

# Leica TCS SP5 Confocal Laser Scanning Microscope User Guide

## 1. BASIC IMAGE ACQUISITION

This manual is the **FIRST** section of a **THREE** part Leica TCS - SP5 User Guide  
edited by **Donald Pottle**

**Leica** True Confocal Scanning - Spectral  
Photometric 5 - Model Upright DM 6000S  
Confocal Laser Scanning Microscope

**Cell Biology - Confocal Microscope Room**

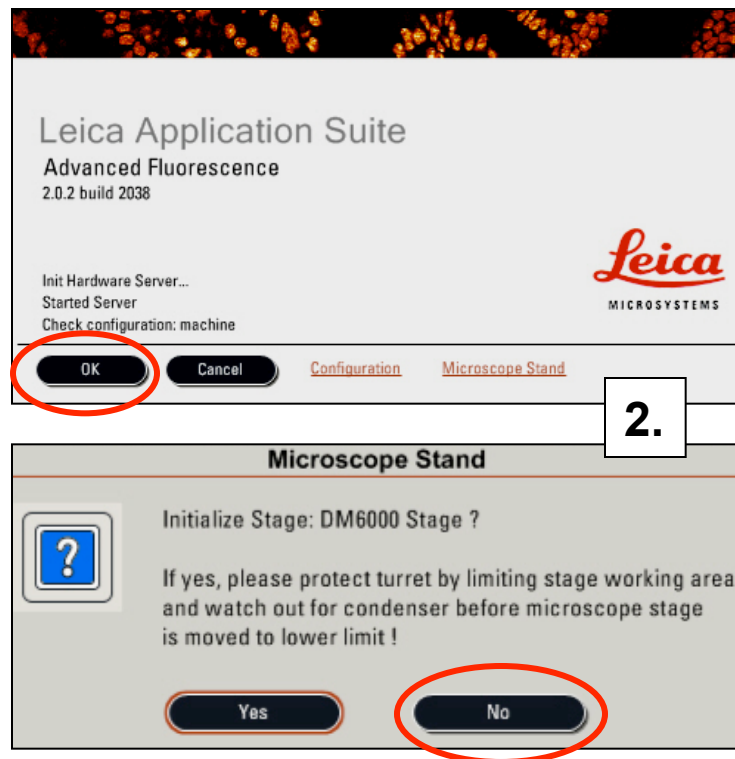
### NOTICE

All users must receive  
legitimate training and  
certification.

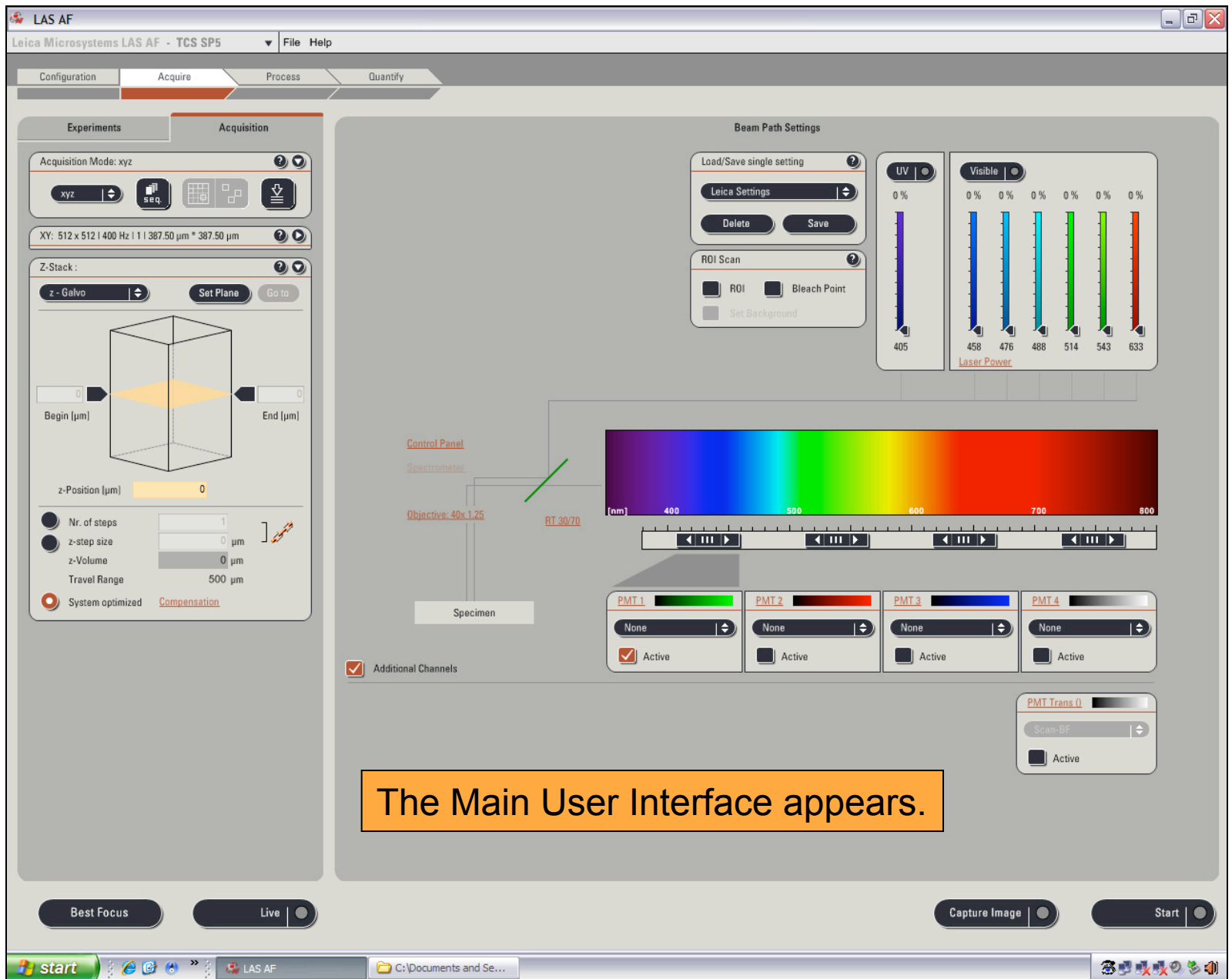
This manual was generously shared with us by the author,  
**Donald Pottle** of the Schepens Eye Research Institute.

# REVIEW: If you have not already done so,

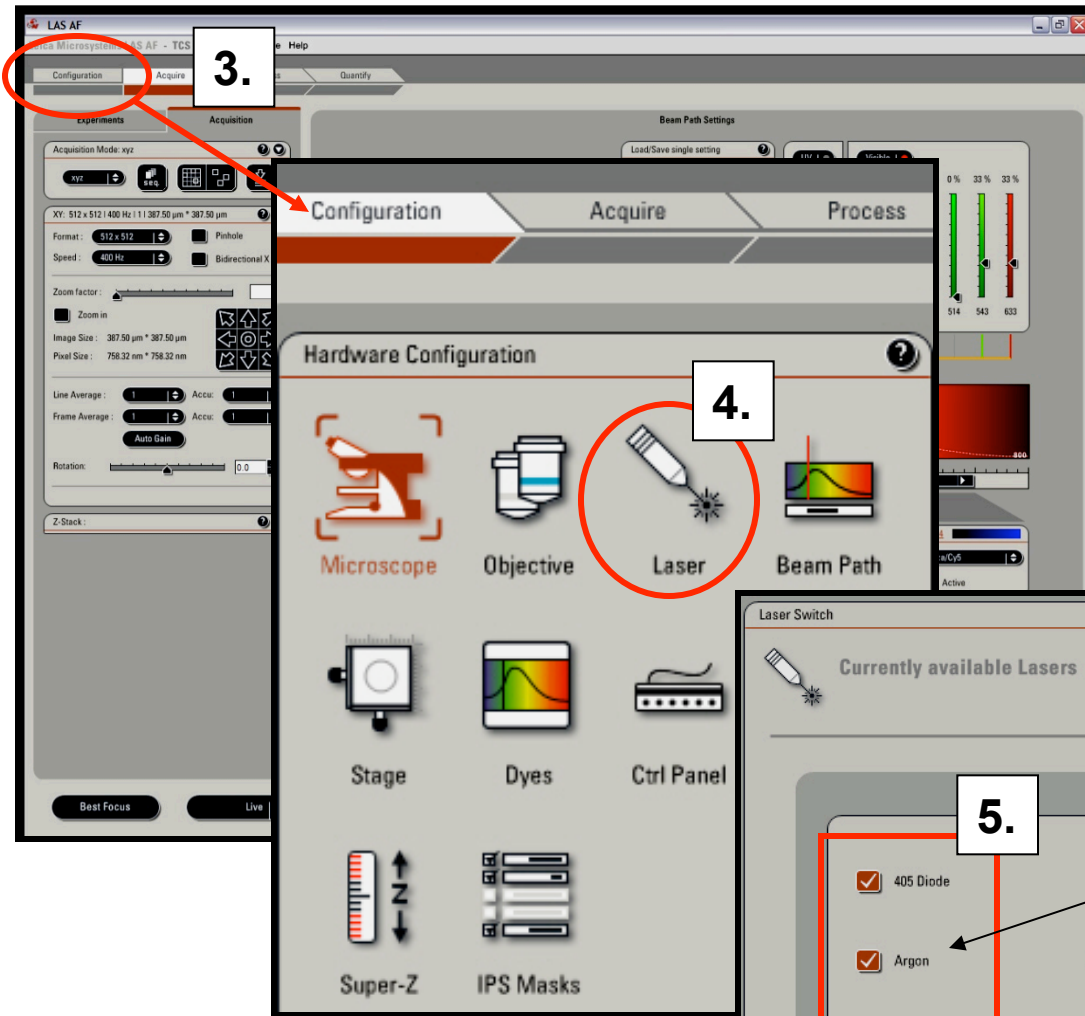
1. Double Click on the Confocal LAS AF Icon on the Desktop
2. Click on <OK> in the Leica Application Suite box. A Microscope Stand initialization box appears. Click <No> if the stage has already initialized.



It takes about two minutes for the software / hardware to complete the initialization process.

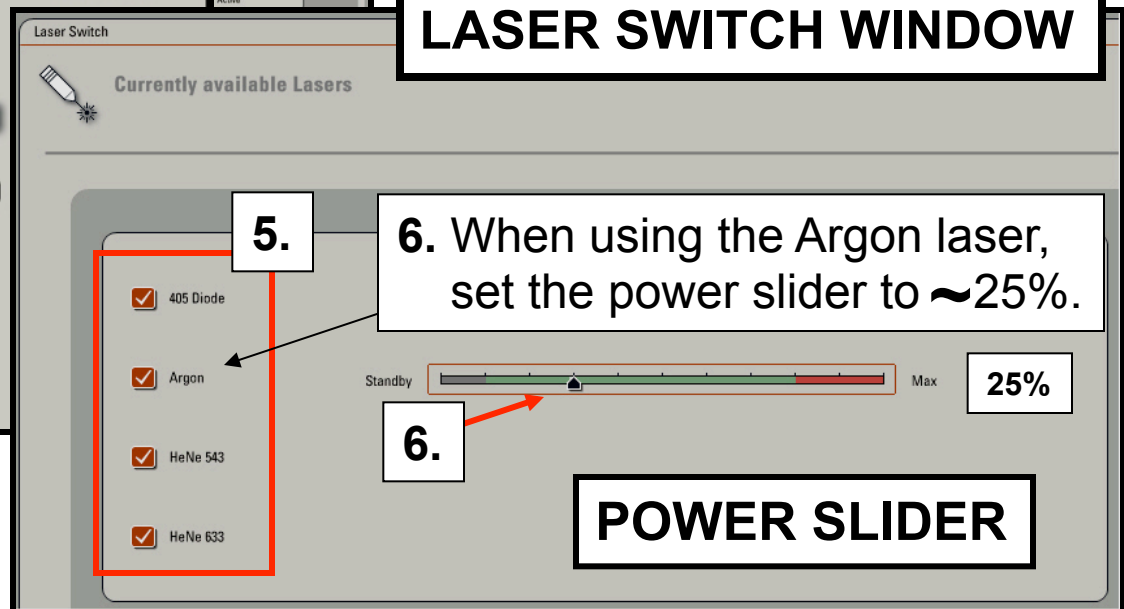


# Configuring the system for Fluorescence



3. Click on <Configuration> in the LAS AF window and the configuration window will open.
4. Click on <Laser> and the Laser Switch Window will open.
5. Check mark the lasers that you intend to use – (The 405 Diode laser is for UV [ e.g. [DAPI](#) ]).

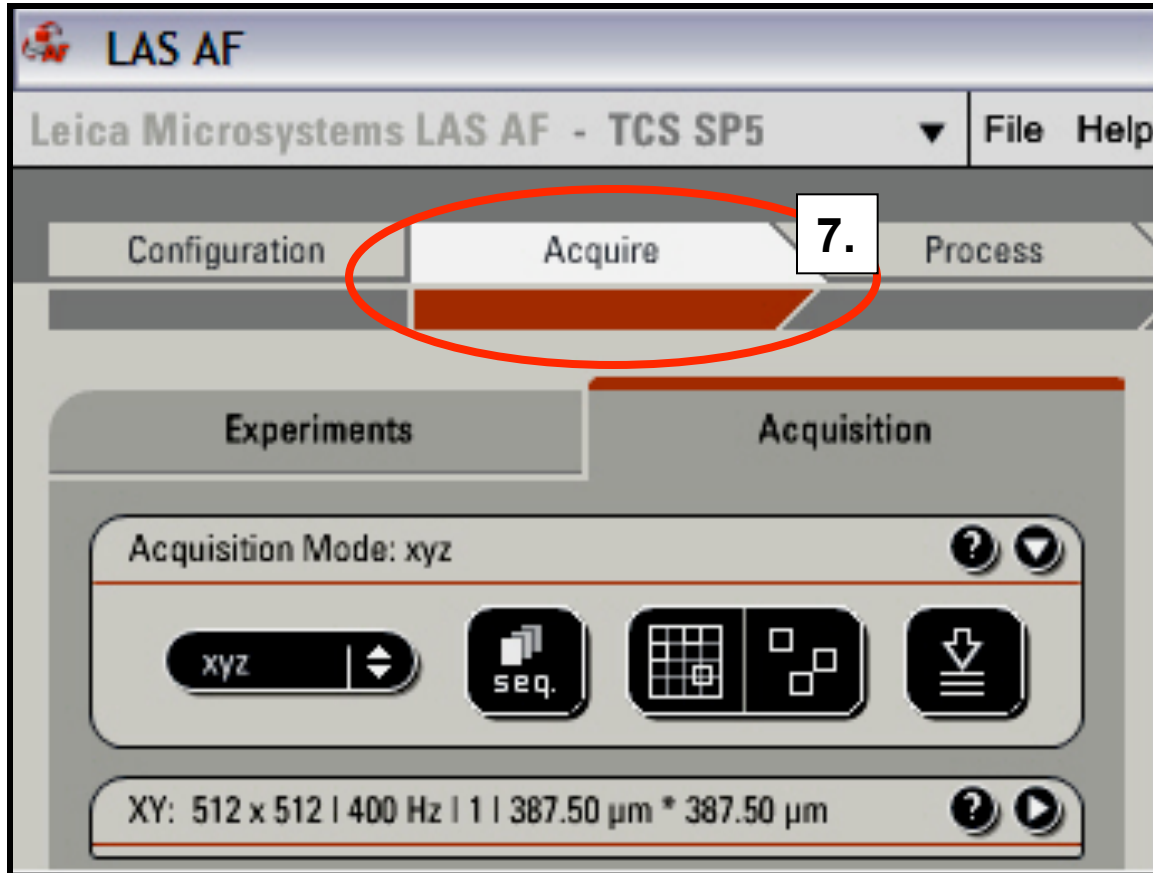
## LASER SWITCH WINDOW



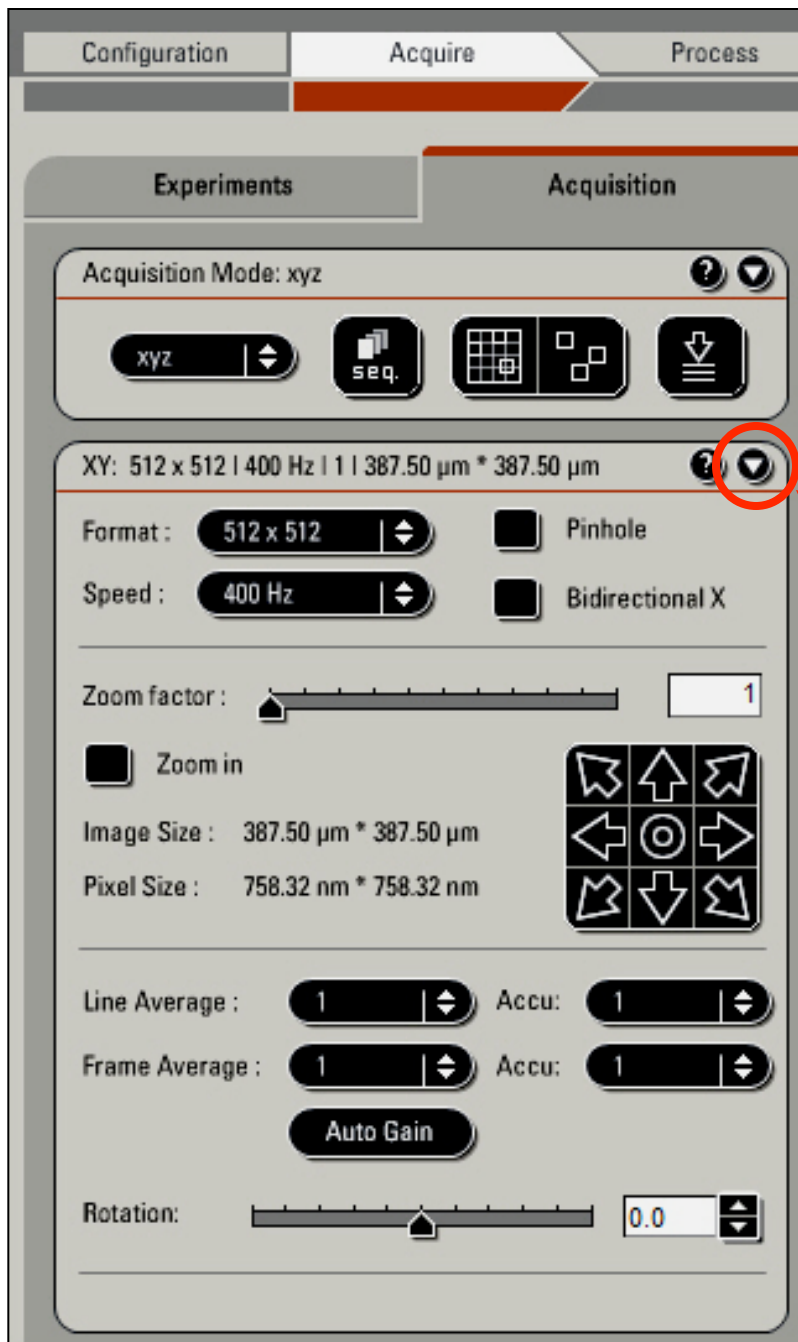


# Acquiring images by simultaneous scanning

7. Click on the Acquire tab in the Main User Interface Window.



The Acquire window opens and displays the default values for:  
Scanning mode – (XYZ) - Format – (512 x 512) - Speed (400Hz)  
Pixel size – (758nm x 758 nm) and Image Size – (387.50µm x 7.50µm)



## Changing Imaging Parameters

8. Click on the XY title bar arrow to display the XY parameter menu.

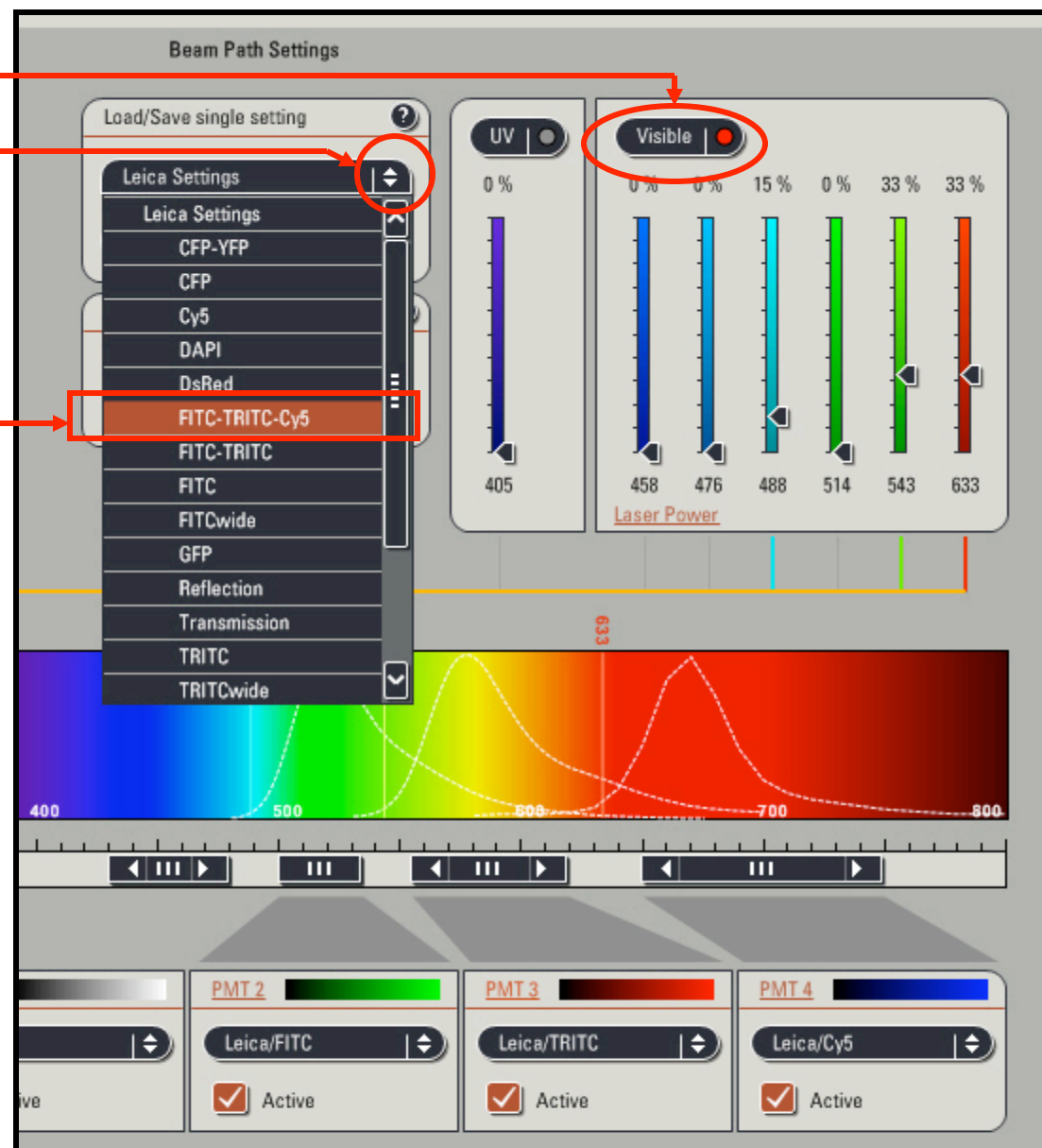
Imaging parameters for **XY** can be changed by clicking on this arrow and selecting values from those offered within the drop-down window.

The default values in this window are usually ideal for most imaging but, with experience, the user may choose to use others.

# SETTING THE BEAM PATH WINDOW

9. Click on <Visible>  
and / or UV

10. Select preset fluorophore  
settings from the Leica  
Settings drop-down menu.  
In this example, the  
combination of **FITC**,  
**TRITC**, and **CY5** is  
selected (and not UV).



## NOTE:

Alexa 488, FITC and GFP are  
excited using the 488 Argon (blue)  
laser line and emit in green. Alexa  
568 is excited using the 568 or 543  
laser line (Green) and emits in red.

**DAPI is excited by the 405 diode  
laser and emits mainly in blue  
but overlaps greatly into other  
spectra and SEQUENTIAL  
SCANNING is required to  
eliminate channel crosstalk.**

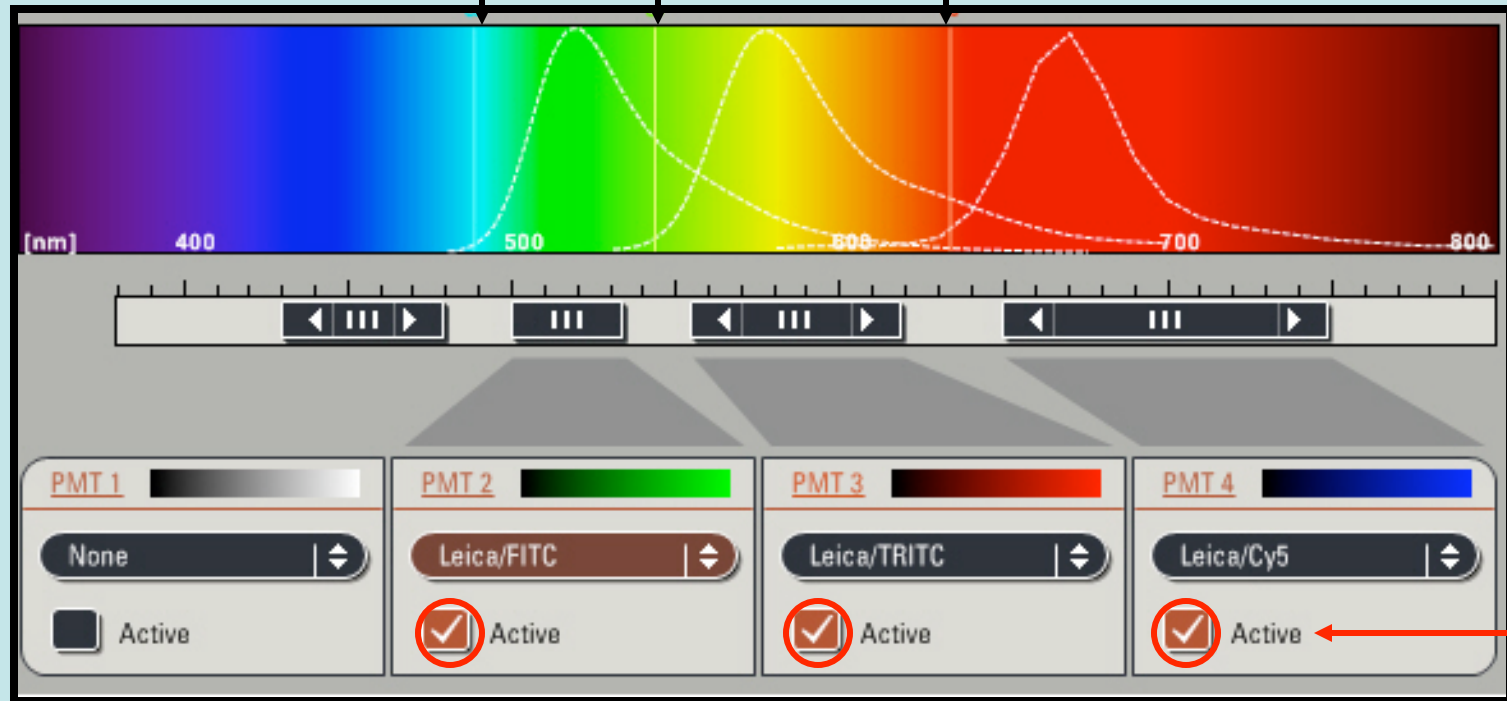
## SPECTRAL GRAPH

EXCITATION LINES FOR:

FITC

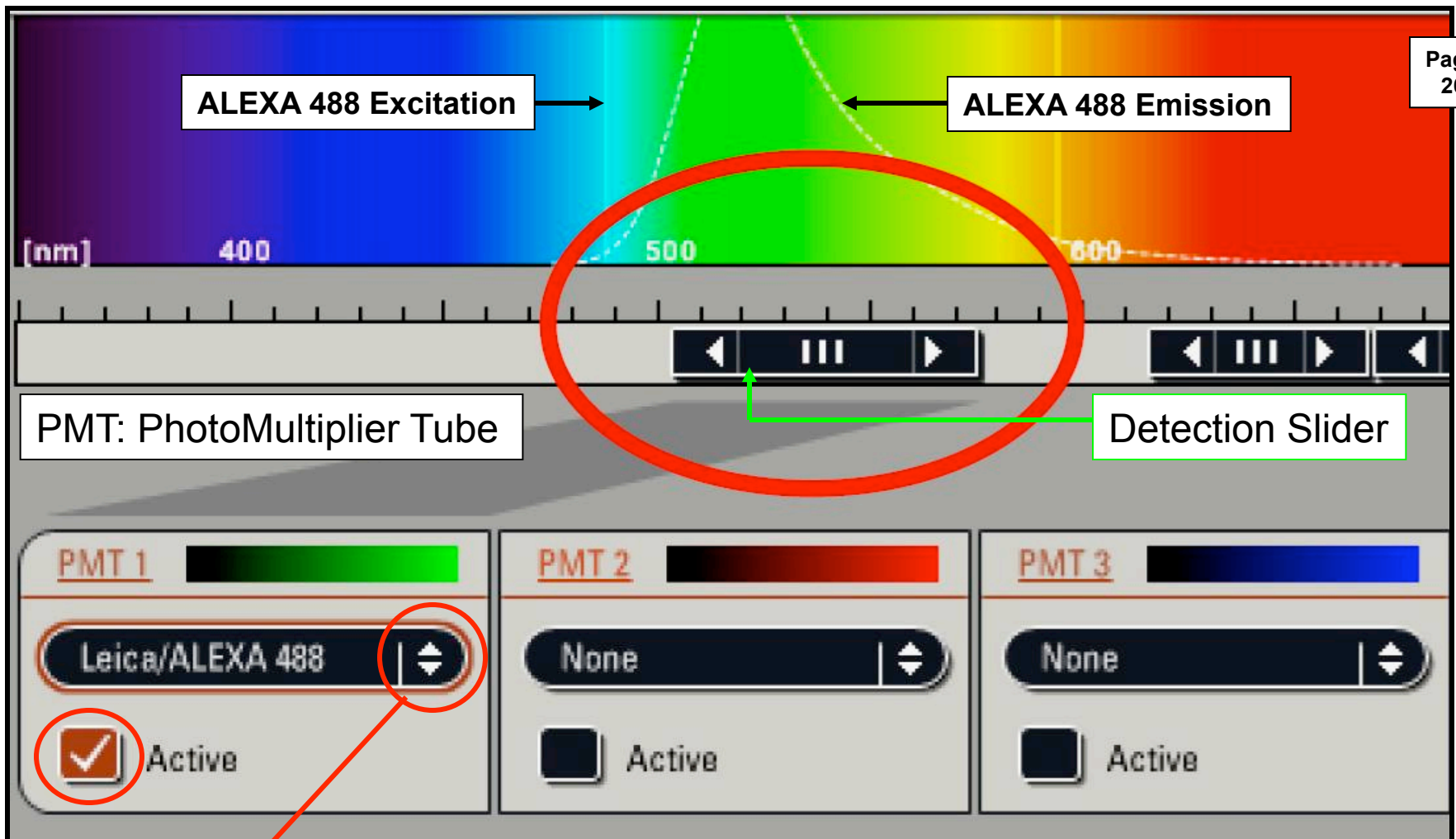
TRITC

CY5



Active lasers (FITC – TRITC – Cy5 in this case) are indicated by thin lines representing excitation wavelengths on the spectral graph.

11. Select and Activate the PMTs (PhotoMultiplier Tubes) by clicking on (check mark) their Active buttons. Gray shadows leading from the PMT slider bars to the activation panels will appear confirming PMT activation.



Fluorophores can be selected manually from a drop-down menu beneath a PMT window. (ALEXA 488 is selected for PMT 1.) Crosstalk among channels is reduced by moving sliders beneath the emission peaks of each fluorophore. PMT Detection sliders can be further refined by clicking and dragging their ends.

LAS AF

Leica Microsystems LAS AF - TCS SP5

File Help

Configuration Acquire Process Quantify

Experiments Acquisition

Acquisition Mode: xyz

xyz

XY: 512 x 512 | 400 Hz | 1 | 387.50  $\mu$ m \* 387.50  $\mu$ m

Format: 512 x 512

Speed: 400 Hz

Zoom factor: 1

Image Size: 387.50  $\mu$ m \* 387.50  $\mu$ m

Pixel Size: 758.32 nm \* 758.32 nm

Line Average: 1 Accu: 1

Frame Average: 1 Accu: 1

Rotation: 0.0

Z-Stack:

Beam Path Settings

Load/Save single setting

Leica Settings

CFP-YFP

CFP

Cy5

DAPI

DSRed

**FITC-TRITC-Cy5**

FITC-TRITC

FITC

FITCwide

GFP

Reflection

Transmission

TRITC

TRITCwide

UV

Visible

0 % 0 % 15 % 0 % 33 % 33 %

405 458 476 488 514 543 633

Laser Power

Control Panel

Spectrometer

Objective: 40x 1.25

TO 468/543/633

Specimen

Additional Channels

PMT 1

None

Active

PMT 2

Leica/FITC

Active

PMT 3

Leica/TRITC

Active

PMT 4

Leica/Cy5

Active

PMT Trans 0

Scan-BF

Active

Best Focus

**Live**

Capture Image

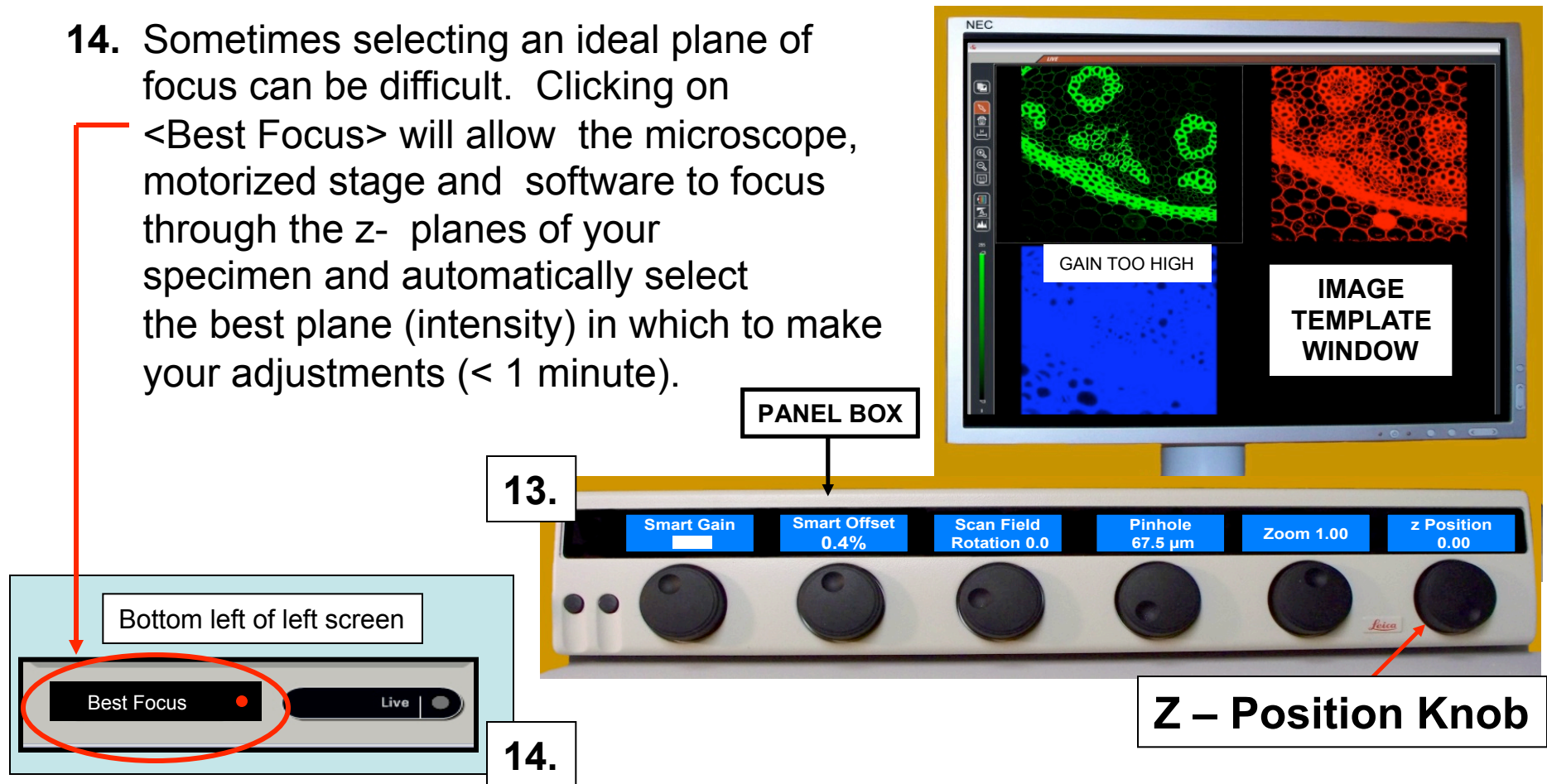
Start

12. Once all of the XY, fluorophore, and PMT selections, activations and adjustments have been made, click on <Live> to produce image(s) of your specimen in the image template window (right screen).



13. Use the Z - Position knob on the Panel Box to select a plane of focus within your XY region of interest for adjusting the brightness (intensity ) and contrast (offset) of the images.

14. Sometimes selecting an ideal plane of focus can be difficult. Clicking on <Best Focus> will allow the microscope, motorized stage and software to focus through the z- planes of your specimen and automatically select the best plane (intensity) in which to make your adjustments (< 1 minute).



Turning the Zoom Knob adjusts the area being scanned so that the image is optically (not digitally) enlarged.

## ZOOM FUNCTION



Clicking on these arrows in the XY window can center the image as you zoom

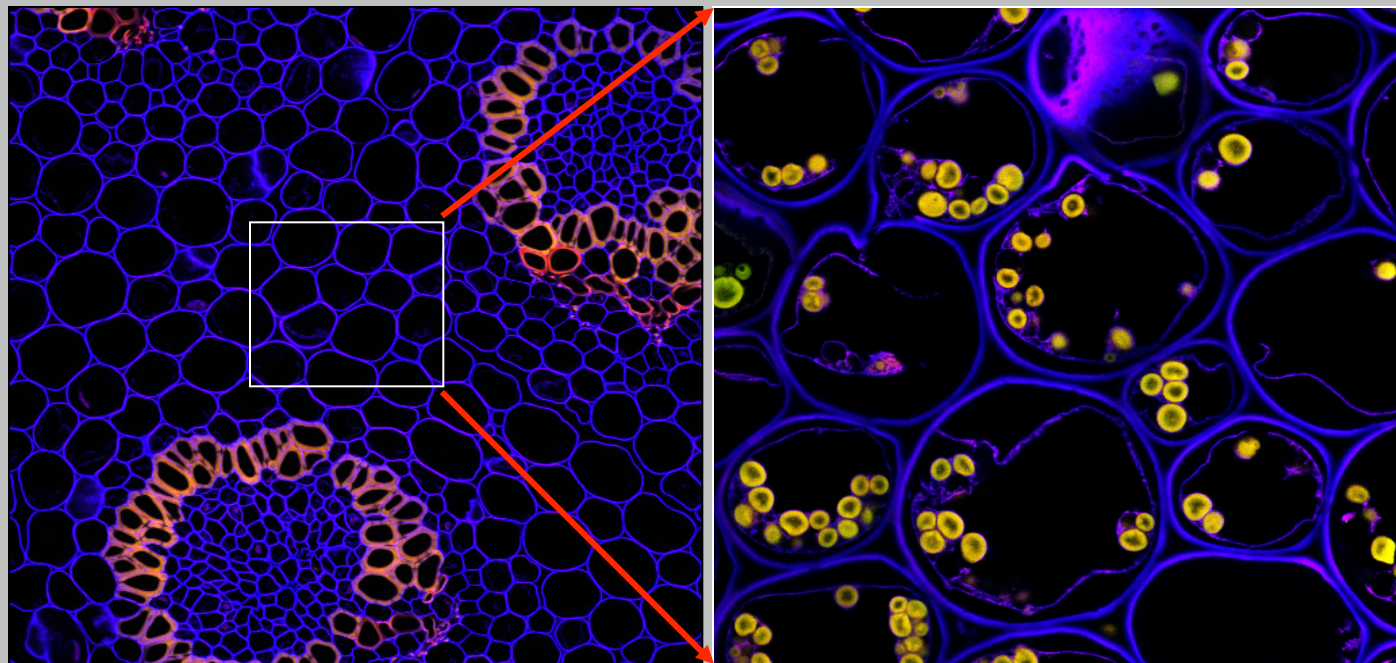


Image (right) Convallaria - projected stack 400 x with 4.26 Zoom

Smart Gain  
0.0

Smart Offset  
0.4%

Scan Field  
Rotation 0.0

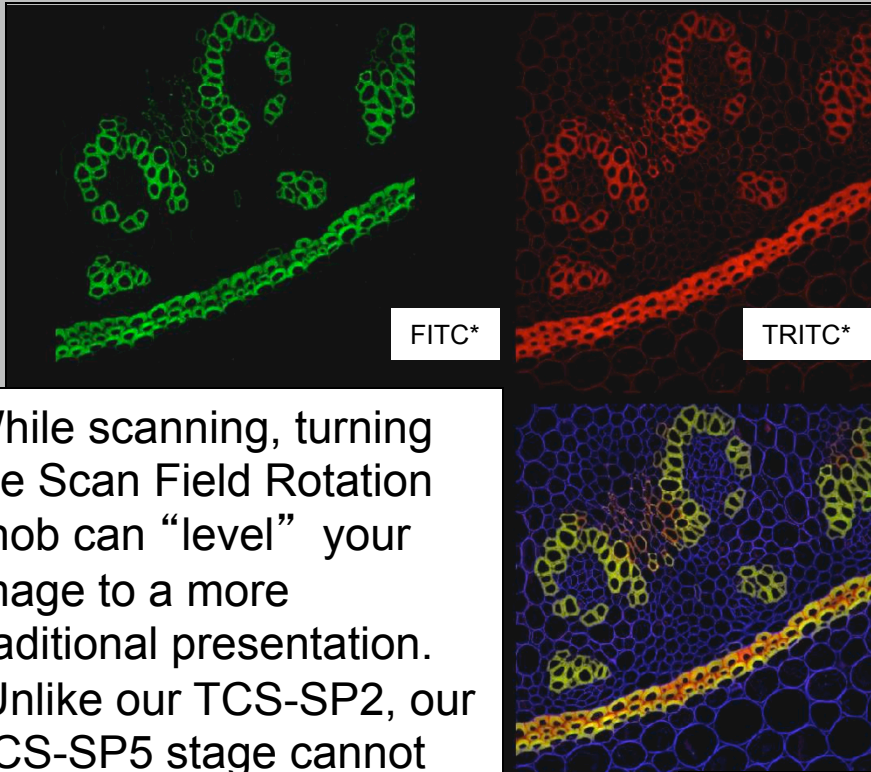
Pinhole  
67.5  $\mu\text{m}$

Zoom 4.26

z Position  
0.00



# SCAN FIELD ROTATION



While scanning, turning the Scan Field Rotation knob can “level” your image to a more traditional presentation. (Unlike our TCS-SP2, our TCS-SP5 stage cannot be turned manually)

Convallaria - \*Leica Settings - 400x

Smart Gain  
0.0

Smart Offset  
0.4%

Scan Field  
Rotation 0.0

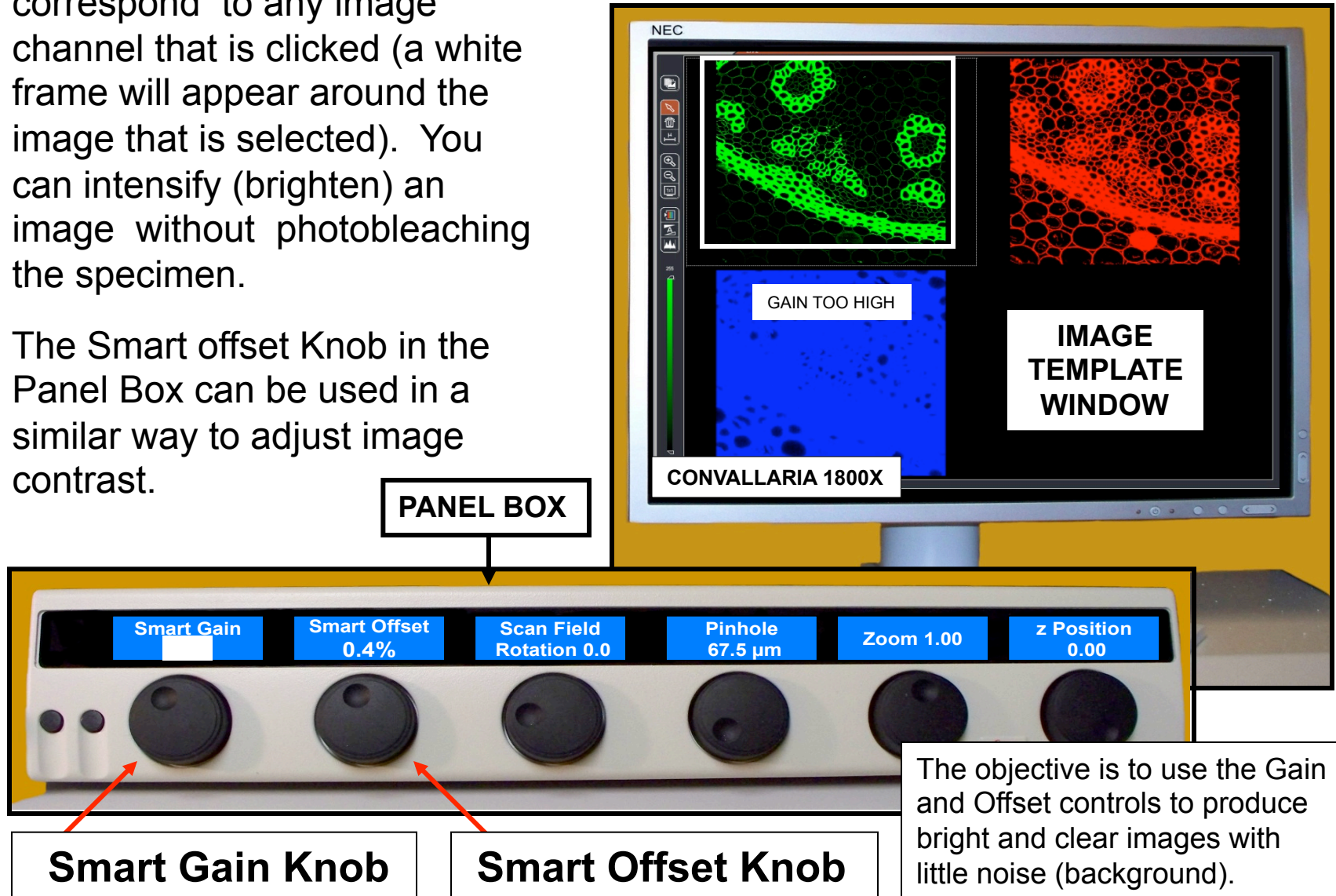
Pinhole  
67.5  $\mu\text{m}$

Zoom 1.00

z Position  
0.00

Leica

15. Click on an image in the image template window and Adjust the Smart Gain knob until the image becomes bright and clear. The single Smart Gain Knob will automatically correspond to any image channel that is clicked (a white frame will appear around the image that is selected). You can intensify (brighten) an image without photobleaching the specimen.
16. The Smart offset Knob in the Panel Box can be used in a similar way to adjust image contrast.





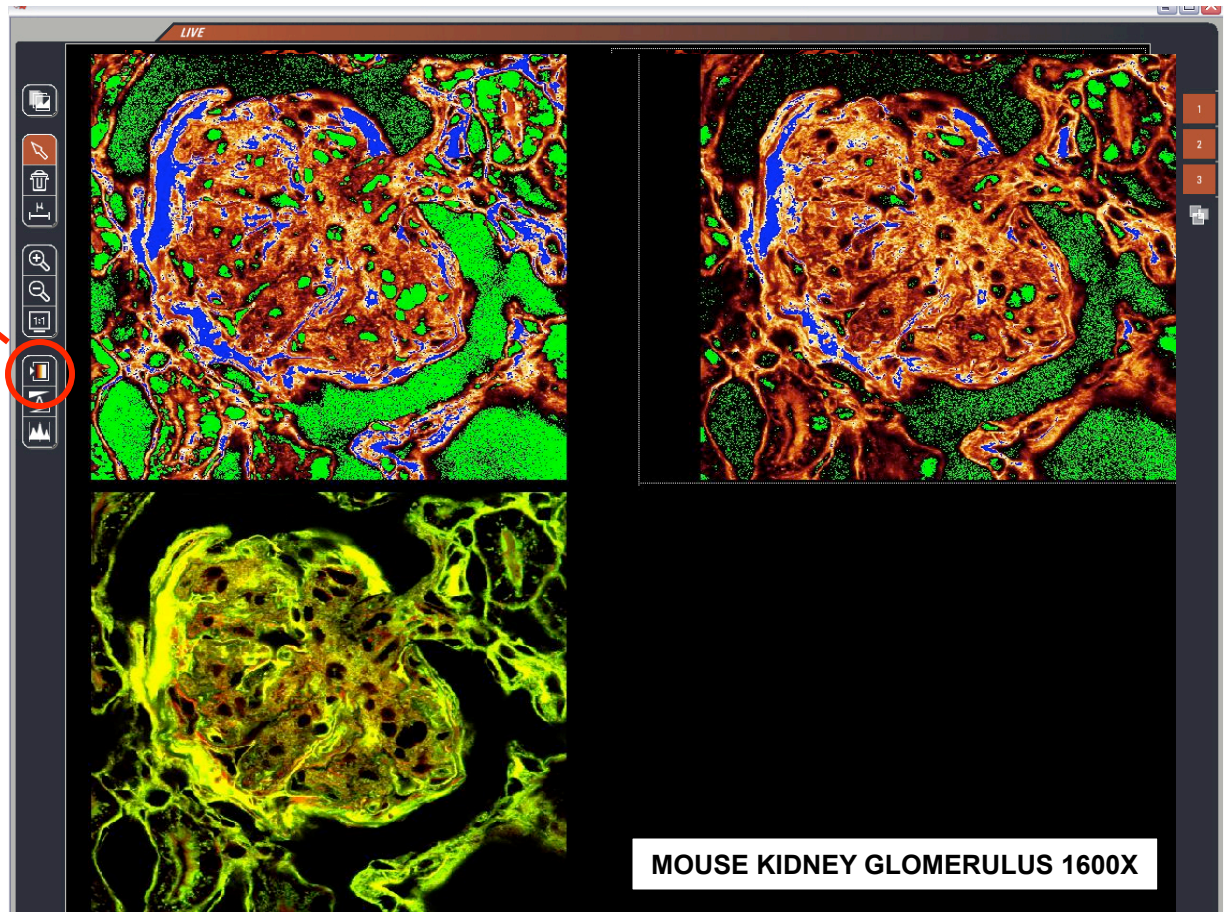
To produce an image of maximum quality, both the gain (brightness) and offset (contrast) must be set to mutually ideal conditions. These settings can often be best and most easily achieved by first changing the colors of the image to intensity values and then adjusting the gain and offset.

**17.** Click on the **QLUT button** to change the colors of an image to intensity values.

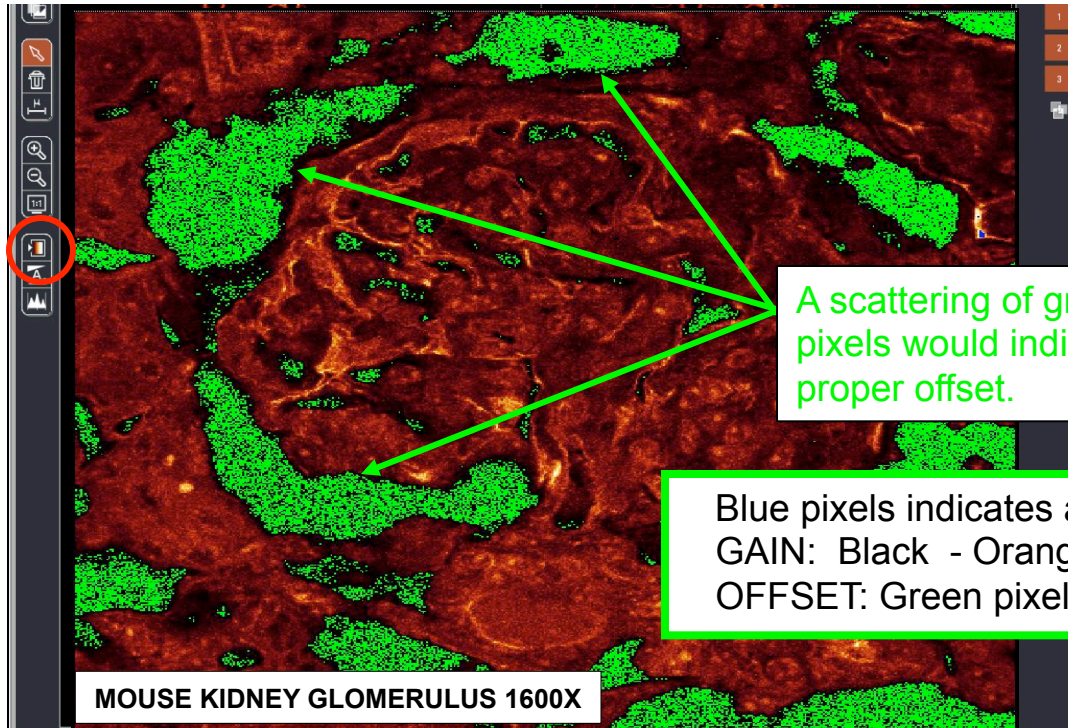


**17.**

QLUT Button  
(Quick Look-Up  
Table)

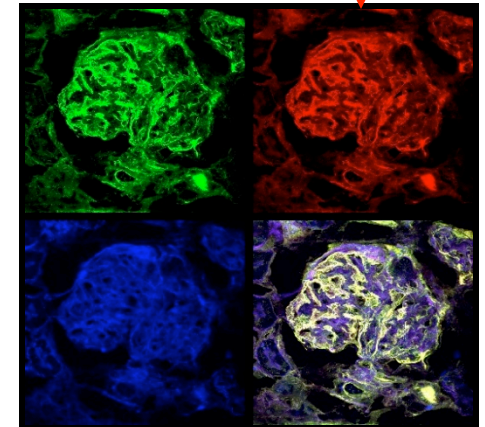
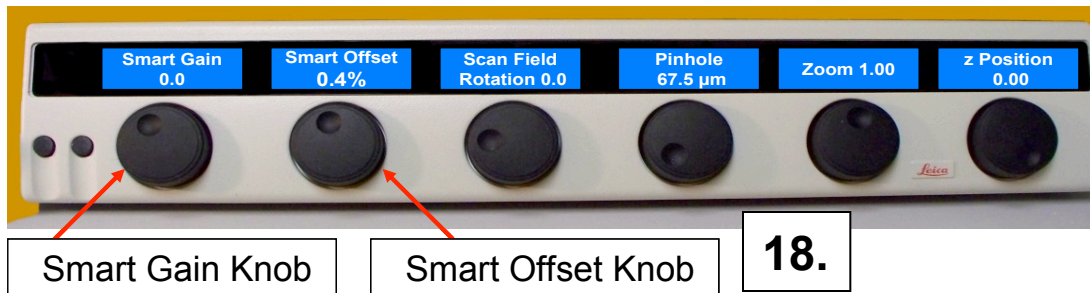


18. Adjust the Gain so that so that the image exhibits mostly black, orange and white components with only a very few, if any, blue pixels. Then adjust the Offset so that the background is represented mostly in green.

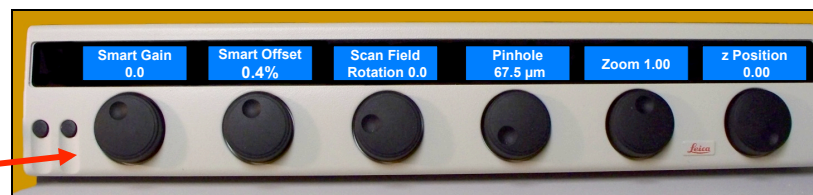
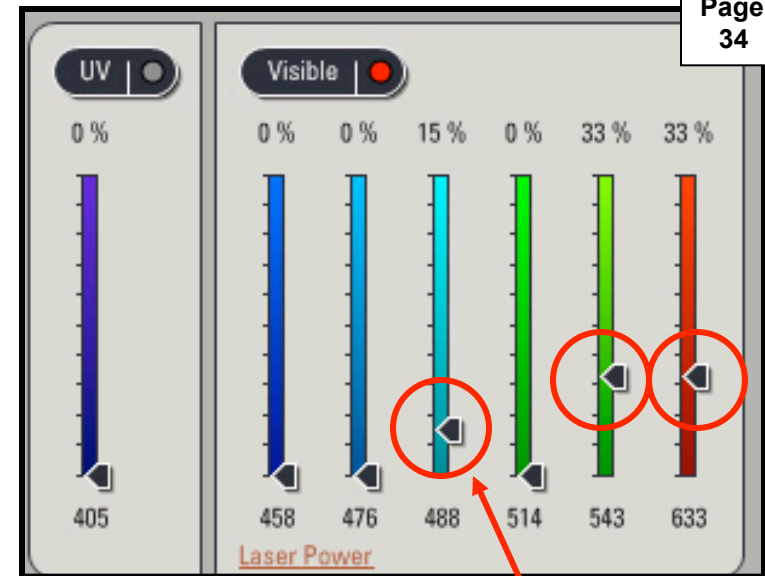
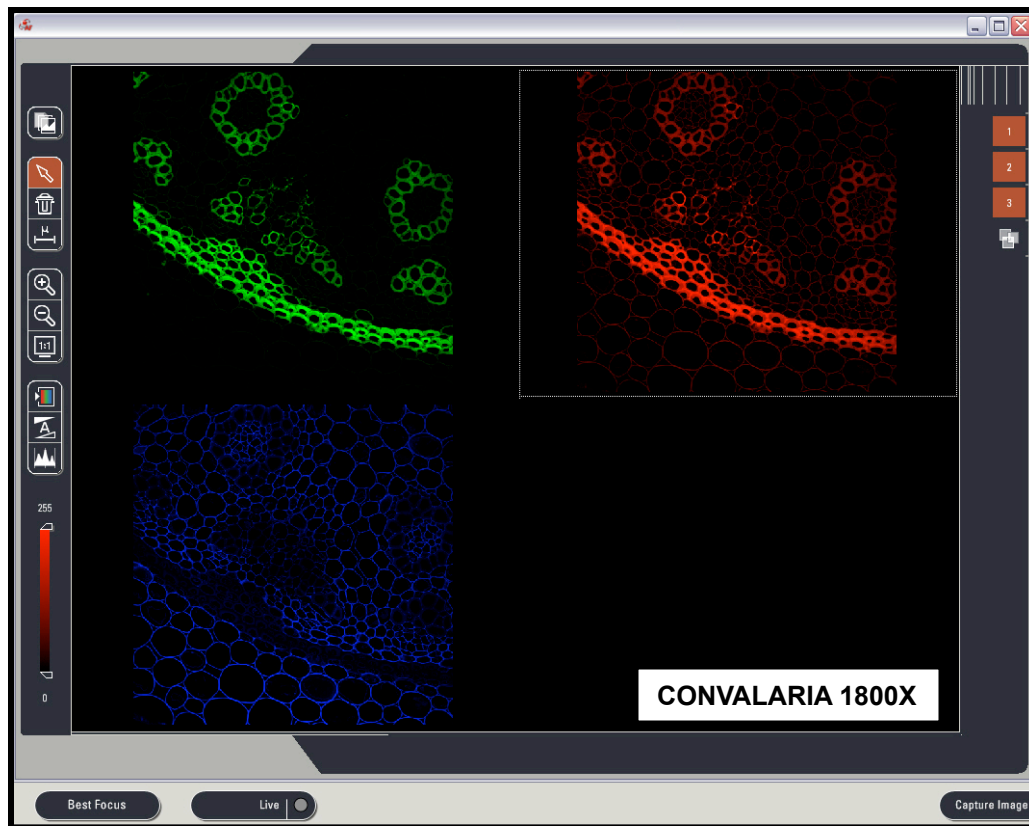


19. Double click on the QLUT button to change the intensity values back to their original color.

19.



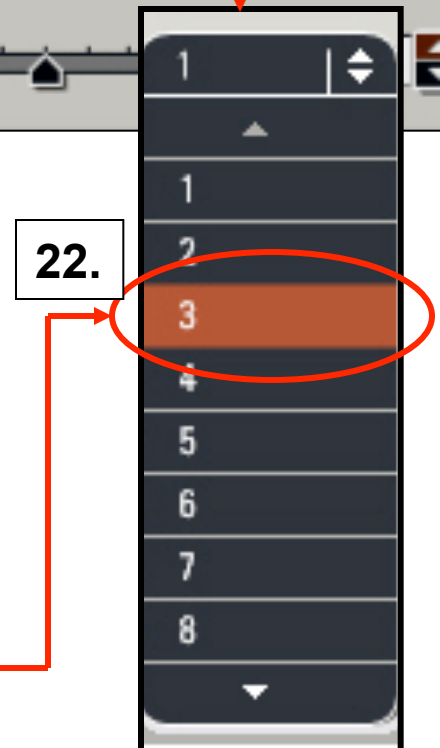
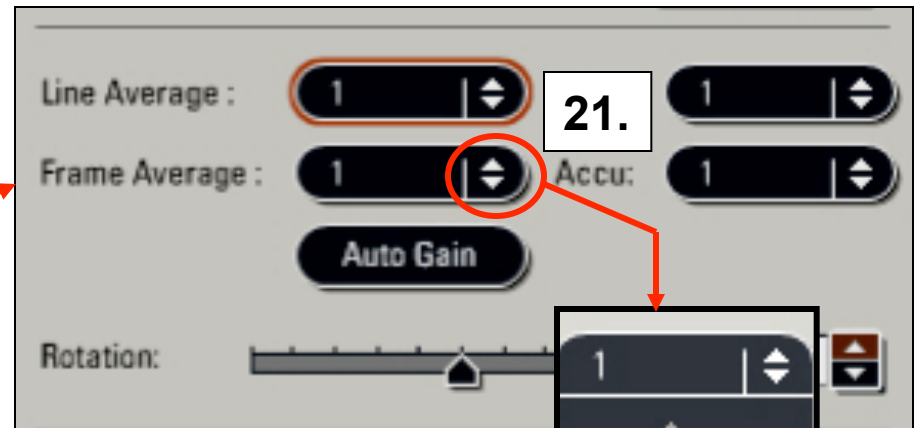




## REMINDER:

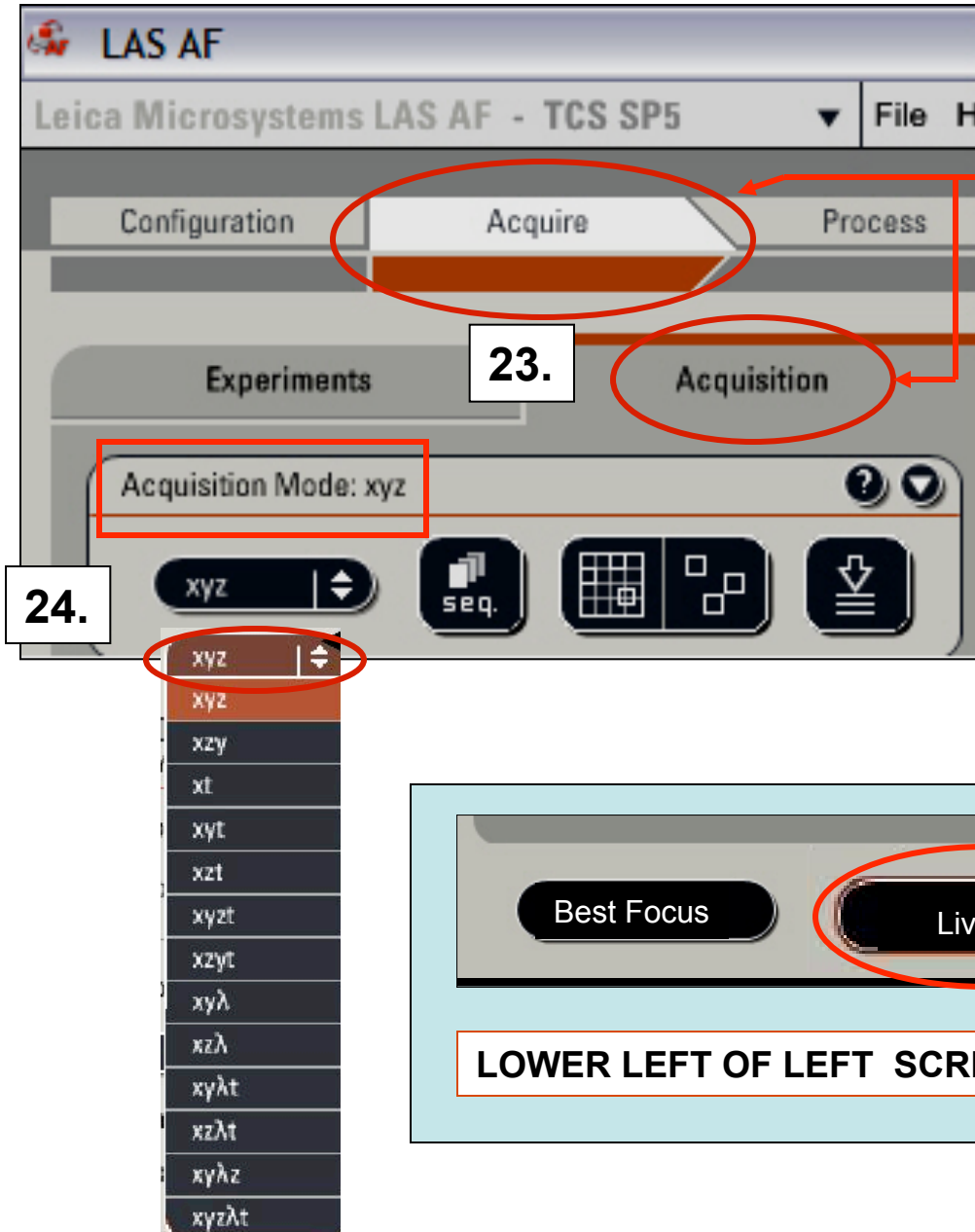
Adjusting the gain on a PMT will brighten an image without photobleaching the specimen. However, if an image is still not sufficiently visible on the monitor, increasing the laser power (vertical slider on the AOTF to a higher percentage) may be necessary, but doing so risks photobleaching the specimen.

Image graininess (noise) can be reduced by resampling / rescanning each specimen slice several times. The software will automatically combine (average) and store the data for each slice.



20. Click on the <Setup tab> in the <Acquire window>.
21. Click on the Line Average and/or Frame Average up-down arrows.
22. Select desired number of averages from the drop-down menu ( e.g. 4).

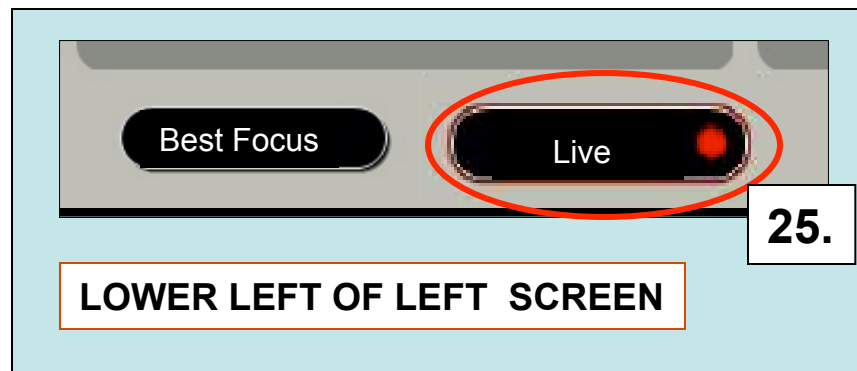
# ACQUIRING A Z-STACK



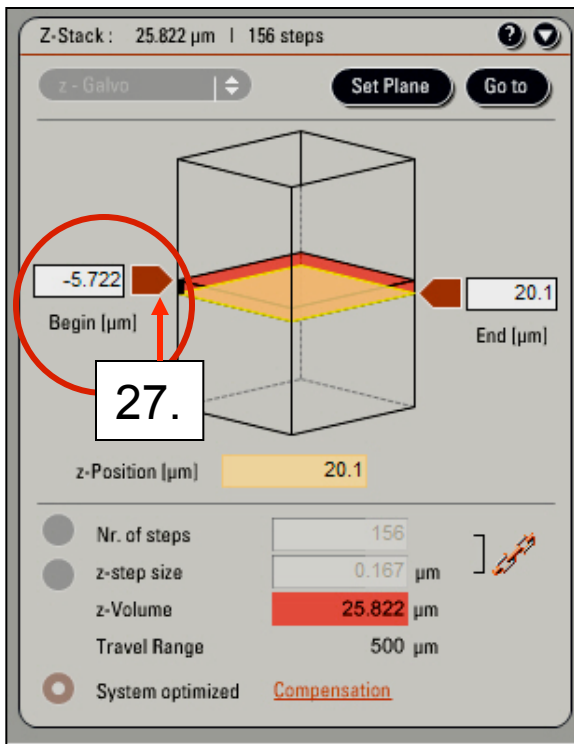
23. Click on the <Acquisition> under the Acquire tab.

24. Click on Scan Modes in the Acquisition Mode menu and select XYZ (usually defaults).

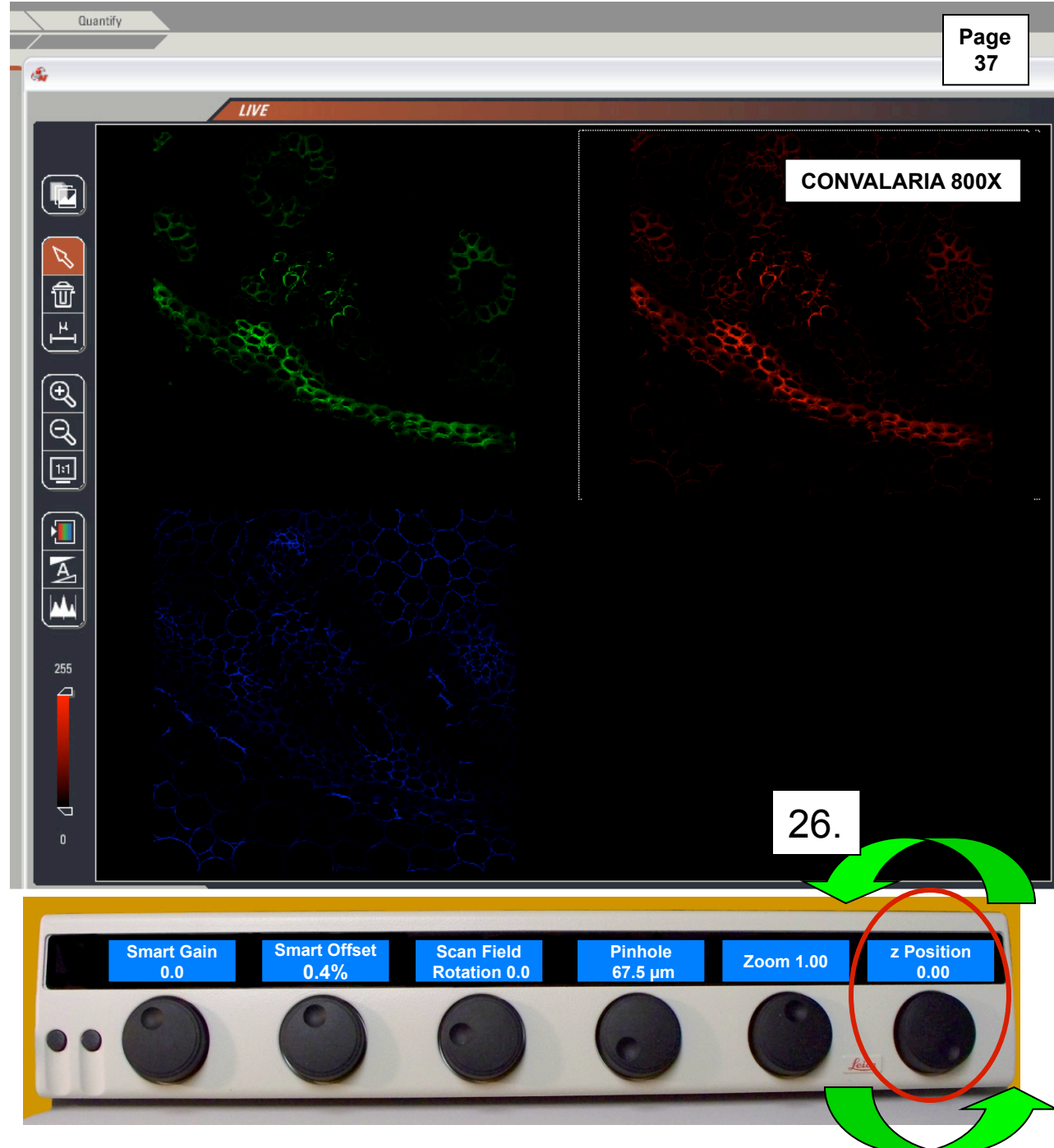
25. Click on live mode (bottom left of the User Interface window).



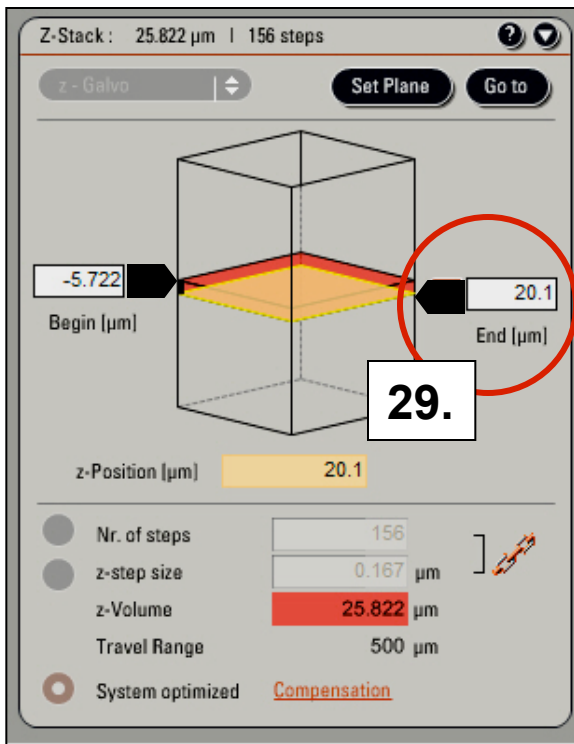
While the lasers are scanning, proceed to next step.



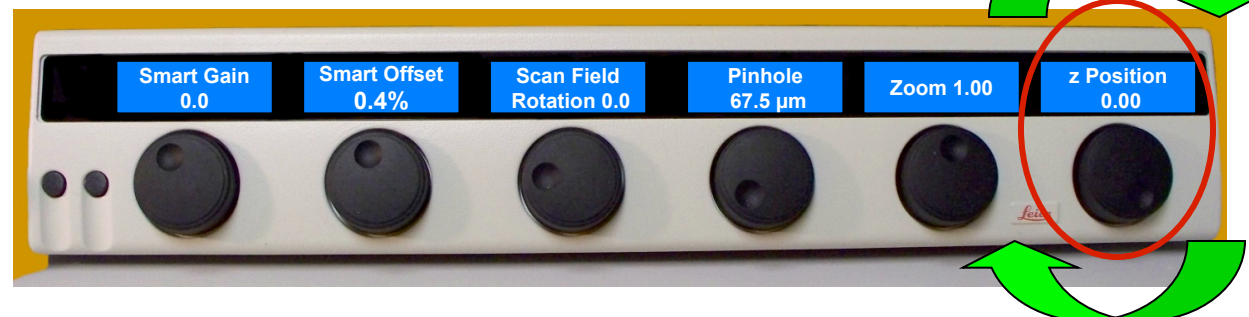
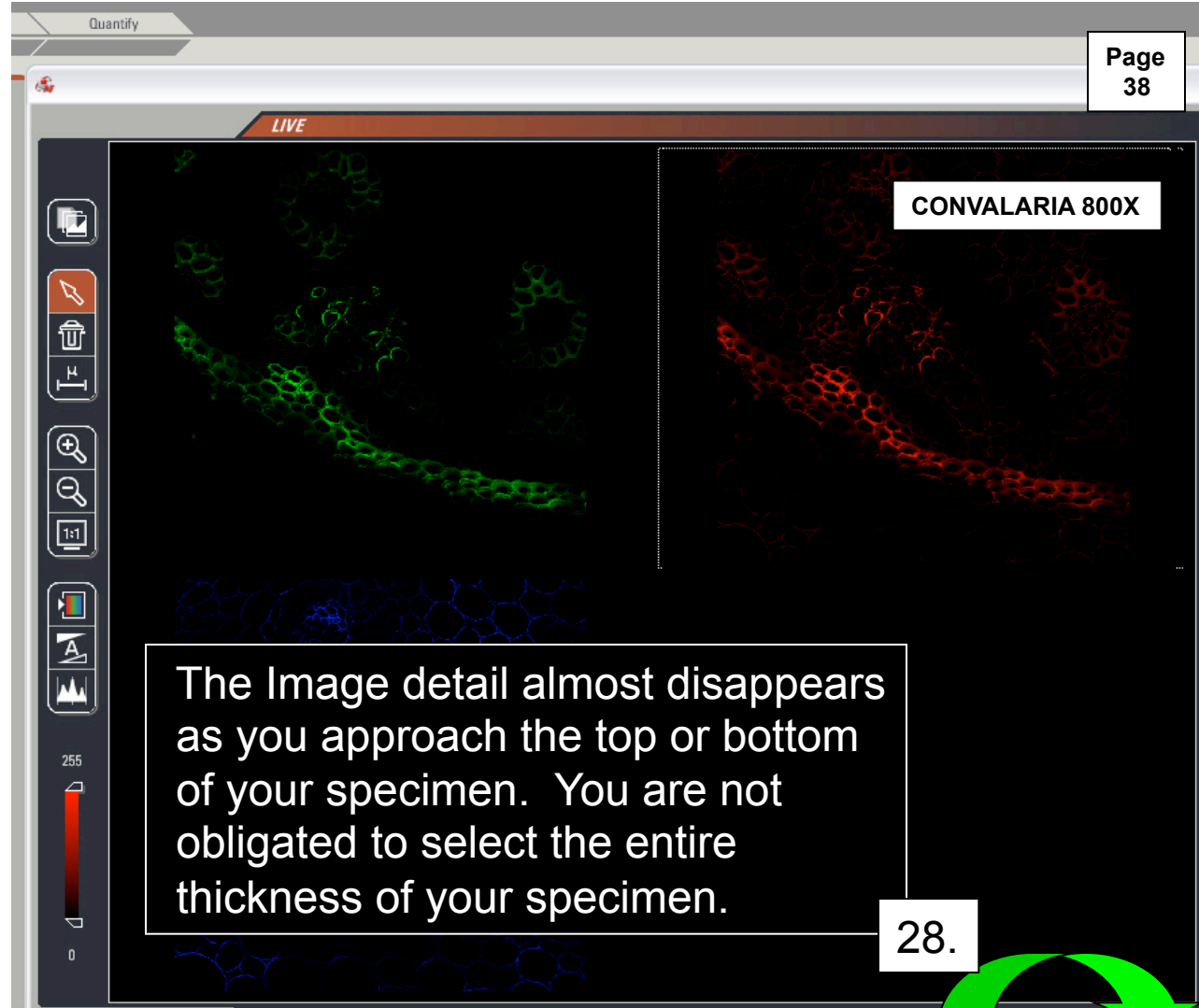
26. While scanning, turn the **Z-Position knob counterclockwise** to move the scan to the **TOP** of your region of interest and then **27. Click on the Begin arrowhead**. This arrow head will turn black.







**28.** While still scanning, turn the Z-Position knob clockwise to move the scan through the specimen to the Bottom of your region of interest and then **29.** Click on the End arrowhead. This arrowhead will also turn black.

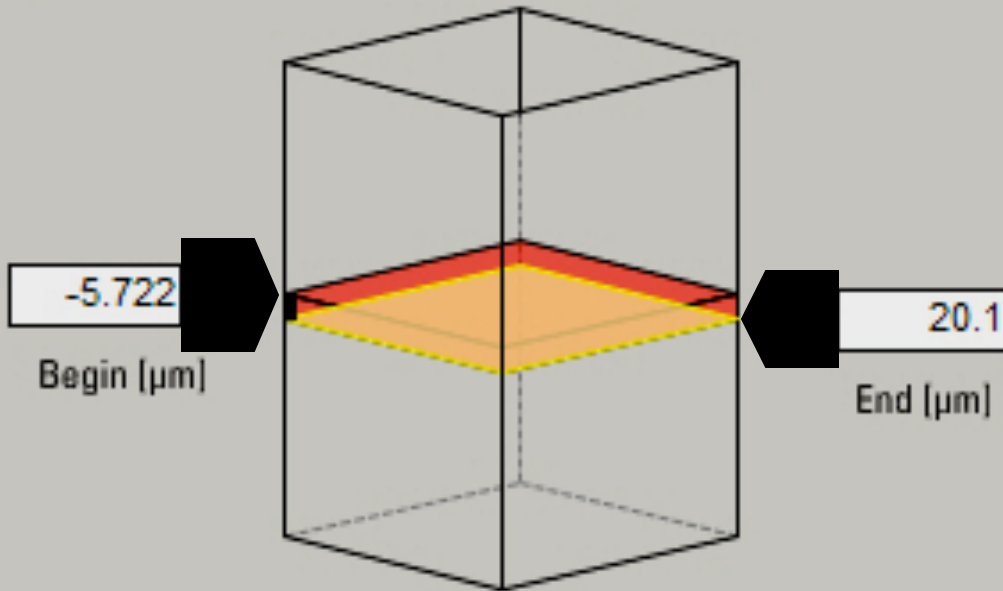


Z-Stack : 25.822  $\mu\text{m}$  | 156 steps

z - Galvo

Set Plane

Go to



Begin [ $\mu\text{m}$ ]

20.1

End [ $\mu\text{m}$ ]

z-Position [ $\mu\text{m}$ ] 20.1

- ☐ Nr. of steps 156
- ☐ z-step size 0.167  $\mu\text{m}$
- ☒ z-Volume 25.822  $\mu\text{m}$
- ☐ Travel Range 500  $\mu\text{m}$
- ☒ System optimized Compensation

The Z – Volume (depth) in microns becomes highlighted.

Page  
39

30. Click on <Stop> to stop scanning and to access the Z – step size window.



LOWER LEFT OF SCREEN

If you **Click** on **System optimized** the software will automatically set the optimum number of **z** - steps and step size based on the selected objective, zoom and image format. **BUT DOING SO RESULTS IN VERY LONG SCAN TIMES and LARGE FILES.** We generally **do not** recommend that you do this. *Instead...*

**Nr. of steps** 50

**Z-step size** 0.5 µm

**z-Volume** 25.882 µm

**Travel Range** 500µm

**System optimized** Compensation

31.

31. Type in the step size of 0.5 and the software will calculate the number of slices (pages) that will be generated. This approach generates a smaller file size, reduces scan time, the risk of photobleaching and produces images that are usually highly resolved.
32. Click <start> and the z-stack image acquisition will begin. A progress bar indicates the remaining time until the scan is complete.
- 32.
- 

Remaining Time: 59 sec

15 %

Configuration

Acquire

Process

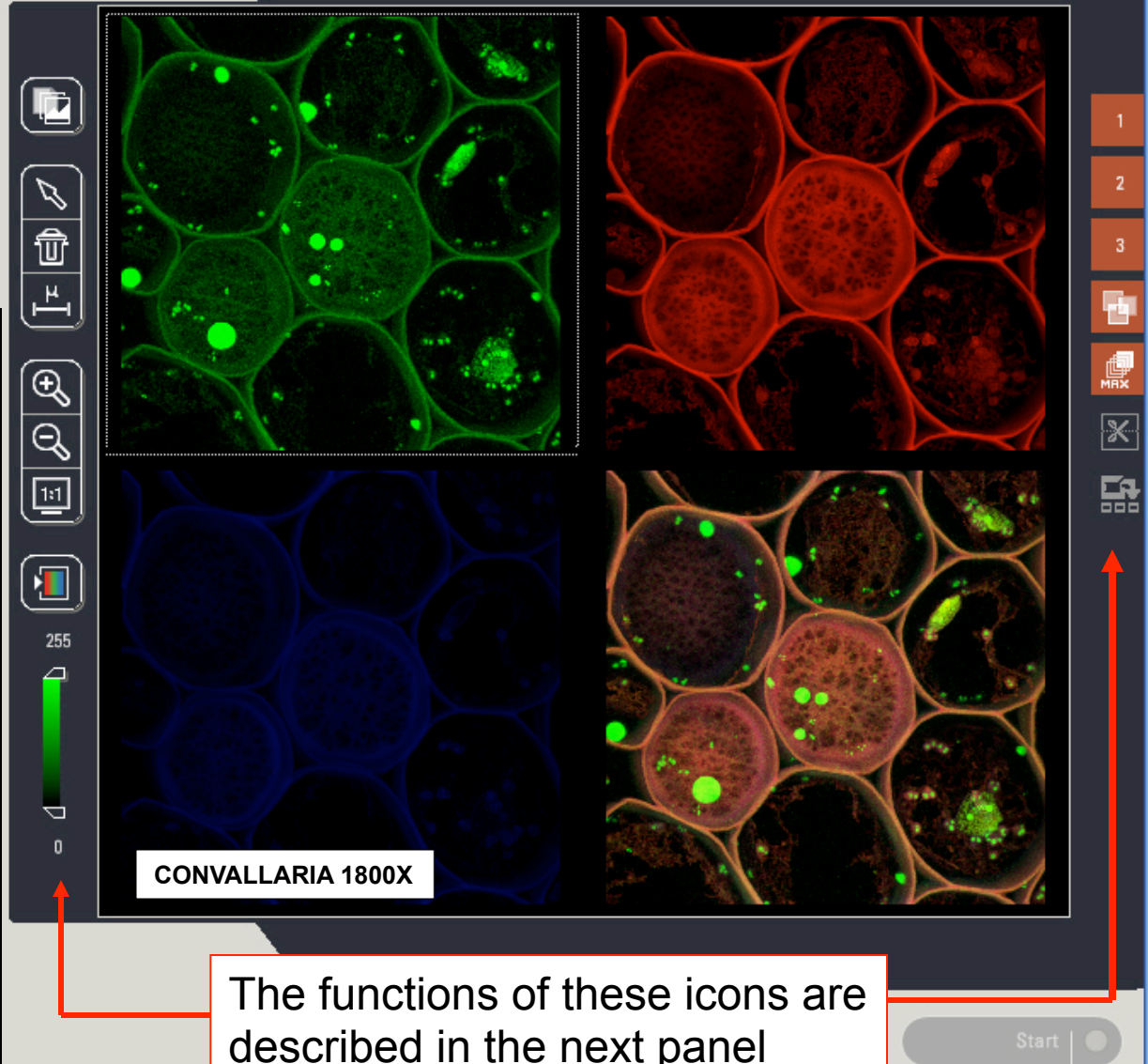
Quantify

33.

Experiments

▼ Experiment Feb 23 09.tif (1.2 GB)  
Image002 (786 KB, xy)  
Series004 (346.0 MB, xyz)  
Series004\_ProjMax001 (453.3 MB, xy\*)  
Series004\_ProjMax002 (372.7 MB, xy\*)

When the collection of a series is complete, **33.** click on <Experiments> and a “Virtual Memory” appears listing the titles of Images and Series that have been collected and automatically stored here. These data can be processed using the tools that are listed on the left and right sides of the image template window.



The functions of these icons are described in the next panel

Configuration

Acquire

Process

Quantify

Experiments

34.

To project an image or series;

**34.** click on a desired title in the Virtual Memory window, and the selected image data will appear in the image template.

Clicking on a tool icon will initiate the functions described.

Expands tool options

Highlights items to be moved on the screen

Trash - delete

Insert a scale & measurement bar (click and drag)

Zoom in or out on a captured image

Resizes images to avoid pixelation when saved

Movie controls and slice slider

Annotation tools

Channel on - off selection (Seq. scan)

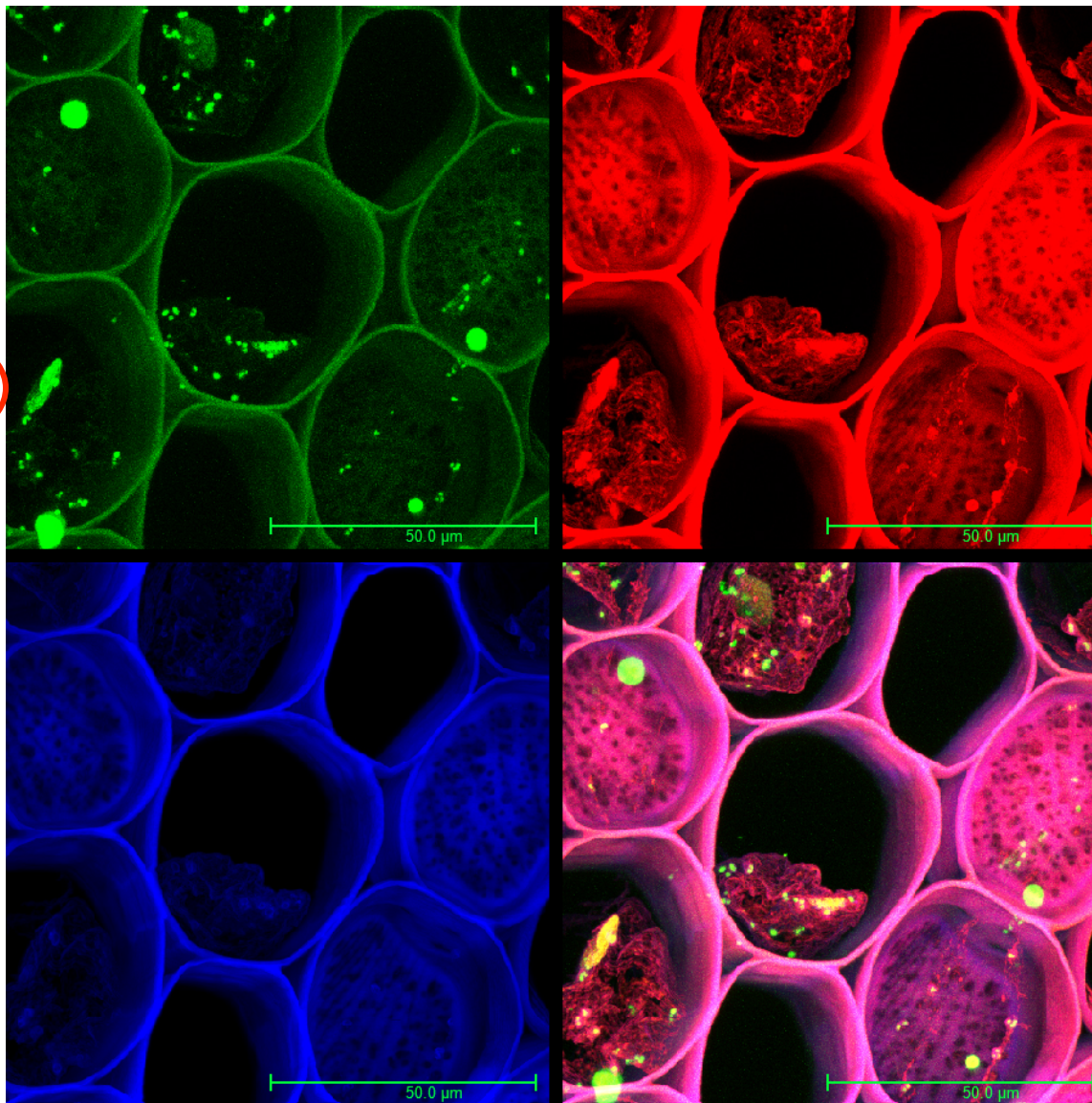
Overlay all channels

Maximum projection  
Projects all images in a series

Orthogonal Projection  
Shows selected X and Y edges of a Z stack series

Allows viewing of images while scanning a series

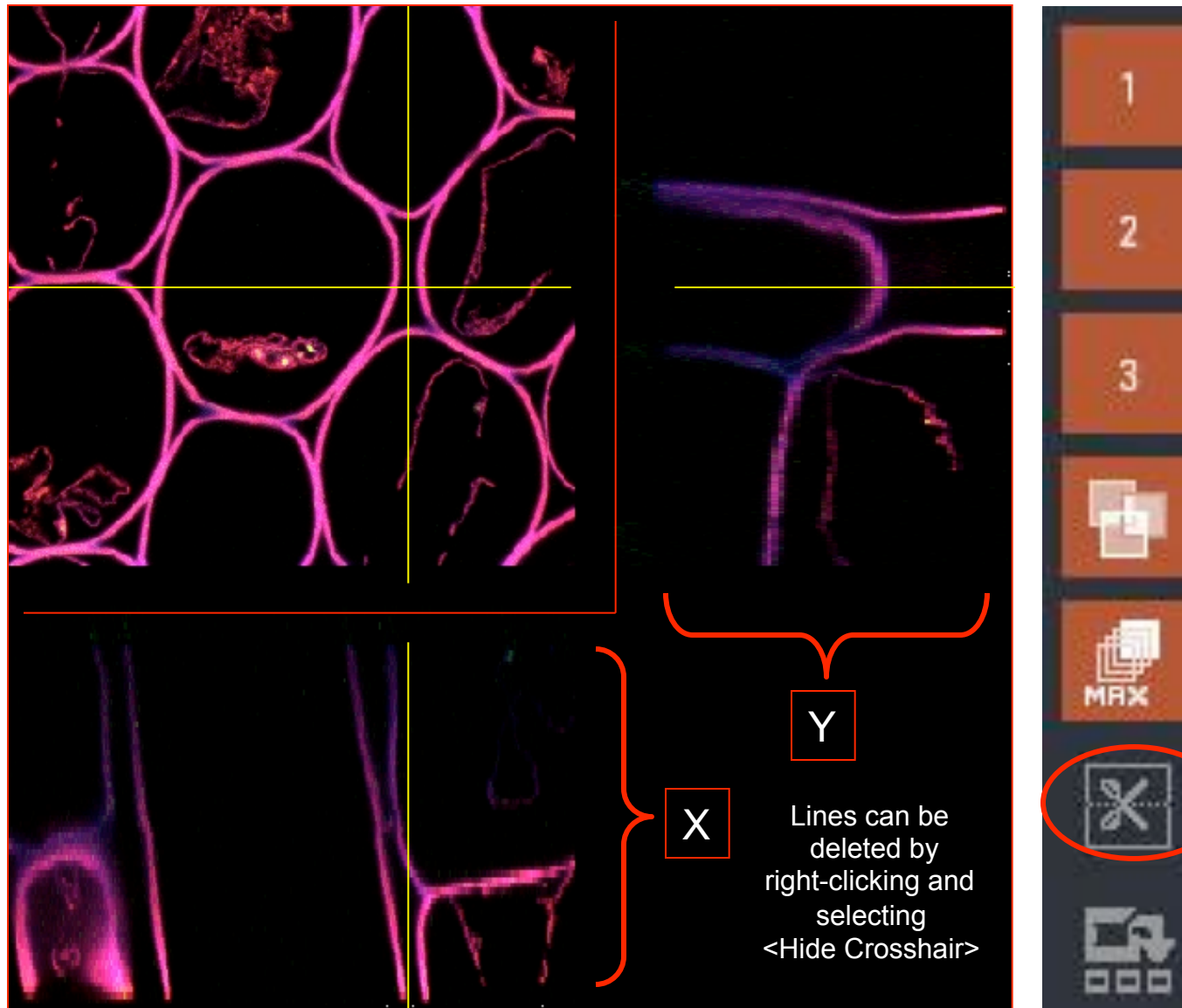




**INSERTING A SCALE BAR**

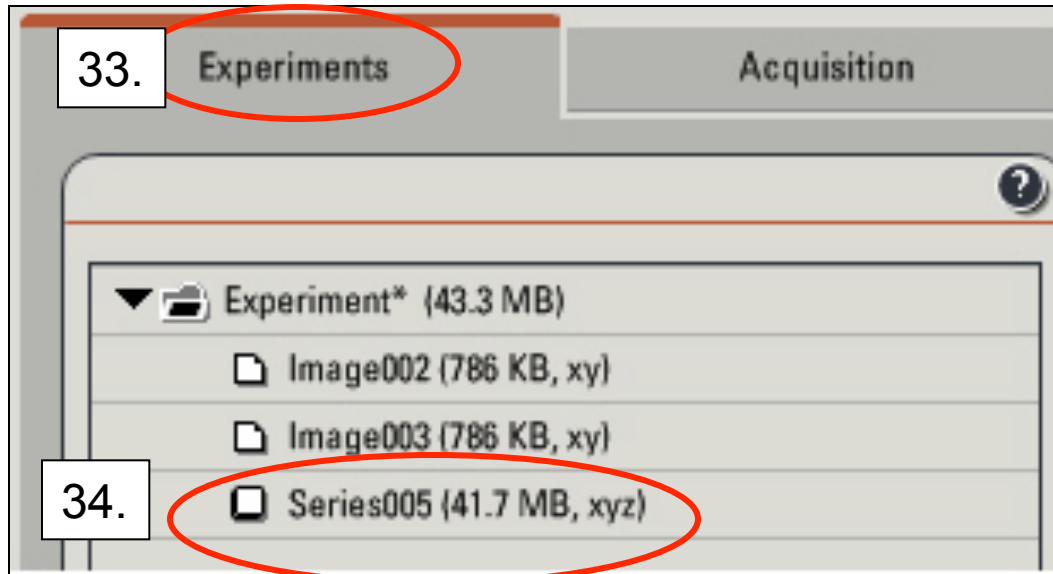
The Scale Bar, Overlay, and Maximum projection tools were used to create this set of images. To save this set of images and lines to the virtual window, right click and click on <All Snapshot>.

# ORTHOGONAL VIEWS



This orthogonal (X and Y) edge views of vertical planes through a Z-Series was constructed through the Orthogonal View icon.

# Creating a 3-Dimensional Projection



33. Click on the experiments tab and click on the name of the desired image series.

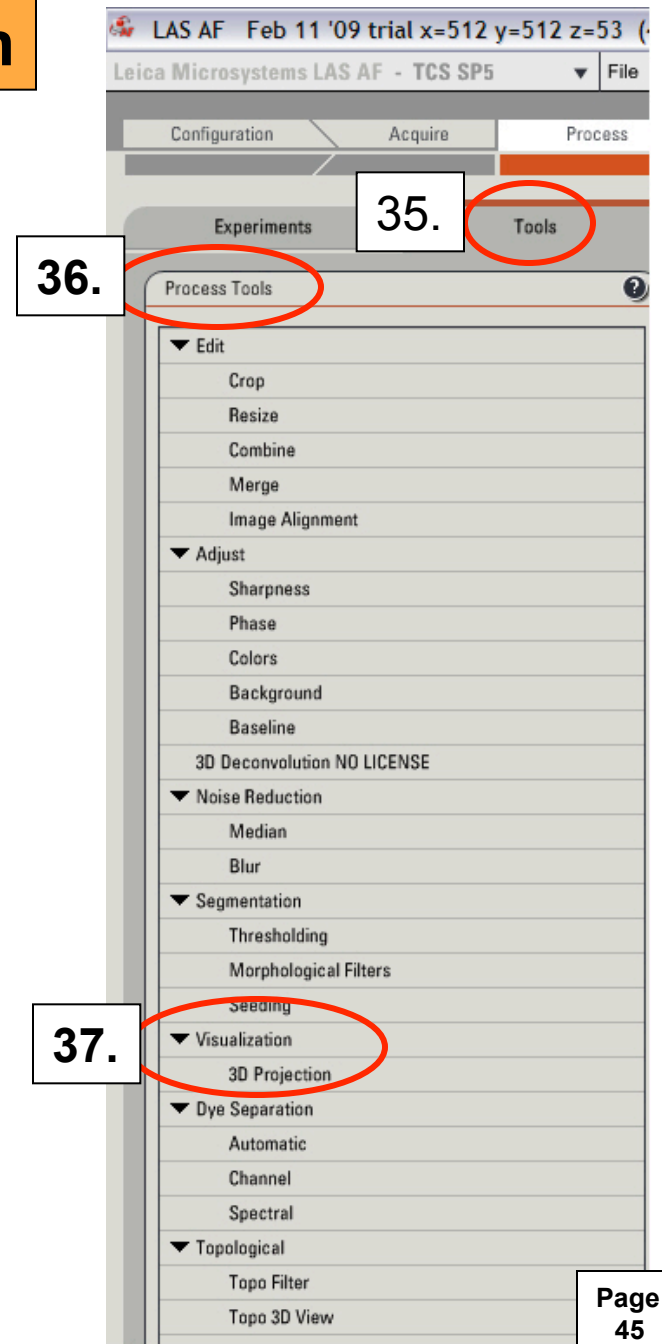
34. Click on the Process tab.

35. Click on Tools.

36. Click on Process Tools

37. In the Process Tools, Click under Visualization and 3D-Projection (near the bottom of the list).

Please use the Off-Line Confocal Simulator work station for complex projections and rotations.





# SAVING IMAGE FILES

**38.****VIRTUAL MEMORY****39.**

Experiment Feb 23 09.tif\* (1.2 GB)

Image002 (786 KB, xy)

Series004 (346.0 MB, xyz)

Series004\_ProjMax001 (453.3 MB, xy<sup>o</sup>)Series004\_ProjMax002 (372.7 MB, xy<sup>o</sup>)

Series004\_ProjMax002Snapshot1 (151 KB, xy)

Series004\_ProjMax002Snapshot2 (151 KB, xy)

Series004\_ProjMax002Snapshot3 (151 KB, xy)

Series004\_ProjMax002Snapshot4 (151 KB, xy)

Close Experiment "Experiment Feb 23 09.tif"

Save Experiment "Experiment Feb 23 09.tif"

Save All

Save Experiment "Experiment Feb 23 09.tif"

Create Collection

Delete Experiment "Experiment Feb 23 09.tif"

Rename "Experiment Feb 23 09.tif"

Copy "Experiment Feb 23 09.tif"

Export "Experiment Feb 23 09.tif" &gt;

As Tiff

As JPEG

AS AVI

**38.** Click on the <Acquire> tab and <Experiments> tab and the titles of the image data sent to this window will appear. This data is stored only temporarily – It **must** be sent to your data folder in the D-Drive or other storage media. **39.** Right-clicking on the Experiment folder and/or image and series titles will produce a drop-down menu containing your file storage options. **40.** Please save your data frequently during your work session.

Configuration

Acquire

Process

Quantify

Experiments

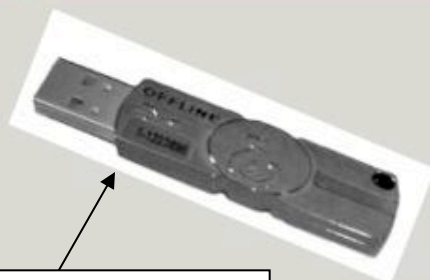
Setup

Acquisition

Light Path

▼ experiment Feb17 '09.lif (131.8 MB)

- Image002 (786 KB, xy)
- Image005 (786 KB, xy)
- Image008 (786 KB, xy)
- Image011 (786 KB, xy)
- Series013 (47.2 MB, xyz)
- Series013\_ProjMax001 (68.4 MB, xy°)
- Series013Snapshot1 (2.5 MB, xy)
- Series013Snapshot All1 (5.6 MB, xy)
- Series013Snapshot All1Snapshot1 (2.5 MB, xy)
- Series013Snapshot4 (2.5 MB, xy)



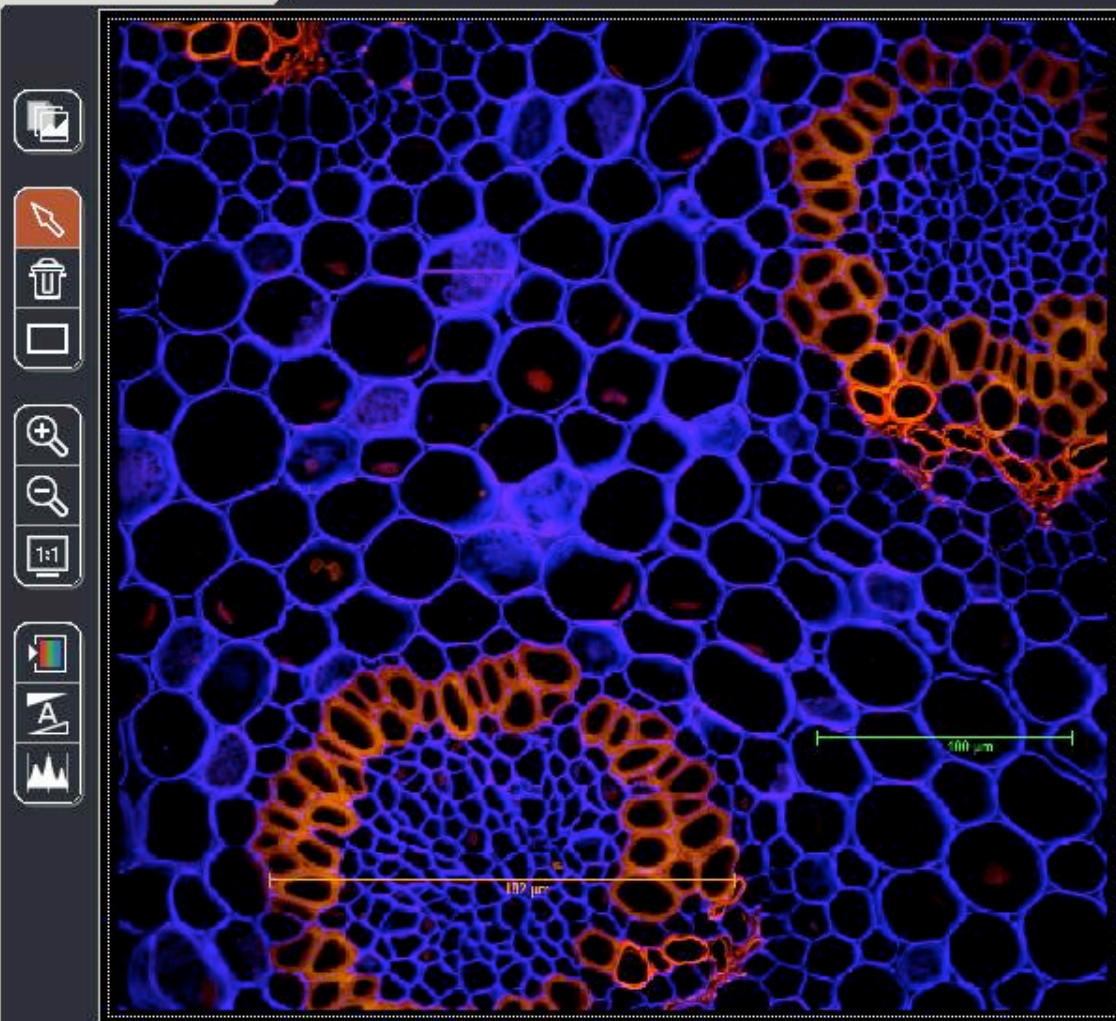
Software Key  
(Dongle)

New

Open

Save All

Apply



Clicking a Leica .lif file can automatically open the Leica Confocal Simulator (LAS v 2.0.2 build 2038) if it is installed on a PC and if the software key is inserted into a USB port. This application will allow the user to manipulate image data in a way similar to the microscope work station.




## TCS - SP5 Off-Line Confocal Work Station - Please see section 2

Processing image data for 3D rotations and movies takes substantial time on our confocal microscope work station. Clients are asked to transfer data to our off-line work station (Confocal Simulator) so that the microscope can be available for other users. Two LAS software versions are available:



Room 249 West  
Full processing version  
Hardware key required



Modified home version  
*No rotation capability*  
single monitor

Please ask for the  
LAS AF Lite  
application if you  
would like to install  
it on your PC computer.  
(Windows® only)

