

Leica SP8

Basic Image Acquisition

Laser Scanning Confocal

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09/14/17

General Information

Laser and Objective Information:

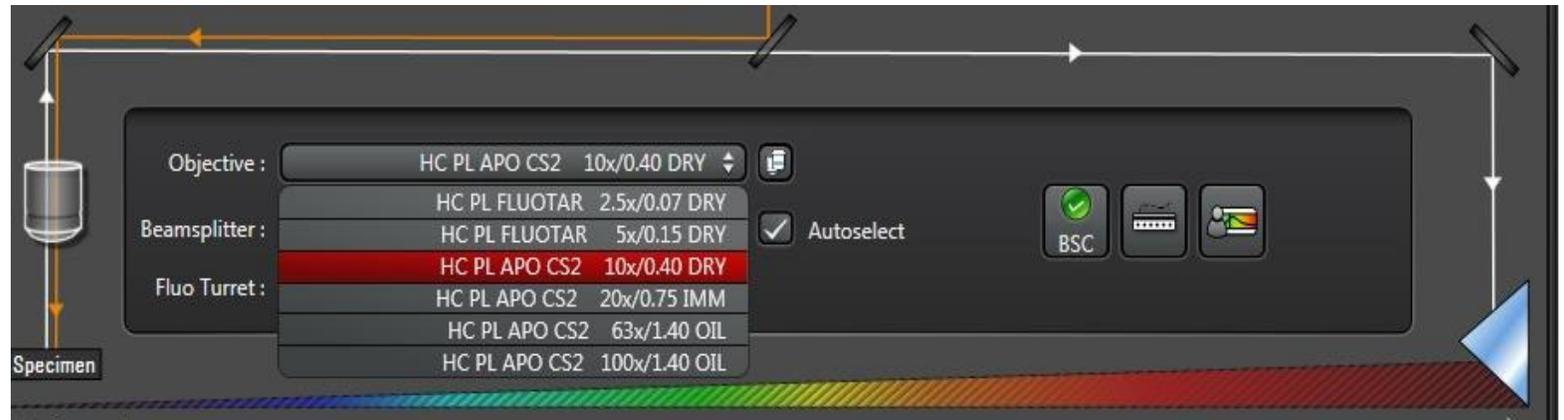
Available Lasers:

- 405nm
- 488nm
- 552nm
- 638nm



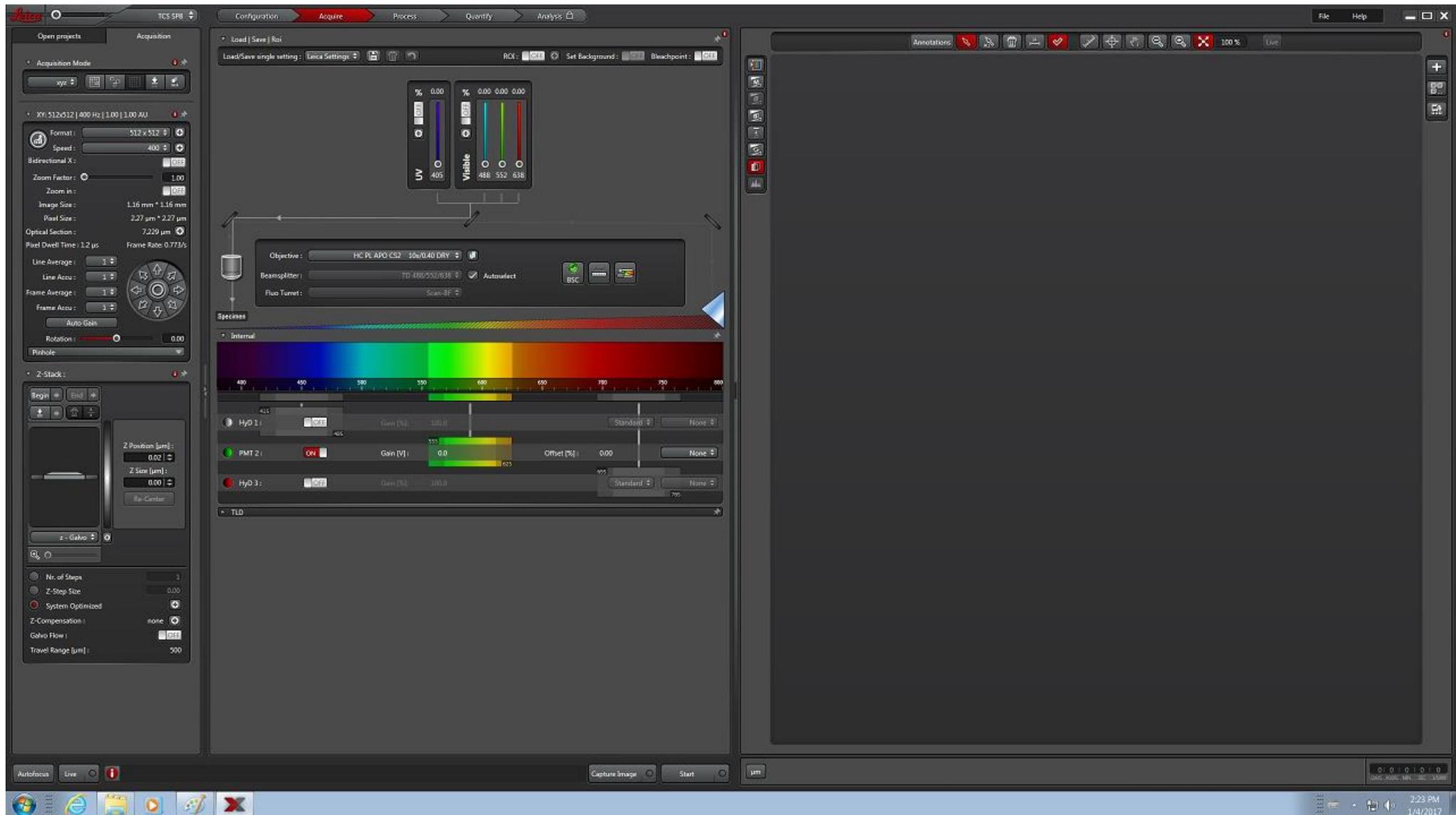
Sample of Available Objectives:

- 2.5x Dry
- 5x Dry
- 10x Dry
- 20x Multi- Immersion
- 63x Oil
- 100x Oil



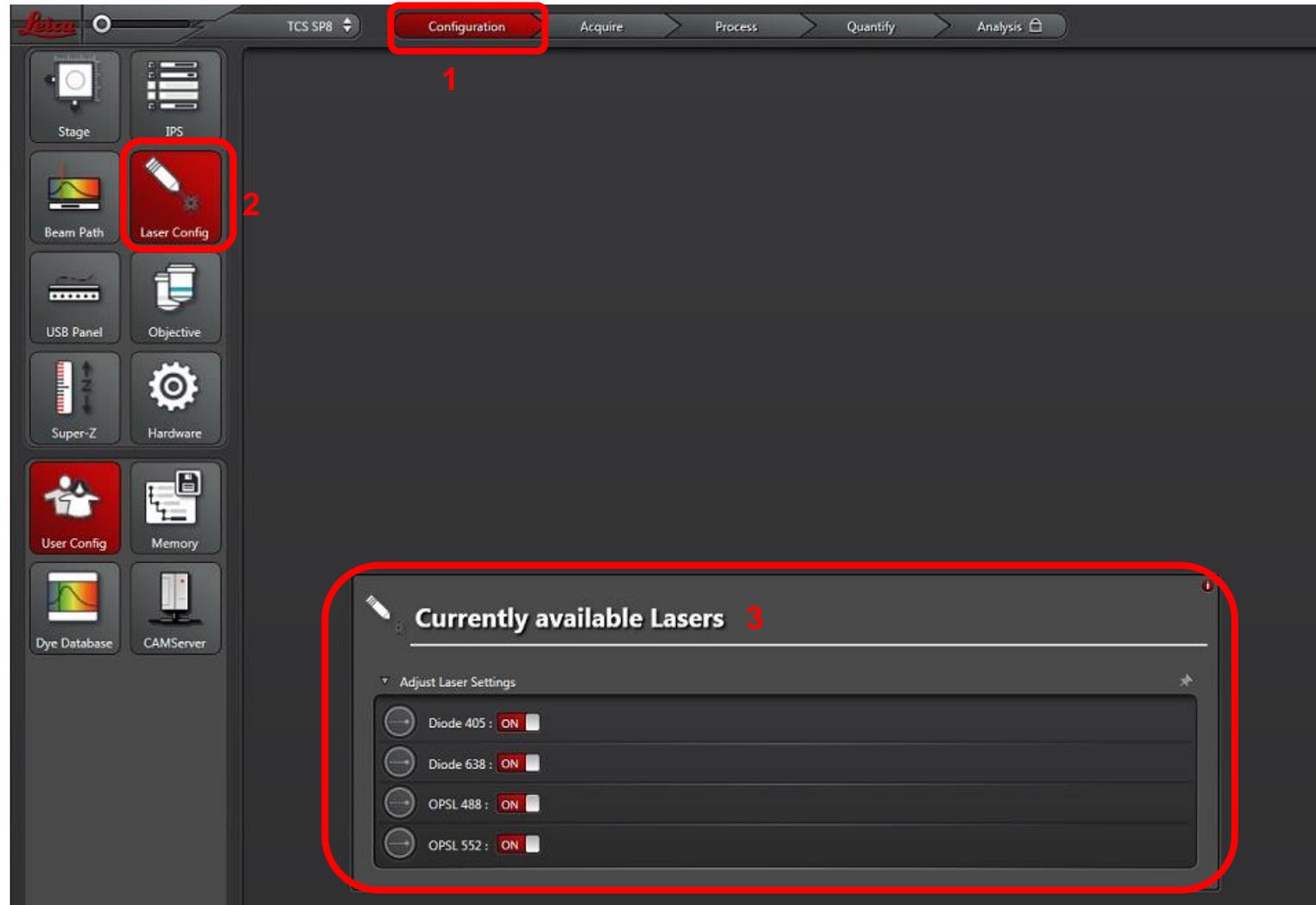
3 Color Image Acquisition

This is the acquisition work area.



1. On the top of the work area - Click Configuration.
2. Click the Laser Configuration button.
3. Turn on all the lasers.

The lasers available on this microscope are: 405nm, 488nm,

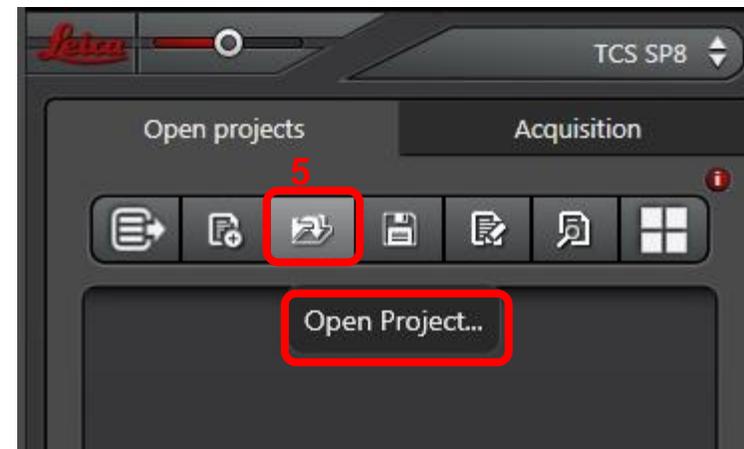
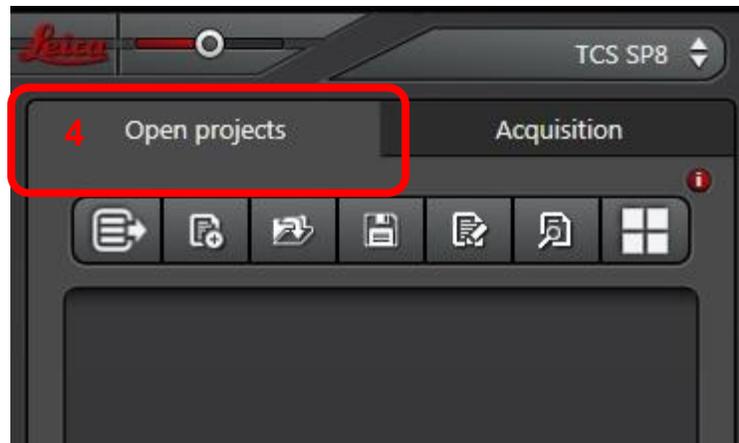


There are 3 different ways you can set your lightpaths:

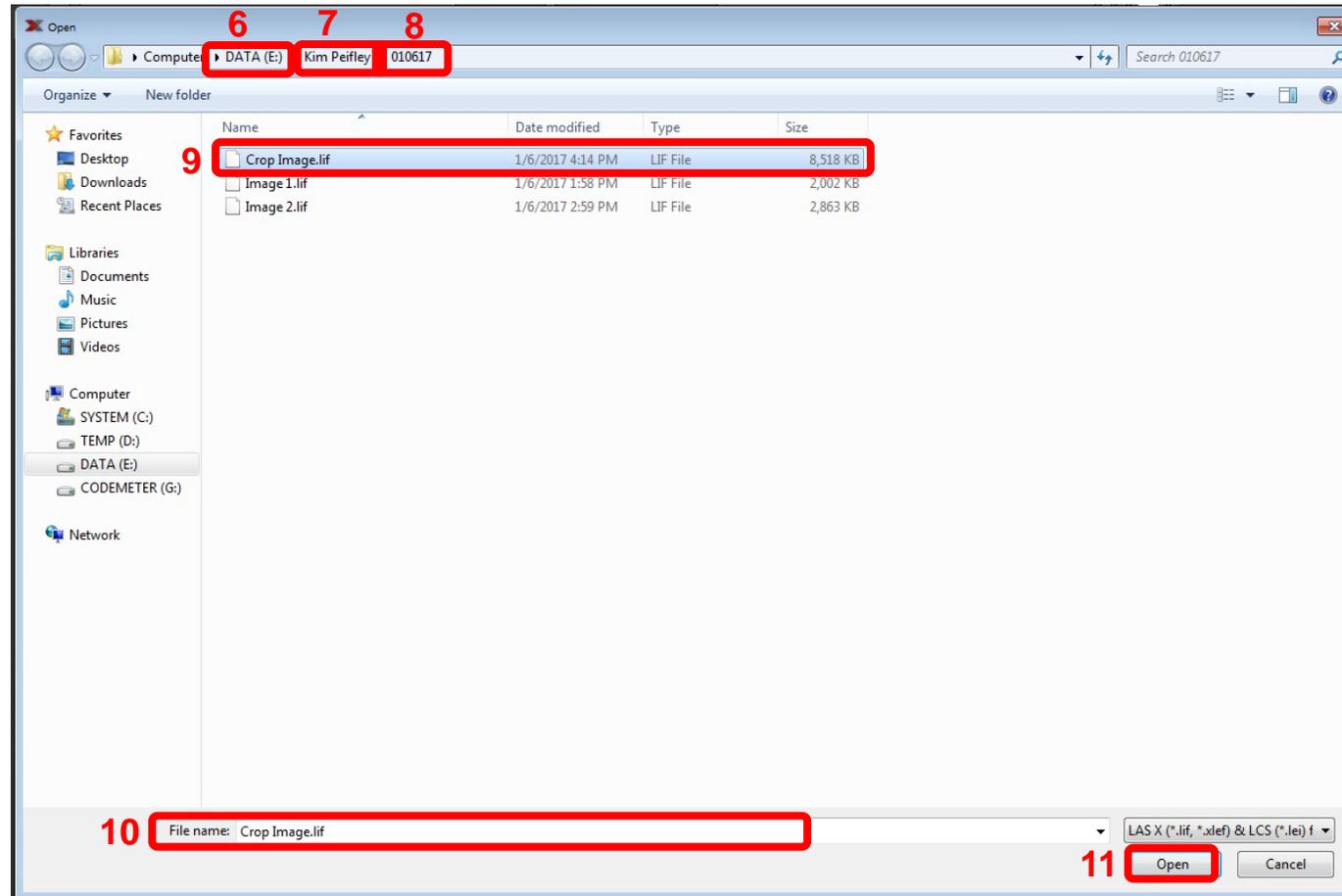
- 1. Reuse settings from an image you previously acquired - Follow Steps 4 – 35.**
- 2. Use preset configurations - Follow Steps 36 – 61.**
- 3. Use dye assistant - Follow Steps 62 - 85.**

To reuse settings from a previous image you acquired:

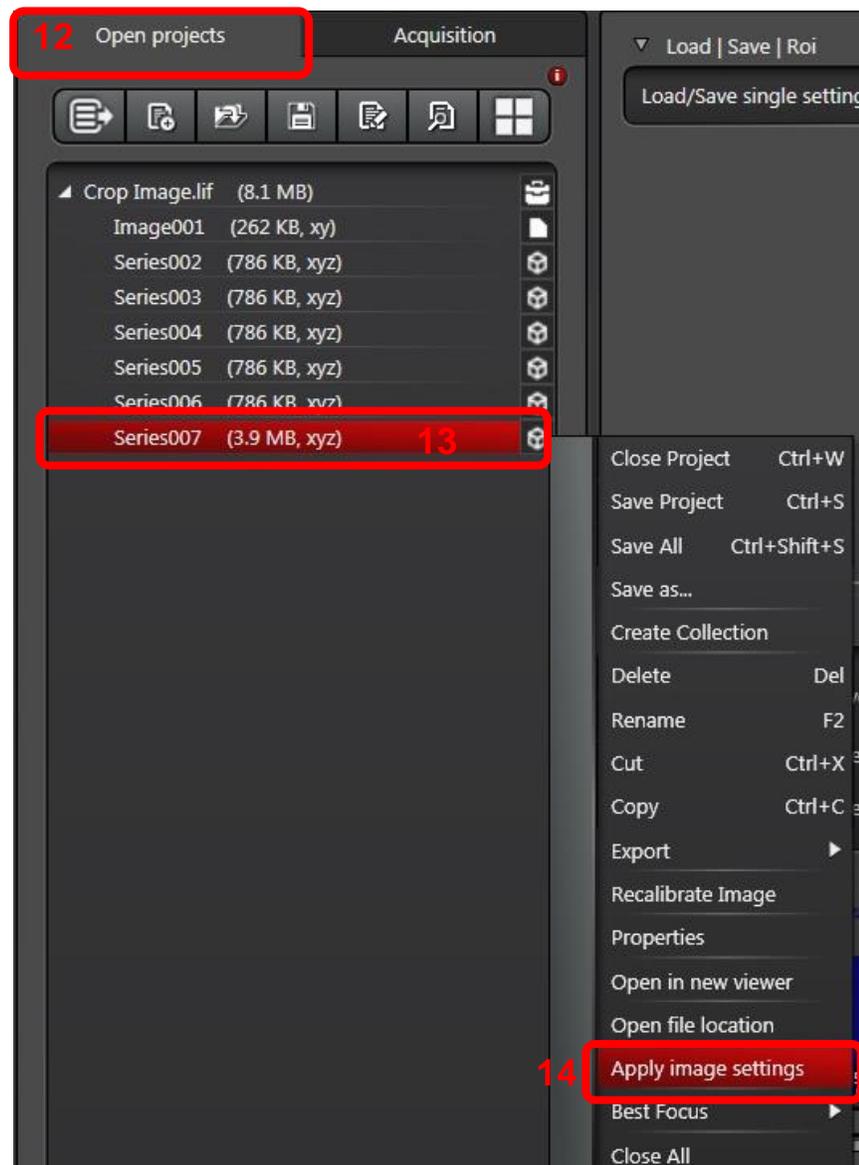
4. Click the Open projects tab.
5. Click the open projects icon. Looks like a folder.



6. Go to the Data (E:) drive.
7. Select your folder.
8. Click on the folder you wish to enter.
9. Select the image file [.lif file] you wish to open.
10. Make sure selected image file [.lif file] appears in the “File name:” box.
11. Click Open.



12. Go back to Open projects tab.
13. Select image you wish to open.
14. Right click and select Apply image settings.

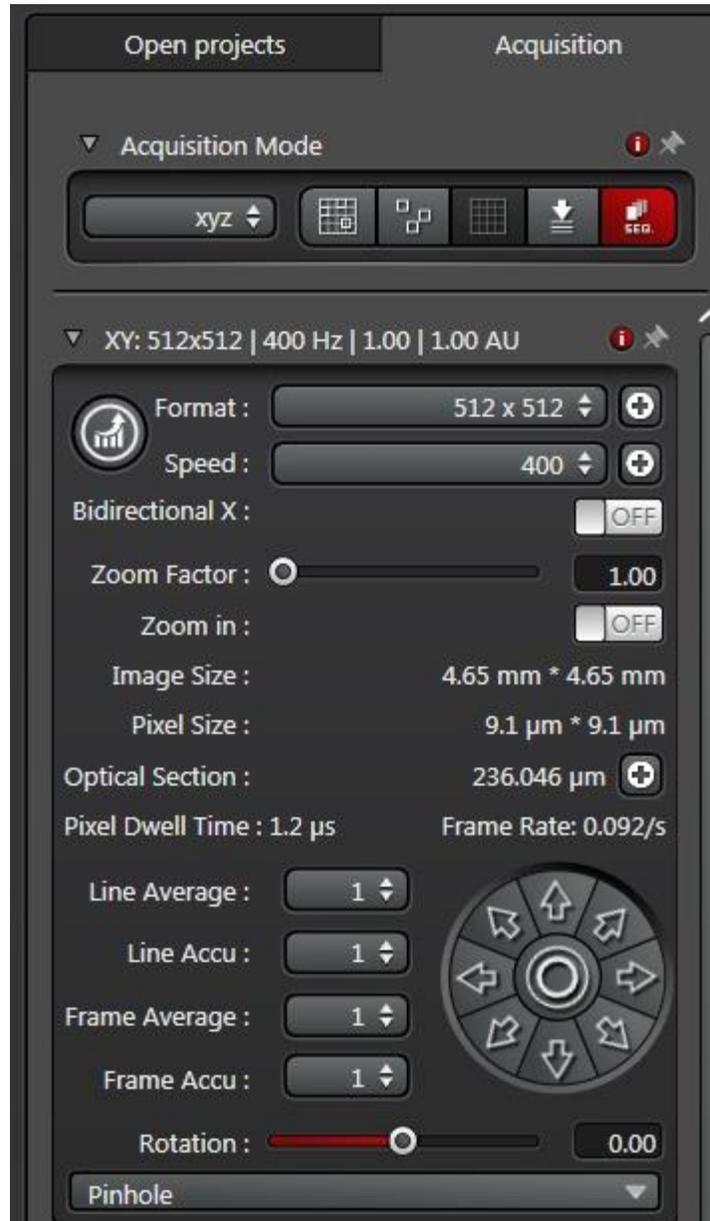




- 15. Go to the Acquisition tab.
- 16. Click on a Sequence and check to make sure the light path settings are as you want them.
- 17. Check the other Sequences.

A screenshot of microscope software. The left panel shows 'Z-Stack' settings with 'Z Position [µm]: 0.00' and 'Z Size [µm]: 0.00'. Below are 'Sequential Scan' options: 'Nr. of Steps: 1', 'Z-Step Size: 0.00', 'System Optimized' (checked), 'Z-Compensation: none', 'Galvo Flow: OFF', 'Travel Range [µm]: 500'. At the bottom, 'Seq. 1', 'Seq. 2', and 'Seq. 3' are visible, with 'Seq. 2' highlighted by a red box and labeled '17'. The right panel shows 'Internal' settings with a spectral graph. Below the graph, 'HyD 1' and 'PMT 2' are OFF. 'HyD 3' is ON, highlighted by a red box and labeled '16'. Its 'Gain [%]' is 100.0 and the filter is 'ALEXA 568', also highlighted by a red box. The 'TLD' section is partially visible at the bottom.

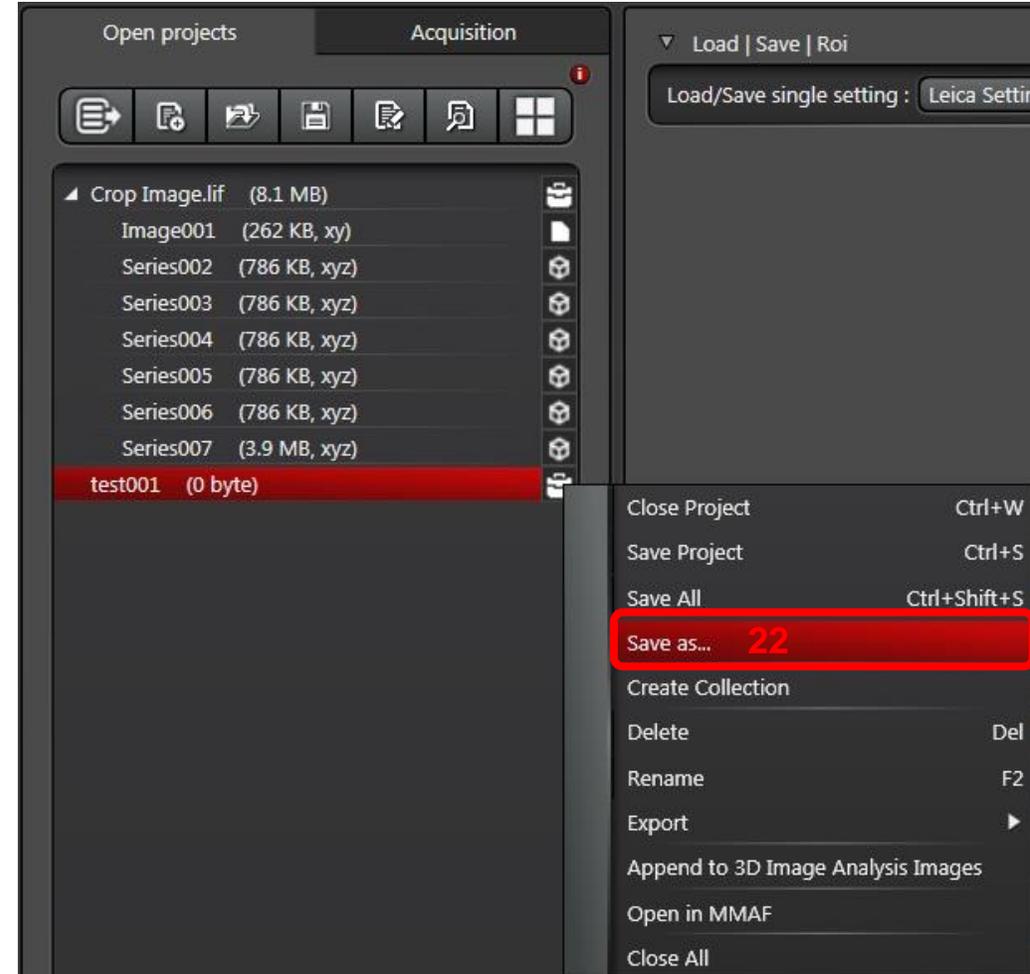
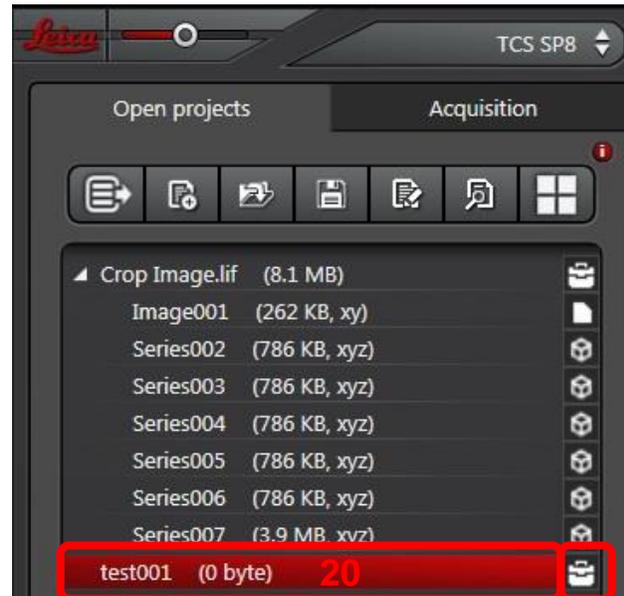
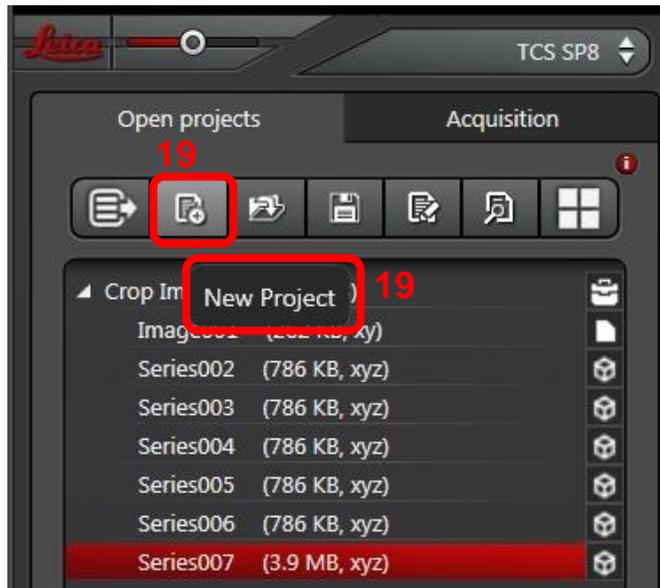
18. Check to make sure Format [frame size], Speed [scan speed], Zoom Factor, Average and Pinhole are as you want them. Apply Image Settings will not set these for you.



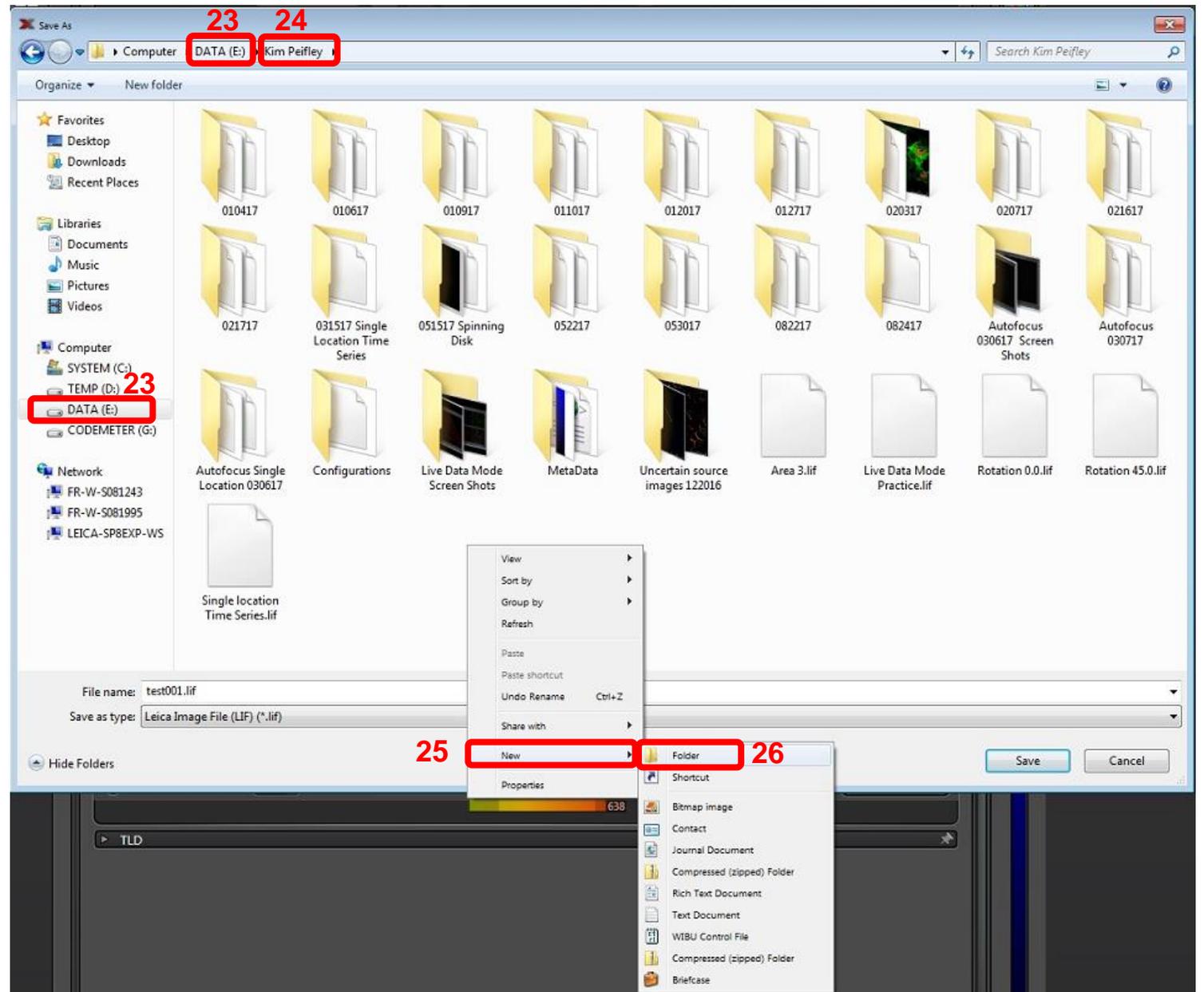
You now want to remove the .lif project file you opened so you don't accidentally

- A. Change the configuration on your old image.
- B. Save your data in the old folder instead of your new folder.

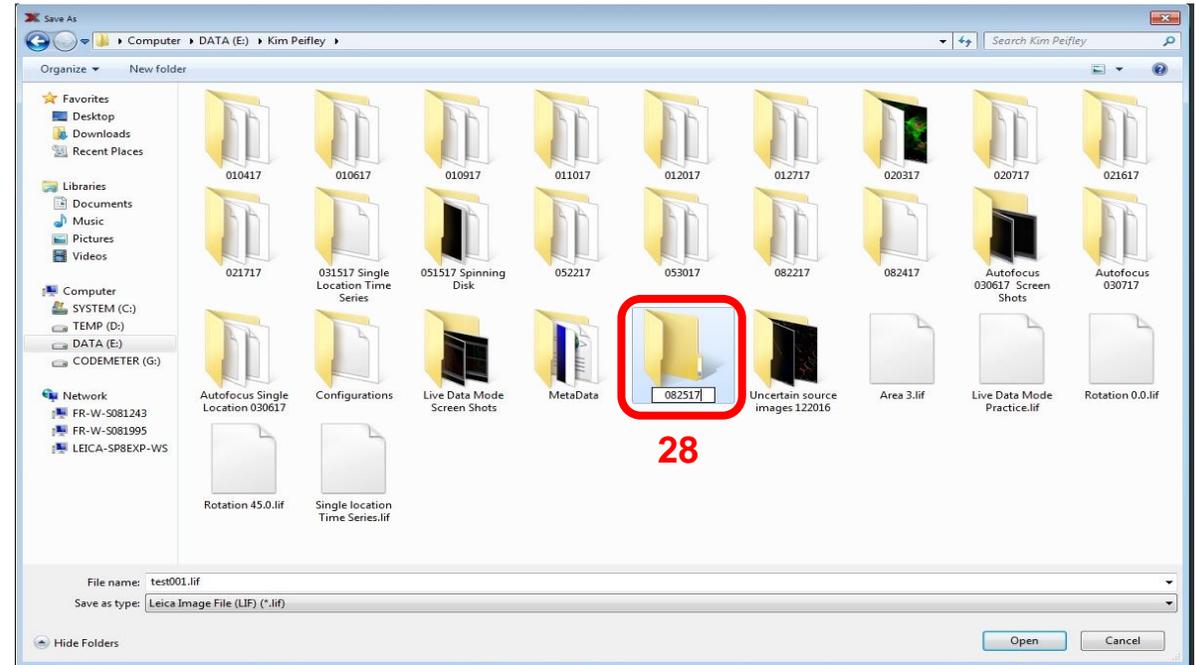
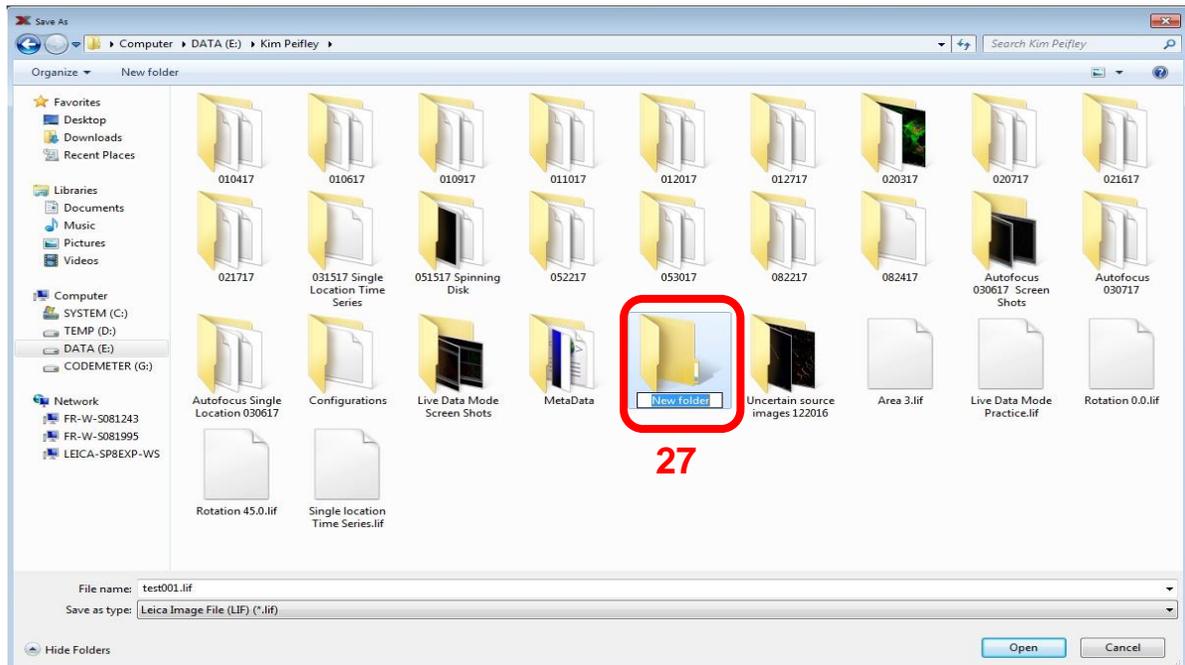
- 19. Click on Paper+ button to create a New Project.
- 20. Highlight new project. In this case it is called test001.
- 21. Right click on the file box icon.
- 22. Select Save as.



- 23. Open the Data (E:) drive.
- 24. Open your folder.
- 25. Right click to highlight New.
- 26. Select Folder.



27. Name your folder.
28. Click on it to open it.

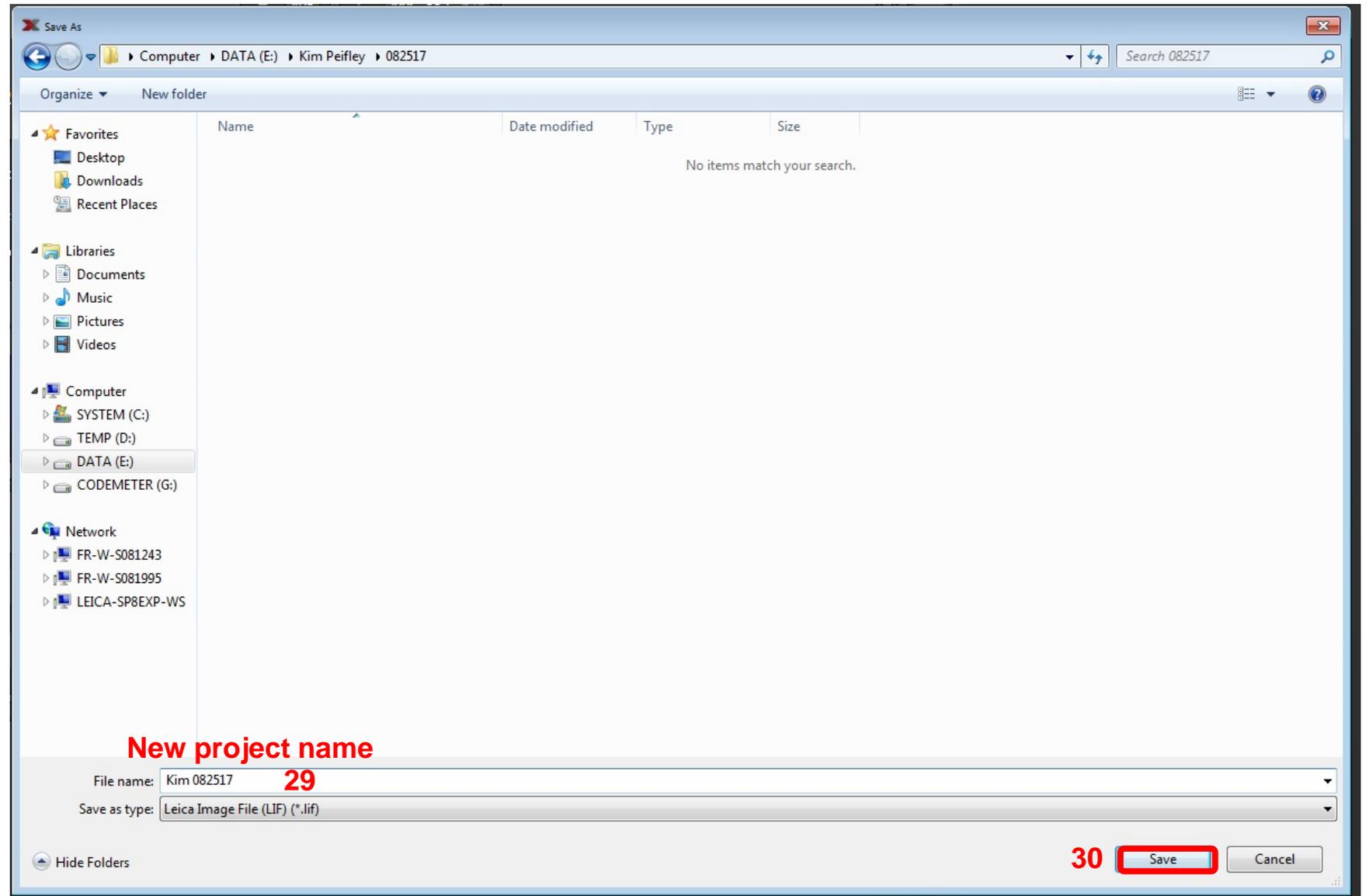


- 29. Name the project.
- 30. Click Save.

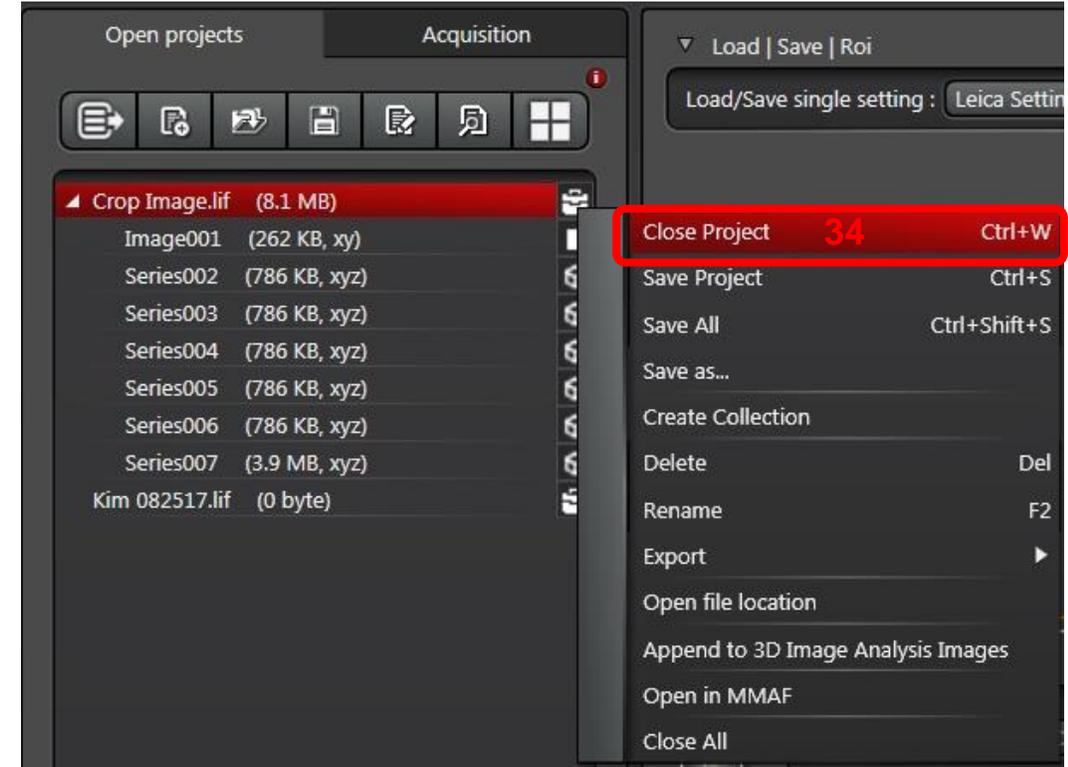
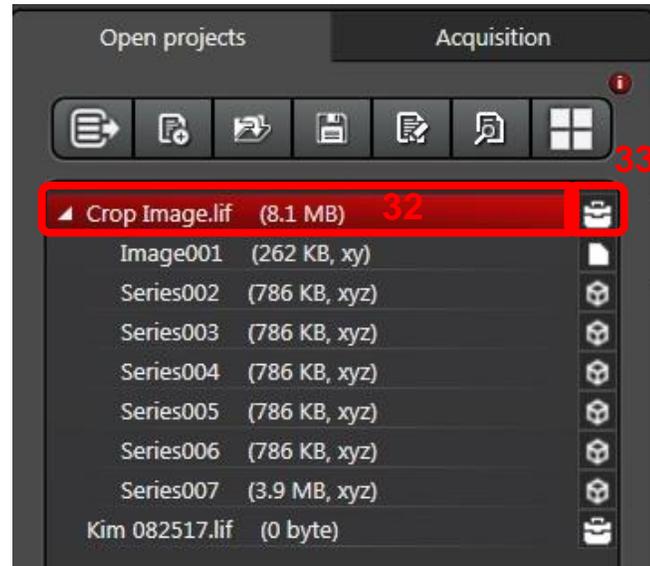
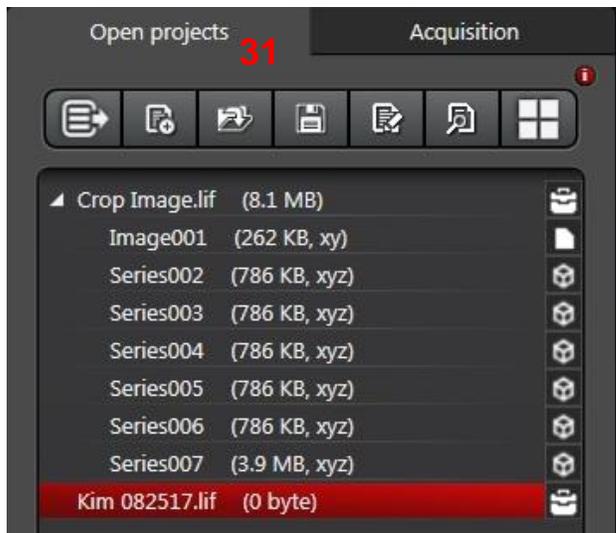
Old name

File name: test001.lif **29**

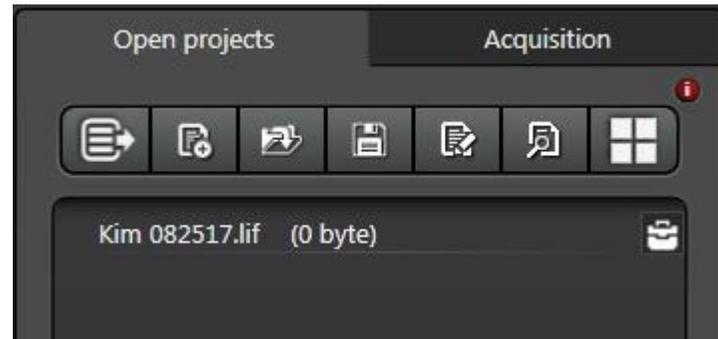
Save as type: Leica Image File (LIF) (*.lif)



31. You now see the project you opened to get the configurations as well as the new project for today's images.
32. Highlight the old project.
33. Right click on the file box icon.
34. Select close project.

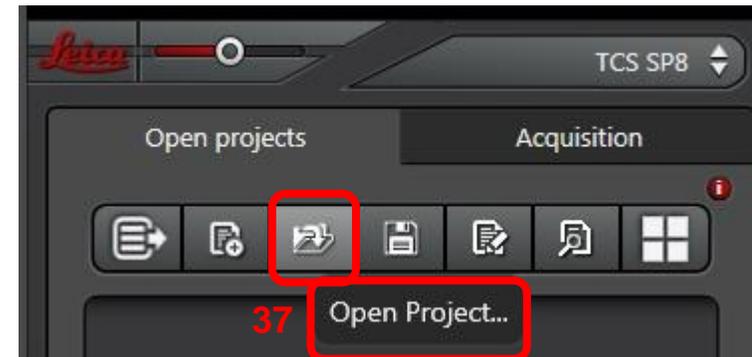
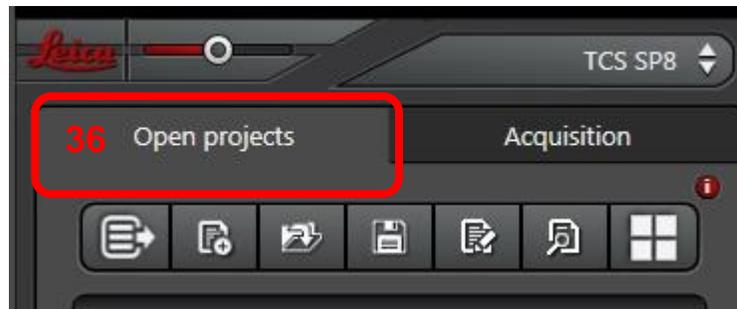


35. You should now see only the project for today's session.



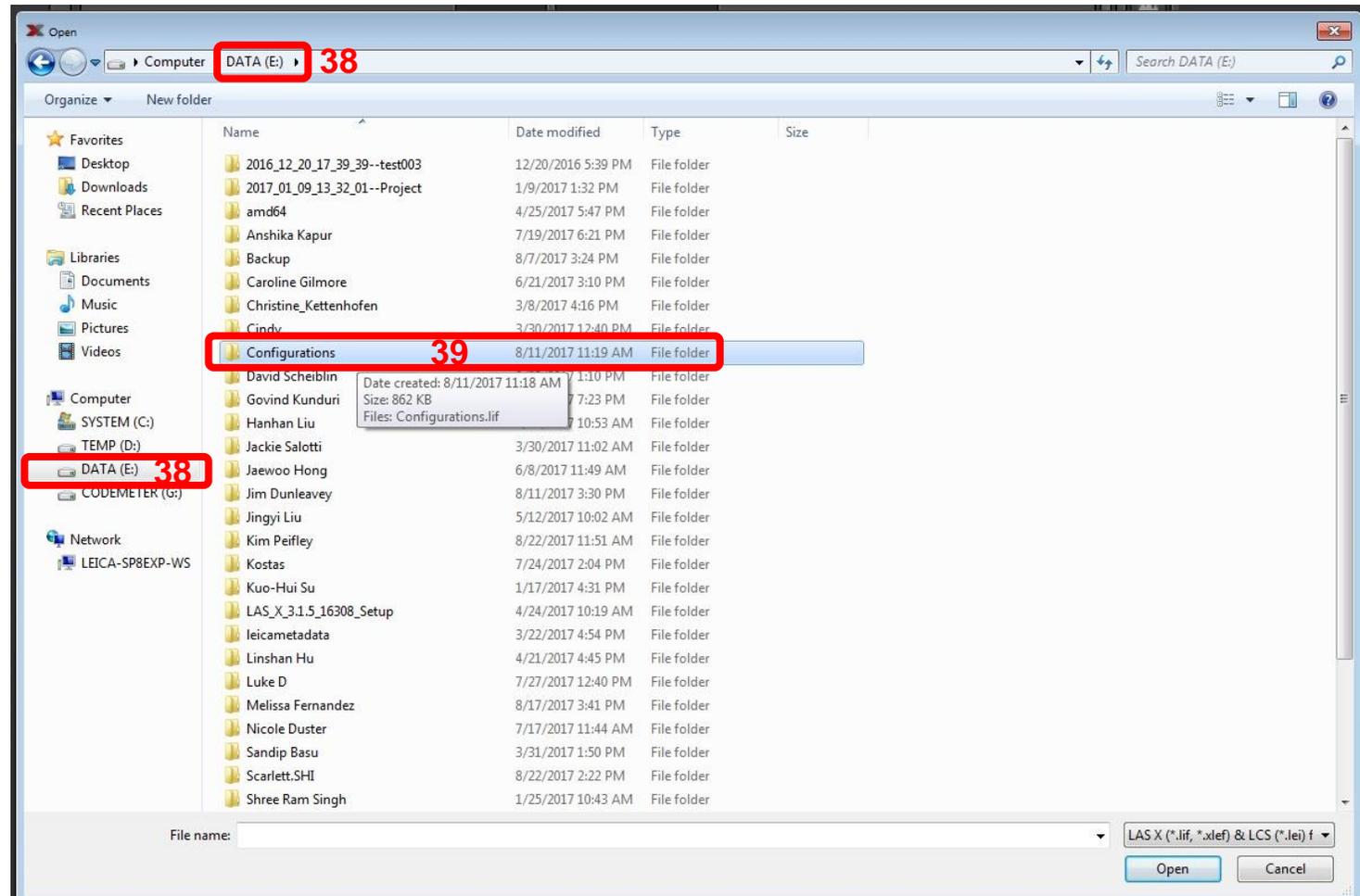
To use the configuration folder to get your lightpath settings:

36. Select the Open projects tab.
37. Click the Open Projects button. It looks like a folder.

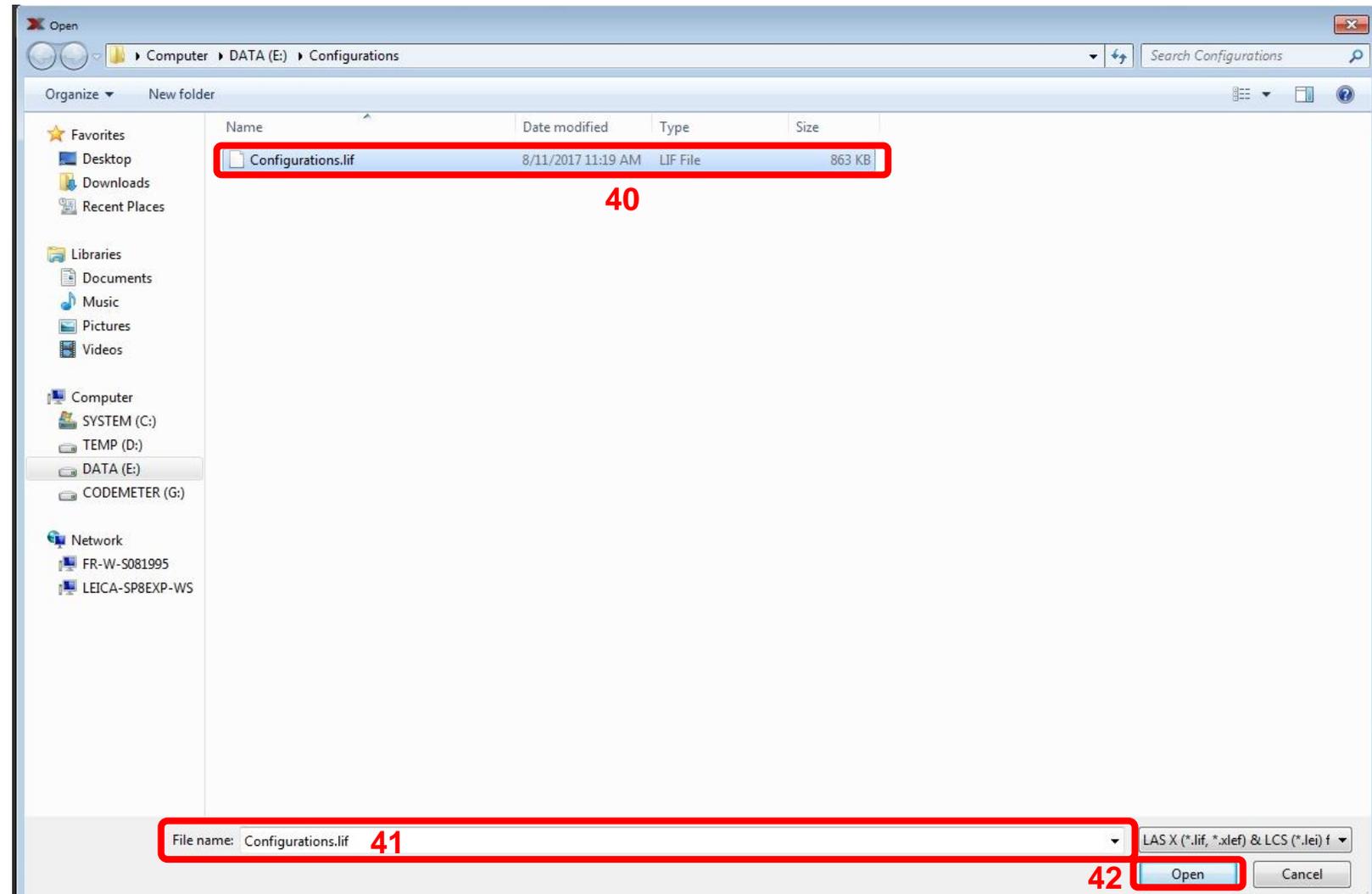


38. Go to the Data (E:) drive.

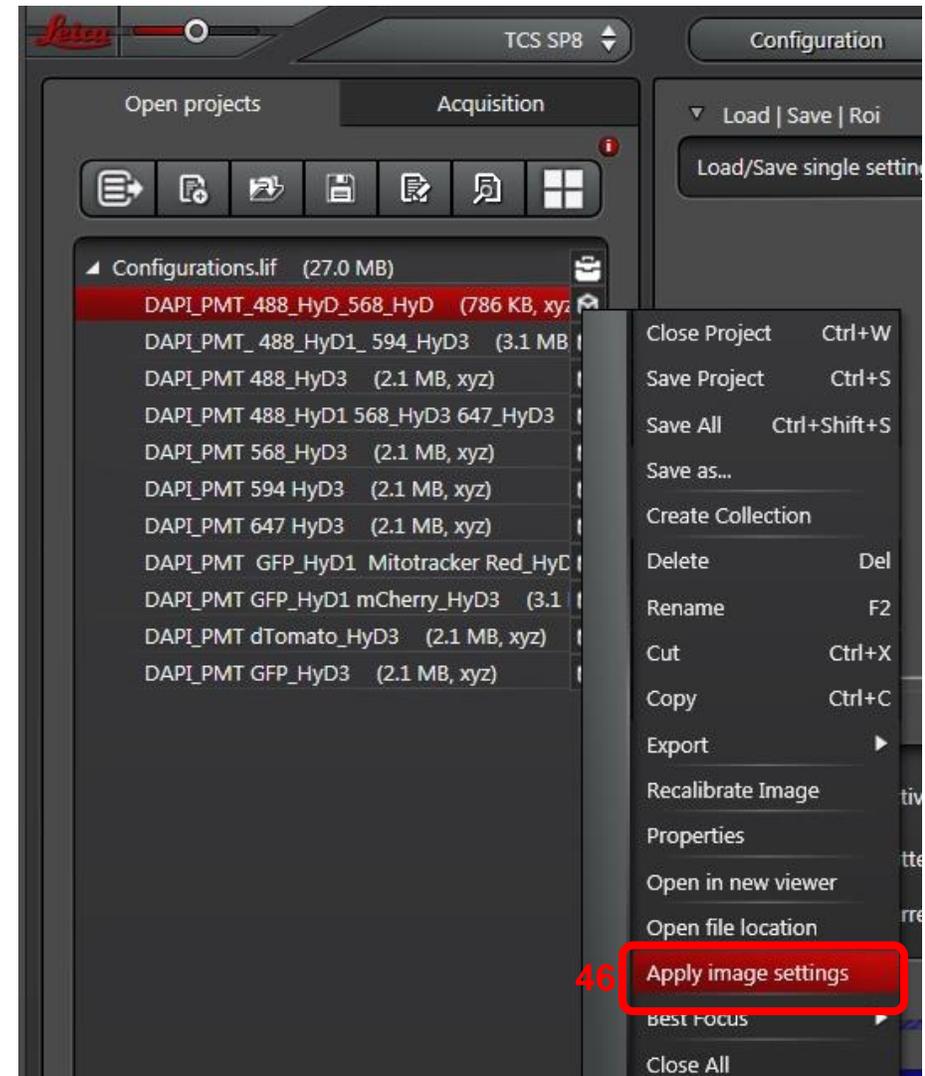
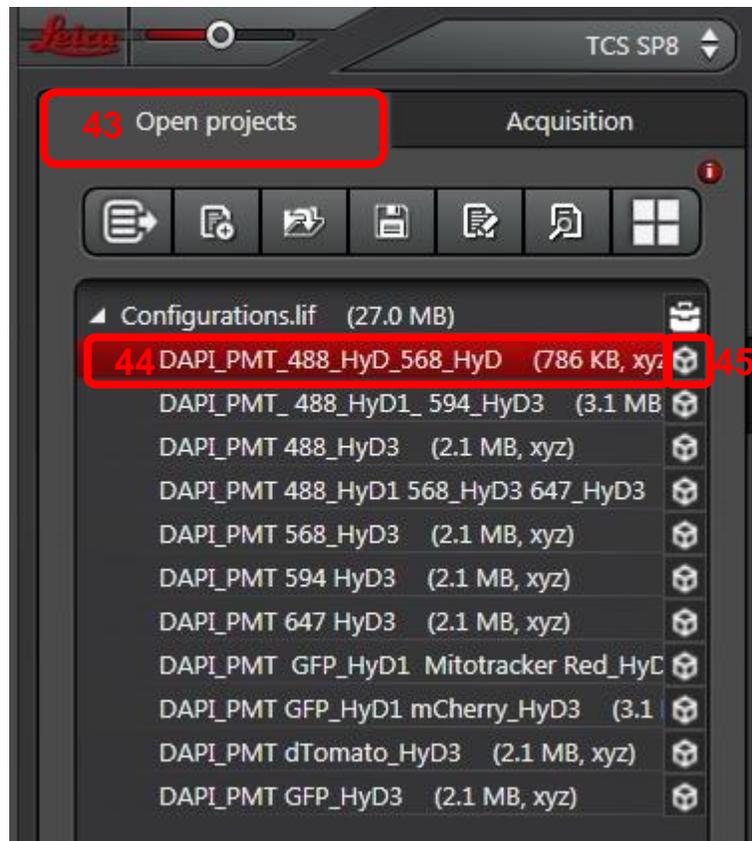
39. Click on the Configurations folder to open it.



40. Highlight the “Configuratons.lif” file.
41. Make sure this file is showing in the File name box.
42. Click Open.



43. Go to Open projects tab.
44. Click on the configuration you wish to use.
45. Right click to on box to open menu.
46. Highlight Apply image settings to select.





- 47. Go to the Acquisition tab.
- 48. Click on a Sequence and check to make sure the light path settings are as you want them.
- 49. Check the other Sequences.

Rotation : 0.00
Pinhole

Z-Stack :

Begin End

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

z - Galvo

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

Sequential Scan

Seq. 1 Seq. 2 Seq. 3

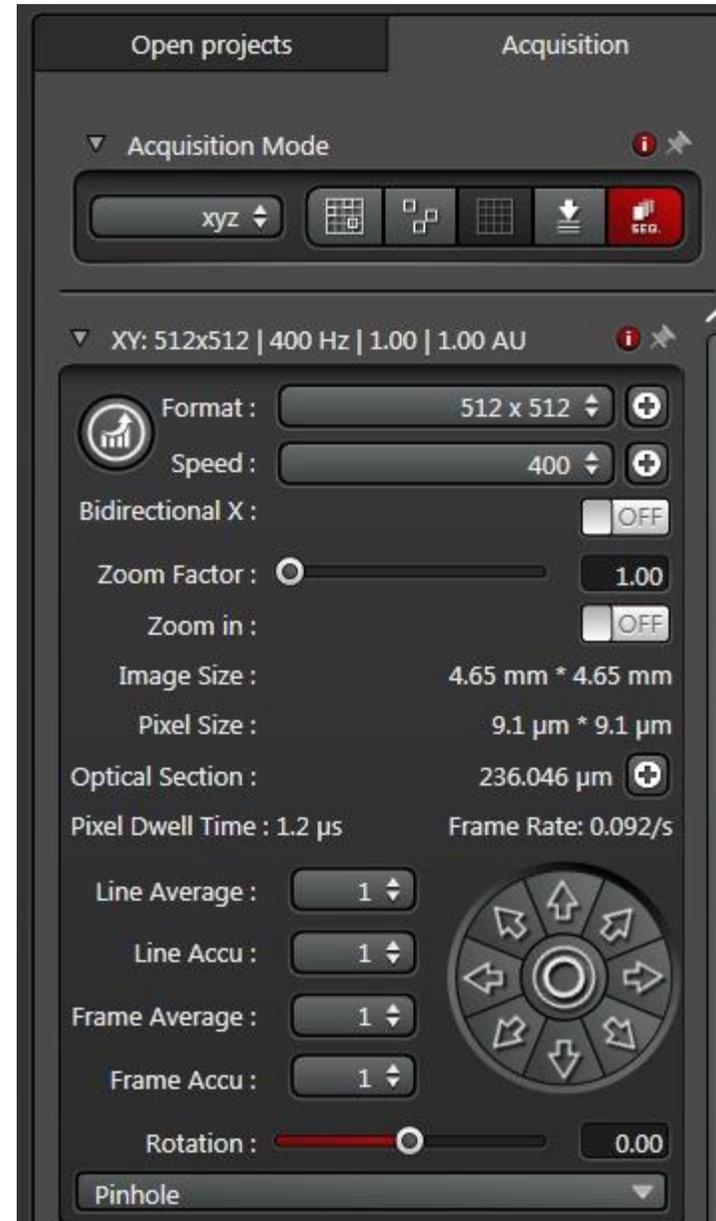
Internal

HyD 1 : OFF Gain [%]: 100.0 Standard None
PMT 2 : OFF Gain [V]: 800.0 Offset [%]: 0.00 None
HyD 3 : **48** ON Gain [%]: 100.0 Standard **ALEXA 568**

TLD

49

50. Check to make sure Format [frame size], Speed [scan speed], Zoom Factor, Average and Pinhole are as you want them. Apply Image Settings will not set these for you.

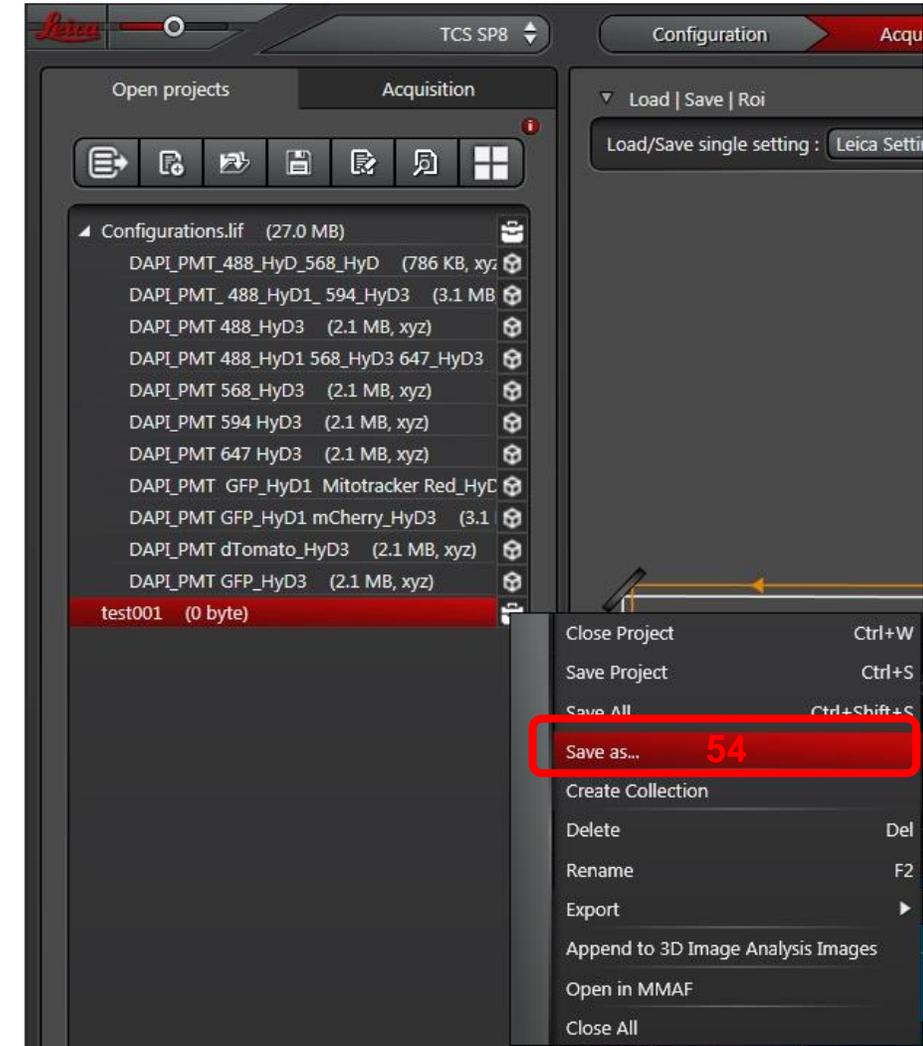
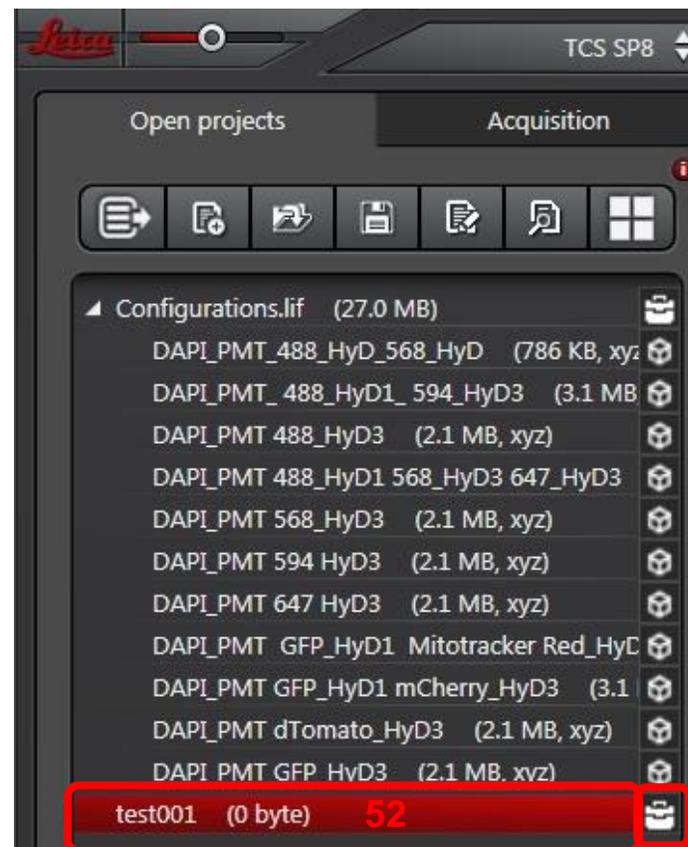
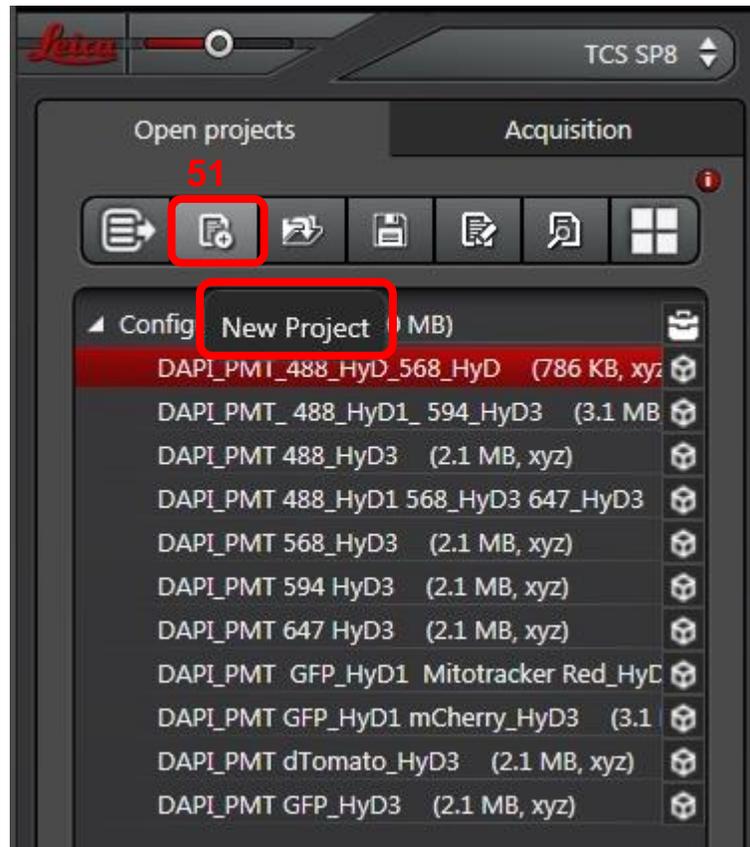


51. Once your configurations are set, click the paper+ icon to create a new project.

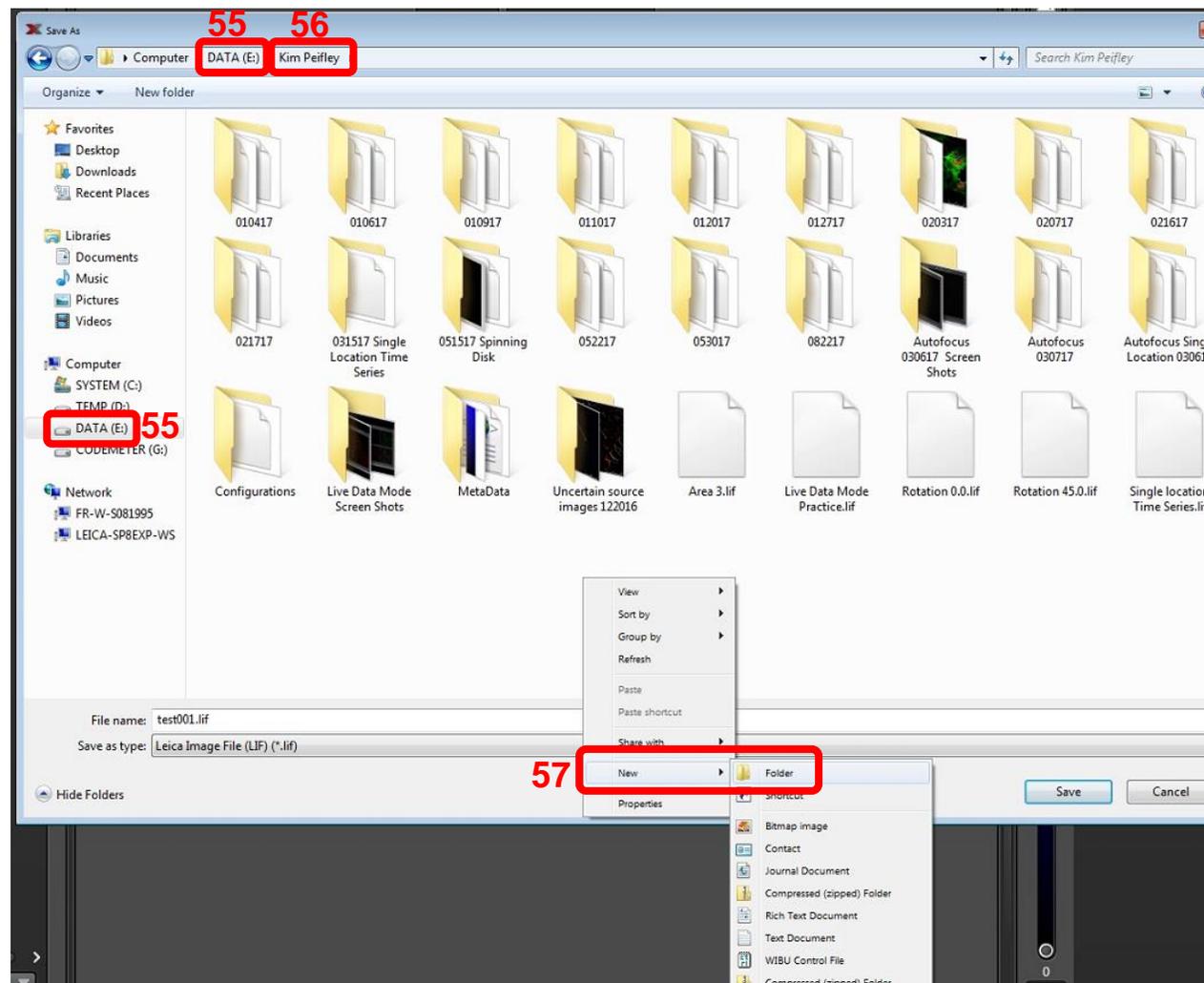
52. Highlight that new project, test001.

53. Right click the box icon.

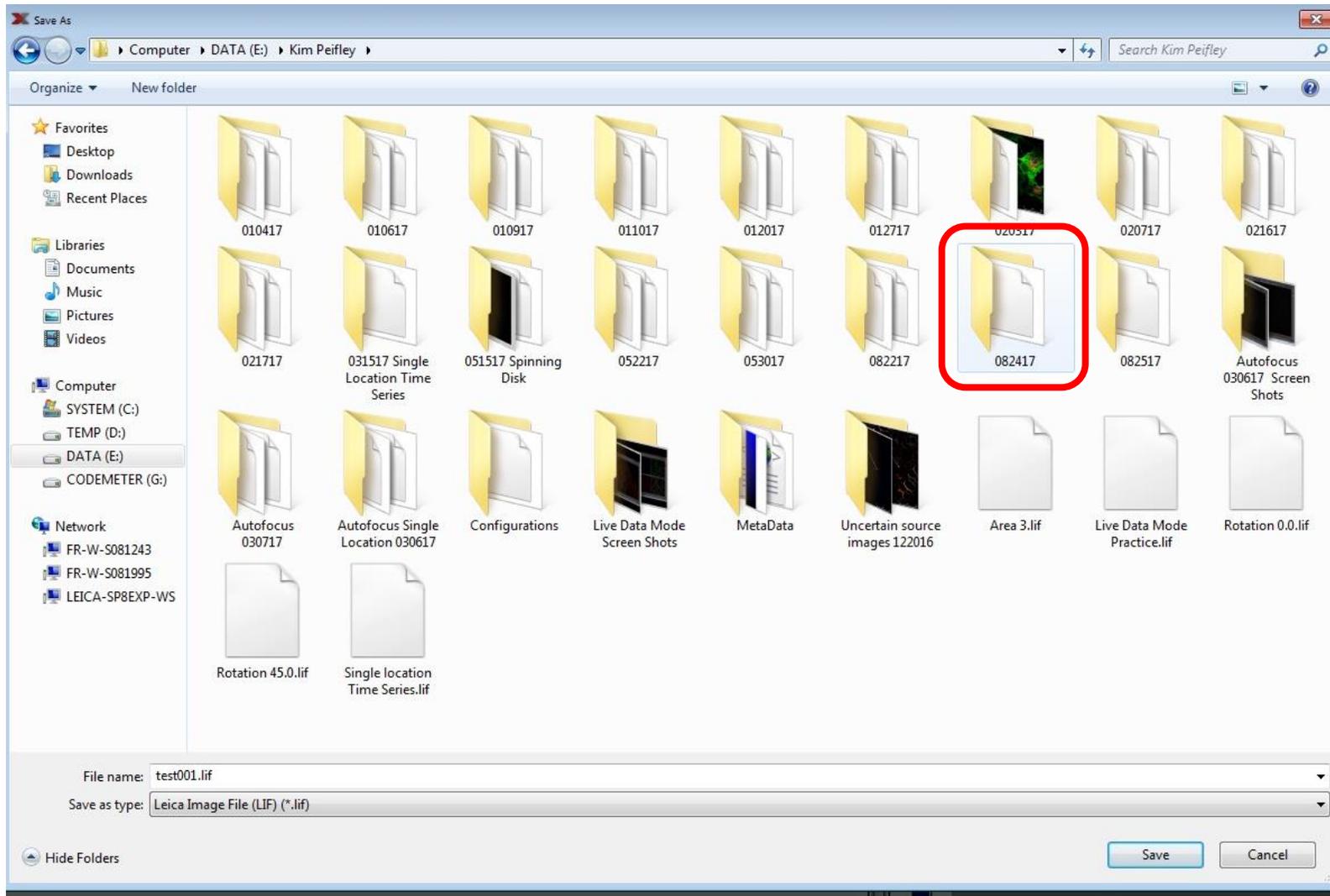
54. Highlight Save as to select it.



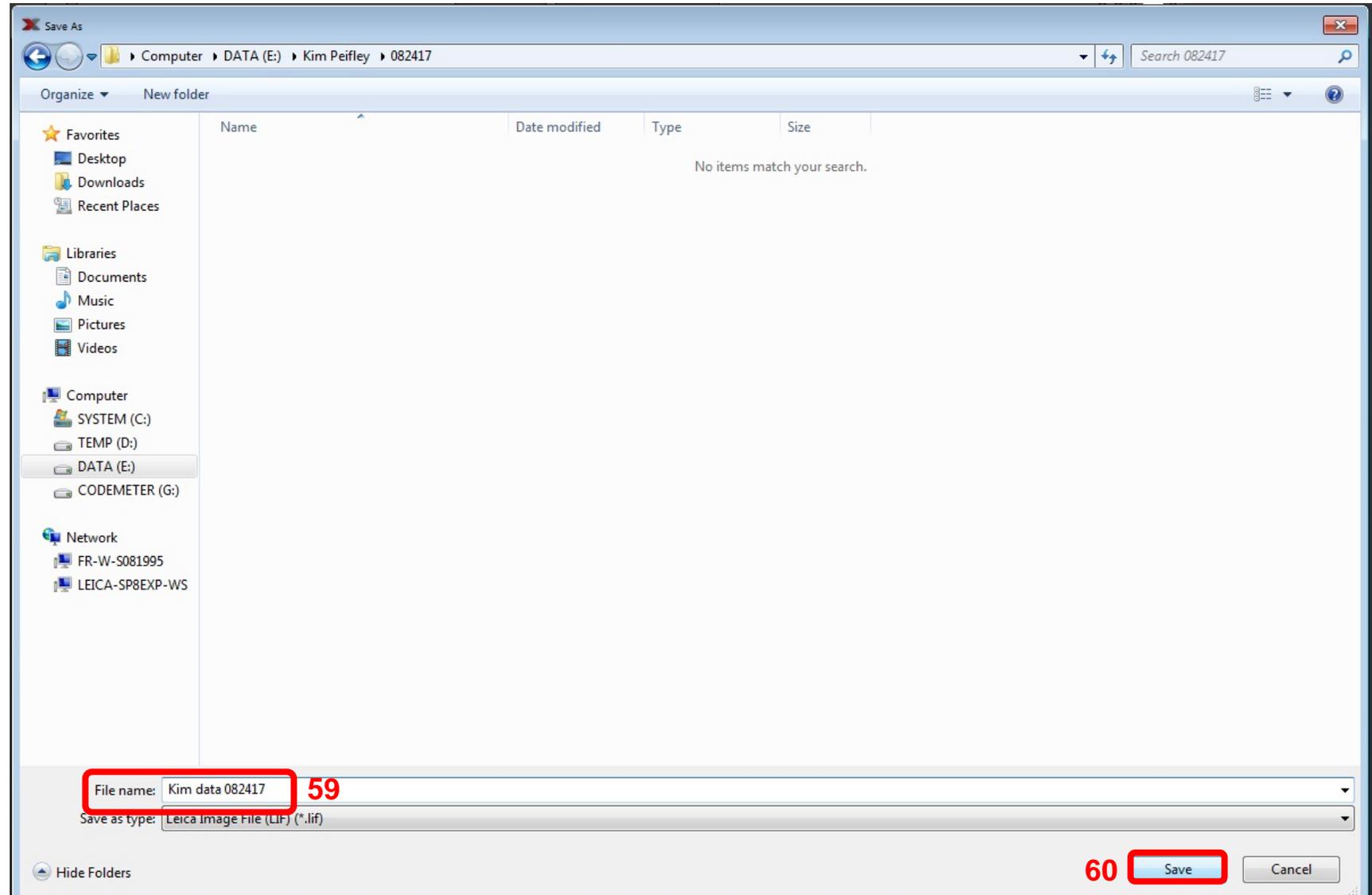
- 55. Select the Data (E:) drive.
- 56. Select your folder.
- 57. Create new folder.



58. Name folder and click to open it.



- 59. Rename test001.lif Project file.
- 60. Click Save.



You now want to remove the Configurations.lif file so you don't accidentally

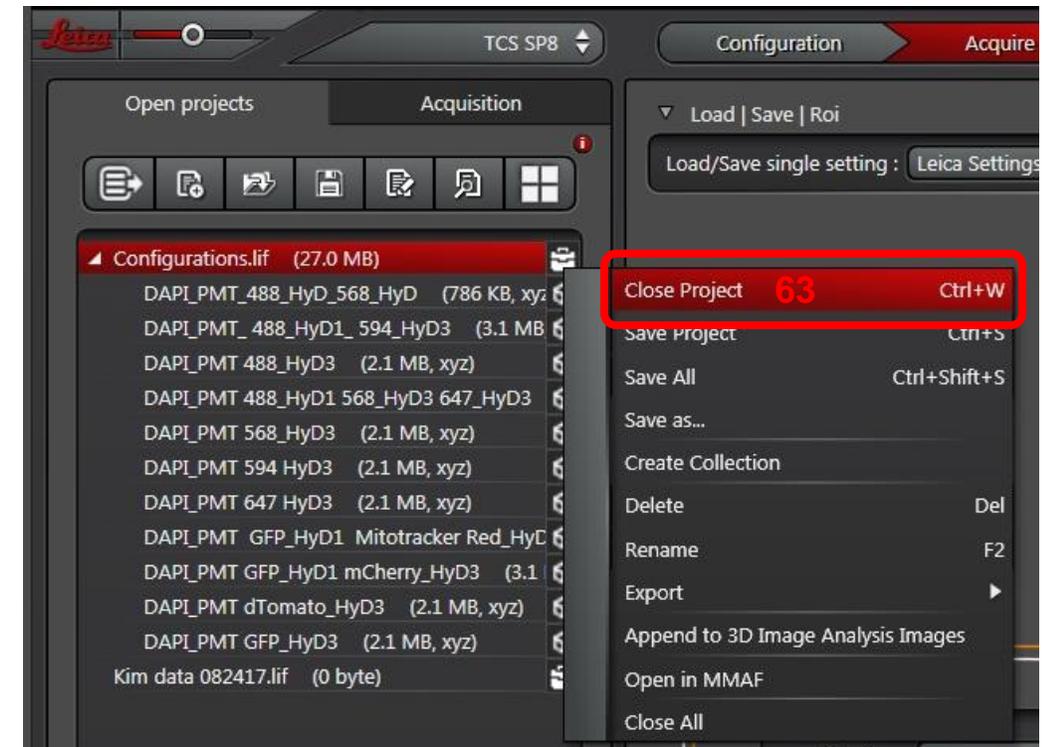
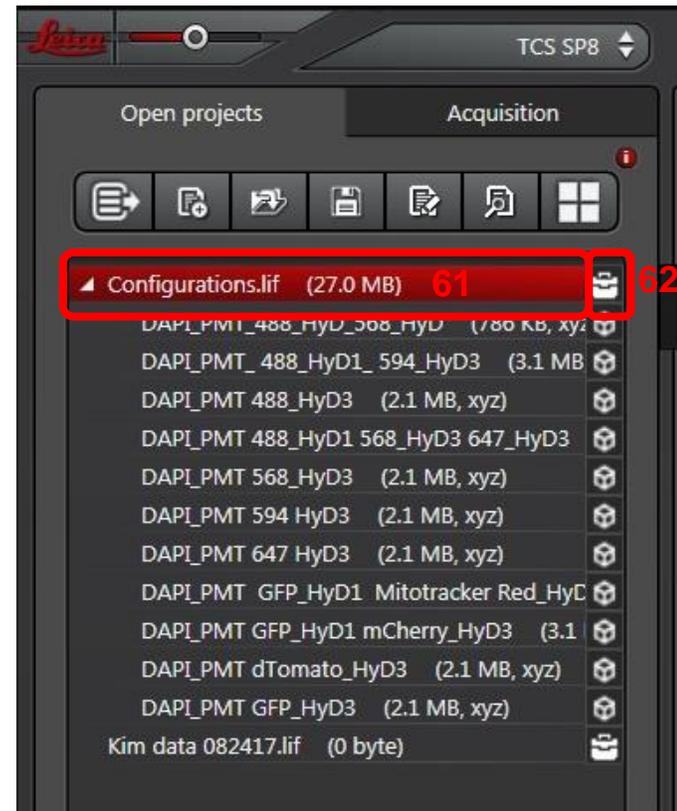
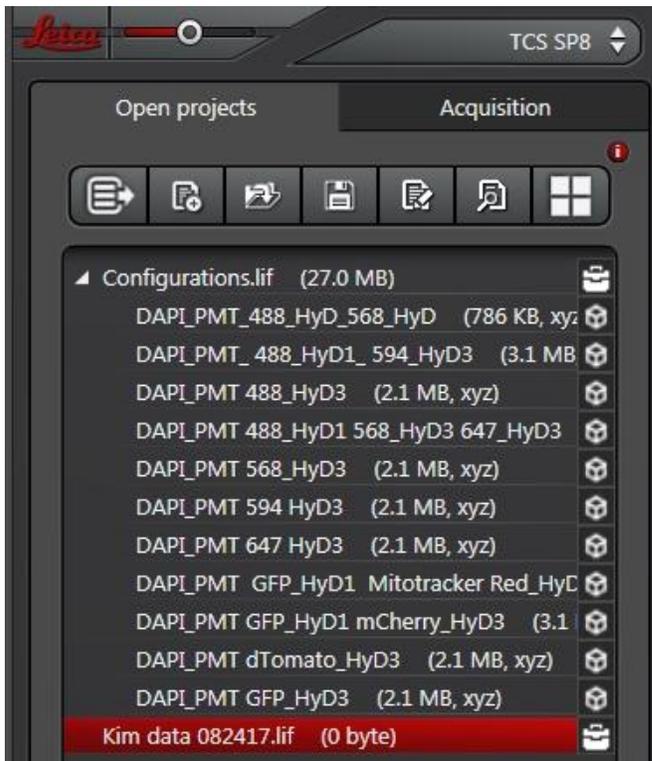
A. Change the saved configuration.

B. Save your data in the Configuration folder instead of your own folder.

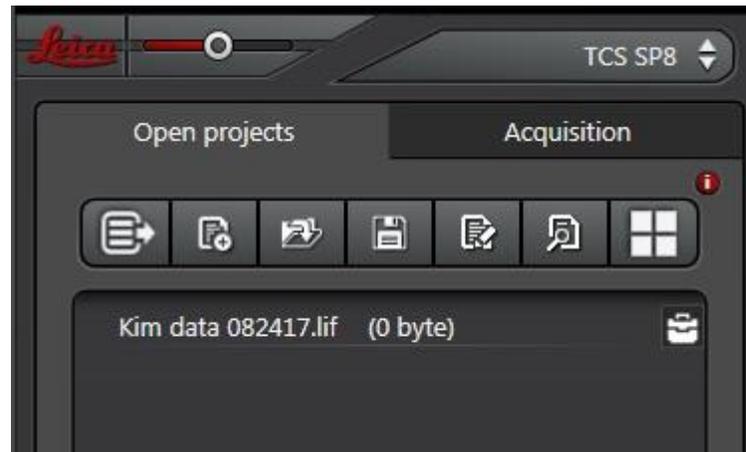
61. Highlight the Configurations.lif project.

62. Right click on the file box icon.

63. Highlight to select Close Project.

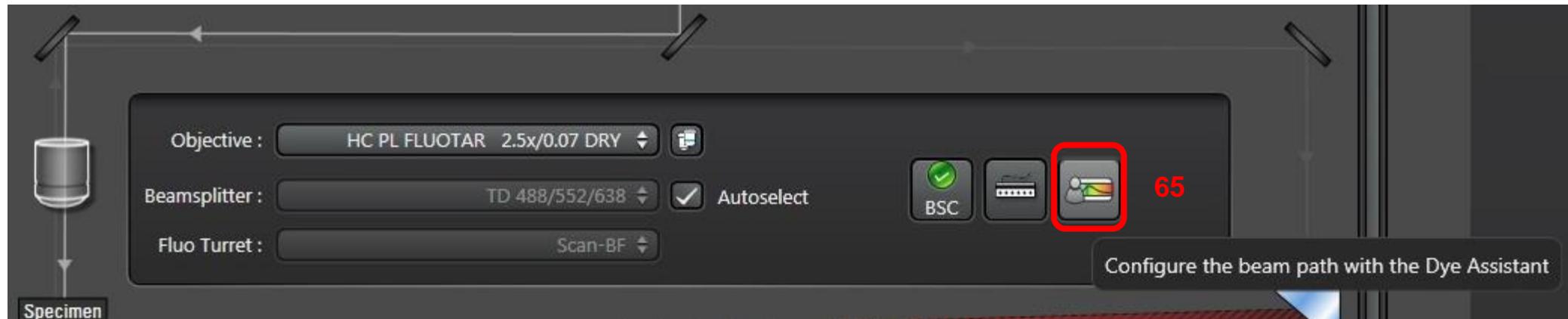


64. You should now see only your current project for today. Your images will appear beneath this project.

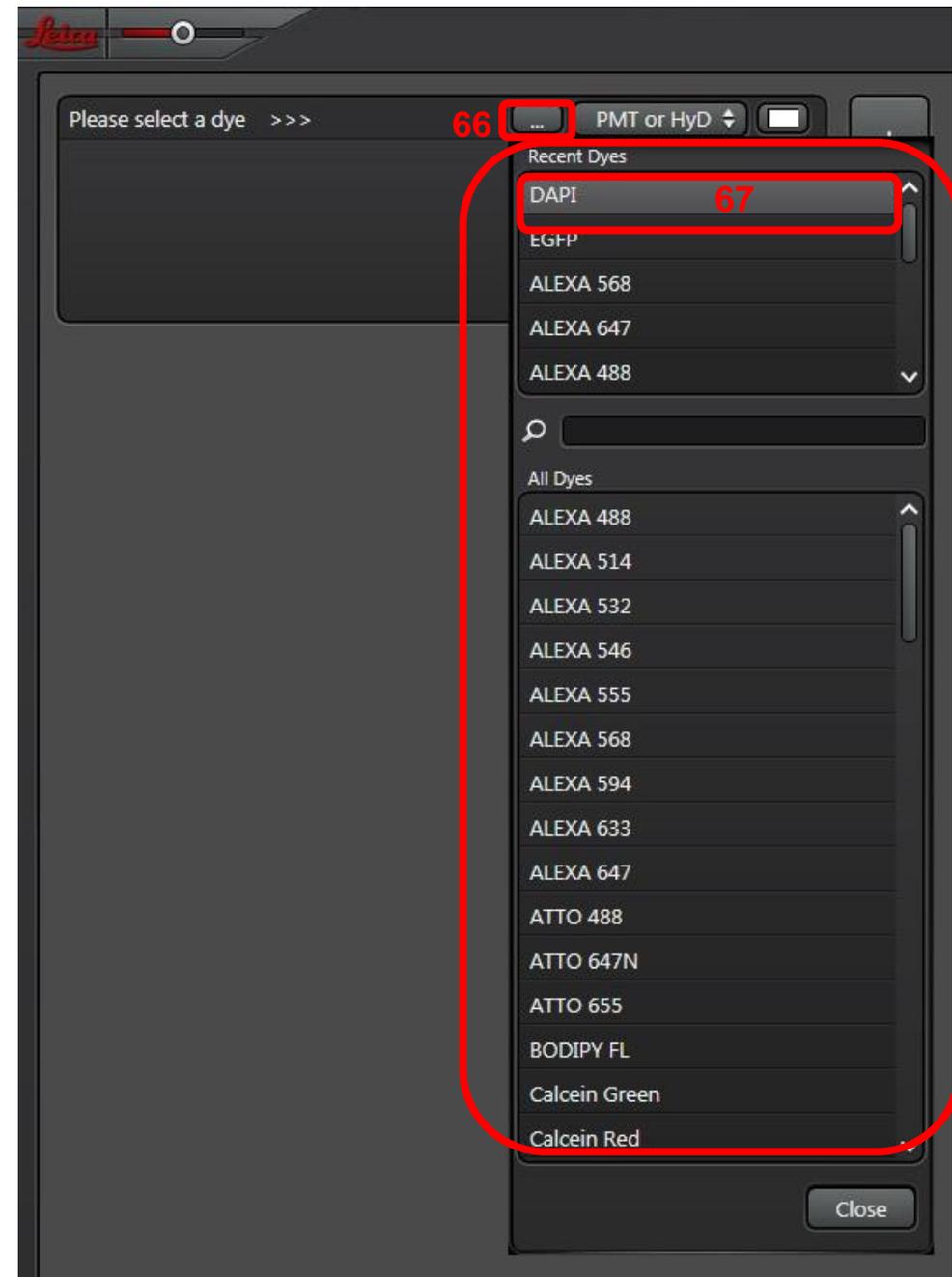


You can use Dye Assistant to set up your beam path [light paths] but you will have to make adjustments to the set up.

65. Click on Dye Assistant.



66. Click on the box with the ...
67. Select dye either in the Recent Dyes box or the All Dyes box below it.



- 68. Continue for other dyes.
- 69. Now you see all your dyes and below the choices for light path settings.
- 70. Frame or stack sequential, 3 sequences is what you want.
- 71. Click Apply in that section.



72. Go to sequence 1.
You now need to
adjust the settings.



The image shows a software interface for microscope control, divided into two main panels.

Left Panel: Z-Stack Settings

- Z-Stack:** Includes 'Begin' and 'End' buttons, a 'z - Galvo' dropdown, and a 'Re-Center' button. Parameters: Z Position [μm] : 0.00, Z Size [μm] : 0.00.
- Sequential Scan:** Features a 'Seq. 1' button (highlighted with a red box and a red arrow), 'Seq. 2', and 'Seq. 3' buttons. Below are radio buttons for 'Between Lines', 'Between Frames' (selected), and 'Between Stacks', along with 'Load' and 'Save' buttons.

Right Panel: Specimen and Internal Settings

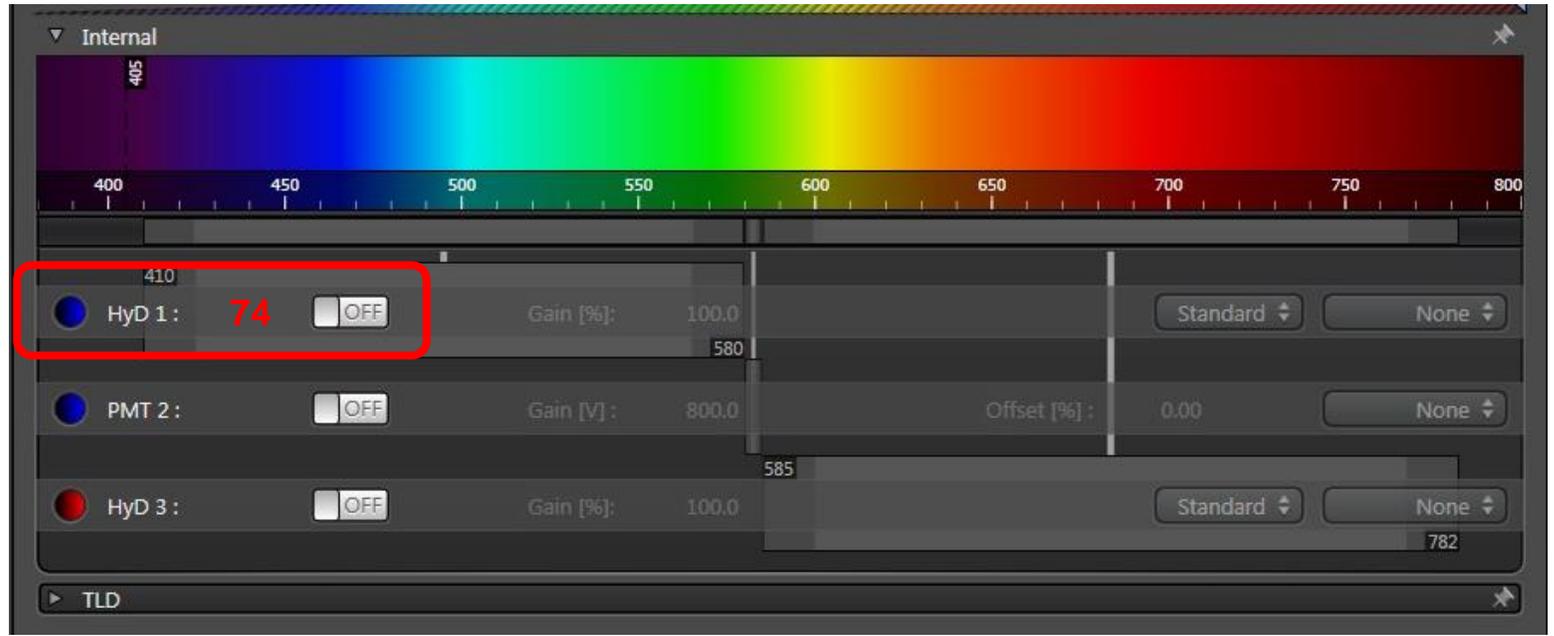
- Specimen:** Shows a color-coded intensity profile across a wavelength range from 400 to 800 nm.
- Internal:** Contains a table of detector and filter settings:

Channel	Status	Gain [%]	Gain [V]	Offset [%]	Filter
HyD 1	ON	100.0	580		Standard / DAPI
PMT 2	OFF	800.0	0.00		None
HyD 3	OFF	100.0			Standard / None

73. On the HyD 1 detector, change DAPI to None.

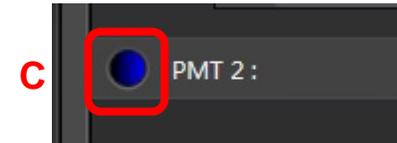
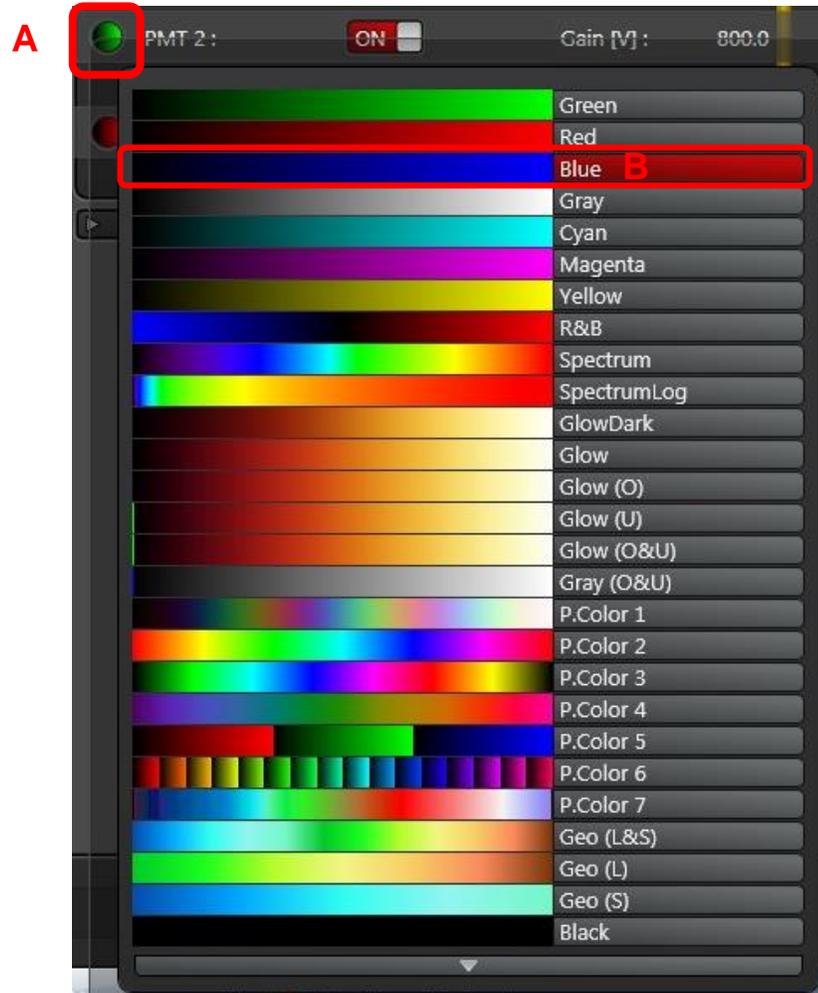
The screenshot displays the 'Internal' settings panel of a flow cytometry instrument. At the top, a spectral plot shows intensity versus wavelength (400-800 nm) with a peak at 405 nm. Below the plot, the 'HyD 1' detector is configured with a gain of 100.0% and is currently set to '73 DAPI'. A dropdown menu is open for this detector, showing a list of available filters: 'None', 'ALEXA 488', 'ALEXA 514', 'ALEXA 532', 'ALEXA 546', 'ALEXA 555', 'ALEXA 568', 'ALEXA 594', 'ALEXA 633', 'ALEXA 647', 'ATTO 488', 'ATTO 647N', 'ATTO 655', 'BODIPY FL', 'Calcein Green', 'Calcein Red', 'Cerulean', 'Chromeo 488 GAR', 'Chromeo 494 GAR', 'Chromeo 505 GAM', 'Chromeo 546 GAR', 'Chromeo 642 GAR', 'Citrin', 'Cy3', 'Cy5', 'Cy7', and 'DAPI'. The 'None' option is highlighted in red. The 'HyD 3' detector is currently set to 'OFF' with a gain of 100.0%. The 'PMT 2' detector is also set to 'OFF' with a gain of 800.0% and an offset of 0.00%. The 'TLD' section is partially visible at the bottom.

- 74. Turn off the HyD 1 detector.
- 75. Turn on the PMT 2.

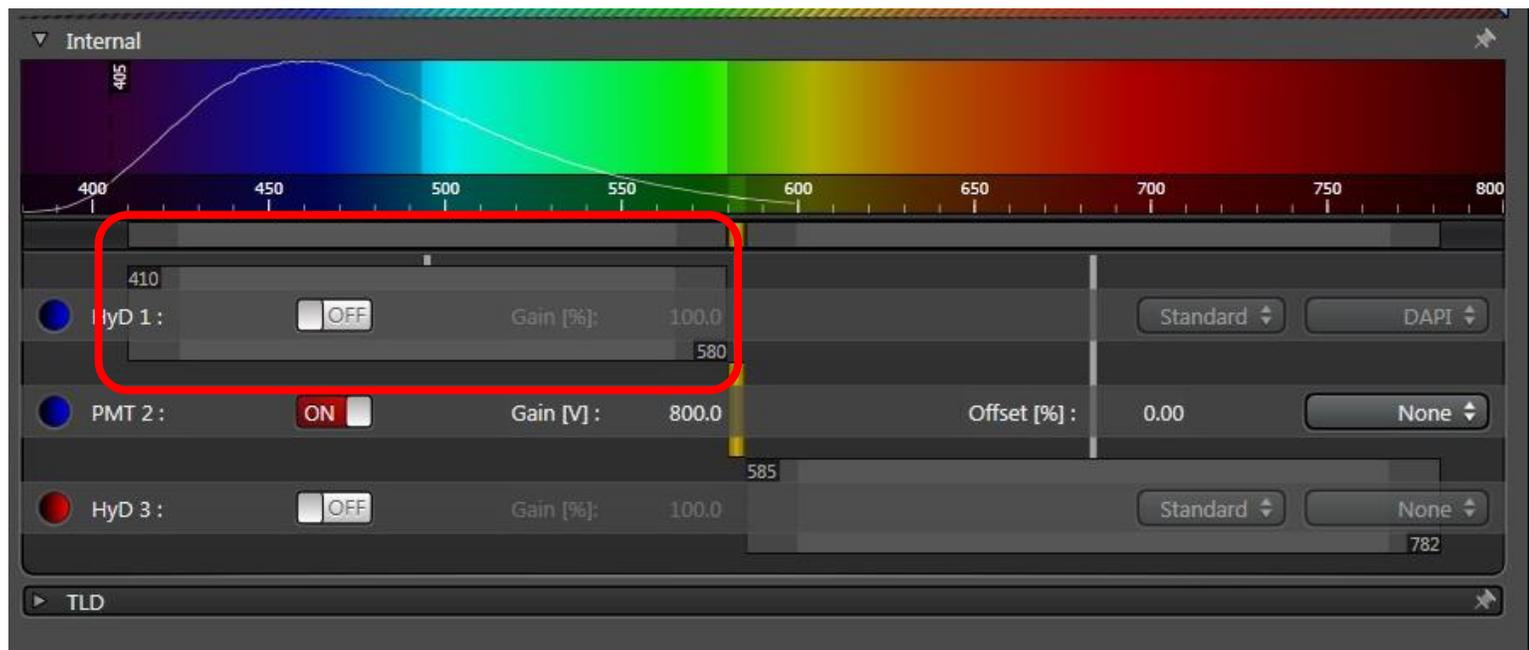


76. Change the pseudo color.

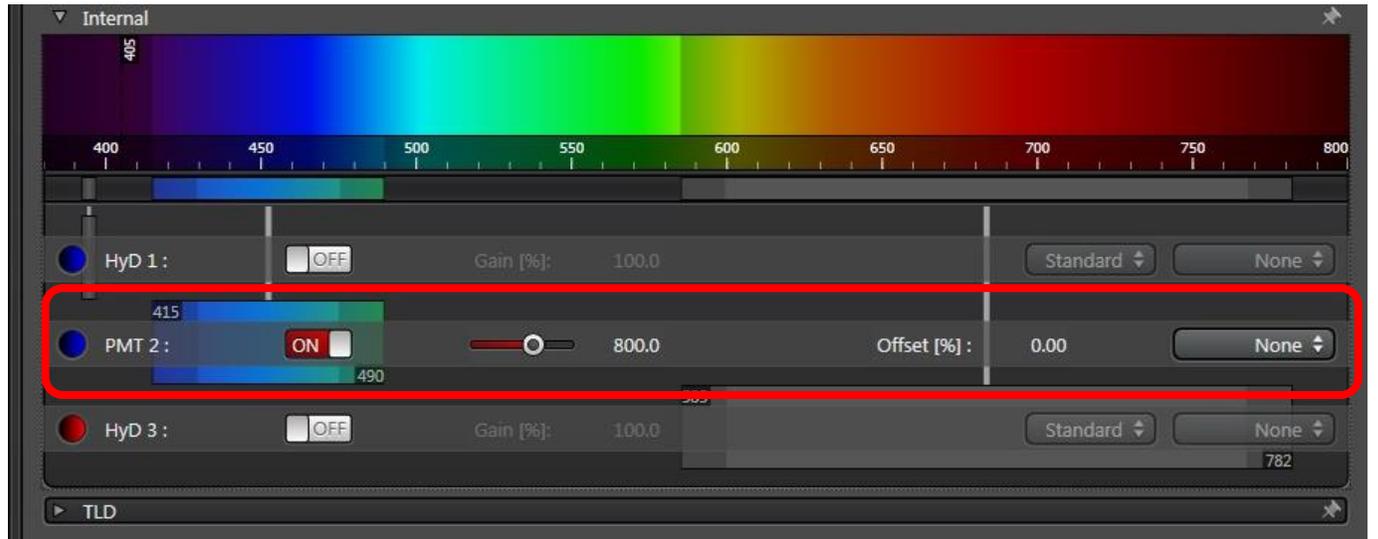
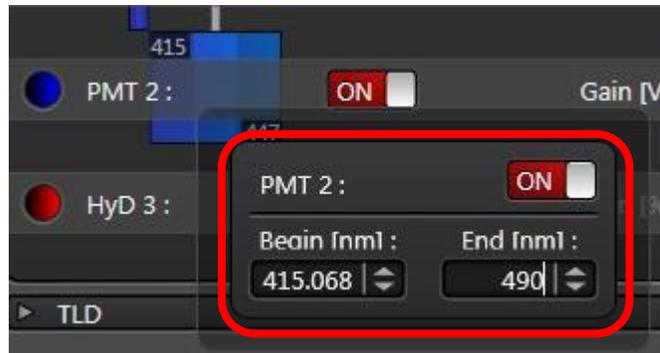
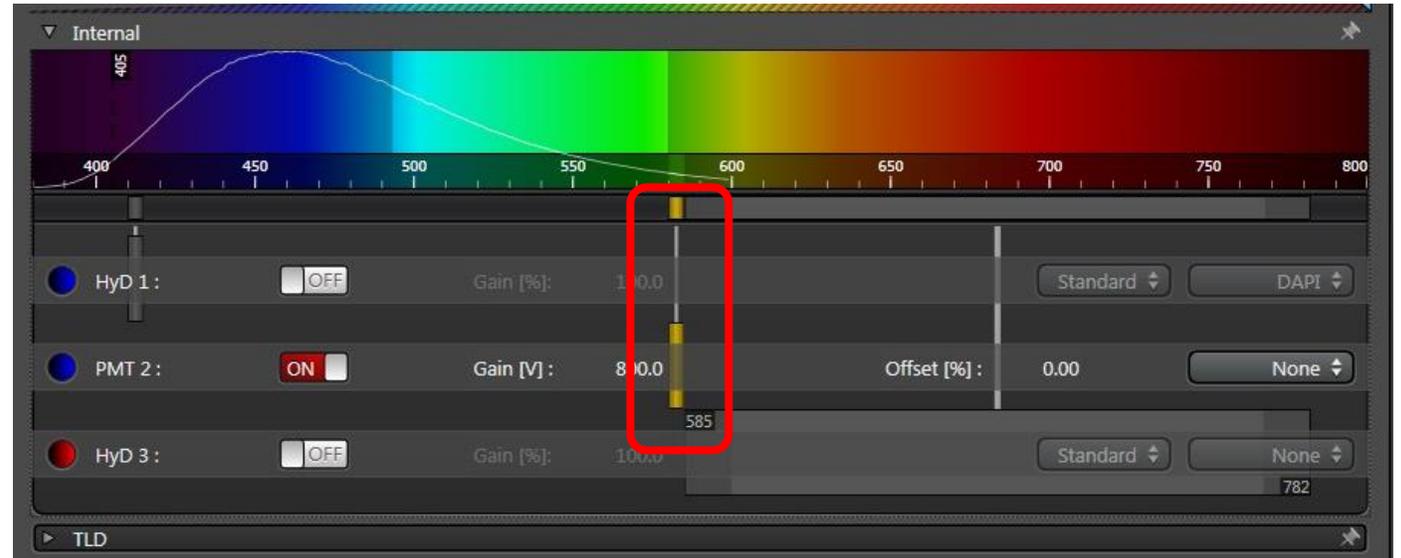
- A. Click on the green circle.
- B. Highlight Blue to select it.
- C. Check that the circle is now blue.



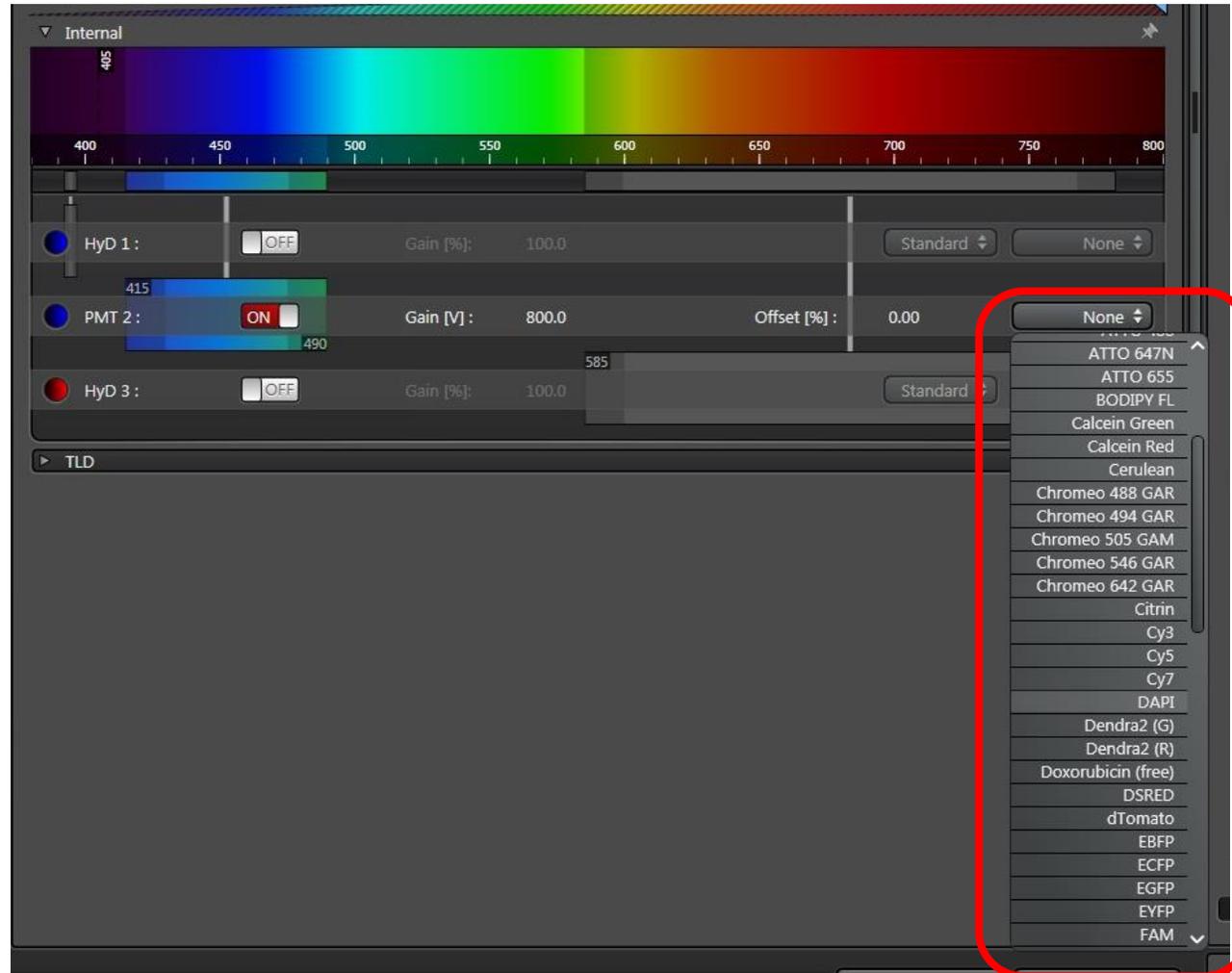
77. Move the sliders to shrink the gray slider area and move to the left out of the way.



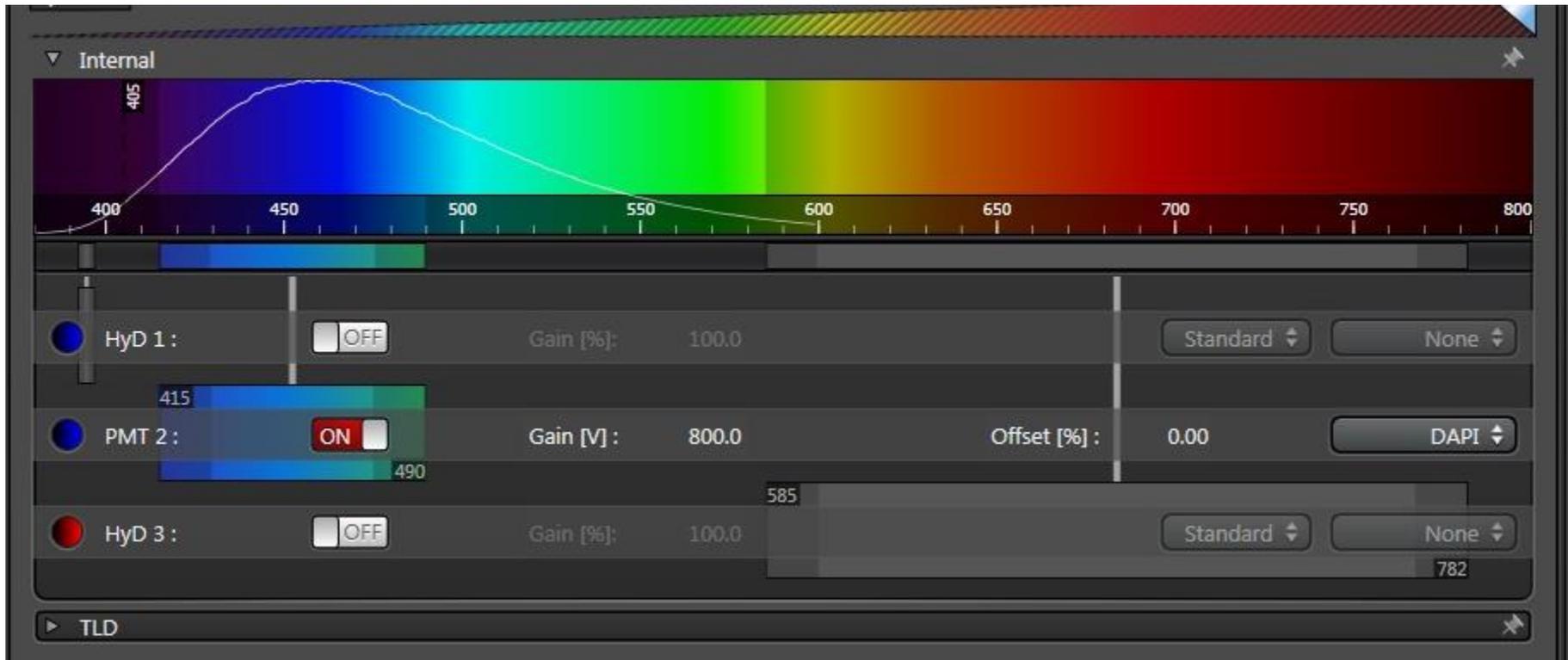
78. Click on the Yellow bars and create your emission filter for DAPI.



79. On PMT 2 detector change the name from None to DAPI.



80. This shows the HyD 1 detector as None and the PMT 2 detector as DAPI.



81. Click on Sequence 2 [Seq. 2].

The image shows a software interface for microscope control, divided into two main panels. The left panel is for Z-Stack configuration, and the right panel is for Sequential Scan configuration.

Z-Stack Panel:

- Buttons: Begin, End, Download, Upload, Refresh, Stop.
- Visual: A small diagram of a microscope stage with a Z-axis.
- Parameters:
 - Z Position [μm]: 0.00
 - Z Size [μm]: 0.00
 - Re-Center button
- z - Galvo: A dropdown menu.
- System Options:
 - Nr. of Steps: 1
 - Z-Step Size: 0.00
 - System Optimized:
 - Z-Compensation: none
 - Galvo Flow: OFF
 - Travel Range [μm]: 500

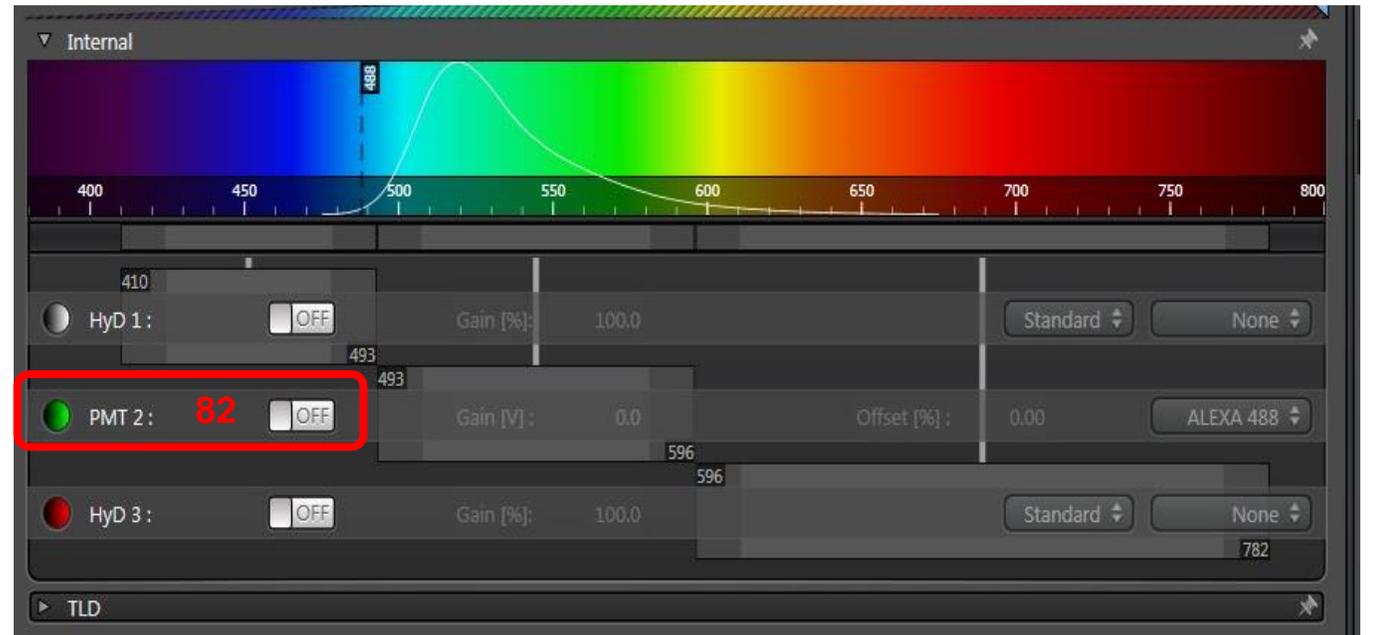
Sequential Scan Panel:

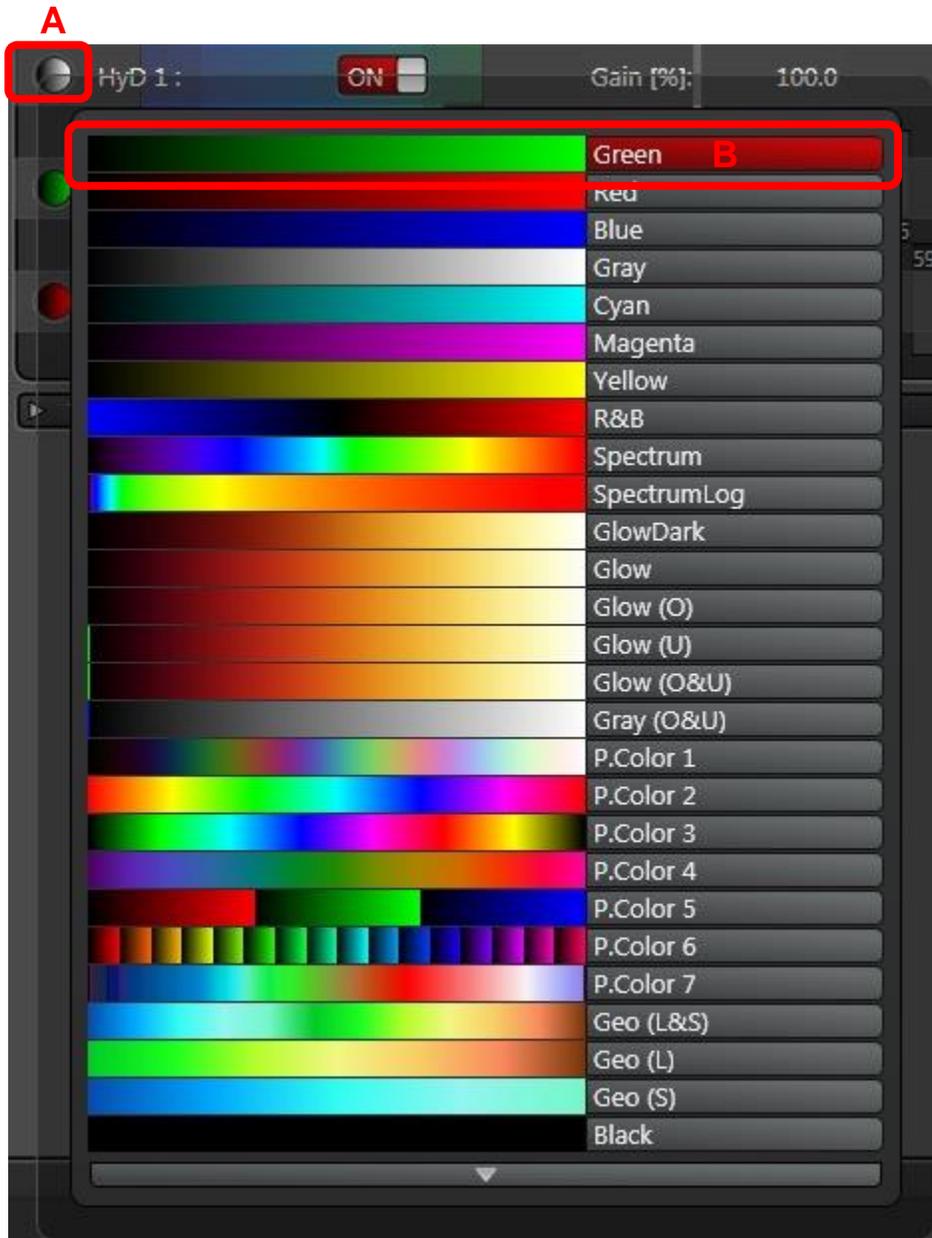
- Buttons: Seq. 1, **Seq. 2** (highlighted with a red box), Seq. 3.
- Options:
 - Between Lines:
 - Between Frames: (Load button)
 - Between Stacks: (Save button)

Internal Panel (Right):

- Specimen: A horizontal bar representing the specimen.
- Internal: A spectral plot showing intensity vs. wavelength (400-800 nm) with a peak at 488 nm.
- HyD 1: OFF, Gain [%]: 100.0, Standard, None.
- PMT 2: ON (highlighted with a red box), Gain [V]: 0.0, Offset [%]: 0.00, ALEXA 488 (dropdown menu).**
- HyD 3: OFF, Gain [%]: 100.0, Standard, None.
- TLD: A section for Total Light Detection.

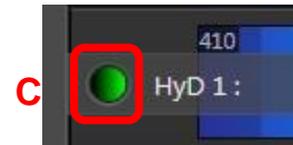
- 82. Turn off the PMT 2 detector.
- 83. Turn on the HyD 1 detector.



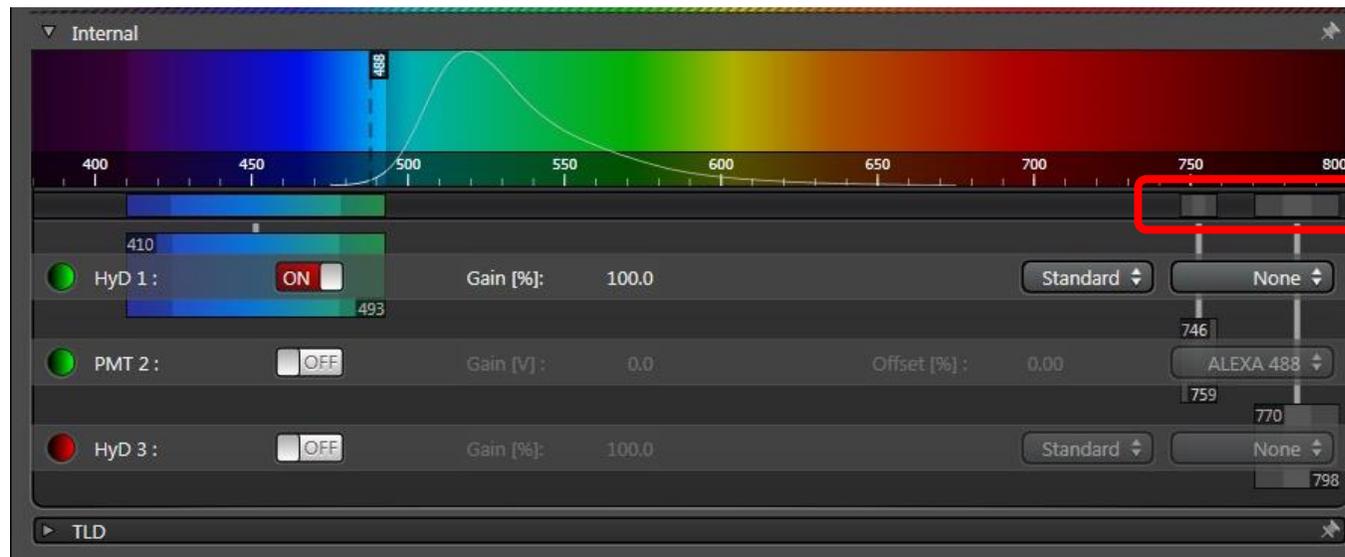
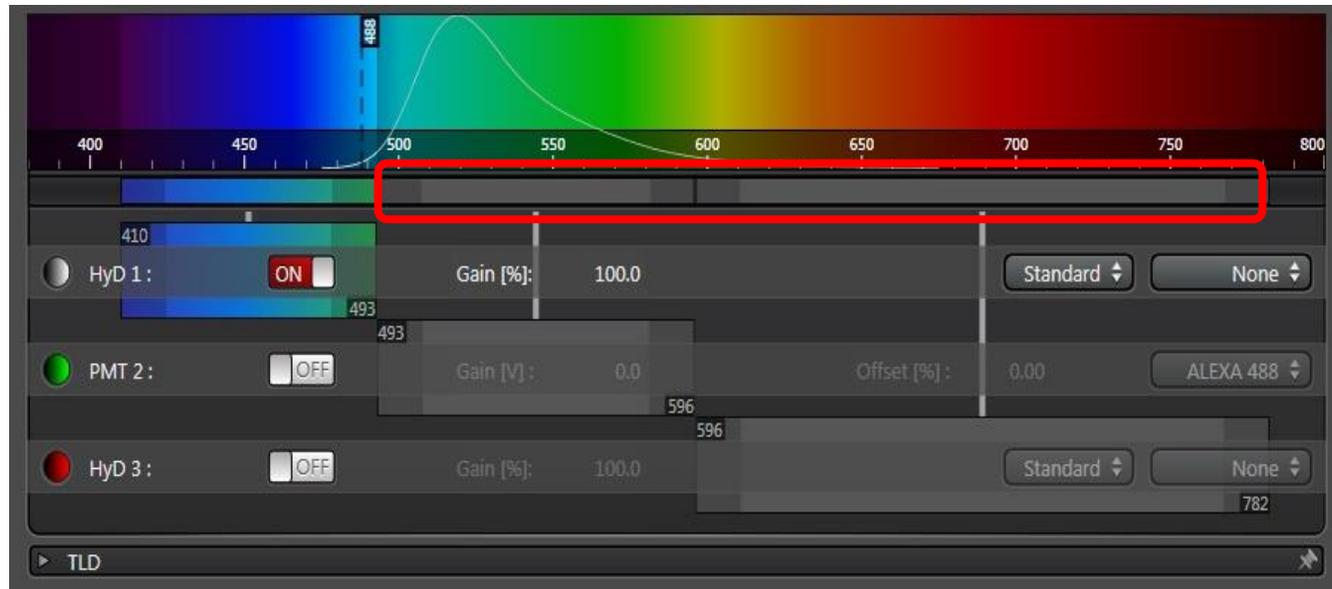


84. Change the pseudo color.

- A. Click on the gray circle.
- B. Highlight green to select it.
- C. Check that the circle is now green.

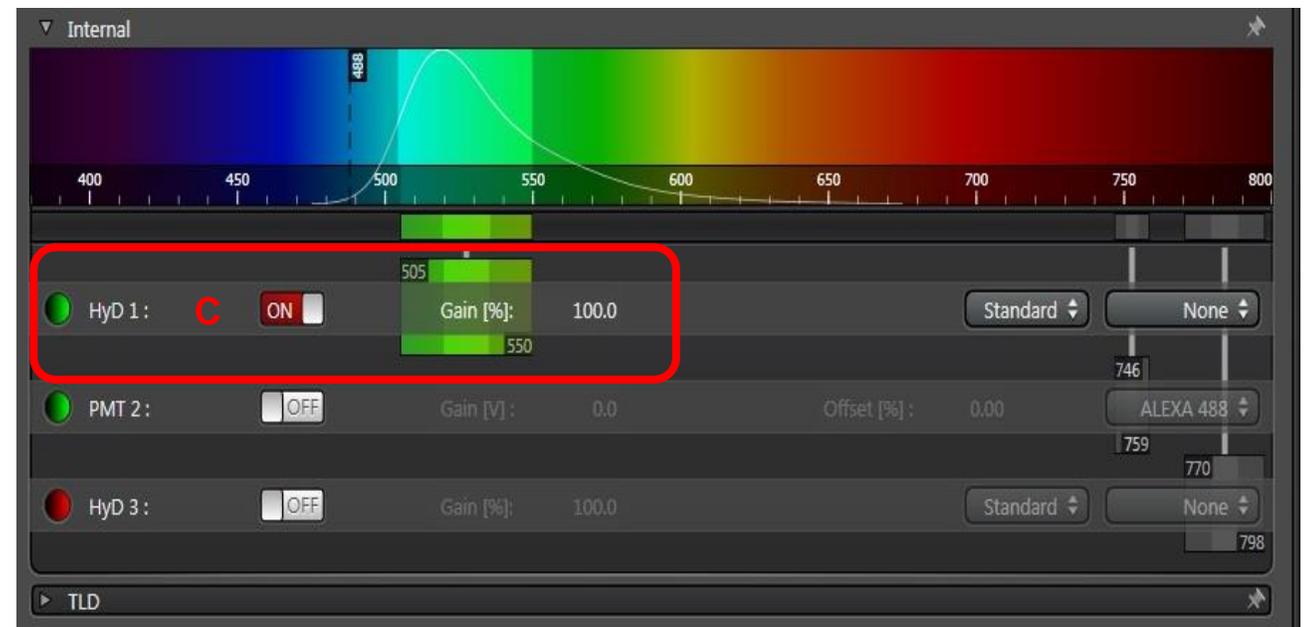


85. Move the sliders to shrink the gray slider area and move to the right out of the way.

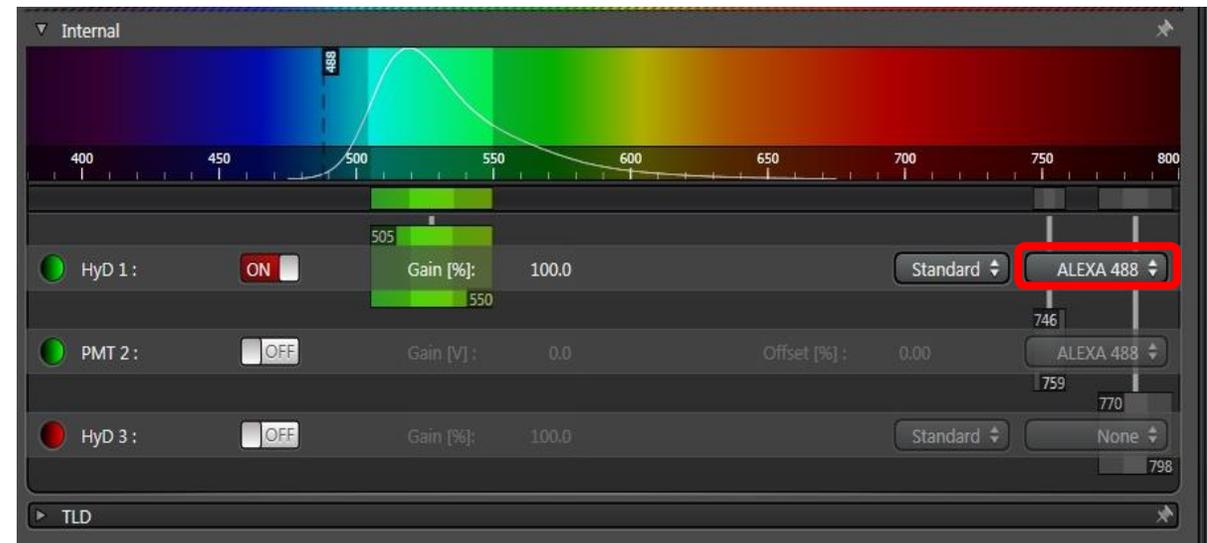
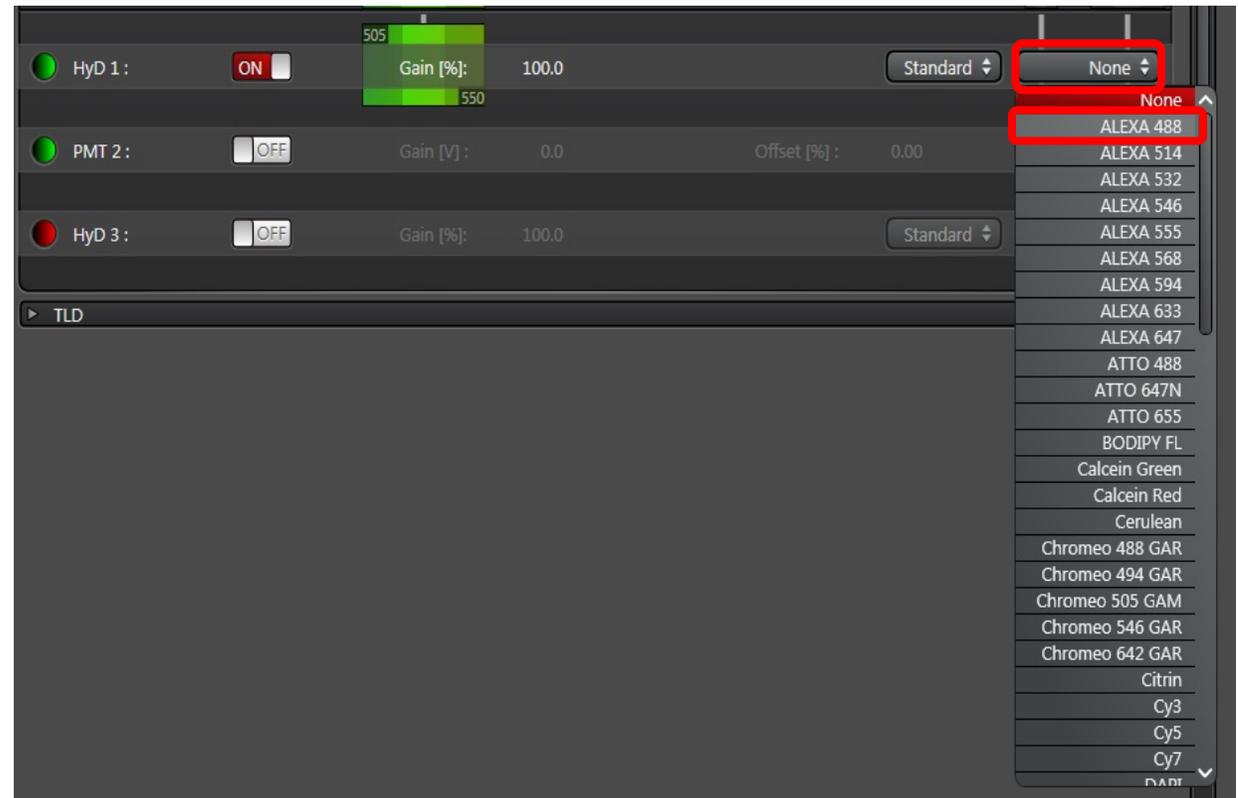


86. Create your emission filter for Alexa 488 on the HyD detector.

- A. Click on emission filter [blue box].
- B. Enter numbers of the emission filter.
- C. See new emission filter.

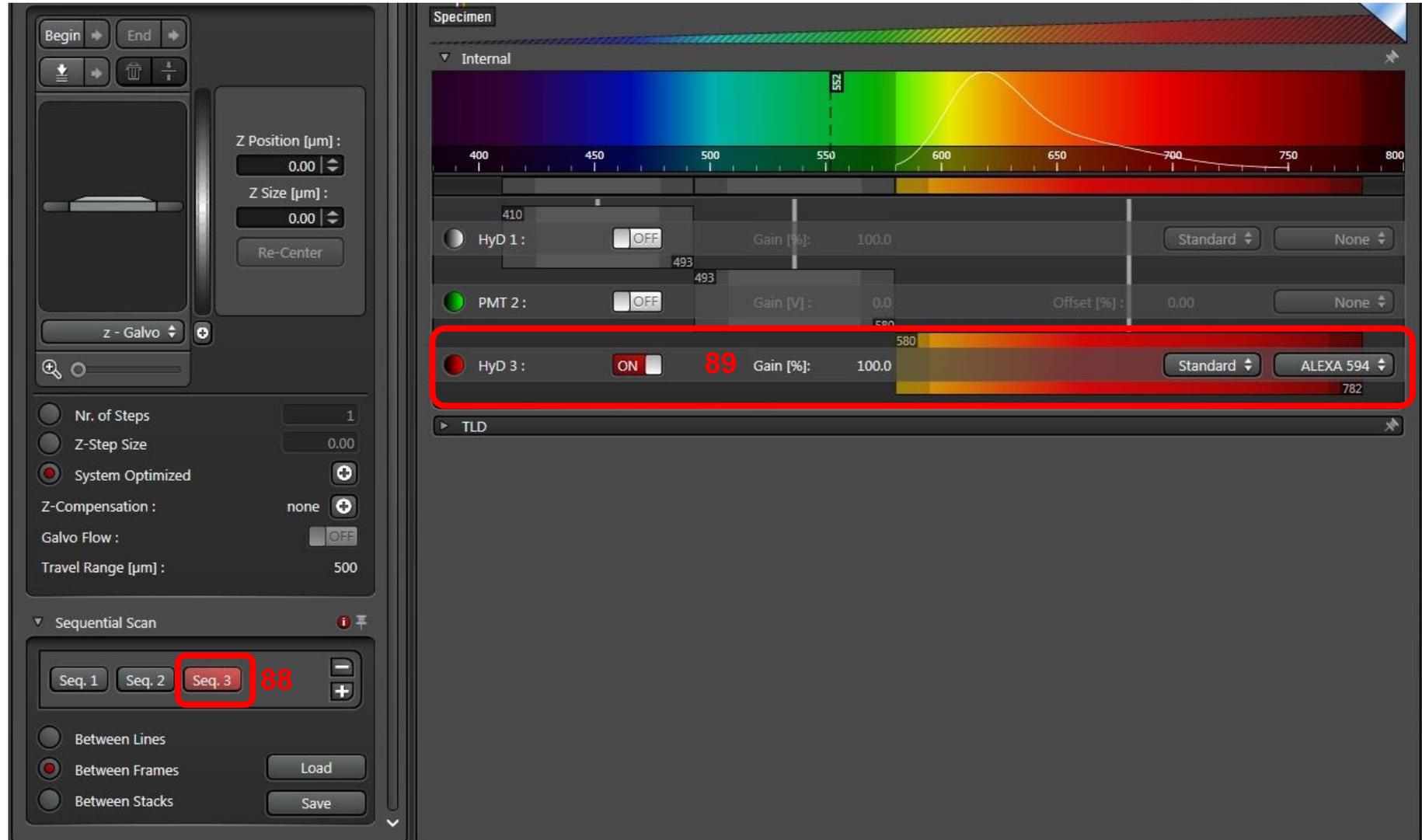


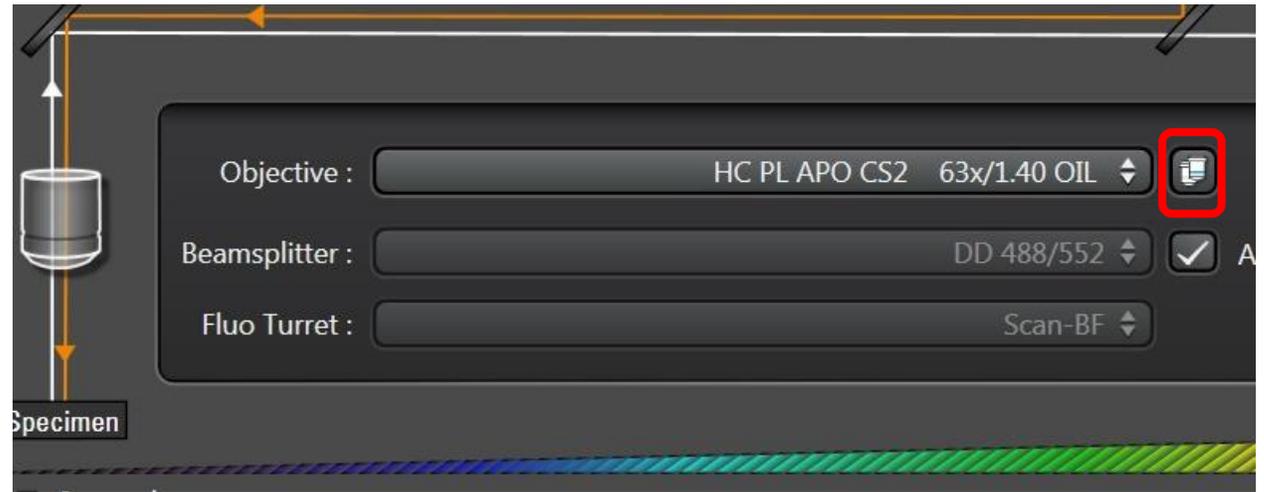
87. On HyD 1 detector change the name from None to Alexa 488.



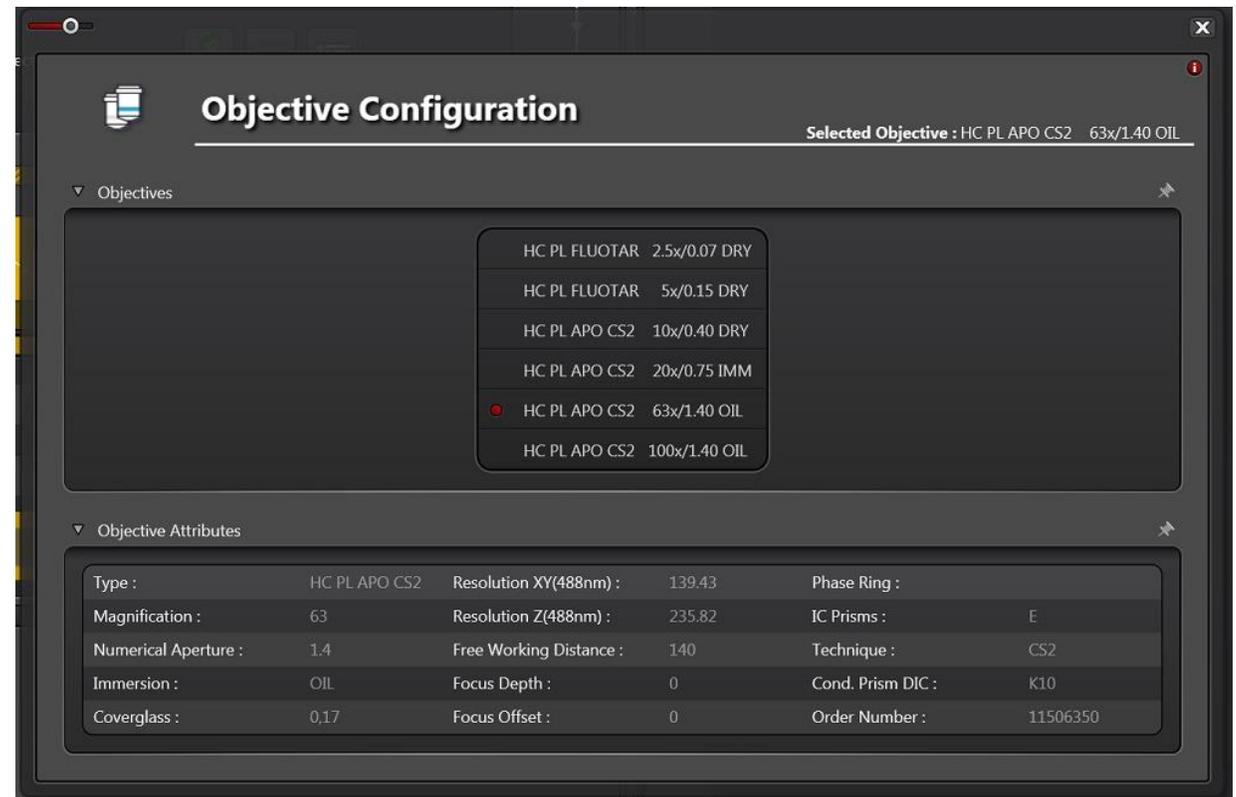
88. Click on Sequence 3 [Seq 3].

89. Check the lightpath to see if it needs to be changed. In this case it does not need adjusting.

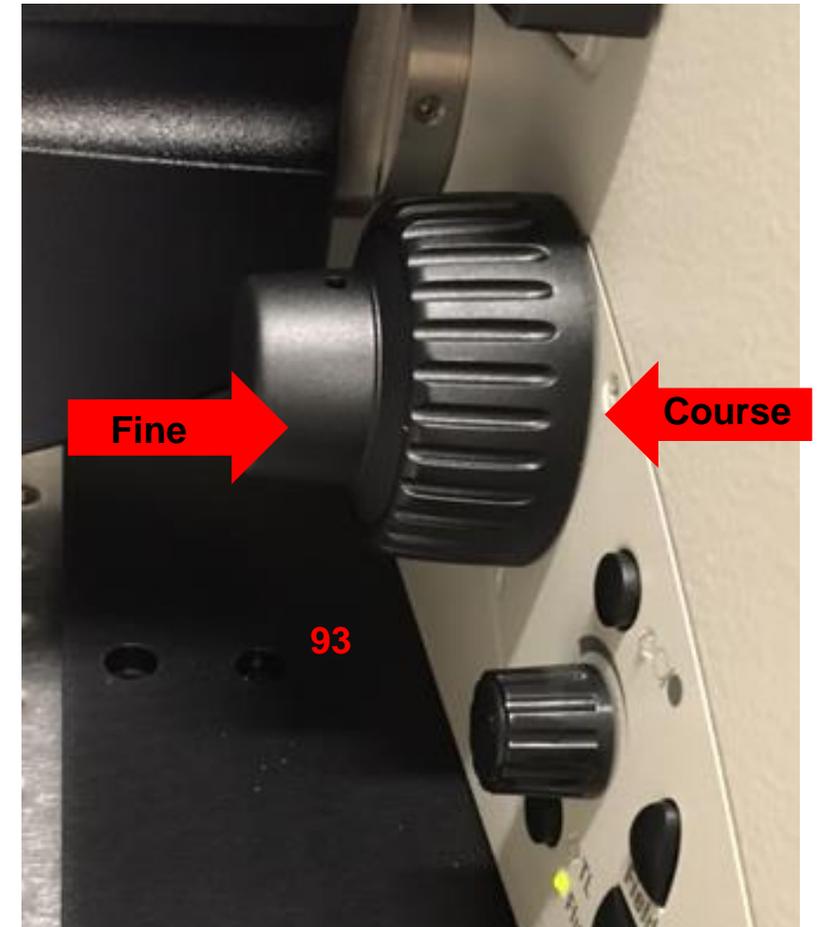
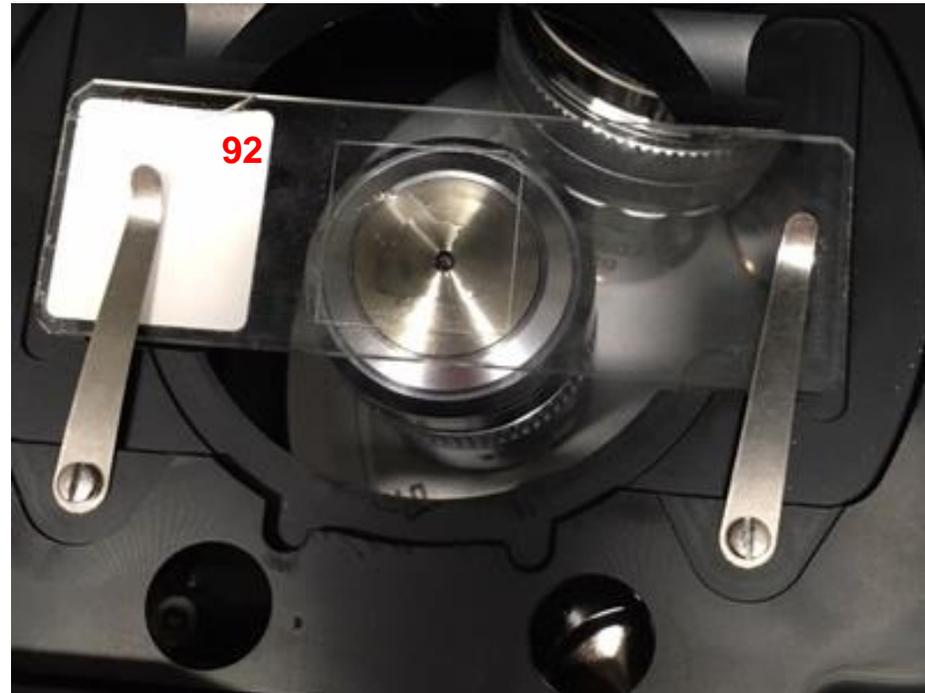
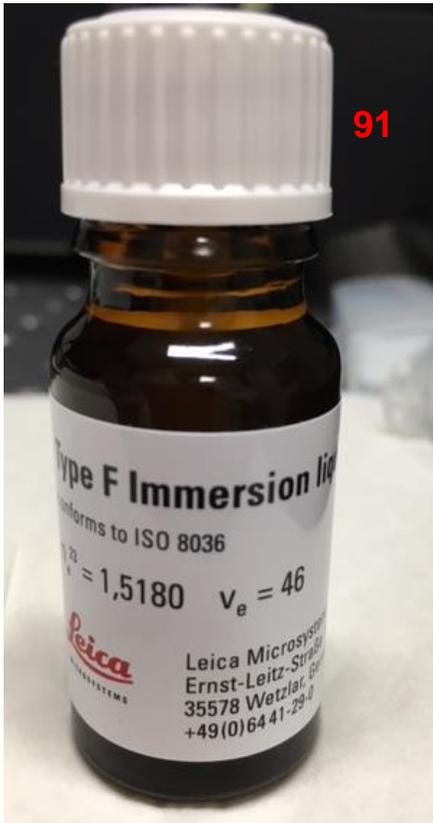




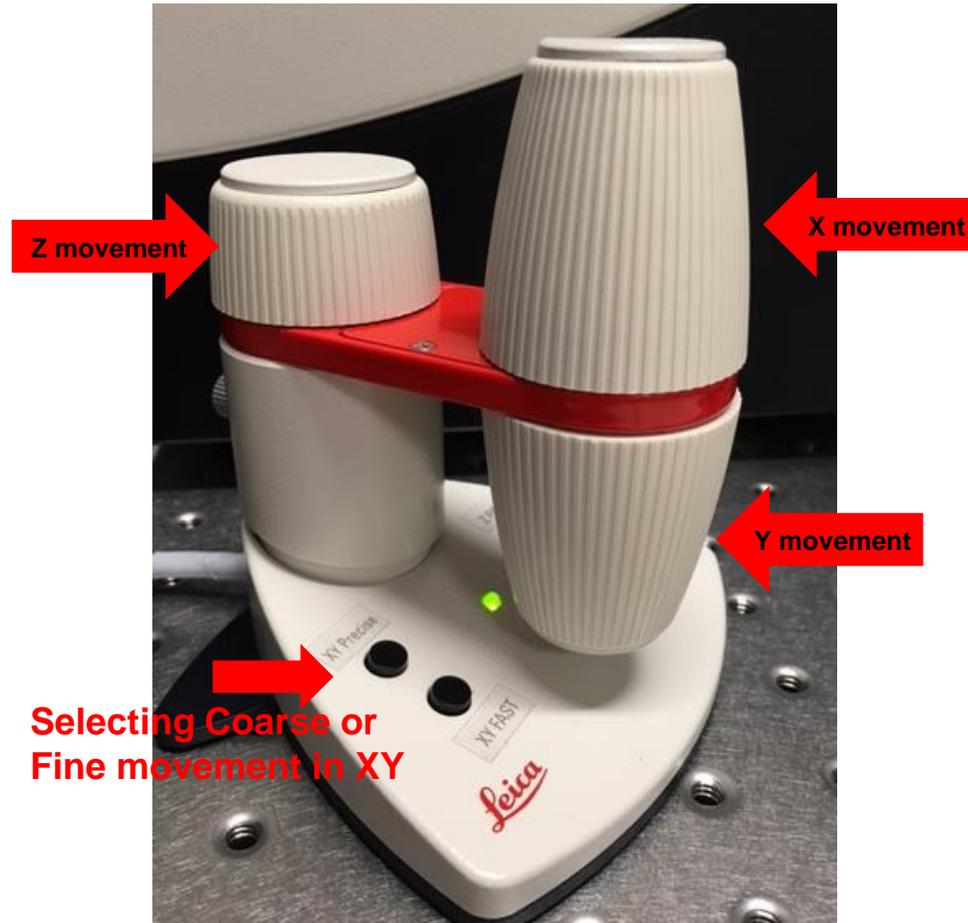
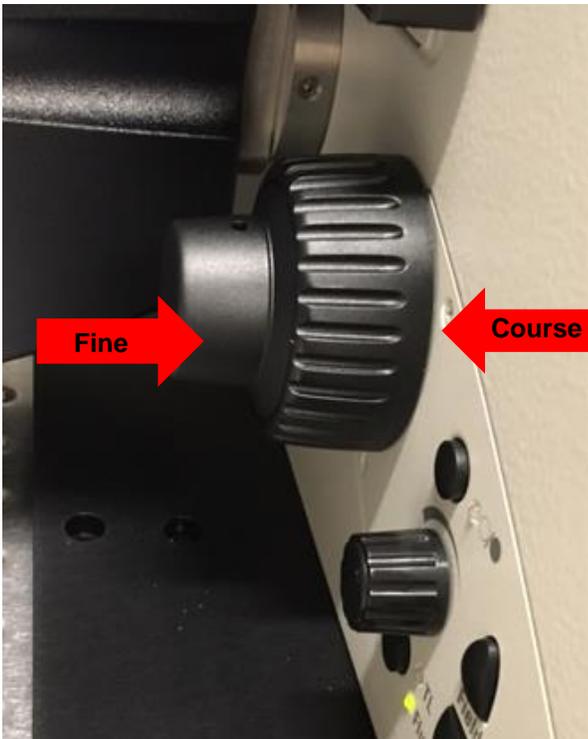
If you click on the objective icon next to the objective window it will open up the Objective Configuration window. This will give you the details of the selected objective.



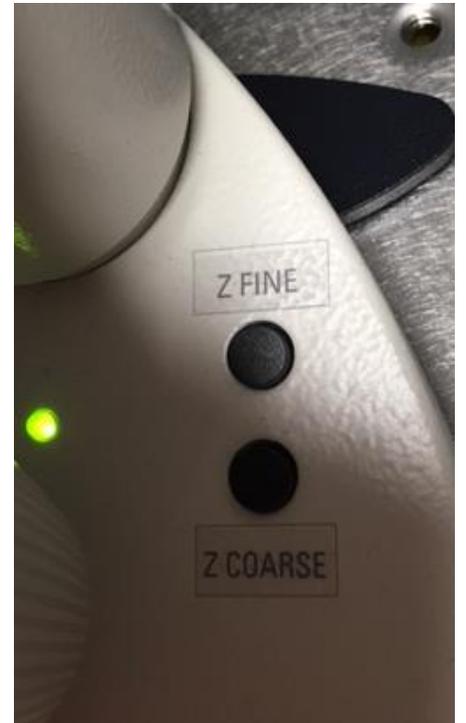
90. Clean the objective using lens paper and Sparkle.
91. Place oil on the slide or on the objective.
92. Place sample on microscope and use the clips to secure it in place.
93. Raise the objective by turning the focus knob away from you.



Stage movement and Focus controls:



Selecting Coarse or Fine movement in Z



If focus seems to be moving too little:

- On the touch screen select the X,Y, Z icon.
- Under Microscope select Coarse.

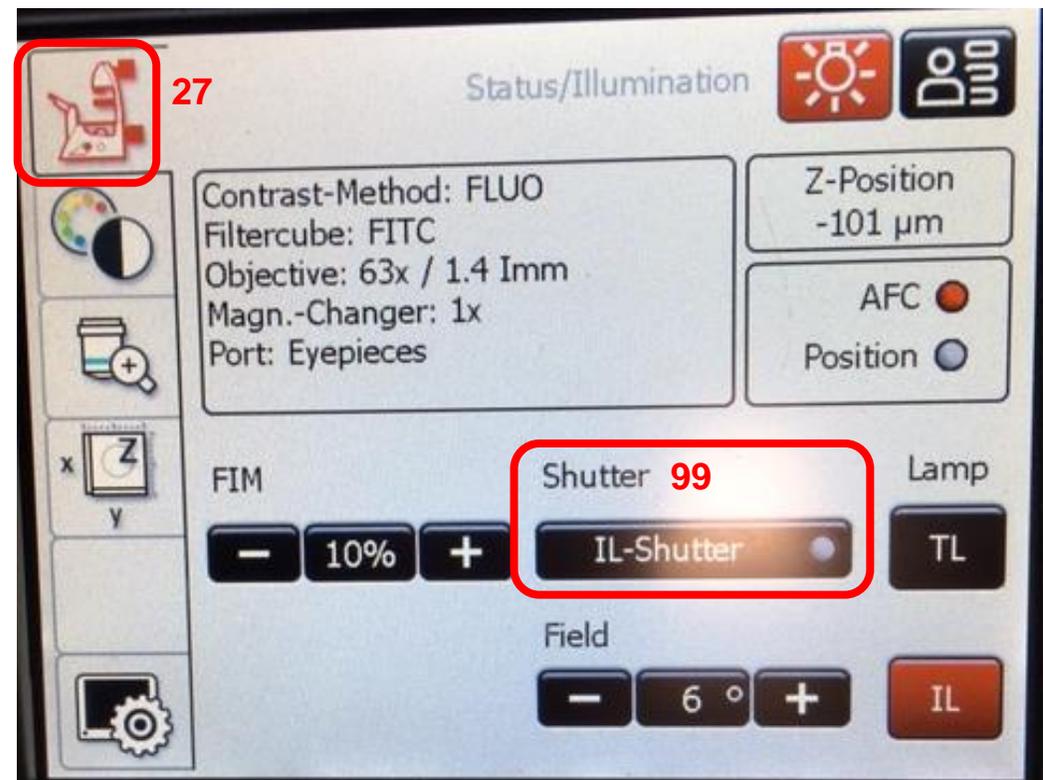
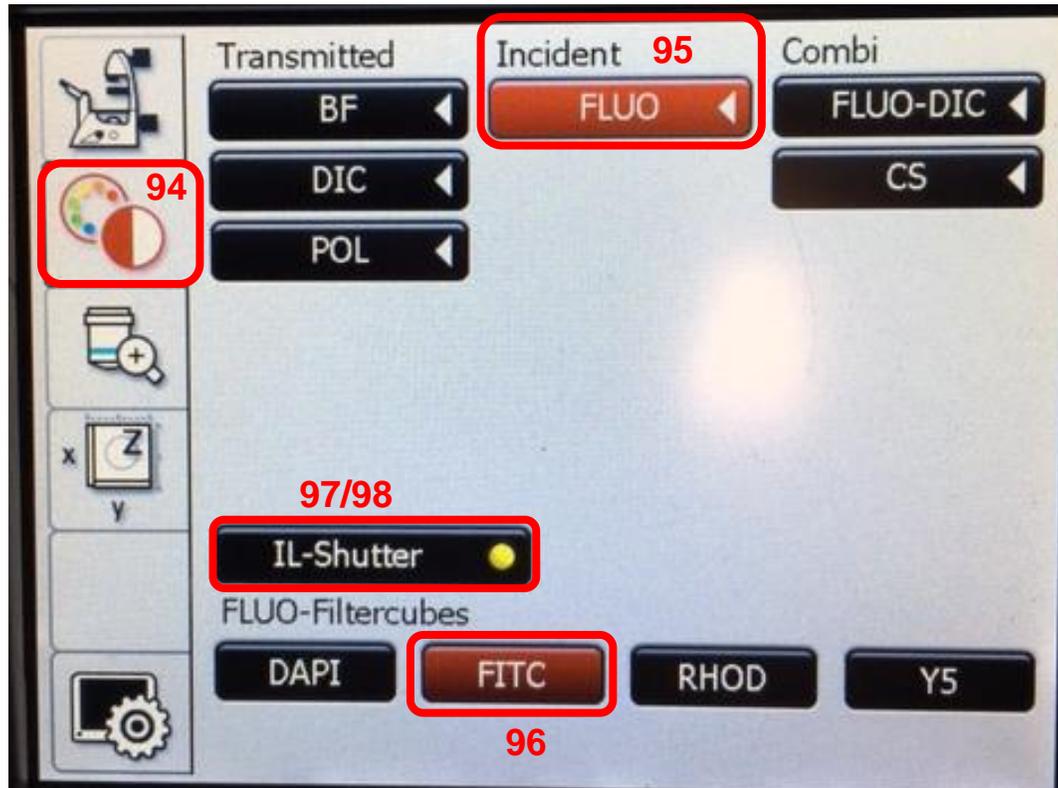


To View Sample Through the Eyepiece

Using the touch pad on the microscope stand:

94. Select the color and contrast icon.
95. Under Incident select FLUO.
96. Select the FLUO-Filtercube you want.
97. Select IL-Shutter. Yellow light indicates light is on.
98. After you get focus, select IL-Shutter to turn off light. Gray light indicates off.
99. You can also turn the light on and off from the Microscope window.

If you do not see any light coming from the objective see the next page to troubleshoot.



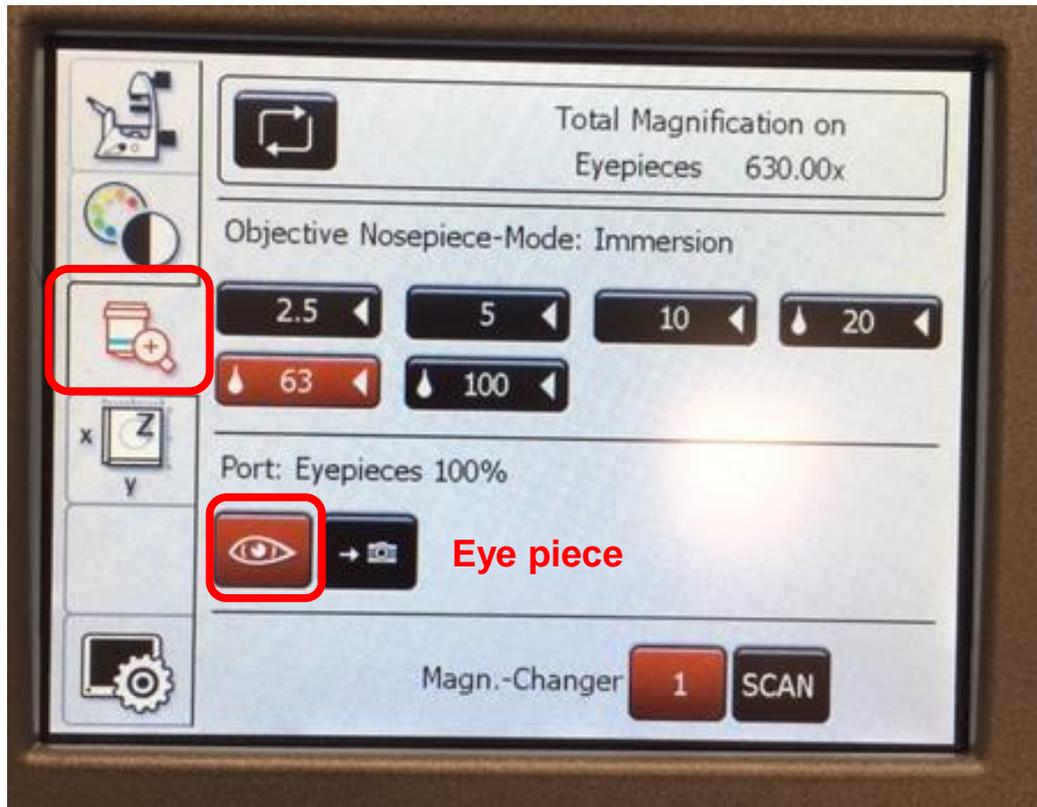
If you do not see any light coming from the objective.

- Check the light source box. The Intensity is likely too low. Turn it up. The second white dot is usually good enough.
- On the microscope check the side of the condenser arm. It should be Brightfield not Digital Lightsheet.



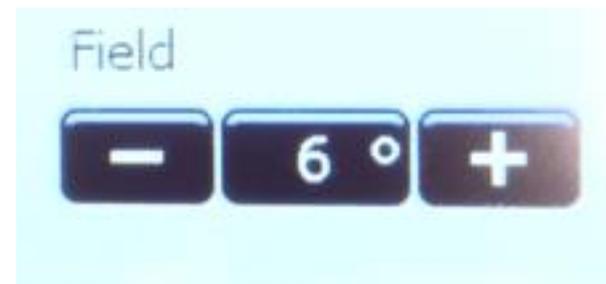
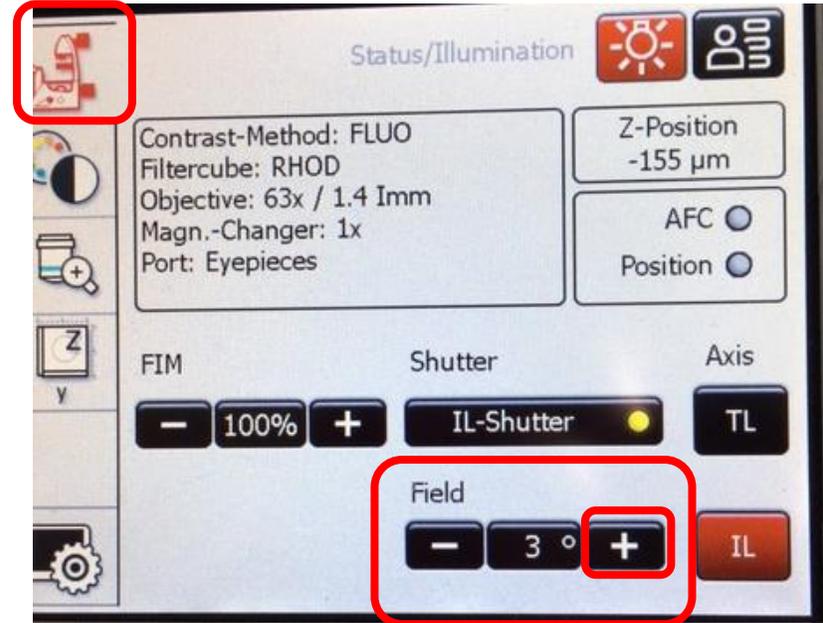
On the Microscope Touch Screen:

- Select the Objective Window.
- Check the “Port:” section – it should be the Eyepiece icon in red not the camera icon.



If you have a very small viewing area:

- **Select Microscope icon on the touch pad.**
- **Adjust the Field by pressing the + button.**
- **A field of 6° is good.**



Control Panel

Located beneath the monitor.

When you hit either high or low limit of any control you will hear beeps.



Smart Gain:
Adjust the gain.

Smart Offset:
Adjust the offset.

Scan Field Rotate:
Rotating the field
of view.

Pinhole:
Adjust pinhole

Zoom:
Adjust the Zoom.

Z Position:
Focus adjustment
and to set Z stack

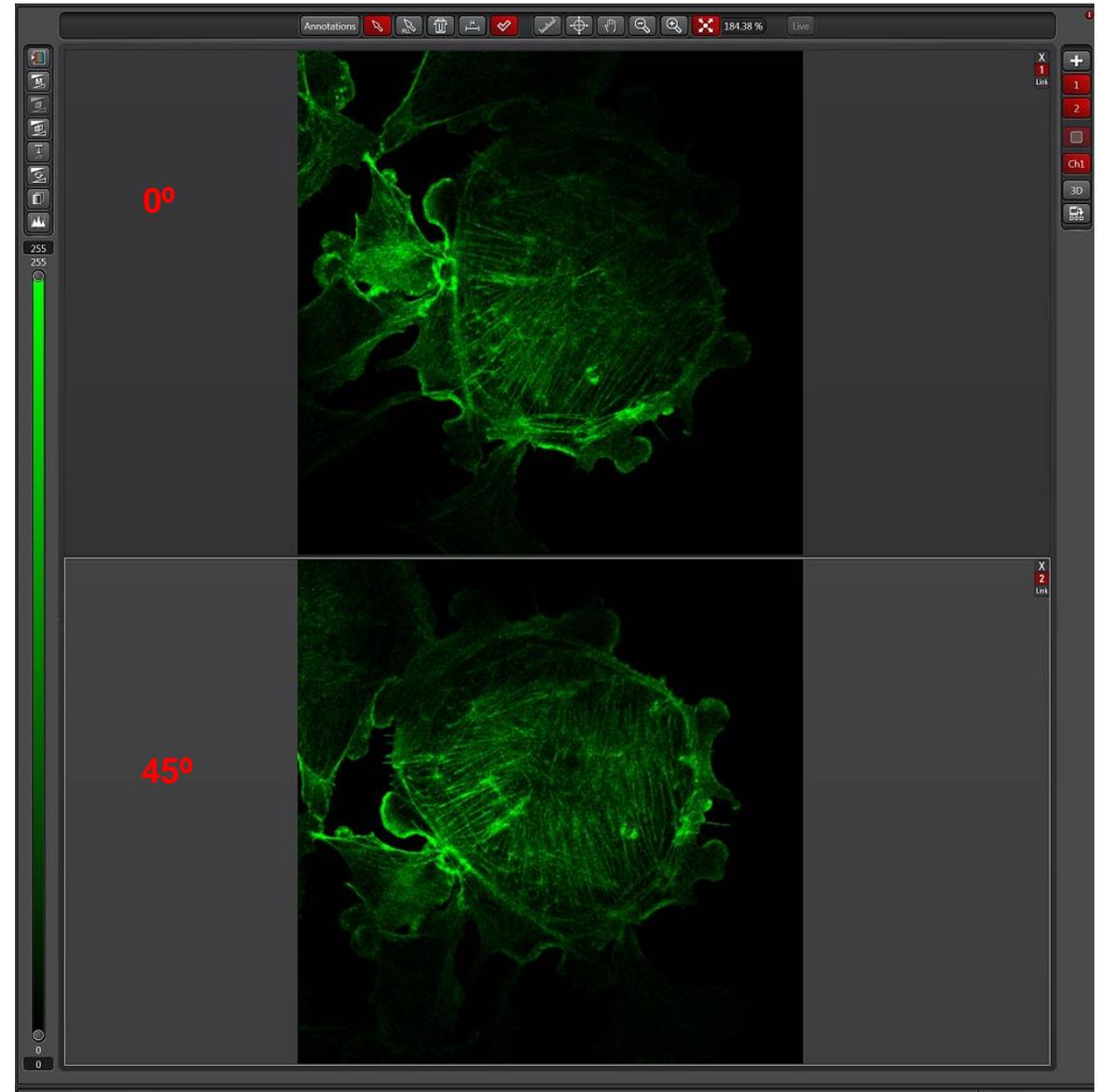
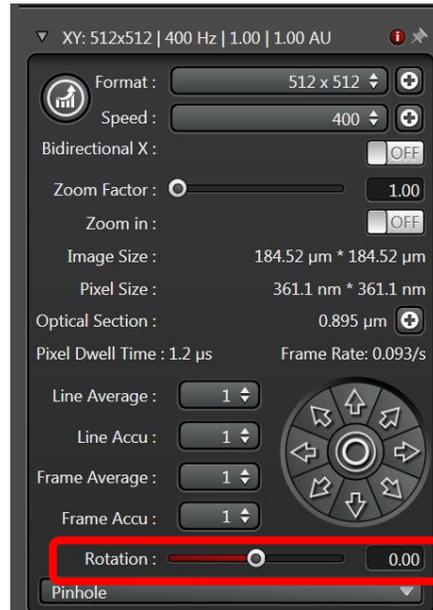


Scan Field Rotate Example:
Scan Rotation of 0° [top] and 45° [bottom].
This can be done from the Control Panel [A] or software [B].

A



B

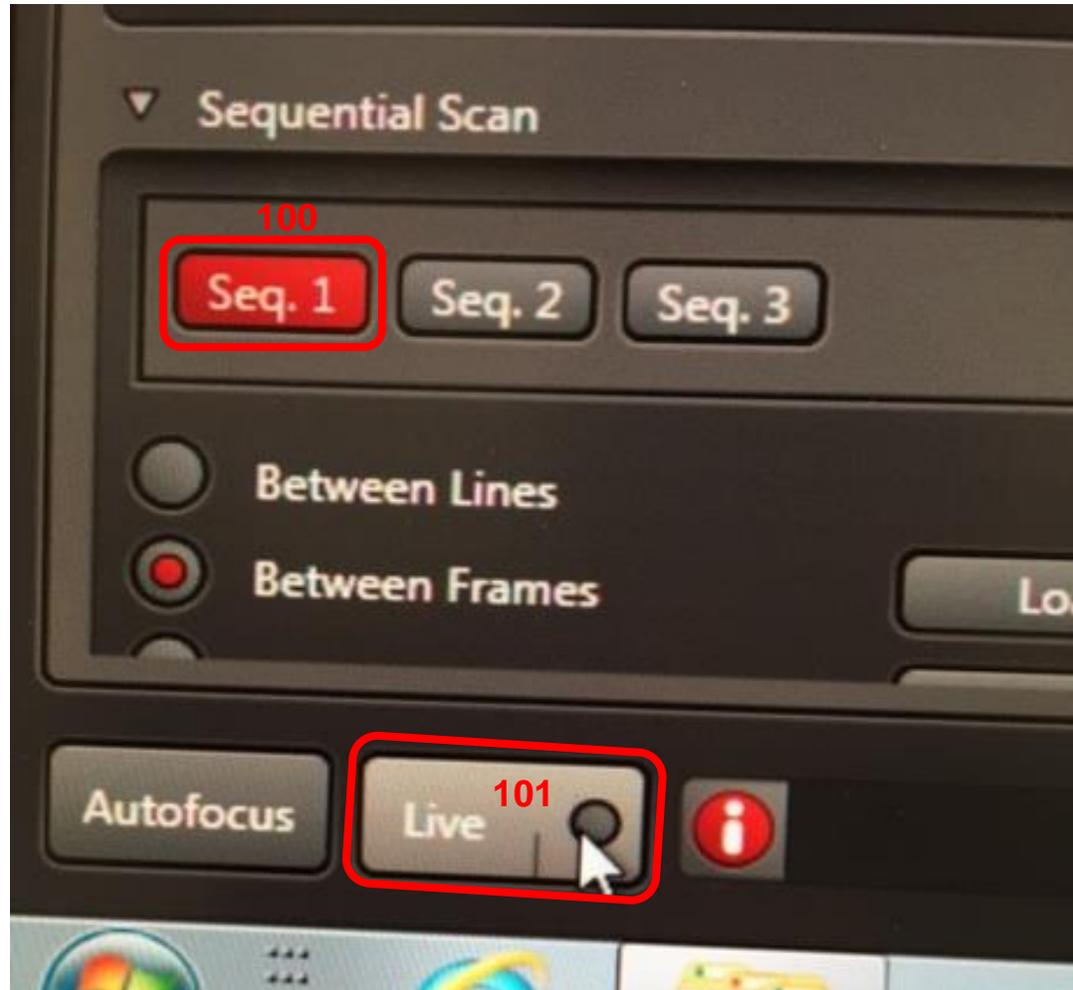


Back on the software:

100. Select the Sequence you wish to scan first.

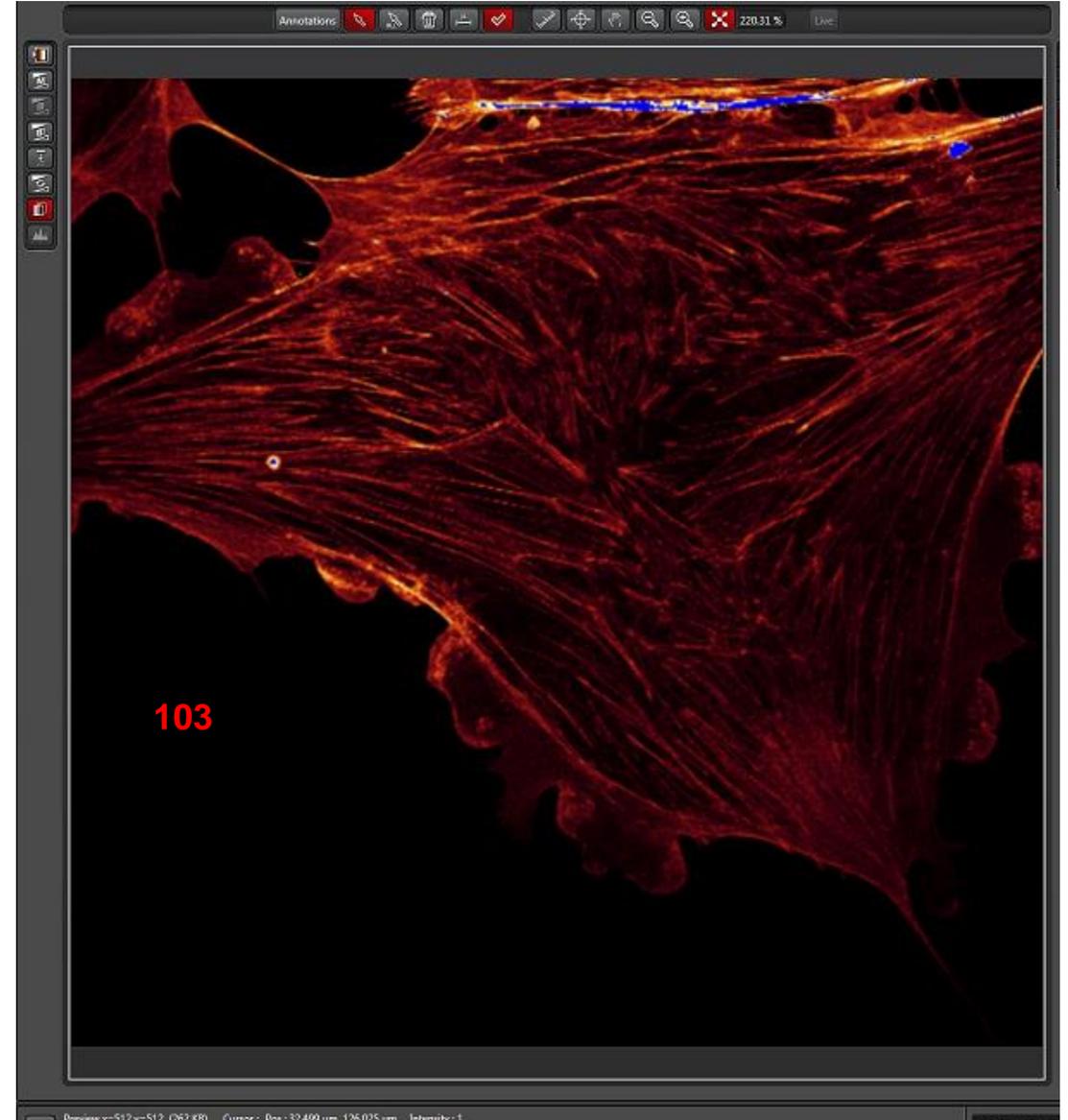
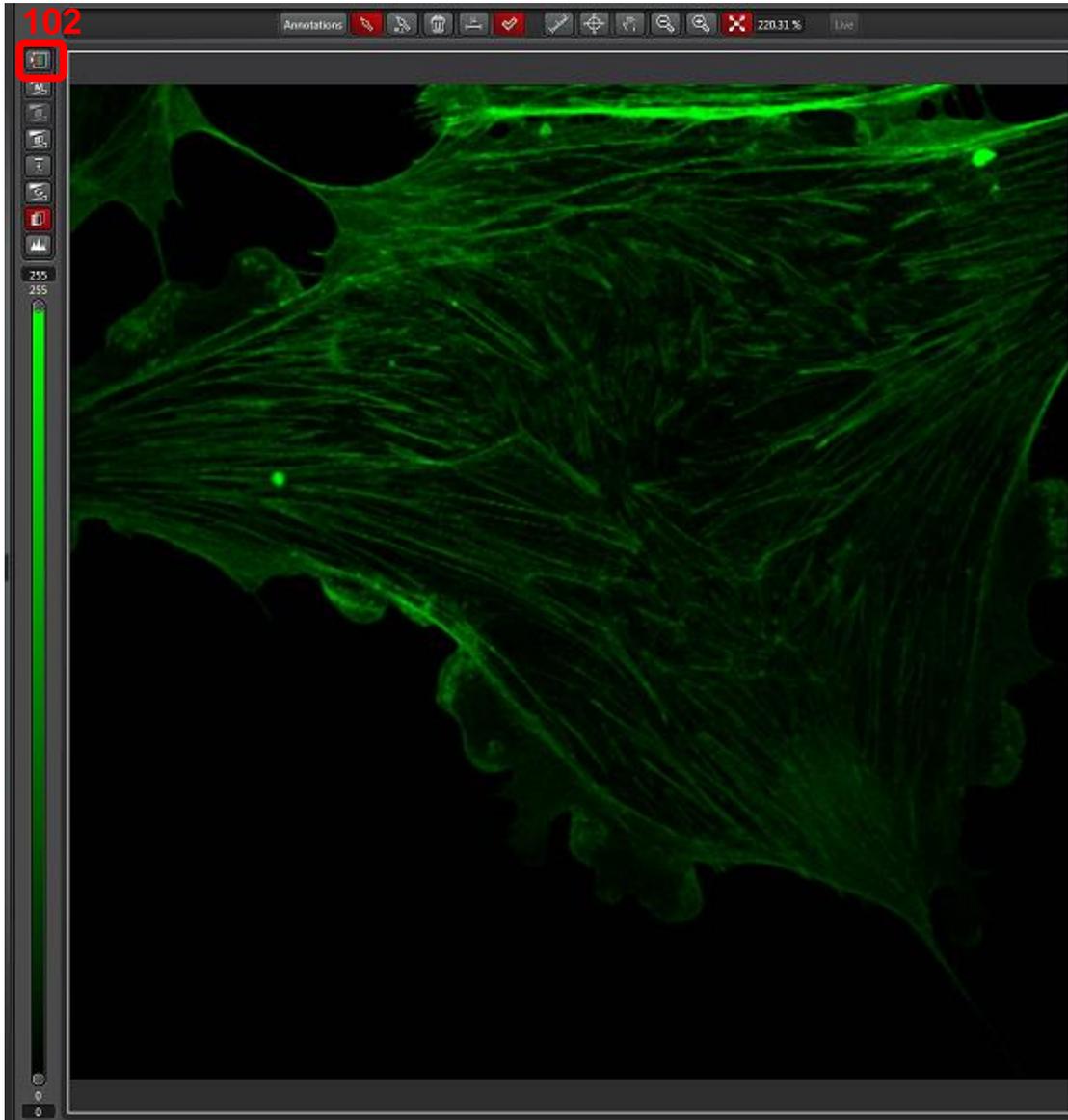
101. Click Live. This is a fast XY scan.

Note: Scan and reaction to adjustments are slower than you may be used to on the Zeiss microscopes. Be patient.



102. Click the LUT button.

103. Blue is saturation. Green is below detection [not seen here].



104. One way to adjust the gain is from the control box.

105. The offset can be adjusted from here too.

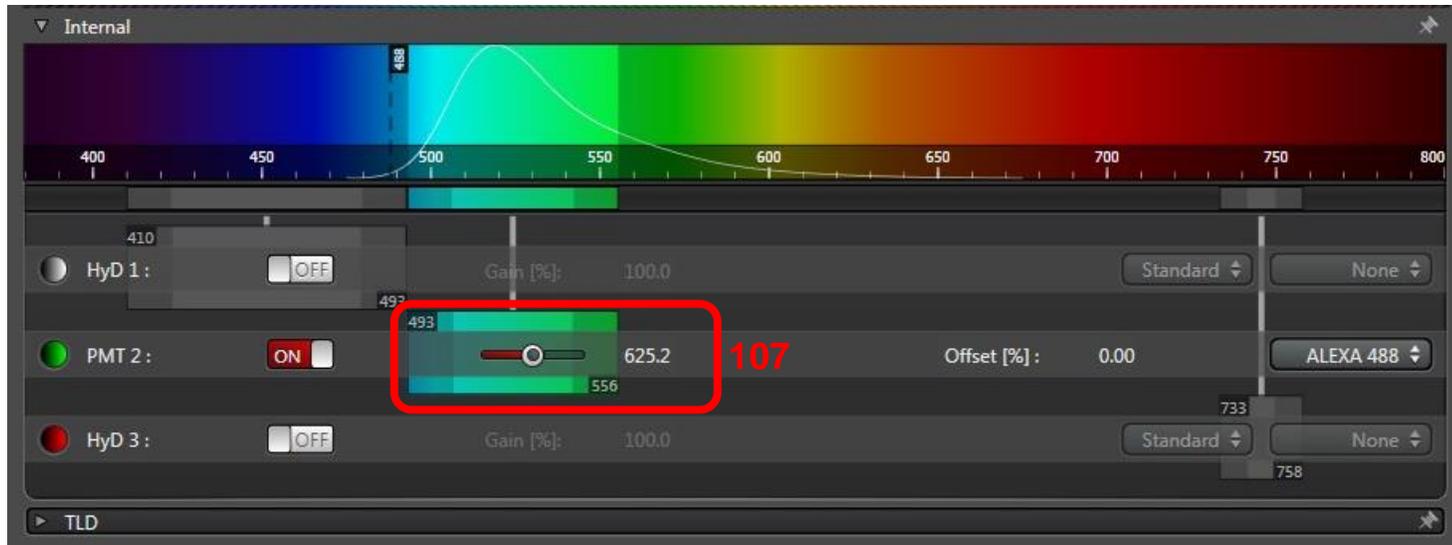
106. If you see Smart Offset disabled it means you cannot adjust the offset. This is usually when you select the HyD detector.



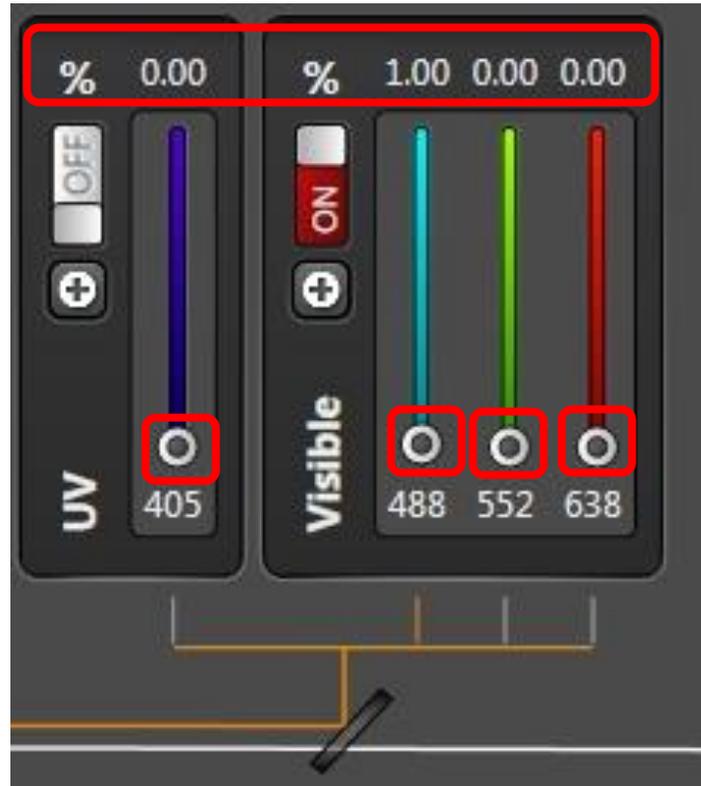
107. Another way to adjust the gain is to click on the gain in the software which brings up a slider. You can use the mouse to move the slider or the scroll bar on the mouse to adjust the gain.

108. Select the next Sequence and repeat the steps.

109. Repeat until you have gone through all your dyes.

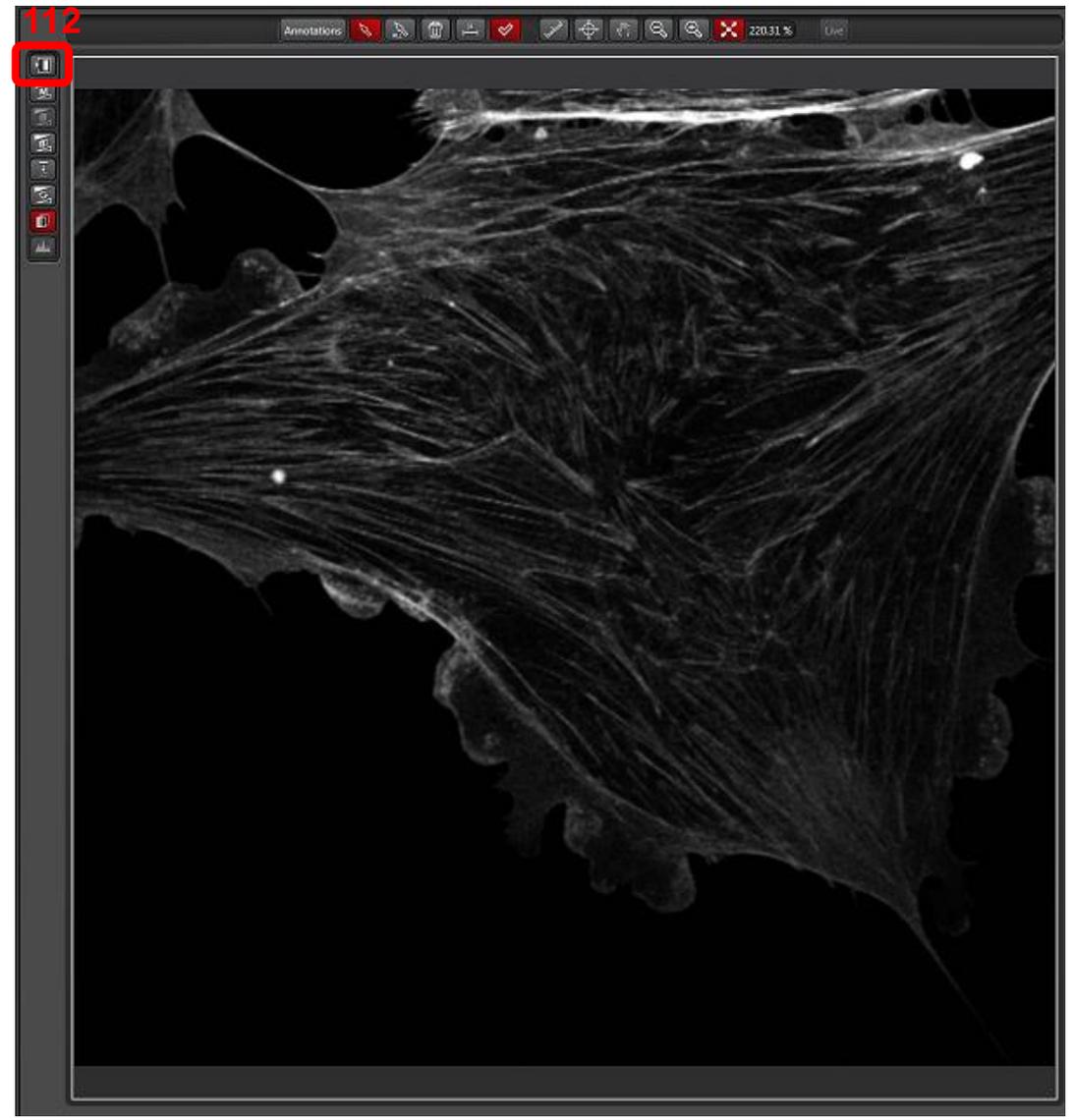
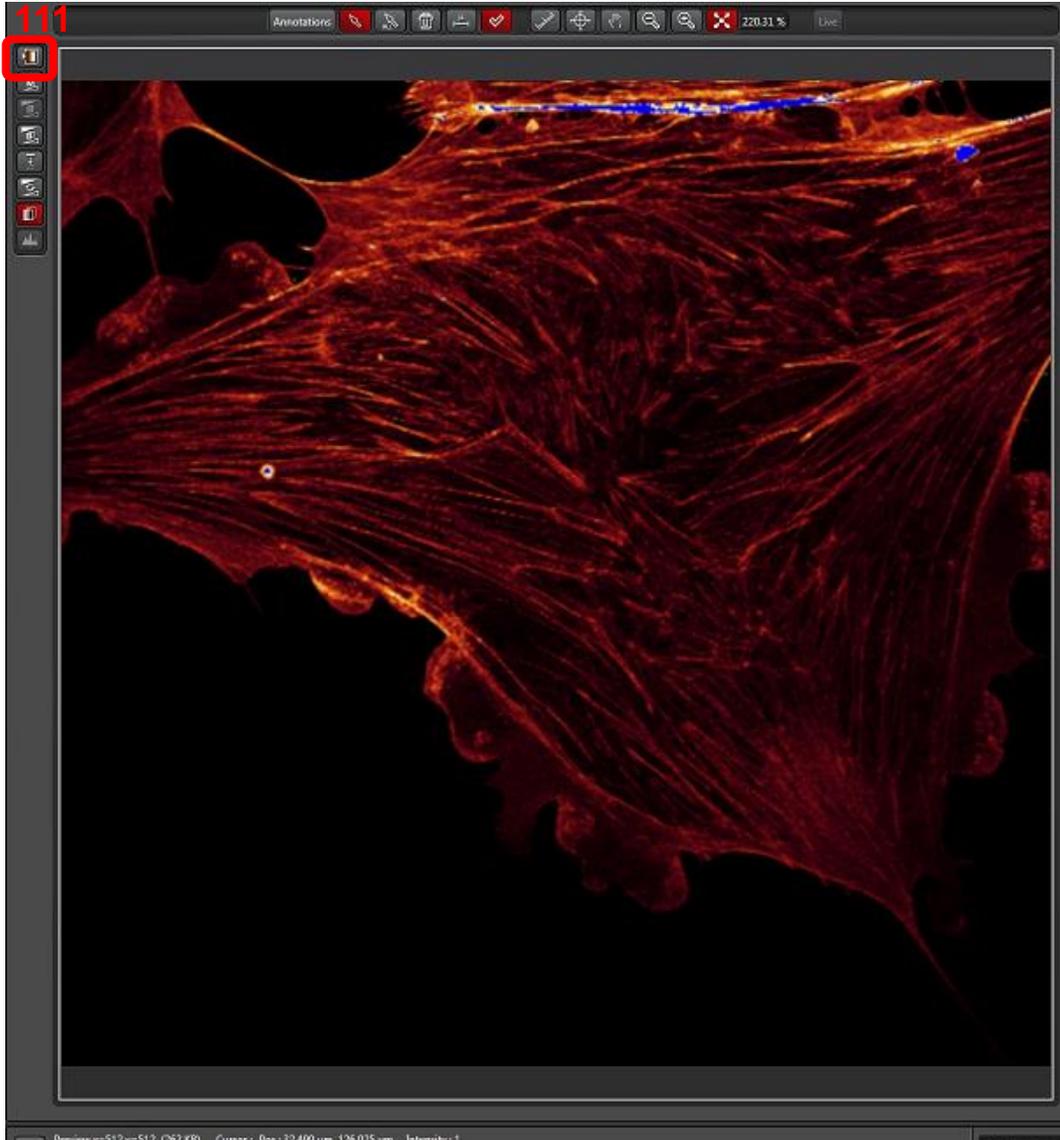


110. To adjust the laser power click on the button of the laser you wish to adjust then either use the scroll bar on the mouse to adjust or click on the laser power % and type in number you want.



111. To get out of the LUT settings box click the box again and you get a gray scale view.

112. Click the gray scale view box to get back to a colored image view.

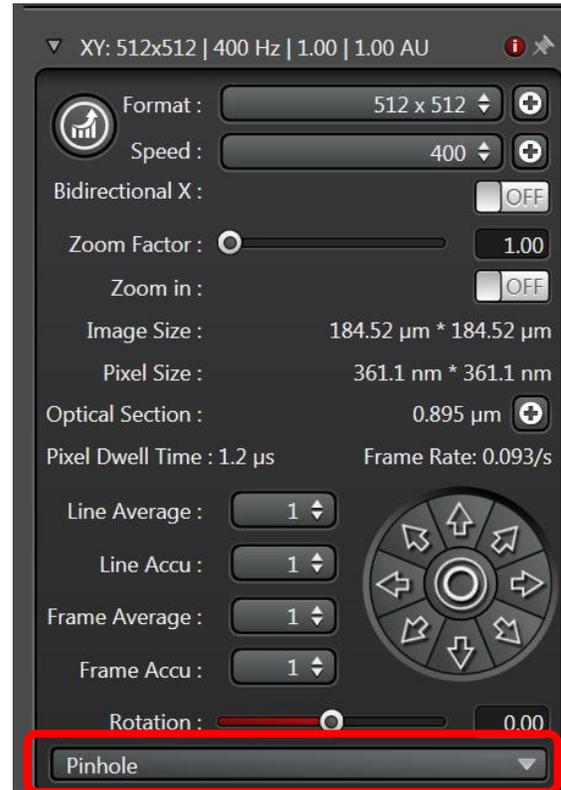


113. The Pinhole can be adjusted from the Control Panel [A] or from the software [B].

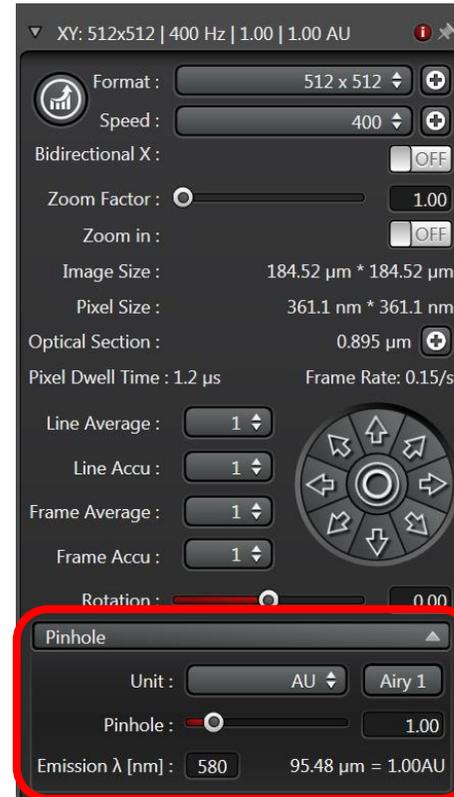
A



B



B



B



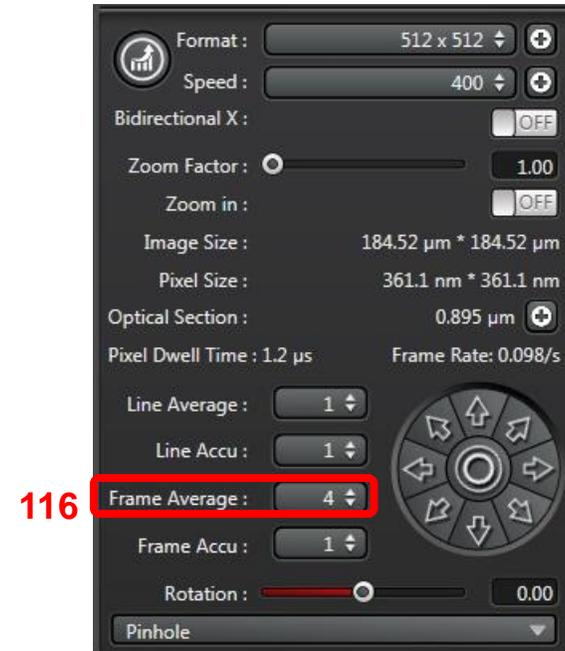
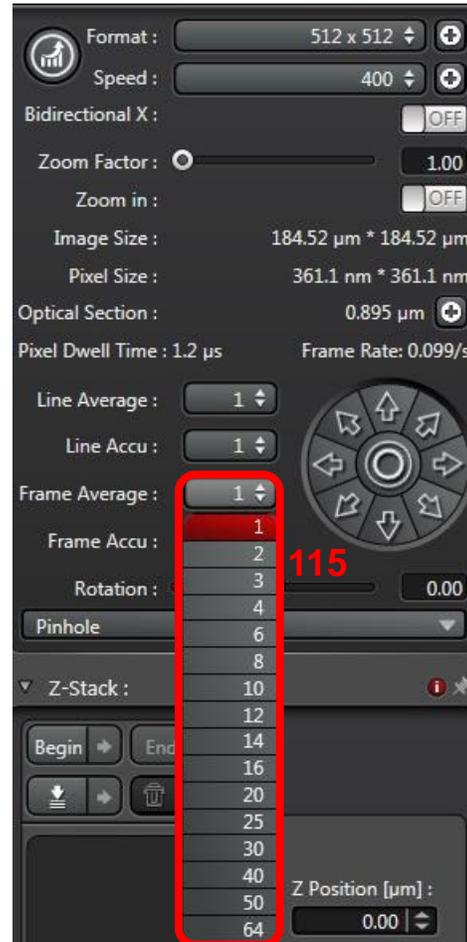
Selecting the Frame Average:

114. Go to the Frame Average window

115. From the drop down menu select 4.

116. See the average you selected in the window.

Frame average will collect sequence 1 then 2 then 3 and repeat this 4 times for the average.



If you wish to Select the Line Average instead:

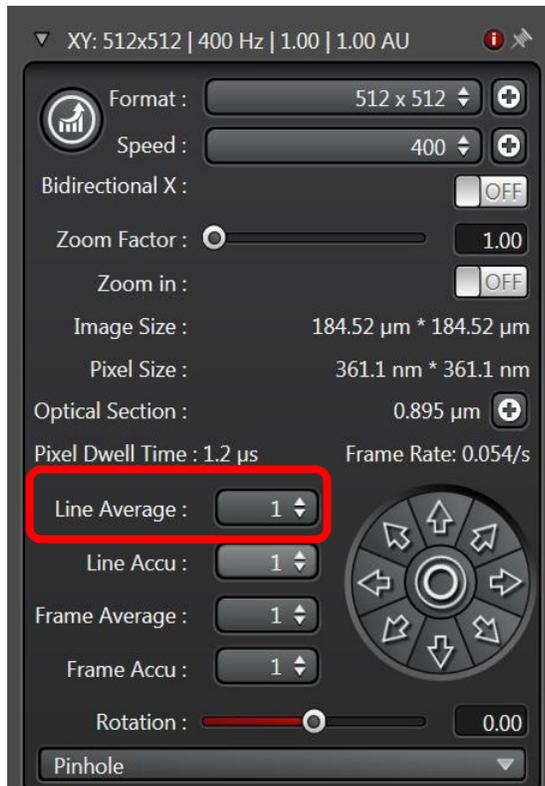
117. Go to the Line Average window

118. From the drop down menu select 4.

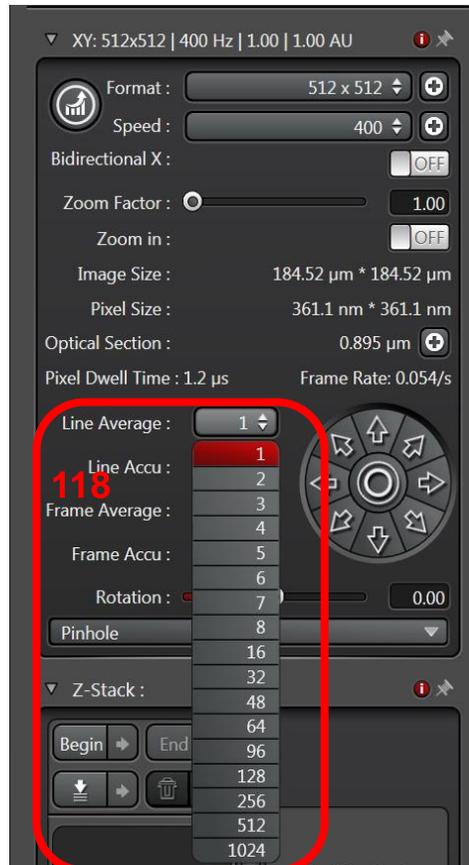
119. See the average you selected in the window.

Line average will create Sequence 1 4 times for the average and then repeat for other sequences.

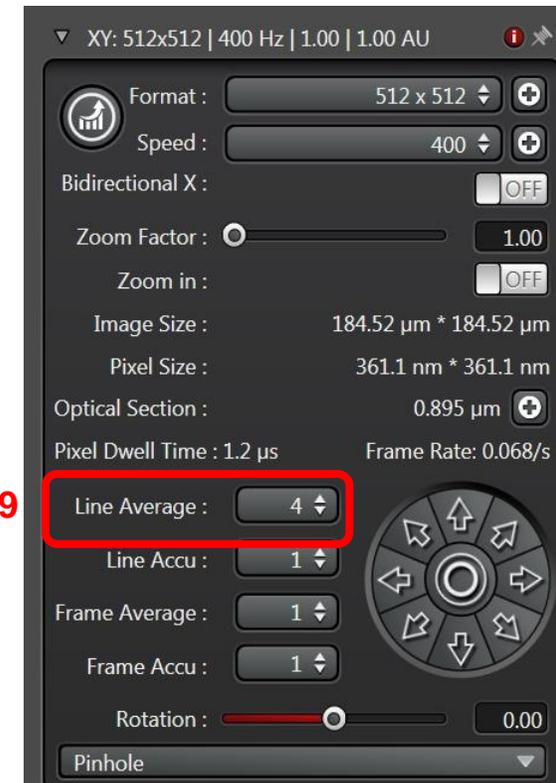
117



118

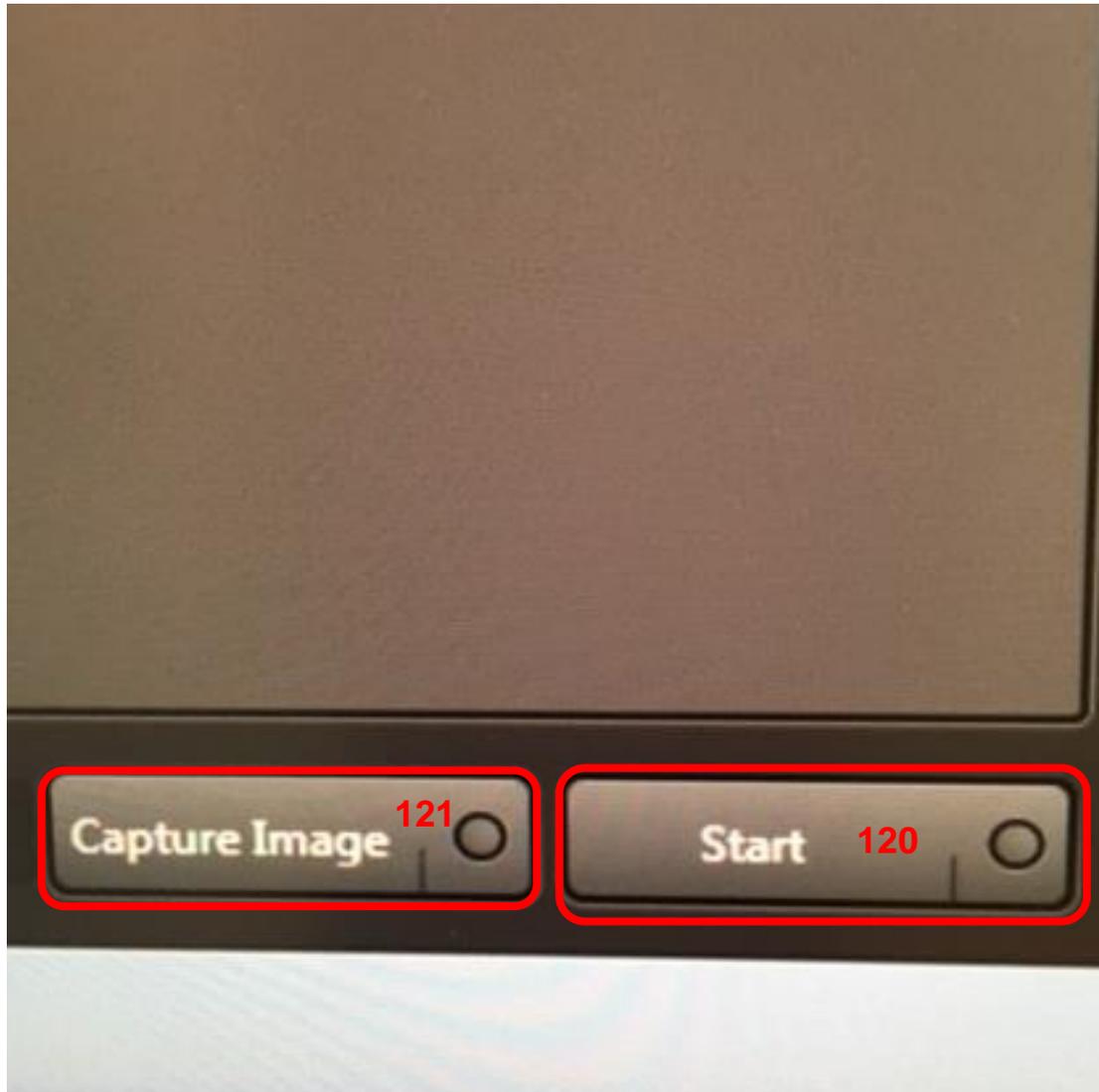


119



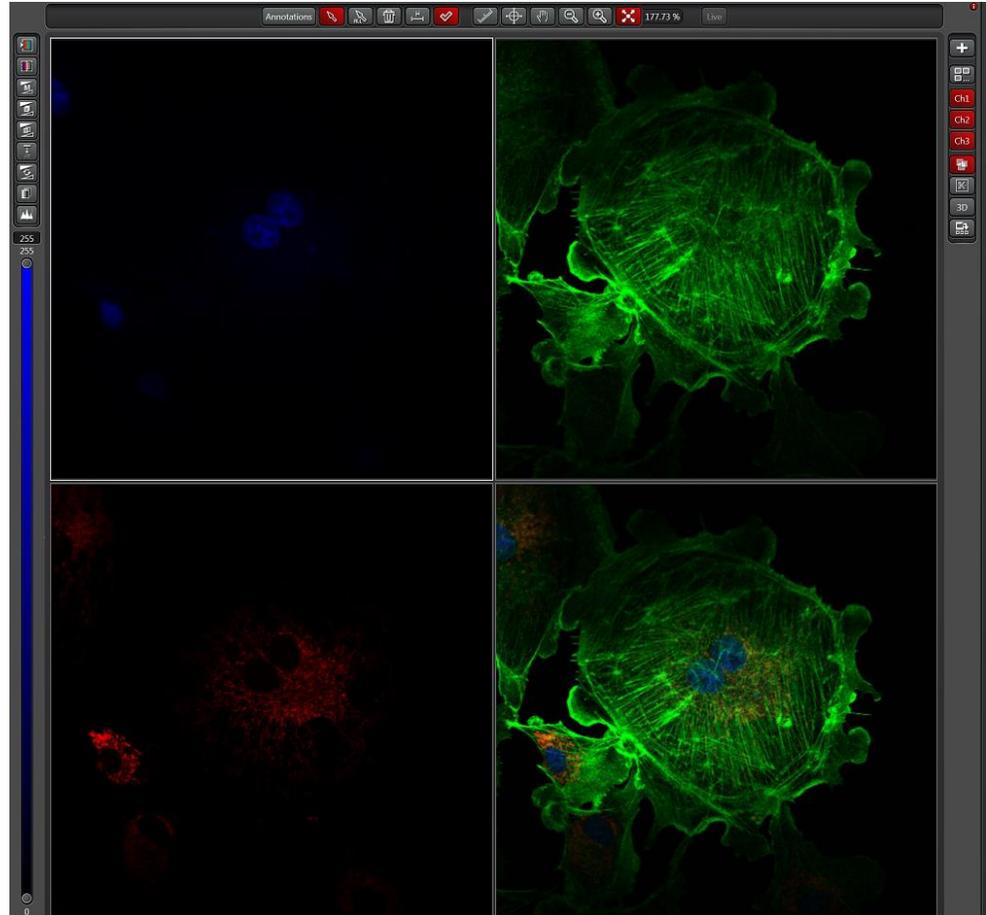
120. Click Start to acquire a multi-color image.

121. Click Capture Image to get a single color image. The color will be the Sequence that is selected. In this case Seq. 2.



122. Image in Split Channel View.

Contrast adjust
the window that
is selected and
highlighted in
white. In this
example blue.



- A. Create another viewing window so you can view multiple images.
- B. Merge Channels.
- C. Ch1 turn off and on. Red is on.
- D. Ch2 off and on.
- E. Ch3 off and on.
- F. Will show split channels as well as a merged channel window.
- G. Gallery view.

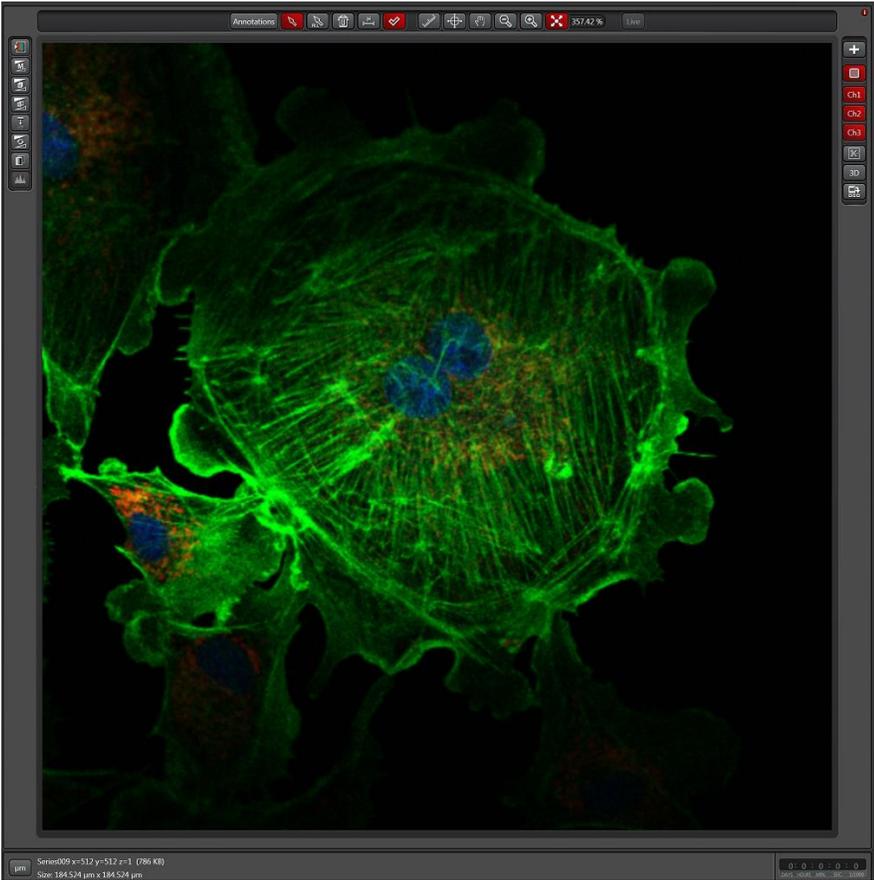
123. Image in Merge View.

Add scale bar

Zoom in and out



Rotate between color, LUT and gray scale view

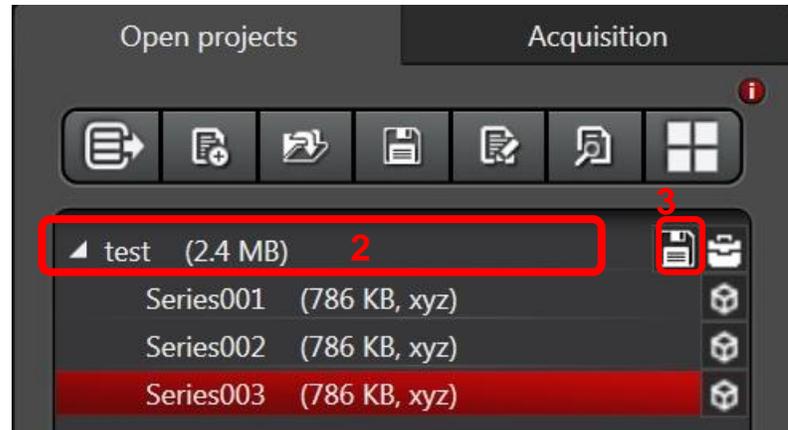
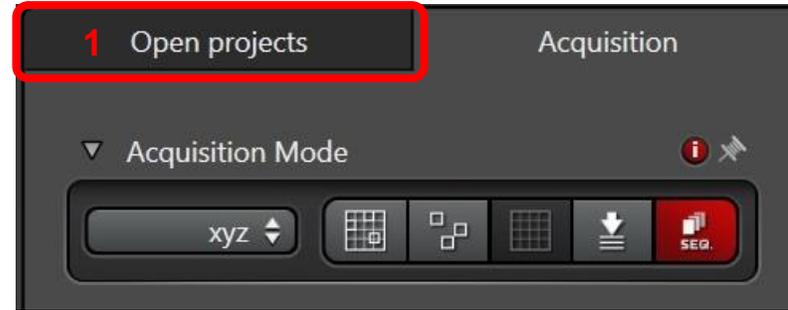


- A. Create another viewing window so you can view multiple images.
- B. Split Channels.
- C. Ch1 turn off and on. Red is on.
- D. Ch2 off and on.
- E. Ch3 off and on.
- F. Gallery view.

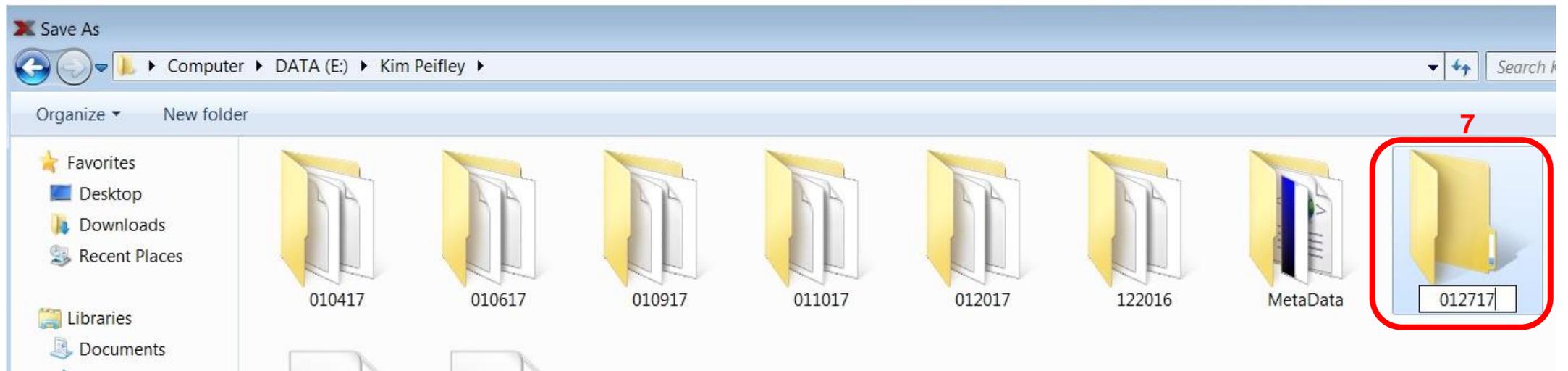
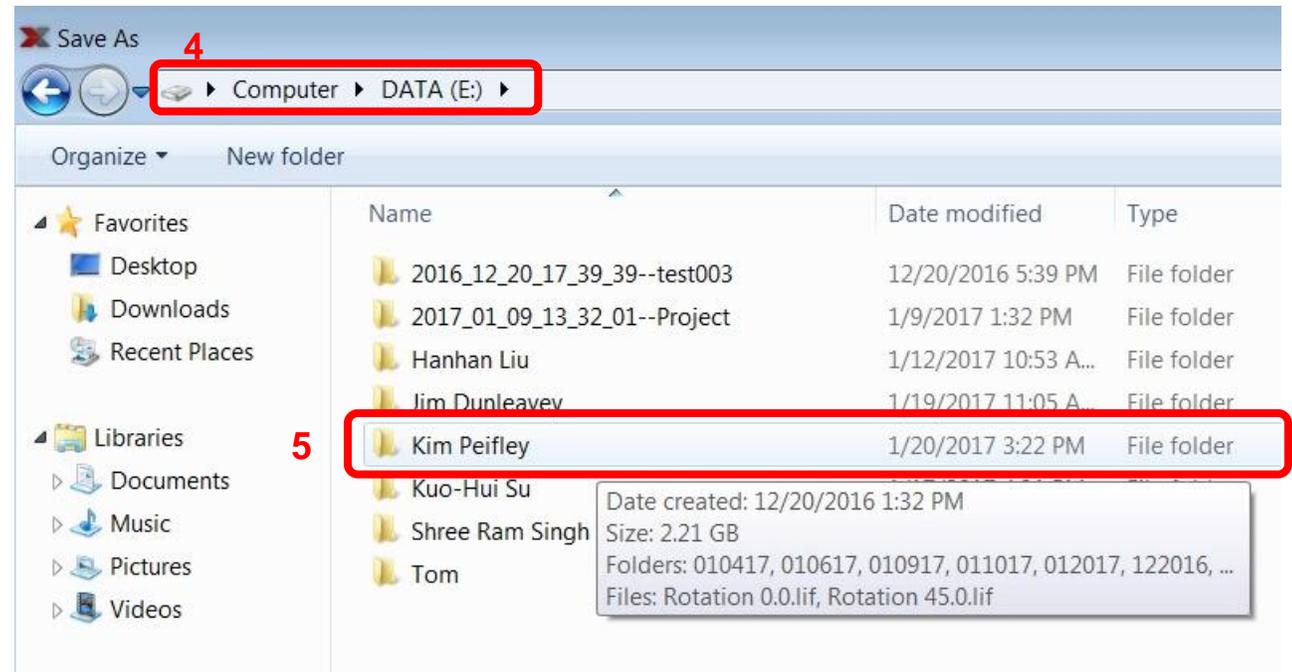
Saving Projects and Images

Saving Image:

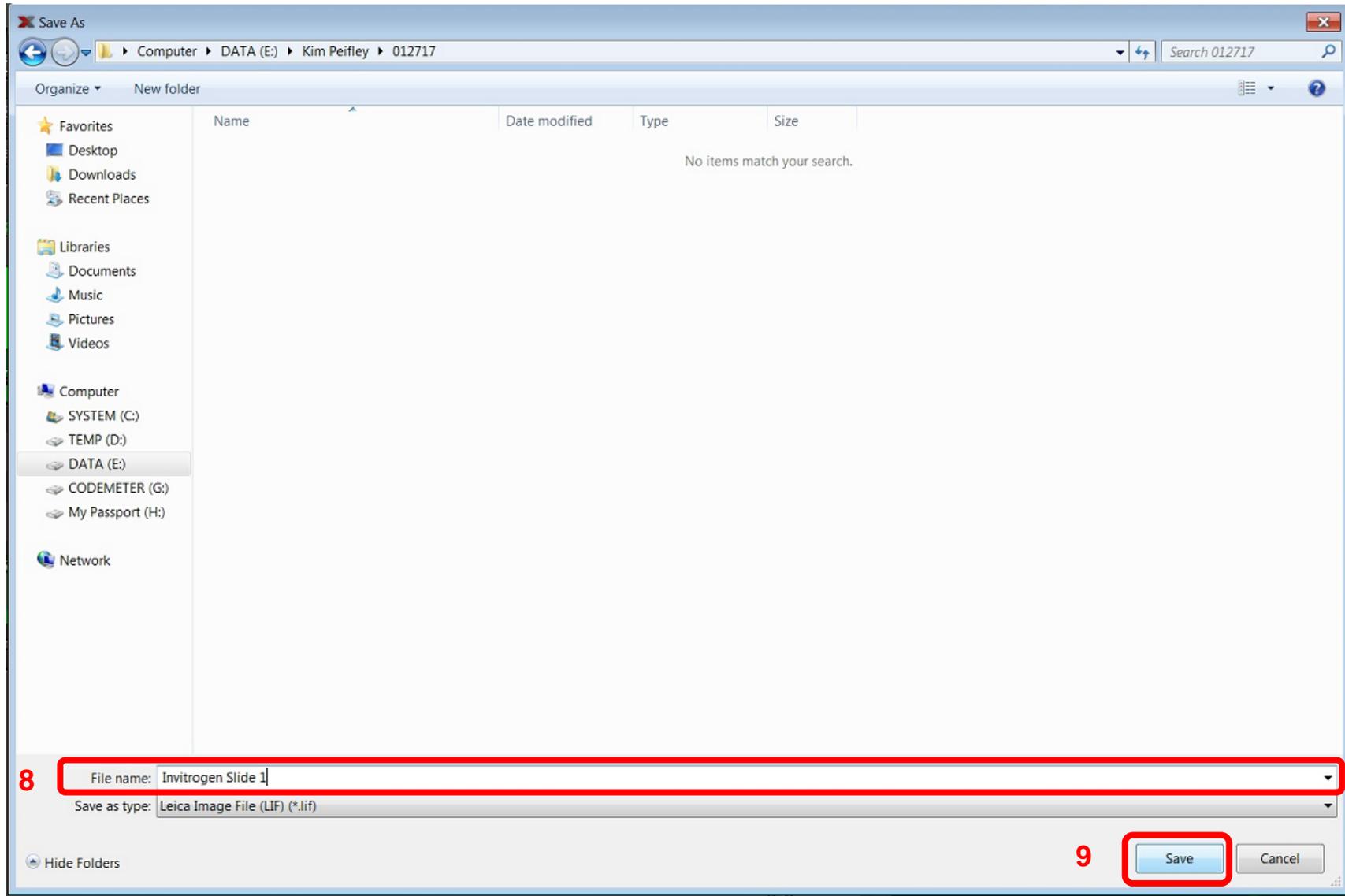
1. Click Open Projects Tab.
2. Highlight Project you wish to save..
3. Click on floppy disc icon to right of Project name. If you hover the mouse over it you will see Save Project.



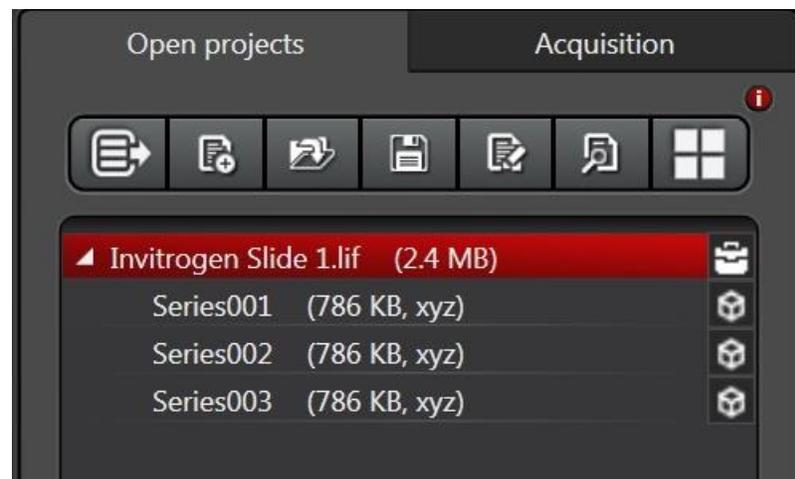
4. Select the Data (E:) drive to save data locally.
5. Select your folder or create a folder with your name.
6. Click on folder to open.
7. Create folder for today's work.
8. Click on that folder to open it.



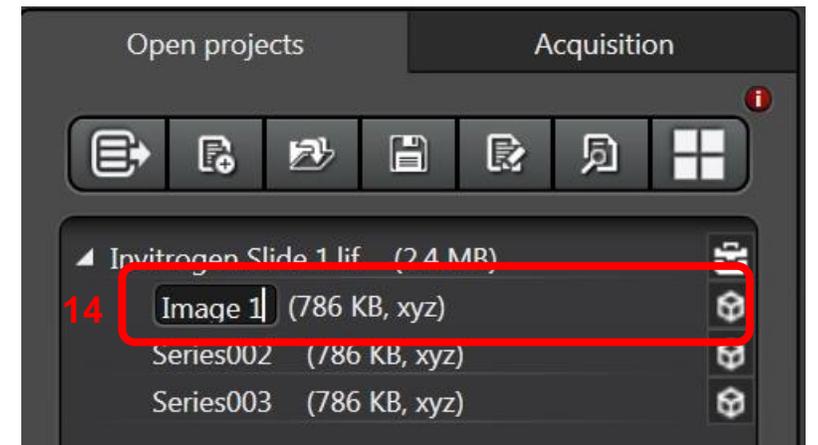
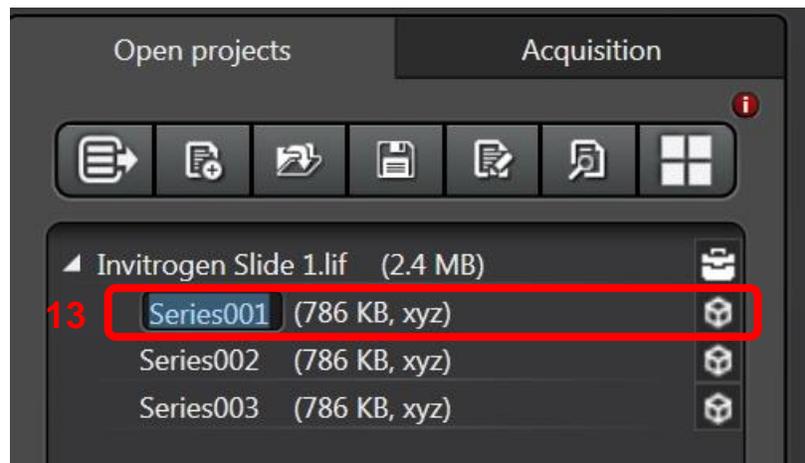
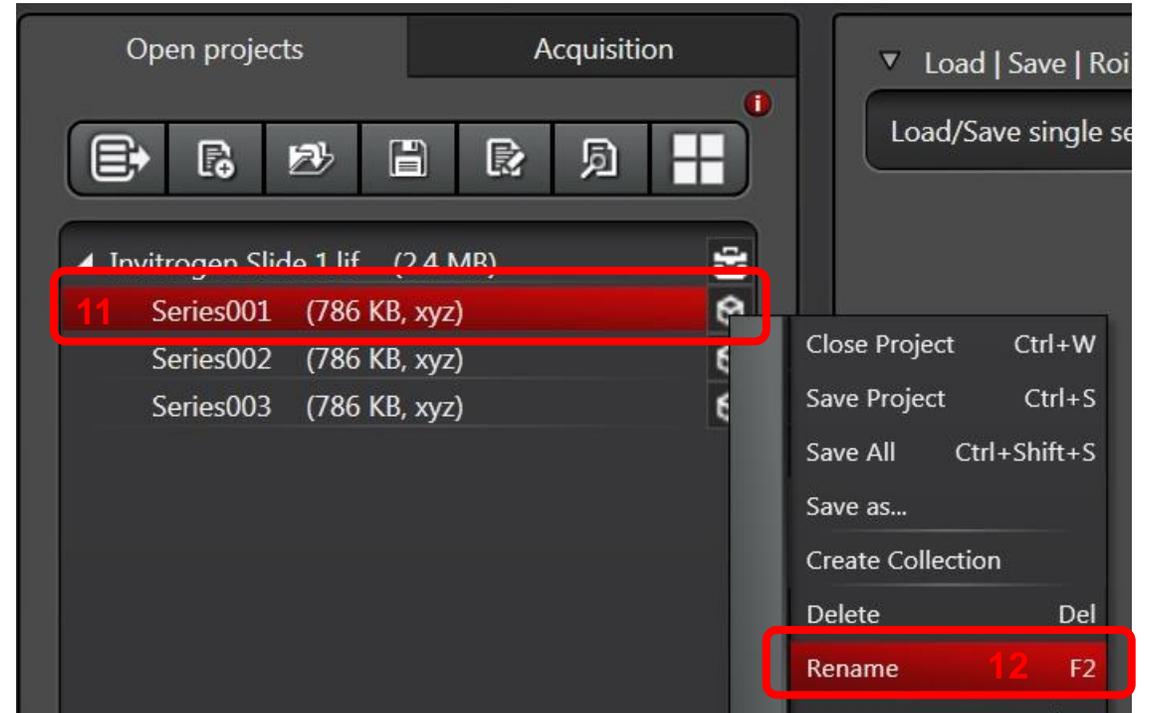
8. Name your project.
9. Click Save.



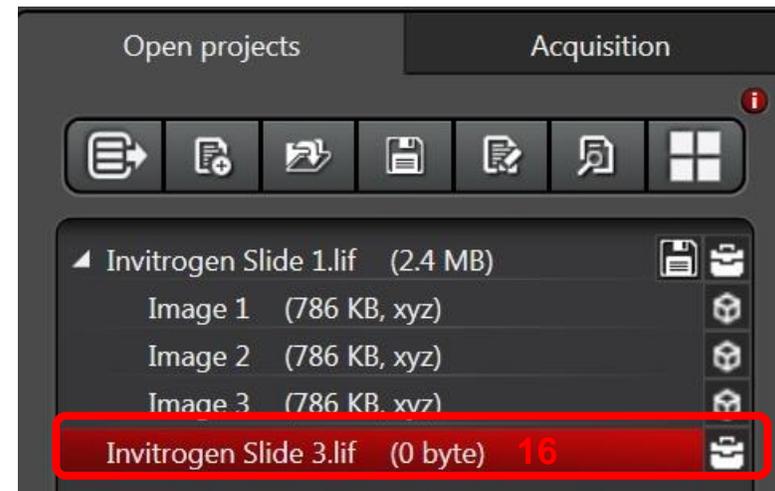
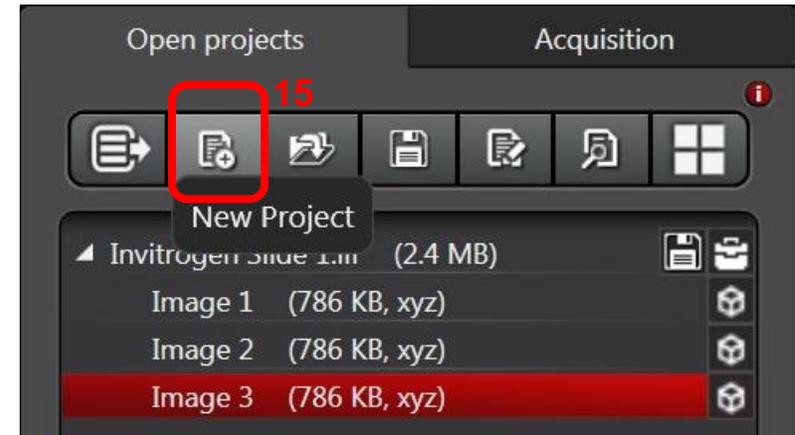
10. The project is now named. In this example Invitrogen Slide 1. All images acquired will be listed under this project.



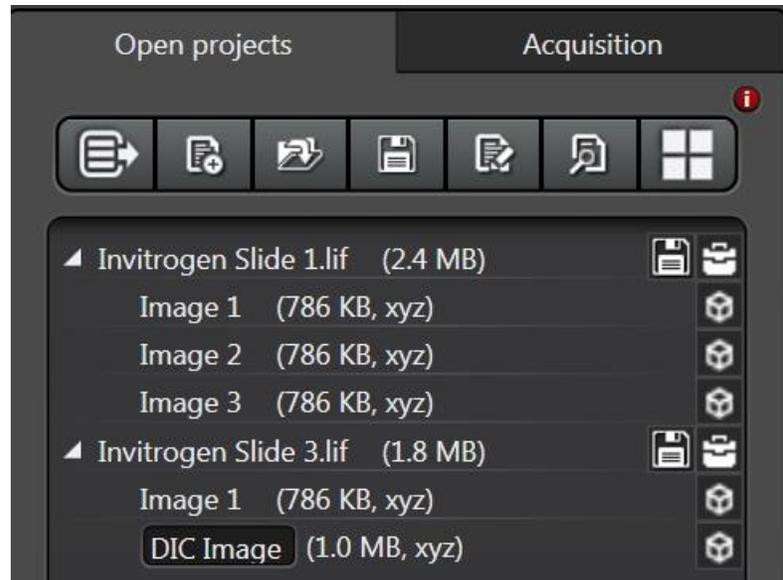
11. To name your image highlight image and right click to get menu.
12. Select Rename.
13. Window will be highlights so you can type in new name.
14. Hit enter to see name.



15. To create a new project click the new project button in the menu bar..
16. Follow previous instructions to name the project.



17. This shows all the projects and images named for one imaging session.



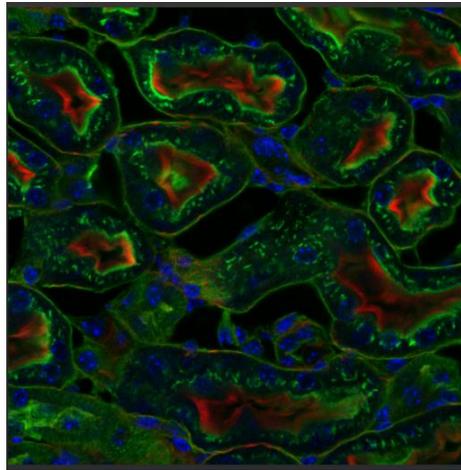
Zoom/Crop an Image

Zoom/Crop Imaging:

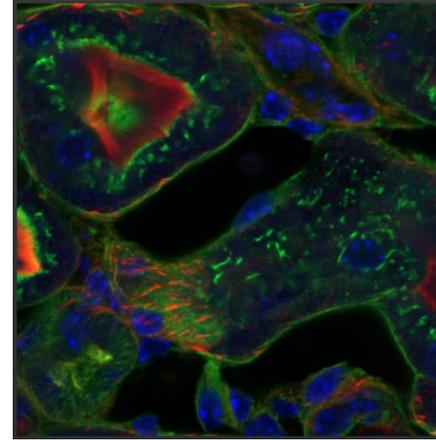
There are two ways to zoom or crop image.

Control Panel:

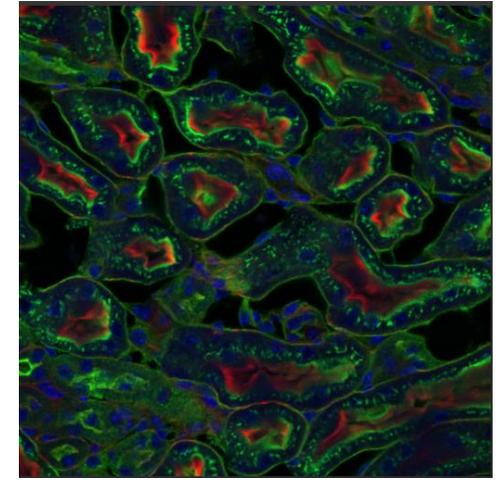
- Adjust the dial to the Zoom you want and take image. You can do this while scanning an image or without scanning.
- When you compare an image Zoom of 1 [A] to Zoom of 2.88 [B] you can see it sets the zoom from the center of the image.
- You can also Zoom out. Maximum zoom out is 0.75 [C].



A



B

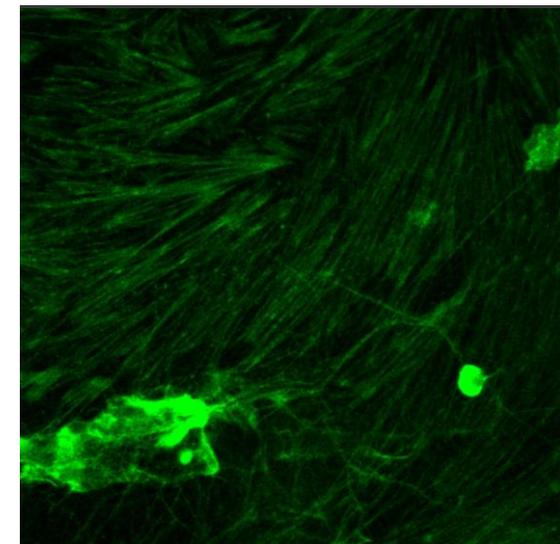
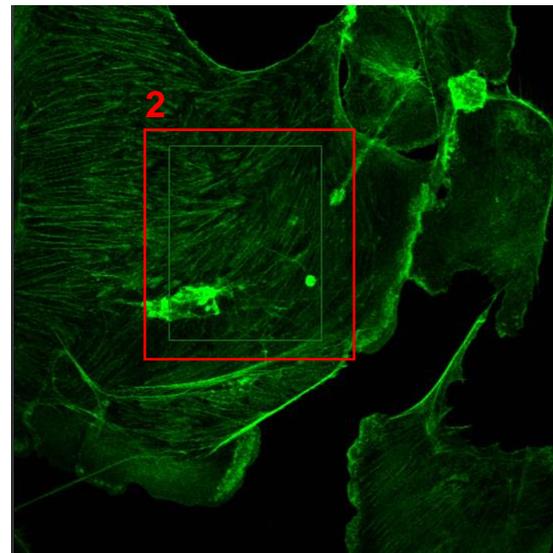
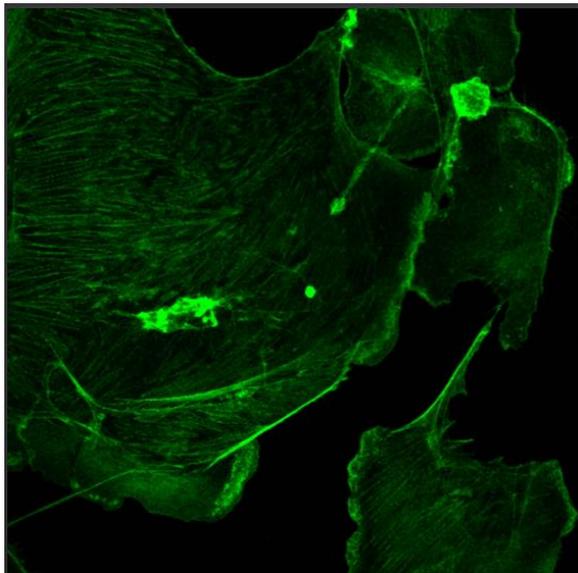
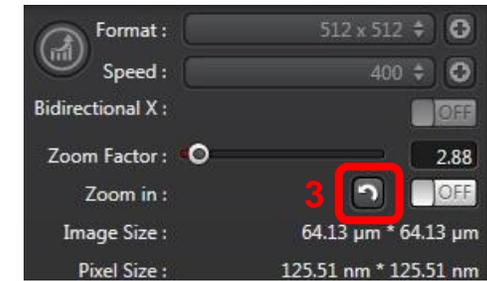
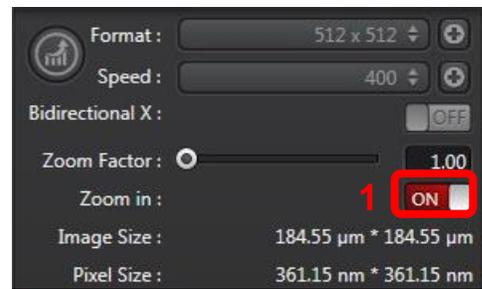
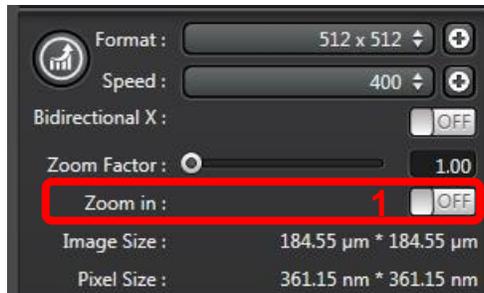


C



Via the software:

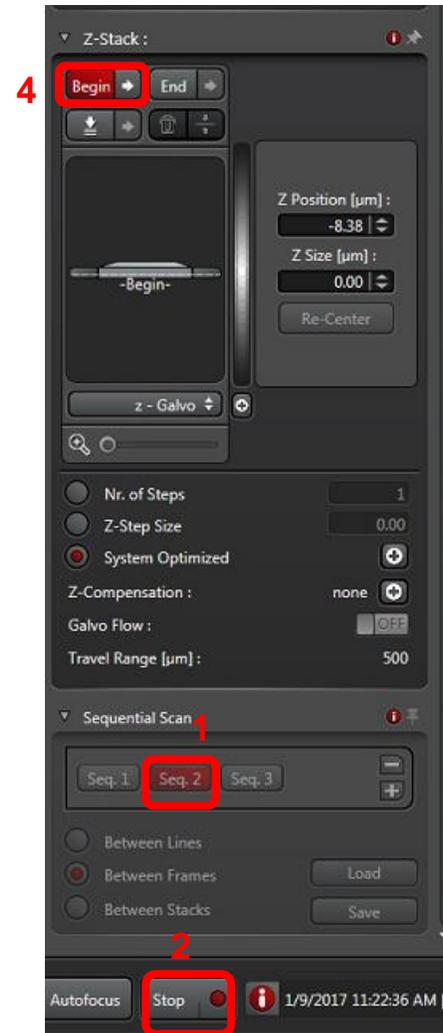
1. Click on Zoom in to turn on.
2. You can now go to the image and draw a zoom window wherever you want. Green box.
3. To return to zoom of 1.0 click the reverse arrow.



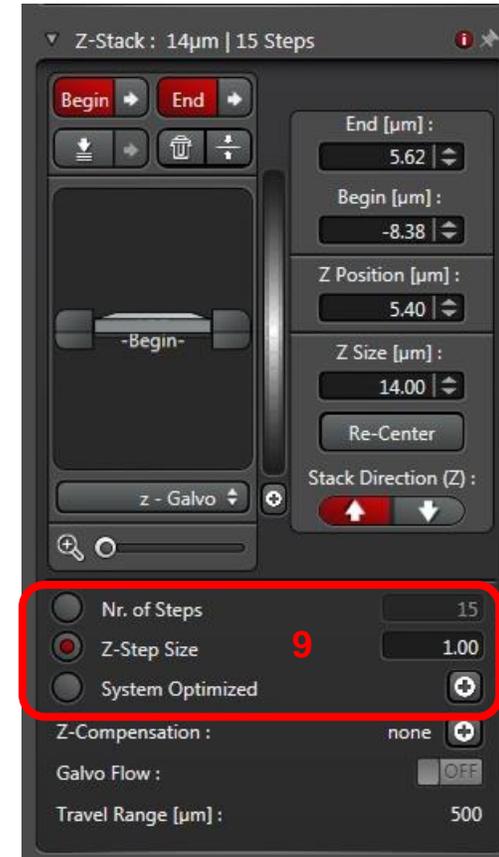
Z Stack Imaging and Maximum Intensity Projection

1. Select Sequence.
2. Click Live.
3. Using the Z position dial on the control panel, change the focus to the point you wish to start imaging.
4. Click Begin.
5. Change focus to the point you wish to stop imaging.
6. Click End.
7. Click Stop.

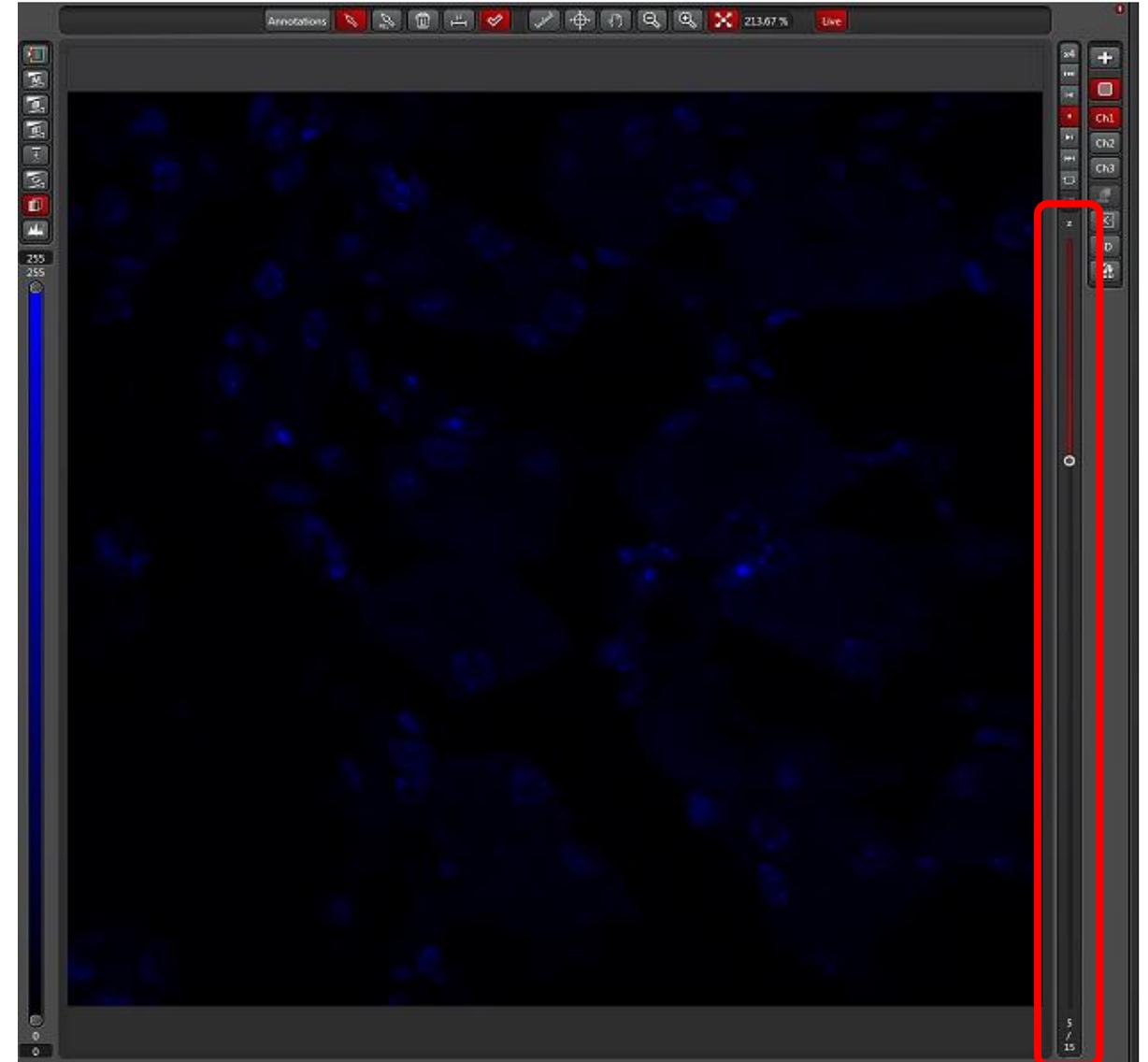
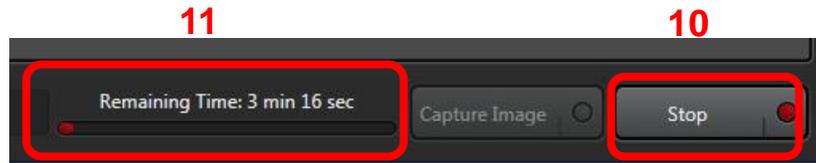
Note: Focus has to be changed from the control panel. If you use the focus knob on the microscope it will not register that with the software.



8. Select the way you want Z stack acquired: Set number of steps, Set Z step size [slice size], System optimized.
9. Z Step Size was selected to set the slice size. Z-Step size 1μ so there will be 15 steps.

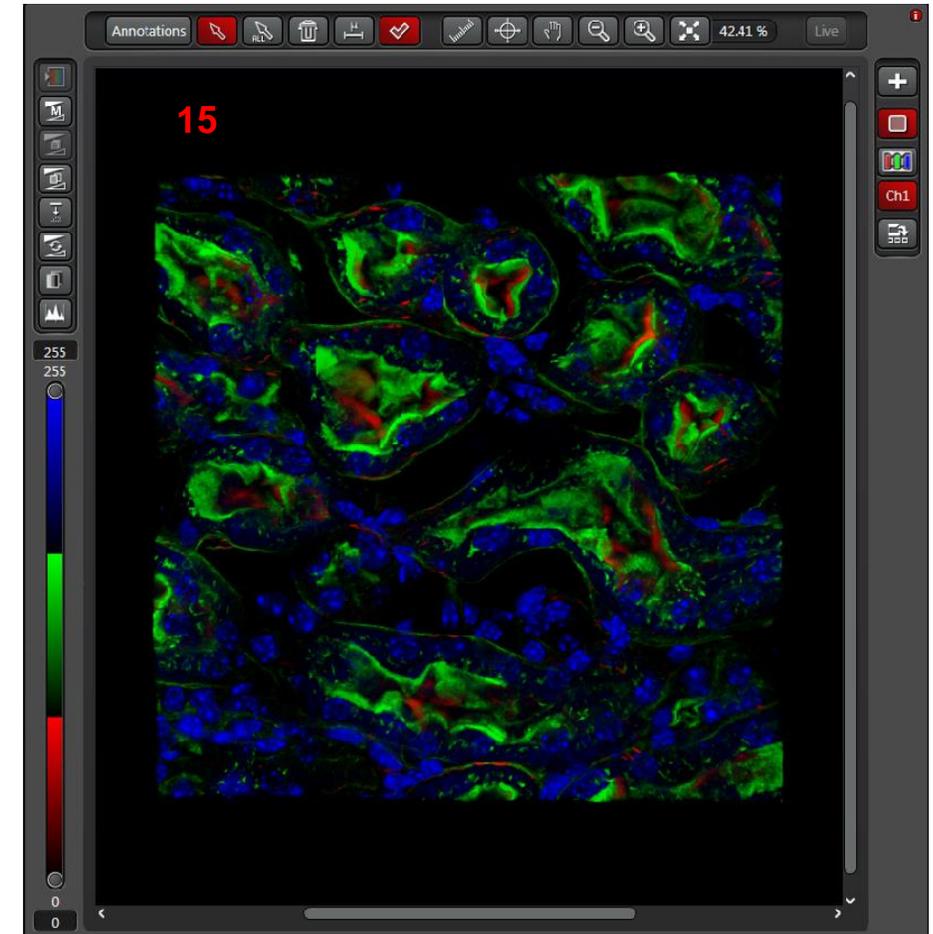
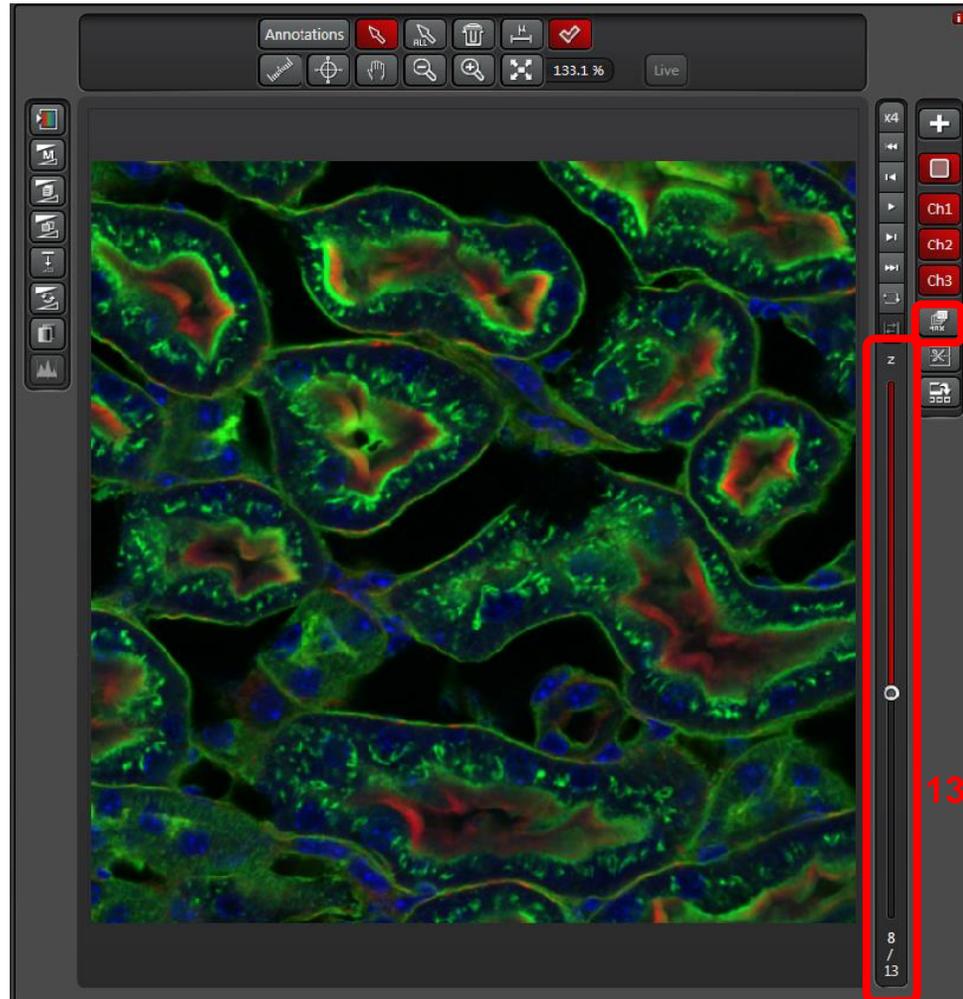


10. Click Start.
11. You can see the remaining time it will take to acquire the Z stack in the bottom menu bar.
12. You can also follow the Z stack progress on the bar on the right size of the image.



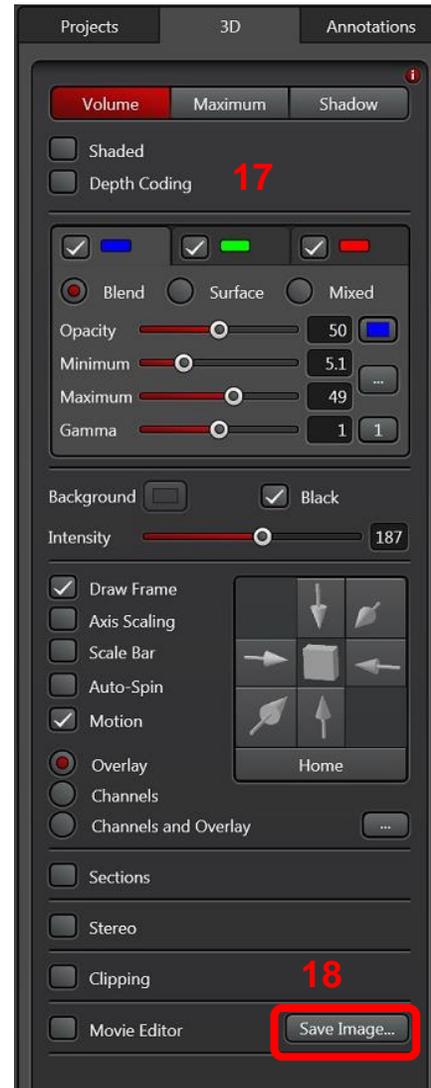
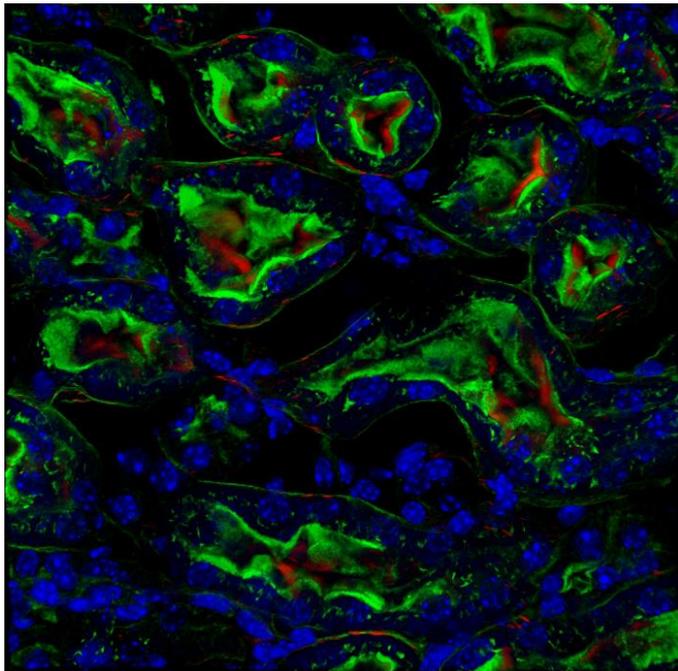
12

13. When viewing your Z stack image you can move through the slices using the bar on the right.
14. To get Maximum Intensity Projection click the box below the last channel button.
15. This is your maximum intensity projection image.

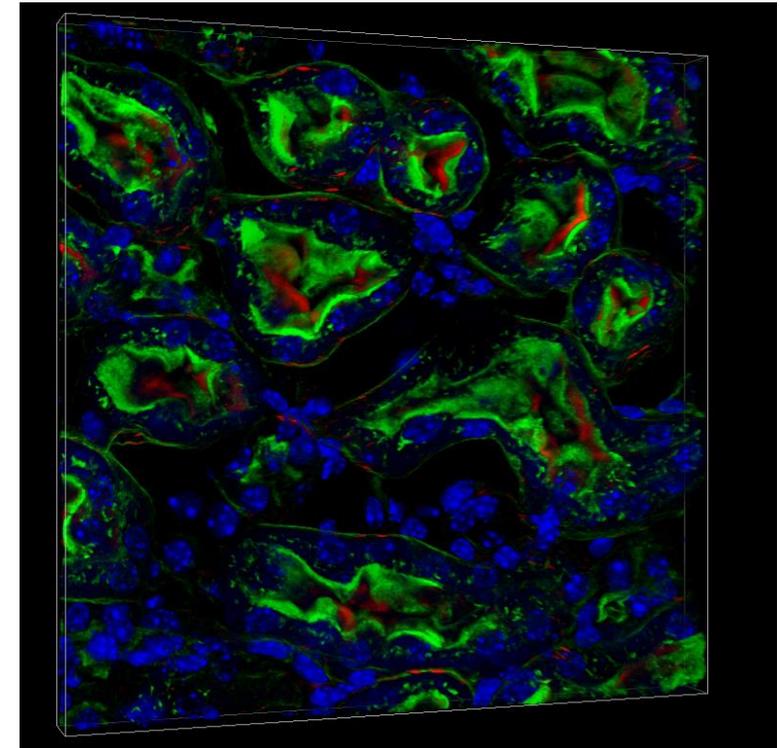


16. To rotate image click the 3D button. This will open a whole new window.
17. Set your parameters.
18. Click Save Image

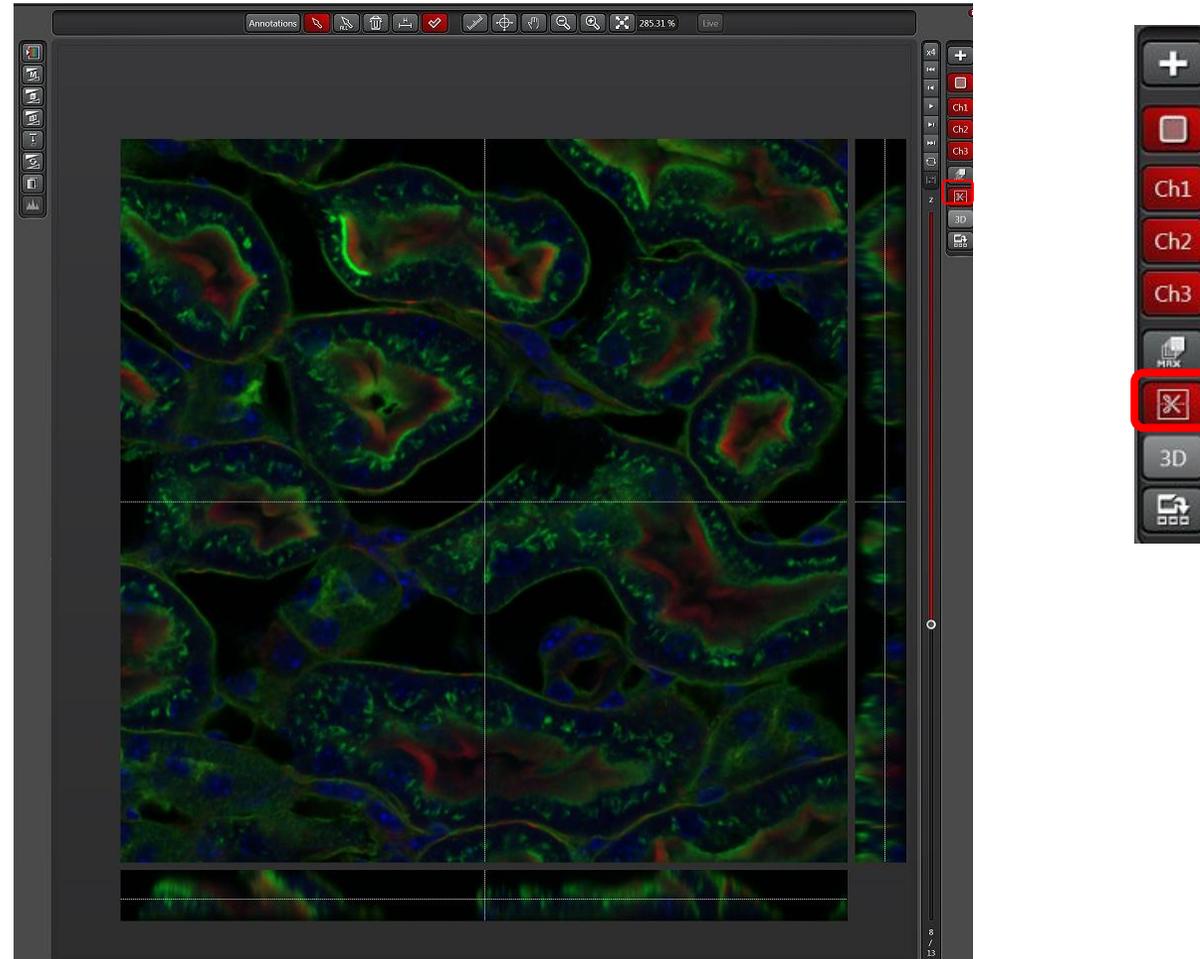
Maximum Intensity Image



Rotational Image



19. For orthogonal view click the Scissors Icon to the right of the image.

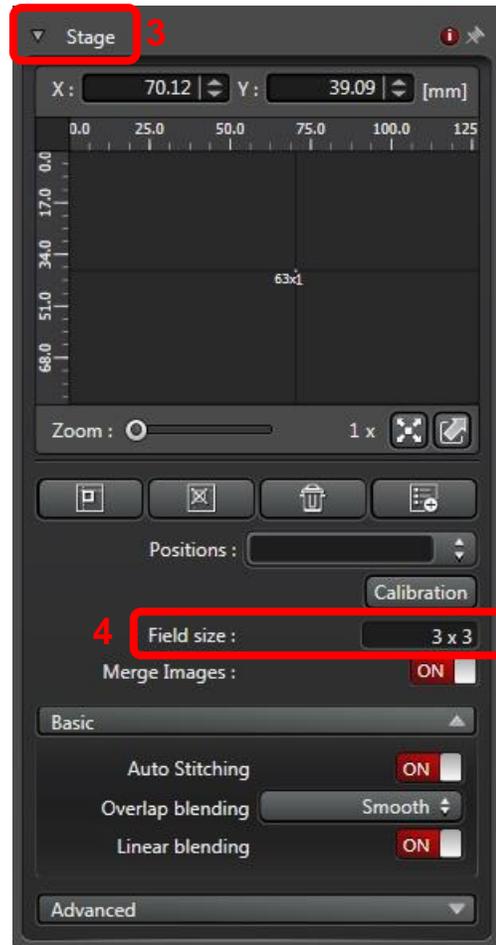
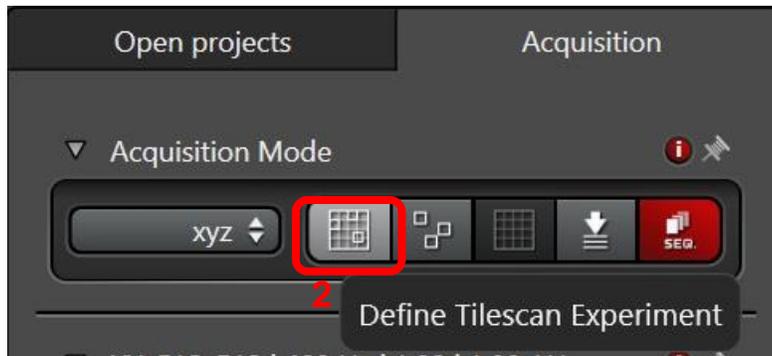


20. To turn off the Z stack click the Trash can icon.

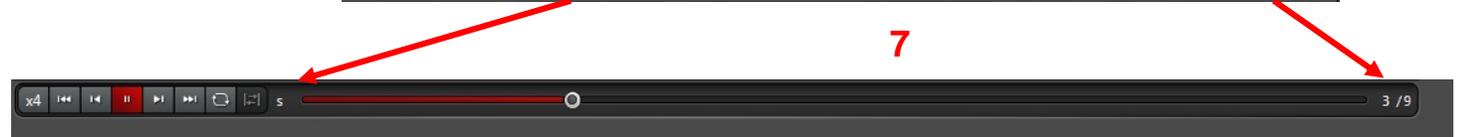
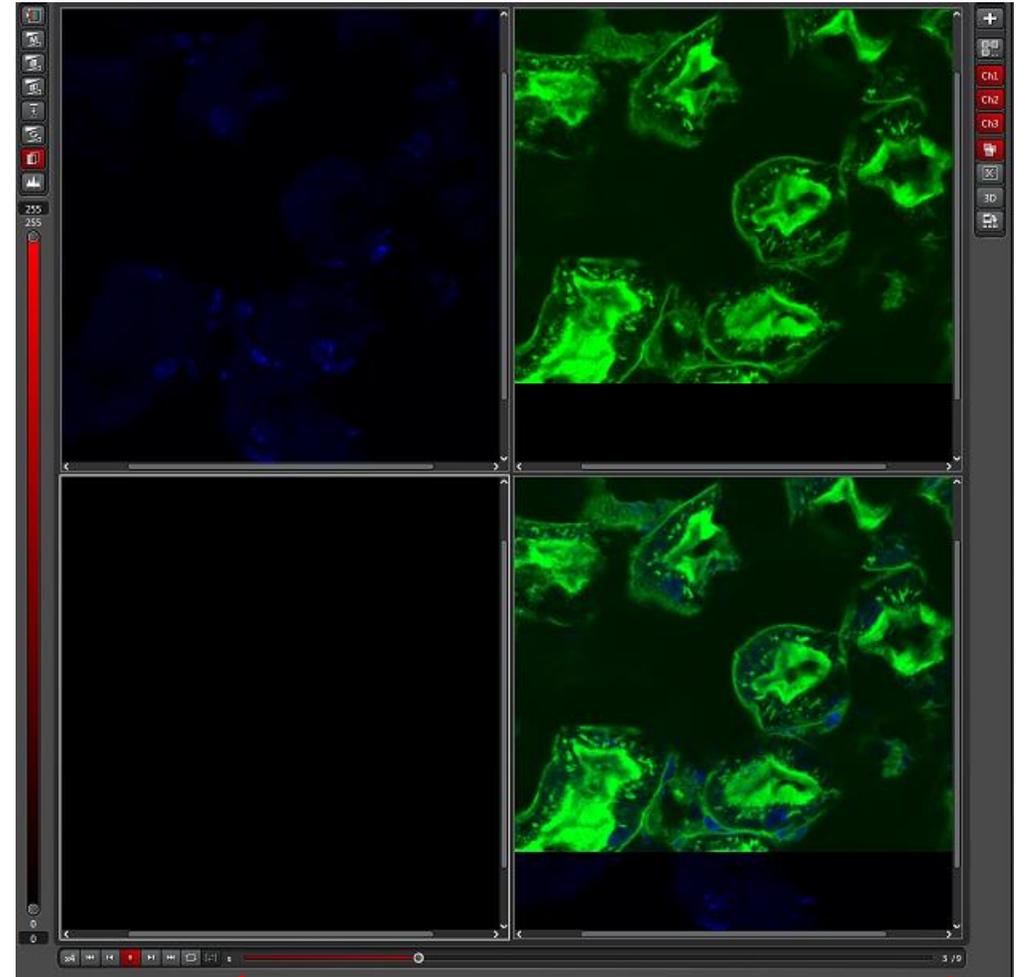
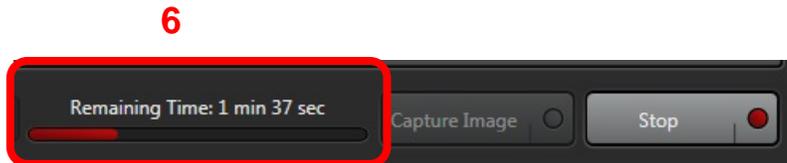


Tiling

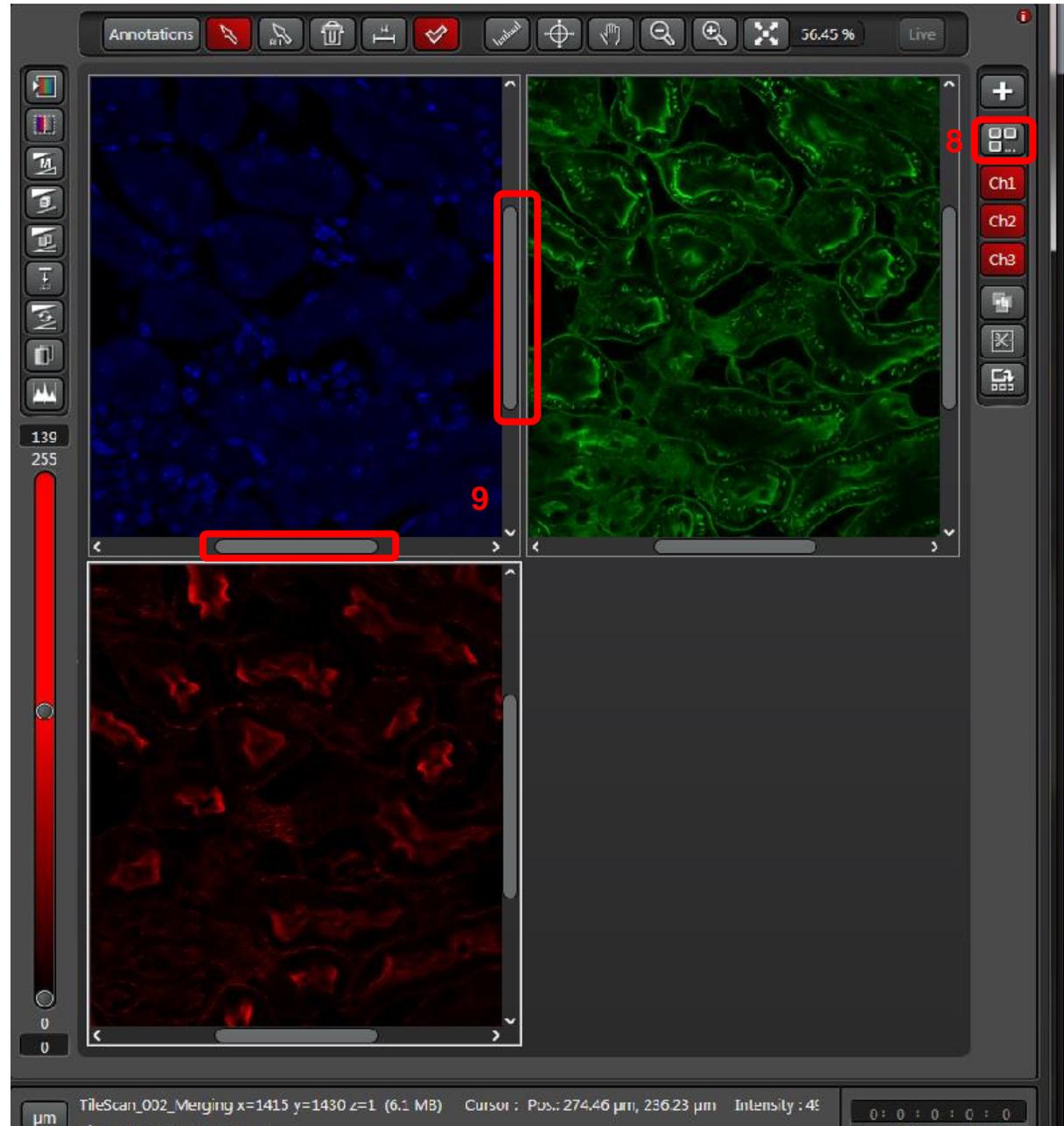
1. Set up your image like normal.
2. In Acquisition Mode window click on the icon for Define Tiledscan Experiment.
3. If the Stage window does not open automatically, click the arrow next to Stage.
4. Set Field size.
5. Slick Start.



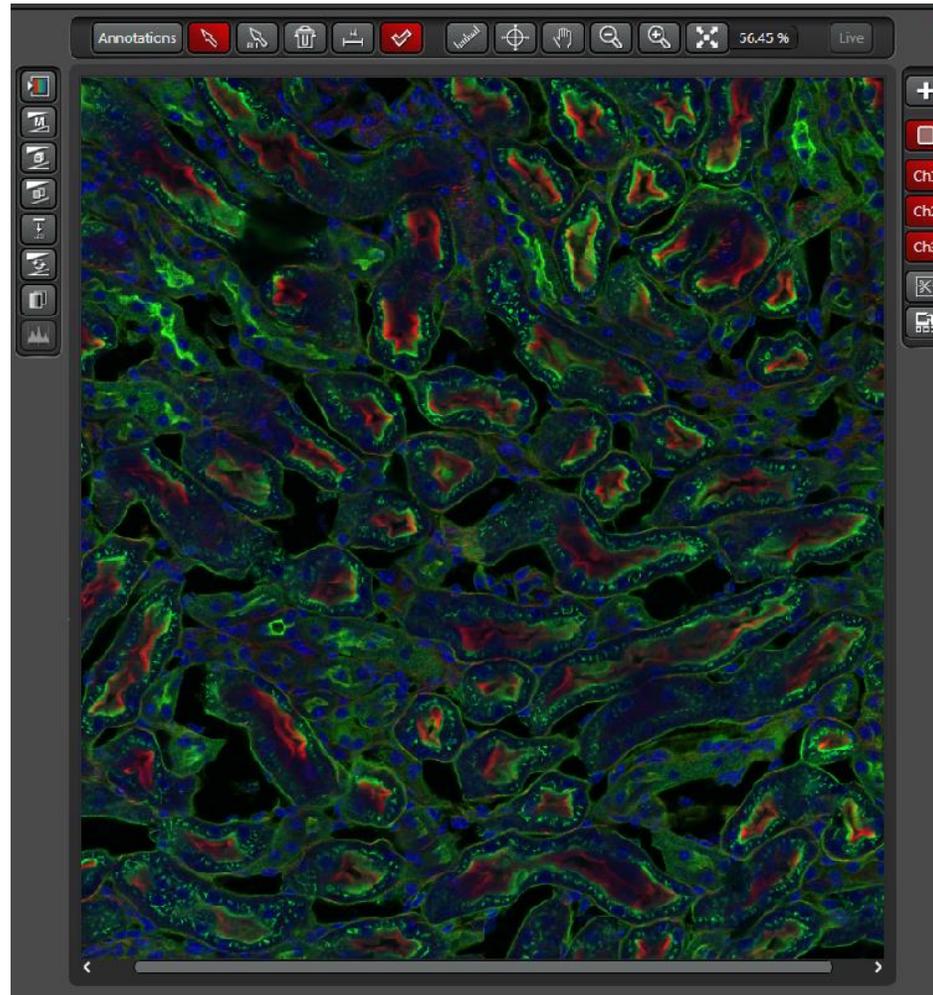
6. The remaining time to acquire the tile is listed next to the start button.
7. You can also follow the progress of the tile from the slider at the bottom of the image window. In this image it is now collecting tile 3 of 9.



8. Clicking on this icon allows you to see one position of the tile in split mode.
9. Use the slide bars to move around the full tile image.

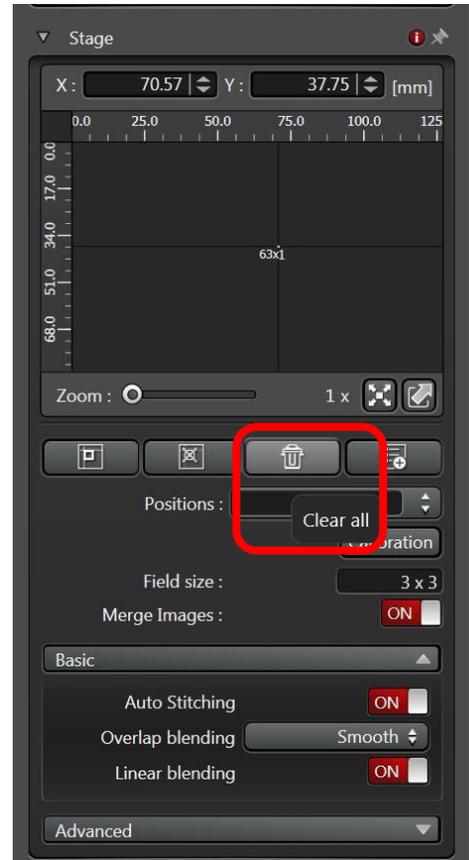
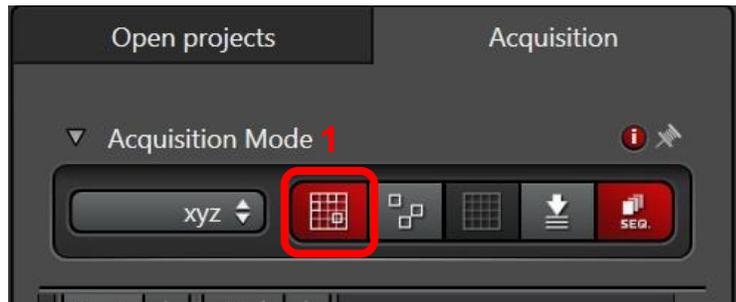


This is the full tile image in merge.



You can turn off Tilescan 2 ways:

1. In Acquisition Mode window click on the icon for Define Tilescan Experiment.
2. In the Stage Window click the Trash Icon aka Clear All. Field Size Window Should now be blank.



2

