

Calibrate the Confocal Volume for and with FCS

Summary

This tutorial shows step-by-step, how to calibrate the confocal volume for FCS measurements with a calibration dye. Therefore, a FCS curve is calculated with the FCS script, that is transferred to the FCS Calibration script.

Background Information

Note: Calibration is a necessary task for FCS measurements. In a calibration procedure, the confocal volume V_{Eff} as well as the structural parameter κ (the ratio of z to xy extension) is determined using a reference dye. This is the prerequisite to determine correct diffusion coefficients and concentrations for the sample of interest. ATTO488 (ATTO-Tec, Germany) is a suited dye to calibrate the confocal microscope at $\sim 470 - 490$ nm excitation and detection around $500 - 540$ nm.

The diffusion coefficient of ATTO488 carboxylic acid has been measured to be $400 \mu\text{m}^2/\text{s}$ at 25°C . Published diffusion coefficients of various fluorophores are summarized in the Technical Note "Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration" available from PicoQuant (see https://www.picoquant.com/images/uploads/page/files/7353/appnote_diffusioncoefficients.pdf). This list is extensive, but does not claim to be complete.

Note that the diffusion coefficients of all dyes are temperature dependent.

FCS measurements are usually point measurements acquired with very sensitive detectors (SPAD or Hybrid PMT detectors).

FCS is a technique applicable over a wide range of concentrations, but ideal FCS concentrations are usually between 1 and 20 nM.

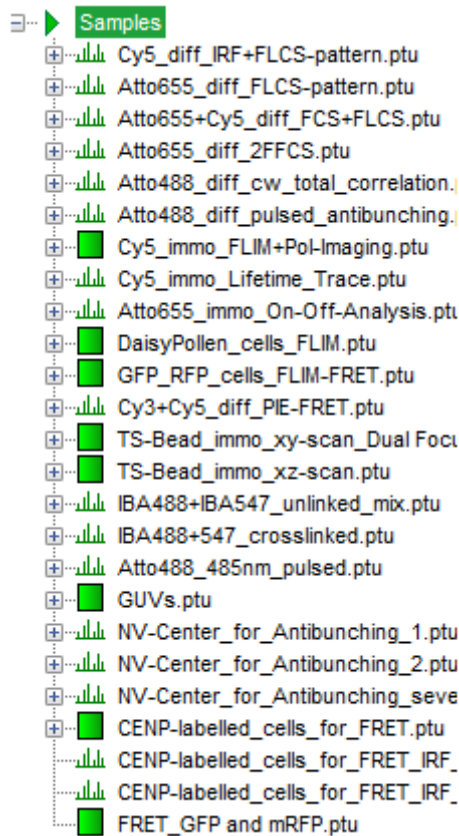
Step-by-Step Tutorial

Select a file and generate a reference FCS curve

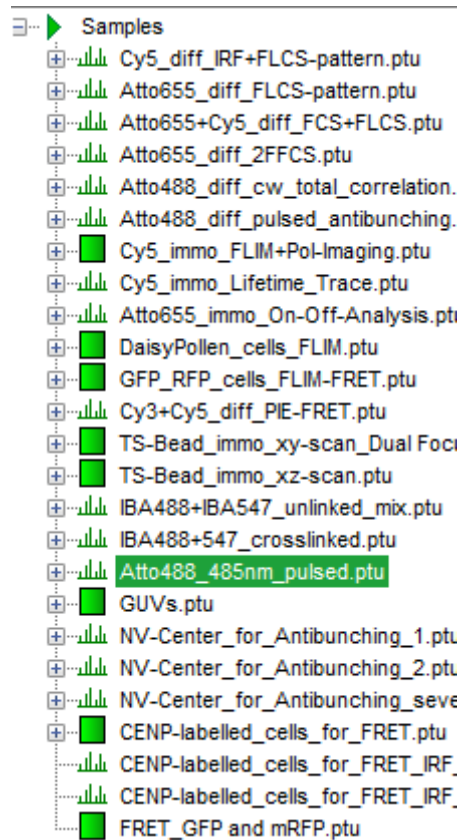
- Start SymPhoTime 64 software.
- Open the "Samples" workspace via "File\open Workspace" from the main menu.

Note: The "Samples" workspace is delivered with the SymPhoTime 64 and on the DVD-ROM and contains example data to show the function of the SymPhoTime 64 data analysis. If you haven't installed it on your computer, copy it from the DVD onto a local drive before going through this tutorial.

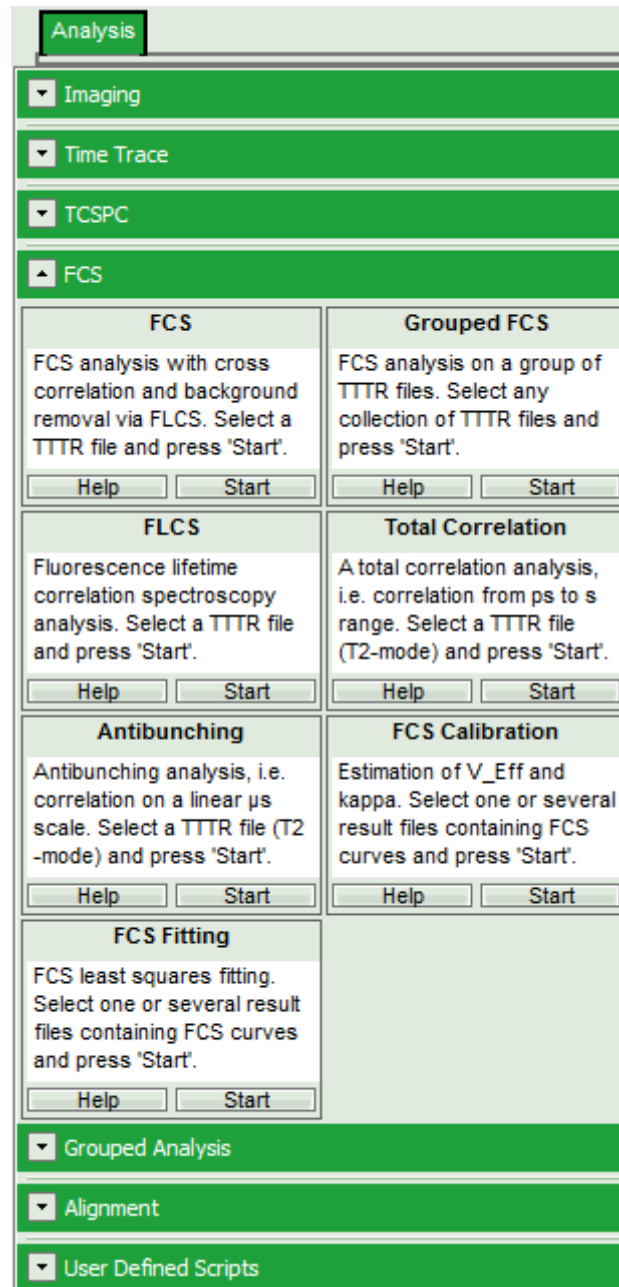
Response: The files of the sample workspace are displayed in the workspace panel on the left side of the main window.




- Highlight the file ATT04888_485nm_pulsed.ptu by a single mouse click.

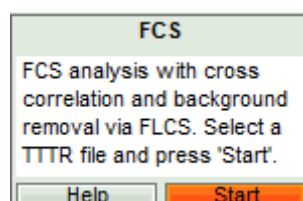


- Select the “Analysis” tab and in there, open the drop down menu “FCS”.

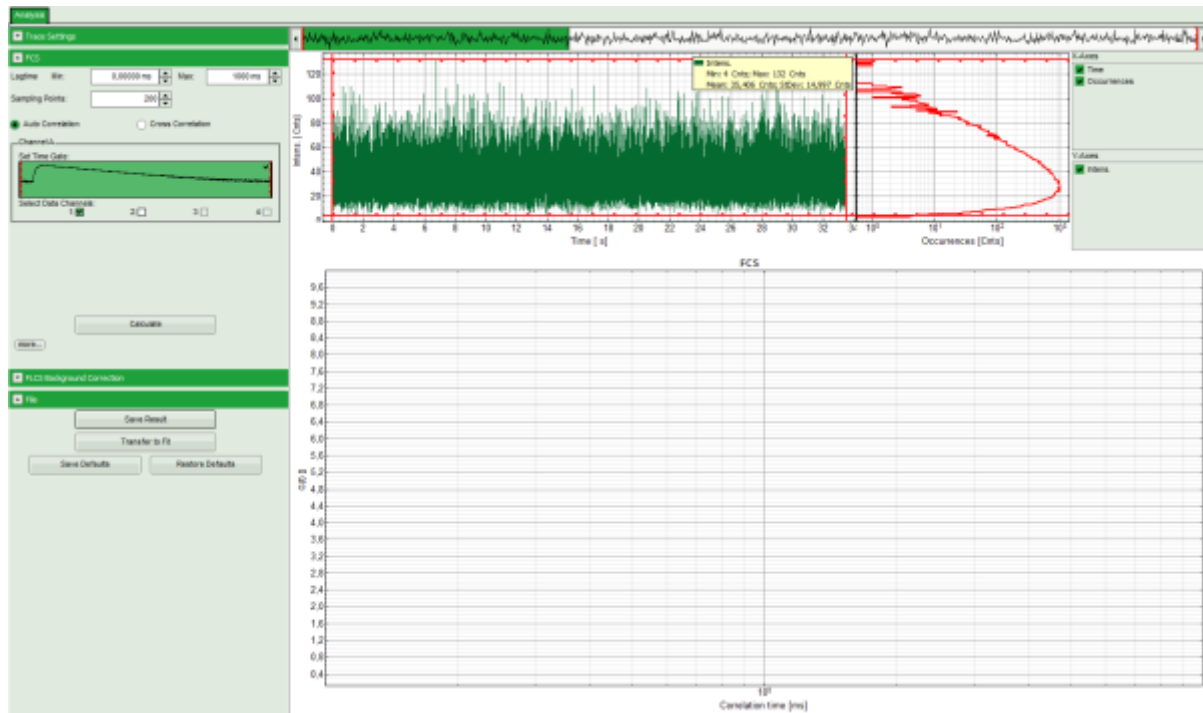


Note: The drop down menu can be opened and closed by clicking on the grey button on the left side of the header of the drop down menu: 

- Start the FCS script by clicking on “Start”.

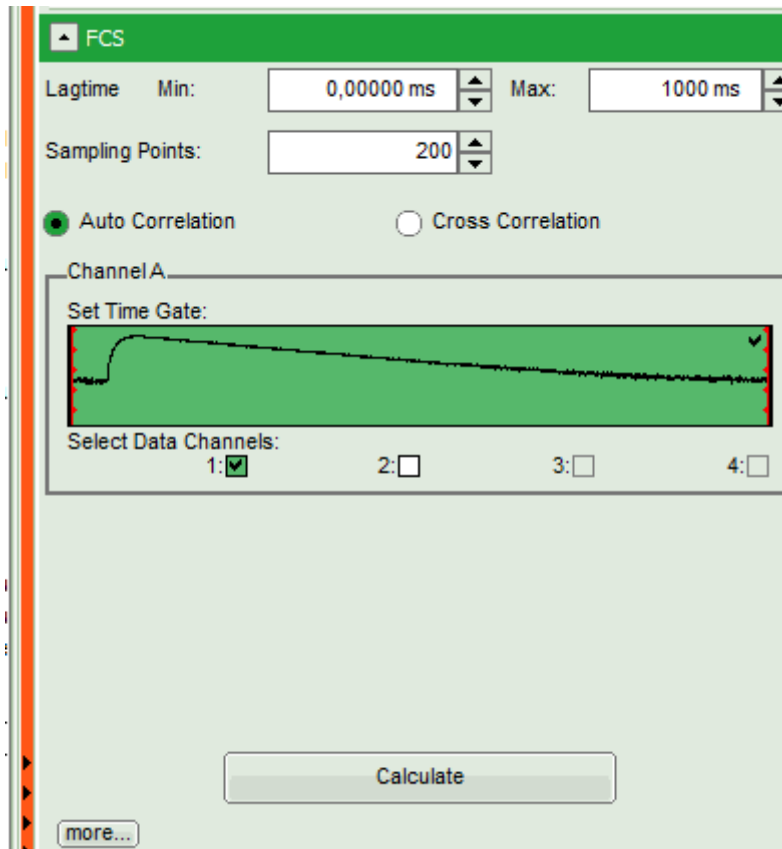


Response: The FCS script is applied to the file ATT0488_485nm_pulsed.ptu. Thereby, a new window opens:

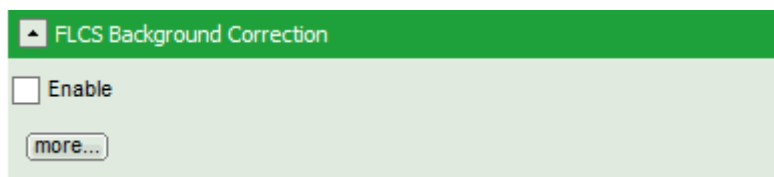


Note: The window contains three different regions:

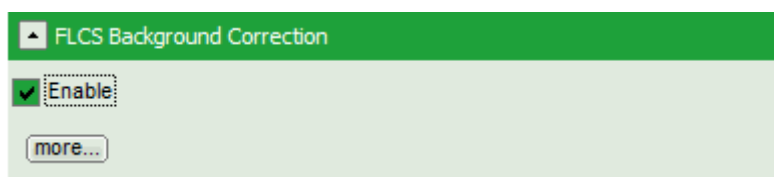
1. **Left:** Analysis and display options. For explanation of the different parameters, place the mouse cursor over this part of the window and press <F1> to open the corresponding help page.
2. **Upper center/right:** Intensity time trace. The display can be changed using the "Trace Settings" of the analysis options. The large window shows the inset of the complete trace above highlighted in green. The photon counting histogram on the right displays the frequencies of the different intensity values. Usually, this trace is used to check, whether the signal is stable during the measurement, which is a prerequisite for FCS. Also, large intensity spikes stemming from aggregation of the fluorescent sample can be identified in this graph. In measurements inside cells, often a slight decrease at the beginning of the trace is noticed. In this case, this region can be excluded from calculating the FCS trace by adjusting the upper vertical red limit bars in the small upper trace.
3. **Lower center/right:** FCS trace window. As first the FCS correlation criteria have to be defined and the curve needs to be calculated, this graph does not contain any trace at this stage.
 - In this file, all light is recorded with detector 1, therefore keep the FCS options as shown.



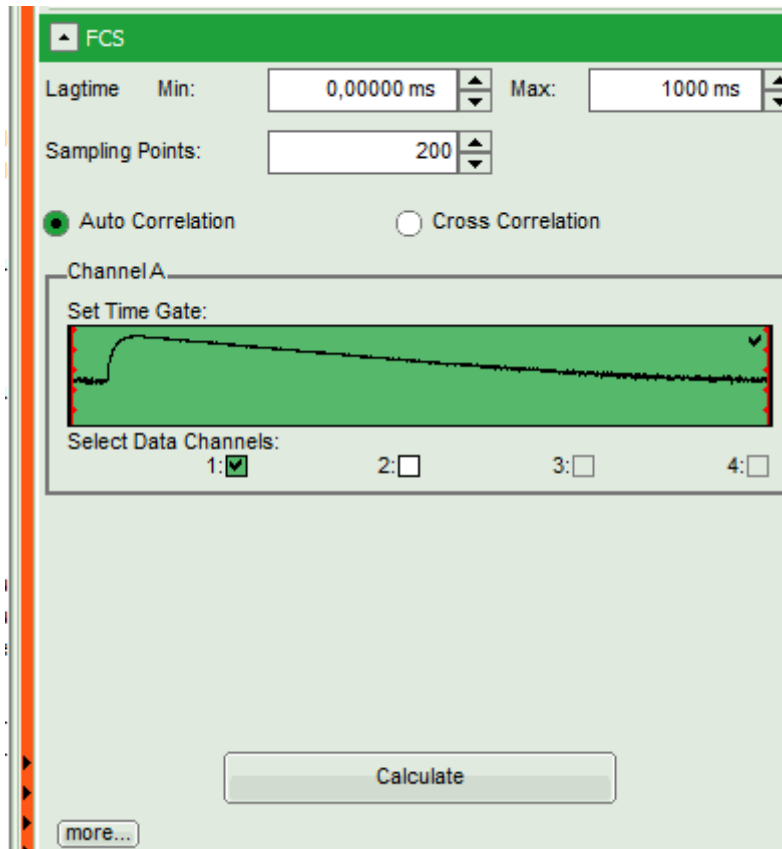
- Open the “FLCS Background Correction” drop down panel and check the “Enable” box.



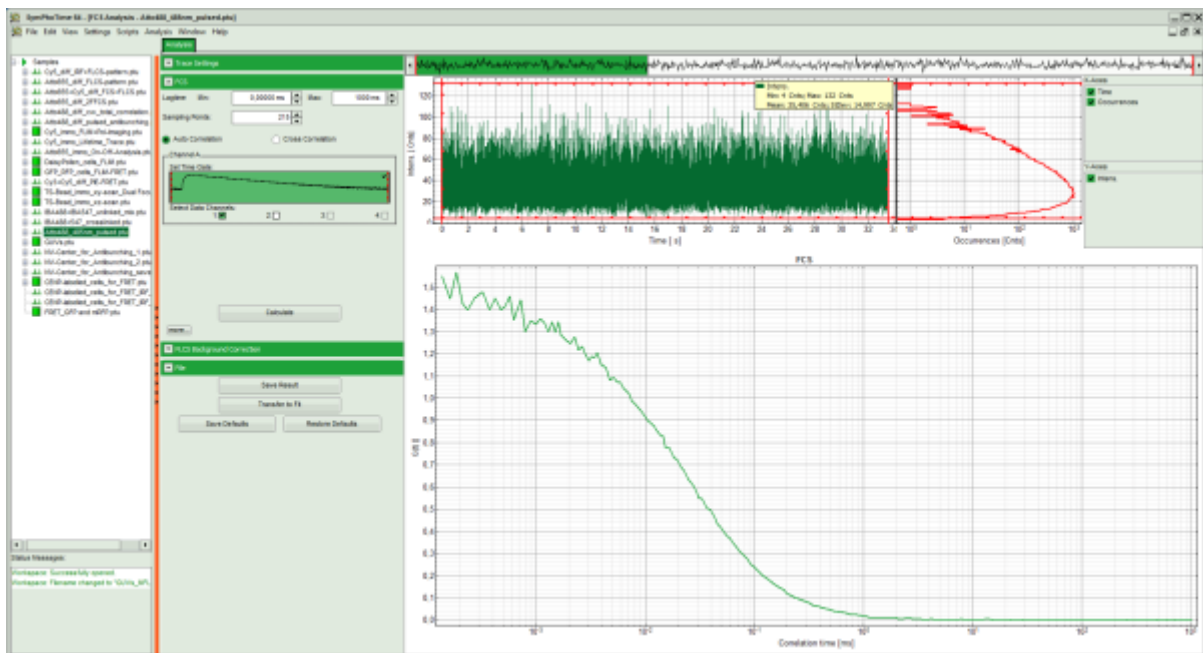
↓



- This button calculates simple FLCS filter on the data: the fluorescence decay versus the background in the TCPSC window. As the detector afterpulsing is not correlated with the laser pulse on the time range of interest, photons caused by detector afterpulsing behave similar as background photons.
- Click on “Calculate” to calculate the FCS trace.



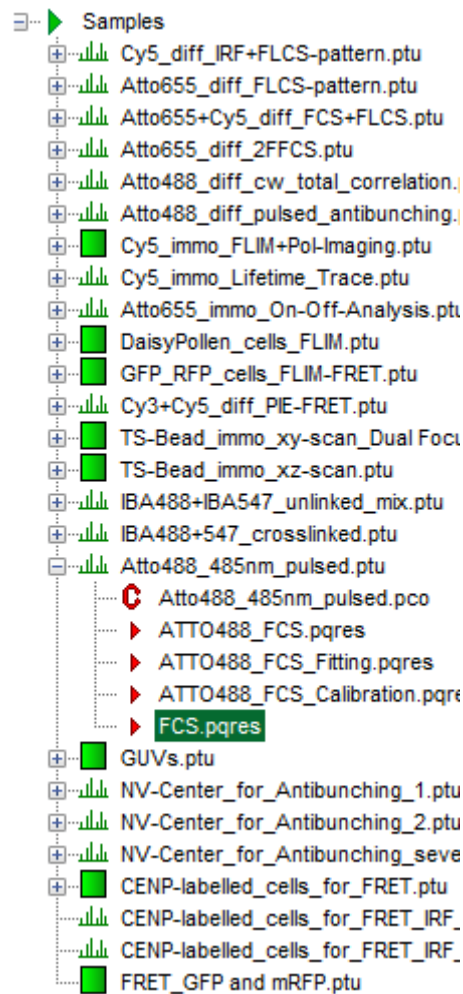
Response: The FCS curve is calculated.



- Save the calculated curve by clicking on “Save Result”.

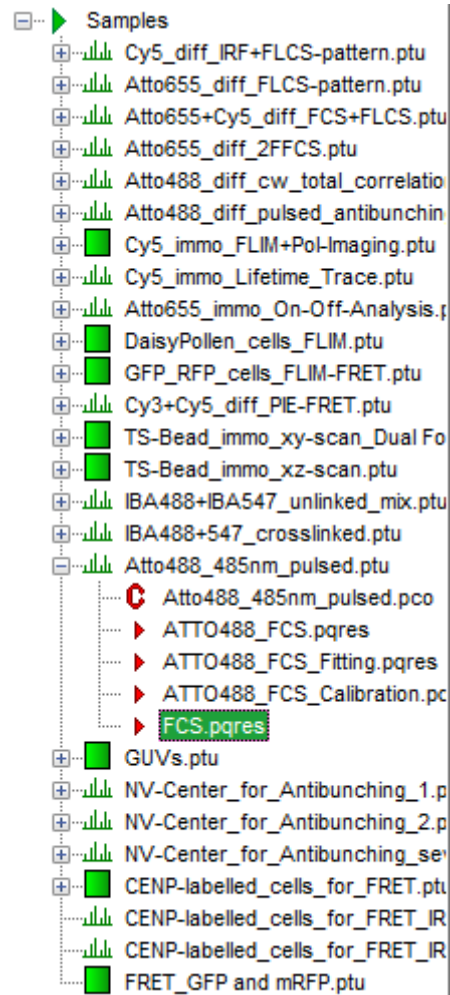


Response: A result file is generated and linked to the raw data file.



Use the generated FCS file for calibration of the confocal volume

- Highlight the newly generated FCS result file.



- Open the FCS pull down menu and start the “FCS Calibration” script.

empty.

- In the parameter panel, enter “400” as diffusion coefficient “D [$\mu\text{m}^2/\text{s}$]”.

Fitting Model: FCS Calibration Help

FCS Curve: FCS; Curve 0 Remove

Exclude Triplet

Parameter	Value	Fit
C [nM]	1,000	
D [$\mu\text{m}^2/\text{s}$]	400,000	
ρ Limits	0,000 \pm 0,000	✓
τ_{DM} [ms] Limits	0,000 \pm 0,000	✓
T Limits	0,000 \pm 0,000	✓
τ_{Trip} [μs] Limits	1,000 \pm 0,000	✓
κ Limits	0,000 \pm 0,000	✓
N	0,7656 \pm 0,0061	
$V_{\text{Eff C}}$ [fl]	1,27 \pm 0,01	
$V_{\text{Eff D}}$ [fl]	0,02575 \pm 0,00073	
$V_{\text{Eff All}}$ [fl]	0,6485 \pm 0,0045	

Note: For calibration two values can be used:

The diffusion coefficient is usually used as parameter. Therefore it must be known in advance.

If no suited diffusion coefficient is known, e.g. because the measurement is performed in a solvent or at a temperature where no calibration values are reported, the concentration can be used instead. Note: when working at very low concentrations in the range of 1 – 20 nM as needed for these kinds of measurements, surface adsorption of the dye at the pipette tips and similar effects may affect the actual concentrations. It is thus recommended to analyze several concentrations and compare if always the same confocal volume is obtained.

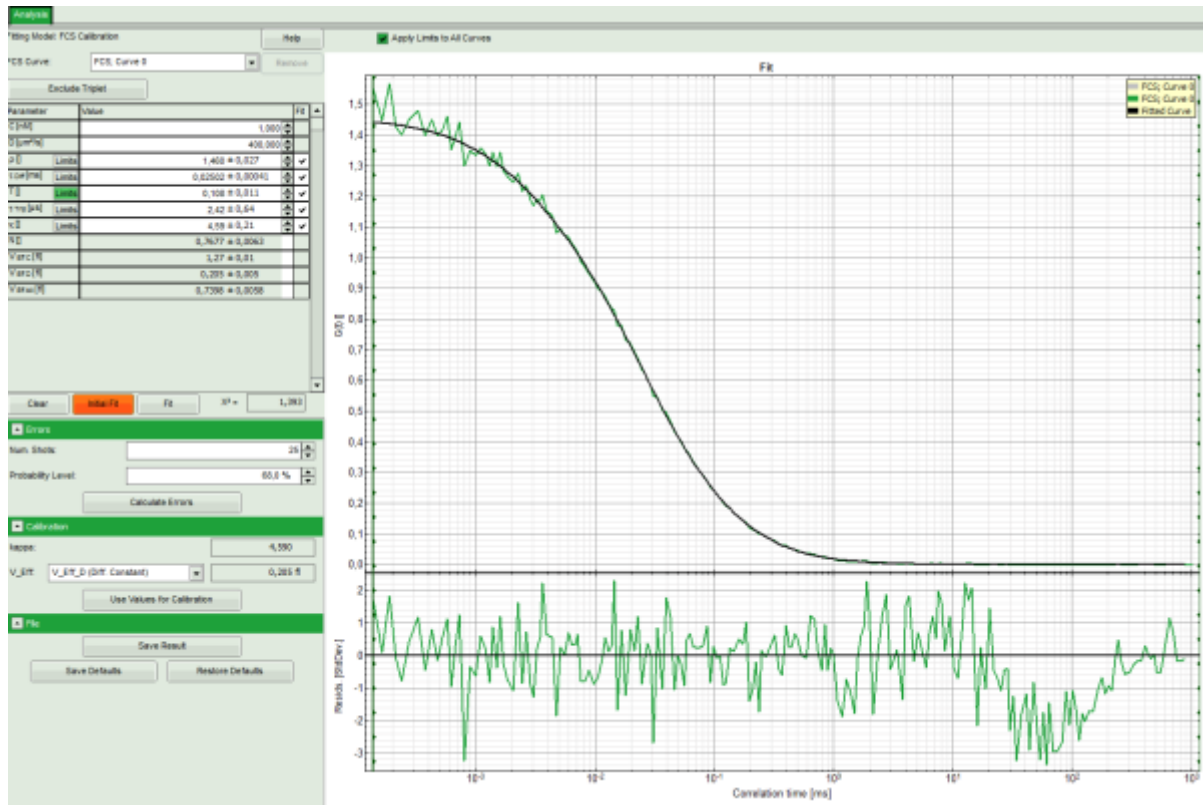
In this case, the diffusion coefficient is known from the literature.

- Click on “Initial Fit”.

Clear Initial Fit Fit $\chi^2 =$ 0,000

Response:

- The curve is fitted and plotted in the main window.
- The residuals (= difference between fitted curve and raw data) are plotted below the curve.
- The fitting parameters are displayed in the parameter panel:
 - In the grey panel, three effective volumes are calculated:
 - $V_{\text{Eff C}}$ (here = 1.27 fl): This would be the value of choice if the concentration was known. As it is not the case for this example, the value is meaningless.
 - $V_{\text{Eff D}}$ (here = 0.2 fl): This value is used for calibration.
 - $V_{\text{Eff All}}$ (here = 0.74 fl): This is the average calculate from the other values. This value could be used, if concentration and diffusion coefficient were both known. As this is not the case in this example, this value is meaningless.
 - The important parameters in this fit are V_{Eff} (= 0.2 fl) and kappa (= κ , 4.59). Both values are reasonable.



Fitting Model: FCS Calibration Help

FCS Curve: FCS; Curve 0 Remove

Exclude Triplet

Parameter	Value	Fit
C [nM]	1,000	
D [$\mu\text{m}^2/\text{s}$]	400,000	
ρ []	Limits 1,460 \pm 0,027	✓
τ_{off} [ms]	Limits 0,02502 \pm 0,00041	✓
T []	Limits 0,108 \pm 0,011	✓
τ_{trap} [μs]	Limits 2,42 \pm 0,64	✓
κ []	Limits 4,59 \pm 0,21	✓
N []	0,7677 \pm 0,0063	
V_{effC} [fl]	1,27 \pm 0,01	
V_{effD} [fl]	0,205 \pm 0,005	
V_{effAll} [fl]	0,7398 \pm 0,0058	

Calibration

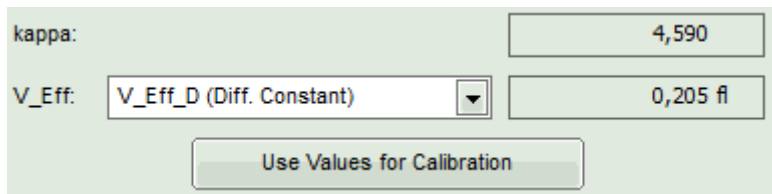
kappa: 4,590

V_Eff: V_Eff_D (Diff. Constant) 0,205 fl

Use Values for Calibration

Note: By default, V_Eff_D calculated from the diffusion constant is chosen as calibration value. If the reference were the concentration, the assignment can be changed in the “Calibration” drop down menu (see image above).

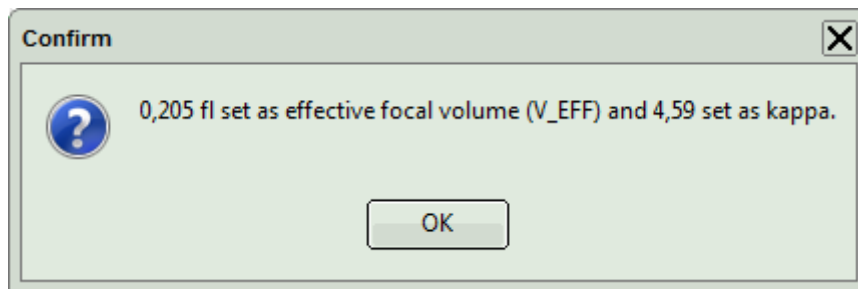
- Click “Use values for Calibration” to store these data as calibration values in the internal memory. Whenever a new FCS curve is fitted, these calibration values will be automatically used.



A window for setting calibration values. It has two input fields: 'kappa' with the value '4,590' and 'V_Eff' with a dropdown menu showing 'V_Eff_D (Diff. Constant)' and the value '0,205 fl'. Below these fields is a button labeled 'Use Values for Calibration'.

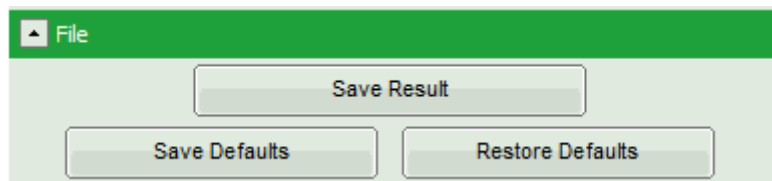
Response: A window pops up asking to confirm the calibration values.

- Click OK to store these values in the SymPhoTime 64 memory.



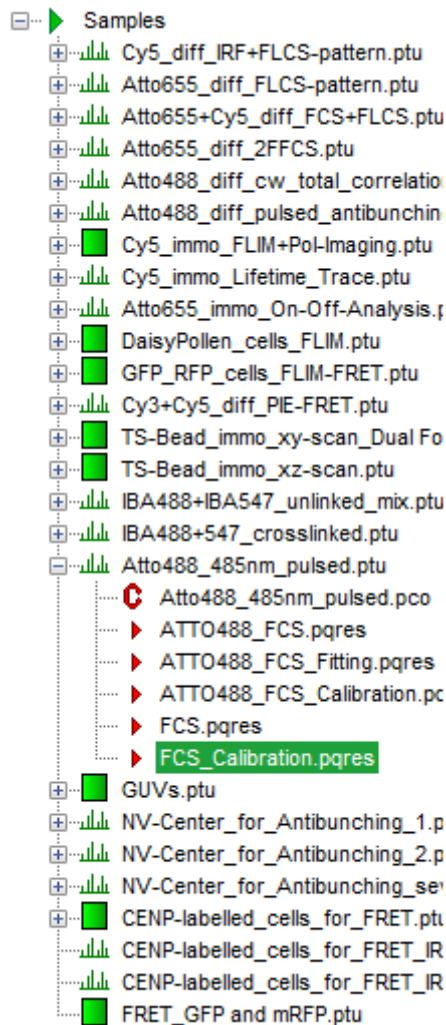
A 'Confirm' dialog box with a question mark icon. The text inside says: '0,205 fl set as effective focal volume (V_EFF) and 4,59 set as kappa.' There is an 'OK' button at the bottom.

- Save the fit by clicking “Save Results”.

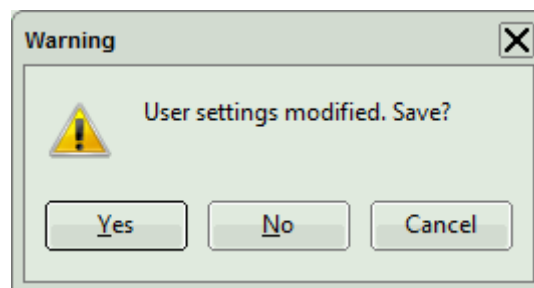


A 'File' menu with a green header. It contains three buttons: 'Save Result' at the top, and 'Save Defaults' and 'Restore Defaults' at the bottom.

Response: A result file is generated and linked to the raw data. The fitting window can now be closed.



- Close the calibration window.
- To permanently apply the changes, the changes in the user profile need to be confirmed when closing the software. A message will pop up when closing SymPhoTime 64:



- Click on “Yes” to store the data, click on “No” if you want to keep the old user profile.

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