Confocal imaging on the Leica TCS SP8
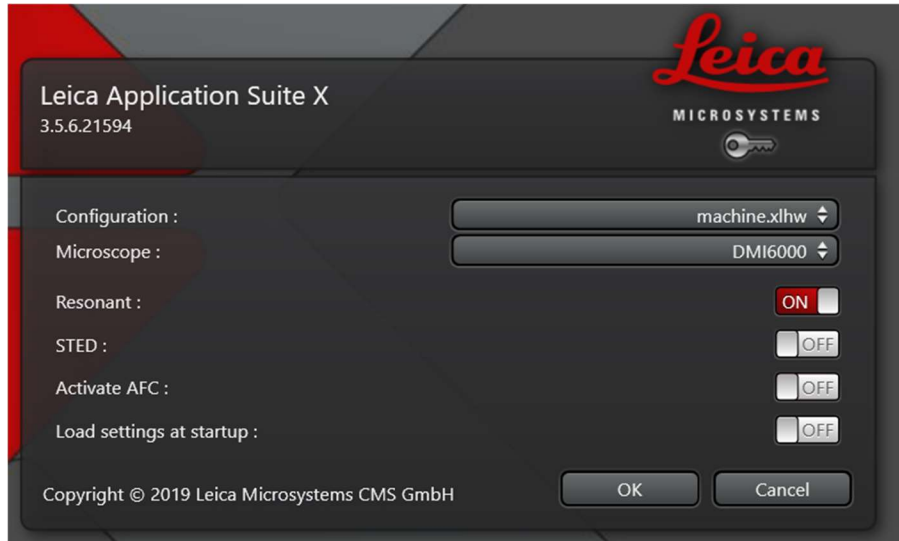
1) Turn the system on.
2) Use "LASX-user" account.
3) Start LAS X software:

4) Do not touch the microscope while the software is initializing. Choose your options:

The default settings are those of the previous user
Click to turn options on or off.

Resonant on uses the fast scanner (8000Hz speed);
Resonant off uses the normal scanner (10-1800Hz speed)
STED on for STED imaging.
STED off for regular confocal imaging
Activate AFC to use autofocus, not compatible with STED.

5) In the top left choose type of acquisition
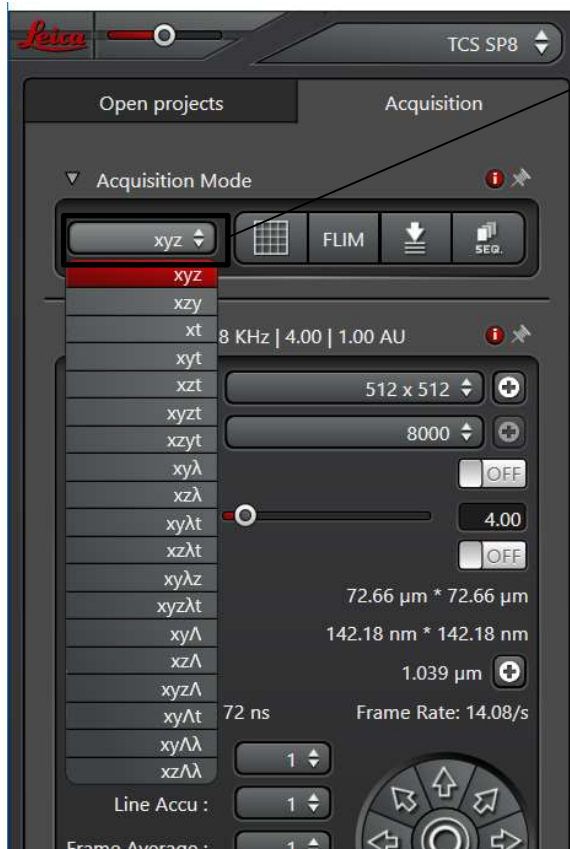
The default acquisition type is xyz, this is the most commonly used, but you can choose any combination of xyzt or excitation (Λ) or emission (λ) scans.

6) In the middle, choose your objective:
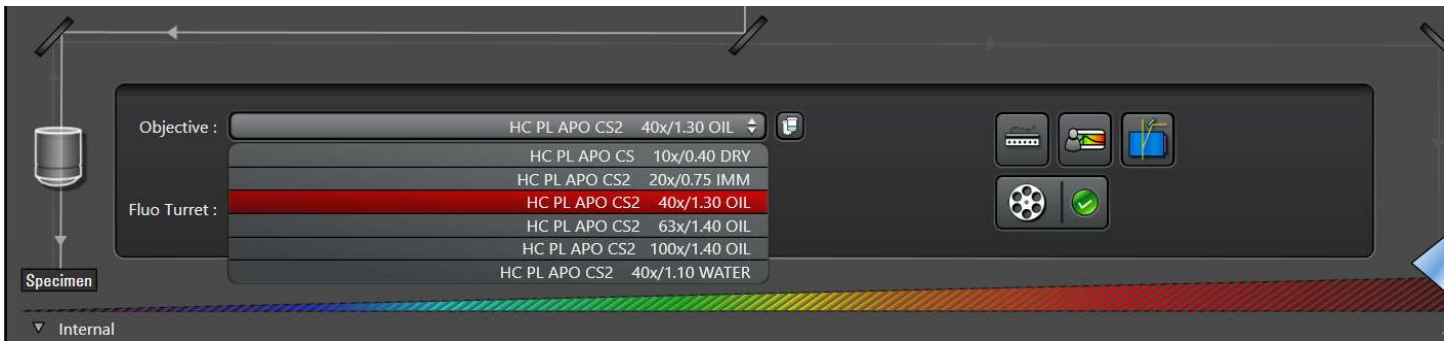
There are six objectives with different immersions: A 10X dry objective, a 20X multiimmersion objective, which we have set to use with oil, 40X, 63X and 100X oil immersion objectives, and finally a 40X water immersion. Please make sure to use the proper immersion liquid; oil and water (distilled water, never use deionized water) are on the table.

7) Once the chosen objective is in place, place your sample using the correct immersion liquid. Find the area you want to image (for instructions on finding your sample thru the eyepieces, see relevant document).

8) Configure the excitation and emission settings.   There are three ways to do it.

   a) If you already have a project with these settings, **open the project and apply the settings**.
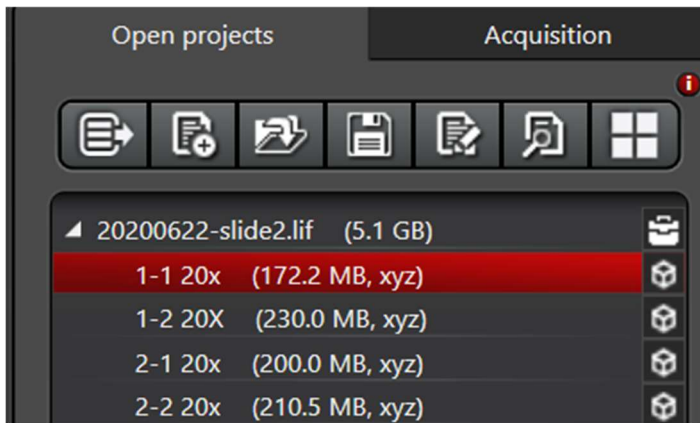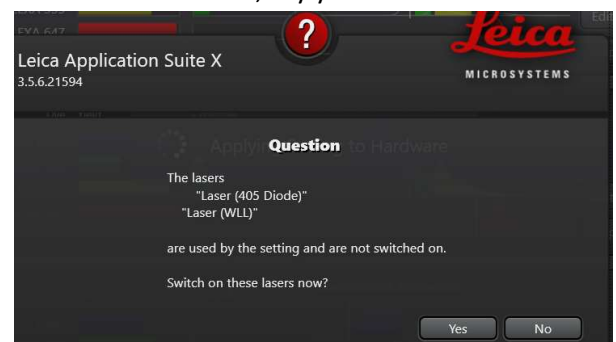

In the left column click on Open projects.

Then click on the open project icon (3rd from left).
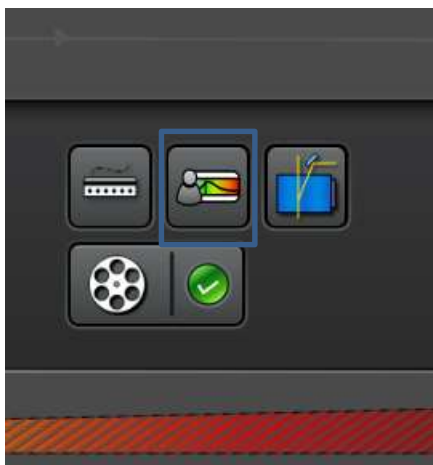
Select the project you want to open.

Then select the specific image you want to apply settings from.

Finally click the apply settings icon (5th from left).
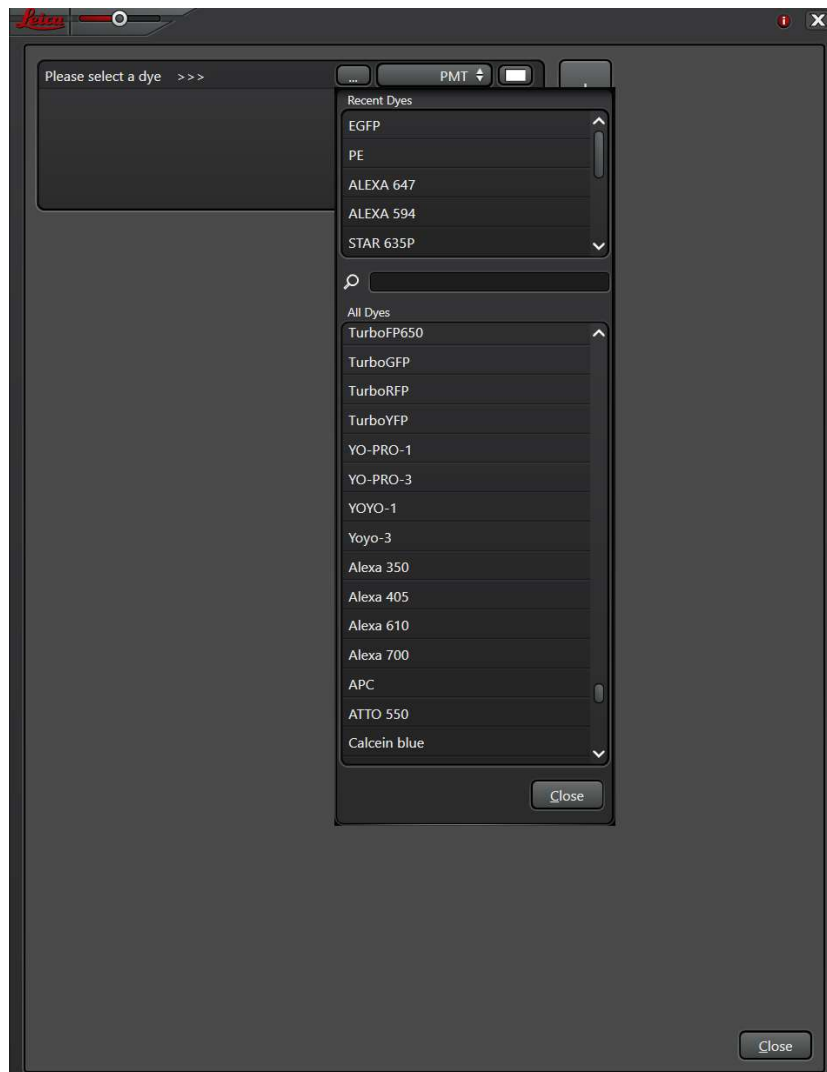
If the lasers are not on, the system will ask if they should be turned on, say yes.

   b) For new settings **use the dye assistant**.

Click on the icon (to the right of the objective choice) to open the dye assistant.

Click on the dots, this will open a list of dyes.

The top window has recently used dyes, the bottom window has all the dyes available. Dyes are in alphabetical order, first all dyes added by Leica, then dyes added by us. If you want to add a dye to the list, please ask.

Select the dye you wish to image from this drop down.

Once you select a dye, a new line will appear, and you can select another dye.

Continue until you have selected all the dyes you need.

Our system has 3 HyDs and 2 PMTs. The HyDs are more sensitive and allow gating when using the white light laser which is pulsed. Next to the dye choice, you can choose detector type, PMT or HyD (the system chooses), PMT, HyD, or FLIM.

The lower part of this window will show the selected dyes' spectra and different options for acquisition.
For each one it shows the yield and cross talk of the dyes.

The system chooses the channel pseudo color automatically, for a single channel it uses gray, for more than one dye it uses the color closest to the corresponding emission spectrum. If you want to change the color, you can click on the color square on the right of this window and choose a different color from the drop down menu.

Options for acquisition:

none sequential- simultaneous acquisition of all channels,

line sequential- lines of each sequence are acquired, all sequences are shown at the same time in live view,

frame or stack sequential- the entire frame or stack is acquired for each sequence, only one sequence is shown in live view.

Choose how you want to image considering possible crosstalk and speed of acquisition required. Click apply.

If the lasers are not on, the software will query, click yes and wait for everything to be set up – this window will close when it is done.
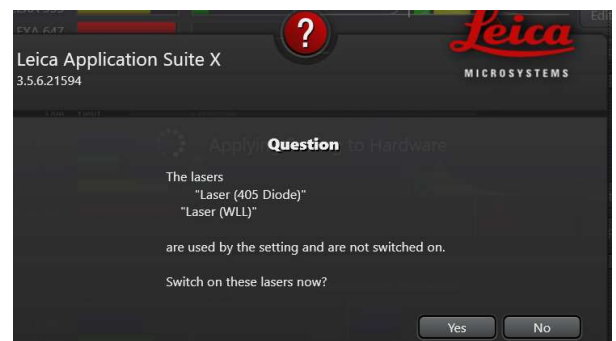
You can now see the sequences and the settings for each sequence. In this example, I chose line sequential, 3 sequences. A new window appears on the bottom left: sequential scan. You can click on each sequence to see the settings chosen for that sequence and adjust them as necessary.
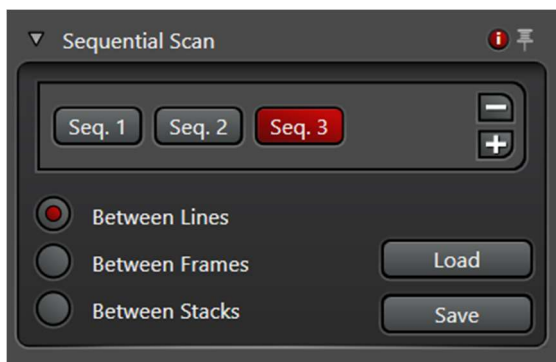
In sequential between lines, laser lines and detector windows are fixed between sequences. In sequential between frames or between stacks, laser lines and detector windows can move. There is generally no need to move laser lines, you can have up to 8 at the time. But if you want to use HyD detectors on more than 3 channels, you could use the same detector in multiple sequences. This will slow down the acquisition.
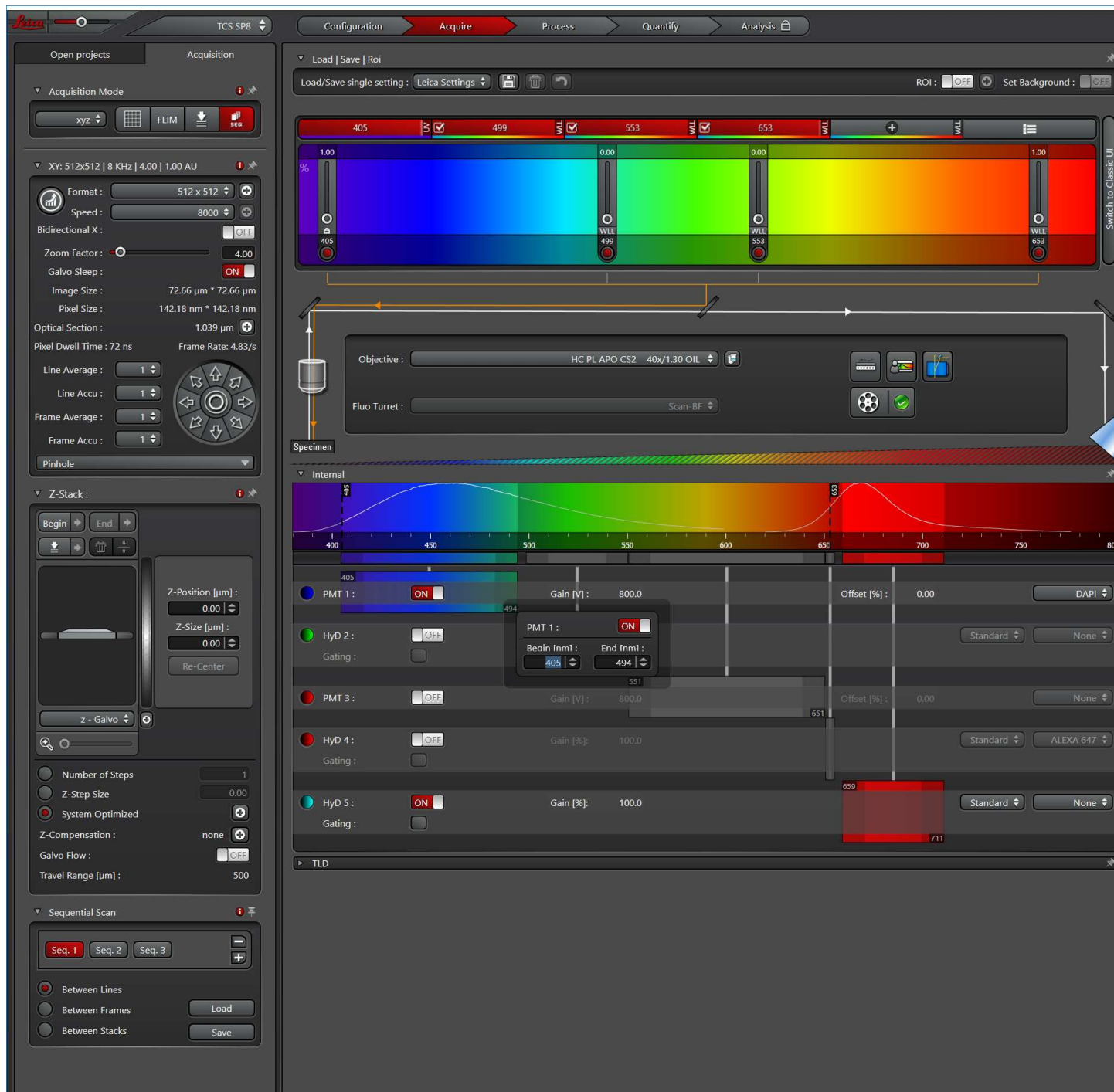
The software will probably select one or two PMTs, you can adjust the settings to use the HyDs as necessary. Always make sure detector windows do not overlap laser lines, in line sequential mode make sure all detectors are 5nm away from all laser lines in any sequence. In frame or stack sequential make sure that in each sequence the detectors are 5nm away from laser lines in the same sequence. You can adjust the detector window by moving the edges or by double clicking on the wavelength at either end and typing in the wavelength in the new window.

By default the emission spectrum for each selected dye is shown, if you want to see the excitation spectrum, right click while on top of the colored band and click on emission spectra.

c) You can also set the acquisition parameters **manually**.

Turn the laser(s) on, choose the wavelength(s), add 1% power to start; turn the detectors on and set the detection window. If you need sequential imaging, click SEQ at the top left, this will open the sequential window at the bottom left.

9) Once the excitation and emission parameters are set, check the scanner speed and format for live scanning:

   a) **Normal scanner**
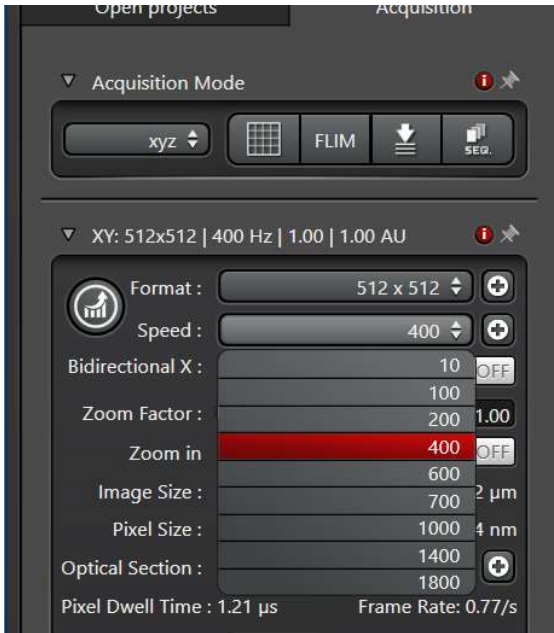
The speed of the normal scanner can be between 10 and 1800 Hz, the default when starting the software is 400. You can change that in the pull down menu. You can increase the speed to 600 without any loss in the number of pixels, if the speed is higher than that, the number of pixels possible decreases and the system automatically increases the zoom to adjust.

By default the scanning is unidirectional, you can turn bidirectional scanning on. The phase is set automatically.
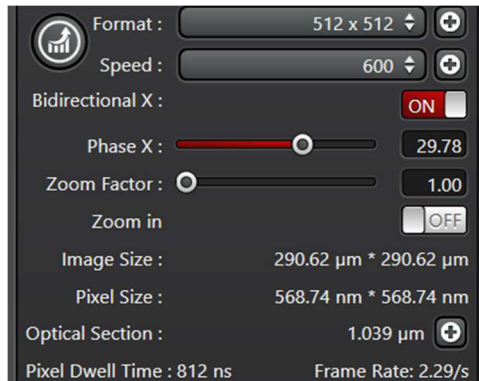
For live scanning keep the format at 512x512.

   b) **Resonant scanner**
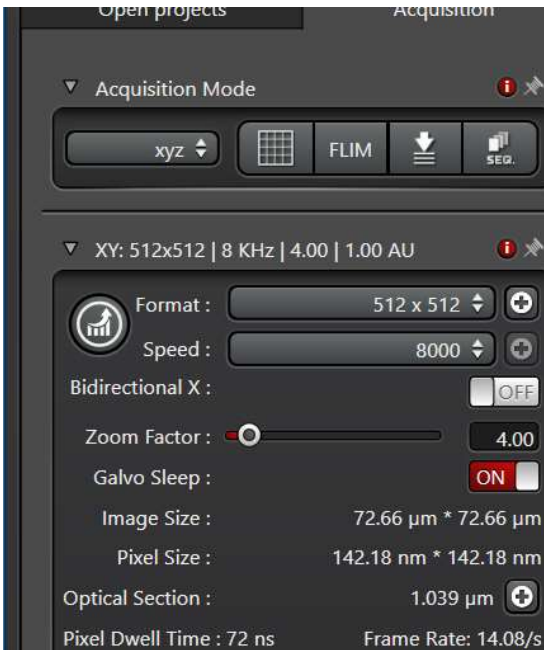
The speed is set to 8000. Because the number of pixels that can be acquired with this scanner is limited, the software automatically zoom in. You can zoom out to 1.25 (lowest zoom).

If you need faster acquisition, you can turn the bidirectional scanning on, however, you will have to adjust the phase.

To adjust the phase, find a vertical structure in your image, as you change the phase, the structure will appear duplicated, adjust the phase to remove the duplicate.

Because the scanning is very fast, the live image (without any averages) may be very noisy, which may make it hard to focus or find the region of interest. To force the system to line average during live acquisition, go to configuration at the top

Then click on hardware.   This will open a new window:
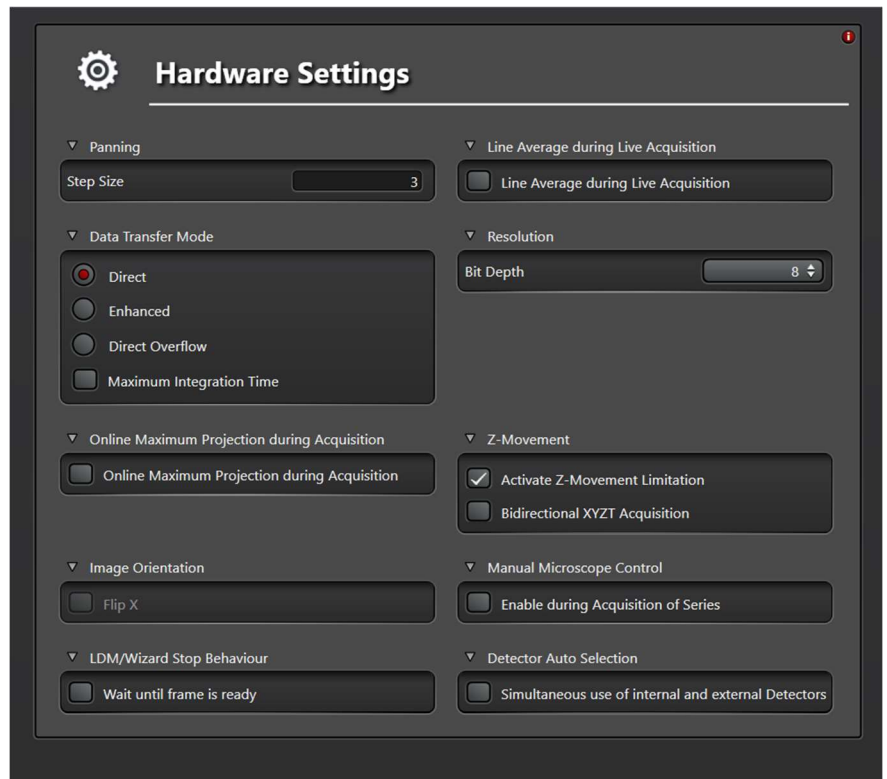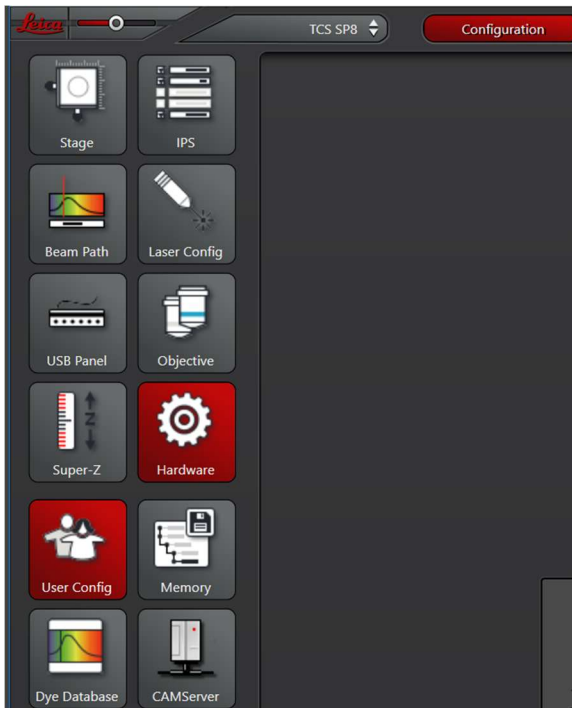
Select line average during line acquisition on the top right of this new window and return to the acquire window.

For live scanning keep the format at 512x512 and set the line averaging to 2 or 3. You want the live window to refresh quickly but be clear enough that you can focus and select the region you want.

10) Click **live** on bottom left to see the image. Focus on the screen using either the focus on the joystick by the microscope or the z-position knob on the bar below the monitors:
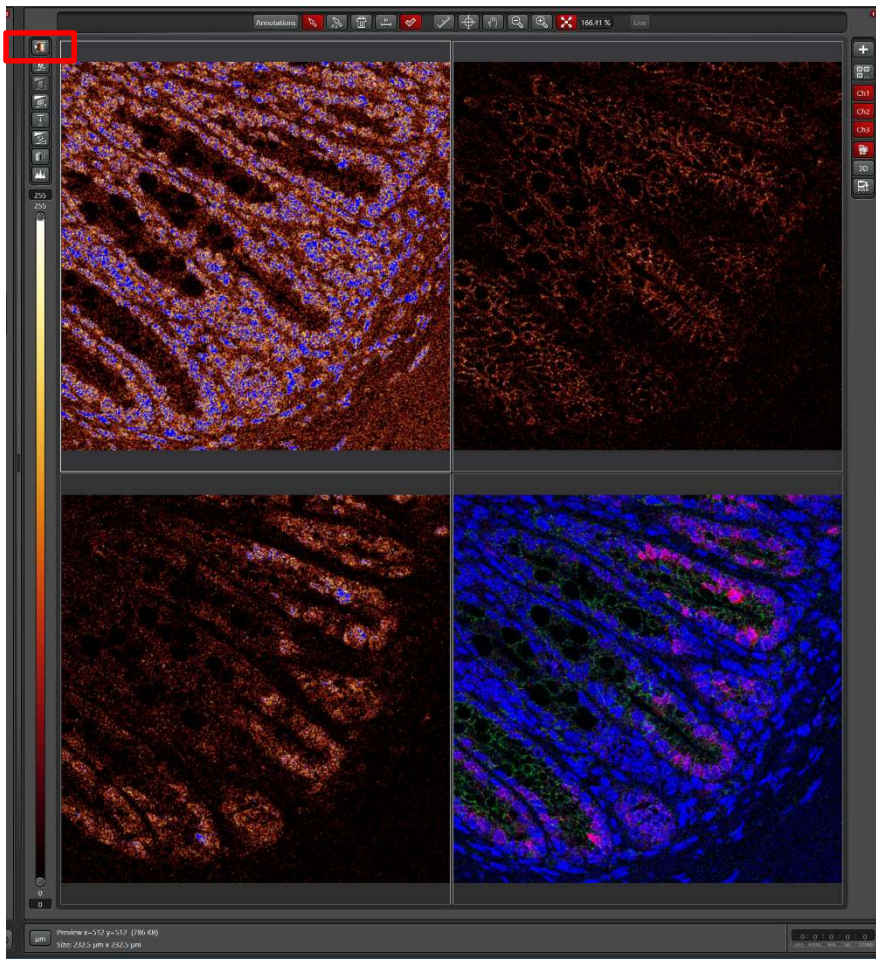
Adjust the laser intensity and/or the gain on the detector to optimize the image. To make sure it is not saturating, use the glow over lookup table:

The top square above the color bar toggles between different look up tables: color, glow over, grayscale.

Glow over as selected here, will show intensities ranging from black to white, blue shows saturation. In this example channel 1 is obviously saturated and you need to lower the laser intensity and/or the detector gain.

Lowering the detector gain reduces the noise, if your laser power is low (less than 1% for the 405 laser, less than 5% for the white light laser) and the image is saturated, lower the detector gain.

11) If you want to **acquire a z-stack**, go to the Z-stack window in the left column.

There are two ways to set the stack:

i)   If you are focused in the middle of the stack and you know how thick a stack you want, set the Z size to the desired thickness on the right. When you refocus, hit re-center; this will keep the same stack size centered around the new focus.

ii)   Move the z position to one end of your sample (either with the z-position button on the USB control panel or by hovering the mouse on the picture of the slice and using the mouse wheel) and click the begin button; then move the z-position to the other end of your sample and click the end button.

When system optimized is selected (default), the system will select the proper step size, which is 1/3 of the optical section. If you do not need the optimized settings, you can select z step size and set it.
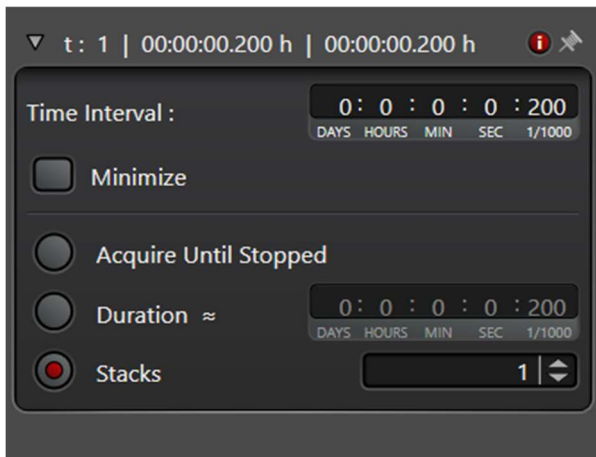
You can also change the stack direction by clicking on the appropriate arrow.

If you are imaging a thick sample, such that fluorescence is decreasing as you go into the sample, you can use z-compensation to increase the excitation laser intensity or the detector gain. Click on the plus sign to open a new window and set this up.

Z-Stack : 12.12µm | 36 Steps

Begin ➡   End ➡

End : [µm] :
6.69

Begin : [µm] :
-5.43

Z-Position [µm] :
6.72

Z-Size [µm] :
12.12

Re-Center

-Begin-

z - Galvo

Stack Direction (Z) :
⬆   ⬇

Number of Steps	36
Z-Step Size	0.35
System Optimized	⊕
Z-Compensation :	none ⊕
Galvo Flow :	OFF
Travel Range [µm] :	500

12) To acquire a **time lapse series**:

t : 1 | 00:00:00.200 h | 00:00:00.200 h

Time Interval :
0 : 0 : 0 : 0 : 200
DAYS HOURS MIN SEC 1/1000

☐ Minimize

○ Acquire Until Stopped

○ Duration ≈
0 : 0 : 0 : 0 : 200
DAYS HOURS MIN SEC 1/1000

⦿ Stacks
1

Under acquisition mode (top left) select xyt or xyzt for a time lapse of a single plane or a z-stack respectively.
A new window will open in the left column at the bottom.

Choose minimize to acquire as fast as possible or a time interval if you would like to acquire slower than the minimum time.

Choose the number of time points to acquire (stacks), the duration of the time lapse or acquire until stopped.
Press start to begin time lapse acquisition.

Note that the minimum time depends on the image format, number of sequences and averages. You can check how long it will take by clicking the minimize in this window after setting the format you would like. If the time interval is good for your experiment you can leave it, if you want to acquire slower, just make the time interval larger, but if you want to acquire faster, you have to change something in the format. There is always a compromise between spatial and temporal resolution.

Acquisition Mode (left panel)

▽ **Acquisition Mode**

xyzt | FLIM | SEQ.

▽ **XY: 512x512 | 8 KHz | 1.25 | 1.00 AU**

Format : 512 x 512
Speed : 8000
Bidirectional X : OFF
Zoom Factor : 1.25
Galvo Sleep : OFF
Image Size : 232.5 µm * 232.5 µm
Pixel Size : 454.99 nm * 454.99 nm
Optical Section : 1.039 µm
Pixel Dwell Time : 72 ns   Frame Rate: 4.99/s
Line Average : 3
Line Accu : 1
Frame Average : 1
Frame Accu : 1
Auto Gain
Pinhole

▽ **Z-Stack :**

Begin | End

Z-Position [µm] : 20.76
Z-Size [µm] : 0.00
Re-Center

z - Galvo

○ Number of Steps   1
○ Z-Step Size   0.00
● System Optimized
Z-Compensation :   none
Galvo Flow :   OFF
Travel Range [µm] :   500

▽ **t : 8000 | 00:26:42.000 h | 00:00:00.200 h**

Time Interval :   0 : 0 : 0 : 0 : 200
DAYS HOURS MIN SEC 1/1000

☑ Minimize

○ Acquire Until Stopped
○ Duration ≈   0 : 0 : 26 : 42 : 0
DAYS HOURS MIN SEC 1/1000
● Stacks   8000

Acquisition Mode (right panel)

▽ **Acquisition Mode**

xyzt | FLIM | SEQ.

▽ **XY: 1024x1024 | 8 KHz | 1.25 | 1.00 AU**

Format : 1024 x 1024
Speed : 8000
Bidirectional X : OFF
Zoom Factor : 1.25
Galvo Sleep : OFF
Image Size : 232.5 µm * 232.5 µm
Pixel Size : 227.27 nm * 227.27 nm
Optical Section : 1.039 µm
Pixel Dwell Time : 36 ns   Frame Rate: 1.9/s
Line Average : 4
Line Accu : 1
Frame Average : 1
Frame Accu : 1
Auto Gain
Pinhole

▽ **Z-Stack : 2.08µm | 7 Steps**

Begin | End

End : [µm] : 21.83
Begin : [µm] : 19.76
Z-Position [µm] : 20.76
Z-Size [µm] : 2.08
Re-Center
Stack Direction (Z) :

z - Galvo

○ Number of Steps   7
○ Z-Step Size   0.35
● System Optimized
Z-Compensation :   none
Galvo Flow :   OFF
Travel Range [µm] :   500

▽ **t : 8000 | 08:10:04.627 h | 00:00:03.676 h**

Time Interval :   0 : 0 : 0 : 3 : 676
DAYS HOURS MIN SEC 1/1000

☑ Minimize

○ Acquire Until Stopped
○ Duration ≈   0 : 8 : 10 : 4 : 627
DAYS HOURS MIN SEC 1/1000
● Stacks   8000

For example, with the resonant scanner, a 512x512 format, 3 line averages and no z-stack, it will take 200ms per image (left) ; but with a 1024x1024, 4 line averages and 7 planes, it will take close to 4 sec per image (right).

To select a slower time lapse, unclick minimize, and change the time interval to a larger time.
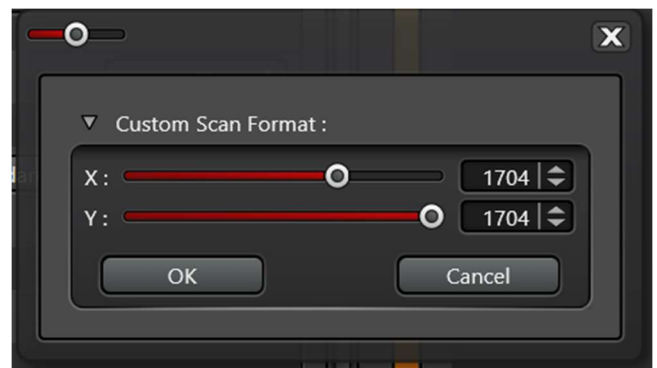
13) Stop the live scanning and **set the acquisition parameters** at the top left:
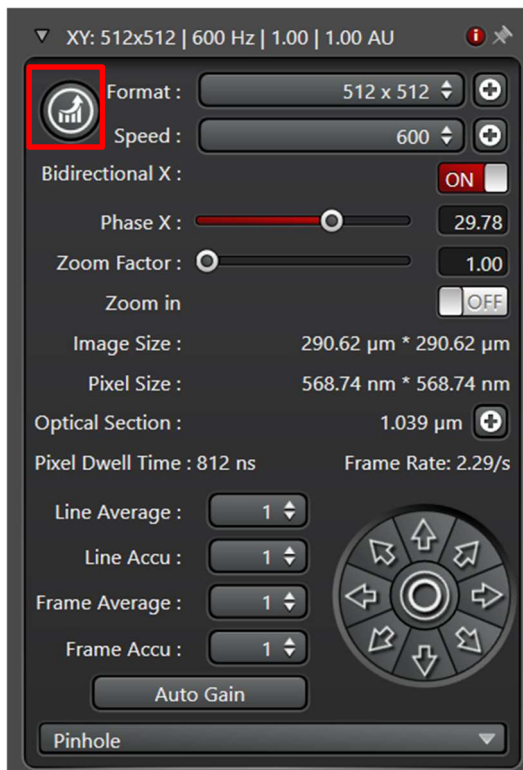
To set the pixel size to the optimal size, press on the optimize format button to the left. If you are using the normal scanner, this will set the number of pixels to achieve the optimum pixel size (3 pixels per resolution). However, if you are using the resonant scanner, there may not be enough pixels. If that is the case, you will see a message to that effect and the number of pixels will not change.
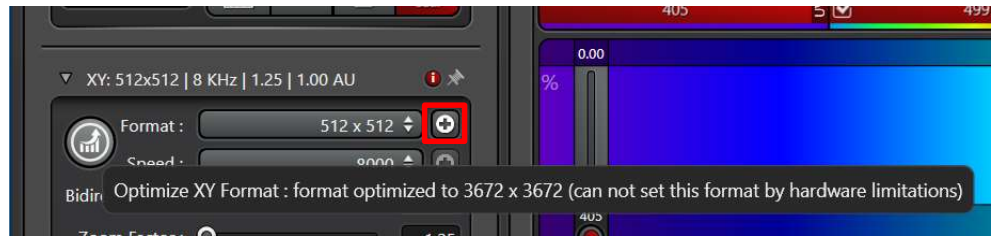
You can increase it manually. The pull-down menu has some format options, but you can choose any format by clicking on the plus sign to the right, this will open a new window:


Note that the number of pixels in x set the pixel size, the number of pixels in y can be any number up to the number of pixels in x.

Finally, to decrease the noise set the number of averages to use. Line average will acquire the same line multiple times and average, frame average will acquire the entire frame multiple times and then average. Generally, you can obtain good images with 2 to 4 line averages with the normal scanner and 4 to 8 averages with the resonant scanner. The brighter your sample, the fewer averages you will need.

If your sample is very dim, you can also set some accumulation, either line or frame, this will acquire either the same line or frame multiple times and add them up. You can only have average or accumulation of the same type, but you could line average to reduce noise and frame accumulate to make your image brighter.

In this window (and on the bar below the monitor, 3rd knob from right) you can change the pinhole. For confocal imaging the pinhole is set at 1 Airy units (the physical size depends on the objective). If you open the pinhole the image becomes "less confocal" with the optical section becoming larger. If you close the pinhole you decrease the optical section and resolution, but also decrease the amount of light collected. If you use the "Lightning mode" the software will automatically adjust the pinhole. In general, leave the pinhole at 1.

14) **Acquire the images**: There are two buttons at the bottom middle right, capture image and start.
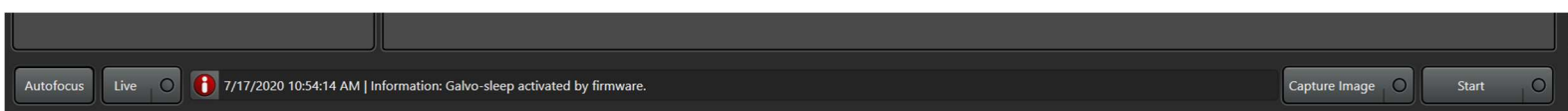
Capture image This will acquire one image, the current plane; in line sequential it will acquire all sequences, in frame or stack sequential, it will only acquire the selected sequence.

> **Start** This will acquire the entire series, all sequences, the z-stack if set, and the timelapse if set.

> Note:
> When you are searching for a new area to image or adjusting the focus, reduce the format to 512x512 before clicking on "live" so the image refreshes fast. If you are using the resonant scanner you can also reduce the line averages for live scanning. Always return to the correct format and number of line averages before capturing the image.

15) Save your images: Images are automatically saved in a temporary directory. Before exiting the software you need to save them.   Images are put in a project; all images go into the same project until you make a new one.
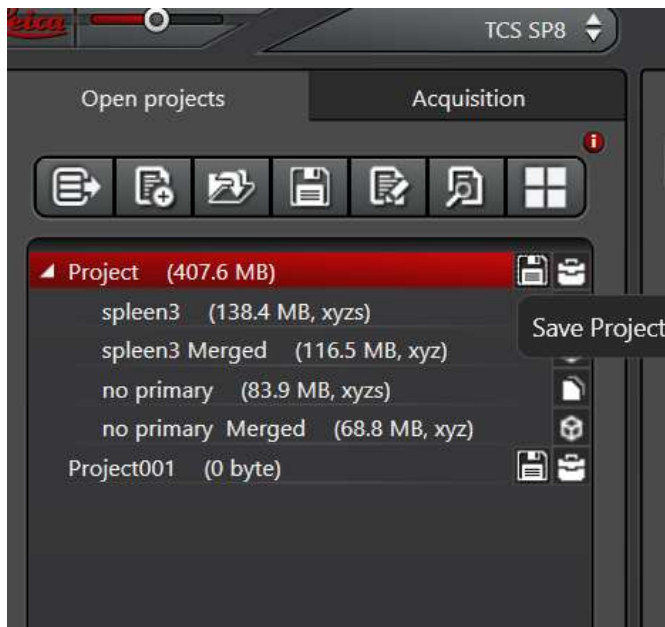

In the open project tab on the left, you can make a new project by clicking on the second from left button.
To save project by project click on the disk shape button next to each project. You can click on the disk at the top (fourth from left) to save all, the software will query for each.

You can rename your images and projects as you go.

Please save your files to the local datadrive (E:). In the folder users, make a folder for your lab with the PI's name. Within that folder you can organize as you wish.
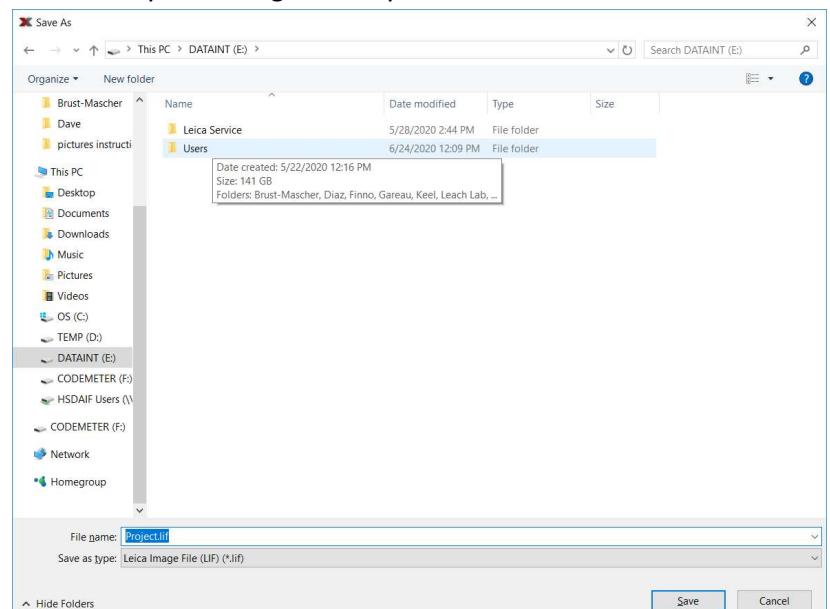
After saving to the local hard drive, copy your files to the server (Y:) for access remotely or from our analysis computers.

16) When finished, remove your slide, lower the objective, clean all objectives you used with lens paper (just blot the oil off) and shut everything down if no one else is using the system within the hour.

Notes:

- Wipe the immersion liquid off the objective after every 2 or 3 slides or if take your slide off and switch objective.
- During acquisition, do not be tempted to do something else on the computer!
- Warning: phone signals may be picked up by hyd detectors, avoid leaving your phone on the table left of the microscope.