

Start up through collecting images

1. X-cite: If you are going to view fluorescence through the eyepieces, turn on the X-cite lamp (1). The box is to the left of the microscope table (figure 1). The X-cite lamp replaces the mercury bulb, and should be treated similarly. When it is switched off, it cannot be reignited until it cools (~20–30 min).
2. Switch on the Main Switch (2) on the control box (figure 2). Note: Never turn or remove the key on the right side of the control box.
3. Switch on Systems/PC (3) on the control box (figure 2).
4. Wait for the microscope to complete start-up. (Look at the microscope control box. When the microscope is ready, it will look like the picture to the right.)
5. If the computer is:
 - a. **Not** already on:
 - i. Once the microscope is ready, turn on the PC (4) using the button on the front of the HP (figure 3). The computer is to the left of the monitor table. Note: the computer can be turned on independently from the confocal system for data transfer, etc.
 - ii. Wait for the computer to start Windows (until it says Starting Windows or you see the Windows icon), then turn on Components (5) on the control box.
 - b. **Already** on: Switch on Components (5) on the control box (figure 2).

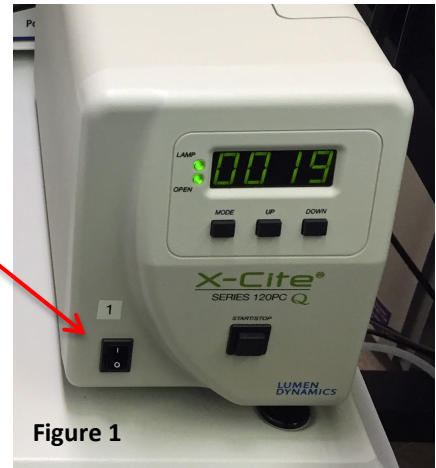


Figure 1

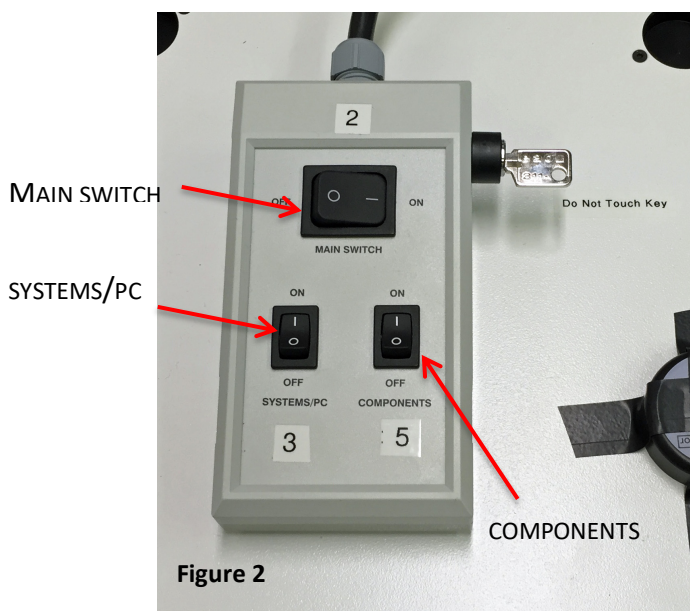
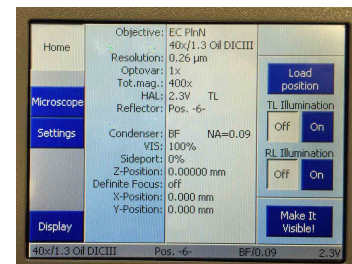


Figure 2

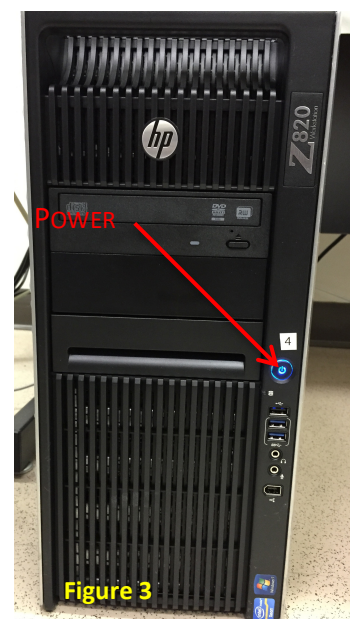


Figure 3

- Log on to your account and click on the Zen Black icon to start the Zen software. (Zen Blue is a software version that has additional imaging processing functions, but it will not run the confocal.)

Click on Start System (figure 4) and expand the main Zen window to full screen. (If you want to see the start-up progress, click on the Boot Status arrow.)

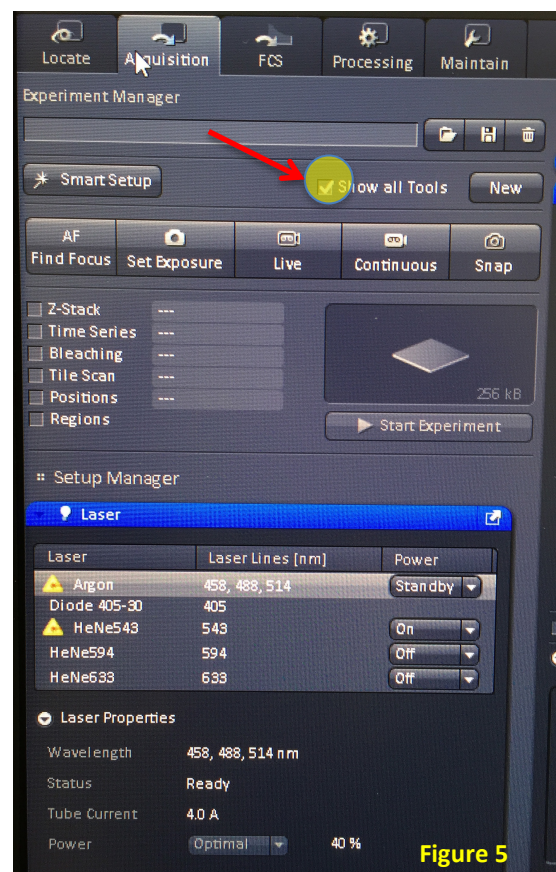


As the software starts you will hear movement in the scanhead as the system self-tests. You should not see any error messages, unless you did not turn on the X-cite lamp. (If you do see any error messages, please take a picture or write down the message and tell Kristi.)

- Click Show all Tools (figure 5). Under the View menu, click Show all (global).
- Turn on the lasers that you will need. At the top left of the Zen window, you will see a series of tabs (Locate, Acquisition, FCS, Processing, and Maintain). Click on Acquisition and go to the Laser menu. Open the laser properties tab under the menu. To turn on the lasers:

- Argon (458, 488, & 514 nm): click on Standby. You do not need to do anything else. The laser will warm up in the background and you will be prompted to turn it on when you load the configuration. It does take ~6 minutes to warm up, so turn it on when you start the software and it will be ready when you need it.

But, if you want to know what is happening in the background, the initial laser status will be WAITING FOR IGNITION. After ~1 min, the laser status will go to WARMING UP. There is a countdown timer for this, and it takes 600 seconds. After that, the status will go to READY and the laser can be turned on and used.

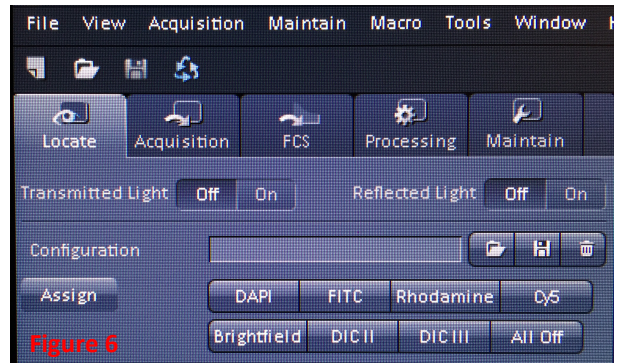


- Near UV 405 nm: now a diode laser. It is powered up with the system.
- HeNe 543 nm: turn ON
- HeNe 594 nm: turn ON
- HeNe 633 nm: turn ON

- While the lasers are warming up, go back to the Locate tab and put your sample on the microscope.

Finding your sample through the eyepieces

1. The microscope can be controlled in the software. Under the Locate tab (figure 6), you can choose the objective (see list in #3 below), select the filter set, and choose the light source. For example, if you chose DAPI, the system will automatically close the transmitted light shutter, put the DAPI filter set in position, and open the shutter for the X-cite lamp. Conversely, if you want brightfield, when you click on Brightfield, the system will open the transmitted light shutter, remove the filter set, and close the X-cite shutter.

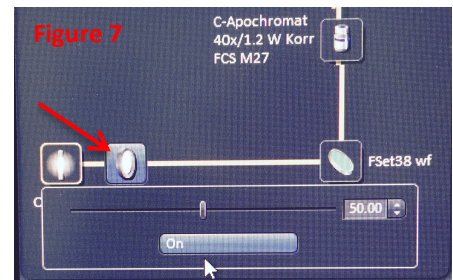


The filter sets available are DAPI, FITC, rhodamine, and Cy5.

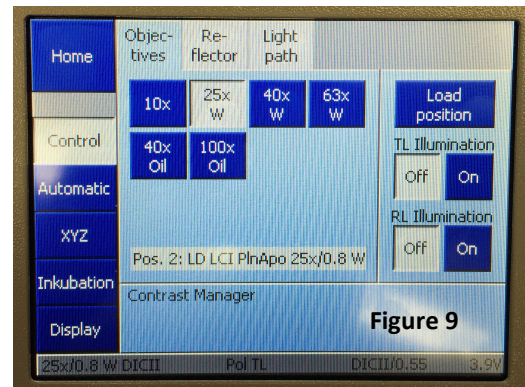
If you are using DIC, you should choose the correct DIC filter (figure 6) to match the objective you are using.

When you are finished, click All Off to close the shutters.

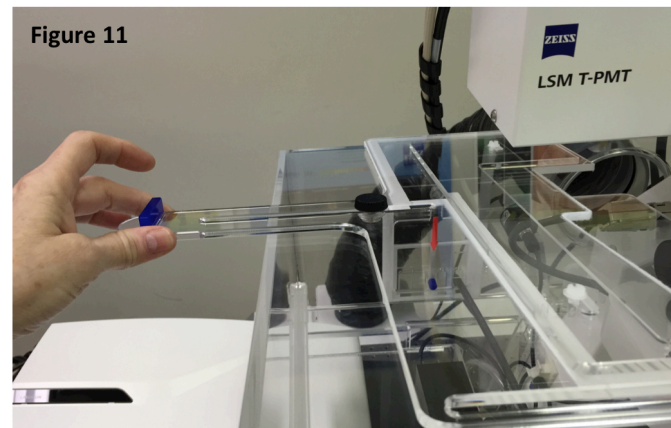
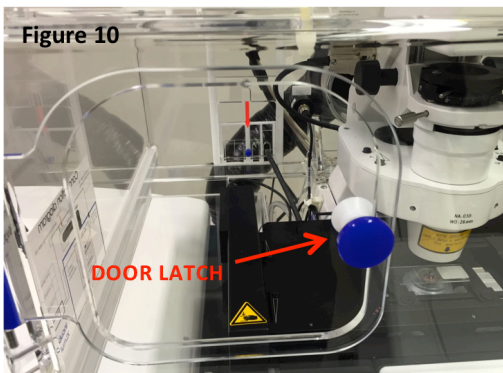
2. Below the area in figure 6, click on Microscope Control to see the visible light configuration. In this area, you may change objectives and filter sets, and change the amount of light coming from the X-cite lamp (figure 7) by opening or closing the shutter (arrow). (You will rarely, if ever need to change this. The default value is 50%..) You may also change the intensity of the quartz halogen lamp (transmitted light) in the upper part of the Microscope Control window, but it is probably easier to do this using the rocker switch on the front of the microscope (red arrow, figure 8).



3. You may also control the microscope using the microscope control box (figure 9), the initial screen will have Home in the upper left corner. Click on the Control tab below that to get the dialog box for the objectives and filters sets.
 - a. Choose your objective. Note: more information on the objectives can be found at the end of this document.
 - i. 5x or 10x (dry)
 - ii. 25x (water immersion)
 - iii. 40x (water immersion)
 - iv. 63x (water immersion)
 - v. 40x (oil immersion)
 - vi. 100x (oil immersion)
 - b. Chose TL for transmitted light illumination (can also be controlled in the software or using buttons on the right side of the microscope).
 - c. For fluorescence, turn off TL Illumination and turn on RL (reflected light) Illumination. Chose your filter set from the Reflector tab.



10. The new microscope has a Plexiglas housing. To load your sample, open the two doors on the front of the housing (figure 10). If you want to tip back the condenser to load your sample, slide the Plexi panes on the top of the housing out away from the microscope (figure 11).



11. The stage on the LSM 880 is totally automated. To move the stage, you must use the joystick (figure 12). It's fairly straightforward.

The F1 button is the only one programmed, switching the speed from fast to slow. The status of the joystick speed is shown on the front of the stage controller box (just to the left of the computer monitor, figure 13). If the slow light is lit (red arrow), the controller is set on slow. If the slow light isn't on, the controller is set on fast. Note: the slow speed is particularly useful for changing your field of view while you are scanning, as you can move the stage in very small, smooth increments with the joystick.

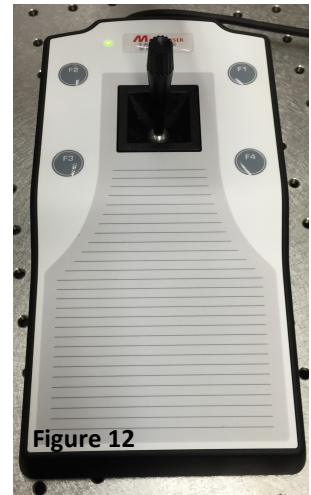


Figure 12



Figure 13

12. The focus knobs are on the right side of the microscope. The larger knob is coarse focus and the smaller knob is fine focus. (There is a coarse focus on the left side of the microscope too.)

Alternatively, while you are on the computer, you can use the remote focus on the right side of the microscope control box (figure 14). The remote focus is an identical control to the focus knobs on the side of the microscope.



Figure 14

Confocal imaging

1. Once you've found your sample through the eyepieces, switch back to Acquisition. There are many configurations stored in the software. To see the list, go to Experiment Manager (at the top left of the Acquisition window) and click on the file folder (arrow, figure 15) and you will get a dropdown menu of the available configurations (figure 16). Chose the configuration by clicking on it. If you are using the argon laser, you will now get a pop-up asking if you want to switch it on. Click yes. If you put it on standby when you first opened the software, it will be ready for use within 1 minute. If you forgot to turn it to standby, you will need to wait 5–7 minutes before it will be ready for imaging.

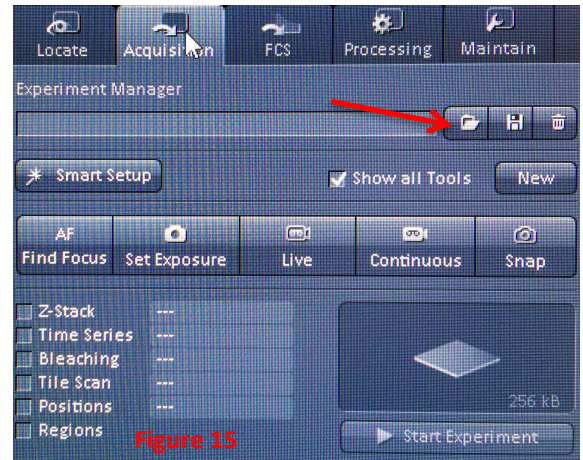


Figure 15

If you need a HeNe laser that you haven't turned on, you will also get a prompt to turn it on, but the helium neon lasers are ready to go when you turn them on.

Note: When you are working with the menus (or, conversely, with an image), you can use Workplace Zoom to make the area of interest larger on the screen. Workplace Zoom (figure 17) is in the upper right of the screen. Use the slider to chose which area to magnify. Click on reset to restore the normal view.

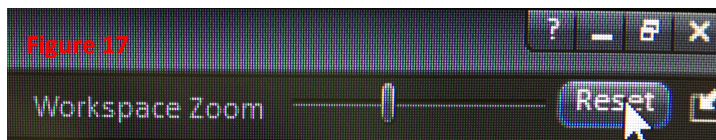


Figure 17

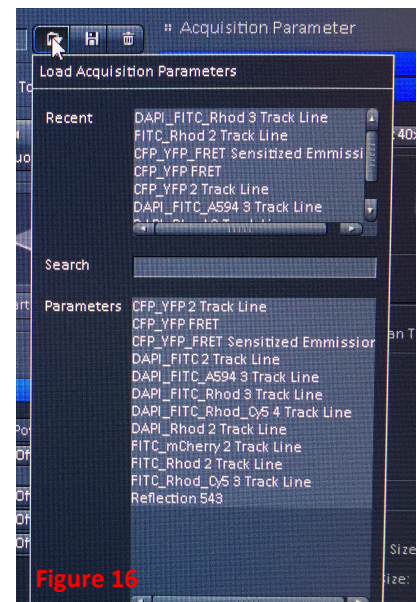


Figure 16

2. Once you chose your configuration, you may choose to turn on the transmitted light detector. At the bottom of the Imaging Setup window, toggle the box next to T-PMT (figure 18). You must turn it on with a visible light channel — it won't work with the 405 nm laser. Note: if you are collecting transmitted light images, there are ways to optimize the images that are not part of the initial confocal training. See Kristi for more details, as the directions are not yet in this manual.

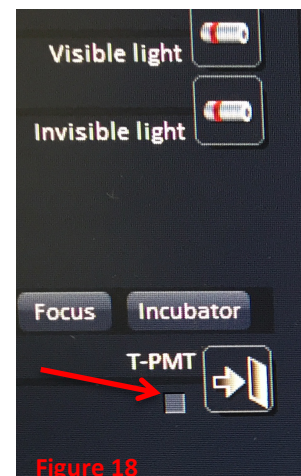


Figure 18

3. The system has a sampling function called Set Exposure (figure 15) in which the system looks at your sample and sets all the parameters. Normally, Set Exposure will be used only when you know nothing about the fluorescence of your sample. It gives you a starting point on the settings, not usually where you will end.
4. There are several features on the 880 that are very helpful for finding and viewing your sample:

- a. In the Scan Area, the system will zoom out to 0.6x to cover a larger area.

- b. Below the image window is a button labeled Crop (figure 19). When you click on the Crop button, you will see a box on the screen. You can move the box and change the size of the box to determine the size and location of the area you will scan. After you place and size the Crop box, click Live (figure 15) and you will now scan the area you have selected.

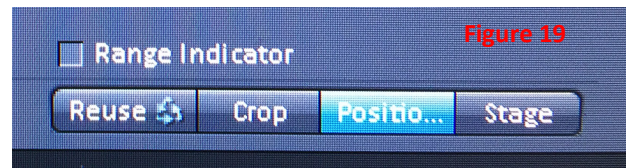


Figure 19

- c. Next to the Crop button is a button labeled Stage (figure 19). Click the Stage button and then click anywhere in the image window, and, when you start scanning, the area you clicked will now be the center of the image. Note: the Crop and Stage functions will not work while you are actively scanning.

- d. There is also a Stage menu (below the Channels window, see figure 20). When you open it, you get a controller that you can use to move the stage in any directions. (For moving the stage small distances, it is easier to use the joystick control set on slow — the movement is smaller.)

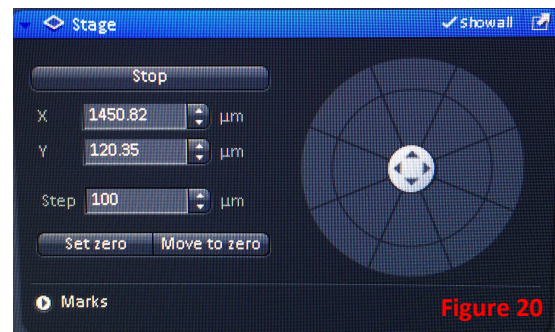


Figure 20

- e. Tiling can also be used as a large area preview. This will be covered later in the manual.

5. Frame size:

- a. The default frame size is 1024 x 1024.

- b. The max on the LSM 880 is 8192 x 8192, but using that frame size would result in huge oversampling for anything on this system.

- c. To set frame size, get your image in the size that you want (e.g., zoomed in or out), then click on Optimal (on the menu near Frame Size). Optimal will give you the optimal sampling for the area that you've defined.

- d. The higher frame size is set, the longer imaging will take. There may times when you may do better with under-sampling and a faster scan speed.

6. Scan speed and averaging:
 - a. Scan speed. While you are optimizing, click on max speed. When you are collecting final images, you can improve image quality by reducing the scan speed.
 - b. Averaging: Averaging scans each line (or frame) from 2–16 times, averages the results at each pixel, and records that value.
 - c. You will need to decide which (reducing scan speed, averaging, or a combination of the two) gives the best image quality for your samples.
7. Other parameters:
 - a. Bidirectional scanning works well on the LSM 880, with no loss of image quality. Bidirectional scanning will cut your scan time by one third to one half.
 - b. Data depth: The default value on the LSM 880 is 12 bit, though 8 or 16 bit are also available. A 12 bit setting will give you 4,096 gray values (8 bit is 256, 16 bit ~50,000).
8. Optimizing your image:

- a. When you are ready to adjust your image, click on Live scanning, then click on the Range Indicator tab (found below the image window, see figure 19). In Range Indicator, red indicates saturated pixels and blue indicates pixels below the limits of detection. Note: make sure that you have separated the channels — do not try to make these adjustments in 2D.
- b. In the Channels window, Master Gain is used to adjust the intensity of the image. You never want the Master Gain to go above **750** (figure 21). If you have the gain set at 750 and the image is still not bright enough, you have several choices:
 - i. Increase the Digital Gain. The signal to noise ratio on the 880 is good enough that you can raise the digital signal without increasing the background.
 - ii. Increase the laser intensity. The default laser settings in the configurations range from 1–5%. For the 405 and 488 nm lasers, raising the intensity to 2–5% is reasonable. For the weaker 543 nm HeNe laser, it is not uncommon to image at 40–50% laser power.
 - iii. Increase the slice thickness a little by enlarging the pinhole. (This would be the last thing to try.) A thicker slice will be brighter, and a few tenths of a micron in thickness will not make a big difference experimentally.

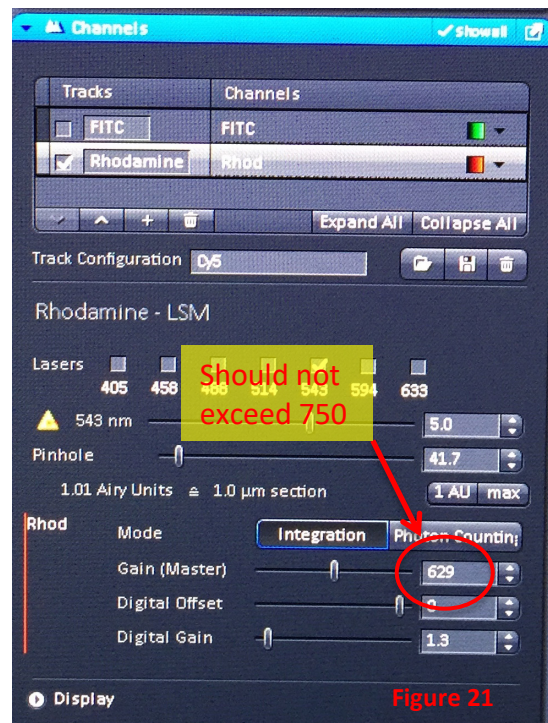


Figure 21

- c. Digital Offset determines the background of the image. In range indicator, blue pixels are below the limits of detection. Digital offset will always be set toward the right edge of the slider, at a value close to zero. Try to match the background intensities of your different channels. Some blue pixels are fine, but you do not want a solid blue background.
 - d. If you use the 1 AU setting in a multichannel configuration, set the longest wavelength to 1 AU. Note: a 1 AU (airy unit) slice is an “optimal” slice thickness for the particular objective lens that you are using.
 - e. Note that this system has only a single pinhole in the light path (even though there is a pinhole setting for each color in the Channels window). The configurations will automatically have matching slice thicknesses. (Do not worry about 0.1 μm differences in slice thicknesses.)
9. Containers: To view images side-by-side, we have a container option.
- a. Above the image window, right-click on the gray bar. You’ll get a pulldown menu that gives you the choice of 1, 2, or 3 containers. Click on 2 or 3 containers (or 1, if you want to get rid of containers).
 - b. Now you can pull an image over to the new container and view images side-by-side.
10. Images are saved in a Zeiss proprietary format, .czi (Carl Zeiss Image). Every user will have a folder in the Images folder (D drive) where they should save all of their images. I suggest that you put each day’s images in a folder with the day’s date so it’s easy to transfer your images to external media.

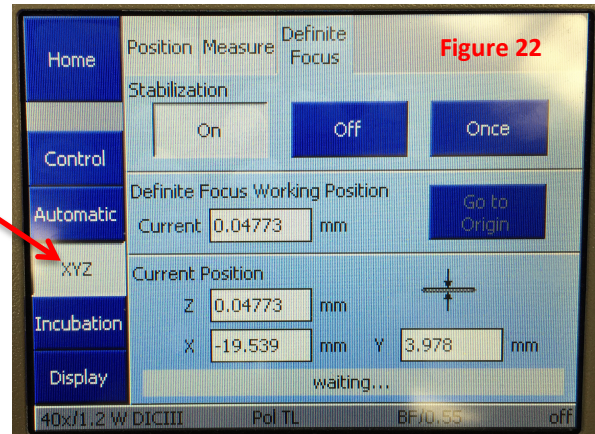
Remember, your images on the confocal computer are not protected and not backed up. You are responsible for your own images, and users are strongly encouraged to take each day’s work with them when they finish. You may leave images on the computer as well, but within reason (i.e., not tens of gigabytes).

Other features

Definite Focus

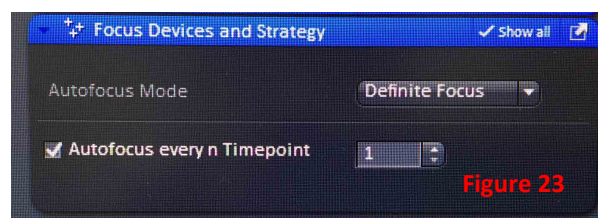
The LSM 880 is equipped with **Definite Focus**. Definite focus allows the system to make automatic adjustment for focal drift and is used for time lapse imaging.

1. To access Definite Focus, click on XYZ on the microscope control box (figure 22).
2. Click On under Stabilization.
3. The status bar at the bottom of the screen will say Waiting until it acquires focus. If it cannot initialize on your sample, you will see an error message at the bottom of the screen. There will also be an error message on the Definite Focus box (behind the control box on the left side of the microscope). If this happens, just turn Stabilization off (on the microscope control box, figure 22).
4. After the system initializes, it will alternate between Waiting and Setting focus (every 3 seconds).
5. Notes:
 - a. Definite Focus works best on samples with a clear difference in refractive index between the coverslip and the slide. It will not work as well on very flat samples, such as fibroblasts on a coverslip.
 - b. Definite Focus works by projecting crosshairs onto your sample. It uses IR, so it will not interfere with imaging.
 - c. You can also control Definite Focus in the software under Focus Devices and Strategy.



Using Definite Focus in Time Lapse

1. After you set up your time lapse settings, open Focus Devices and Strategy (figure 23). Under the Autofocus Mode, select Definite Focus.
2. Next to Autofocus every n Timepoint, choose how frequently you want the system to autofocus. For example, if you are collecting images minutes apart, you might choose to have it focus immediately before each timepoint (select n=1). If you are collecting images more frequently, you might choose to set to a larger number, so it doesn't autofocus before each image.



Tiling

Tiling is used to image a large area of your sample. The system collects a number of tiles (usually around a center tile), and the subsequent images (tiles) are combined using stitching to create one large image. The number of tiles possible depends on the objective lens that you are using. It is also possible to combine tiling with other functions, such as z-stack and time lapse.

1. In the Experiment Manager window (figure 15), click on Tile Scan. This will open a new menu window under Multidimensional Acquisition (figure 24).
2. After you have optimized your image, decide if you want that image to be the center of the tiled area. (You could also make it one edge of the tile using Bounding grid or one end of the scan using Convex hull; see Zeiss LSM 880 manual.) If you want it to be the center, select Centered grid.
3. Next, select the number of tiles, for example, 3 x 3 or 5 x 8. (If you exceed the maximum number of tiles available, the gridded area in the Tile Scan window will become an open box.)
4. Set overlap at 10%. That insures that you will be able to stitch the tiles together correctly.
5. Click Start Experiment in the Experiment Manager window to start the tile scan. Note: if image has horizontal lines, do not use bidirectional scanning.
6. To stitch the tiles together, go to the Processing tab (to the right of the Acquisition tab).
 - a. Under ICS, select Stitch (figure 25).
 - b. Set Correlation threshold to Medium.
 - c. Click Select to choose your image. The most recent image should open below in the preview window. Select it.
 - d. Click Apply (at the top of the window) and the image will be stitched together.



Figure 24

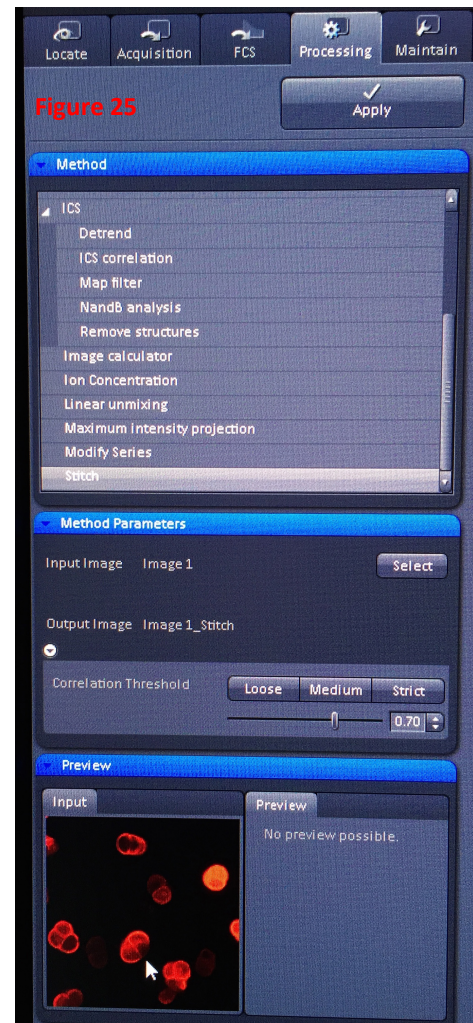
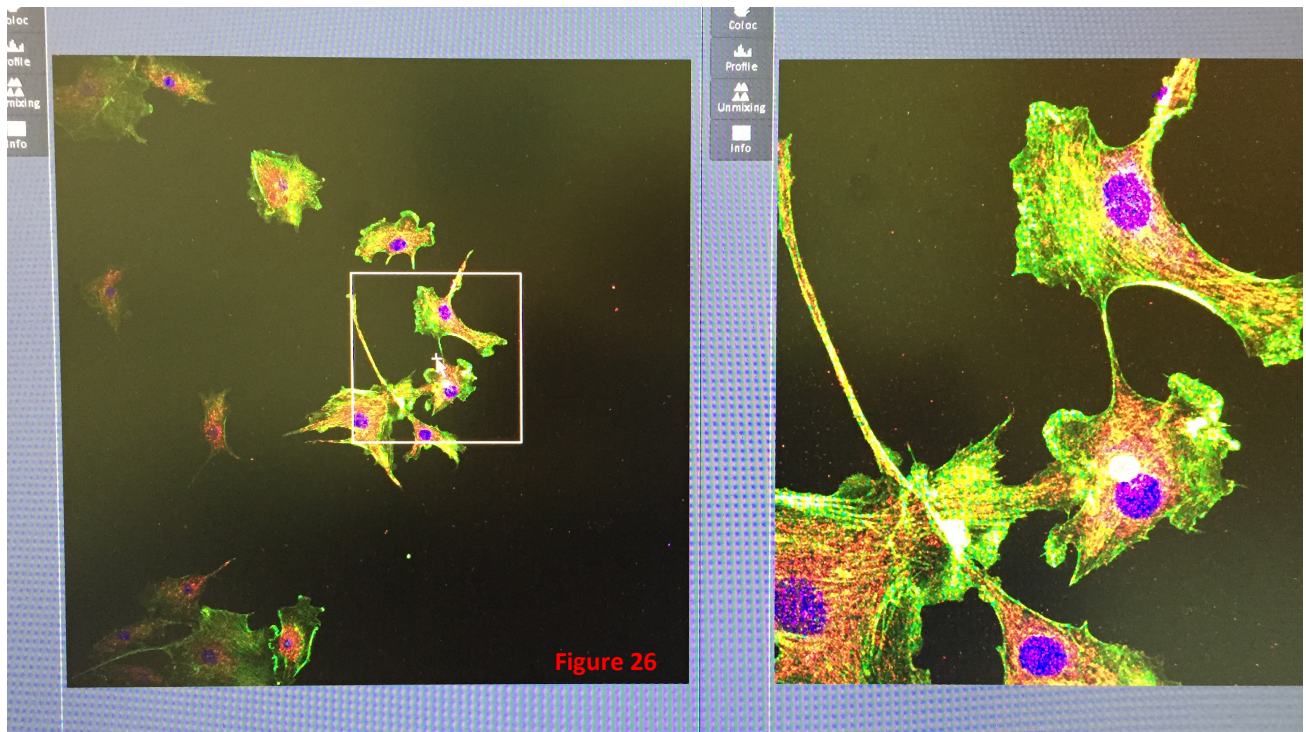


Figure 25

Using tiling for sample preview

You can do a rapid tile scan of a large area, then use Stage to choose areas to look at under normal scanning. In other words, if you do a rapid, large area tile scan, you can use that image as a preview, then scan in and highlight particular areas. So if you're looking for something like one transfected cell in a field of cells, you can tile a large area, then go back and use the tiled image to find the cell you want to image.

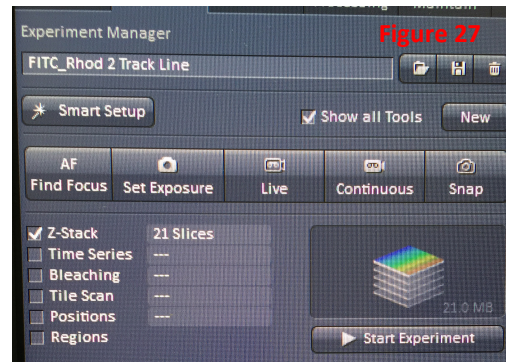
1. Chose an area of interest and optimize as normal.
2. Set up tiling for the larger area you want to examine.
3. Set all of your options for fast scanning. For example, drop the frame size down to 128 x 128, turn on bidirectional scanning, etc.
4. Perform the tile scan. (The example in the left container in figure 26 took 10 seconds to do the entire tile scan.)
5. Open a second container and set your parameters for normal scanning (for example, resetting frame size to 1024 x 1024, slower scan speed, unidirectional scanning, etc.)
6. Click on the Stage button (below the image window), highlight your new container (to make it the active image window, and click Live to start scanning. Note: alternatively, you could use Continuous scanning for a high quality image in the right container.
7. As you move the Stage box around your original image (the tiled image), you will see the area within the box in the right container (figure 26).



Z-stack

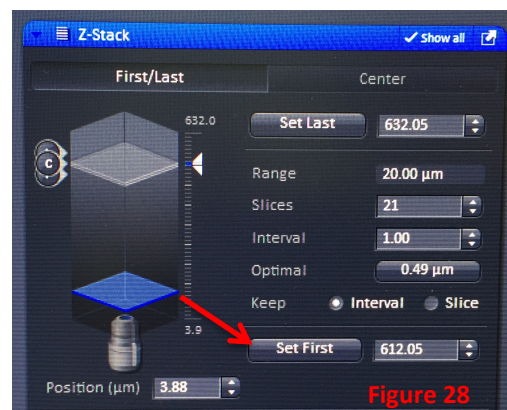
Z-stacking, or collecting a series of slices in z-axis, provides data for 3D sample analysis. Z-stack can be combined with other features, such as tiling, time series, or positions.

1. Optimize your image as usual, except you must think 3D. Focus up and down on the area to be scanned to be sure you've chosen the correct slice for optimization. (If you optimize on a lightly stained slice, you may find that other slices in your z-stack are oversaturated and useless.
2. Choose Z-stack in the Experiment Manager window (figure 27) and click on Live to start fast scanning.



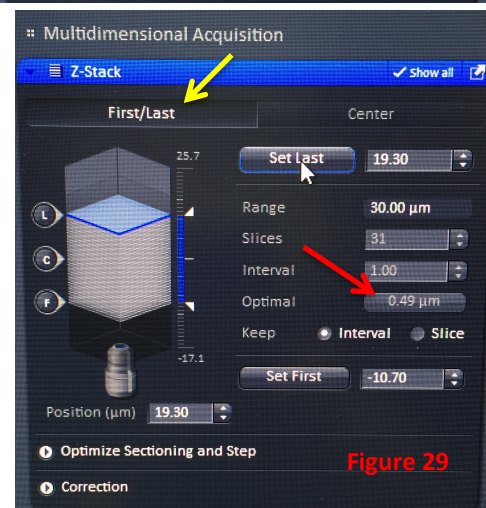
3. Focus to the bottom of your sample.
Note: the "bottom" will be the area closest to the coverslip. Turn the microscope focus knob counterclockwise to lower the objective and view the "bottom" of the sample.

4. When you are at the bottom, go to the Z-stack window under Multidimensional Analysis (note: this window is not visible unless Z-stack is toggled in Experiment Manager) and click on Set First (see arrow, figure 28).



5. Focus to the top of the sample (turning focus knob clockwise) and click Set Last.
6. Stop scanning.

7. Click on the button next to Optimal in the Z-stack window (arrow, figure 29). That will set the number of slices needed to capture the distance you have defined. Note that number of slices will be more than expected for the distance on the z-axis. That is because the slices overlap by about 50% so that data at the interface of the slices will not be lost.



8. Go to the Experiment Manager window and click Start Experiment.

These directions are for the First/Last mode in Z-stack (yellow arrow, figure 29). Alternatively, z-stacks can be collected in Center mode. In Center mode, you select the center slice, set Optimal, then add slices to cover the z-range that you want to image.

Time lapse

Time lapse images can be collected over minutes to hours. Time lapse can be combined with other features, such as tiling or z-stack. Definite Focus is an added feature (see above) to manage any sample drift during a time lapse experiment.

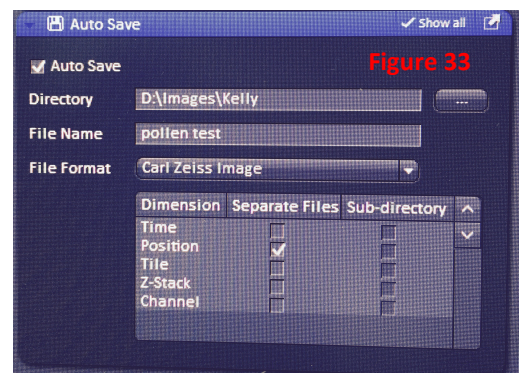
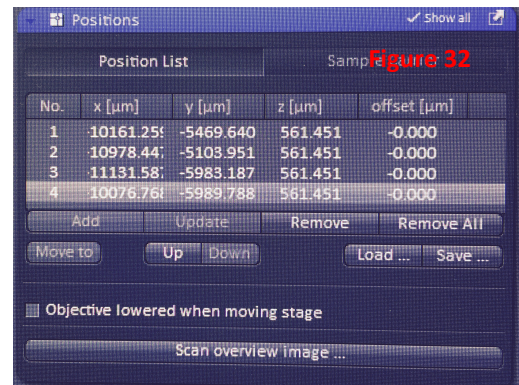
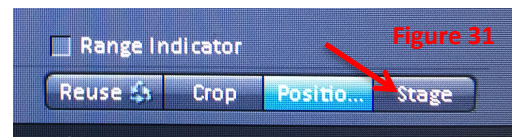
See Zeiss LSM 880 manual for more details on time lapse imaging parameters.

Positions

Multiple positions can be defined using Positions. This function is particularly useful for imaging replicates, and can be combined with other functions such as z-stack and time lapse.

1. Perform a tile scan preview as described above.
2. Click on Positions in the Experiment Manager window (figure 30).
3. Click on the Stage button (below the image window, see figure 31). Place the stage box in the first area that you want to image and add the position by clicking Add in the Positions window (figure 32). Note: this window will not be available until you toggle Positions in the Experiment Manager window.
4. Continue to add positions by moving the stage box and clicking Add.
5. Before you start the experiment, turn on Auto Save (figure 33).
 - a. Toggle Auto Save
 - b. Choose your directory and give the files a name
 - c. Toggle Position in the box at the bottom of the window.

Doing this will save each position as a separate image file. If you do not do use Auto Save, all positions will be saved in a single .czi file.
6. Click on Start Experiment in the Experiment Manager window.



Temperature control

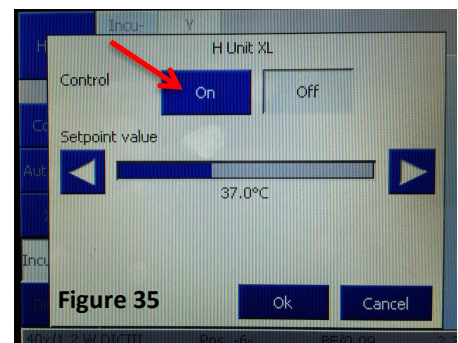
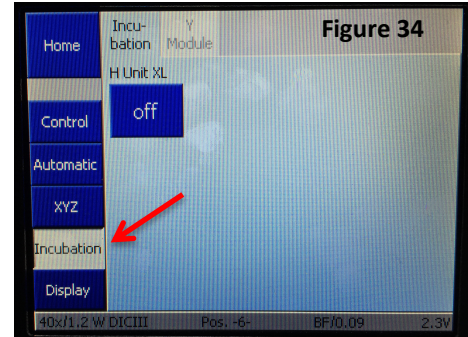
If you want to use temperature control, you need to turn on the heat at least two hours before you begin imaging. (Note: it takes 2 h for 37°C. If you don't need it that warm, then you don't need to pre-warm quite as long.) The pre-warming period lets the entire system to get to the correct temperature, which includes the microscope components.

To preheat the system:

1. Turn on the system as usual (Main Switch, Systems/PC, and Components). You do not need to turn on the X-cite lamp or open the Zen software.
2. Click on Incubation (or Inkubation) on the microscope control. Touch On (red arrow, figure 35). This turns on the fan only. It does not turn on the heat.
3. Select Control to open a window to set the temperature (figure 35). The default setting is 37°C.
4. On the right side of the microscope table, there is a power strip on top of the real-time computer. To turn on the heat, turn on the power strip (figure 36). There is only a single switch on the power strip.
5. Be sure that all of the doors on the Plexiglas enclosure are closed and let the system equilibrate for 2 hours before imaging.

When you are finished imaging, turn off the heat, but you **must** allow the fan to continue running for 20–30 minutes. To shut down the heating system when you are finished imaging:

1. Turn off the heat by switching off the power strip (to the right of the microscope table, figure 36).
2. After 20–30 minutes, turn off the fan in the Incubation tab on the microscope controller. Under H Unit XL Control, select Off (figure 35). Note: during the 20 minute cool down, you can turn off the X-cite bulb and exit the software, but do not complete the shutdown until after the cool-down period.



Shut down

As always, check the online calendar before beginning shut down. The shutdown procedure is different if there is another user coming ≤ 1 h after you.

No user coming after you

1. When you are finished imaging, turn off the X-cite lamp. (This can be done at any time. It does not have to be before you turn off the lasers, etc.)
2. Turn off the lasers in the software.
3. Exit the software. **Wait 2 minutes, then log out of your user ID.** Leave the computer on.
4. If you used the argon laser, you must wait for the fan that cools the argon laser to shut down. The fan housing is the black box right behind the system control box (see * in figure).
5. Once the argon laser cooling fan has stopped running, turn off Components, Systems/PC, and the Main Switch (in that order).
6. For the microscope itself, clean lenses as always. Please be careful not to get oil anywhere it doesn't belong. Also, always remember that the stage is fully motorized and cannot be moved manually.
7. After you have removed your sample and cleaned the objective lens, close all the doors in the Plexiglas housing, including the sliding panels on the top.
8. Sign out on the clipboard.



User coming after you in 1 hour or less

1. Exit the software, leaving the lasers on. **Wait 2 minutes, then log out of your user ID.**
2. For the microscope itself, clean lenses as always. Please be careful not to get oil anywhere it doesn't belong. Also, always remember that the stage is fully motorized and cannot be moved manually.
3. After you have removed your sample and cleaned the objective lens, close all the doors in the Plexiglas housing, including the sliding panels on the top.
4. Sign out on the clipboard.

Troubleshooting

- If things start acting oddly in the software (or if you really mess things up and don't know how to fix it), the easiest first step is to restart the software. This will fix most problems.
 - Leave the lasers on. They will automatically go to standby.
 - Exit the software. You will get a pop-up asking if you want to save any unsaved images. The second pop-up will warn you that the lasers are still on. Click okay.
 - Wait 2 minutes, then restart the software. You will need to reload your configuration. If you are using the argon laser, you will get a pop-up asking if you want to turn it on. Click okay.
 - Usually, your problem will be gone.
- Beyond this, call Kristi for assistance. If it is after hours or if she is not there, call the cell phone number written on the white board.
- Power outages:** The system has its own uninterruptible power supply. If the power goes out in the building, you should have 15-30 minutes from the UPS in which to shut down the confocal following the normal procedures. So, if this happens (a very rare occurrence), save your images and shut down everything normally. Do not restart until the building power is on and stable (not flickering).

Note: the computer is on its own UPS that should give at least 15 minutes of power. Don't waste time if there are power issues — shut off lasers, save data, and shut the instrument down.

Objectives

Name	Magnification	Immersion	Working distance	N.A.
Fluar 5x/0.25*	5x	None	12.5 mm	0.25
Plan-Apochromat 10x/0.45*	10x	none	2 mm	0.45
LD LCI Plan-Apochromat 25x/0.8 Imm Corr DIC	25x	water, oil, or glycerine	570 μ m	0.8
C-Apochromat 40x/1.2 W	40x	water	280 μ m	1.2
LD C-Apochromat 63x/1.15 W	63x	water	600 μ m	1.15
Plan-Apochromat 63x/1.40 Oil DIC f/ELYRA	63x	Oil	190 μ m	1.40
Alpha Plan-Apochromat 100x/1.46 Oil DIC	100x	Oil	110 μ m	1.46

* Either the 5x or the 10x objective will be on the microscope, never both at the same time.