with the application of range indicator (see instructions below). The higher the gain, the higher the noise; it's especially noticeable when the master gain is at 1000 or higher. If you are using multiple ChSx channels, these channels share a same value for the master gain, but still you can adjust the saturation for each of the ChSx channels by the adjusting the digital gain (the third option, immediately below the digital offset).

Range Indicator and Color Palette / optimizing the dynamic range

Range indicator can be applied to help to visualize the points of saturation (red pixels), and points of zero intensity (blue pixels). With the application of range indicator, a decent image should have a few red pixels and a few blue pixels because it indicates that the image is at full range.

Look for a row of buttons for selecting display of the channels (Merged, Ch1, ChS1, Ch2 . . .), near the bottom of the image window. Click on bottom immediate below the Merged button. To remove the range indicator palette, click on the same button.

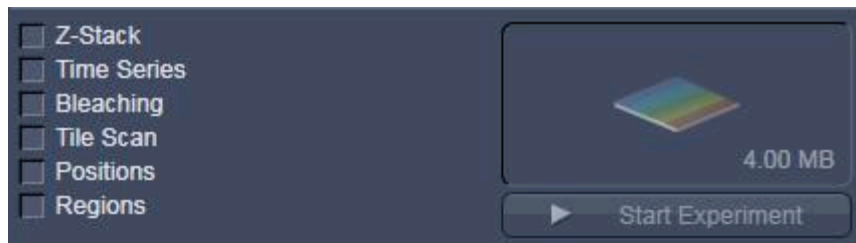


Click on the inverted triangle on each of the buttons in this row allows you to choose a color palette to apply to the channel displayed on the screen; you may choose a preferred false color.

Optimizing the dynamic range suggestions:

- >You can use continuous or live imaging so that the image you are trying to optimize is constantly updated
- >In the bottom left side of the picture, check the range indicator box.
- >Adjust the master gain until little red is seen and the digital offset until small amount of blue is visible (usually 0.0)
- >Snap to take images for all selected tracks

Z-Stack



>Check the **Z-Stack** box one above the Time Series box, make sure **show all** is enabled

>Select **First/Last** imaging mode

>Click **Live** button to scan at the maximum speed. While it is scanning Live, move focus wheel to the top of your sample and click **Set Last**. Now move the focus wheel to the bottom of your sample and click **Set First**. (always focus down to Set First. You should collect against gravity)

>Click the Live button (which is now a Stop button) to stop scanning.

> Click **smallest interval**

Notes: In order to achieve Nyquist sampling, the optimal interval is half the slice thickness (determined by the pinhole size settings and objective)

For best results you should check each channel in the **Channels** tool, to ensure that all of them have the same optical section thickness. This means you should adjust the pinhole settings slightly for each channel (you should check the gain settings after doing this)

Click on Optimal Sectioning and Setup, choose either optimal slice interval (this is the choice in most cases), or a fixed number of slices (if you want the same number of slices for all the stacks).

Click on the C (or Center) button in the Z-Stack panel to scan check the representative middle slice of image, before commit to acquire the whole stack of images. A useful tool: apply range indicator as a color palette to check saturation against the background (refer to notes above).

>Click **Start Experiment** to start acquire the stack of images.

Viewing options: 2D (composite image), Split (to the several channels), or Gallery view.

Time Series

Check the Time Series box one below the Z-Stack box.

In the Time Series tab, choose the number of time points (total number of scans), and the duration of interval between the time points. If you have a z stack checked, you will get xyzt series.

Press Start Experiment.

For a time series of Z-Stacks, the final images are presented in a 4D format that can be saved into a single *.czi file.

Bleaching (FRAP) or Photoactivation

>Choose from menu **Bleaching**. Time series and regions should also be activated.

- > Choose # of scans before the bleaching
- > Iterations are the number of scans during the bleaching (more iterations will bleach longer)
- > Set laser and laser power (slower scan speed will give better bleaching, use fewer iterations), Zoom bleach can be faster but it will be less spatially accurate
- > Define **Regions**
- > Set up time series (time series does not include the bleach step!! – time stops)
- > **Start Experiment**

Tile scan

Centered grid- the computer will center on the current stage position and you must specify number of images in horizontal and vertical direction.

Boundary grid- manually move to the corner/edge of the field you want and press add. Move to diagonal corner and press add. The computer will calculate the tiling as a square/rectangle.

Convex hull- select the region as in Boundary grid but computer reduces the number of tiles, avoiding the excess of a square shape, which may save some time by selecting convex hull over a centered grid.

Overlap: set at 10% or more. This is how much overlap there is between the each tiles.

Click Start Experiment to start capturing the tile scan.

Note that the tile scan images cannot be saved individually.

To stitch tiles together, go to Processing tab and select “stitch” function. Highlight the tile scan images, then click select to choose these images to be processed. Strict setting (correlation threshold at 0.9) is recommended.

Positions

Use Position to image two or more positions using the motorized stage.

Click Live imaging, then click Add to remember the x-y-z coordinates.

Click “Start Experiment”.

For Z stack, you can only set the position at the center of the stacks.

Cf. For Z stacks of different Z sizes, use the advanced multi-time macro module.

Regions of interest / ROIs

It is used to image a small part of the image frame or for bleaching

Note: the smallest the Y axis (height) the faster the scanning

- > click on the **regions** toolbox

Updated by Sanny Papanikou, june 2015

- > choose the region shape
- > Draw the region on the image
- > select the “fit frame size to bounding rectangle of regions” box
- > always have the acquisition box checked
- > click snap or continuous to image the region of interest

ADVANCED ACQUISITION

Macro Module: Multiple Time Series (Multi-Time)

Press Alt+F8, or click on Macro (fourth in the text menu on top).

If using Multi-Time for the first time, follow the steps below. Otherwise, choose Multi-Time from the menu.

Click assign macro tab.

Choose an empty entry from the menu, then click on the ellipses “. . .”.

Find “C:\ZEN\Macros\Multitime*.lvb” and let it load, and name it in the text box.

Specify the parameters, such as the number of cycle which is the number of time points within a block.

After specifying the parameters, save the configurations, by clicking on update, then apply the configurations in the Multi-Time macro module.

Recipe means setting at a high level in Multi-Time. You may store or apply each of the recipes.

Most likely you are using Multi-Time to image multiple (two or more) locations, so click on Multiple Locations tab.

Choose the parameter that you want to change, then click on apply to apply that parameter to *all* the locations. Otherwise, any changes to the parameters will not take effect. Click on Select parameters to find the button to apply the parameters.

Alternatively, go to stage > marks > and mark each of the locations; *not* positions.

There can be only one common list of locations, so each of the blocks shares the same list of locations.

The following illustrates the hierarchy in descending order:

Experiment	< Recipe A	< Group I	< Block 1	< Location a < Location b
			< Block 2	< Location a < Location b
		< Group II	< Block 3	< Location a < Location b
	< Recipe B	< Group III	< Block 4	< Location a < Location b
			< Block 5	< Location a < Location b . . .

For example, you may define the time interval between each groups, blocks, or locations. Similarly, you may use block when you want to image in the green channel for a set of locations, then later to image in the red channel for the same set of locations. Also, blocks allow you to define Z stacks of different Z sizes at different locations.

The final images are presented in stacks for each of the locations.

PROCESSING

- Choose the Processing tab.
- Here is the popular post-acquisition tool “Maximum Intensity Projection”, which allows you to flatten a stack of images.
- Also available here is the stitch function for stitching images of a tile scan.

FINISH

Check the microscope’s online calendar at <http://pondside.uchicago.edu/oba/Cal/booked/Web/>?

If someone has signed up after you, simply close the Zen2010 program, and log off the computer. If no, see instructions below.

Argon laser: Make sure that the red toggle on the small black box is in the down position on “idle” (or standby).

However, if the microscope will not be used in over 2 hours, please turn off the argon laser by turning the key on the large black box to “off” position.

If you are the last to use the microscope:

- After exporting and transferring your data, close the Zen2012 program
- Shut down computer
- Argon laser Step One: turn the red toggle on the little LASOS box to “idle” (or standby)
- Argon laser Step Two: turn the key on the black LASOS box from horizontal “1” to vertical “0” (off) position
- Wait about three minutes for the lasers to cool
- While waiting for the lasers to cool, clean up and put cover on the microscope
- On the power remote control, turn off the System/PC and the Components switches, and then turn off the Main Switch.
- Turn off X-Cite, the epi-fluorescence light source.

TROUBLESHOOTING

The system or program is stuck . . .

Computer is beeping and the power button is flashing red.

Press and hold the computer's power button until it ceases to beep or flash. Wait for 10 seconds or so before pressing the power button again to start the HP computer.

Computer is stuck at the HP blue screen.

Two possible solutions: First, if under the logo, the screen says "press any key . . ." simply press any key to continue. Second, if it does not respond after you press on any key, then restart the computer by press and hold down the power button for a few seconds.

I cannot login.

Make sure that you are using your Hullbiolabs' ID and password for login to the Hullbiolabs domain, and not the local domain on the HP confocal computer. To set up your Hullbiolabs ID, please contact Ian Miller at ianm@uchicago.edu

Program does not start up after clicking on the "Start System" button.

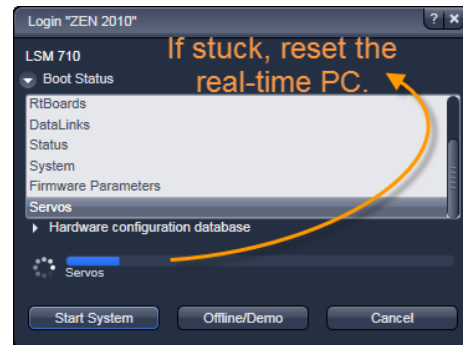
If the starting process is stuck for quite some time, then reset the real-time PC, which is located behind and perpendicular to the HP PC. Open the cover, press and release the bigger button.

Stage is stuck at a level lower than focal plane. *OR*

The touchscreen says that software limit reached.

If the system has been turned on for a while and the argon laser has stabilized, you may reset just the microscope itself by pushing off, wait, and on again the power button on the lower left side, on the front of the microscope stand.

Otherwise, restarting the whole system should reset the stage position. Turning off just the microscope stand and its peripherals are less drastic than a full restart.



The laser does not turn on . . .

The argon (Ar) laser does not turn on.

- (a) There is no red LED light at all, next to where the power box says Power and Interlock.

Check that both of the sub-switches are turned on, and the key on the side of the main power switch are still in the horizontal ON position. Also, check that the switch right below the metallic output connector on the LASOS power box is turned on and hasn't been inadvertently turned off by the last user.

- (b) There is only one red LED light, and turning the key does not turn on the Ar laser.

Keep the toggle on idle; find the switch right below the metallic output connector; this can be difficult to locate because of the poor design. Flip the switch off and on (requires only one repetition) to reset the LASOS power box. This problem is fixed when there are two red LED lights. If so, you may turn the key clockwise from vertical to horizontal, and flip the toggle from idle to run.

- (c) There are two red LED lights on the power box, but turning the key does not turn on the Ar laser. OR (d) The green LED on the small panel does not light up.

Depends on the room conditions, it may take about 10 minutes before the green LED lights up to indicate that the Ar laser's power is stabilized. However, the Ar laser could be on and could be used even without stabilized the Ar laser's power (that's when the green LED light is on).

Not on the power box, but look on the LASOS RMC 7812Z2 small panel, check that small toggle has been flipped from idle to run. Check that the knob for Light Control has been turned up until just a little before the red LED comes on. The red LED (higher power mode) on the small panel should not be on, unless you are using high power to ablate or bleach cells.

Also, in the ZEN software, look under the Channels panel to check that the laser excitation is set at 0.5% or higher; it's usually set higher at 1% or even more.

Cf. If you can see that the laser is on but you are not getting an emission signal, check that the PMT detector's Master Gain is at 600 or above.

If problem persists, please let the facility manager know about this problem.

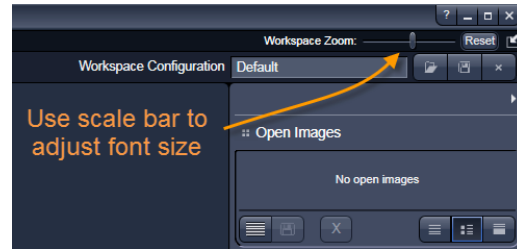
The ultraviolet (UV) laser does not turn on.

The ZEN software interface does not allow turning on or off the UV laser. The UV laser is connected and ready when the system is turned on. To use the UV laser for excitation, set up the pathways under the section of CONFIGURATION. Simply checking the 405nm UV laser under the Channels panel may not work properly because the correct dichroic and beam splitter have to be in place along the optical pathway.

Other problems . . .

The font size is too small to read.

You may increase the display font size by sliding the scale bar to the right. Look for the “Workspace Zoom”.



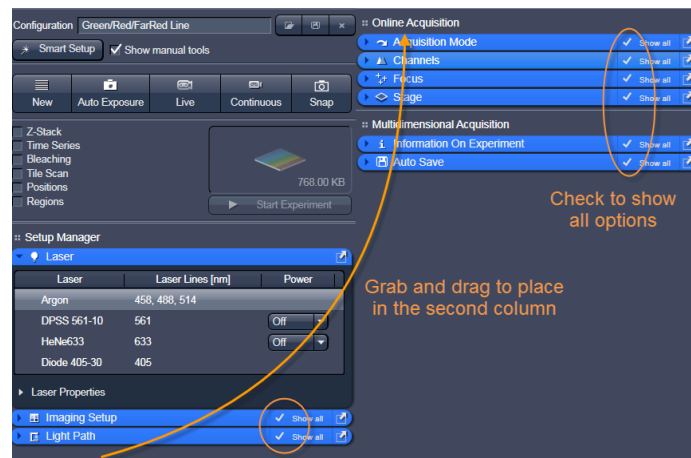
Where is the Laser panel? Imaging Setup panel? Light Path panel?

Under Acquisition tab, next to the Smart Setup button, check on the box to show manual tools.

Some of the functions are missing.

Check on the boxes to show *all* options; otherwise, some of the versatile functions are hidden by default.

How to put the Acquisition panels into a separate column?



For a two-column view, grab and drag the menu bar to place in the second column.