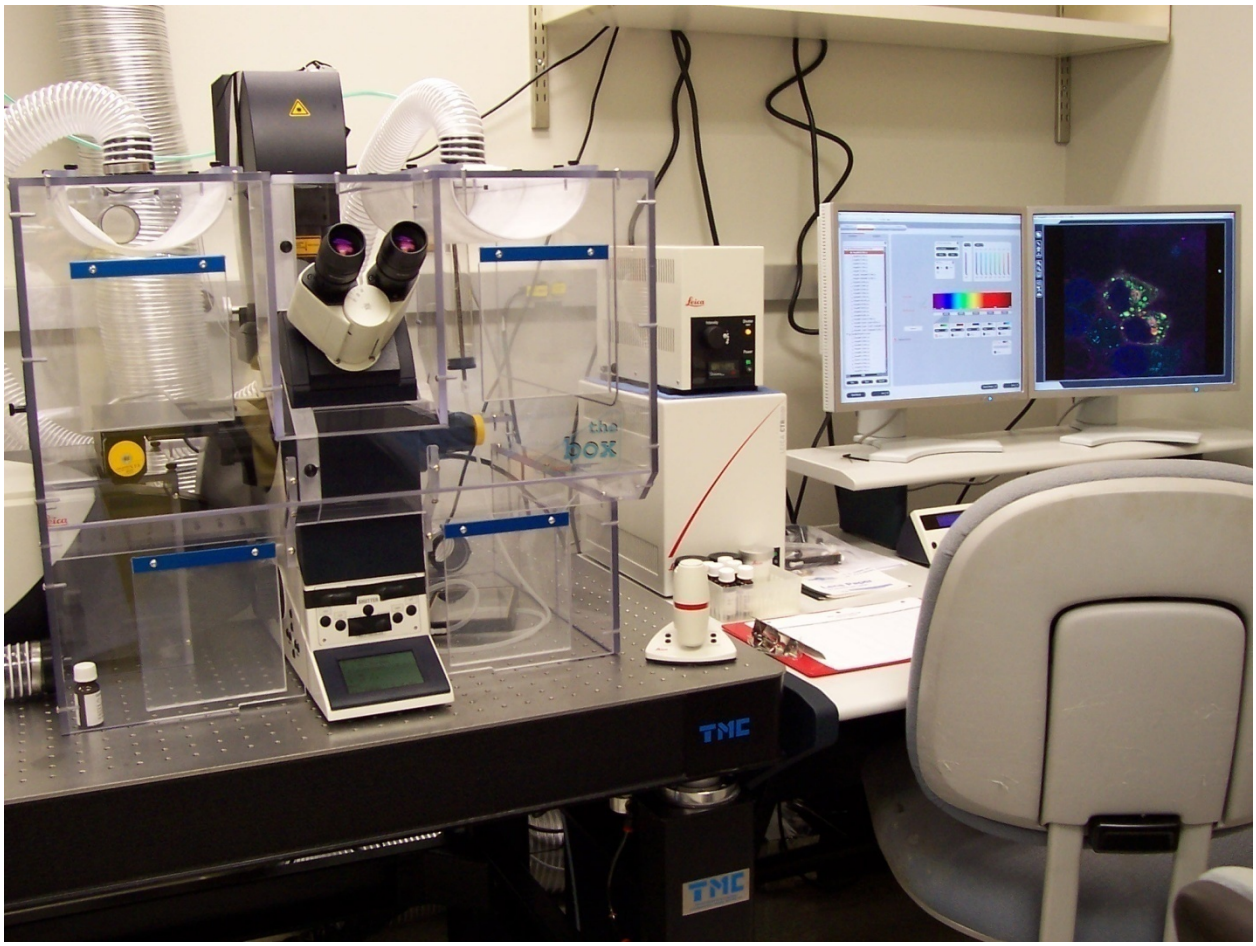
Leica TCS SP5 Confocal Microscope

Quick Start User Guide



LSU Health Sciences

Research Core Facility

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1. START UP THE SYSTEM

Turn on the mercury lamp power source. The green power light and the yellow shutter light will come on. The first and second intensity levels are sufficient.

Once turned on, the lamp should remain on for a minimum of 30 minutes. Do not turn the lamp off if another user is scheduled within 2 hours after your session.

Switch on the buttons/key in the following order:

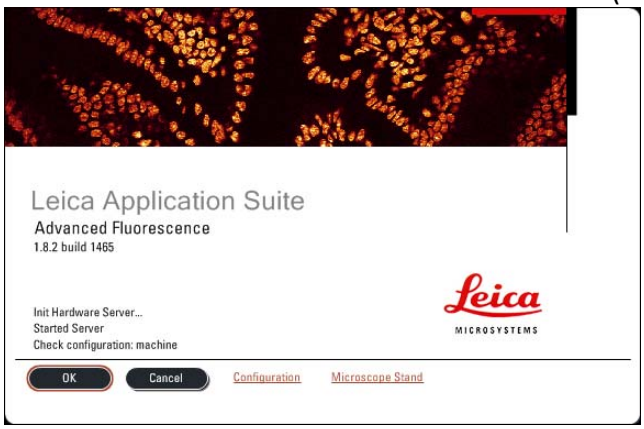
1. PC/Microscope
2. Scanner Power
3. Laser Power
4. Turn the laser key clockwise from OFF to ON

Wait for the login screen to appear and log in with your LSUHSC ID and password. Make sure the domain is set to LSUMC-MASTER, selected from the drop-down menu.

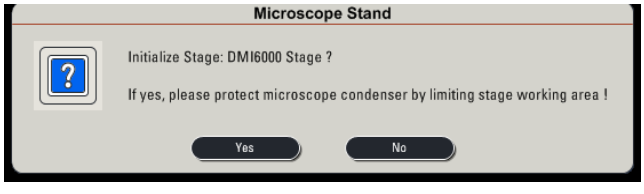
2. START UP THE LAS AF SOFTWARE

Double-click on the LAS AF icon to start the program.

Click OK and wait for the hardware to initialize (About 2 minutes)



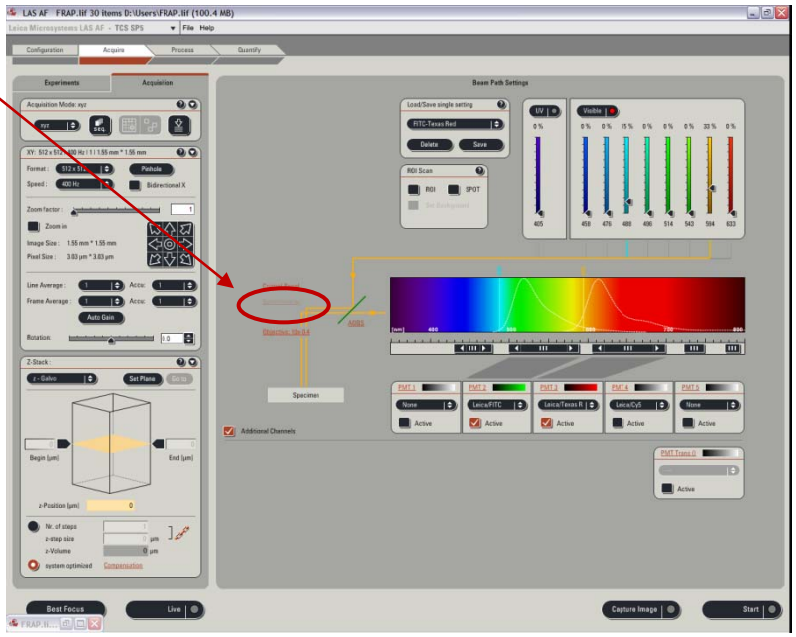
After the Microscope Stand window opens, click No.



The main window will then open.

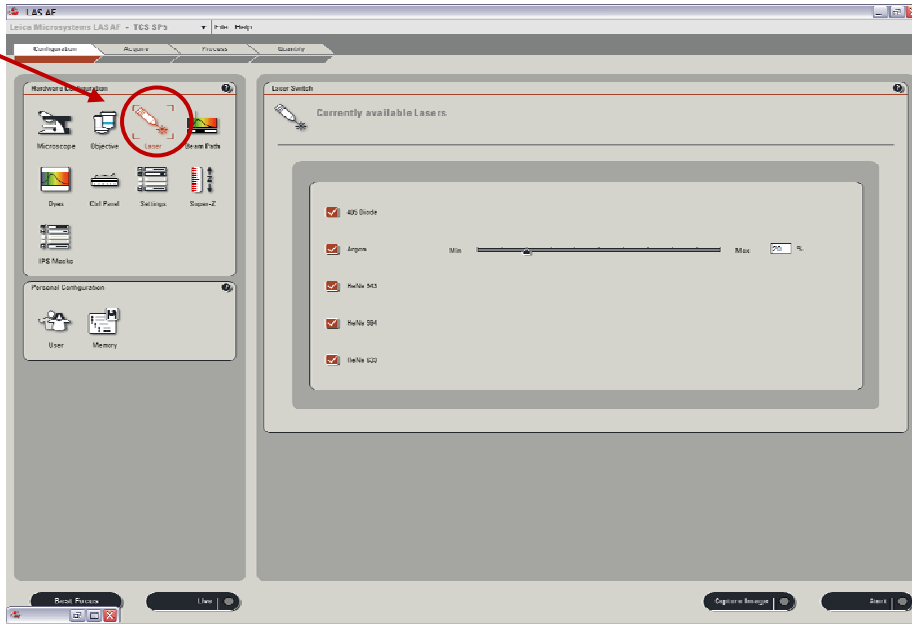
2.1 Chose an objective

Click on the current loaded objective in the main window and select the objective you prefer from the pull-down menu. The objective turret will automatically lower and swing the chosen objective into the light path. It would be a good idea to read more about the objective you are working with, and that information can be accessed by clicking on **objective** in the **configuration** window.



2.2 Turn on lasers

Turn on the lasers by first selecting the laser icon in the configuration window. Tick the lasers you need. If you are using the argon laser, drag the slider to 20%.

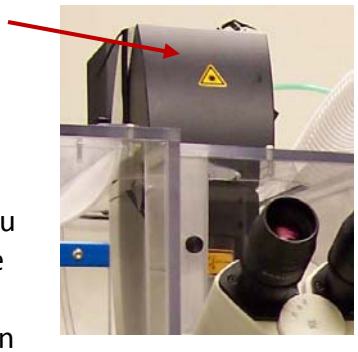


3. SAFETY

- Do not look into the eyepieces during the scan operation.
- Do not look into the eyepieces while switching the beam path of the microscope.
- Never look directly at the laser beam or a reflection of the laser beam.
- Do not introduce any reflective objects into the laser path.
- Never change a specimen during scanning.
- Do not change objectives during scanning.
- Make sure all positions on the objective turret are occupied or closed with a protective cap.
- Do not change any filter cubes during scanning.

4. MOUNT AND VIEW THE SAMPLE THROUGH THE MICROSCOPE

Gently tip the transmitted light arm backwards for easier access to the stage.

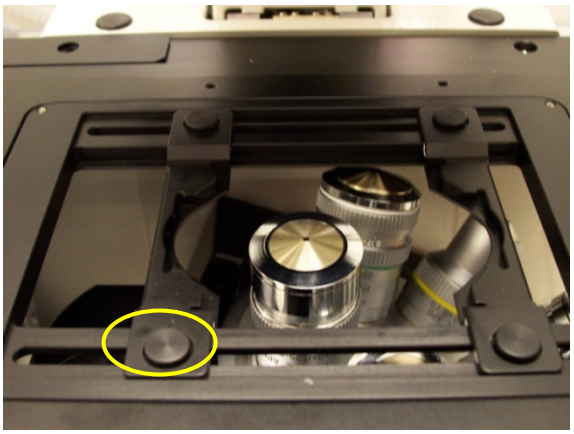


If you are using an immersion objective, place a VERY small drop of the appropriate immersion fluid on the objective face, or the coverslip. Be sure to only use the immersion fluids located next the microscope. If you accidentally use the wrong fluid, please contact staff right away. For the 20x IMM, the default fluid is oil – ask staff for assistance to use other media. If you are using a slide, insert it coverslip down into the specimen stage template.

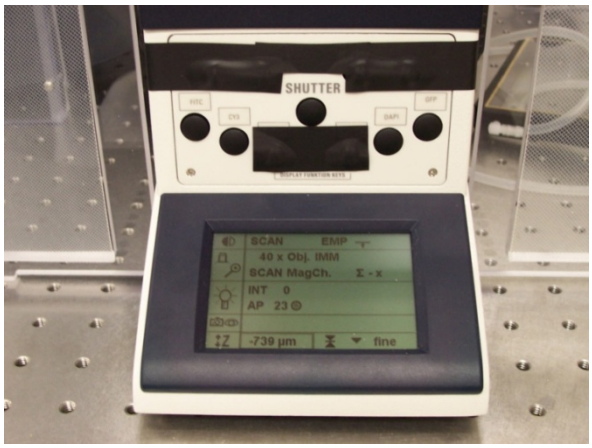
Objective	Imersion media	DIC Compatible?
Plan neoflar 2.5x/0.07	air	No
Plan Apo 10x/0.40 CS	air	Yes
Plan Apo 20x/0.70 CS Immersion correction	Multi-immersion (oil, glycerol, water)	Yes
Plan Apo 40x/1.25-0.75 oil	oil (type F)	Yes
Plan Apo 40x/0.75 U-V-I	air	No
Plan Apo 63x/1.4-0.6 oil	oil (type F)	Yes
Plan Apo 63x/1.30 Glycerol Immersion 21^{o}C	glycerol (type G)	Yes
Plan Apo 100x/1.40-0.70 oil	oil (type F)	Yes
Plan Apo 100x/1.46 oil temperature correction	oil (type F)	Yes

The 4 buttons on the template move the support brackets along the track. The template will hold a variety of shapes. If you need a multi-well plate template, please ask staff.

After your sample is loaded, gently return the transmitted light arm to the down position.



To illuminate your sample with fluorescent light, press the appropriate filter button and then the shutter button on the front panel of the microscope. Pressing the shutter button once, opens it, and again, closes it.

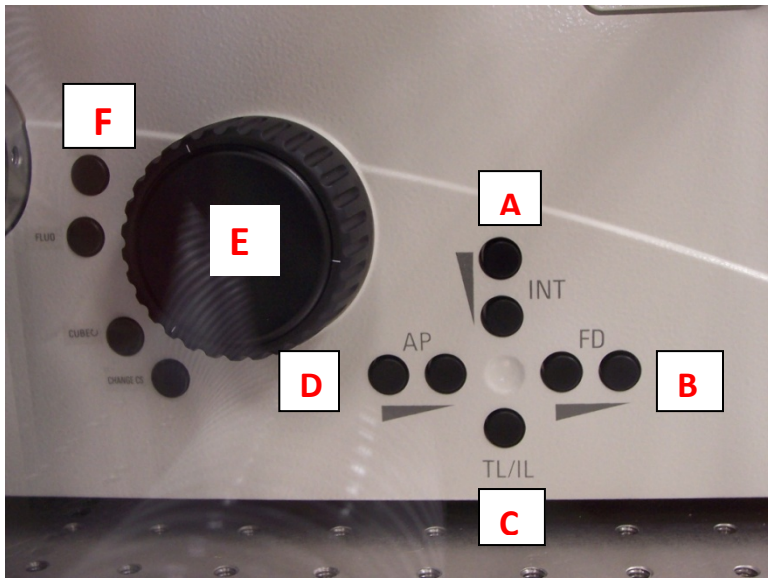


Filter choices are labeled as:

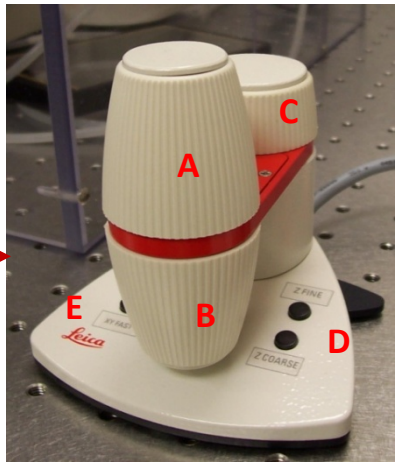
FITC (EX 450-490, EM LP 515) – for FITC, Alexa 488, Cy2
Cy3 (EX 515-560, EM LP 590) – for Alexa 543, Cy3
DAPI (EX 340-380, EM LP 425) – for DAPI, Hoerchst, AMCA
GFP (EX 450-490, EM 500-550) – for GFP, and prevents red emission bleed through

To illuminate your sample with brightfield light, press the buttons on the lower left side of the microscope.

A= intensity adjustment (pressing both intensity buttons at the same time toggles between coarse and fine adjustment)
B= Field iris diaphragm
C= Switch between transmitted light and incident light (fluorescence)
D= condenser aperture diaphragm
E= Focus knob
F= Switches among BF/DIC/Polarized



To focus and move your specimen on the stage, use the axis controller unit.



A Move along y axis (clockwise = front to back)

B Move along x axis (clockwise = left to right)

C Focus -move along z axis (clockwise = objective up)

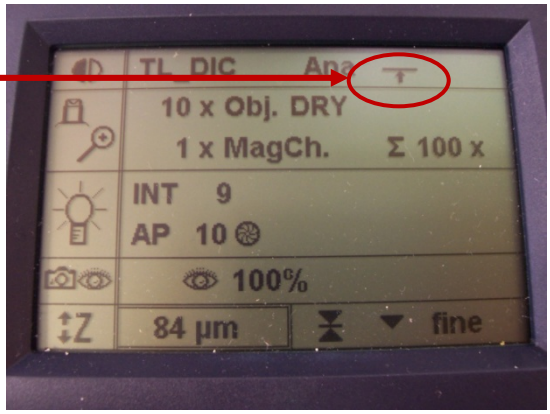
D Select between coarse and fine focus

E Select between fast and slow xy

Having trouble focusing on your sample with an immersion objective? Try this:

- Illuminate your sample with brightfield light
- Tilt the transmitted light arm back slightly so you can see your sample
- Set the focus to coarse and raise the objective just until the immersion fluid makes contact between the sample and the objective
- Return focus control to fine and lower the transmitted light arm

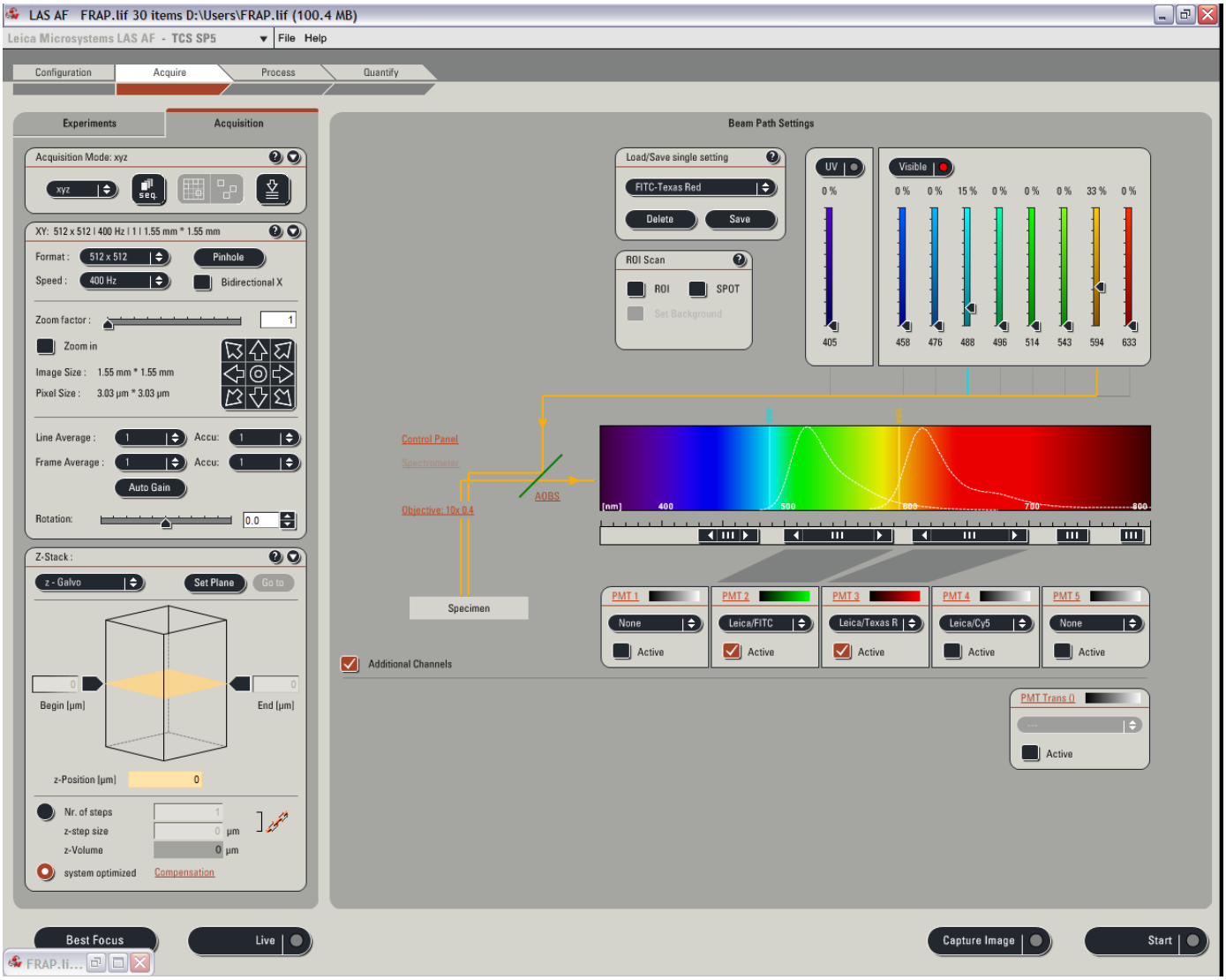
The current button positions are displayed on the **microscope screen.**



In this setup, the light path TL_DIC is transmitted light with DIC,
the fluorescent shutter is **closed**
The objective is a 10x.
The intensity of the halogen bulb is 9.
The field aperture is set at 10.
100% of light is directed to the eyepieces.
The focus knob is set to fine instead of coarse.

5. INSTRUMENT SETTINGS

Click back on Acquire. The main Acquire panel will open.



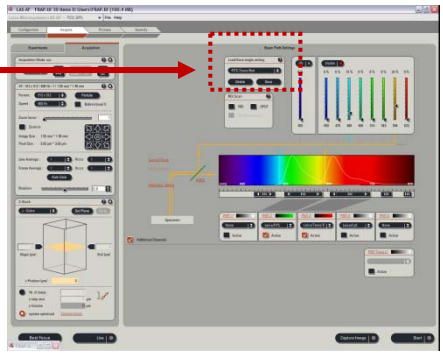
The right panel controls the lasers and the light path. The settings above are for a simultaneous scan of FITC/Texas Red, with the argon laser on at 15% to excite FITC and the FITC emission going to PMT2, and the 594 HeNe laser on at 33% to excite Texas Red and the emission going to PMT3.

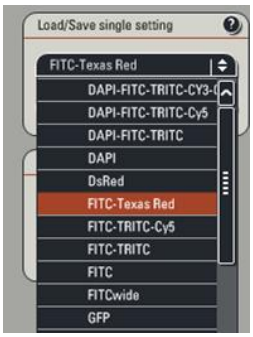
The left panel controls image acquisition parameters, and will usually open with certain default settings:

Acquisition as xyz	also possible: xzy ,xt, xyt, xyzt, xzyt, xyλ, xzλ, xyλt, xyλz, xyzλt
Format or pixel number at 512 x 512	more pixels = increased resolution, but slower acquisition, more photobleaching
Speed at 400HZ	slower speeds = less noise and improved image quality
Zoom at 1	zoom should be used to match pixel size with Nyquist sampling
Averaging at 1	more aver. = improved image quality, slower acquisition and more photobleaching
Accumulation at 1	used at >1 when signal is weak.
Rotation at 0	rotation at +90 will match the image to what you see through the eyepieces
Pinhole at 1 airy unit	values > 1 increase signal at the expense of increased section thickness and more out-of-focus light; values < 1 may increase resolution, but significantly reduce signal

5.1 Choose a setting

From the Acquire panel, select the correct settings for your fluorophores from the pull down menu. In the **Leica Settings** group, you can select predefined settings. In the **User Settings group**, you can select user-defined settings you have saved earlier.





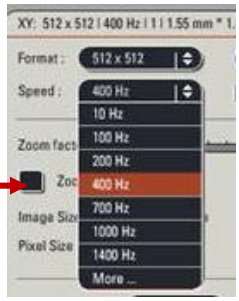
You can modify a predefined setting and save it with a new name, and these settings will only become accessible by you in the User Settings Group. All settings in these menus are simultaneous scans.

Staff can help you configure a setting for your experiment that can be saved and reused.

*Note: For multi-labeled experiments, sequential scanning settings are a better choice. The lasers and PMTs are turned on sequentially as you scan different fluorophores, and this significantly reduces artifacts from cross-excitation and from bleed- through of emission into other PMTs. Staff can walk you through creating a sequential method.

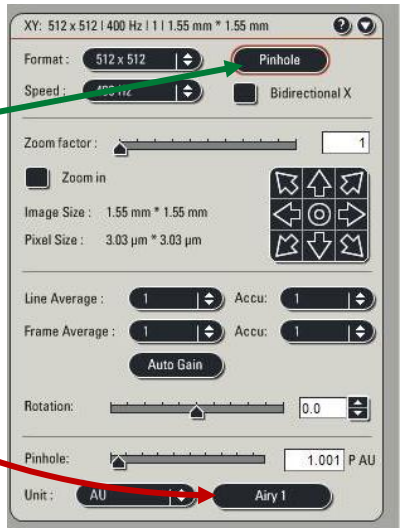
5.2 Set scan speed

Have acquisition mode at XYZ while you are setting up scan parameters. Select 400Hz from the Speed drop down menu. This is a medium speed which will give you good quality images . 700Hz will give you a noisy image, but allow for faster setting of detectors with less bleaching. Quality images are usually collected at slow speeds, 100 to 200Hz.



5.3 Set pinhole

The pinhole determines the thickness of the optical slice (the z plane section thickness that the PMTs collect signal from). It also affects image resolution.
The optimal size of the pinhole is determined by the objective's numerical aperture and the wavelength of light you are acquiring. The software does the math, and all you need to do is click the **Pinhole button** and select **Airy 1.**



6. ADJUST DETECTOR SETTINGS

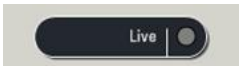
6.1 Set up the display screen

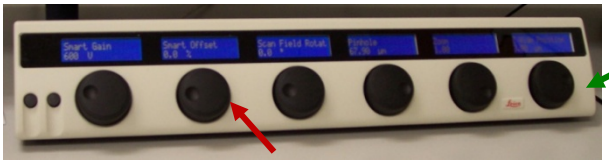
In the display, a pane is set aside for each channel selected in the scan configuration. To show the merged image, click on the **merge** icon.

The pseudocolors of each channel correspond to colors selected in the scan configuration. To adjust the detector settings correctly, change the colors to the range set LUT by clicking the **LUT button** until it cycles to the range set LUT. This LUT will reveal saturated pixels as bright blue and black pixels will appear green (LUT refers to color lookup table). This will allow you to collect image data from the full dynamic range of your sample.

6.2 Adjust offset

Click the Live button which scans continuously. This button also becomes the Stop button.





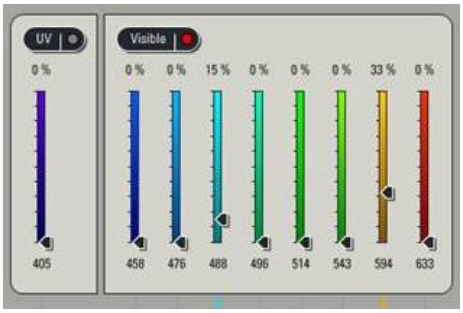
Click inside image pane of channel 1 to activate it. While scanning, adjust the **offset dial** until there are a few green pixels in the background of channel 1. Repeat for the other channels.

Adjust the focus with the **z level dial** until you have the focal plane of interest.

6.3 Adjust Intensity

Adjust image intensity until there are very few blue, saturated pixels in each channel. This can be controlled in two ways, by increasing laser power or increasing detector gain, and the settings for each are determined empirically while scanning. Increase the **laser power** by clicking and dragging the **sliders.** Increase gain with the **smart gain dial**. Turning up the laser power will increase photobleaching, and turning up the gain may result in a noisier image. An optimal gain setting is usually around 600-800V. Generally, you should set your gain in this range and reduce laser power to remove saturated pixels

Smart gain dial

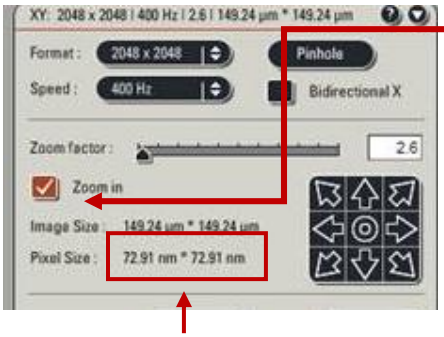


Laser sliders

6.4 Adjust pixel size

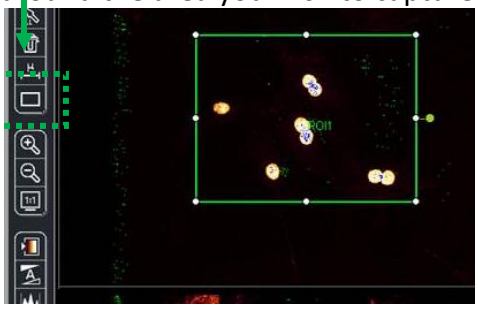
Pixel size is very important for successful imaging. Pixels smaller than optimal result in oversampling, more photobleaching, and longer scan times. Pixels larger than optimal produce images with reduced spatial resolution. High resolution may not be important to you – you decide. Remember that more pixels means a larger image file. Optimal pixel size should conform to the Nyquist sampling rule for digital imaging, which requires 2.7 pixels across the smallest resolvable object. This varies with objective numerical aperture and wavelength. Pixel size is managed by the format and zoom features.

First, crop the image to include only the desired field of view:



Pixel size shown here.
The zoom factor and pixel size will be automatically updated.

Do a Live scan, then Stop. Select **Zoom in** from the XY panel, then select the **rectangular region of interest (ROI) tool** and drag the pointer around the area you wish to capture.



You can resize and reposition the ROI , if needed. Do a Live scan to determine if the ROI is correct, then stop the scan.

Now adjust the **format size** to reduce the pixel size. Increasing the number of pixels in a defined area (the ROI) decreases the pixel size. If you select **More** from the bottom of the format window, you can design your own pixel format.

Examples of appropriate pixel sizes:

Objective	Resolution in xy with 488 nm laser	Pixel size (nm)
20x plan apo IMM	279nm	103 nm
100x plan apo oil 1.46NA	134nm	49 nm

*Note: Objective resolution declines with increasing laser wavelength. With multichannel imaging, pixel size will need to be a compromise.

6.5 Using averaging or accumulation

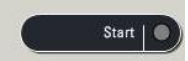
You can improve your image signal-to- noise ratio by averaging repeat scans together, either by line or by frame. For weak signals, repeat scans can be added together to accumulate signal.

With Auto Gain, the software will do a test scan to determine an appropriate gain setting for you.

7. ACQUIRING AND SAVING AN EXPERIMENT

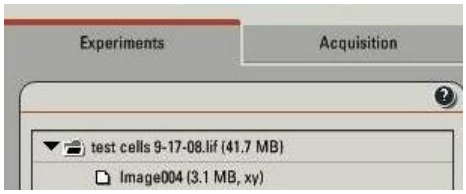
7.1 Acquire

After adjusting all the settings above, click **Capture Image** to acquire an image. This will be an XY image of finite z depth.

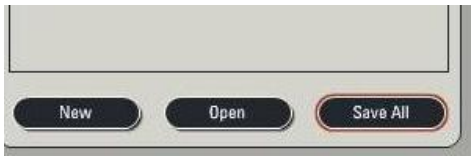


Use **Start** if you have selected an image sequence (z- stack, time series, etc.).

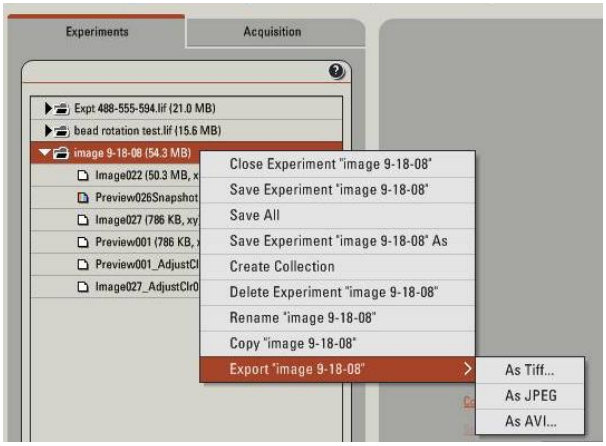
Your experiment will now be visible in the Experiments tab. Each time you click Capture or Start, the new image or image series will be placed in the same database file.



If you want to separate images into a different group, you need to click **Open** from the bottom of the Experiment window to open a new database file.



7.2 Save



To save an experiment, right click on the main experiment heading and select **Save Experiment As** . it will save every image or image series under the heading in a file of type .LIF, a Leica proprietary file format. It is strongly advised you save your experiments in this format. You may also export in other formats.

Subsequent acquisitions will be added to this experiment, but not automatically saved. Right click on them to save them and/or rename, or click on the main heading and select **Save All.**

It is also advised that you save at regular intervals to protect your images from an unexpected software crash.

YOU MUST SAVE ALL IMAGES ON THE D: DRIVE IN A FOLDER WITH YOUR LAST NAME. DO NOT SAVE ANYTHING ON THE C: DRIVE. THESE IMAGE FILES WILL BE PERIODICALLY REMOVED BY STAFF, SO YOU MUST BACK UP YOUR FILES ELSEWHERE.

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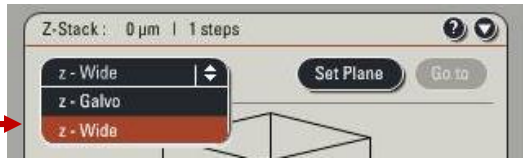
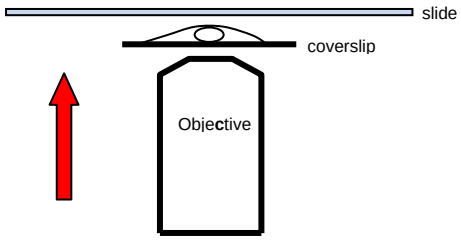
8. Z -STACK ACQUISITION

Acquire a z-stack only after setting up all the imaging parameters in the steps above. Have the acquisition mode set at XYZ . Then open the Z-Stack window and select **z-Wide.**

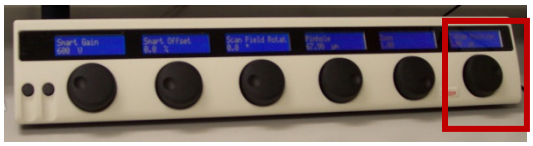


The system can acquire a z-stack in either the top-down or bottom-up direction, but it is best to work the objective against gravity, making it move in the up direction.



Best Direction for Setting a Z-Scan

Do a Live scan using a fast scan speed and adjust the Z level with the **wheel button** to find the bottom of your sample, and click the **Begin arrow** to mark the start position.

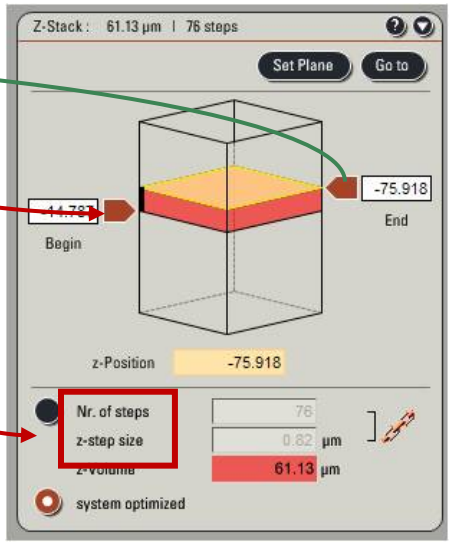


Use the wheel button again to reach the top of your sample and click the **End arrow .**

Define the **number of slices or the z-step size.** By default, the software calculates the optimal z-step, or slice thickness, based on the axial resolution of the chosen objective and wavelength, and then determines the number of steps. You may override this by clicking on the button and entering a different step size or number of steps in the field.

Click Start to begin the acquisition sequence. After capturing a z-series, you will need to click on the begin and end arrows to deactivate them (brown = active, black = inactive).

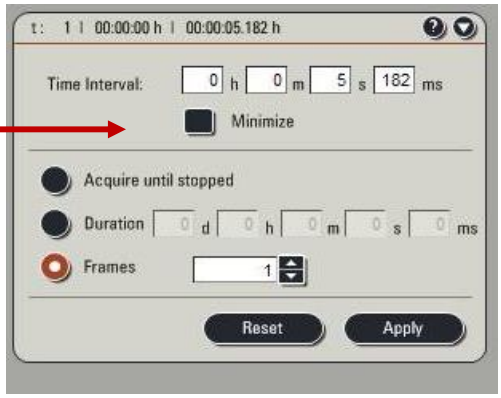
Note: There is a software glitch that occasionally maintains the z settings after deactivation. If this happens, work around it by setting the number of steps to 1.



9. TIME COURSE

In the acquisition mode window, select **XYT** from the drop down menu.

This will open the **time course panel.** Most buttons are self-explanatory.
Clicking **Minimize** will set the time interval to as fast as possible (the time it takes to do a single scan without any delay before the next scan).



Each image is given a time stamp and is visible in the image display after clicking Gallery View.

10. SHUTDOWN

If no one else is signed up to use the system for 2 hours, shut the system down in the following order:

- Save your images and exit the software.
- Shut down the computer via the Start menu of Windows
- Turn off the mercury lamp power supply and write the elapsed time on sign-in sheet.
- Turn the laser key counterclockwise. Leave the laser power button on (button #3).
- Switch off the Scanner power button (button #2) .
- When the computer is completely shut down, switch off the PC/Microscope button (button #1).
- Wait 5 minutes, then switch off the laser power button (button #3). This button controls the fan that cools the lasers, and laser life will be significantly shortened if this rule is not followed. You can be doing other housekeeping things while you wait – cleaning off the objective and your slides, etc.

11. SPECIFICATIONS FOR PUBLICATION

The system is a Leica TCS SP5, a point scanning spectral confocal with five channels. The system runs on a Windows XP platform with Leica LAS AF system software. The microscope is enclosed in a Ludin environmental chamber with temperature, humidity, and CO2 control. It has the following lasers and objectives:

LASERS

Blue diode 50mW 405nm
Multiline argon: 5mW ,458 nm, 5mW 476 nm, 20mW 488 nm, 5mW 496 nm, 20mW 514 nm
HeNe 1mW 543 nm
HeNe 2mW 594 nm
HeNe 10mW 633 nm

OBJECTIVES

PL Fluotar 2.5x/0.07
HC Plan Apo 10x/0.40 CS
HC Plan Apo 20x/0.70 CS Immersion correction
HCX Plan Apo 40x/1.25-0.75 oil
HCX Plan Apo 63x/1.4-0.6 oil
HCX Plan Apo 63x/1.30 Glycerol Immersion
HCX Plan Apo 100x/1.40-0.70 oil
HCX Plan Apo 40x/0.75 U-V-I
HCX Pl Apo 100x/1.46 oil temperature correction 23-37°
HCX Pl Apo 100x/1.46 oil temperature correction 23-37°