Overview

This SOP describes the routine operation of the Zeiss LSM 800 Confocal Laser Scanning Microscope at the Nano-Engineering Research Core Facility (NERCF), University of Nebraska–Lincoln.

Safety Notice：Users must complete instrument training before independent operation.

Microscope Lens list

Position	1	2	3	4	5	6
Magnification	10x	20x	40	63	EMPTY	5x
Numerical Aperture	0.3	0.8	1.4	1.4		0.16
Free Working Distance [mm]	2 mm	0.55 mm	0.13 mm	0.19 mm		18.5 mm
Coverglass Thickness [mm]	0.17 mm	0.17 mm	0.17 mm	0.17 mm		0.17 mm
Immersion	Air	Air	Oil	Oil		Air
						

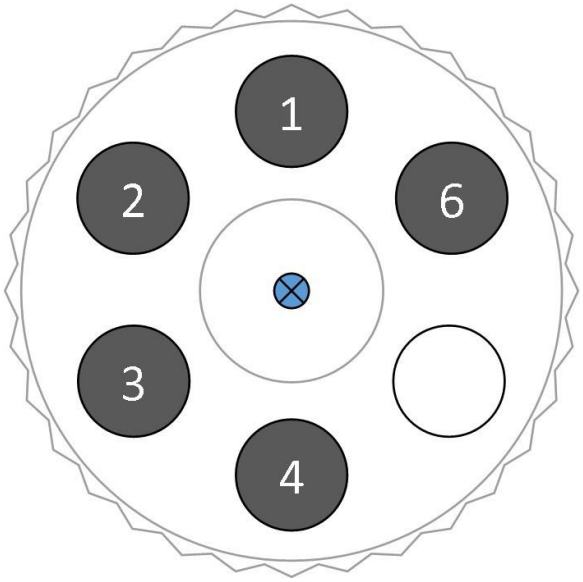


Diagram showing arrangement of objective positions. Position 5 is reserved for special application lens thus will not be populated at all times.

Objective	Application
5X	Whole slide
10X	Overview
20X	General fluorescence
40X Oil	High resolution
63X Oil	Highest resolution

System start up

Preparation

1. Be sure to know the excitation and emission spectra of all fluorophores for your dyes you are using. before imaging
2. Turn off the room lights to minimize ambient light.

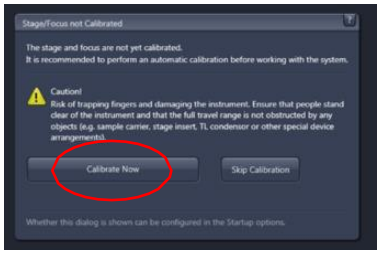
Start the microscope

1. Turn on scope by flipping the STEP 1 (System) switch, then, STEP 2 (Components) switch.
    a. Note, to turn off, this must be done in the opposite order, turn off step 2, then step 1.

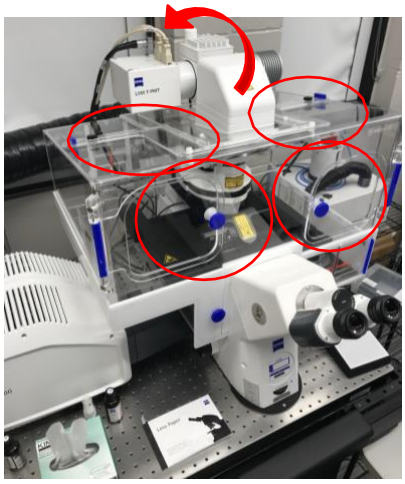
Start Zen software

1. Log into computer using your FOM ID and password
2. Once on, open the ZEN 2.0 program on desktop

3. Click “ZEN system” to access the confocal



4. A “stage focus” dialog box will open, and asking you to calibrate the slide. Hit “calibrate now”
   took out sample frame
   <u>adjusted objective lens to 10X or lower**</u>**
   before you proceed with “Calibrate Now” button.



Load sample

1. Once calibration is done, open all four doors
2. Gently tilt and lift the scope head up
3. Load your sample on the stage of the microscope
    a. Note: If the stage is not secure, sure the stage is securely in place with the stage springs in the bottom left corner

DIC (Differential Interference Contrast)

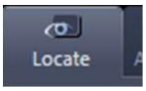
- Creates a pseudo-3D relief image by converting gradients in optical path length into intensity differences.
- **Best for**: relatively thick, fixed samples—gives crisp edge definition of tubules, glomeruli, etc.

Phase Contrast (Ph1, Ph2, Ph3)

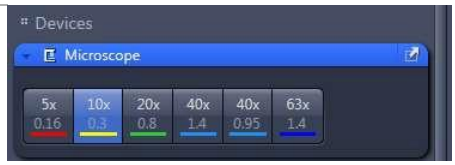
- Relies on shifting the phase of un-diffracted vs. diffracted light; you need to match the condenser annulus to the objective's phase ring.
- **Ph1**: small annulus—optimized for very thin, low-contrast samples (e.g. live cells in culture dishes).
- **Ph2**: intermediate—live cells on coverslips or moderately thick sections (~20–50 µm).
- **Ph3**: large annulus—thicker samples, but still <100 µm; beyond that the optical sectioning and light scattering start to limit contrast.

Locate specimen, focus, find cells / region of interest (**Always locate your specimen using the 5× or 10× objective before switching to higher magnification objectives)**

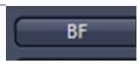
1. You should be on "Locate" tab if the software has just finished calibration. If not navigate to "Locate" tab.



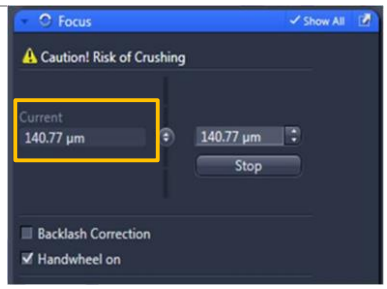
2. Select objective lens with software / on microscope control panel.
   40x and 63x objective lens needs immersion oil.

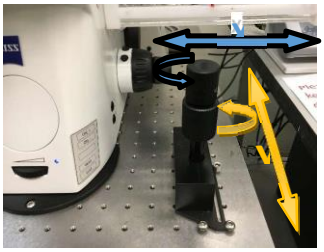


3. Push back condenser column.
4. Mount your sample on sample carrier. Ensure your specimen holder is level.
5. [Optional] Click "Bright Field" on software for easier sample finding and bright-field

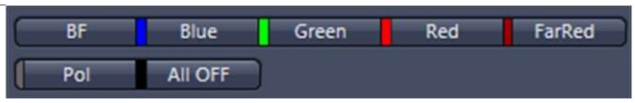


6. Move the area of the slide you wish to observe above objective lens with X-Y manipulator on air table.
7. Focus onto your specimen. Notice "Z-position" value shown on the microscope control panel and software, larger number means higher focus height.

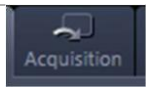




8. Click "B" = Blue; "G" = Green; "R" = Red; "Far- red" on software for fluorescent observation.
   **Note: fluorescent dye will eventually be bleached under intense excitation light. Find your region of interest quickly. When you turn to other tasks turn of ALL illumination by clicking on "All off" button. **



9. Click "Acquisition" tab when you arrive at region of interest.



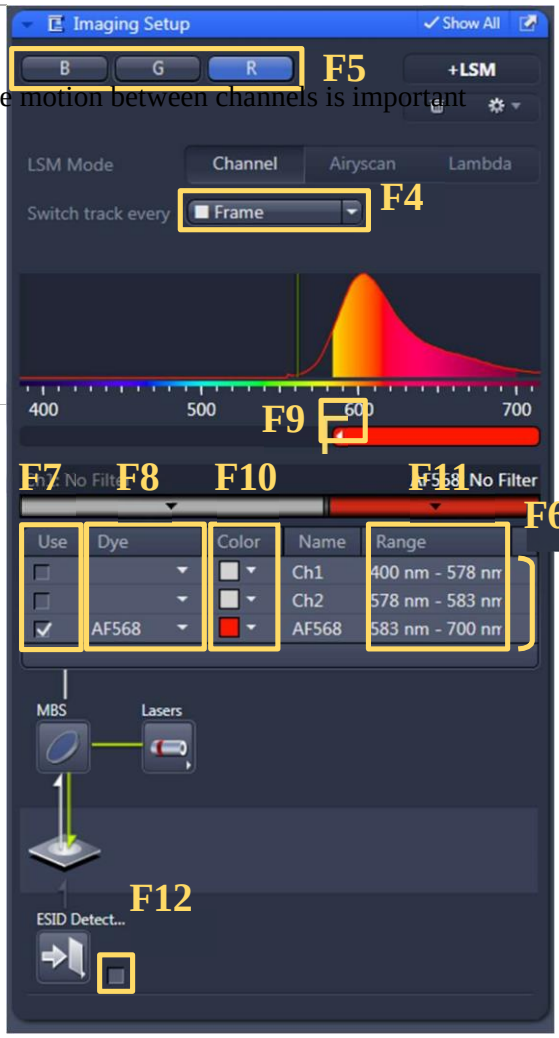
Confocal imaging set up

Prerequisites: Region of interest is centred in field of view and have clicked 'Acquisition' tab.

1. Select one of the pre-set made by Imaging and Flow Cytometry Core.
2. Go to dropdown menu in "experiment settings" area. Usually you can use the "**Confocal-General Training Test Aug 2025**"
3. Please note "Blue" "Green" "Red" are general broadband wavelength setting you will need to fine tune the detection range in Imaging set up window. (Step F8)

Frame
Recommended for most multi-color imaging.
Line
Use only when minimizing sample motion between channels is important.
Frame Fast
Faster acquisition with reduced flexibility.

4. Go to "Imaging setup" window
   Decide on switch track every:
   "Line" = Use only when minimizing sample motion between channels is important
   "Frame" = Each color per frame sequential.
   "Frame Fast" = lock up pinhole and wavelength splitter but still change color per frame.
   "Full Z-stack" = (only available when 'z-stack' is activated. Will change track after finishing whole z-stack.
5. Select any one track for set up.



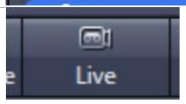
6. Table's rows indicate detector choice.
   Row 1 = PMT1
   Row 2 = Airyscan detector
   (when idle will work as confocal)
   Row 3 = PMT2
7. 1st column indicates activation.
8. 2nd column indicates dye reference and name (click on ▼ for dye selection window).
9. After setting up dye selection, spectra window will be updated. Use slider to have detector detect most of the emission wavelength range.
10. 3rd column indicates pseudo-color choice. Click ▼ to change.
11. Last column indicates detection range.
12. If a bright-field image is required, check the 'TPMT' option in 'Imaging Setup' window.

13. Go to 'Channels' window. While you make changes please observe histogram in 'Display' tab under preview area.
14. For detailed description of 'Display' tab please read ZEN Blue 2.3 SOP on Imaging and Flow Cytometry Core website-> Protocols -> Imaging

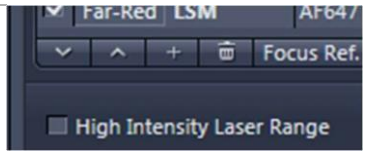
15. Activate all channels you need by ticking checkboxes.
16. Highlight a track you wish to adjust.
17. You may use the '1 AU' hotkey 

to adjust pinhole size. Alternatively balance pinhole size so that every channel's optical thickness are the same (applicable when multi-channel imaging is required).
18. When adjusting laser intensity and master gain values pay attention to the 'Display' histogram. Lower both settings if you see a peak at 'White' indicating PMT over-saturation. Turn on 'Auto' checkbox in 'Display' tab and select 'Min/Max' option for real time display. 8-bit image do not exceed 250 units. 16-bit image do not exceed 65500 units.
**Important information. Preventing damage to PMT units. Avoid over-saturating the detector. **

19. Turn on 'Live'



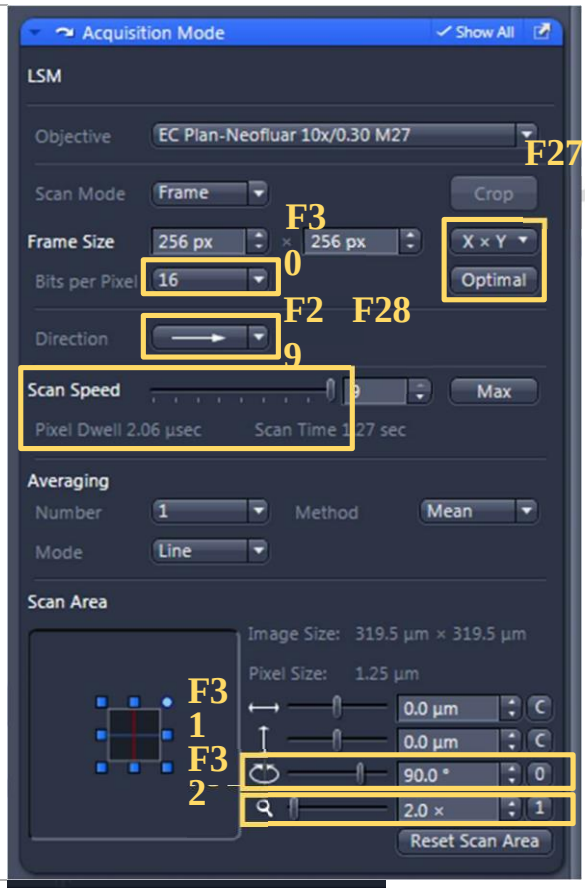
20. Adjust laser power. You can use value between 0.01% and 5% in normal mode.
21. Adjust 'Master Gain' to tune PMT sensitivity (recommended range between 500V to 800V).



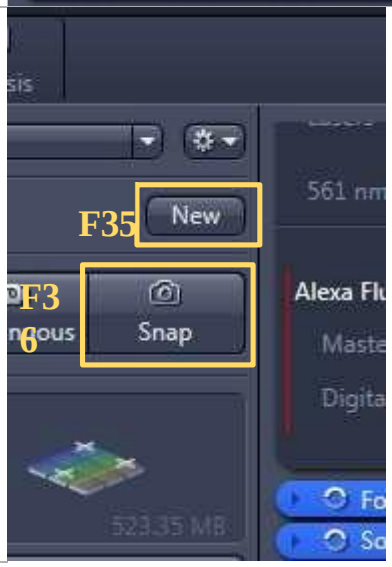
22. **For extremely weak fluorescent signal even at 5% laser power and 800V 'Master Gain' please workout labelling parameter (preferred) or turn on 'high intensity laser range' checkbox option. This will open up the laser from 3.5% to 100% range.
**High chance of sample bleaching use with caution. **
23. Other options in 'Channels' window:
24. 'Digital Gain': for mathematically enhance apparent intensity of image. Typical setting = 1.0. All pixel values will appear as:
(original value) * (Digital Gain factor)

25. 'Digital Offset' for mathematically subtract apparent intensity of image typical setting = 0. All pixel values will appear as: (original value) - (Digital Offset value) = image pixel intensity.

26. Go to 'Acquisition Mode' window:
27. Adjust 'Frame Size' if you are not sure and do not mind performing 2X Nyquist sampling, click 'optimal' option.
28. Adjust 'Scan Speed' recommended value from (slowest) ~4 µsec to (fastest) ~1 µsec 'Pixel Dwell'
29. Switch between scan directions unidirectional [insert image] for quality; bi-directional [insert img] for speed.
30. Switch between bits per pixel [N.B.: different degrees of intensities a pixel can represent] 8-bits for presentation, representative images, reports for faster work-flow; 16-bits for intensity quantification / segmentation if required.
31. Adjust 'Scanner rotation' angle if applicable
32. Adjust 'Zoom' if applicable. [N.B.] This function scans a smaller area through objective lens, consult 'optimal' frame size after adjusting this parameter.



33. After all parameter has been set up do the following to acquire an image.
34. Ensure again all channels you need to acquire is checked in 'Channels' window. (Refer to step F15)
35. Click 'New'
36. Click 'Snap'



Parameter	Recommendation
Pinhole	**Keep at 1 AU whenever possible.**
Master Gain	**Use the lowest gain that provides sufficient signal.**
Digital Offset	**Keep close to 0.**
Digital Gain	**Keep 1.0–1.2.**
Laser Power	**Use the lowest laser power that provides sufficient signal.**

(1) Pinhole Adjustment

Set the pinhole to **1 Airy Unit (1 AU)** whenever possible. A larger pinhole increases signal but reduces optical sectioning and confocal image quality.

---

(2) Master Gain Adjustment

Master Gain amplifies both signal and noise. **Use the lowest gain that provides sufficient image quality.**

---

(3) Digital Offset Adjustment

Digital Offset reduces background but may also remove weak signals. **Keep the offset as close to 0 as possible.**

---

(4) Digital Gain Adjustment

Digital Gain amplifies both signal and noise digitally. **Keep the Digital Gain between 1.0 and 1.2 whenever possible.**

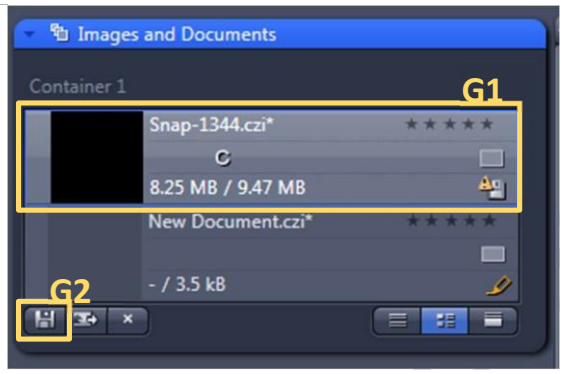
---

(5) Laser Power Adjustment

Adjust laser power for each fluorescence channel individually. **Use the lowest laser power that provides sufficient signal to minimize photobleaching and phototoxicity.**

Saving an Image file

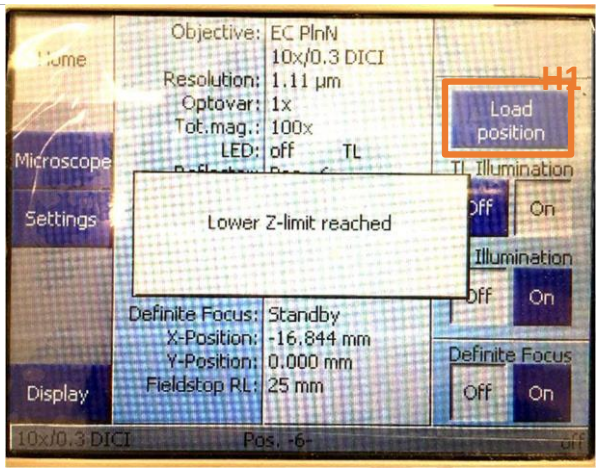
1. Go to right hand side Image Library column to find your latest acquired image. Double click on the image you wish the save. Click on 'Save' button.
2. Alternatively,
   Right click on the image you wish to save and then click 'save selected' from the pull down menu appeared.
3. Navigate to D:\\ () User\
4. Create a folder with your PI's name and + 'Lab' wording suffix. Applicable if no such folder exists and you are the first one in your lab using LSM 800.
5. Navigate into the folder you have just created.
6. Choose file format as '.czi' acronym for 'Carl Zeiss Image'
7. Type in name (preferably no spacing).



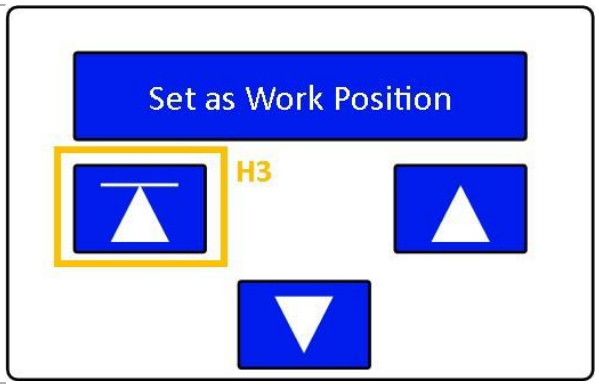
8. Click 'Save'
9. Warning Icon  or  indicates unsaved or changes has been made to your .czi file.

Removing sample from microscope stage.

1. On control panel press 'load position' button. Objective lens should be moved to lowest position allowing clearance for you to operate with the stage area.
2. Move slider on sample carrier apart to retrieve your specimen, then install a new specimen.



3. Press 'return to work position' on control panel.



Turn off

1. Close ZEN software first.
2. Set focus back to 5x
3. Lower the objectives till the lowest z has been reached
4. Turn off the scope by flipping the step 2 switch, then, step 1.
5. Remove immersion oil from objective. Remove immersion oil from coverslip.

Oil immersion Lens clean procedure

Pre-requisite: you should know how to clean objective upon novice training. Seek technical support if you ae not sure.

- [N.B.] Always take lens tissue one by one from the original case. Discard any lens tissue in contact with other surfaces (e.g. bench / air-table / microscope stage)
- [N.B.] Use only lens cleaning tissue instead of Kimwipe or any coarser material to prevent scratches on optical surfaces.

1. Fold a piece of lens tissue into a long strip.
   Never use Kimwipe to clean objectives.
   Never use ethanol or acetone unless instructed by Zeiss.
2. Drawn the folded lens paper across objective lens surface ONCE ONLY.
3. Move to a new clean area of the lens tissue. Repeat last step. If you run out of space on the lens tissue pick up another one from the container.
4. Repeat this process until oil residue is no longer observed on used lens tissue.
5. Water / Glycerol / Silicon objectives, please follow the same clean procedures (not installed on LSM 800 routine use. Request from technical staff for objective lens list and assistance)

Appendix I

Problem	Possible Cause	Solution
No fluorescence signal	Incorrect laser or detection settings	Verify the selected laser, fluorophore, and detection range.
Image is saturated (all white)	Master Gain is too high	Reduce the **Master Gain** until saturation is eliminated.
Image is too dim	Laser power is too low	Increase the **laser power** gradually while avoiding saturation.
High background	Digital Offset is not optimized	Adjust the **Digital Offset** to reduce background while preserving weak signals.
Blurry image	Pinhole is too large	Set the **pinhole to approximately 1 AU**.
Cannot find the specimen	Objective magnification is too high	Start with the **5× or 10× objective**, then switch to higher magnification after locating the sample.
DIC image is not working	Analyzer or DIC prism is not engaged	Check that the **A/F slider is set to Analyzer (A)** and the correct **DIC prism** is selected.
Cannot find exported images	ZEN uses the default export directory	Check the **Export Folder** or use **Save As** to specify the destination folder.