Title: Bumblebee FCS User Manual (Assumes proficiency in confocal mode)

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Office: CG07 or 05
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Download a PDF copy of manual:

\\cmvm.datastore.ed.ac.uk\igmm\Imaging-Users\Microscope User Manuals\Confocal\Bumblebee

Facility Usage Policy

1. **You must have the relevant Risk Assessment/COSHH form for the work you are undertaking before using imaging facility resources**
2. **Users must be trained before using facility equipment**
3. **Please leave the microscope clean and tidy for the next user**
4. **Please report any issue, even if it seems minor, to facility staff**
5. **Any clinical waste must be placed in the orange bins provided**

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Mounting the FCS sample holder

1. Ensure that the FCS stage insert is mounted either while the entire system is turned off or if it is already turned on, switch off the confocal scanner via the middle green switch. This will ensure there is no power to the z galvo which is advised when securing a sample holder to it.

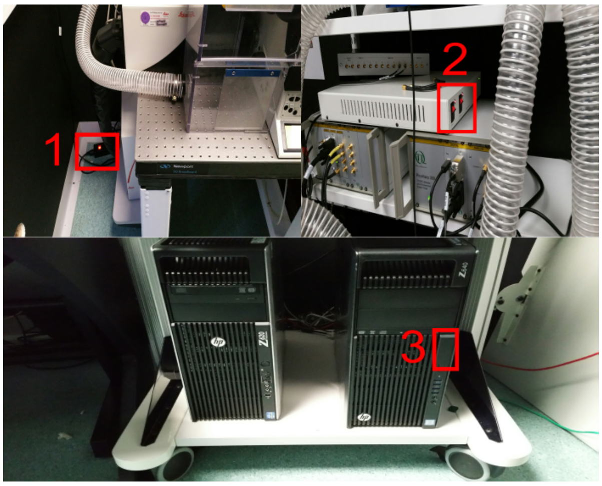
2. The sample holder for FCS is kept in the drawer, place your dish/slide in the holder first before sitting the holder into the Z Galvo stage. Do not apply any downward pressure to the z galvo insert. Secure it using the screws provided. Ask a member of facility staff if you are unsure.

Switching on the system for FCS use

Turn on the incubation chamber heater, ideally 3hrs prior to experiment start to ensure focus drift is minimal

Turn on the confocal hardware following the standard procedure

WARNING APD'S ARE EXTREMELY SENSITIVE AND CAN BE DAMAGED BY BRIGHT ROOM LIGHT OR TOO INTENSE LASER ILLUMINATION, ENSURE THE ROOM AND INCUBATION CHAMBER LIGHTS ARE OFF BEFORE PROCEEDING TO THE NEXT STEP. IF YOU HEAR AN AUDIBLE WARNING SOUND TURN OFF ANY SOURCES OF LIGHT AND WAIT FOR THE APD'S TO RESET THEMSELVES (SEVERAL SECONDS).



1. Power to the PicoQuant FCS hardware is via the red switch on the extension cord on the floor (left side of the microscope), see image below. You will hear an audible sound when its turned on, this is normal

2. The two APD detectors used for FCS have independent switches found on the APD detector unit on the shelf behind the confocal, If you are only doing FCS with one wavelength & are not doing FCCS then you only need to turn on one of the detectors

3. Turn on the FCS analysis PC

1. Log on to the confocal PC using your Windows account

2. Go to Input detector on the Bumblebee PC and click this. This activates Master + Slave control of the Bumblebee (master) and FCS (Slave) PC. So you can move from one to another.

3. Log on to the FCS analysis PC as "FCS User"

4. Start the Leica LAS-AF software

5. At the first pop-up select the “Configuration” text then select the “c_FCS” option

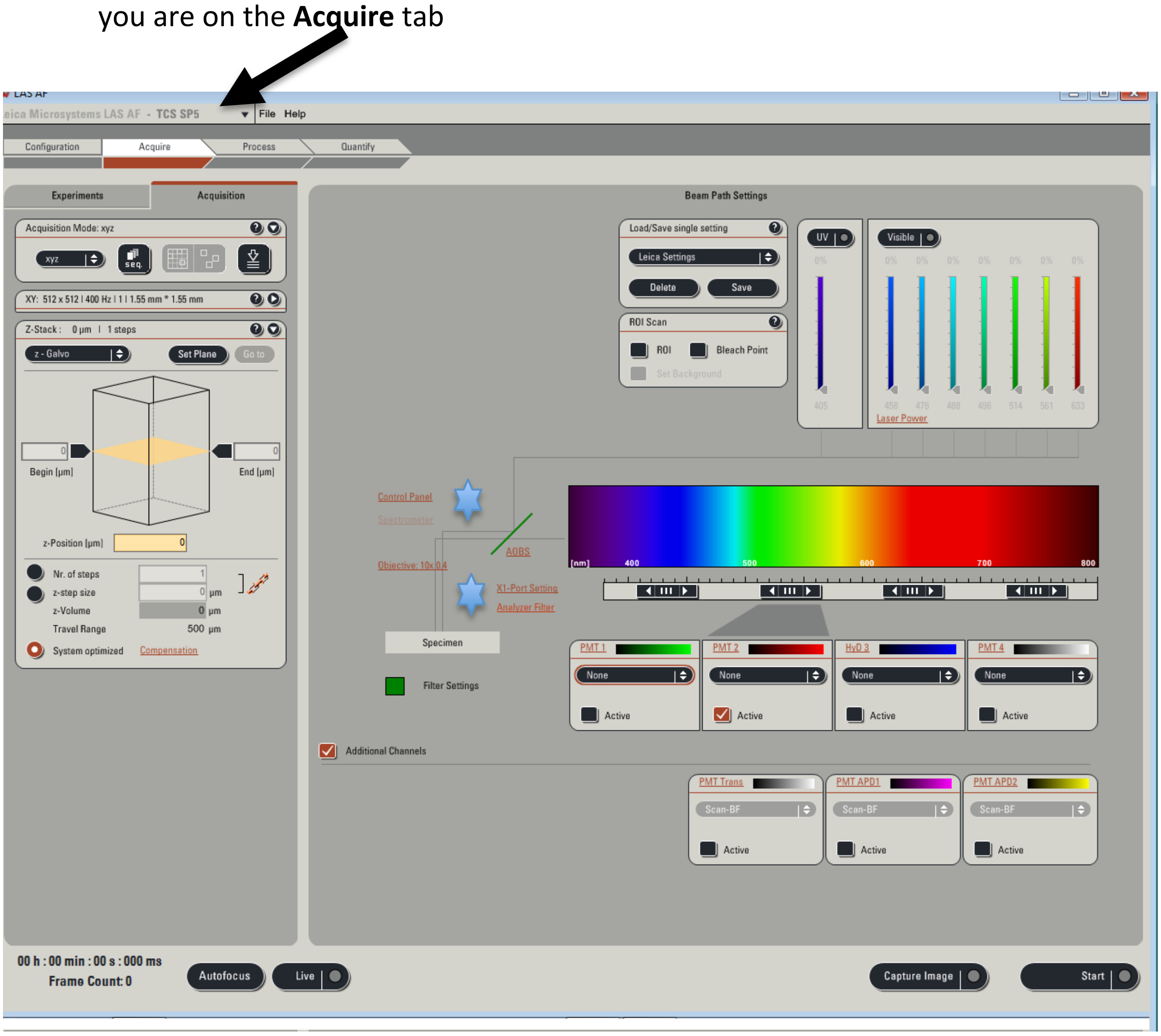
6. Select yes to the stage initialisation

7. On the FCS analysis PC start the Symphotime 64 software from the desktop shortcut icon

8. Rotate the collar on the lens so that the dot is half way between the 0.16-0.18 marks (~170µm, thickness of the cover glass)

9. In LAS-AF switch on the lasers you require from the **Configure** menu. Generally this will be the Argon and 561 laser. The argon should be set at 30 – 50% power

10. Enter the **SMD FCS Wizard** by clicking on the top left corner of the menu bar when you are on the **Acquire** tab



Acquisition Mode: xyz

XY: 512 x 512 | 400 Hz | 1 | 1.55 mm * 1.55 mm

Z-Stack : 0 µm | 1 steps

z-Position [µm] 0

Nr. of steps 1

z-step size 0 µm

z-Volume 0 µm

Travel Range 500 µm

Objective: 10x 0.4

00 h : 00 min : 00 s : 000 ms

Frame Count: 0

This indicates where the controls relevant to FCS are.

- X1 port sends the light to the APD detectors.
- Analyser Filter determines which filters sit infront of the two APDs for FCCS

Locate sample and correct collar

Water immersion lenses have correction collars that provide correction for spherical aberration when imaging at different depths of a sample and/or to adjust for different thickness cover glasses. A change in imaging depth will lead to a change in shape/size of the confocal volume. Ensuring the collar is in the right position for your FCS sample chamber is essential. It should be checked each time you use the system or if the environmental temperature varies significantly

1. Ensure the sample holder is secured to the z galvo stage using the grub screws provided (ask a facility staff member to do this)

2. Place a drop of distilled water onto the 40x or 63x water immersion lens using a pastette

3. This can be done either with the sample or with a 1nm solution of AlexaFluor 488 dye on a glass bottom dish or as a drop on a glass coverslip or in a well of a Greiner / Labtek2 chamber. (See technical notes)

4. Raise the lens until the water contacts the cover glass

5. You may be able to focus on the cover glass looking down the microscope but its not strictly necessary for the dye solution. It may help for a biological sample.

6. In LAS AF, start the FCS Wizard from the top menu if it isn't open already

7. Select the **Set Corr-ring** tab at the bottom of the screen. This automatically configures the necessary acquisition settings and sets the AOBS to Reflection mode

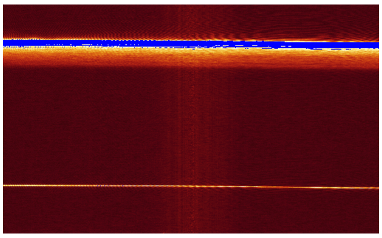
8. Check that the **X1 Port** is set to "**Mirror**"

9. Set the **QuickLUT** to Glow over/under

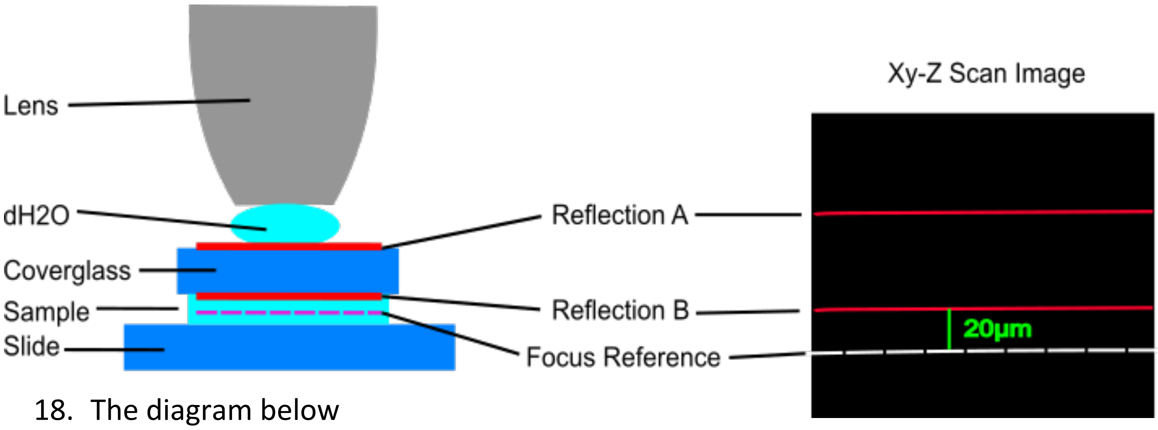
10. Press the **AutoContrast** button on the left side of the image window, the icon will change from "M" to "A" so that even a low intensity reflection can be seen.
11. Check the scanning is in XZY mode
12. Scan speed 600MHZ and bidirectional
13. Check PMT is active and set to about 1000 Gain
14. Ensure that the imaging mode is set to reflection
15. Check there is No notch filter in place

☑ Auto Select

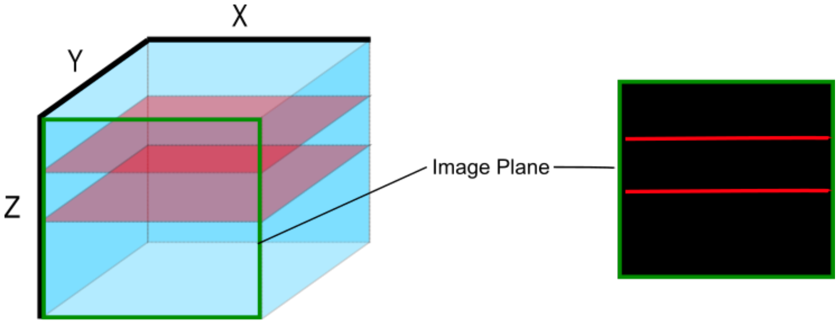
16. Press **Live**, then focus by moving the lens (not the Z Galvo) upwards slowly until you see two horizontal lines on the screen. The upper line is the reflection of the laser from the water cover glass interface, the lower line is the reflection from the cover glass and specimen. See image of the reflections below:



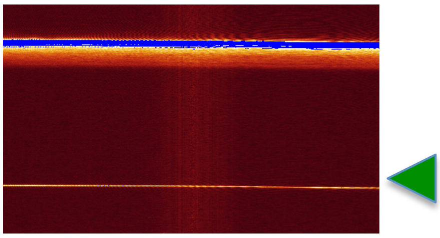
17. The diagram below is to help explain the orientation of the image on the screen which is inverted relative to how the sample is mounted, therefore the diagram shows the optics/sample inverted for easy interpretation of the lines:
Reflection A---Immersion water/Coverglass
Reflection B---Coverglass/Specimen
Focus Reference---Denotes the z plane where FCS measurements in solutions could be measured from, i.e. a point below the lower reflection line is within the specimen solution, set to around 20µm



18. The diagram below illustrates how to interpret the XZ-Y image. Instead of the image showing the XY face, you're seeing the ZX face and the z galvo is moving up/down very rapidly to generate the XZ scans throughout a 500um travel range

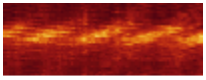


19. The lower reflection line (Reflection B) only is used to optimize the position of the correction collar so change the microscope focus (using lens not z-galvo) while live so it is in the middle of the xz image

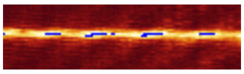


20. The system autozooms to make line easier to see. While scanning Live in xz mode rotate the correction collar on the lens in either direction until you achieve the thinnest line with highest brightness & contrast, no secondary lines should be visible

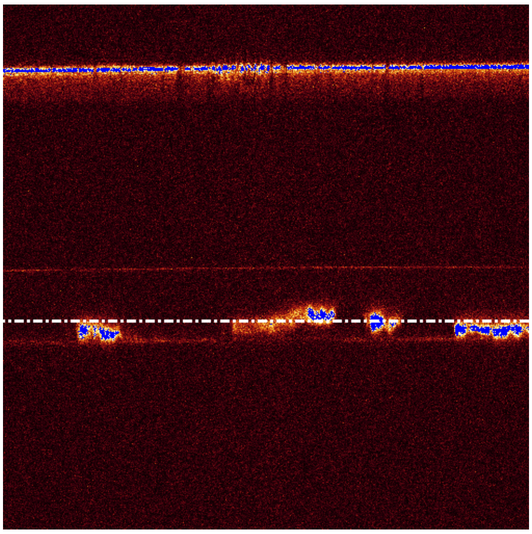
Incorrect collar position



Correct collar position



21. When you have found this point the calibration is complete
    a. If you can't see Reflection B focus the correction color on reflection A as described. Go back to XYZ scanning and with the galvo stage move 200nm into the sample.



22. Select the **Setup Imaging** tab and you'll notice a white dashed line on the frozen xz image, this line can be clicked and dragged up and down the image enabling you to choose a Z plane for the standard xyz scan

23. Move the white dashed line on the frozen xz live image to a plane where you can see the sample, if you are imaging a dye solution position the line at least 20um below the lower line

Capturing a confocal reference image

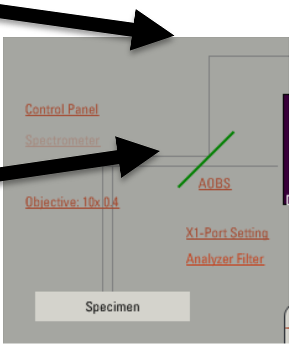
1. Place the sample into the z galvo insert, you may need to slide open/close the mounting brackets depending on the length of the coverglass to support it

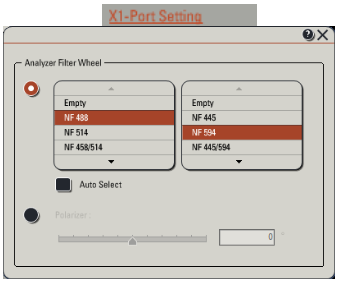
2. Raise the lens until the water contacts the underside of the coverglass. Focusing using the eyepieces is difficult for a homogenous dye solution so switch back to LAS-AF

3. At the bottom of the wizard window are a series of tabs, select **Setup Imaging**, by default you should have the **Workflow** tap selected in the left hand column

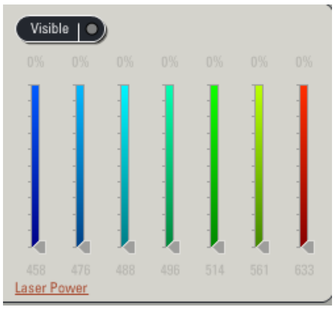
4. Ensure the X1 port is set to "**Mirror**"

5. Check the Analyser Filter is set to NF 488 and NF 594 to enable GFP and RFP collection

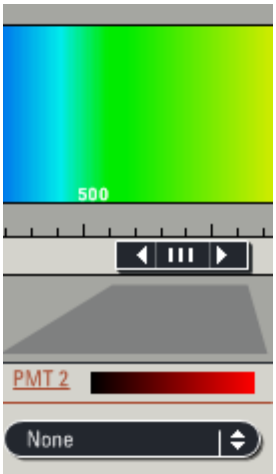




6. The acquisition settings will be set to default values for relatively fast confocal imaging but you may need to alter the gain, laser power etc



7. For a better comparison of the confocal image with the FCS measurement data you can set the spectral detection window for the PMT's to match the emission filters in the X1 port (I.e. GFP--500-530 or RFP--607-683). Note that you can capture the reference image with any of the available laser lines but the FCS measurements are limited to the emission bands stated above



8. Press **Live** to focus and select an area of interest that you will ultimately acquire FCS measurements from

9. Press **Capture** to acquire your 2D xy scan at the Z plane of interest. Do not change any acquisition settings from this point onwards

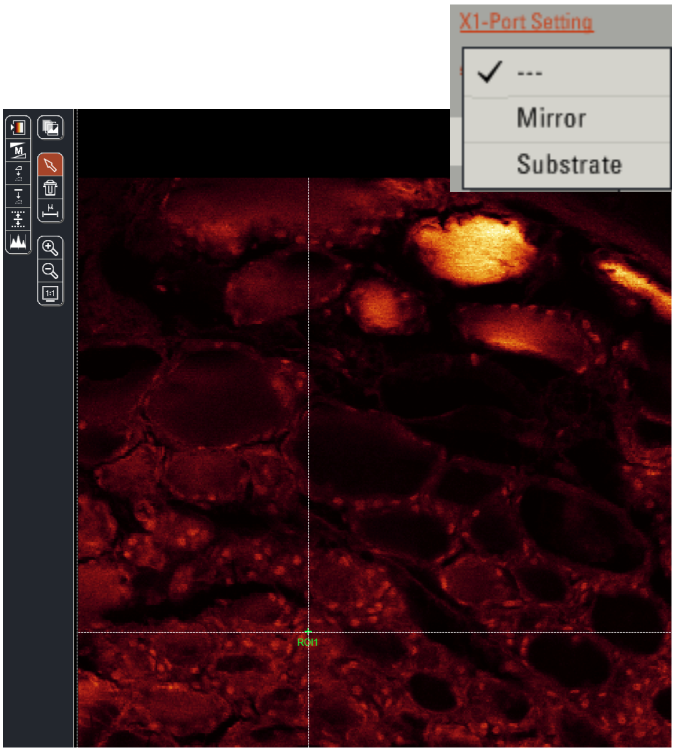
Acquiring FCS Measurements

1. Ensure that the room and incubation chamber lights are off and that the laser isn't scanning

2. Select the **Setup FCS** tab at the bottom of the wizard window

3. Check that the **X1 port** has been set to the **"---"** position, this should happen automatically when selecting the Setup FCS tab. Emitted light will now be directed to the APD detectors (NO ROOM or CHAMBER LIGHTS SHOULD BE ON)

4. Move the crosshair ROI1 on the image to a desired location to acquire FCS measurements

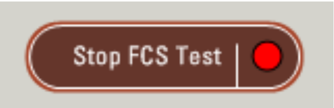


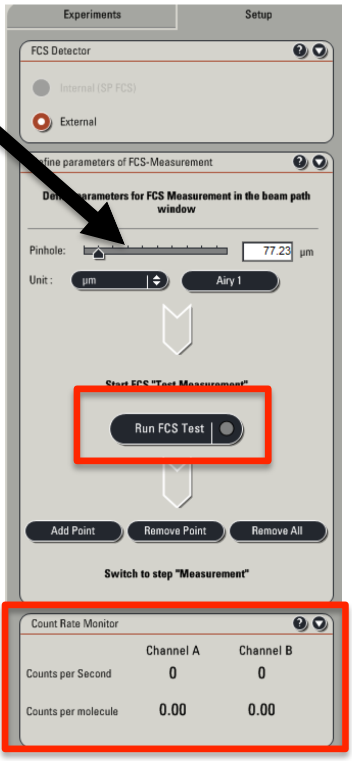
5. Set the **Pinhole** to desired size. Airy 1 recommended. Needs to be kept consistent in all experiments

6. Press **Run FCS Test** to start the illumination at the ROI point

7. You will see on the **Count Rate Monitor** both the Counts per second and Counts per molecule readouts. Adjust the laser intensity until you attain the desired figures

8. Press Stop FCS Test when you have finished optimizing the signal





9. The count rate per molecule read out is calculated from the correlation curve that is generated while the Run FCS Test button is active. The higher the value, the better the signal to noise ratio of the correlation curve. Choose the minimal laser intensity which achieves the maximum count rate per molecule
    a. Tips – Check the laser is actually on and that the correct notch filters are set

10. If you observe photobleaching or fluorophore saturation this will affect the shape of the autocorrelation curve which you will learn how to identify. In such cases continue to reduce the laser power

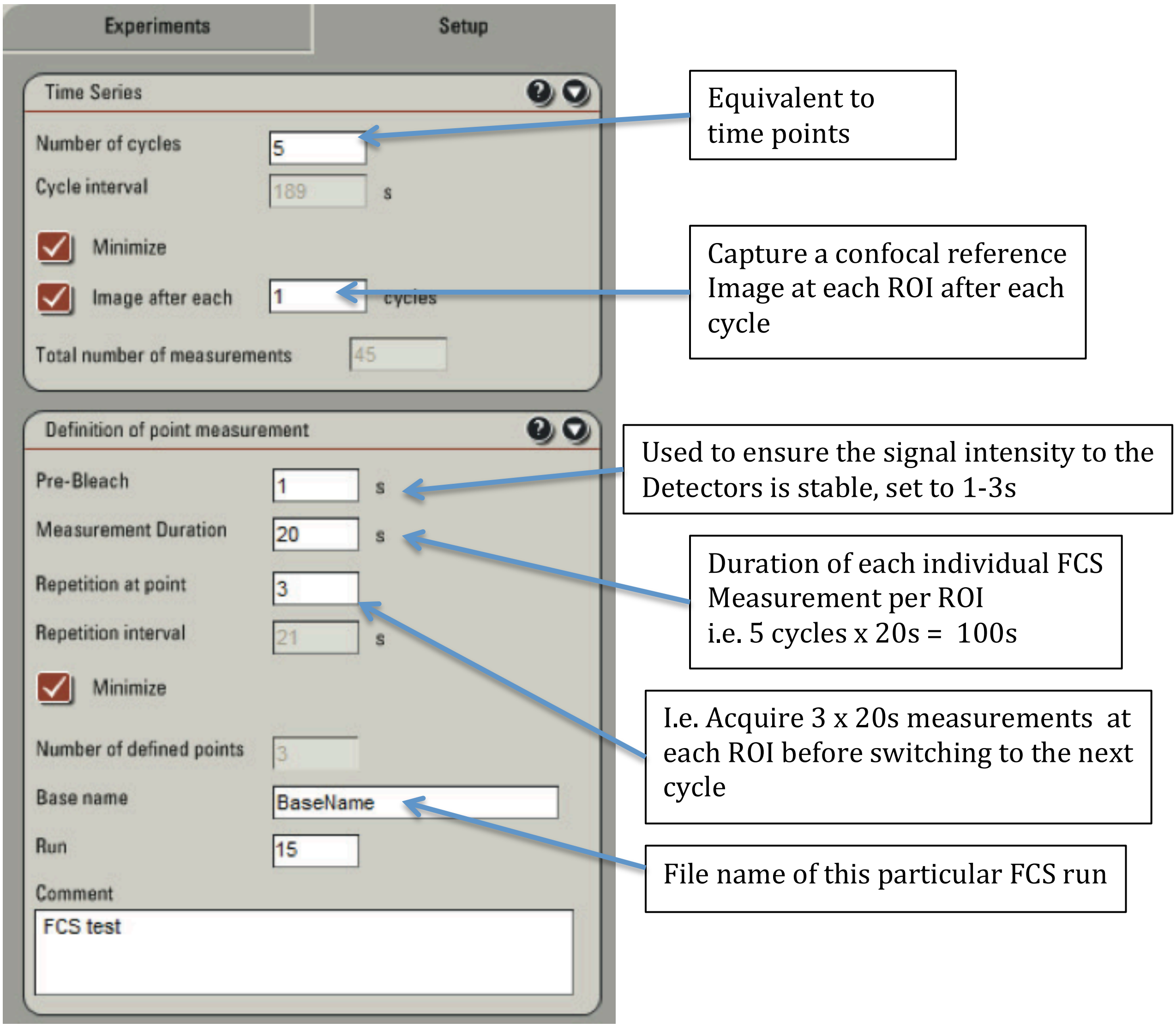
11. Select the Measurement tab at the bottom of the wizard window

12. If you would prefer to add multiple FCS measurement ROI's and carry out a time lapse you can do this here

13. Move the crosshair to a point of interest as before then press the **Add Point** button, repeat this process for further points

1: frame 1, z 0 µm
1: frame 1, z 0 µm
2: frame 1, z 0 µm
3: frame 1, z 0 µm

14. The image below describes the options in the FCS Measurement menu



Number of cycles: 5
Cycle interval: 189 s
☑ Minimize
☑ Image after each: 1 cycles
Total number of measurements: 45

Pre-Bleach: 1 s
Measurement Duration: 20 s
Repetition at point: 3
Repetition interval: 21 s
☑ Minimize
Number of defined points: 3
Base name: BaseName
Run: 15
Comment: FCS test

Equivalent to time points

Capture a confocal reference Image at each ROI after each cycle

Used to ensure the signal intensity to the Detectors is stable, set to 1-3s

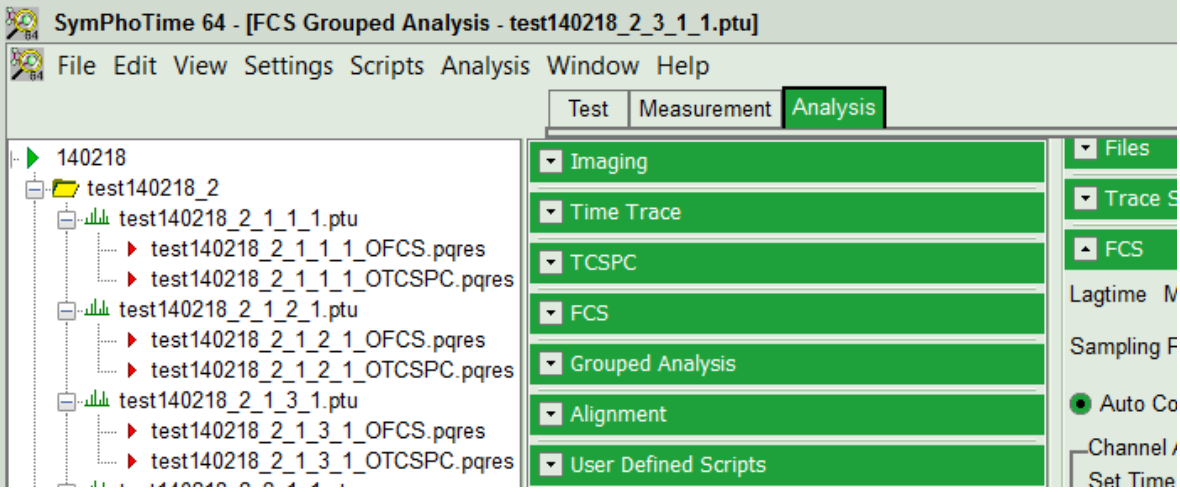
Duration of each individual FCS Measurement per ROI i.e. 5 cycles x 20s = 100s

I.e. Acquire 3 x 20s measurements at each ROI before switching to the next cycle

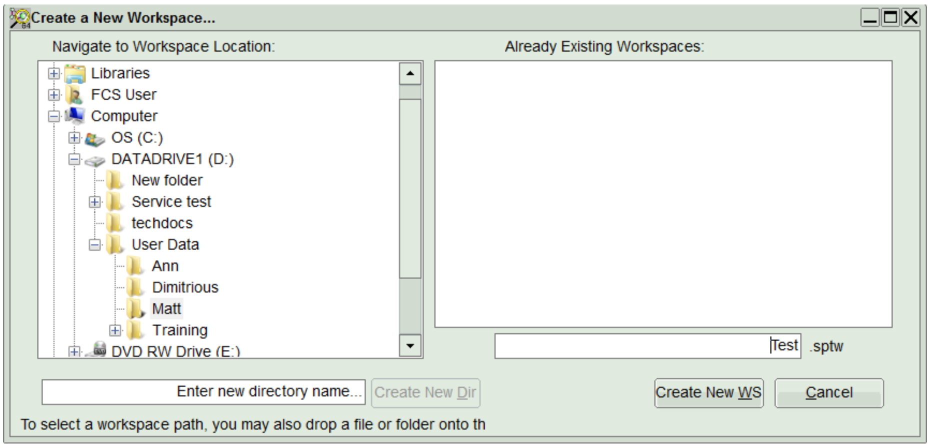
File name of this particular FCS run

15. **In Symphotime:** Select File>New Workspace. Please save your data to the local data drive, DATADRIVE1 (D:)>User Data>Your Folder and then move the data to a network share at the end of the imaging session.

16. It is not recommended to save to a network share as there are some inconsistencies in how Symphotime can read and store data and there is a risk of data loss.

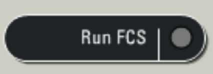


17. Enter a name for the workspace then press Create New WS



18. This is saved on the Symphotime PC not the Bumblebee PC. Eunsure the naming of the experiment on the Bumblebee PC is the same as on the FCS PC. Ideally data should be transferred into the same Datastore location asap so the images and FCS quantification stay in the same place.

19. Press **Run FCS** to acquire the measurements

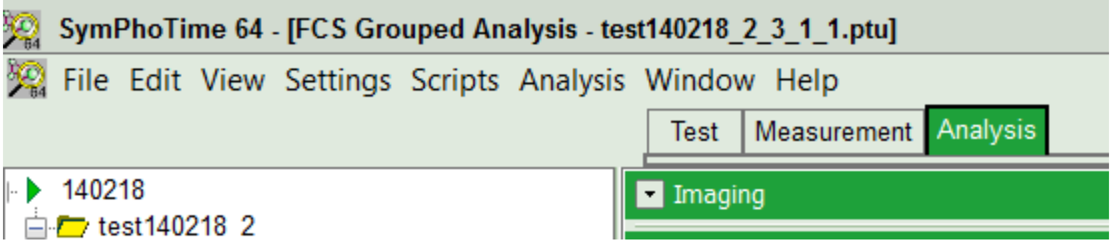


20. The confocal PC is directly connected to the PicoQuant analysis PC meaning when an FCS run starts in LAS-AF a file with the same base name is generated in the Symphotime software so long as a Workspace has been created/opened.

Fitting FCS Curves using Symphotime

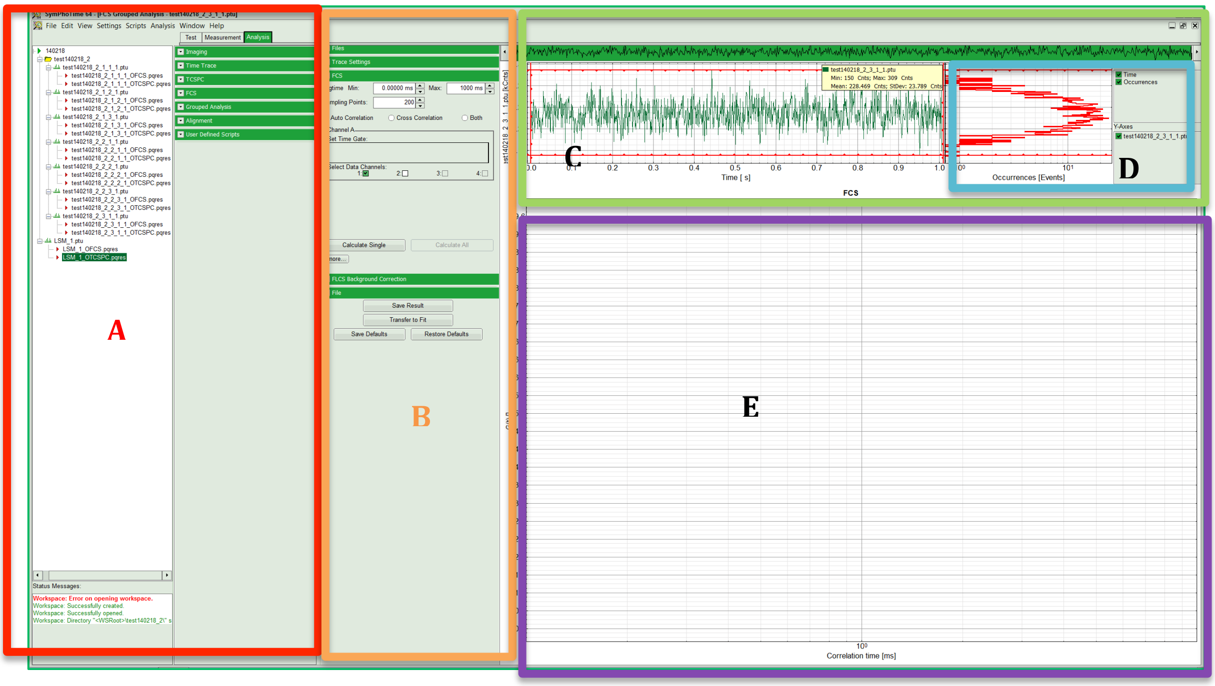
1. Open the Workspace or click on the relevant ptu file you want to analyse

2. Select the **Analysis** tab then Expand the green **FCS** dropdown menu

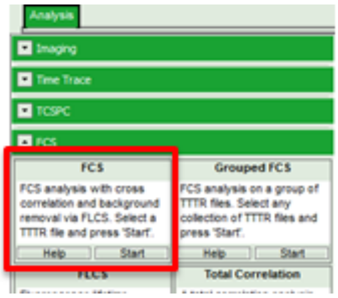


The workspace comprises:

a. **Data area**
b. **Analysis control pad**
c. **Intensity vs time trace**- The thin top trace shows the intensity across the entire measurement time. The lower larger trace can be used to display a subset of the total trace as highlighted by the green boxed area. You can change which segment of the whole trace is displayed using the left and right pointing arrows
d. **Photon counting histogram** - Displays the frequencies of the different intensity values. This can be used to check the signal is stable during the measurement period, a prerequisite for FCS. The histogram will normally represent a poisson distribution. Large intensity spikes here can reveal aggregation of the fluorescent sample.
e. **FCS Trace window**



3. In the Analysis tab select the FCS box & press the **Start** button.



FCS analysis with cross correlation and background removal via FLCS. Select a TTTR file and press 'Start'.

FCS analysis on a group of TTTR files. Select any collection of TTTR files and press 'Start'.

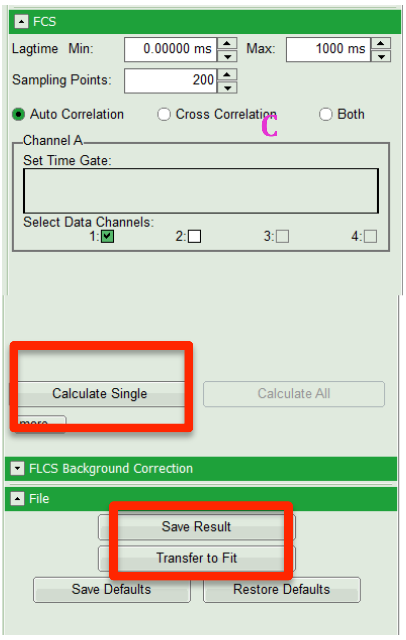
4. In the FCS Analysis window which appears select Autocorrelation and check and ensure that Data Channel 1 is selected.

5. Press **Calculate Single**

6. The resultant autocorrelation curve will be shown in the FCS trace window

7. The numerical result can be appended to the FCS raw data by pressing **Save Result.**

8. The data can then be moved onto the fitting step by pressing **Transfer to Fit.**



Lagtime Min: 0.00000 ms Max: 1000 ms

Sampling Points: 200

● Auto Correlation ○ Cross Correlation ○ Both

Channel A

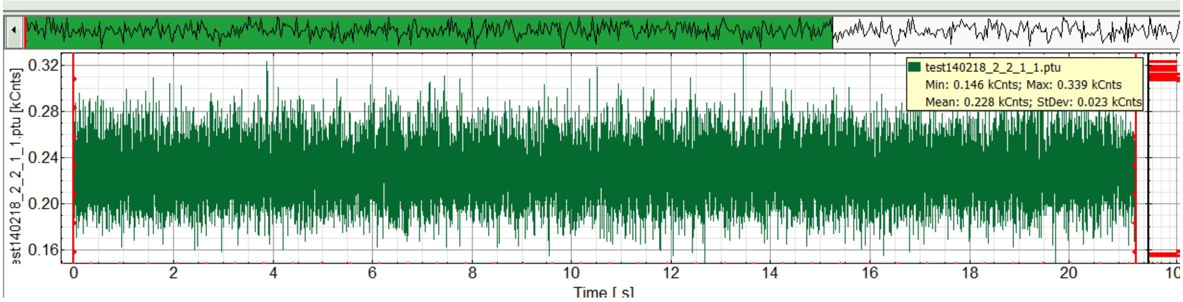
Select Data Channels: 1:☑ 2:☐ 3:☐ 4:☐

Artefacts in autocorrelation analysis

9. Artefacts are caused by afterpulsing and dyes being promoted into the triplet state.

10. You can use a region of interest to exclude the afterpulse section of the curve from the fitting process. Simply select on the fluctuation plot (Counts vs Time) Which section to fit. The green area highlighted will be fitted.

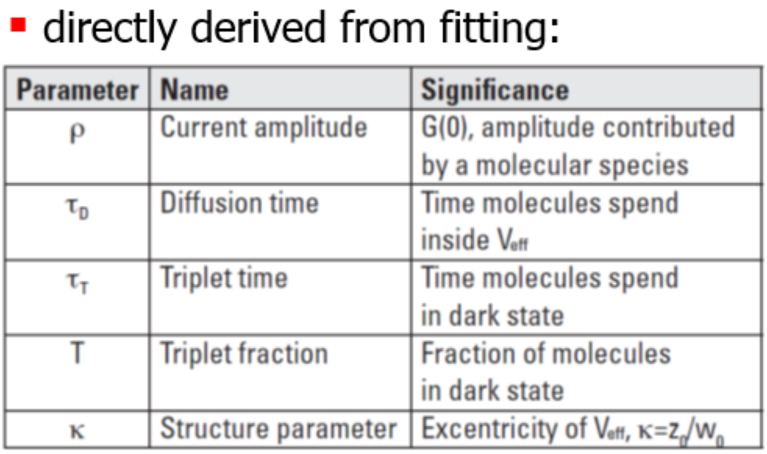
11. When selected only the data required can be transferred to fit. Or in this case 'Recalculated'



12. The Fitted curve can also be rescaled.
Please see the technical notes for a more full discussion of this phenomena

Fitting Data

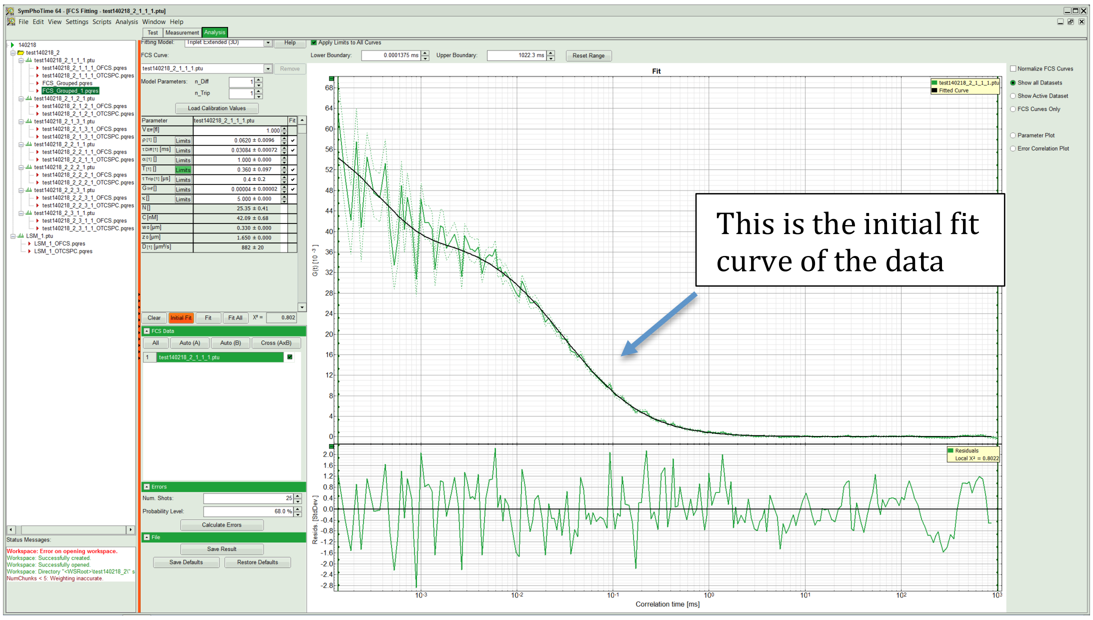
To obtain useful metrics from the FCS data the curves need to be fitted. This enables calculation or extrapolation of time constants, rate constants and other Michaelis Menten parameters. Symphotime has some fitting models already stored which can be used. It is also possible to export the data to other software to be fit with more complex equations. It is possible to either measure or assume the effective volume (Veff), concentration ©, beam waist (w_0) and beam height (Z_0)



- directly derived from fitting:

Parameter	Name	Significance
ρ	Current amplitude	G(0), amplitude contributed by a molecular species
τ_D	Diffusion time	Time molecules spend inside V_{eff}
τ_T	Triplet time	Time molecules spend in dark state
T	Triplet fraction	Fraction of molecules in dark state
κ	Structure parameter	Excentricity of V_{eff}, $\kappa = z_0/w_0$

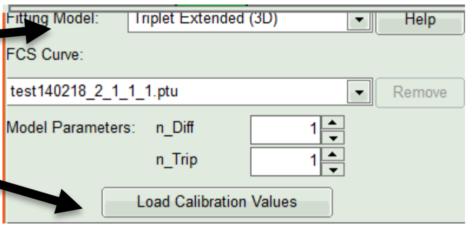
Once the data has been transferred to fit the fitting window appears. Its possible to either use assumed values for the effective values or load values obtained when calibrating the system. See technical notes for calibration for this.



This is the initial fit curve of the data

The fitting model is chosen from a list of 6 models. Triplet extended 3D is recommended as a start point for Biological experiments.

If the system has been calibrated load values here:



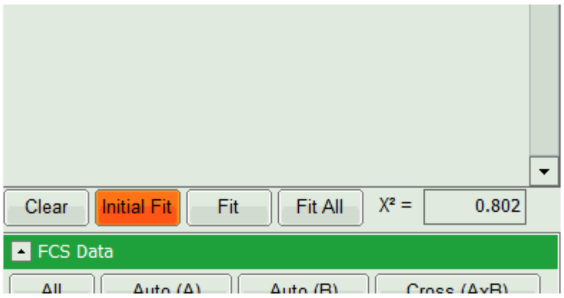
Fitting Model: Triplet Extended (3D)

FCS Curve: test140218_2_1_1_1.ptu

Model Parameters: n_Diff 1

n_Trip 1

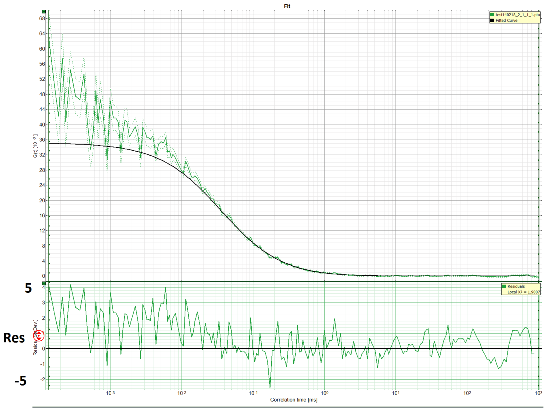
To fit the data.



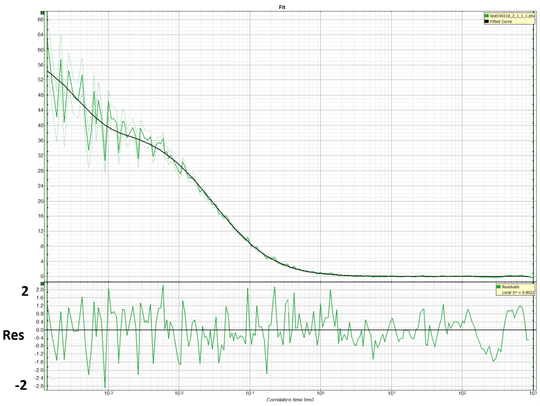
- Select the dataset to be fitted from left hand side menu
- Select the fitting model
- Press **Initial fit**
- Look at how well the curve fits the data and the residuals
- Determine if the fitting model is acceptable. If not change it and try doing an initial fit with a different model

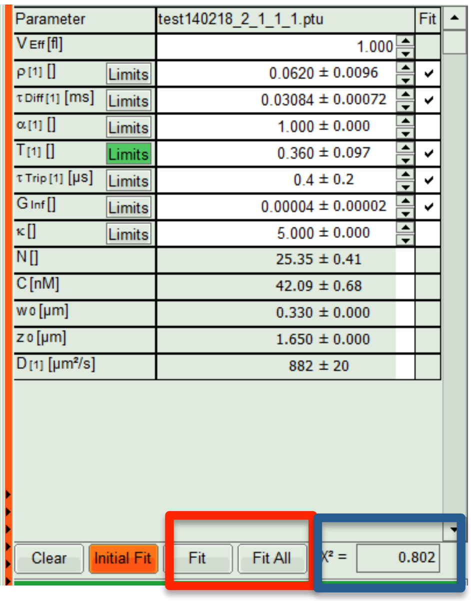
To determine whether the fit is good the curve fitting needs to be compared with the data. The results are fairly obvious and study of the residuals should give an indication of goodness of fit. (Res = Residuals)

Poor Fit



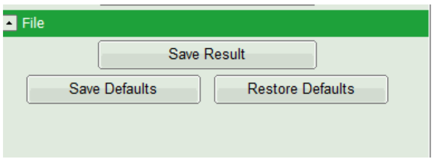
Good fit





Parameter		test140218_2_1_1_1.ptu	Fit
V_{Eff} [fl]		1.000	
ρ [1] []	Limits	0.0620 ± 0.0096	✓
$τ_{Diff}$ [1] [ms]	Limits	0.03084 ± 0.00072	✓
α [1] []	Limits	1.000 ± 0.000	
T [1] []	Limits	0.360 ± 0.097	✓
$τ_{Trip}$ [1] [μs]	Limits	0.4 ± 0.2	✓
G_{Inf} []	Limits	0.00004 ± 0.00002	✓
κ []	Limits	5.000 ± 0.000	
N []		25.35 ± 0.41	
C [nM]		42.09 ± 0.68	
w_0 [μm]		0.330 ± 0.000	
z_0 [μm]		1.650 ± 0.000	
D [1] [μm²/s]		882 ± 20	

- Once the fitting model has been determined the data can be fitted. If a time series has been carried out all of the data can be fitted. Using either **Fit** or **Fit All** as appropriate.
- The parameters which are kept constrained are checked in the box
- A Chi Squared value is given to the fitting which is indicated in the bottom right (blue box)
- These results can then be saved to file although the result can be only read in symphotime.



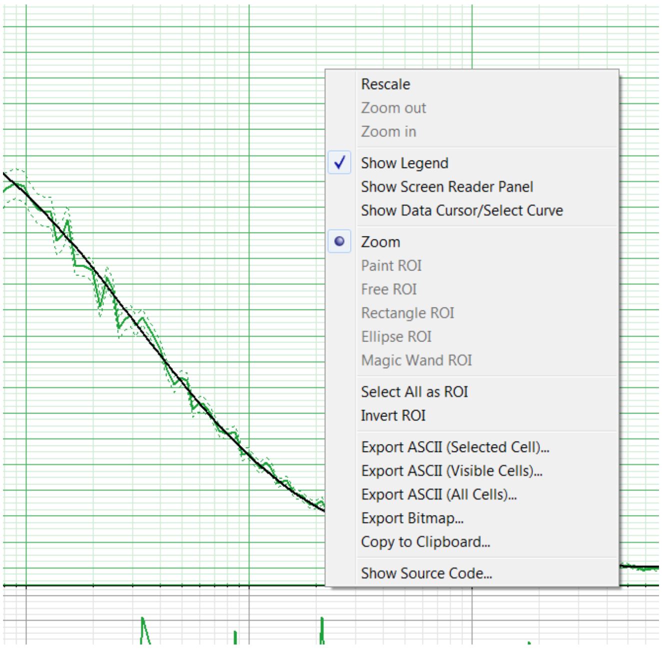
Data can be exported from Symphotime as follows (see next page)

Numerical data can be exported into ASCII file format which can be imported into Excel, Origin, Prism or Graphpad for fitting and modelling.

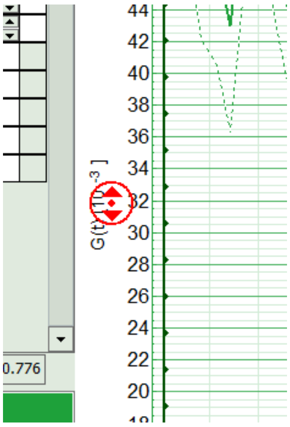
To do this right click in the FCS trace window and click export to ASCII (all cells).

The graph can be exported to a BMP format.

However this data can't be saved on the Bumblebee PC or transferred to it. To save data and your sanity have a folder on the datastore everything can be put into.



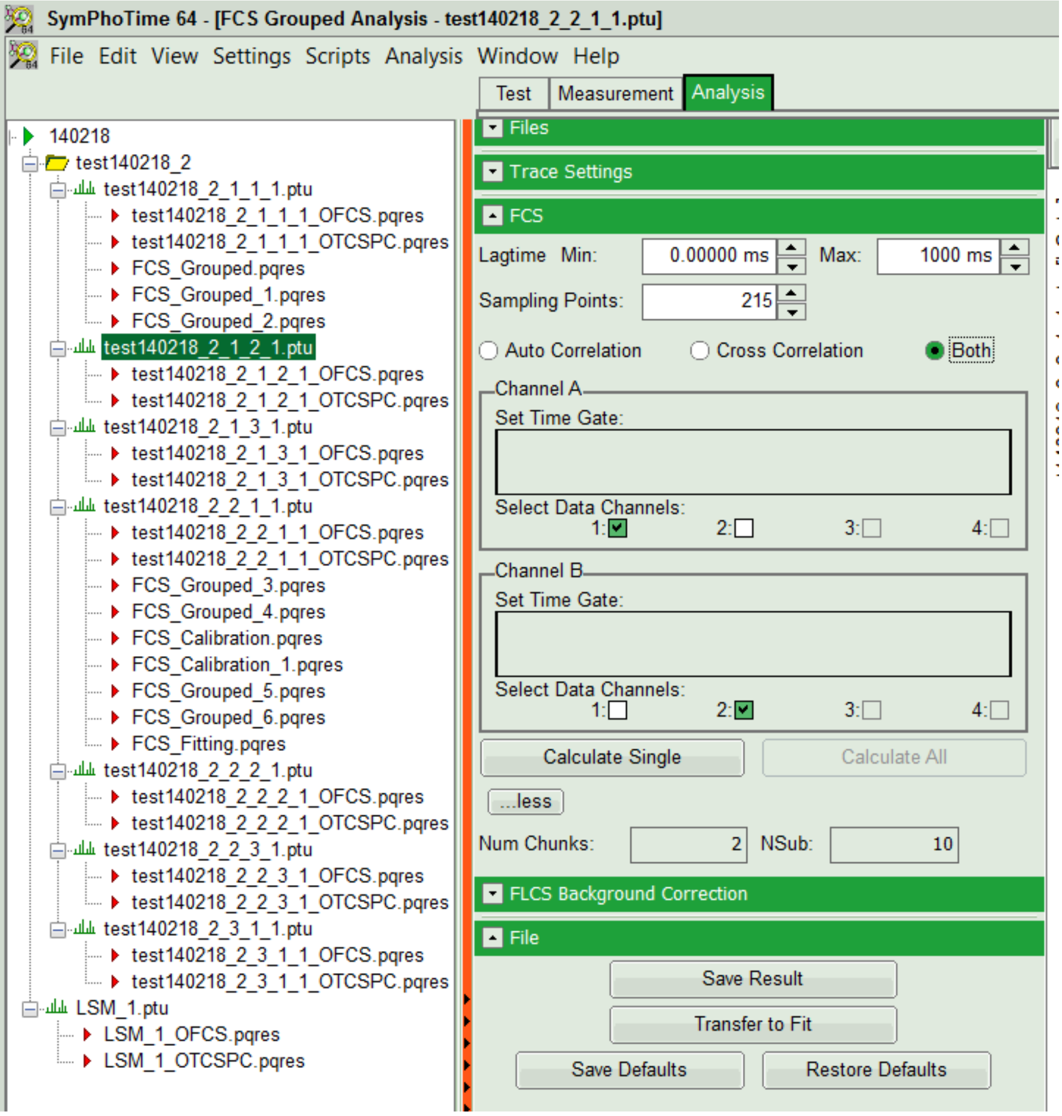
For the plot the x and y axis can be rescaled by clicking on them there is usually a scale icon to help with this.



FCCS

This works very similarly to FCS in Symphotime.

FCS fitting should still be selected. However this time one is calculating Both Auto Correlation and Cross Correlation and this needs to be selected in the software.



SymPhoTime 64 - [FCS Grouped Analysis - test140218_2_2_1_1.ptu]

Lagtime Min: 0.00000 ms Max: 1000 ms

Sampling Points: 215

○ Auto Correlation ○ Cross Correlation ● Both

Channel A

Set Time Gate:

Select Data Channels: 1:☑ 2:☐ 3:☐ 4:☐

Channel B

Set Time Gate:

Select Data Channels: 1:☐ 2:☑ 3:☐ 4:☐

Num Chunks: 2 NSub: 10

The Data Channels then need to be set up. Channel A which will be the 488 / GFP channel needs to be set to Data Channel 1.

Channel B, the AF594 / RFP, channel needs to be set to read Data Channel 2. See above

The method of using 'Calculate Single' Saving result, transferring to fit. Initial fitting and robust curve fitting stays the same. However there is now cross correlation as well as autocorrelation data to export.