

# Time-Resolved Confocal Microscope ALBA

## User Manual

*SMART Lab – July 2015*



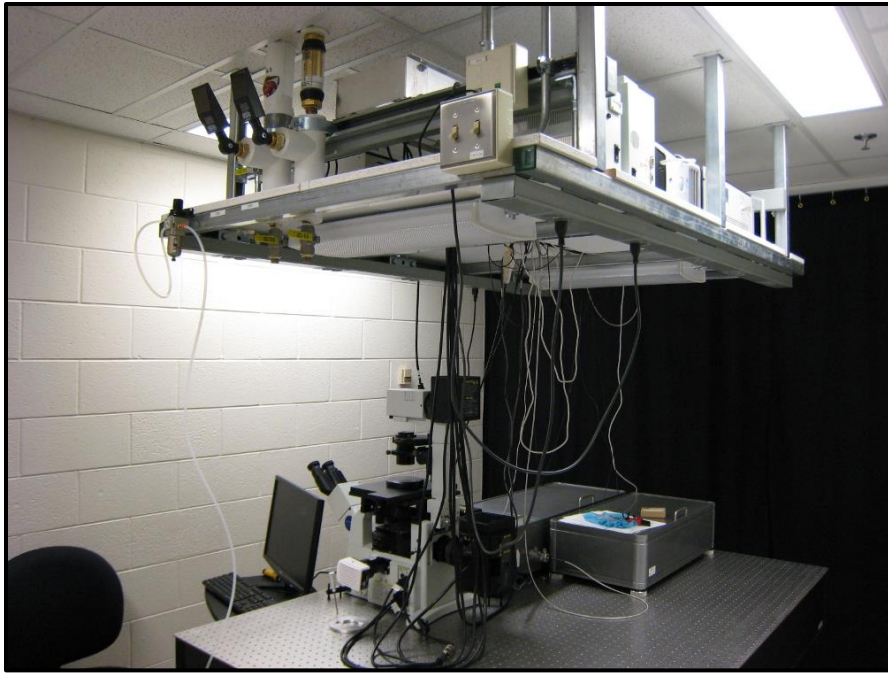
This is a **class IV laser** – its use can result in serious damage. Before using this laser you must have read the chapters on safety in the laser manuals. Use protective goggles when necessary. If you run into any problem or you don't understand this protocol, contact a supervisor.

### ***Laser Safety Reminders:***

- Take the laser safety class offered by Steve Katnik (skatnik@umich.edu).
- Do not look directly into a laser beam.
- Do not look directly at a reflected or scattered laser beam.
- While the objective safety cover is removed, make sure the shutter is blocking any active laser beams.
- Always have the black curtain closed and 'LASER IN USE' light on when working with lasers.

### ***Biosafety Reminders (BSL1):***

- Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiments or work with cultures and specimens are in progress.
- Persons wash their hands after they handle viable materials, after removing gloves, and before leaving the laboratory.
- ***Eating, drinking***, smoking, handling contact lenses, applying cosmetics, and ***storing food*** for human use ***are not permitted in the work areas***. Persons who wear contact lenses in laboratories should also wear goggles or a face shield. Food is stored outside the work area in cabinets or refrigerators designated and used for this purpose only.
- Mouth pipetting is prohibited; mechanical pipetting devices are used.
- Policies for the ***safe handling of sharps*** are instituted.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- ***Work surfaces are decontaminated*** at least once a day and ***after any spill*** of viable material.
- ***All cultures, stocks, and other regulated wastes are decontaminated before disposal (use bleach)*** by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory are to be placed in a durable, leakproof container and closed for transport from the laboratory. Materials to be decontaminated outside of the immediate laboratory are packaged in accordance with applicable local, state, and federal regulations before removal from the facility.



**Fig. 1:** Time Resolved Confocal Microscope ALBA

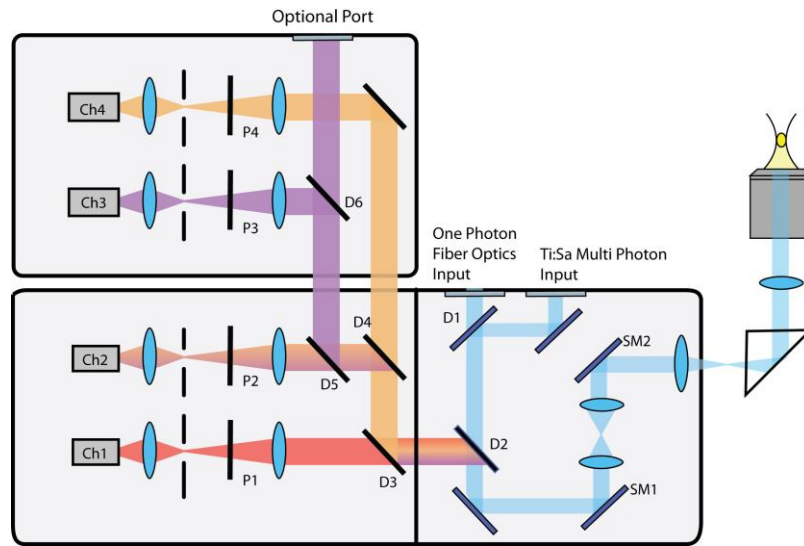
***Instrument description:***

The ALBA microscope system from ISS (Illinois) combines fluorescence lifetime imaging (FLIM, FRET) with fluorescence fluctuation spectroscopy (FCS, FCCS, PCH) capabilities.

- Microscope: Olympus IX-81  
60X 1.2 NA water immersion objective  
ALBA module attached to the left side port
- Spatial Range: 200um x 200um (galvanometers) x 100um (piezo-positioner)
- Excitation: Fianium WL-SC-400-8, 8W, up to 80 MHz, <6ps pulse width  
currently 20MHz with ~100 ps pulse width after optical fiber  
excitation wavelength pick with an AOTF
- Detection: 2 x PMTs Hamamatsu H7422P-40, 40% quantum efficiency at ~550nm  
2 x APDs Perkin Elmer SPCM-AQRH-15, 65% quantum efficiency at 700nm  
Becker & Hickl SPCM-830 board, up to 8MHz detector count rate
- Optical pathway: Filter sets are optimized to do dual color illumination and detection for either PMTs or APDs.

***Nomenclature and wheel position description (Fig.2):***

SM1, SM2: scanning mirrors



**Fig. 2:** Optical pathway

D1: empty

D2: pos1: 490 shortpass dichroic mirror  
 pos2: 405/470/532/635 quadband dichroic mirror (Alluxa)  
 pos3: 405/488/561/635 quadband dichroic mirror (Z405/488/561/635rpc, Chroma)

D3: pos1: glass  
 pos2: mirror  
 pos3: 50/50 beam splitter  
 pos4: 561 longpass dichroic mirror (FF562-Di03, Semrock)  
 pos5: 650 shortpass dichroic mirror (655DCSPXR, Chroma)

D4: pos1: glass  
 pos2: mirror  
 pos3: 50/50 beam splitter  
 pos4: 561 longpass dichroic mirror (FF562-Di03, Semrock)  
 pos5: 650 shortpass dichroic mirror (655DCSPXR, Chroma)

D5: pos1: glass  
 pos2: M

D6: mirror

P1: pos1: FITC emission filter for 470/488nm excitation (FF01-531/40-25, Semrock)  
 pos2: Cy3 emission filter for 532nm excitation (FF01-582/75-25, Semrock)  
 pos3: TRITC emission filter for 561nm excitation (FF01-593/40-25, Semrock)

P2: pos1: FITC emission filter for 470/488nm excitation (FF01-531/40-25, Semrock)  
 pos2: Cy5 emission filter for 635nm excitation (FF01-697/75-25-D, Semrock)

P3: pos1: FITC emission filter for 470/488nm excitation (FF01-531/40-25, Semrock)  
 pos2: Cy5 emission filter for 635nm excitation (FF01-697/75-25-D, Semrock)

P4: pos1: FITC emission filter for 470/488nm excitation (FF01-531/40-25, Semrock)  
 pos2: Cy3 emission filter for 532nm excitation (FF01-582/75-25, Semrock)  
 pos3: TRITC emission filter for 561nm excitation (FF01-593/40-25, Semrock)

Ch1, Ch2: APDs

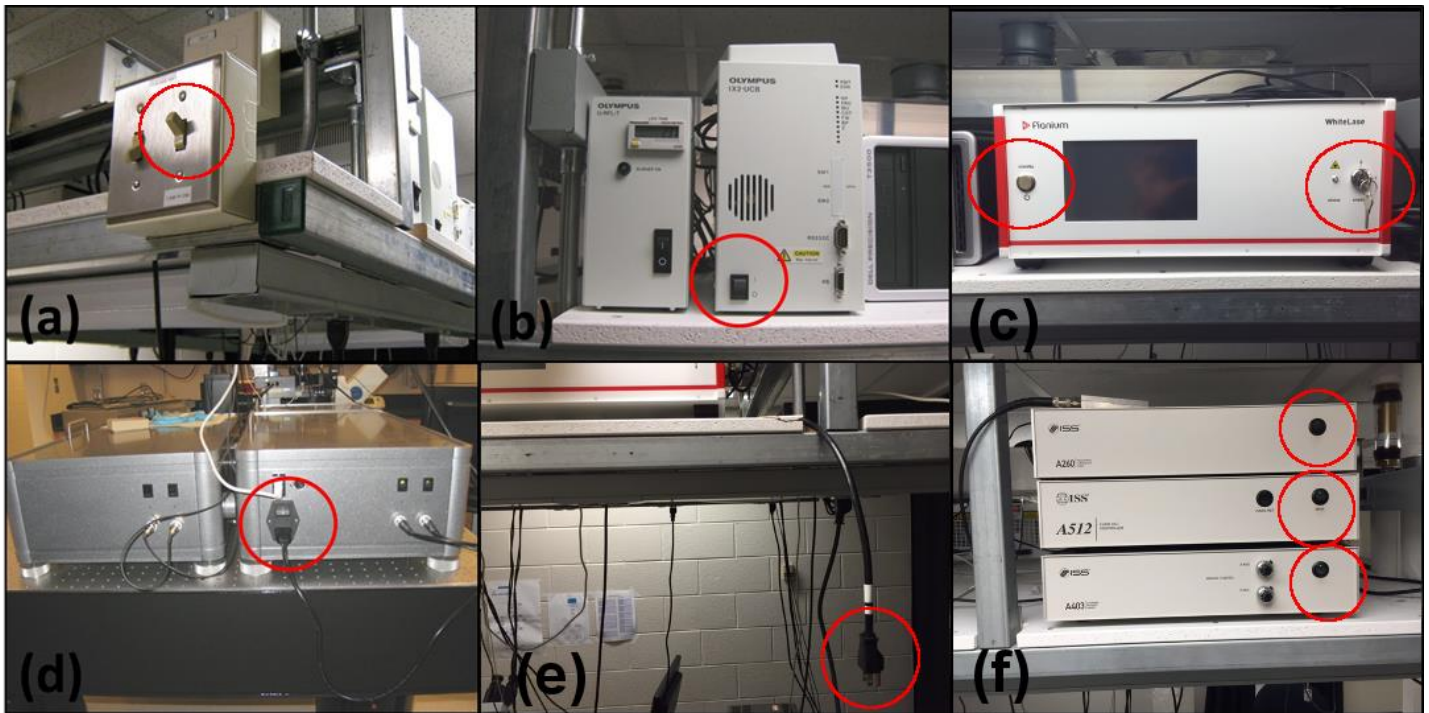
Ch3, Ch4: PMTs

### ***Starting up the microscope:***

Standard start-up: turn on the following, in this order (following **Fig.3.x** order):

1. Switch “LASER ON” sign – light switch located on table shelf, near entrance (**Fig.3.a**)
2. Microscope controller – located on table shelf facing the hallway (**Fig.3.b**) (NOT the epifluorescence controller on the left)
3. Laser power switch – located on the left side of the front panel (**Fig.3.c**) – will need to wait 2 minutes for system to boot before turning key switch on the right
4. Detection unit master switch and detectors – located at the back (**Fig.3.d**)
5. AOTF power cord plug – located on table shelf above the detection unit (**Fig.3.e**)
6. 3-axis DAC controller (A512) main switch – located on table shelf above the IX-81 microscope *CENTER position* (**Fig.3.f**)
7. Start AOTF control software (see ‘Additional Information’ for more details). Choose wavelength(s) needed.
8. When laser 2 minute countdown is finished, switch front panel key
9. On Laser touch panel, set laser power to 50%
10. Start ALBA software (VistaVision or VV, see ‘Additional Information’ for more details) using “Alba 5” or “Daily Start-Up Alba 5” (use the latter if the detection unit was off)
11. Once VistaVision start-up routine is finished, switch on the piezo stage controller z-axis (A260) *TOP* and scanning mirrors driver (A403) *BOTTOM*

*If you already know how to use the VV software and select optics, jump to **Optimization of the detection unit***

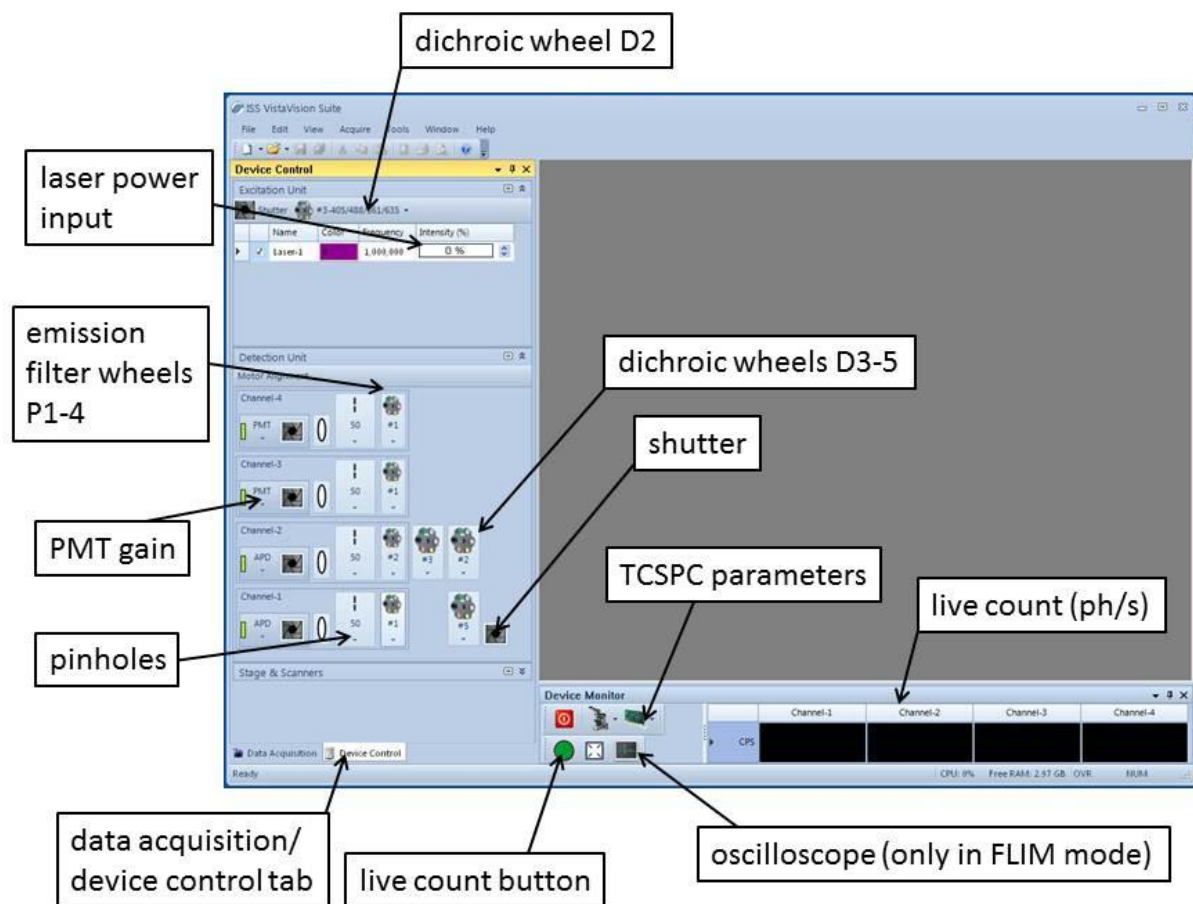


**Fig. 3:** Start-up procedure locations

## *VistaVision software (VV) description and commands:*

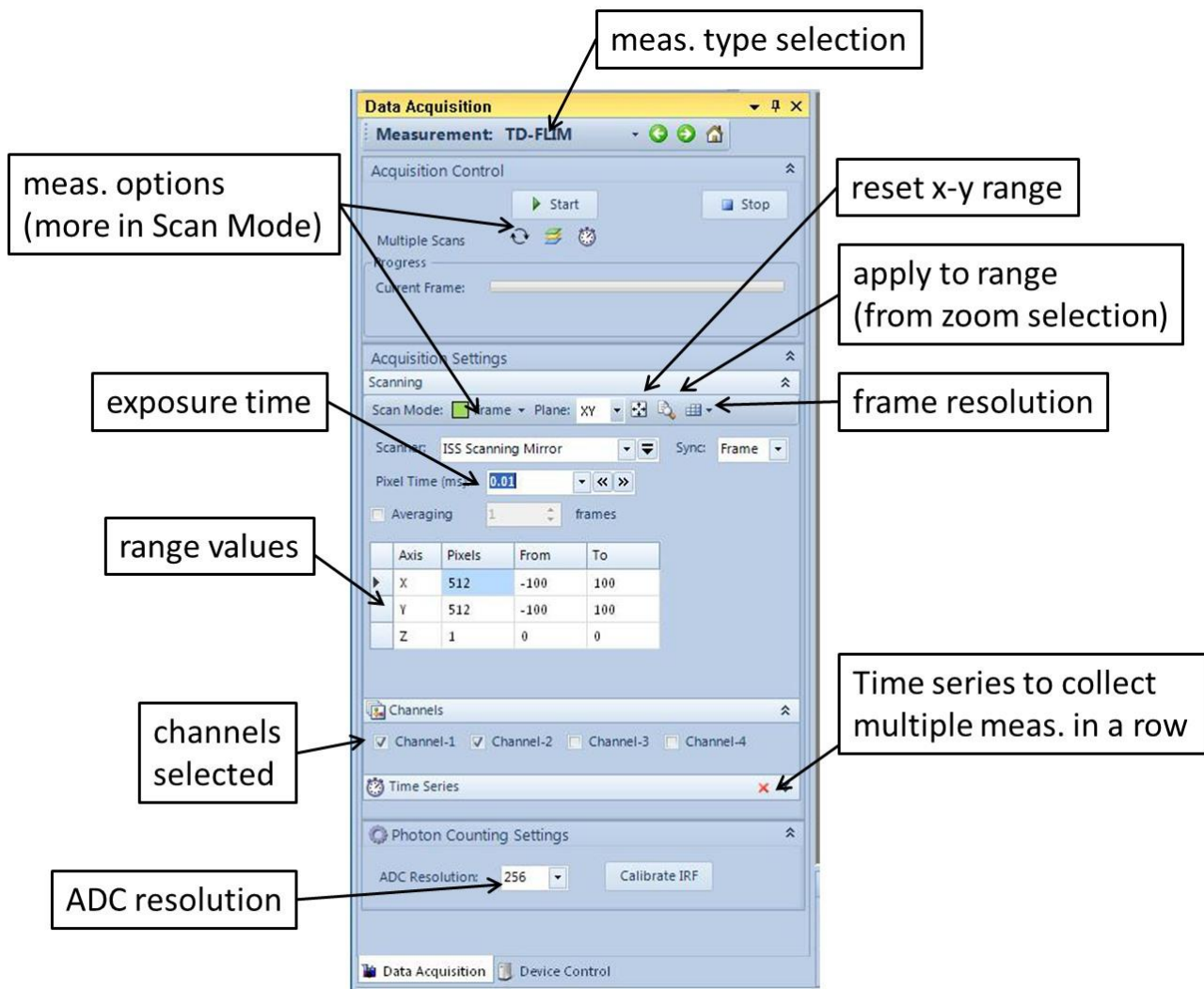
1. Requirements and limitations:
  - a. This software is only working under Windows XP; stability is not guaranteed for any other environment. As of August 2011, there are still several bugs and limitations. Make sure you save your data often!
  - b. It requires sufficient free memory random access memory (RAM) to run properly. **IT WILL CRASH IF TOO MANY WINDOWS (IMAGES, FCS DATA ETC) ARE OPEN IN THE SOFTWARE! So SAVE your data as soon as you are done recording it!**
2. Use “Daily Start-up Alba 5” mode if you are the first one to use the microscope that day or if the detection unit was switched off (this mode re-adjust all wheels position). Otherwise use “Alba 5”.
3. Layout (**Fig.4**):
  - a. Parameters are on the left, for either data acquisition or device control (bottom tab)
  - b. Various indicators are on the bottom right: live count button start a continuous photon count displayed for each channel. In addition, when on FLIM workspace – see below, the live oscilloscope display (fluorescence lifetime decay) can be turned on.
  - c. TCSPC parameters are accessible there too, but record any change you made and input initial values before switching off!
  - d. On the top right, measurement windows are displayed
4. Device control mode (**Fig.4**): used to choose and align optical components
  - a. You can access each dichroic and emission filter, pinhole size
  - b. The laser input power is controlled at the top. It is the overall laser intensity; use the AOTF to adjust intensities from one wavelength to the other.
  - c. Gain on the PMTs need to be 100% for single-photon counting. Use RESET to switch the gain off – not typing 0 because it needs to decrease gradually (function of the reset button)





**Fig. 4:** Device control mode and general layout

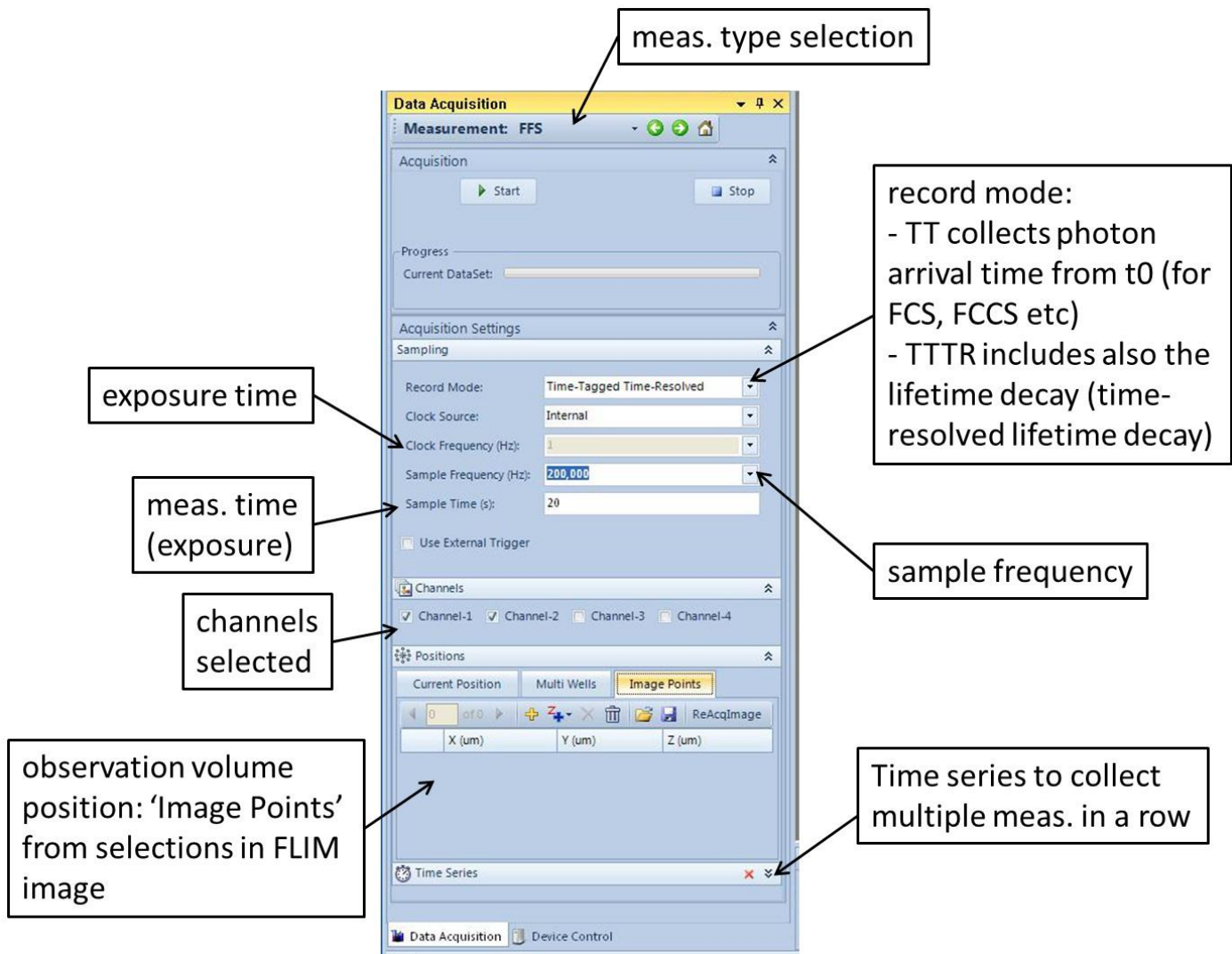
5. Data acquisition mode: TD-FLIM workspace – time-dependent fluorescence lifetime imaging (**Fig.5**):
  - a. Multiple scans switch on one of these options: continuous, z-stack, time series
  - b. Scan mode: spot, line, frame (xy-plane), z-stack
  - c. When in frame mode, you can adjust the frame resolution and reset the travel range to  $-100\mu\text{m}$ ,  $100\mu\text{m}$  for x and y. Also after selecting a new area zooming onto a picture, the “apply to range” will update the range values table for subsequent measurement
  - d. The channel selection applies to the device control mode. However it is not remembered when changing from one measurement mode to the other!
  - e. Time series allows multiple measurements to be collected in a row, with the possibility to add a delay between each
  - f. ADC resolution represents temporal binning of the lifetime decay (higher ADC means higher temporal resolution but the pixel intensity is lower)



**Fig. 5: TD-FLIM**

6. Detection mode: FFS workspace – fluorescence fluctuation spectroscopy (**Fig.6**):

- a. Record mode:
  - i. time-tagged collects photon arrival time from  $t=0$  (for FCS, FCCS, PCH etc)
  - ii. time-tagged time-resolved includes also the lifetime decay
- b. ADC resolution (for the lifetime decay histogram) in FFS is fixed to 4096
- c. Sample frequency needs to be adjusted (lowered) if diffusion is much slower
- d. The observation volume position
  - i. is at the center of the image if not specific
  - ii. “Image Points” come from selections in the FLIM image. Multiple points mean FCS measurements are taken following the displayed order
- e. It is a good practice to remove image points after a measurement as they are saved even after the VV software is closed
- f. Use Time series to collect multiple measurements in series (not linked to any image – aimed at the current position)



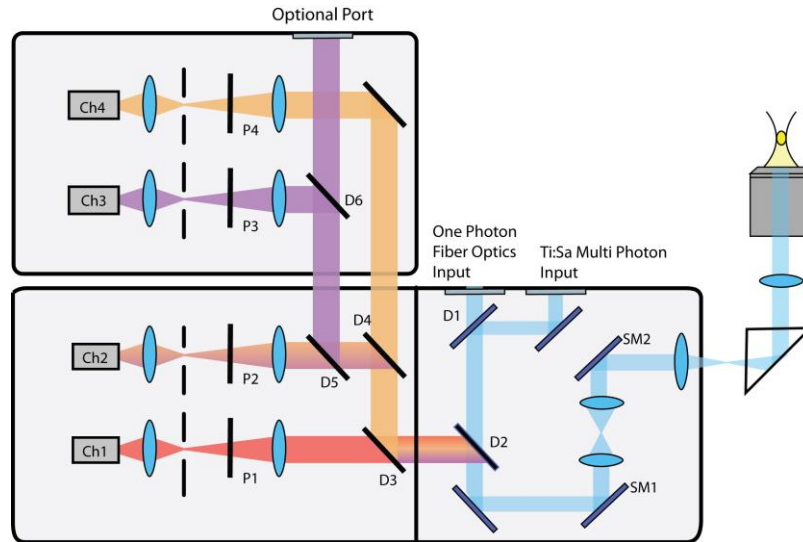
**Fig. 6:** FFS workspace

***Illumination/Detection optics selections (Fig.2):***

1. The system is optimized for dual color using either APDs or PMTs: same set of filters per type of detector. But it is also possible to record simultaneously 3 colors. Note that APDs are more sensitive (quantum efficiency) and even more so in the red spectrum whereas PMTs have a faster response (better for lifetime and high count) and are more sensitive in the blue-green spectrum.
2. Select the necessary dichroics:
  - a. change D2 if you either need 532nm or 561nm illumination line
  - b. change D3 to record on APDs using dichroics or on PMTs using the mirror
  - c. change D4 to record on PMTs using dichroics or on APDs using the mirror
  - d. Pos5 for D3 and D4 separate 630nm (Cy5) from other lines and Pos4 separates 405nm and 488nm (FITC) from the other lines
  - e. change D5 to record on Ch2 (APD) using glass or Ch3 (PMT) using the mirror
3. Select channels and corresponding filters to fit your need. Note that there is only one APD or PMT which can record Cy5 emission (from 630 excitation) and the other only greens or Cy3/TRITC emission (from 532/561 excitation)



4. Select the pinhole: use 50  $\mu\text{m}$  for typical confocal imaging/FCS, increase to 100 or 200 to get more count. 1000  $\mu\text{m}$  almost corresponds to an open aperture.



**Fig. 2:** Optical pathway

***Optimization of the detection unit:***

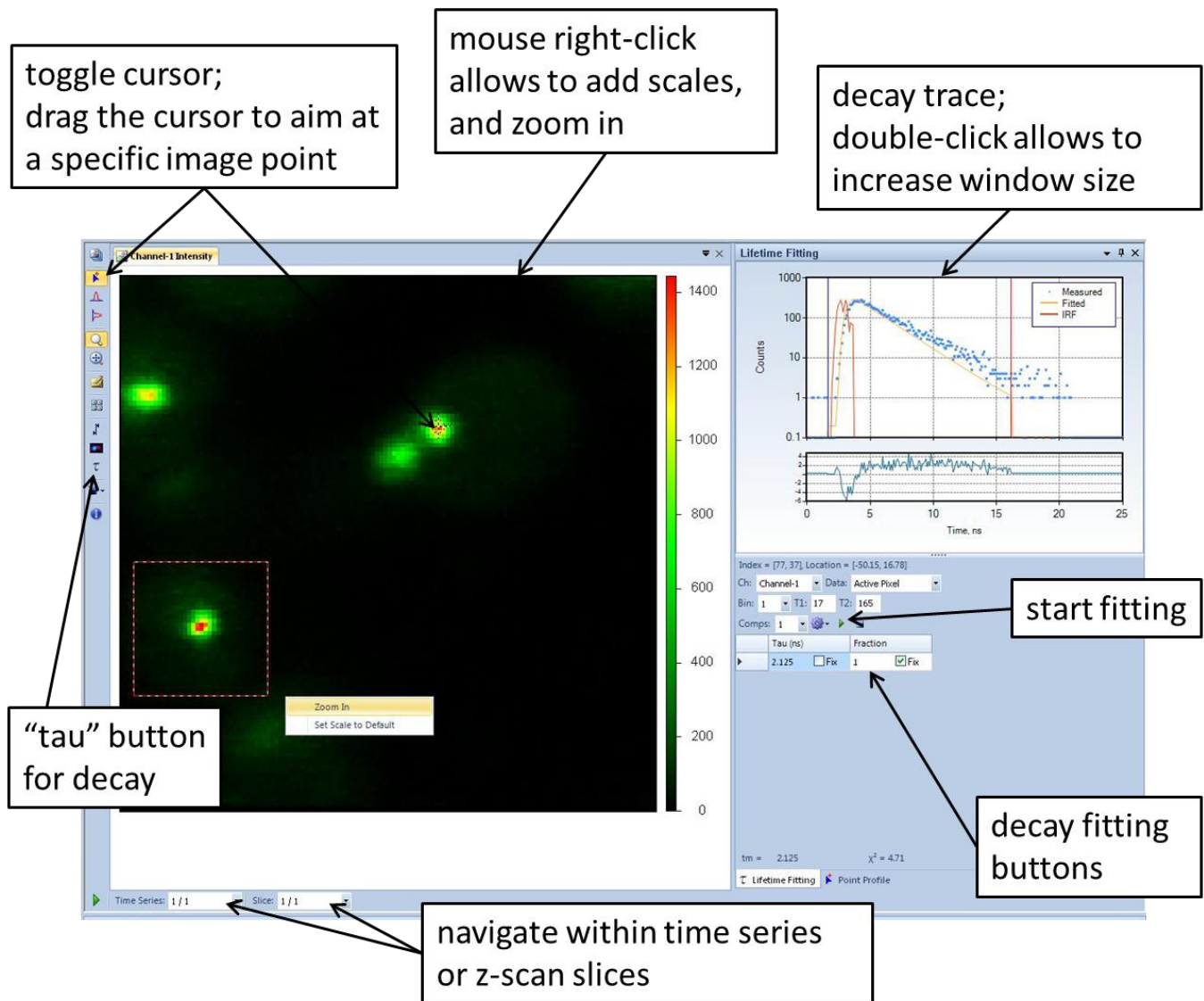
1. Maximize count for lifetime (FLIM) and additional standard calibration for fluctuation spectroscopy (FCS)
  - a. Add a drop of ddH<sub>2</sub>O on the objective
  - b. Place a coverslip on the stage (we use #1 thickness; adjust the objective ring if you are using another thickness; and put it back to its original position afterward please!)
  - c. Add a minimum of 20  $\mu\text{L}$  of dye solution
  - d. Place the sample cover (don't look at the sample while the laser is ON)
  - e. Switch off room lights – DETECTORS CAN BE DAMAGED BY ROOM LIGHT!
  - f. Select the detector of choice in VV
  - g. If using PMTs, increase Gain to 100 (don't forget to RESET it before shutting the system down!)
  - h. Switch live count on in VV
  - i. Increase laser input power from VV. Remark that all lines are actually off below a non-zero input power value (usually  $\sim 35\%$ ). Start with a low value if uncertain ( $\sim 50\%$ ).
  - j. Switch the microscope focus knob to 'coarse' (F/C button, it is on fine at start-up) and locate your sample: the count will jump once in the sample
    - i. If no count increase occurs, increase the input power progressively and keep focusing on the microscope
    - ii. If the count increases above 200,000 ph/s decrease the input power in VV
  - k. Switch the focusing to 'fine' on microscope and adjust sample location to stay within working distance of the objective (280 $\mu\text{m}$  for the 60XW). Note that the fine focus moves the sample 1 $\mu\text{m}$  per graduation.
  - l. Run 'Motor Alignment' in the 'Device Control' tab. Stay in the 'Pinhole & Lens' tab.
    - i. Start with a live count < 20,000 ph/s because it can drastically increase when aligned.
    - ii. Select the detector used (it already needs to be selected in the 'Data Acquisition' tab) and pinhole/lens.

- iii. Run the auto optimization at 16 steps for pinhole, then lens. Always use ‘binary’ for pinhole and ‘cross’ for lens.
  - iv. Run again if improvement occurred.
  - v. Try with 8 steps and repeat if improvement occurs.
2. Standard calibration for fluctuation spectroscopy (FCS, FCCS)
    - a. Need to complete maximizing the count for lifetime first (3.)
    - b. Use low concentration (1-100nM) of standard dye (known diffusion coefficient). Try to prepare a fresh sample. Vortex and/or sonicate if necessary to dissolve aggregates.
    - c. Take a 20s FFS measurement. Export the autocorrelation curve and import it in FCS workspace
    - d. Fit it after restricting the time window (e.g. from 2E-5s to end) and fixing the diffusion coefficient to the known value.  $w_0$  should be  $< 0.5$  and  $z_0/w_0$  should be  $< 10$
    - e. If it does not fit, try re-optimizing the detectors or using a freshly prepared or sonicated sample.

### ***Collect and Process TD-FLIM data:***

VistaVision requires sufficient free memory random access memory (RAM) to run properly. IT WILL CRASH IF TOO MANY WINDOWS (IMAGES, FCS DATA ETC) ARE OPENED IN THE SOFTWARE! So SAVE YOUR DATA as soon as you are done recording it!

1. Eyeball the sample focus using the coarse setting of the microscope (F/C button – on Fine at start-up)
2. Start ‘live count’ and adjust the focus using Fine. The focus should be at the highest count. Beware of reflections at the coverslip interfaces with water which should rise above standard noise
3. Select ‘measurement – TD-FLIM’
4. Select your choice of spatial and temporal resolution:
  - a. 512 x 512 pixels and -50 to 50  $\mu\text{m}$  for x and y ensures that you illuminated the whole area (useful for single molecules)
  - b. Keep the Z values from 0 to 0 for 2D imaging. If need to use the piezo-z to move, replace that value by how many microns away from where you currently are (absolute value)
  - c. 0.02ms is sufficient to have a quick look at an averagely bright sample
  - d. you can type in much higher exposure time values than available in the rolling menu (<10,000ms)
5. Select the ADC resolution: higher gives better lifetime decay resolution but takes longer to gather contrast in the picture. Typically, use  $\leq 16$  for simple imaging and  $\geq 250$  for lifetime decays.
6. Start the measurement. **Fig. 7** shows a sample FLIM measurement (*TD-FLIM workspace*).
7. Add axes scales by right-clicking  $\rightarrow$  plot  $\rightarrow$  axes
8. Zoom in by right-clicking  $\rightarrow$  zoom  $\rightarrow$  square selection. Use ‘apply to range’ button (Fig.5) to declare the new image range and take another picture. Adjust resolution as needed



**Fig. 7: FLIM measurement**

9. The right panel shows once the "tau" icon is selected.
10. Select a point using the cursor by toggling it first, and then drag it.
11. Decay data and fit are shown on the upper window, which can be magnified by un-pinning it.
12. You can drag the fitting boundaries on the graph itself. Select the binning you want ('1' means the sum of all photons collected over 1 pixel in each direction => 9 pixels total)
13. The instrument response function is built from the derivative of the rising edge of the decay of the 3 brightest pixels in the picture. Note that this fitting technique requires sufficient amount of photons and a relatively intensity-even image (to fit the whole image)
14. Save an image by right-clicking on it
15. To access the raw data, you can right-click on the decay image → properties → series → data
16. Alternatively, we can provide a Matlab code to select the data of a particular point and let you fit at your ease.



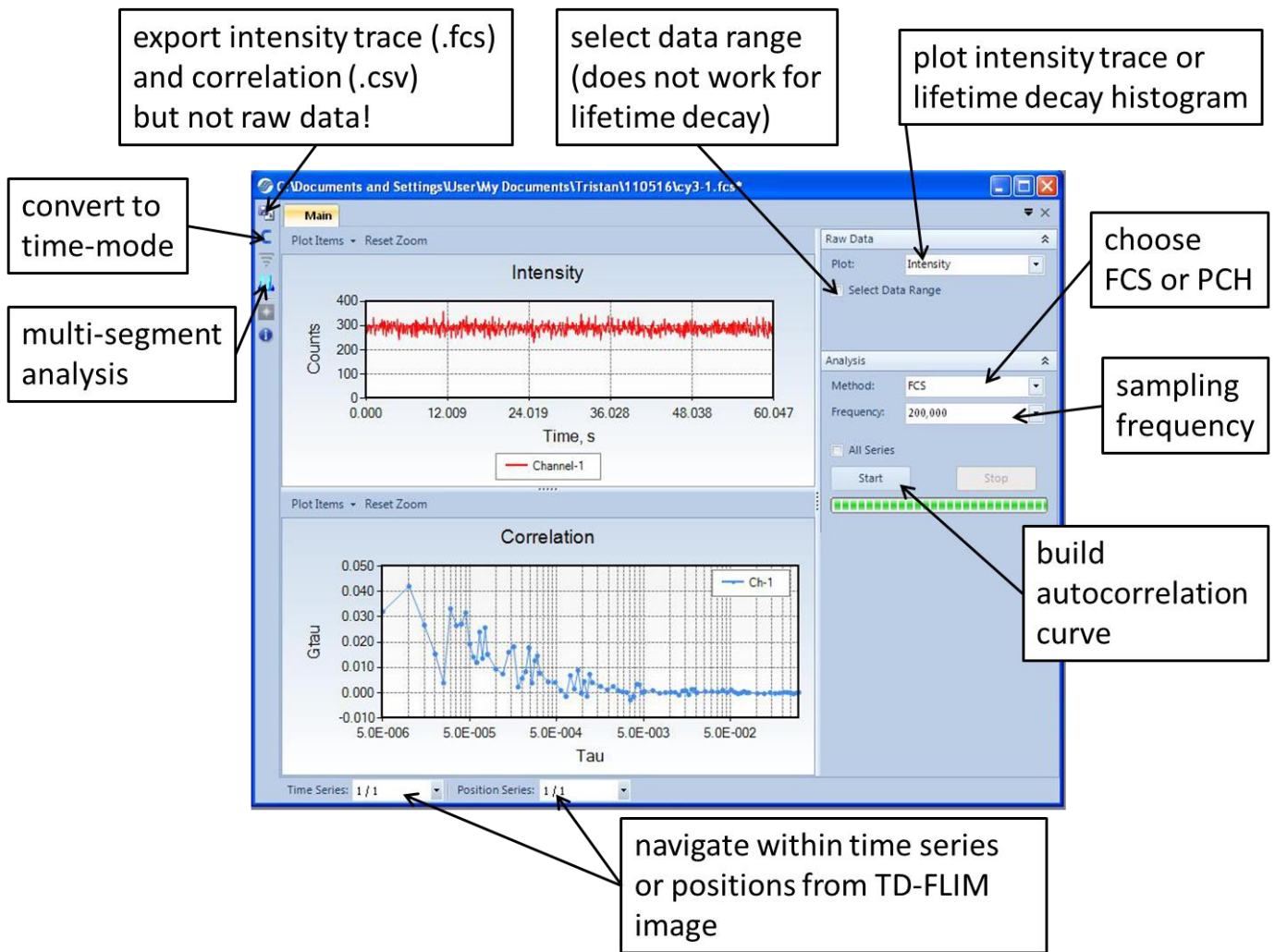
**Fig. 8:** Z profile and FFS options in FLIM

17. To see the z-profile at an image point, and know if we are properly focused, select 'acquire Z profile at cursor position' (**Fig.8**). Note that it will bring the interface to 'point-mode' and you will need to change back to 'image' if need (AND the Z-position!)
18. To take FCS measurements from a FLIM image, select 'acquire FFS at cursor position' or 'at multiple points' (**Fig.8**). For this last, select various points and click to select them. Select 'acquire FFS at multiple points' again and the software will start measurements in the same order. Note that you need to select all FFS parameters before-hand in the FFS workspace (exposure time etc.)

***Collect and Process FFS data:***

VistaVision requires sufficient free memory random access memory (RAM) to run properly. IT WILL CRASH IF TOO MANY WINDOWS (IMAGES, FCS DATA ETC) ARE OPENED IN THE SOFTWARE! So SAVE YOUR DATA as soon as you are done recording it!

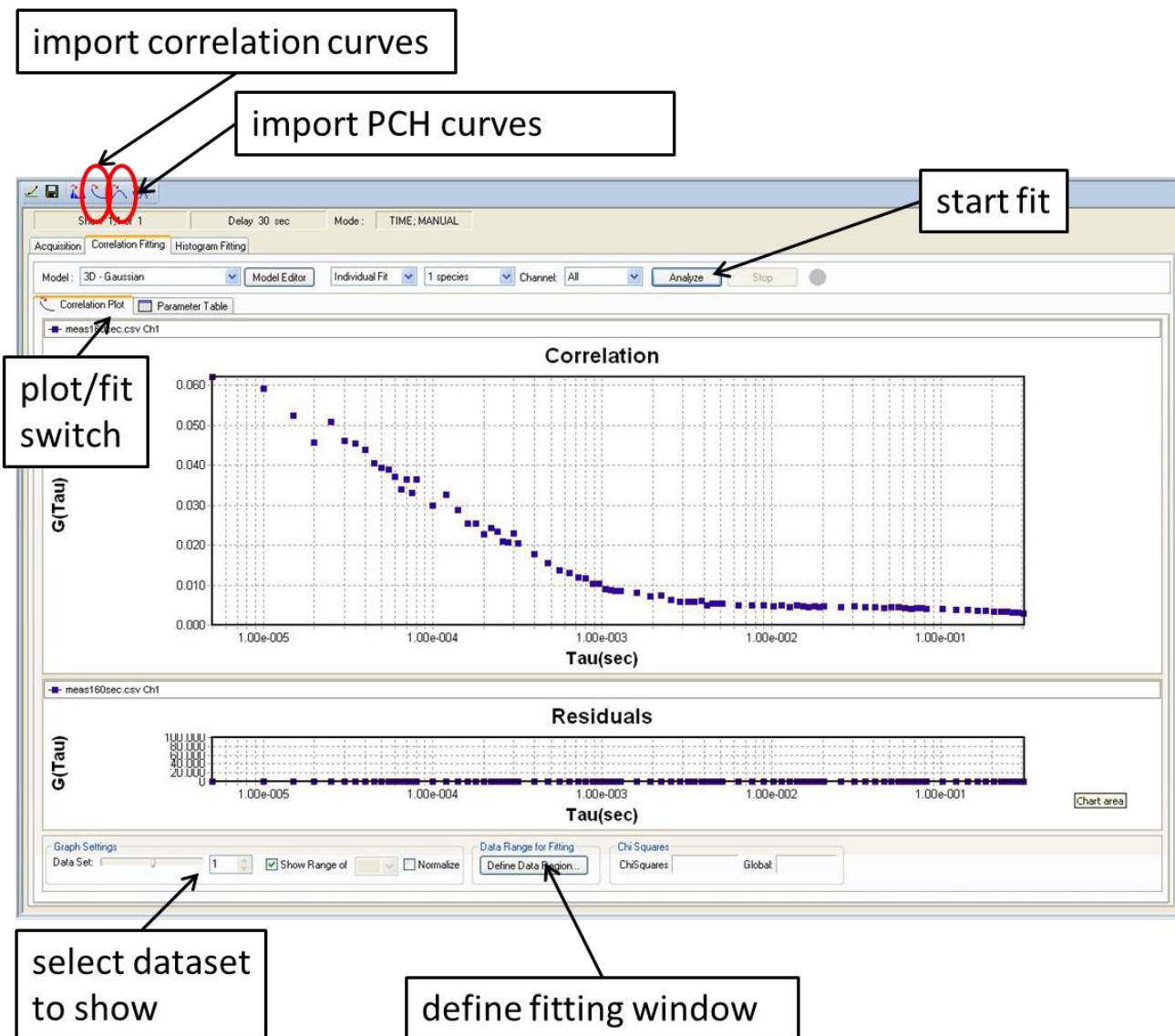
1. Data are recorded in FFS workspace but processing is done in FCS workspace. Note that the software saves data from both workspaces in .fcs formats. Make sure you rename files from one workspace to the other
2. Eyeball the sample focus using the coarse setting of the microscope (F/C button – on Fine at start-up)
3. Start 'live count' and adjust the focus using Fine. The focus should be at the highest count. Beware of reflections at the coverslip interfaces with water which should rise above standard noise
4. Select 'measurement – FFS'
5. Select your parameters:
  - a. time-tagged time-resolved that includes lifetime decay
  - b. sample frequency: 200kHz for most small to 5-10kD molecules (low viscosity)
  - c. exposure time: minimum 20 sec.
  - d. positions: make sure there are no unwanted locations from previous measurements
6. Start the measurement. **Fig. 9** shows a sample FCS measurement (*FFS workspace*). FCCS (cross-correlation) shows curves (intensity and correlation) from both detector channels as well as a 3<sup>rd</sup> correlation curve: the cross correlation



**Fig. 9:** FFS measurement

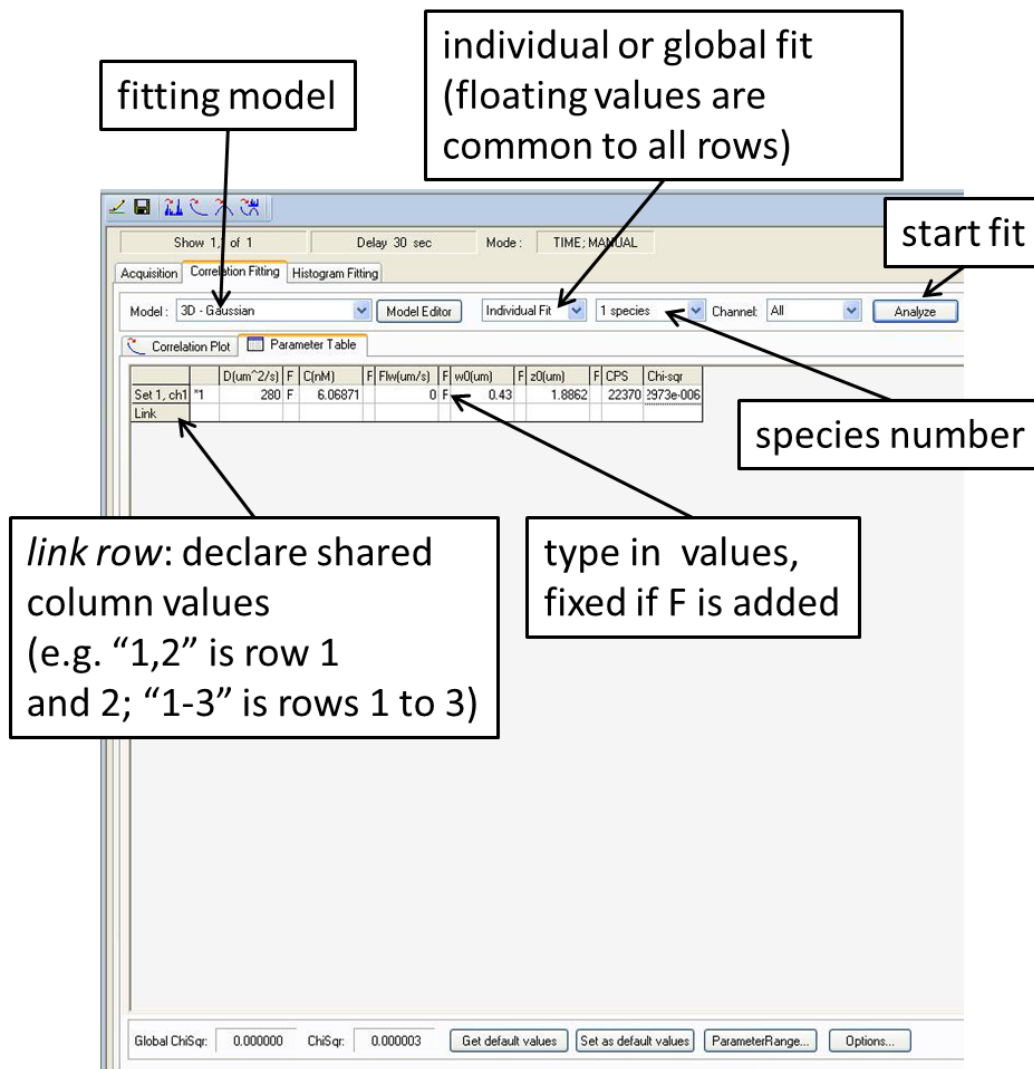
7. Save an image by right-clicking on it
8. Save the raw data from the 'save' in the main menu (main window)!!
9. Click on Start to build correlation curves (method - FCS) or histograms (method - PCH)
10. If you need to access time-dependent behaviors, use multi-segment analysis
11. Convert to time mode converts time-tagged time-resolved data to time-tagged.
12. Use the 'save' icon window to export data and correlation curves that can be imported and fitted in FCS workspace. Note that it saves exports in .fcs as well, so make sure to change the file name accordingly (e.g. "data-exp.fcs")
13. Alternatively, we can provide a Matlab code to access the raw data – works for multiple channels but not for multiple point positions yet.
14. From the main menu, open a FCS file. An 'open' window shows up. Click cancel. Click on the 'import icon shown in **Fig. 10**. Select the file(s) and import





**Fig. 10:** FCS workspace – correlation plot

15. Define the fitting window. To avoid triplet-state with most dyes, take 2-3E-5sec as lower boundary
16. Switch the view to the parameter table shown in **Fig. 11**.



**Fig. 11:** FCS workspace – parameter table

17. Choose fitting model (3D – Gaussian for calibration) and species number ('1' for calibration). Use the model editor if necessary
18. Set approximate values and fix known ones: D during calibration and w0 and z0 after that
19. Link curves (rows) to share same values between rows
20. Use 'individual fit' for each curve or 'global fit' to share floating fitting variables
21. Use the save icon in this window (**Figs 10 & 11**) to save the fitting results
22. Save correlation and fitting curve data from the main menu: export FCS data → correlation with fit

### ***System shutdown:***

Switch everything off in reverse order:

- a) Reset PMT in VV if used
- b) Make sure all shutters were closed properly (if VV crashes, it can leave some open)
- c) Set laser input power to 0 in VV
- d) Close VistaVision software
- e) Unselect lines in the AOTF Controller software and close
- f) Switch off piezo stage controller z-axis (A260) *TOP* and scanning mirrors driver (A403) *BOTTOM*
- g) Switch off the 3-axis DAC controller (A512) main switch *CENTER*
- h) Unplug the AOTF
- i) Switch off detectors and detection unit master
- j) Switch off laser front panel key
- k) Switch off the microscope controller
- l) Wait 10 min, then switch off the laser master switch (at the back)
- m) Back up your data on USB key or server

### ***Additional Information:***

1. Laser frequency change:

- a. Switch off the front panel key (**Fig.3.g**)
- b. Change frequency in VistaVision: 1, 2, 5, 10, 20, 40 or 80MHz
- c. Switch back on the front panel key
- d. NOTE: physical delay lines between photon arrival (APD/PMT) and sync (laser) have to be adjusted

2. AOTF controller software:

- a. Output power can be adjusted for each line. However 30% is the maximum value possible. Damage for the AOTF and potentially the laser can occur
- b. Also, due to the nature of the supercontinuum laser, power is function of the wavelength. Relatively, if **488nm** outputs 50 $\mu$ W (e.g. 30% at 20MHz), then **532nm** outputs \_\_ $\mu$ W ( **$\times 1.$**  more), **561nm** outputs 100 $\mu$ W ( **$\times 2$**  more) and **630nm** outputs 200 $\mu$ W ( **$\times 4$**  more). Adjust each used line accordingly – keeping the weakest at 30% maximum – to reach even spectral intensity. The **405nm** only outputs ~12 $\mu$ W and you can do so by combining 3 lines
- c. Optimal wavelengths (AOTF vs. dichroic bandwidth) are:
  - i. for 405nm: 405/408/411 (add the 3 lines)
  - ii. for 488nm: 488
  - iii. for 532nm: 532
  - iv. for 561nm: 557
  - v. for 630nm: 629