Bring: slides

 Coverslips used for sample

 Atto488 10-8 and 10-9

 diH2O

 p200

 p200 tips

1. Turn of 1, 2, 4-7 on Laser Scanning Confocal
2. Check FCS filter for 520/35±(if you are using 485 laser)
3. Make sure the 485 laser is in the FCS box port, and turn on the FCS box and laser (power strip, and turn key to 3:00)
4. Make sure the key is in the “slave” computer, and turn on both computers.
5. Open elements, Client\_SymPhoTime, and SymPhoTime (Operational)
6. In elements under calibration 🡪 objectives 🡪 change 10x position to position “---“ and change the PLAN APO IR 60x WI DIC N2 to position “1” 🡪 okay
7. Install the PLAN APO IR 60x WI DIC N2 objective in the turret. Clean with a little water and lens paper. Start collar at 0.17.(for #1.5 coverslips)
8. Go set up slides as the system and room warms up.
9. Once slides are ready, go back to the confocal and add 30µL Atto488 10-8 onto a coverslip and onto the objective.(add water on objective first and you may skip Atto488 10-8 step and directly add Atto488 10-9)
10. Under confocal presets 🡪 remove interlock 🡪click “Map” button and only check the GFP channel (make sure it says eGFP) 🡪 select transmission out 🡪 click Auto 🡪 okay
11. Start with laser power=5, size=512, 1 frame/s, gainhv)=200, laser offset=0
12. Make sure when you click 1AU it equals 31.9. If it does not then something is set wrong and repeat/check steps 6-10 (normally you might have laser other than 488 selected, defult laser for such calculation is the one with the larger wavelength).
13. Turn on perfect focus (PSF) and focus until PSF engages.
14. Make PSF offset=7000
15. Scan 🡪 capture 🡪 autoscale.
16. Now scan 🡪 scroll up and down with PSF to make sure you are inside the sample (this is easier with Atto488 10-8, but it can be done with Atto488 10-9)
17. Once you are sure you are in the sample switch to a 30µL drop of Atto488 10-9.
18. Scan 🡪 stop scan 🡪 select “ROI on/off” 🡪 “simple ROI editor” 🡪 “pt for AUX” 🡪 place a point in the middle of the field of view 🡪 finish
19. On the “slave” computer in SymPhoTime (Operational) 🡪 File 🡪 New Workspace 🡪 drive (D:) 🡪 PQ users 🡪 your folder 🡪 place new folder name in tops section that says “new untitled”
20. In elements, right click background 🡪 “Acquisition Control” 🡪 “A1 settings” 🡪 “AUX” 🡪 when dialog pops up click “okay”
21. In Client\_SymPhoTime, click the “FCS” tab 🡪 set the seconds to 300-500
22. On FCS box make sure the large knob is set to 0 (max laser power) and the fine knob is all the way up (max laser power)
23. In A1 settings under AUX, start laser.
24. In Client\_SymPhoTime, click “Test”
25. Ideal cpm for the Atto488 10-9 >25K at largest laser power
26. Now magnify the SymPhoTime test window on G(0) and adjust the collar of the objective until G(0) is as high as possible (above 1), or cpm is as larger as possible
27. Stop the test and turn off the laser.
28. Make a new 30µL drop of Atto488 10-9, in Client\_SymPhoTime, click the “FCS” tab 🡪 set the seconds to 120 🡪 start the laser 🡪 and “record” that Atto488 10-9 for 2 mins for a good calibration.
29. When recording is done stop the laser.
30. Write comments in the window about what was measured and save the measurements.
31. Now in SymPhoTime (Operational) click “FCS trace” 🡪 check “FCLS” 🡪 “calculate FCS filters” 🡪 draw the magenta line to the left a tiny but and see the filter 🡪 okay
32. Now check the raw data, click “MCS/photon filter” 🡪 “Trace” 🡪 drag line to show all data points, check for bleaching, and check the histogram.
33. Now click “accurate” 🡪 “Nsub8” 🡪”Calculate FCS Trace” 🡪 “Fit” 🡪 “Triplet State” 🡪 start
34. Click “add to Veff calibration” 🡪 remove Z0, W0, and <C> 🡪 change Di expected to: 360 if room is 21°C or 400 if room is 25°C 🡪 “Calculate” 🡪 “Accept” 🡪 “Yes”
35. Now the values on the left of the graph should update.
36. Fit the calibration is good the values should be in the following ranges:
	1. K ~ 5-6
	2. Veff ~ 0.2-0.3
	3. Z0 ~ 1
	4. W0 ~ 0.2
37. If calibration is not good, then talk to Ann about aligning the laser.
38. If calibration is good, save and export what you need.
39. Now add on sample (HisprGFP is the best to image next). In confocal preset find the cells.
40. Focus on the cells of interest, and drag the cells to the AUX point in the screen. Make sure to put the point in a non-nuclear site.
41. In Client\_SymPhoTime, click the “FCS” tab 🡪 set the seconds to 300-500
42. In A1 settings under AUX, start laser.
43. In Client\_SymPhoTime, click “Test”
44. Ideal cpm for the proteins is 2000-4500 (should not record if cpm is below 2000). You may need to adjust the laser power. The laser power used for HisprGFP should be the same as the laser power used for sample.
45. Now record a measurement for 10 seconds. Then stop laser so you do not bleach or kill the cells.
46. Write comments in the window about what was measured and save the measurements.
47. Now in SymPhoTime (Operational) click “FCS trace” 🡪 check “FCLS” 🡪 “calculate FCS filters” 🡪 draw the magenta line to the left a tiny but and see the filter 🡪 okay
48. Now check the raw data, click “MCS/photon filter” 🡪 “Trace” 🡪 drag line to show all data points, check for bleaching, and check the histogram. You can select a range of data to correlate, to avoid initial bleaching for example
49. Now click “accurate” 🡪 “Nsub8” 🡪”Calculate FCS Trace” 🡪 “Fit” 🡪 “Triplet State (Extended)” 🡪 start
50. Uncheck K on left of graph, click “load calibration” 🡪 “Calculate” 🡪 “Accept”
51. Ideally the Di for protein diffusion is ~20, and the α=0.8-1
52. Take multiple HisprGFP for comparison to sample.
53. Continue with sample using same laser power as HisprGFP.