FLIM
Fluorescence-Lifetime Imaging Microscopy

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09/25/15

Fluorescence-lifetime imaging microscopy [FLIM] generates an image based on the difference in the exponential decay rate of a sample's fluorescence. It is the lifetime of the fluorescence signal rather than the signal intensity that is used to create the image.

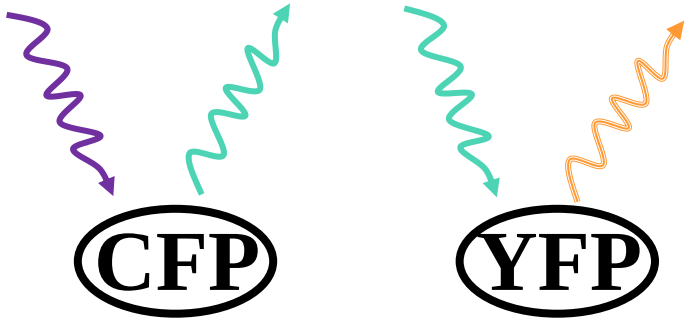
FLIM can discriminate between spectrally similar fluorophores and used to measure FRET. FLIM-based FRET measurements can filter out artifacts introduced by variations in concentration and emission intensity across a sample. Something intensity-based FRET cannot measure.

FLIM uses a pulsed laser so it requires a 2 photon laser. The LSM780 located in Building 560 Room 32-27 has a 2 photon laser.

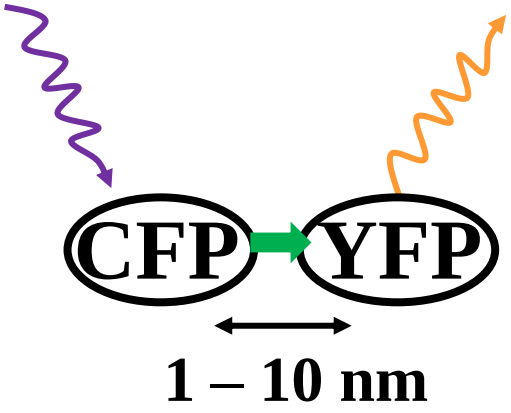
Forster Resonance Energy Transfer [FRET] detects the direct binding of two fluorescent labeled proteins.

Each protein is tagged with a fluorescent label . One label [the donor] is designed to emit in the wavelength that excites the other label [the acceptor].

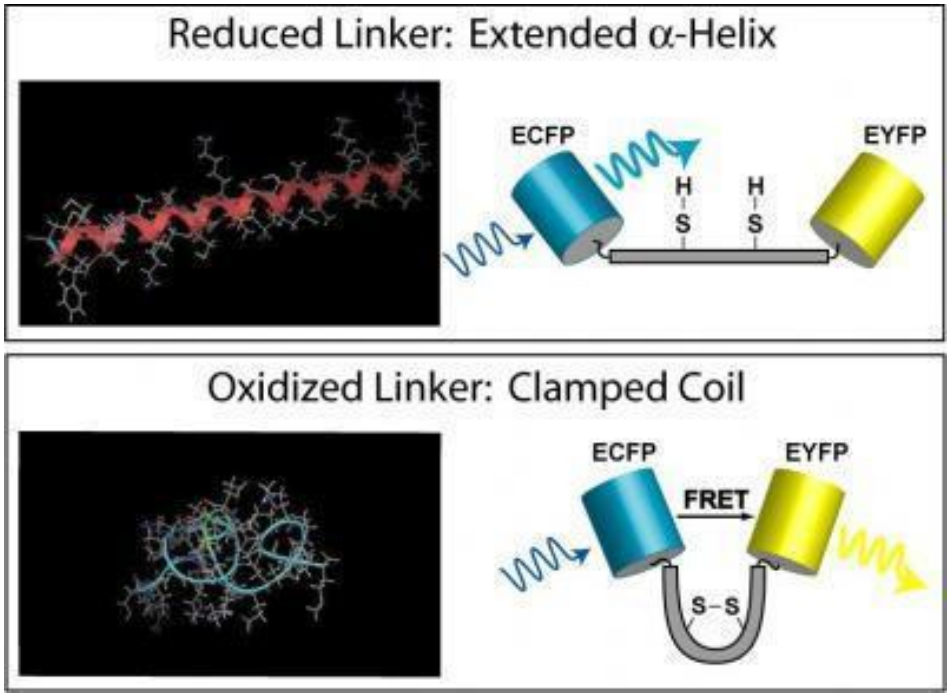
In this illustration we have CFP and YFP. Blue light excites CFP which emits Green light. Green light excites YFP which emits Red light.



If the proteins are within 10nm of each other then instead of CFP emitting Green light it becomes the donor and transfers the energy of the Green light to excite the acceptor, YFP, and emits a Red light. Green light is not seen when FRET occurs.



This is another example of FRET. In the reduced linker the ECFP and EYP are more than 10nm apart so excitation of ECFP will not excite EYFP so we get a Green signal. When the linker is oxidized it brings the two closer and we have FRET or Red Signal.



*(*Fig 1 in Exp Biol Med Feb 2008 vol 233, no 2, vii – viii*)*

1. **Turn on the live cell components using the Live Cell On/Off Instructions.**
2. **Turn on the 780 using the standard Turn On instructions.**
3. **If you are not doing DIC, remove the DIC slider from underneath the objective you will be using.**

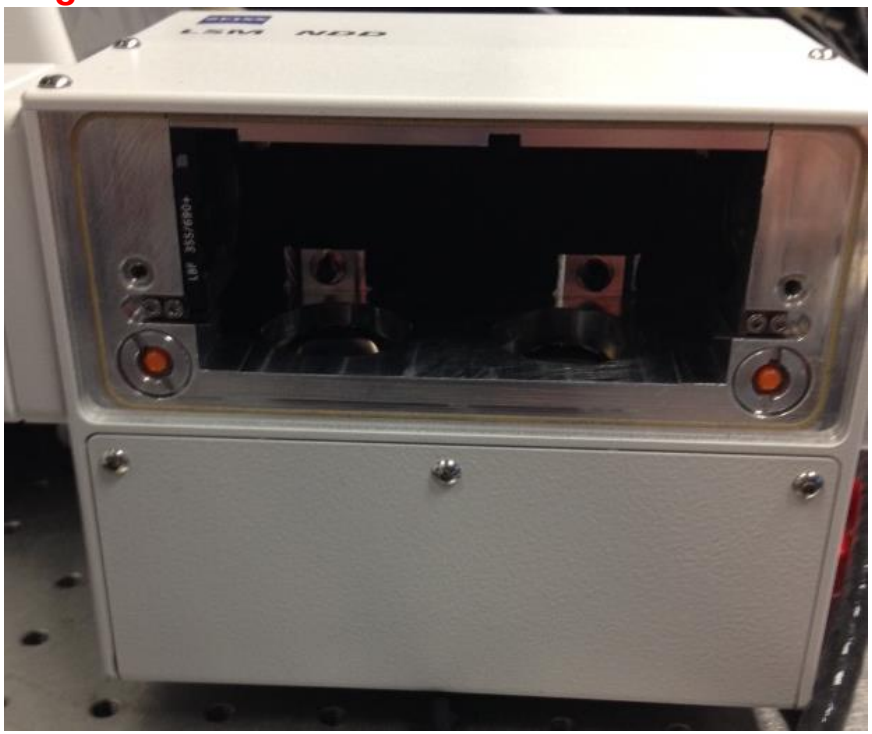
4. **The microscope is turned on following standard turn on protocol.**
5. **The ZEN software is opened same as for a standard imaging session.**
6. **Turn on the 2 Photon Laser. The key is the only thing needed to turn it on.**

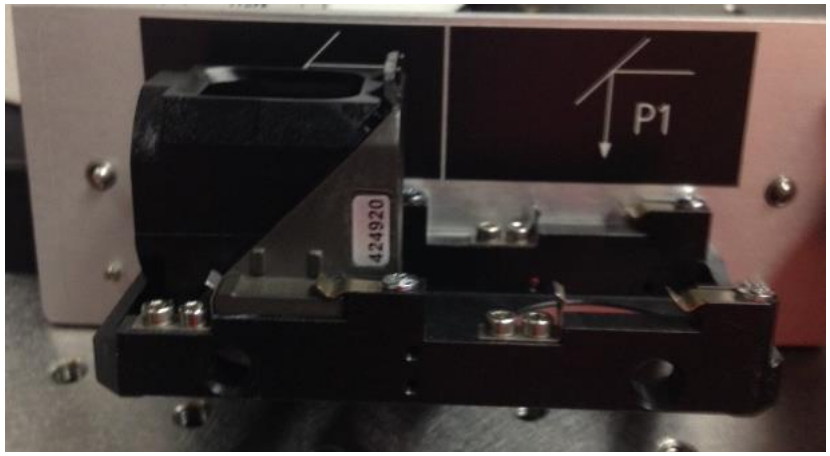
7. **Check the chiller to make sure the temperature is 20º C.**

Note: If temperature is not 20º C please contact a member of the OMAL Lab. The chiller should remain in "Run" mode at all times. There is no need to push the Run/Standby button to put it in "Standby" mode when not in use or as part of a turn off procedure.

8. **The Non-Descanning Detector [NDD] has a filter cube for TFP. You can remove the cover to see if the filter cube is in place. There is also a GFP filter cube that can be used. The filter cube not in the NDD unit is in a plastic bag in a cardboard box labeled “FLIM”. The box can be found on the shelf.**
9. **The cube should be in the P2 or left most position.**

8





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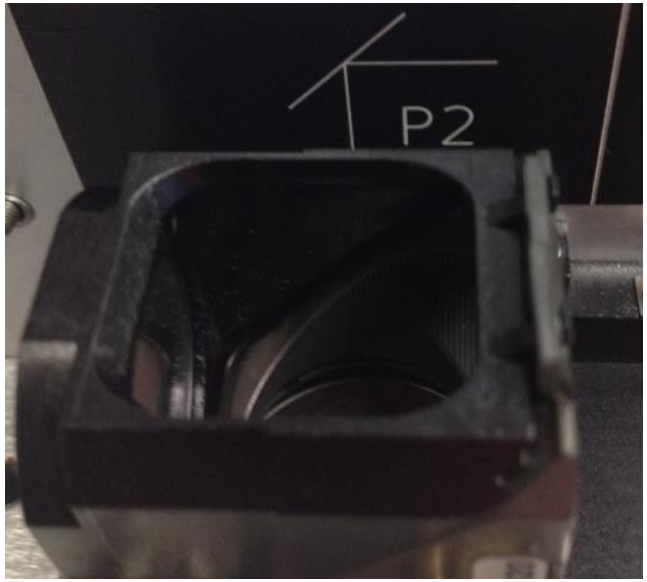
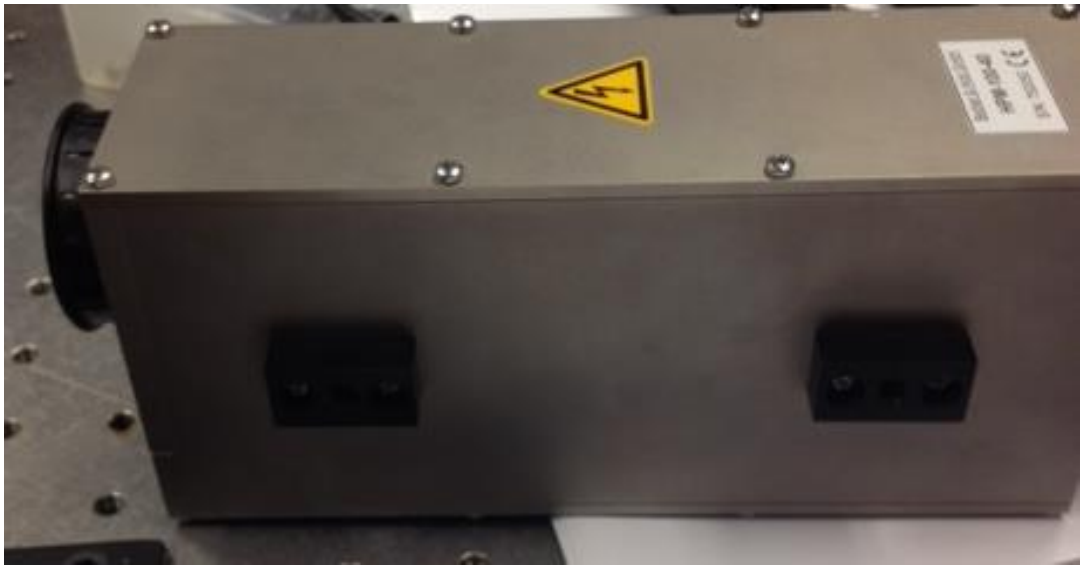
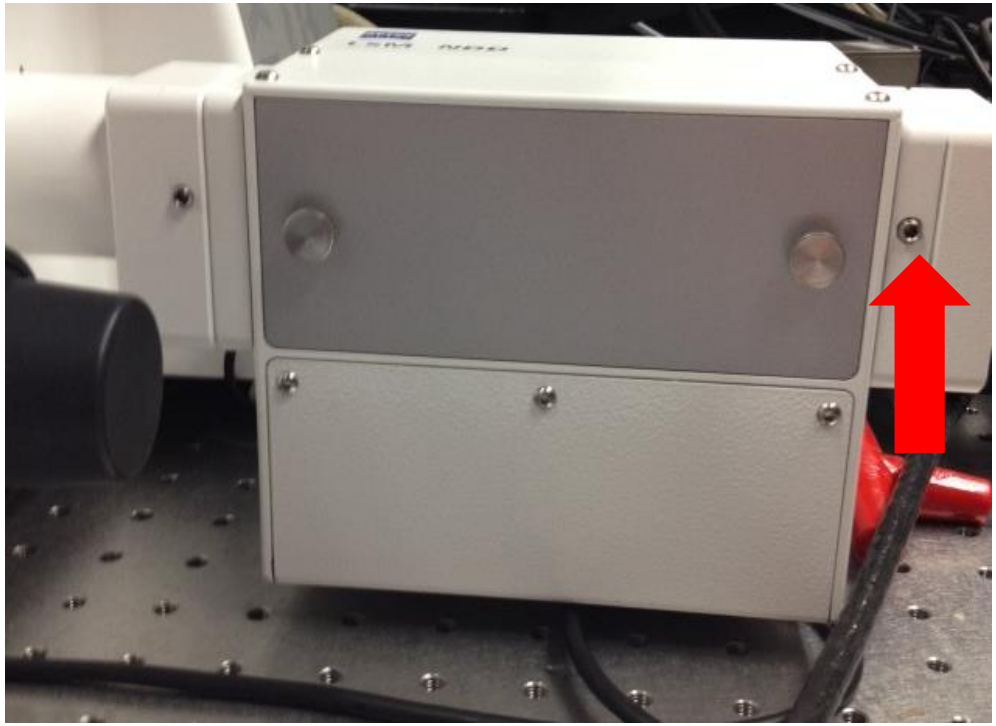


10. Connecting the FLIM detector to the NDD unit.

This is the FLIM Detector. It's stored on the microscope table towards the back. Double check label on the detector to confirm you have the right one. There is another detector stored there that looks similar.



11. **Carefully loosen the screws of the cover and remove the cover. Do not let the screws fall off the NDD.**



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12. **Be aware of the laser safety interlocks. They are located on the inside of the cap as well as the NDD. If they are not secured in place both with correct pin alignment and tightened with the screws the lasers will not fire.**

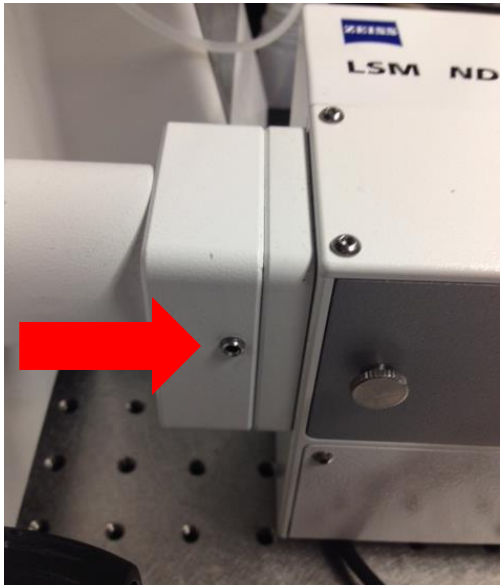
NDD

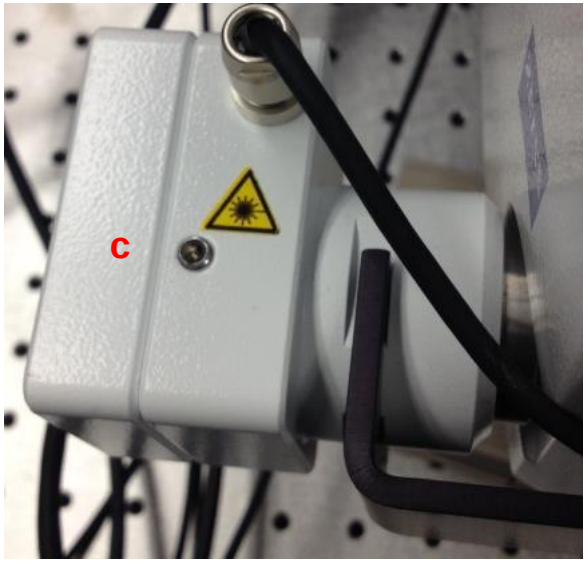
Cap

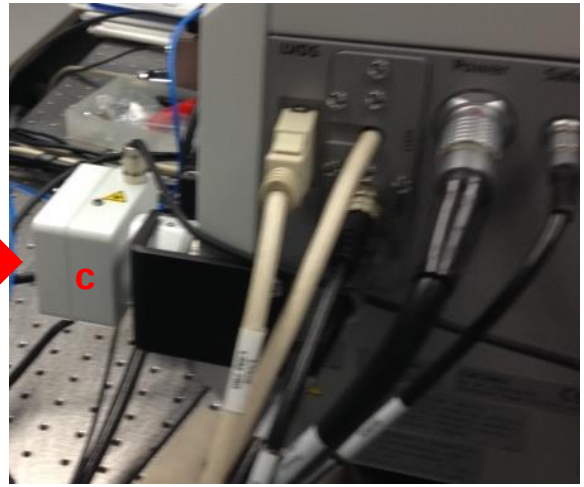
12. Continued. For additional reference there are 3 other locations of laser safety interlocks.
    a. One is located on the base of the microscope stage where it meets the turret.
    b. Another is located at the other end of the NDD closest to the microscope base.
    c. On the back of the scan head on the side facing the wall with the air conditioner.

a

b

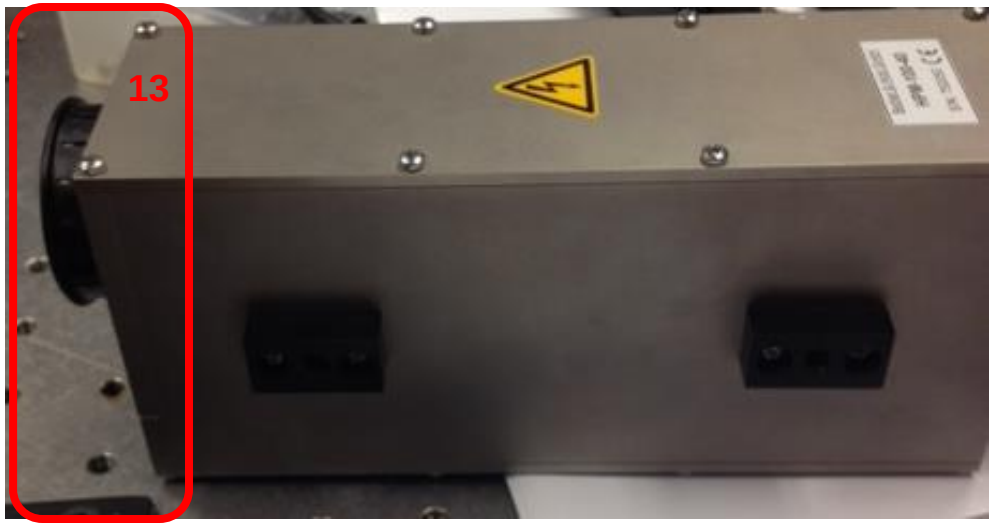


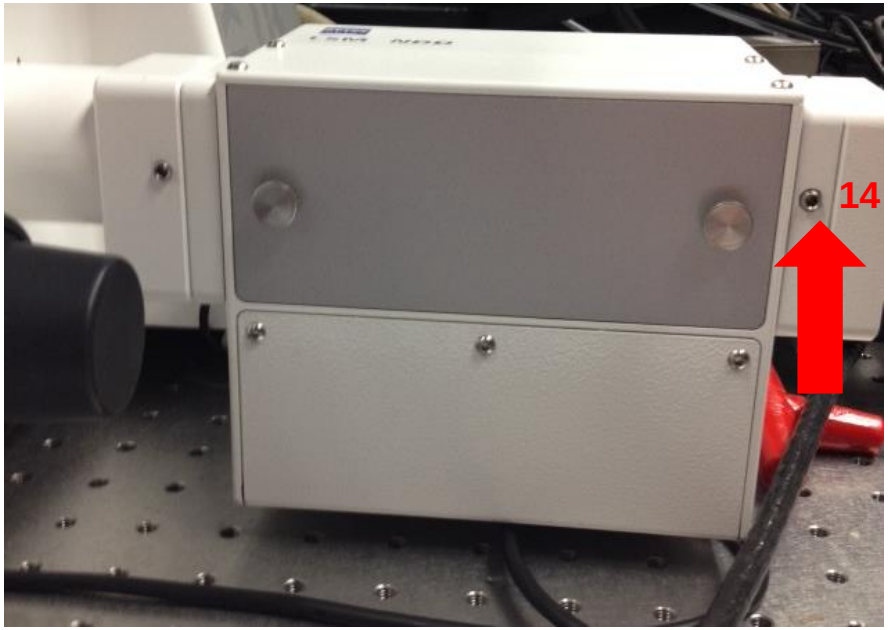




These steps must be performed in the dark!
It is also best to have two people when doing these steps.

13. **Carefully remove the cap of the FLIM detector and place on the NDD.**
14. **Once securely in place, turn on the lights and tighten the screws to attach the FLIM detector to the NDD.**

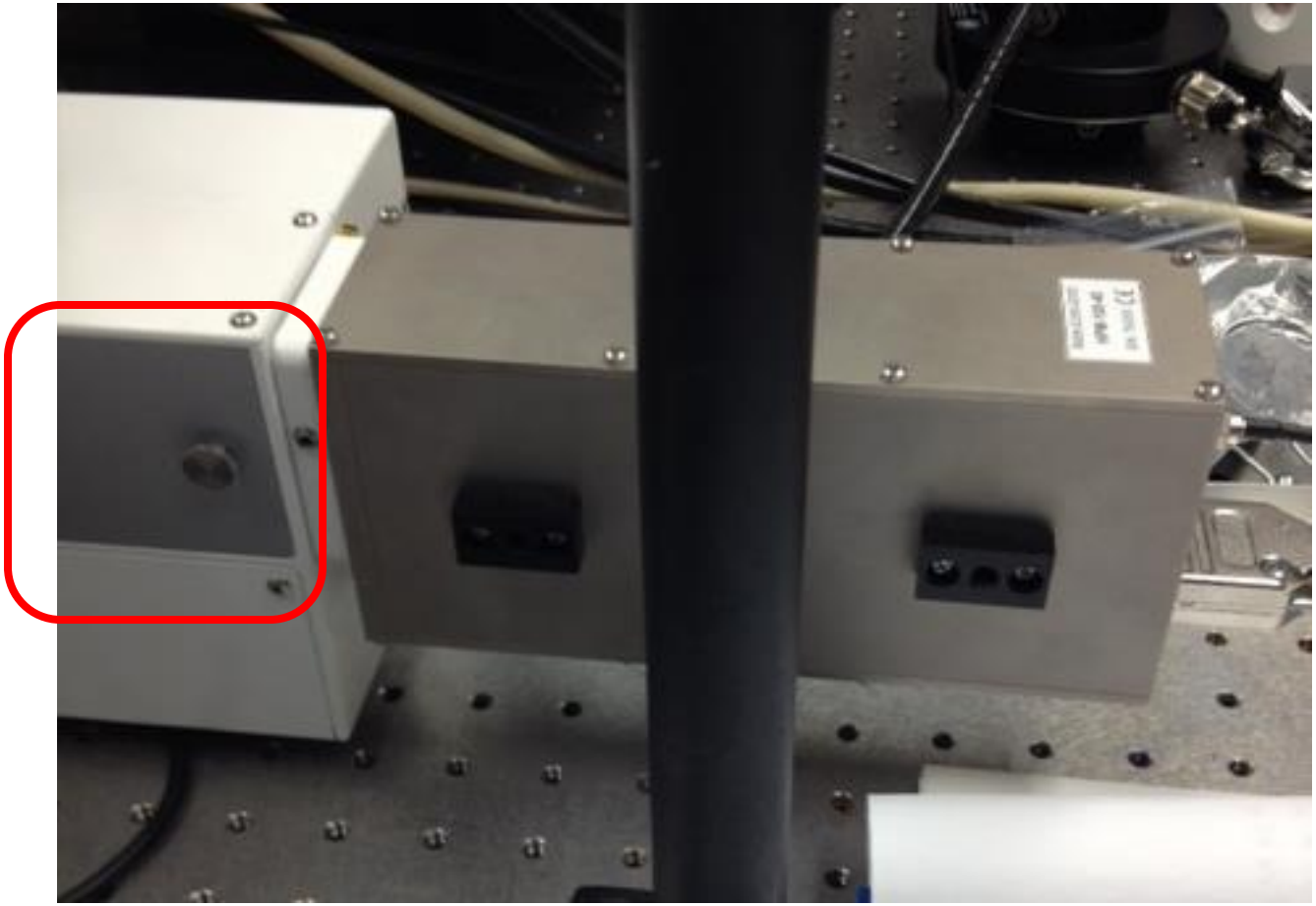


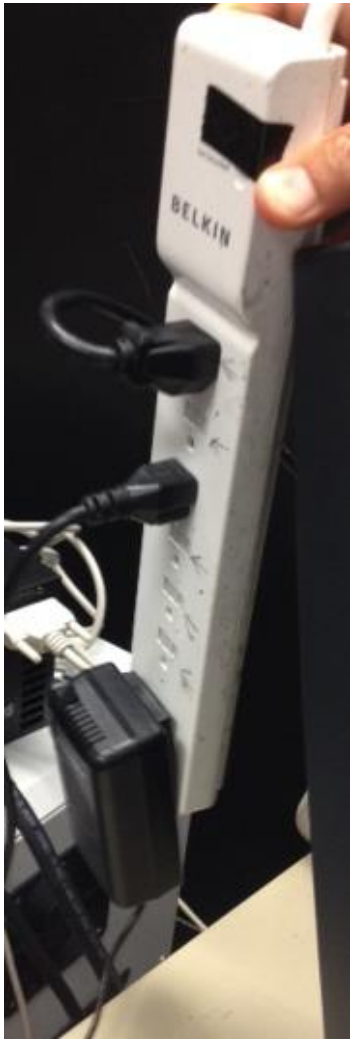


13

15. This is how the FLIM Detector looks when attached to the NDD.

Once FLIM Detector is attached do not remove the door to the NDD. This will allow light into the FLIM detector and potentially damage the FLIM detector.



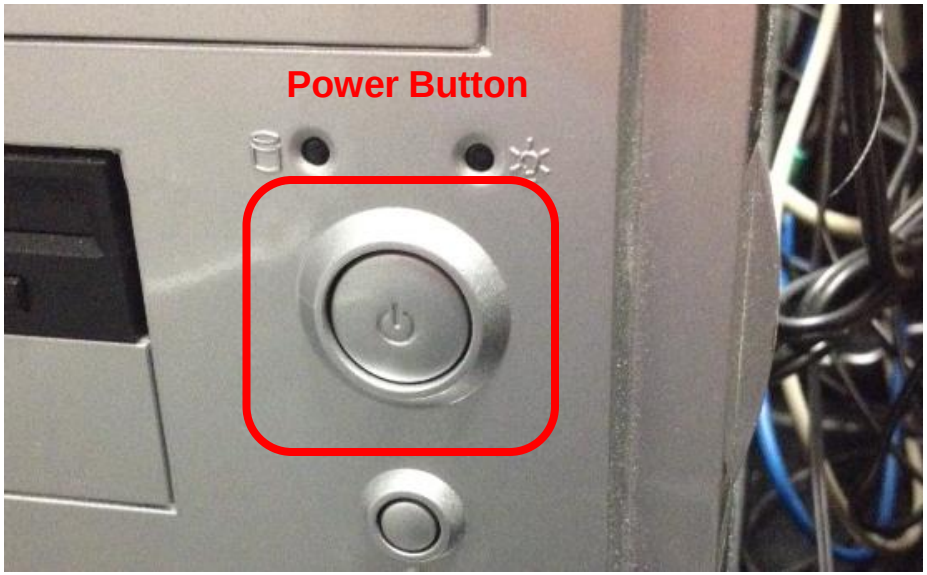


16. **Locate the power strip behind the FLIM Monitor and turn the power switch on. The Power Switch is located at the side of the power strip.**

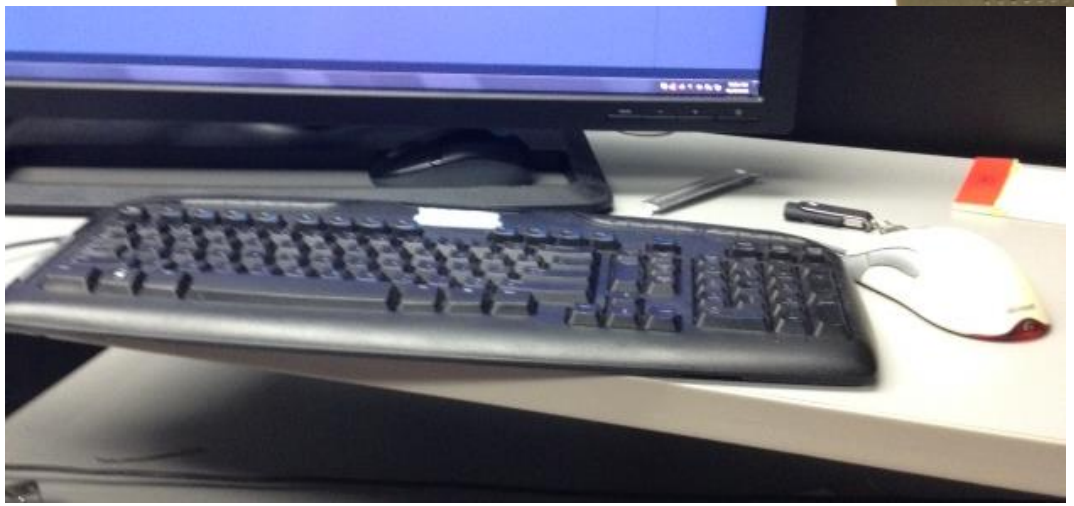
This powers the hardware of the FLIM detector.

17. Power up the FLIM computer.

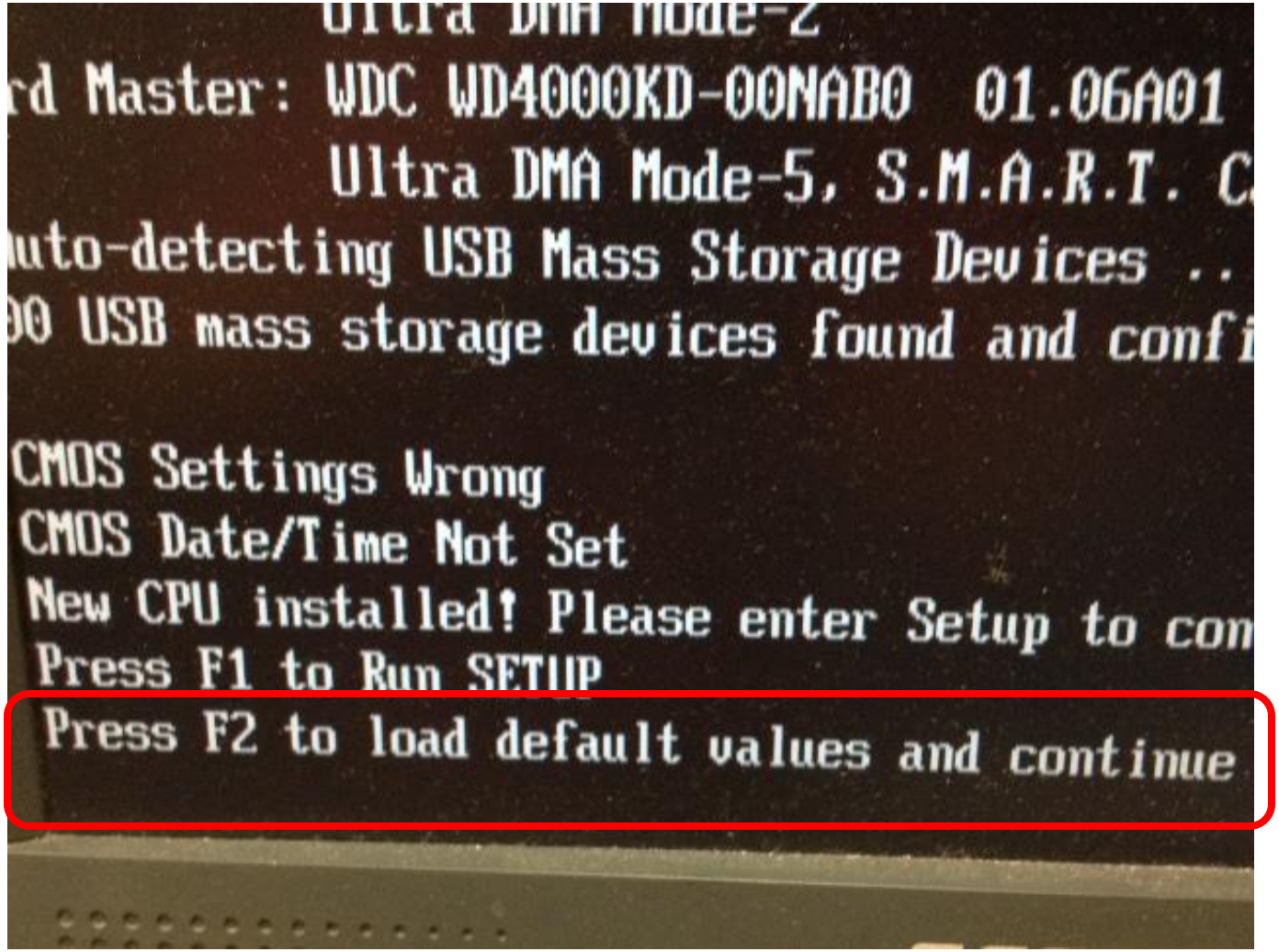
Open Door



This is the FLIM monitor along with the keyboard and mouse. This is separate from the monitor, keyboard and mouse used to operate the LSM780 computer.



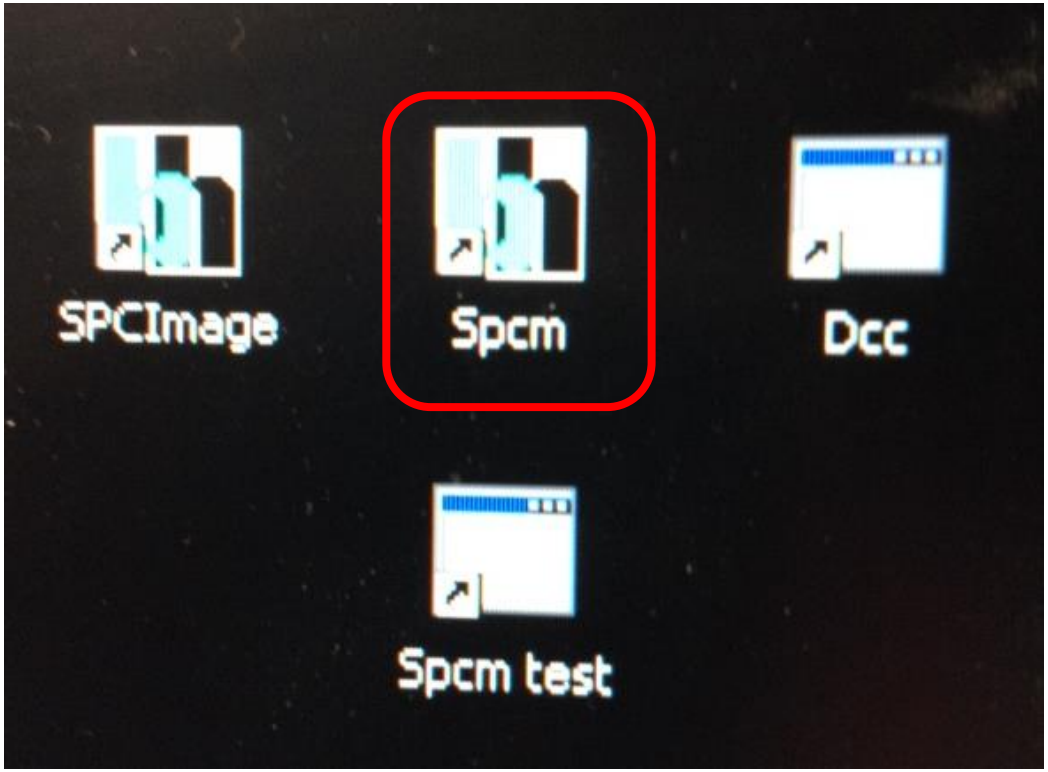
18. Press F2 to start up the FLIM computer.



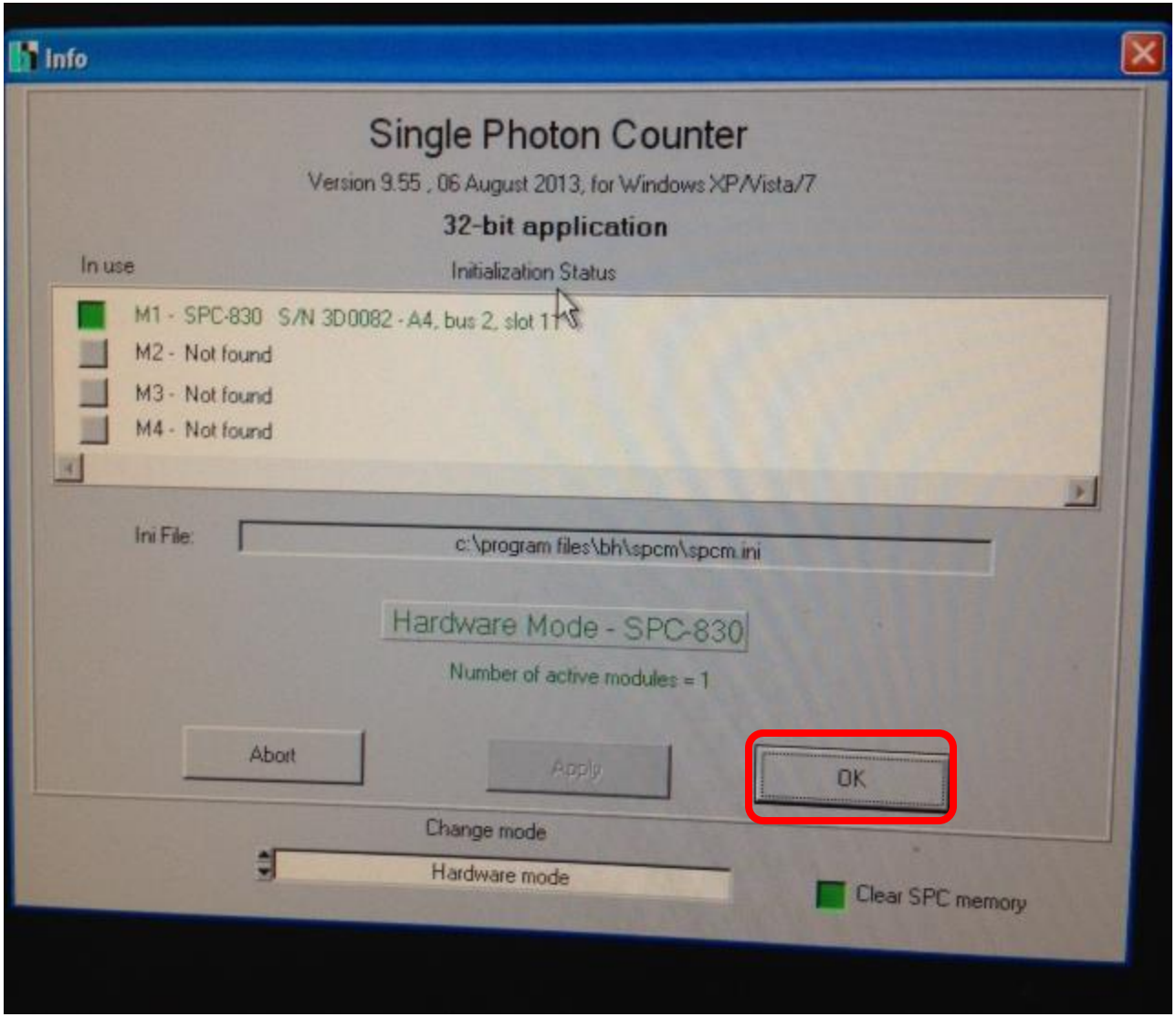
19. **Click FLIM.**
20. **Password is: phil551100**

21. Quit “AiBooster” by clicking on the “x”.

22. Click Spcm.

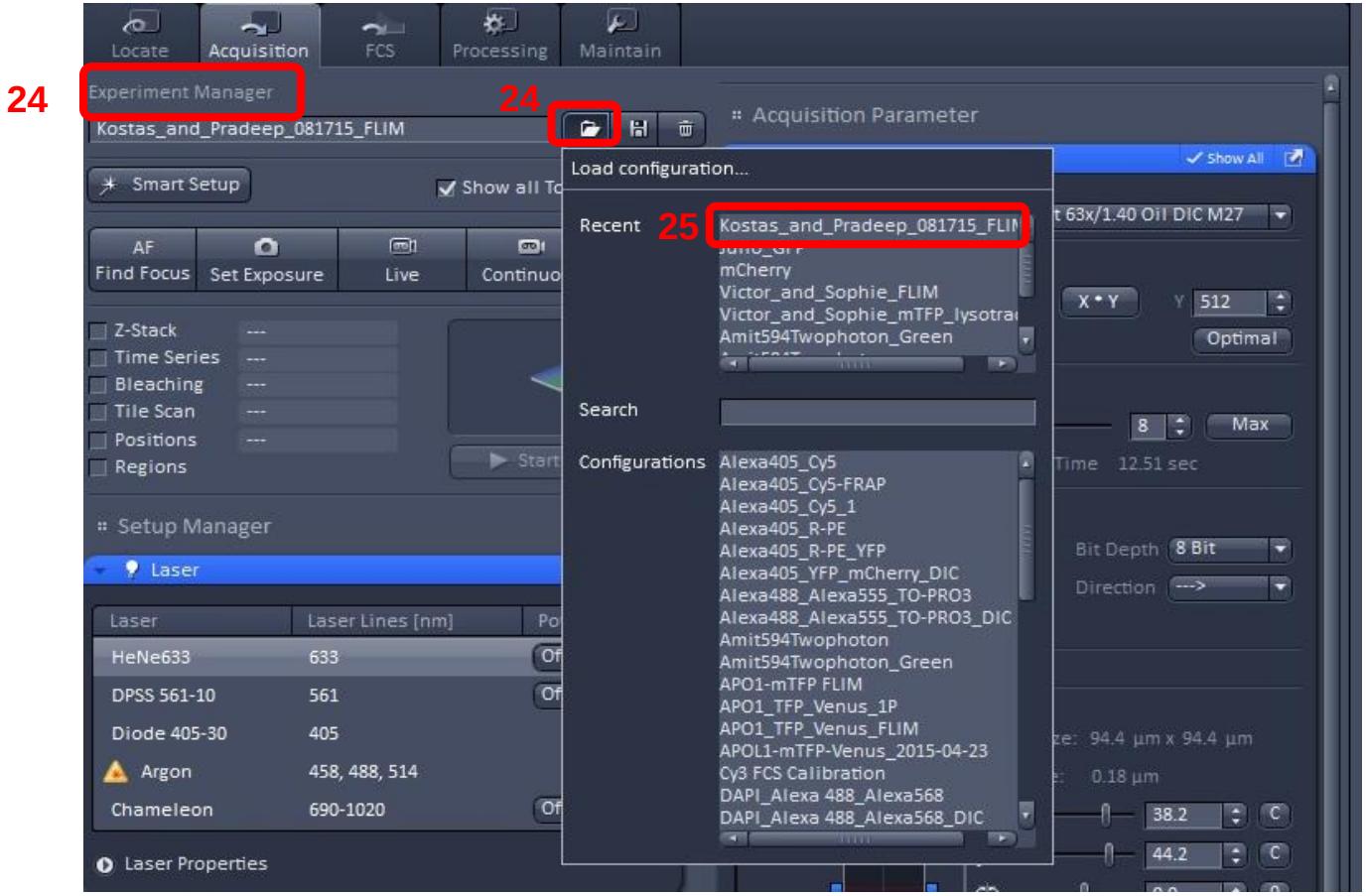


23. In Single Photon Counter window click OK.



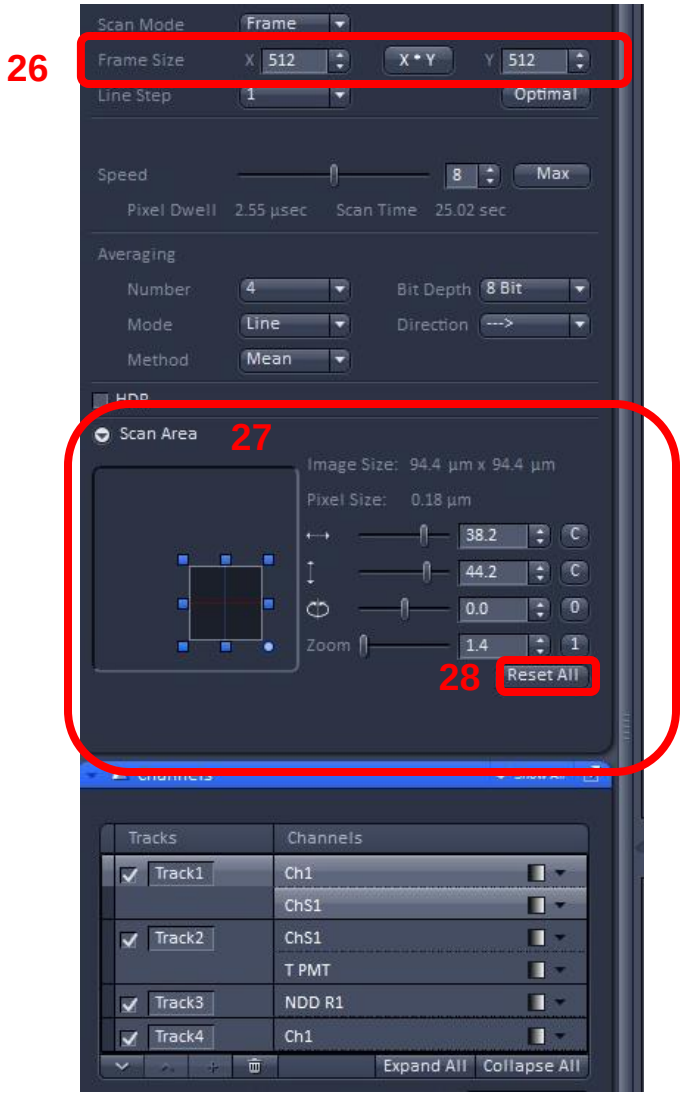
Back on the LSM780 Monitor and Computer.
In the ZEN Software:

24. **In Experiment Manager Click on the Folder Icon.**
25. **Build your configuration or select your configuration in order to load the configuration.**



Loading the configuration also sets some other settings that you may not wish to use. Be sure to check these areas:

26. **Frame Size.**
27. **In Scan Area see that the Zoom is 1 and location of the image area is centered.**
28. **Zoom and centered can be fixed by clicking the Reset All button.**

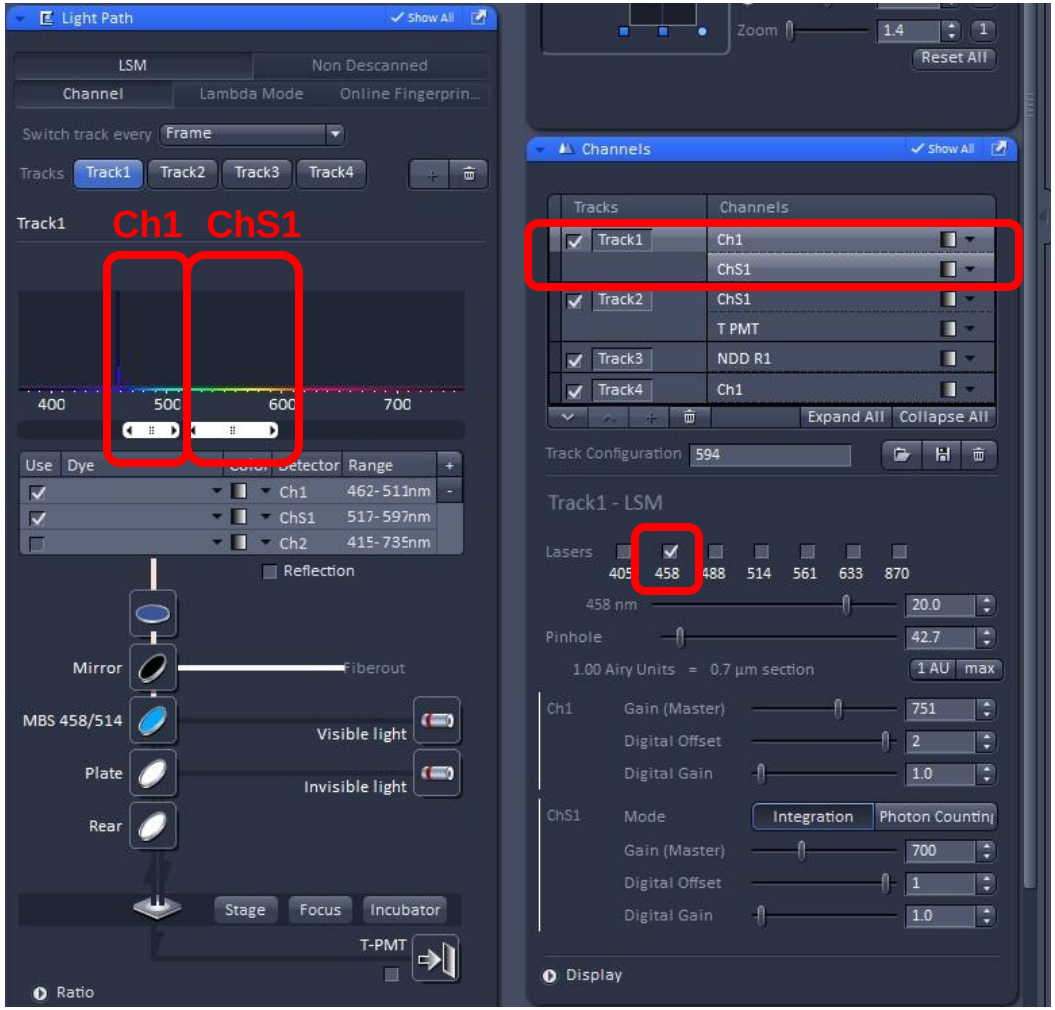


This is a review of what each track is set up to acquire.

Track1 is the excitation of the donor. It is acquired in confocal mode.

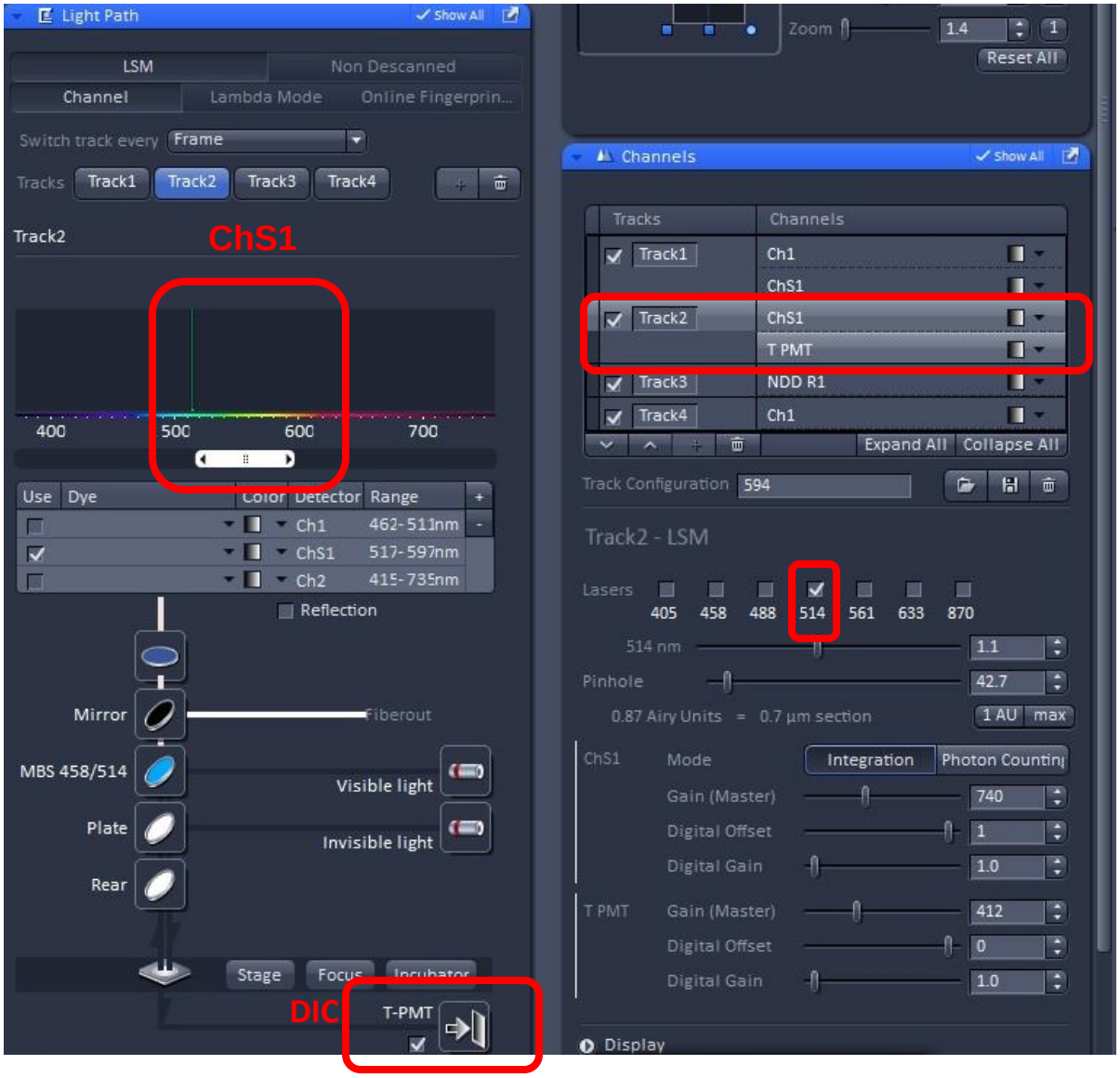
Excitation is with the 458nm laser line. The emission is collected through 2 channels:
Ch1 = Emission filer set to acquire direct excitation of the donor. In this case green light emission.
ChS1= Emission filter set to acquire acceptor emission. The FRET.

This is where one would get data to calculate FRET ratio.



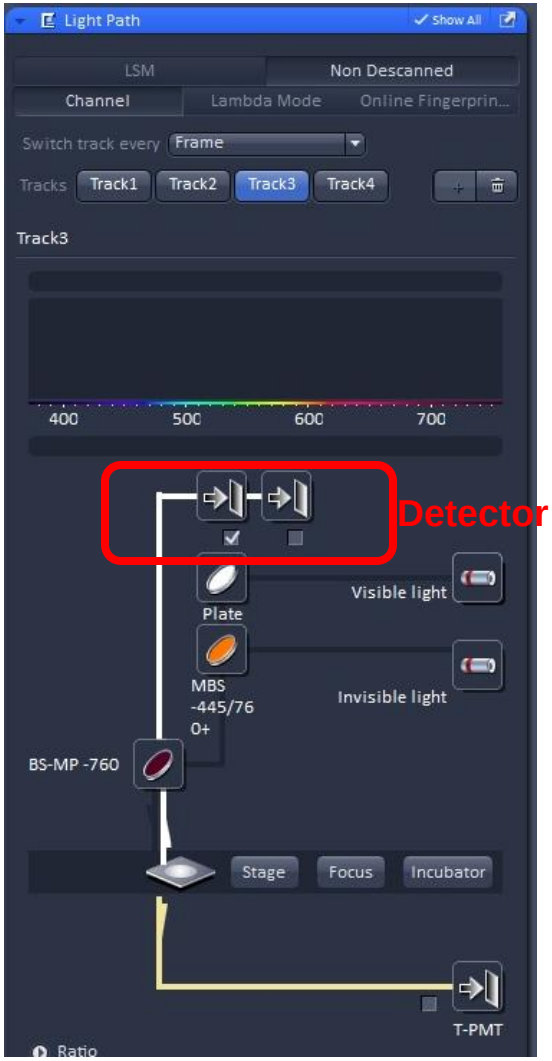
Track2 is the acceptor by itself and DIC image. It is also acquired in confocal mode.

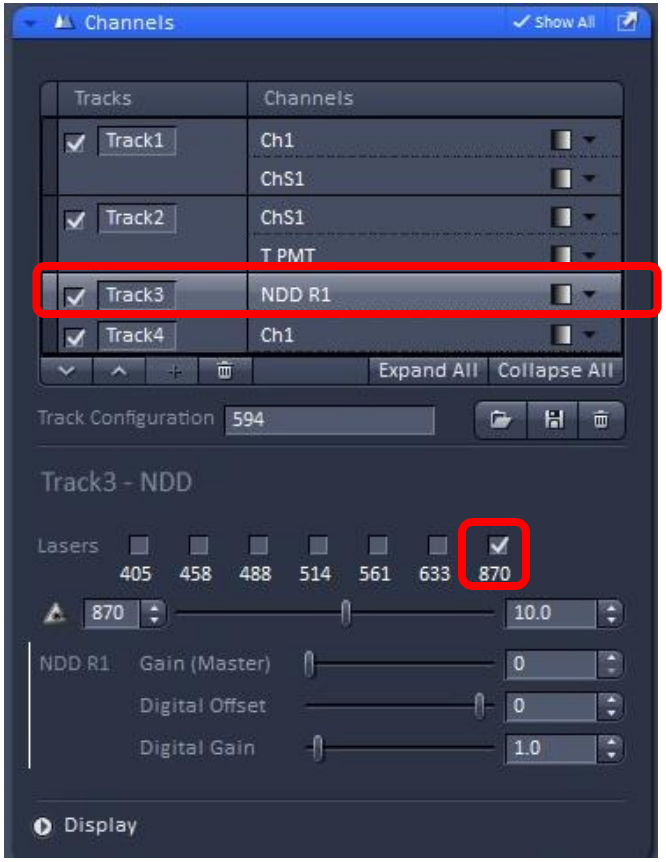
ChS1 is the acceptor emission filter and excited using the 514 laser. This is the way to directly see the acceptor. If you wish to see the morphology of the cell you use DIC. To see this the DIC slider must remain in place [See Step 3].



Track3 is the FLIM track. It is checked and acquired in confocal mode but will appear black in that image. The image is actually acquired on the FLIM computer. You must save this blank image in order to be able to save and later access information on settings, wavelength and other data. The FLIM computer has no knowledge of the settings.

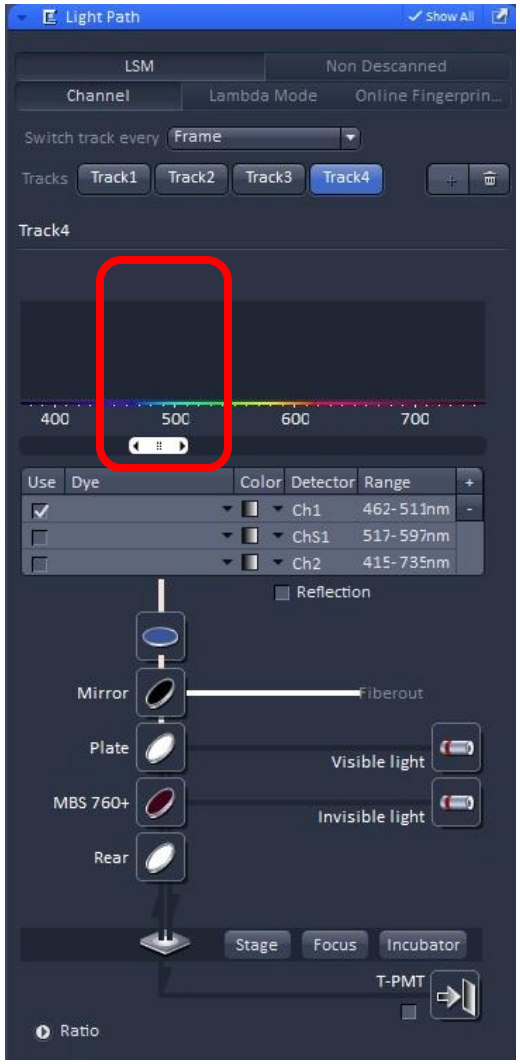
Two Photon light is set at 870nm laser for this experiment but the laser is tunable. The light goes to the FLIM Detector but in the light path configuration a detector must be checked so the image is acquired.

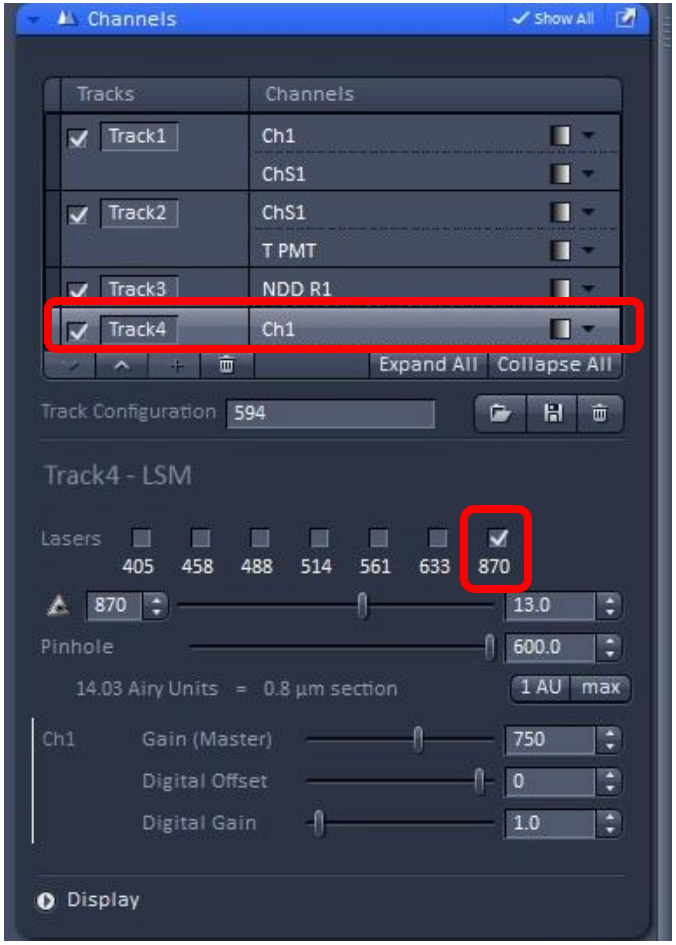




Track4 is set up to test the two photon laser. It is not used when acquiring either the confocal or FLIM image.

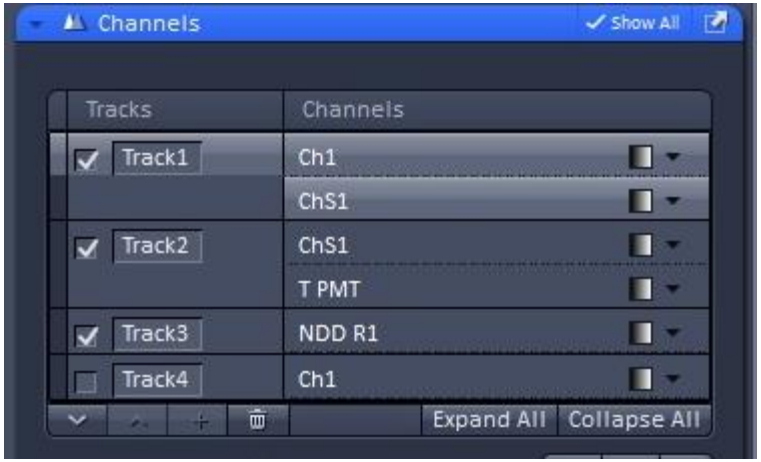
This is just to make sure the 2 photon laser is working and that you can get an image from the donor. Two photon light is used at 870 nm in this case to excite the sample. Ch1 is set up the same as Ch1 in Track1.





29. **Put sample on microscope.**
30. **Get focus through eye piece.**
31. **If doing DIC set up DIC. See the DIC tutorial for instructions on how to set up DIC.**
32. **In the ZEN Software. Check the tracks you wish to acquire.**

32

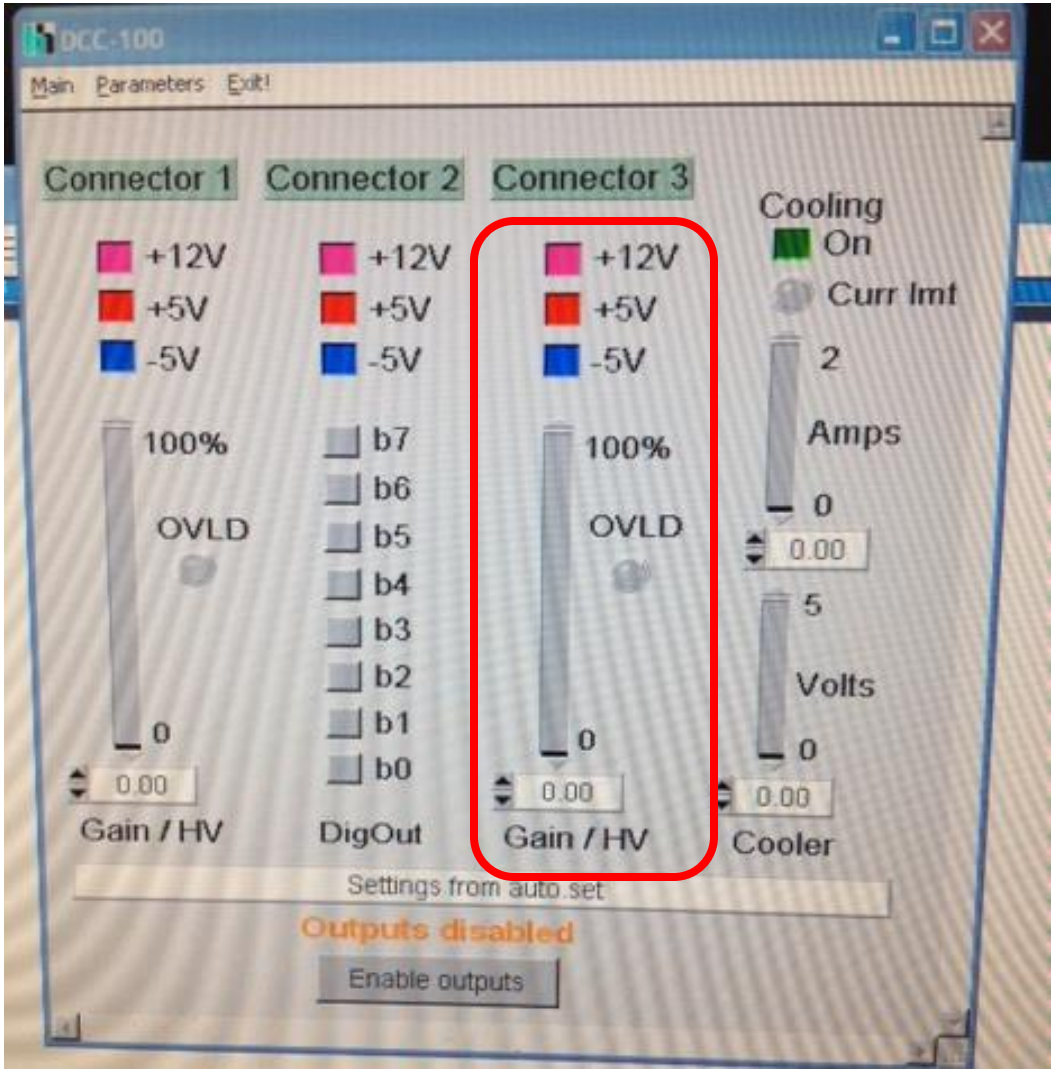


33. **Make sure the microscope is well covered so as not to allow any light through. Check that the stage controller is not covered by the curtain thus allowing the LED light to be inside the curtain.**

Go back to the eye piece and make sure you didn't move the sample or lose focus while putting the curtain over the microscope.

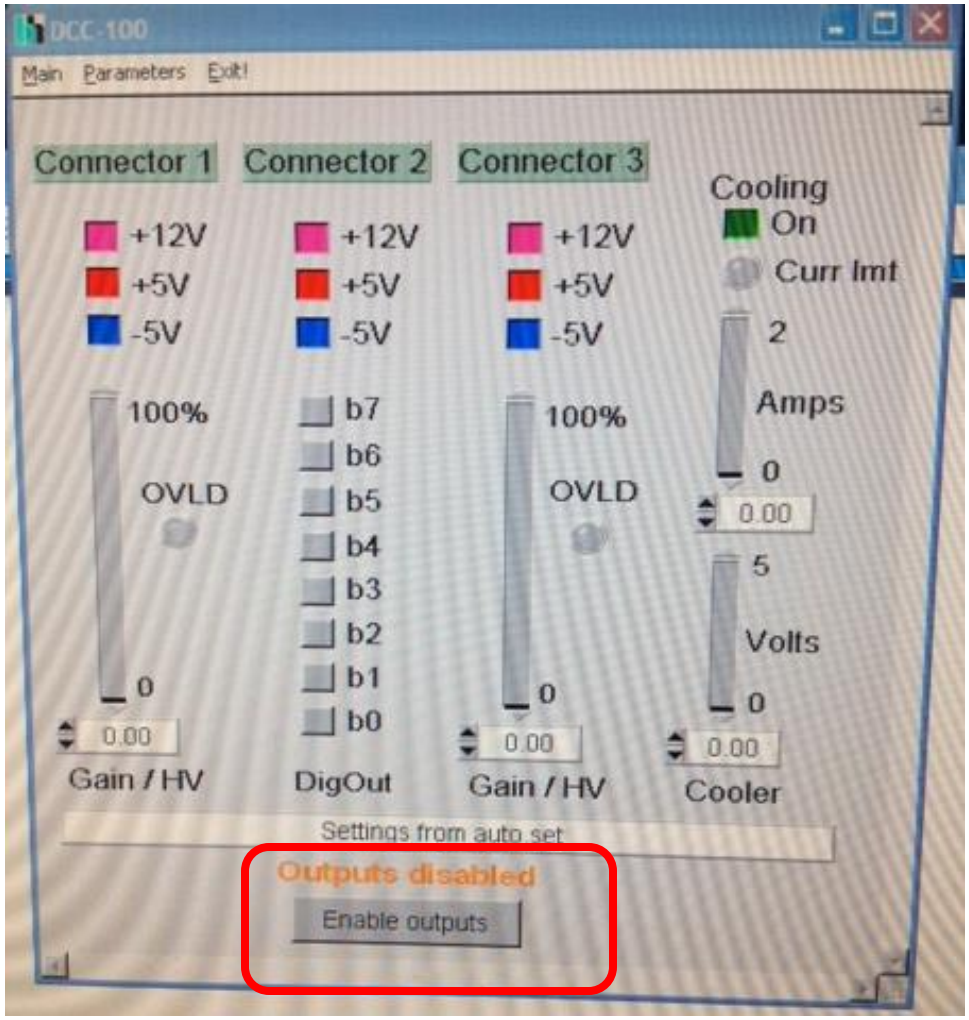
In the FLIM software:

34. Make sure Voltage is at zero.

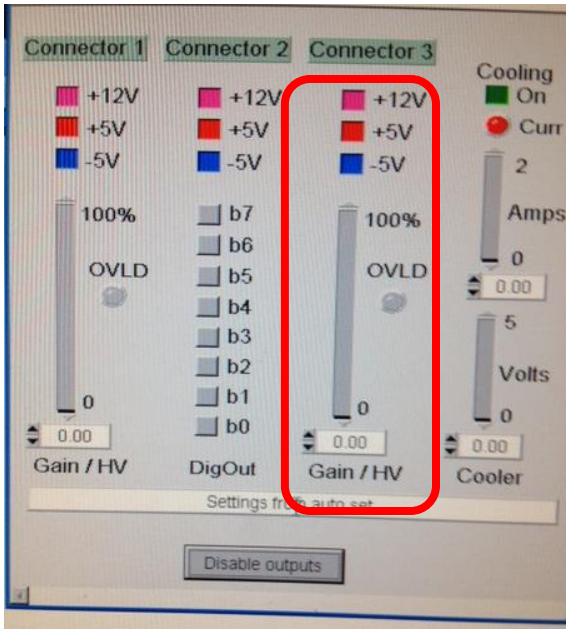


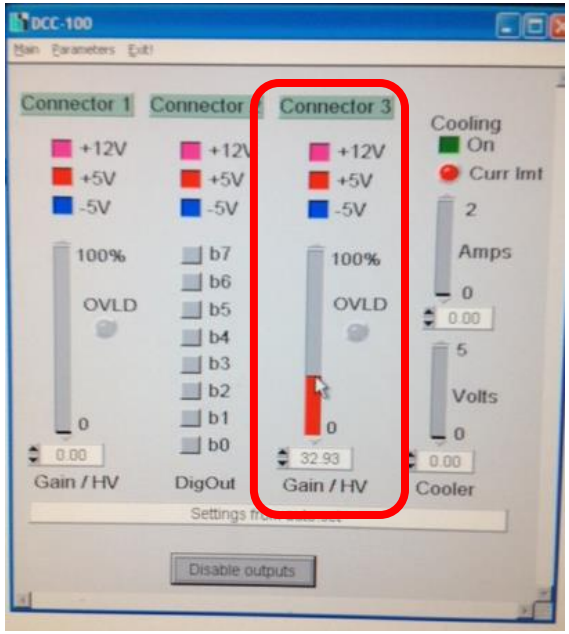
35. **Check that pulse rate indicated in SYNC is high, at 80MHz. If not, there is a problem with the 2 photon laser (not mode locked).**

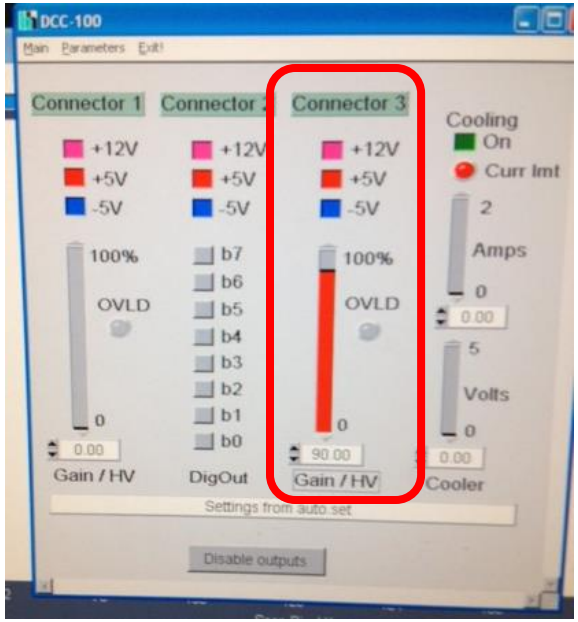
36. **Turn off overhead lights and desk lamp. Make sure curtain is closed in case someone comes in and turns lights on while you are imaging.**
37. **Click Enable Outputs.**



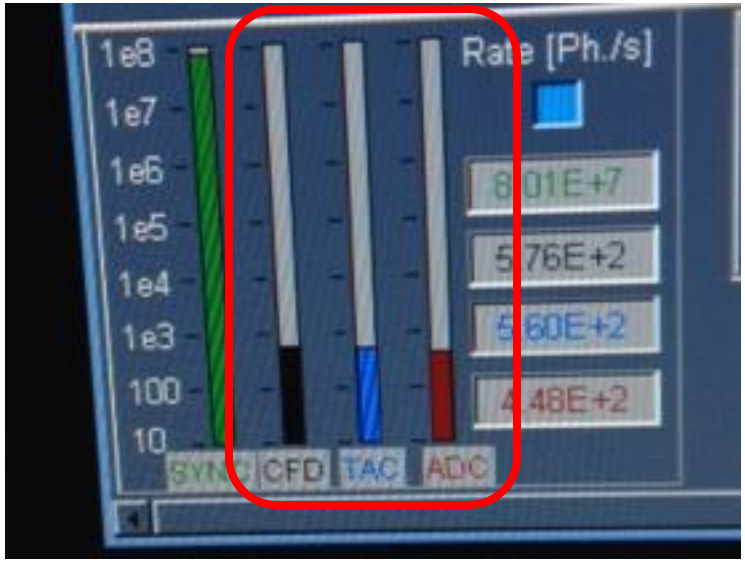
38. <u>Slowly</u> raise Gain to 90%.



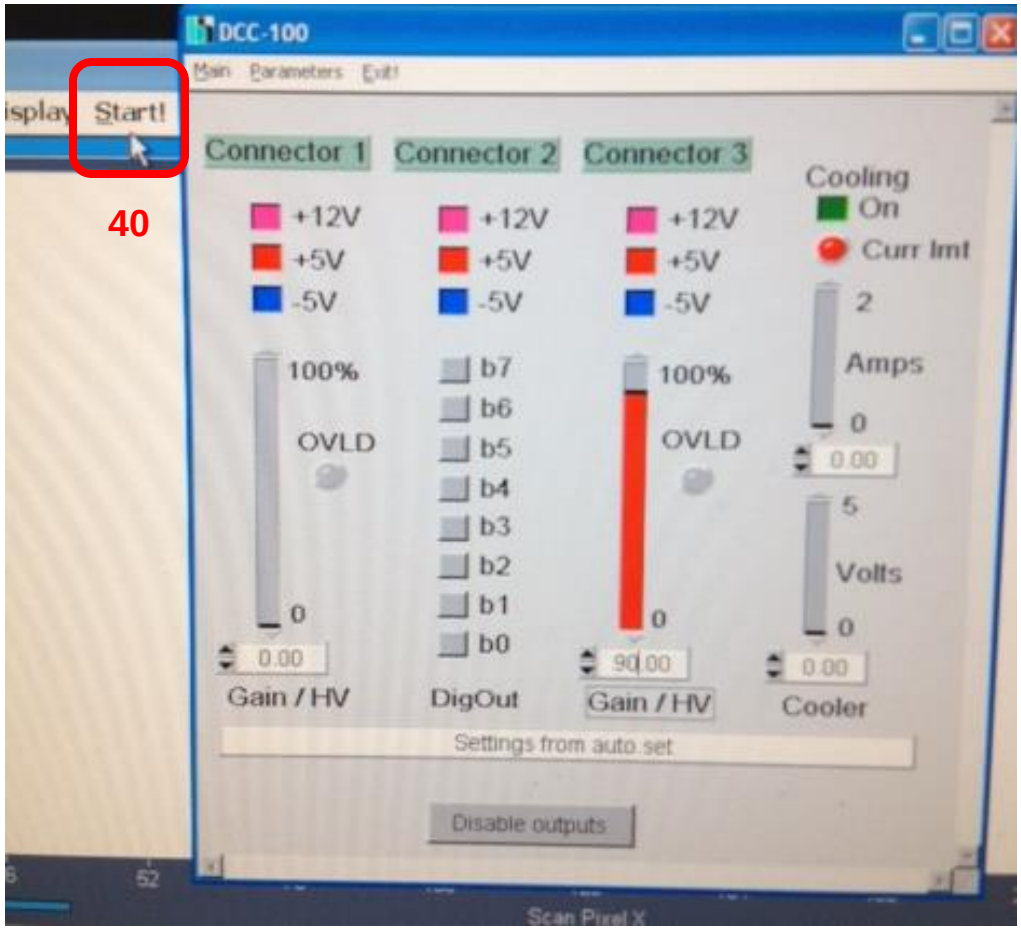


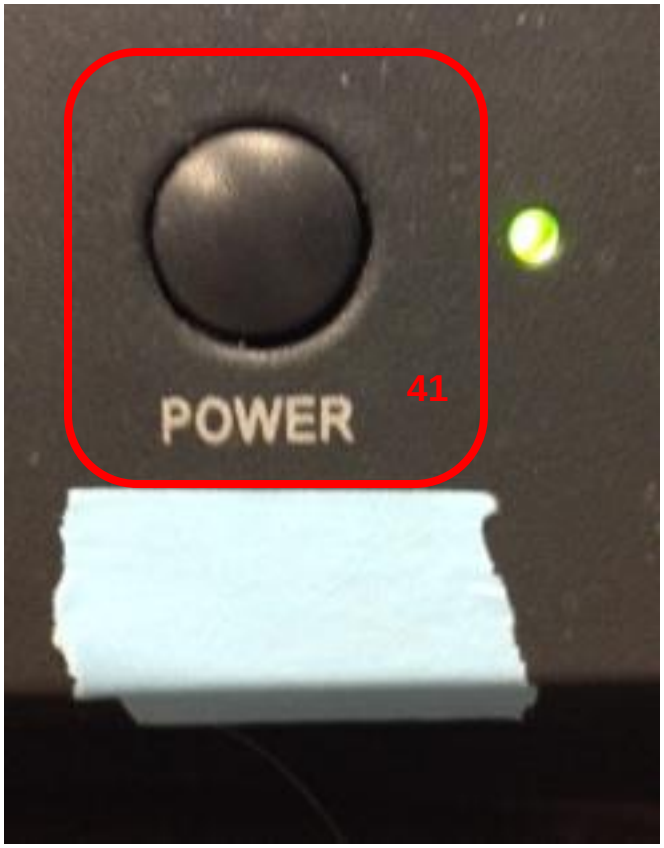


39. Check background levels indicated by CFD [black], TAC [blue] and ADC [red].



40. **Click Start.**
41. **Turn off monitor.**

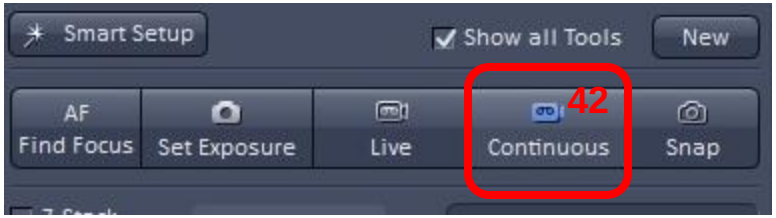


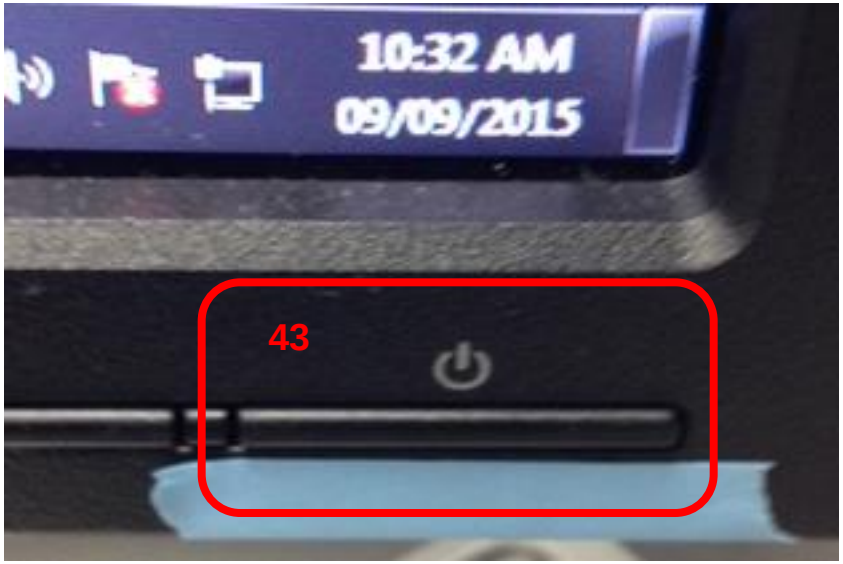


Tape has been placed below the power button to help you feel where it is when the lights are out.

In ZEN Software:

42. **Click Continuous.**
43. **Turn LSM780 monitor off.**

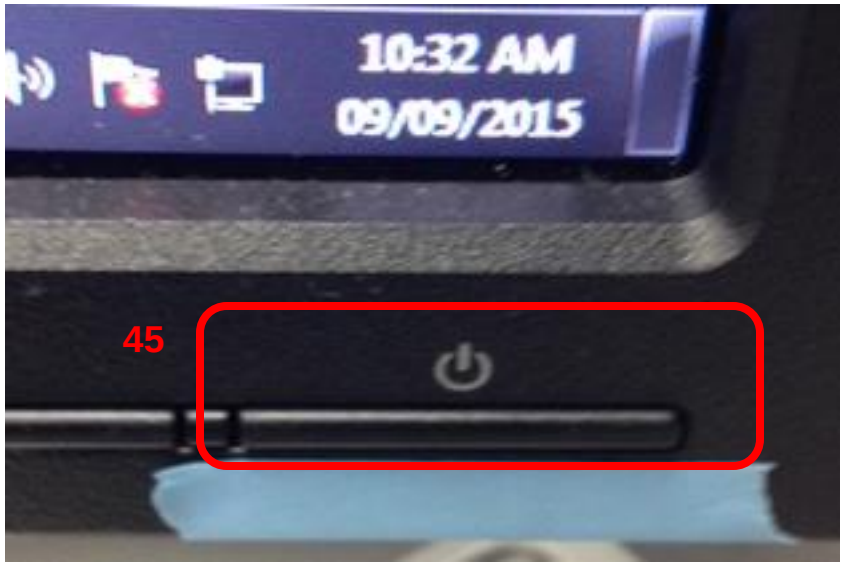




Tape has been placed below the power button to help you feel where it is when the lights are out.

In ZEN Software:

44. **Wait until you hear the beep.**
45. **Turn ZEN Monitor on.**
46. **Stop Continuous.**



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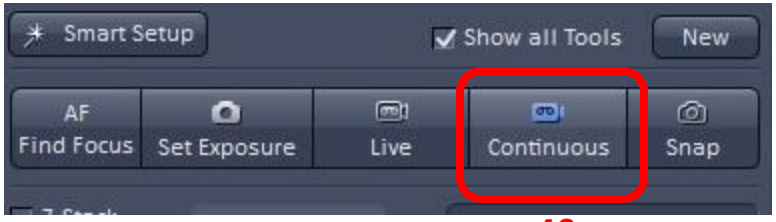
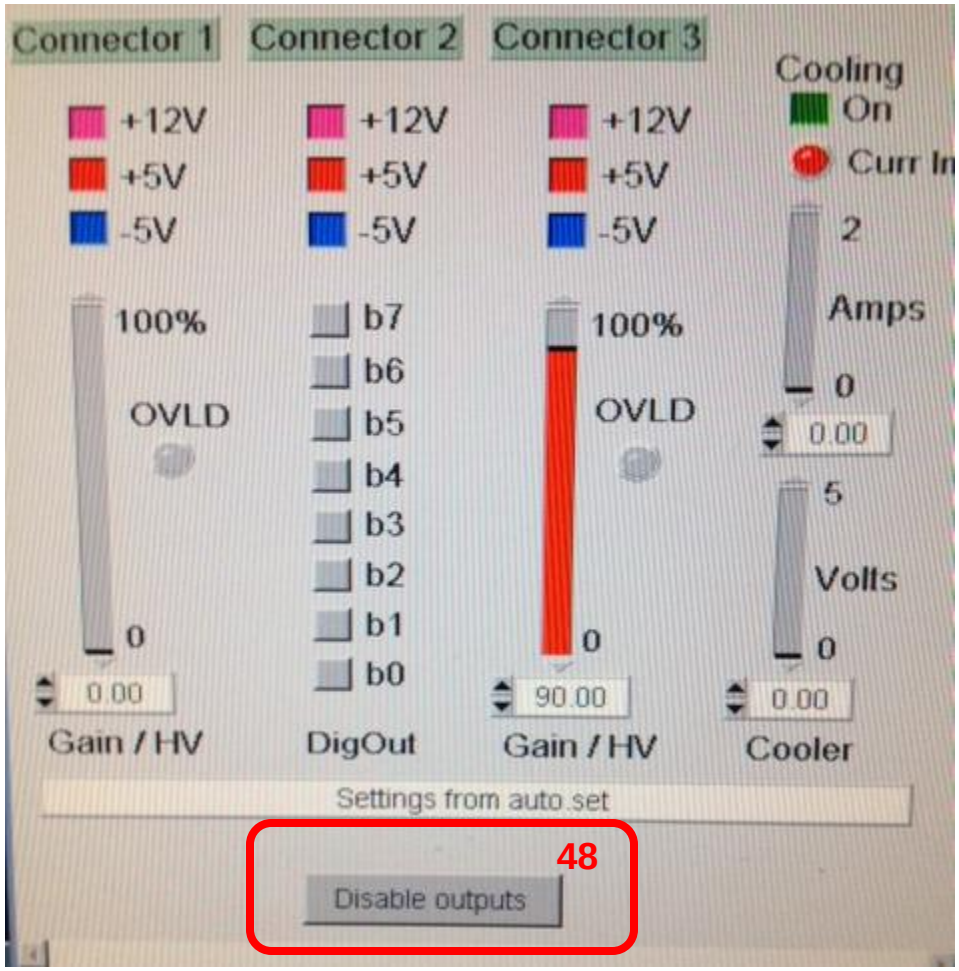
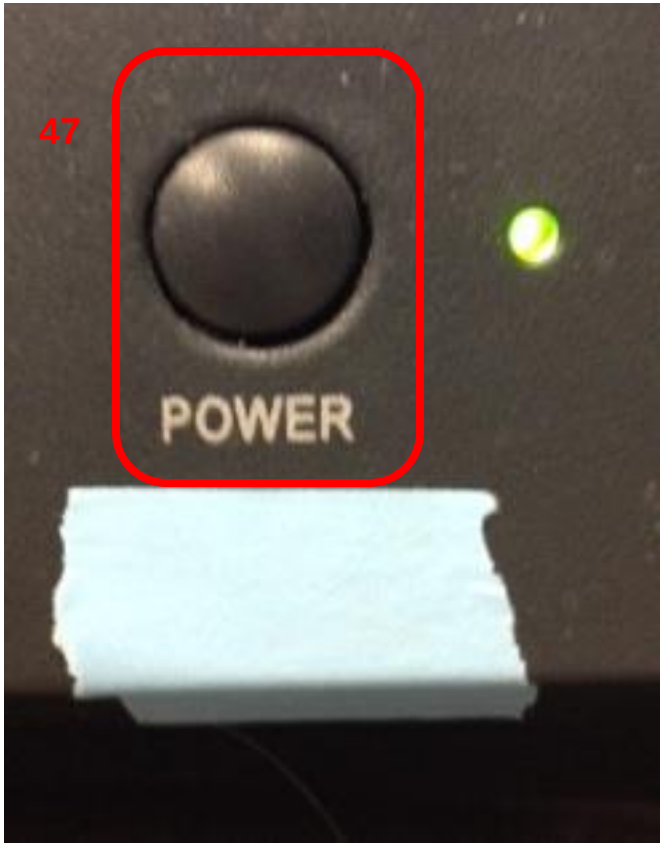


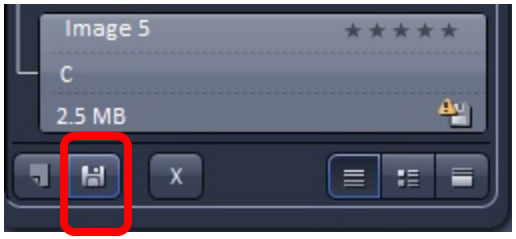
47. **Turn FLIM Monitor on.**
48. **Click Disable Outputs to disable detector.**



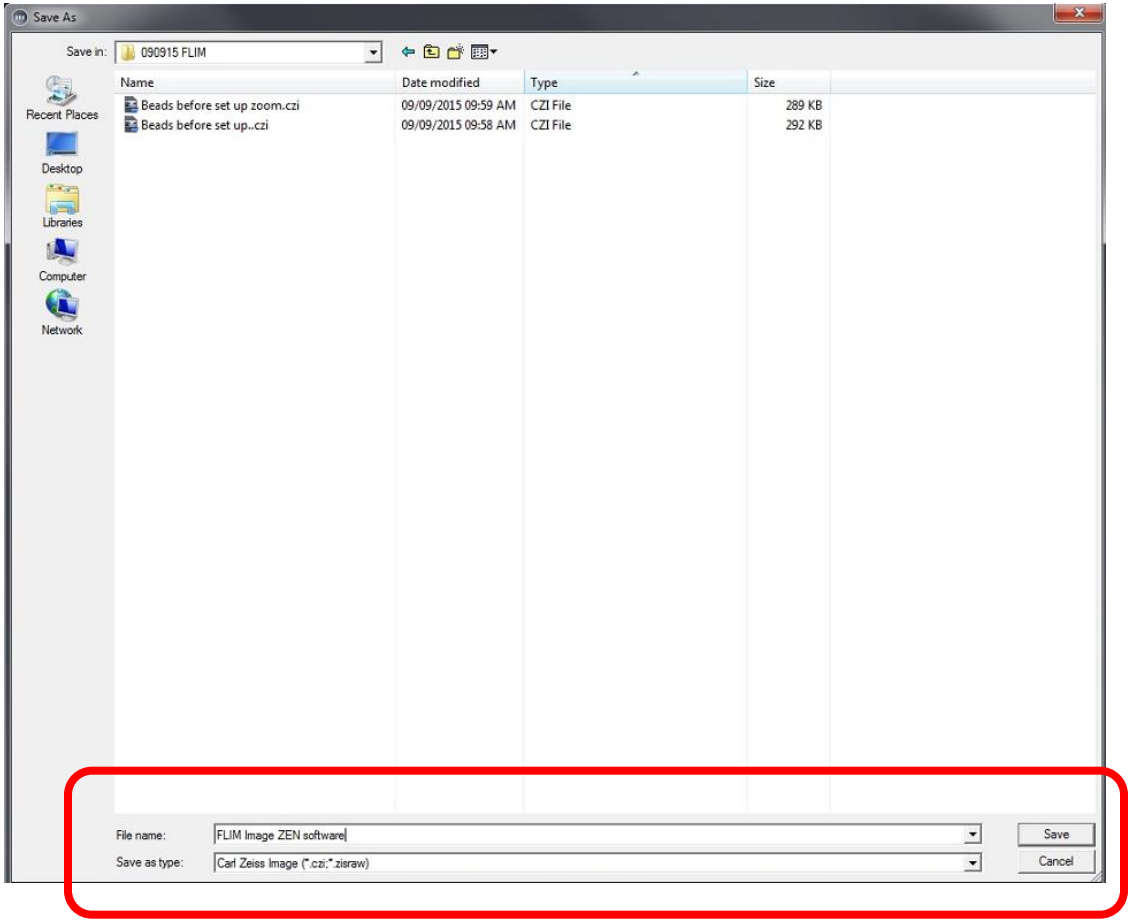


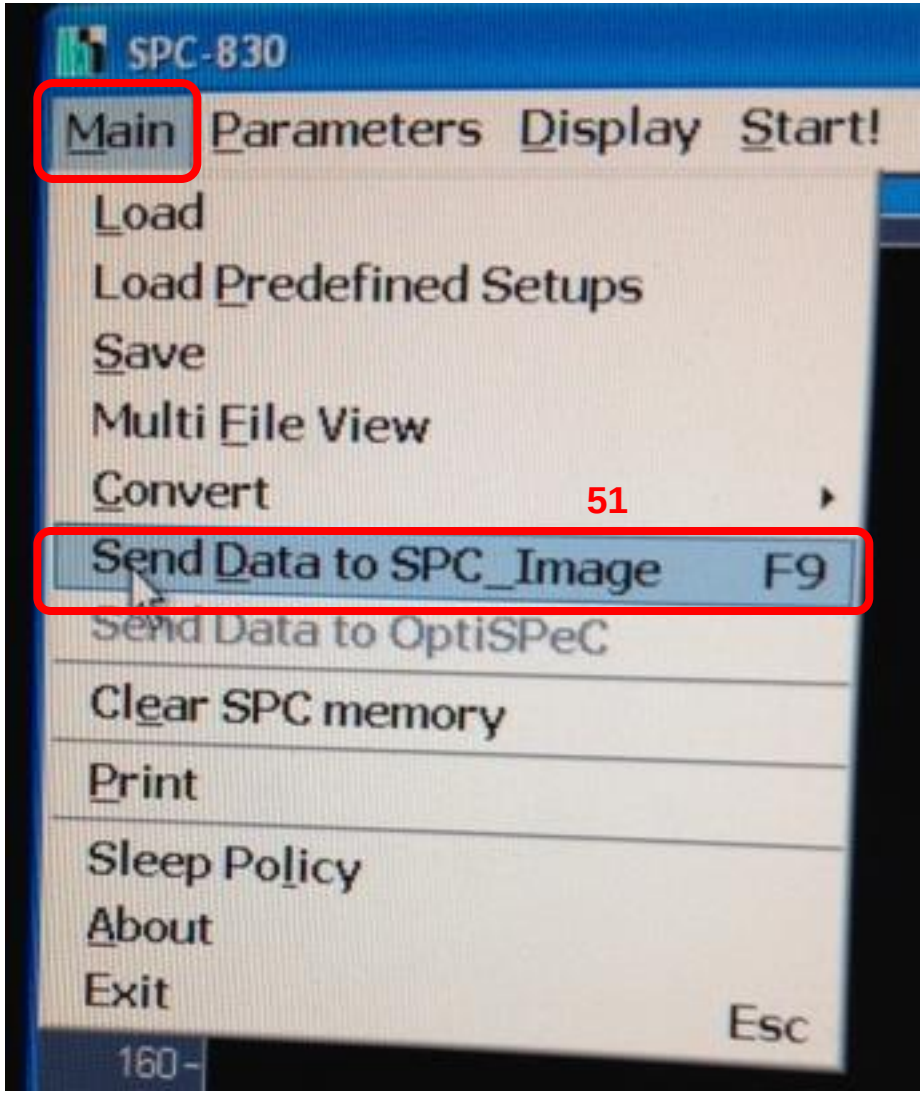
Tape has been placed below the power button to help you feel where it is when the lights are out.

49. Save ZEN Image.



It is best to save directly to the omalshare1 server so your FLIM and ZEN software data is saved together in the same place.





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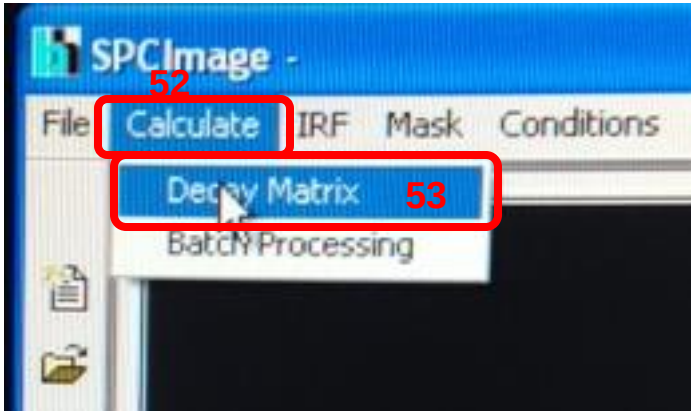
In FLIM Software:

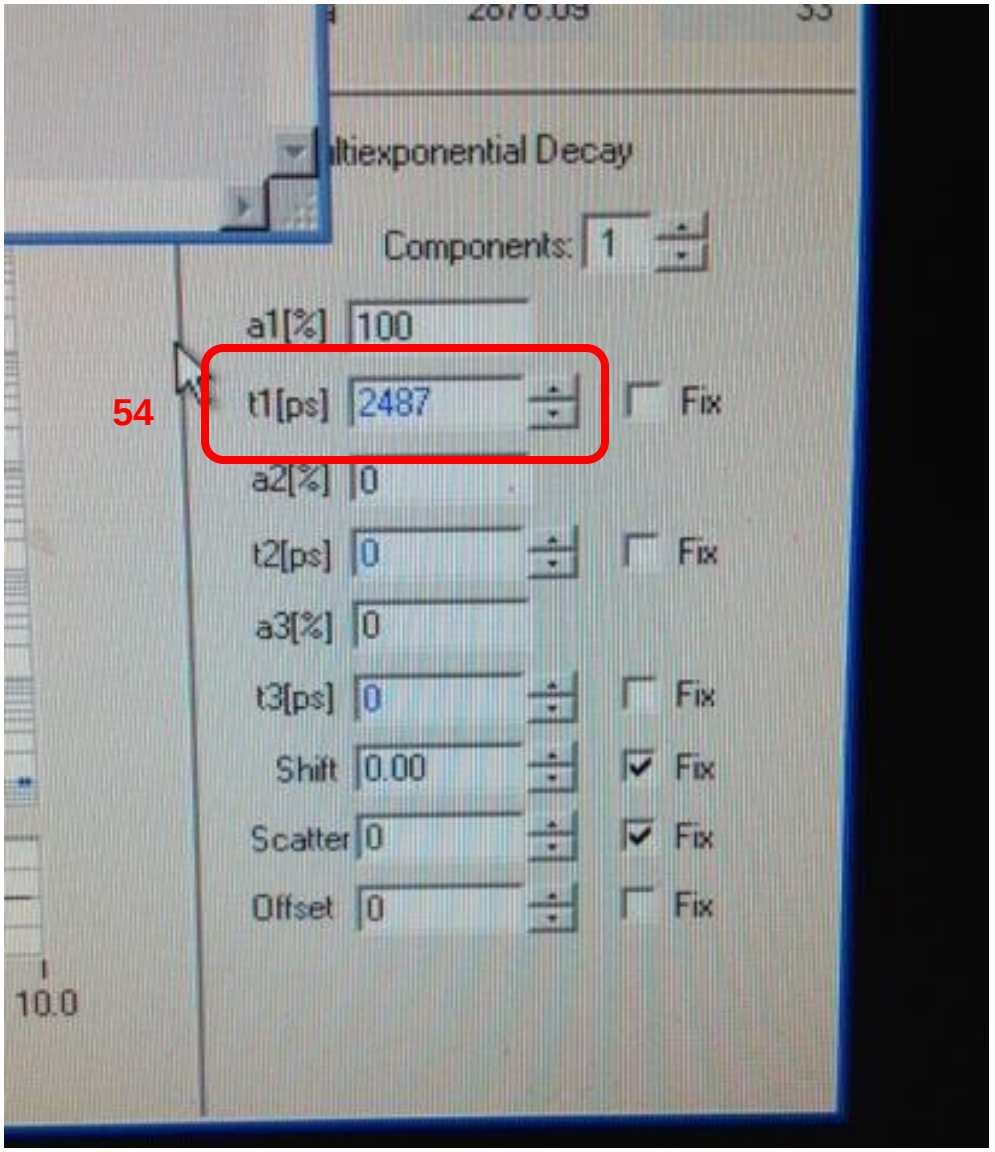
50. **Select Main.**
51. **Click Send Data to SPC_Image.**
52. **Select Calculate.**
53. **Click Decay Matrix.**



52

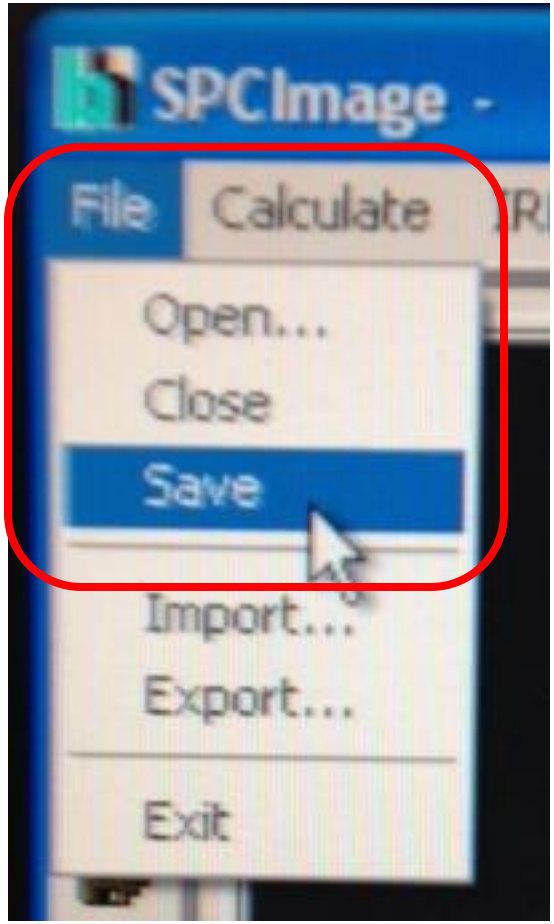
53

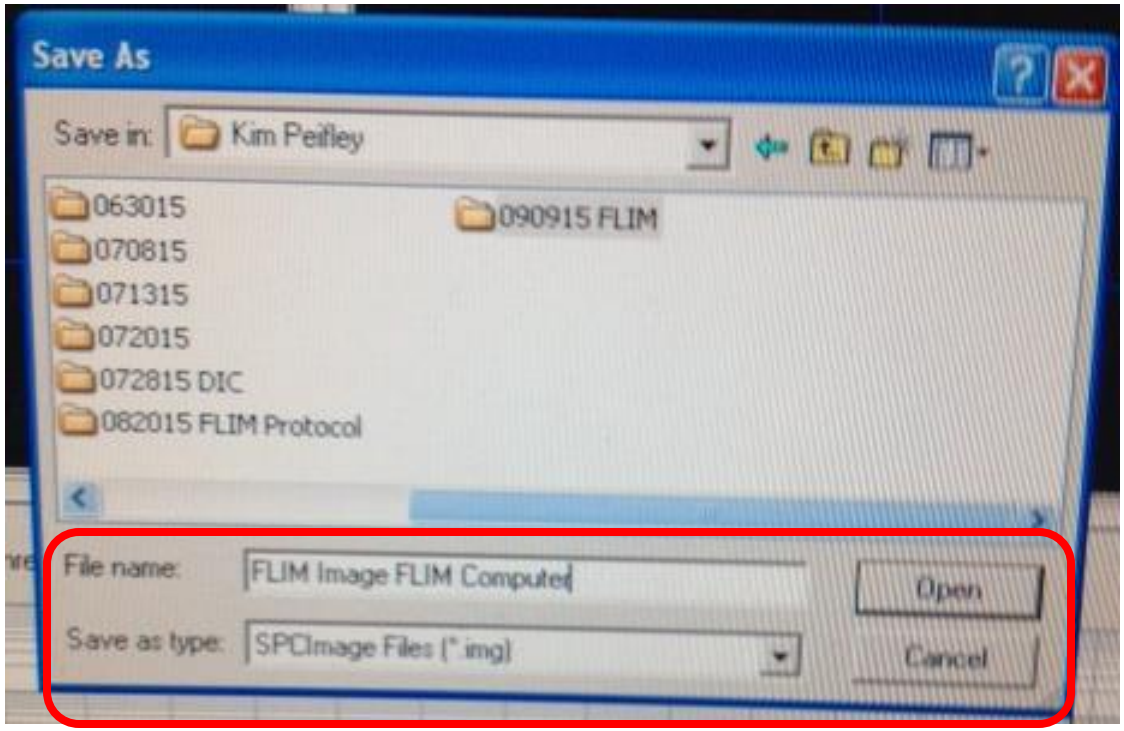
54. **Check the number of t1[ps]. The number depends on the fluorophore. In this case it should be 2,500 or $2.5x10^3$.**



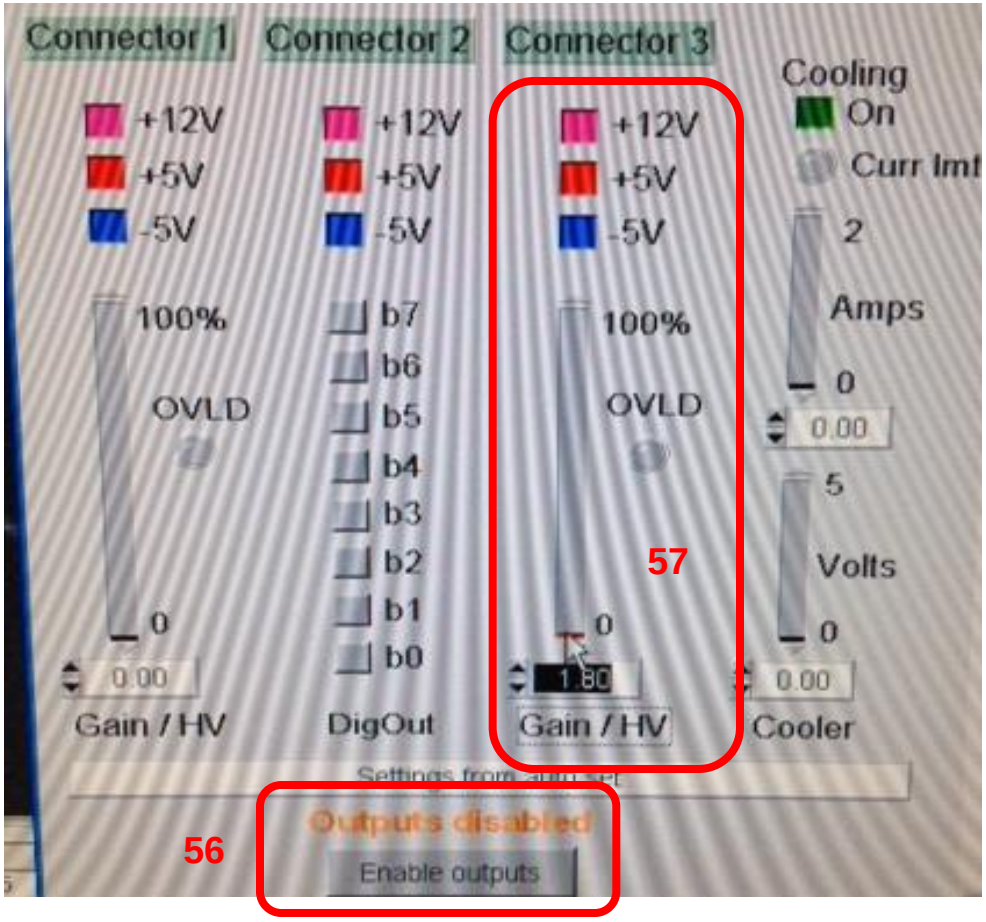
55. Save FLIM Image.

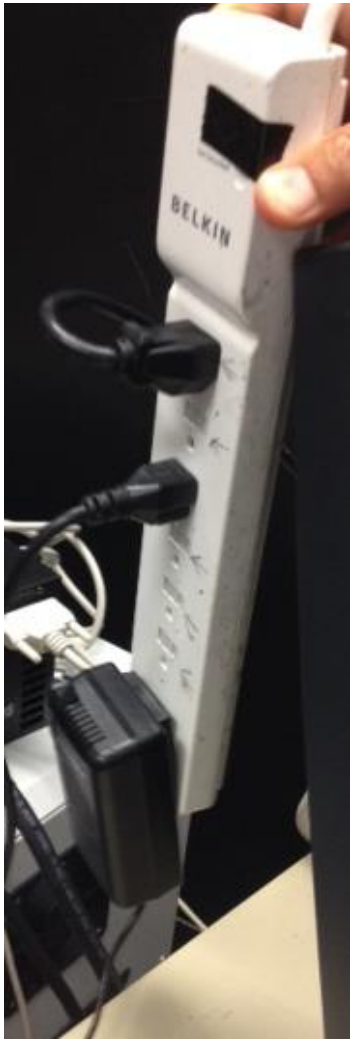
It is best to save directly to the omalshare1 server so your FLIM and ZEN software data is saved together in the same place.





When imaging session is complete.
Turn off all FLIM elements.
56. Click Enable outputs to off [Outputs disabled].
57. Turn Voltage down to Zero.

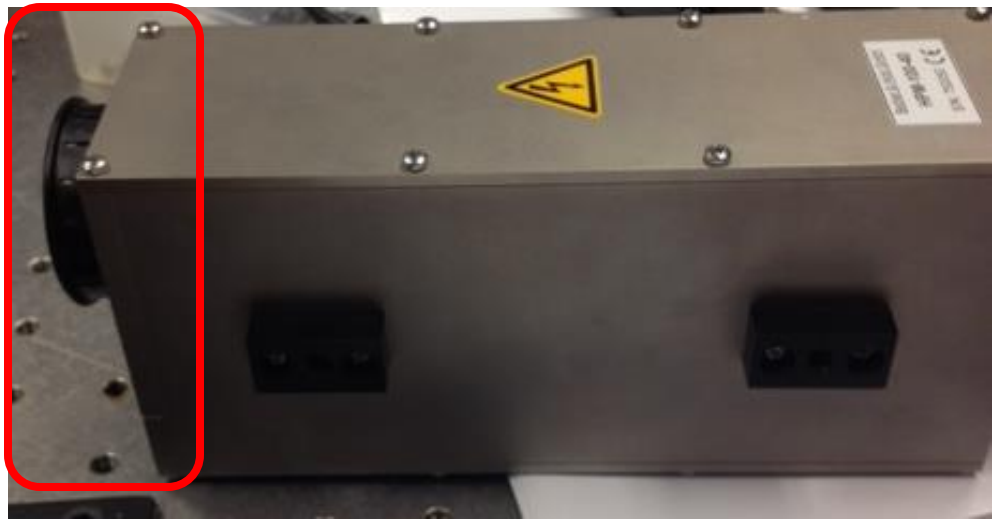




58. Turn off FLIM computer.
59. Turn off power to the FLIM detector at switch.

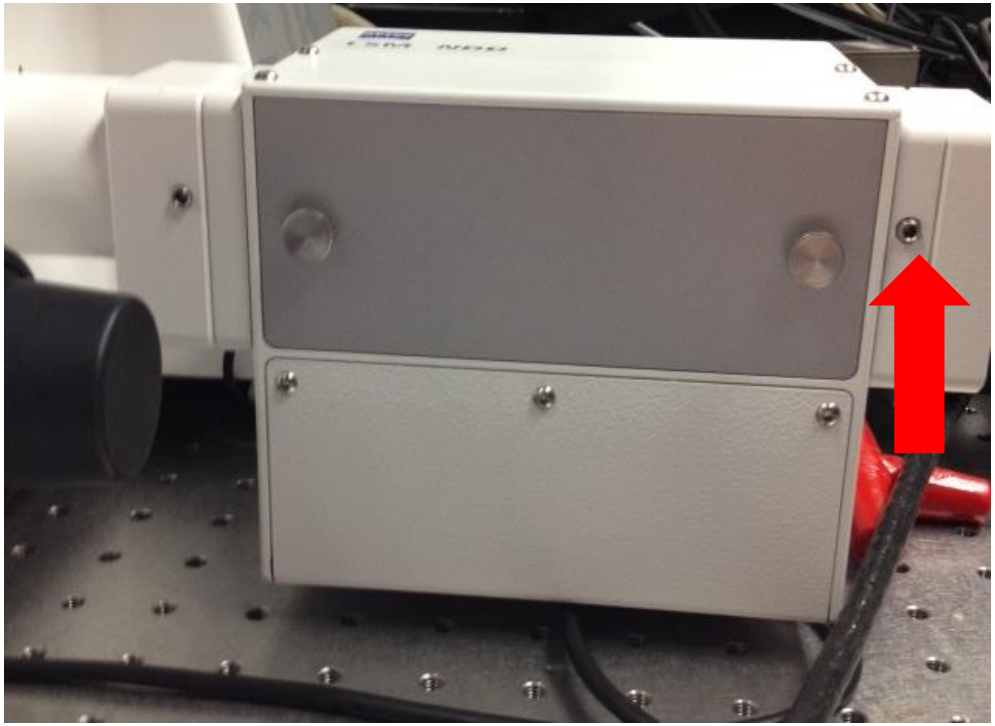
59

60. Turn off lights.
61. Remove the FLIM detector and replace cap on detector.



62. Replace the cap on the NDD detector.

Make sure the cap is placed properly and tightened so as not to trigger the laser safety lock.



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63. Turn off 2 photon laser.

64. **Move your data to the omalshare1 server if you did not directly save your data there.**
65. **Remove your sample from the microscope.**
66. **Check the microscope schedule to see if the person following you is doing live cell.**
67. **If no live cell experiment follows you, turn off live cell elements at the touch pad.**
68. **Remove live cell elements from microscope.**
69. **Turn off live cell switches.**
70. **Turn off CO2**
71. **If no one is following you on the microscope, turn off the microscope.**