From Eye to Insight

Leica
MICROSYSTEMS

FALCON FCS

Manual

20200730_EN

手順

Tips

＊The calibration is a part of the optimization of the FCS measurement conditions.
The calibration routine uses a solution with known parameters which must be inserted in the stand before each calibration.

＊PMT、Standard HyD are not available for FCS

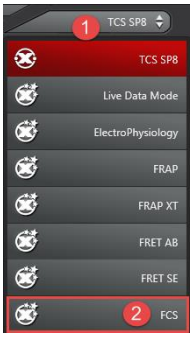
＊FCS is available for 0.5 to 100 nM

＊Due to the STED-FCS reduce the measurement volume, higher concentration sample is available

＊ Click the 「LAS_X_SingleMoleculeDetection.exe shortcut」 

open the An evaluation in LAS X FLIM/FCS.　LAS X Small is not available for the analysis

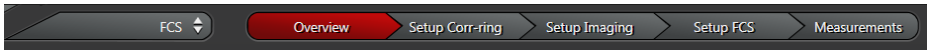
FCS Wizard



①Click the TCS SP8 in the main menu bar

②Select the FCS option

The structure of the FCS wizard is described below.



Overview	The procedure for the steps to be carried out is displayed here
Setup Corr-ring	An optimum adjustment of the correction ring correlates with the best possible optical resolution of the systems (A basic prerequisite for FCS)（Ref. page 3）
Setup Imaging	Establish and optimize the parameters for image acquisition.（Ref. page 4）
Setup FCS	Define and optimize the conditions for the FCS measurement.（Ref. page 7）
Measurements	Define multiple FCS measuring points in the previous acquired XY, XZ, XYZ or XZY images (Measurement condition is defined in the Setup FCS step.)（Ref. page 10）

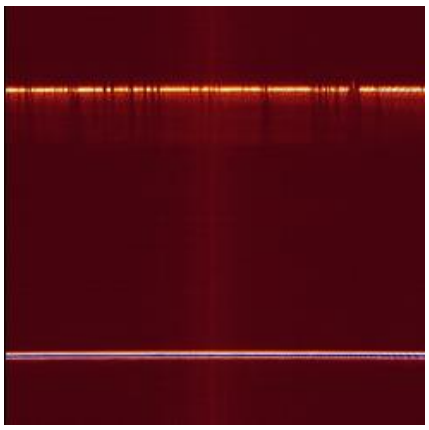
Setup Corr-ring

you must always make this adjustment when you change the specimen carrier, even if it comes from the same batch.

Scan mode	XZ-y（Z-Galvo）
Format	512x512
Zoom	1
Lazer line	488 nm
AOBS	Reflection
Detector	PMT

＊Super Z Galvano stage

Move the focus using the z-drive of the microscope stand until you see the 2 reflection lines that appear between the immersion and the glass and the between the glass and the specimen.



Immersion（water）

Cover slip

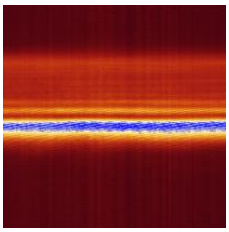
Medium

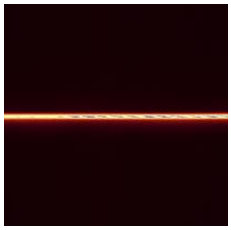
Focus on the below.　Use a higher zoom（e.g. 4 to 8）

Rotate the correction ring on the objective into a position that provides the signal with the highest contrast in the reflection between cover slip and medium

Left image: Reflection with poorly set position of the correction ring.　The image is out of focus, several secondary peaks are visible

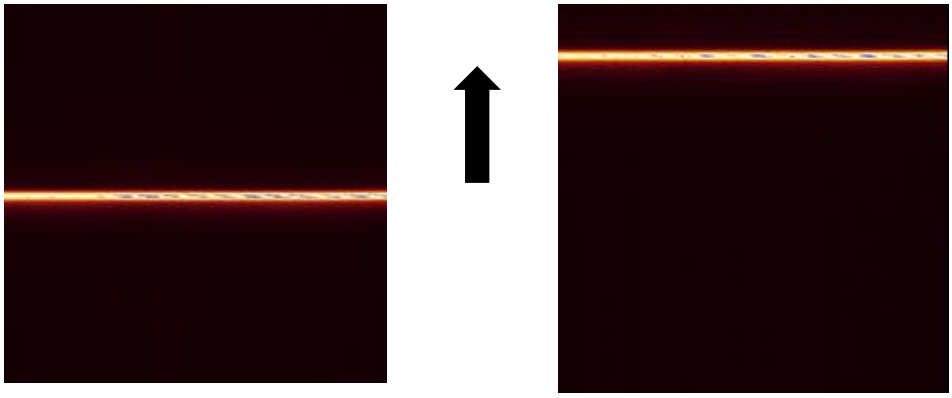
Right: Reflection with correctly set position of the correction ring:　The image is sharp, and secondary peaks are scarcely visible.



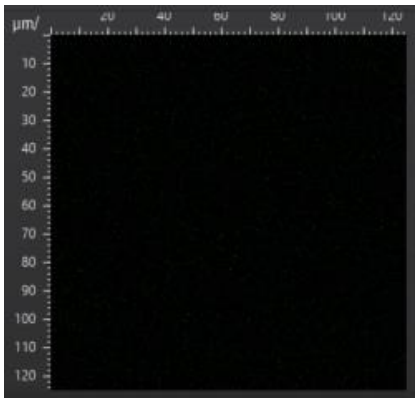


＊Without Super Z Galvano: Rotate the correction ring on the objective into a position that provides the signal with highest contrast in XY image.　The Z position move accompany with the correction ring rotation.　Then, keep the z position.

Move the focus about 10 – 20 μm from the center of the image toward to the specimen



 The tool on the top edge of the display window enable to show ruler like below image

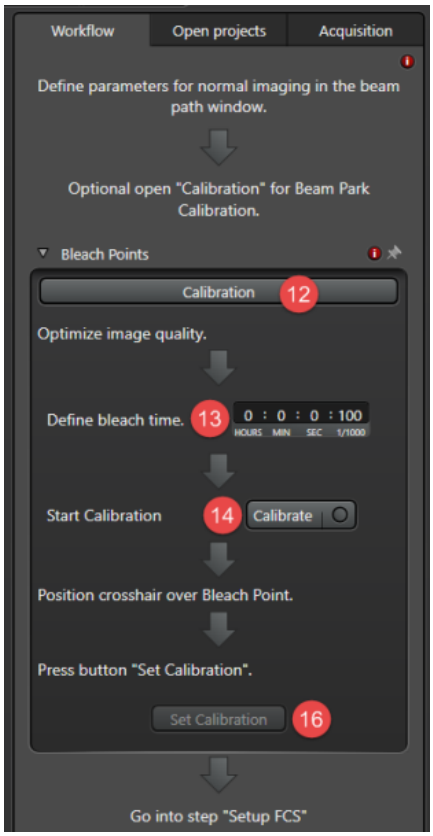


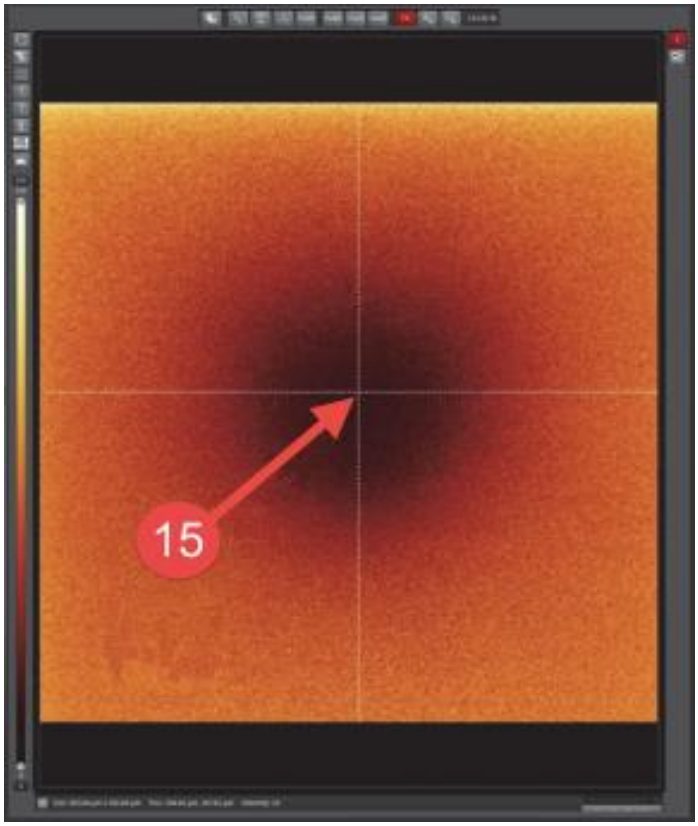
Calibrate Beam Parking

Before starting an FCS experiment, you must calibrate beam parking in order to ensure the positioning accuracy of the FCS measuring point. Repeat this procedure about every 3 days. Use a bleach specimen with fixed fluorescence that is capable of being excited with a wavelength in the visible range and can also be photobleached with higher laser intensity. （e.g. Leica standard sample, Convallaria）

1. Select the ②Setup Imaging
2. Focus on the bleach specimen（Press Ctrl + T to show crosshair）

3. In the ⑪Acquisition tab, set the ⑥xyz mode, ⑦scan speed 600 Hz, Bi-directional
4. Select ⑧laser line、suitable ⑨detector and the detection range
5. Select Glow(O and U) in the color look up table (LUT)
6. Switch to the ⑪Workflow tab





7. Click the ⑫Calibration button, open the upper left dialog. The system automatically determines the following values: Zoom 32 and pixel format 1024x1024.
8. Activate live mode by clicking the Live button. Configure the gain and offset
9. At ⑬Define bleach time, you can enter the bleaching duration: the default value is 100 msec. For thin specimens easy to bleach, a bleaching duration of 500 msec or less is recommended.
10. Click the ⑭Calibrate button. An image will be acquired automatically. Following this the previously selected laser lines are automatically set to 100% AOTF. Following this, one more image is acquired with the previously configured settings.
11. Now, position the crosshair in the center of the bleach point ⑮
12. Click the ⑯Set Calibration The systems saves the calibration data and display it under X and Y

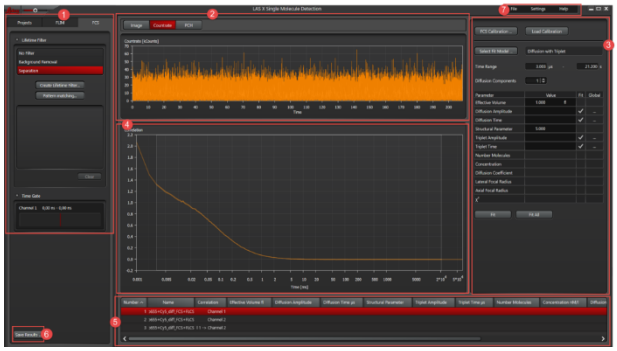
13. Acquire a reference image

Setup FCS

(Ref. page 19)

Click Setup FCS to open the FCS window

Ref. page 17



①Select the detector

②Setting the lasers

③you can check which notch filter is currently in the beam path（Automatically introduces a barrier filter into the beam path）

⑦Prepare the FCS measurement by configuring all required setting under Acquire – Beam Path Settings.

Set the diameter of the detection pinhole using Pinhole, Airy 1.

Λ	If you would like to acquire an excitation spectral series at a defined FCS point. Its purpose is to determine the excitation spectra of fluorochromes. The wavelength of the excitation laser continuously changes and documents the resulting emission intensity
λ	If you would like to acquire an emission spectral series at a defined FCS point. Its purpose is to determine the emission spectra of fluorochromes. The wavelength of the excitation laser continuously changes and documents the resulting emission intensity
Λ λ	If you would like to acquire an excitation/emission spectral series at a defined FCS point. Its purpose is to determine the excitation spectra of fluorochromes. The wavelength of the excitation/emission laser continuously changes and documents the resulting emission intensity

⑥Correlation Assignment：In this process, the first 4 FCS detectors that have been activated in the beam path setting are each assigned an FCS correlation channel. Thus, correlation curve is generated for each correlation channel.

FCS（Auto-Correlation）

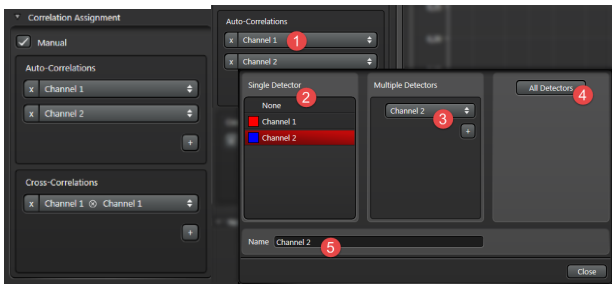
①The dropdown menu represents an FCS correlation channel.

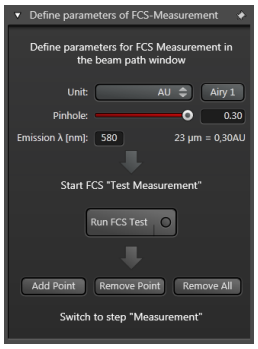
②When click on a dropdown menu, anew dialog opens in which you able to assign an individual detector or ③multiple detectors simultaneously.

④All activated FCS detectors are assigned to the correlation channel by means of the All Detectors button

FCCS（Cross-Correlation）

In this dialog, 2 auto correlation channels are selected to calculate the cross correlation between both.





1. Define an FCS measurement point. A crosshair appears in the display window. Use the mouse drag the crosshair to the position where you wish to carry out the measurement and click on Add Point at every point.
2. Click on Run FCS Test to start the measurement.

The measurement results are displayed and evaluated on the sub-monitor.

After configuring the setting for the FCS measurement, switch to the Measurement operating step.

Tips

- When FCS measurements are taken in solution, the measuring point must always be defined with the aid of a reference point. The reference that is generated at between cover slip and medium. move the focus on the reflection about 10 to 20 µm inwards towards the medium.

・Adjust to the count rate between 10 to 500 kcps (count per second)

・Higher cpm value give S/N of Correlation curve

・To avoid the effect of the photobleach, reduce the laser power to 2/3 of maximum cpm

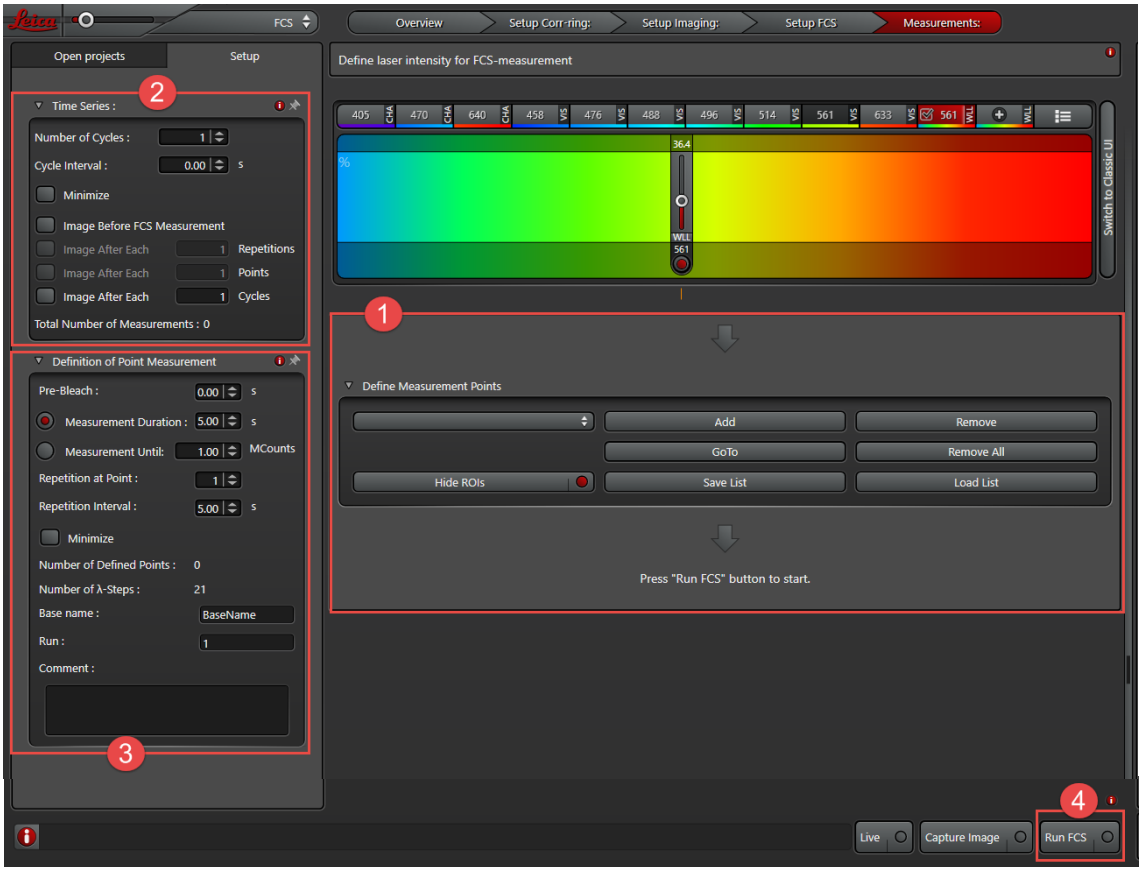
Then, still you can observe the effect of the bleach or triplet, more reduce the laser power

Count per second: The number of photon detected per second is displayed

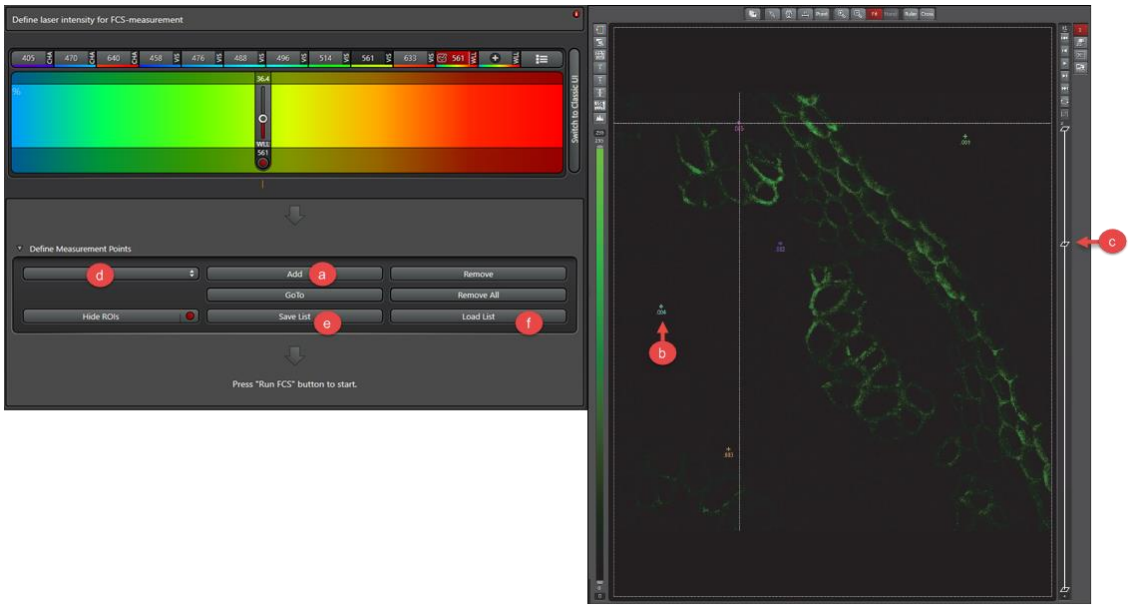
Count per molecule: The number of photon detected per molecule is displayed

Measurements

Define the measurement point （Ref. page 16）



①Define the FCS measurement point



1. Define the FCS measurement point.　Drag the crosshair to the measurement position

2. ⓐClick Add, then the position was shown like ⓑ

（Max 200 points）

Also you can define the position of XYZ.

3 . You can save/load defined positions from ⓔSave List or ⓕLoad List.

Remove: Remove current point, Remove All: Remove all defined point

4. You can move the focus point form ⓓMove to.

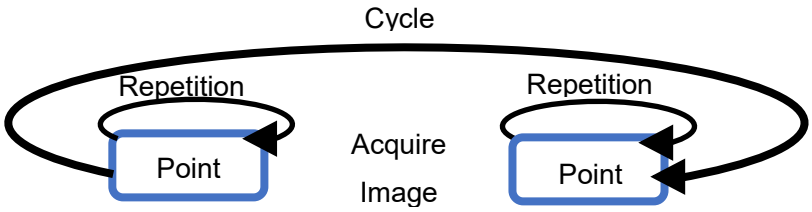
②Time series

Number of Cycles	How many measurement run will be carry out within an FCS experiments.
Cycle Interval	The time interval for a measurement run.
Minimize	Automatically use smallest possible time interval
Image Before FCS Measurement	An image acquisition is to take place before the actual FCS measurement
Image After Each　Repetitions	Acquire image after a certain number of repetitions
Image After Each　points	Acquire image after certain number of points
Image After Each　Cycles	Acquire image after certain number of runs
Total Number of Measurements	Total number is calculated from the number of defined measuring point time series and repeats

③Definition of point measurement

Pre-Bleach	You can first photobleaching the specimen at a measuring point before beginning the FCS measurement.　Pre-bleaching serves to reduce photobleaching effects during the FCS measurement
Measurement Duration	Specify the duration of the FCS measurement 1 sec to 10 min
Measurement Until	Specify the number of the photons that must be detected during the FCS at a particular point
Repetition at Point	How often the FCS measurement will be repeated
Repetition Interval	The time interval for a measurement run.
Minimize	Automatically use smallest possible time interval
Number of Defined Points	Define the measurement points for FCS in the ①
Number of λ-Steps	The number of the steps for spectral series
Base name	Enter the name which the FCS data

Run	The Run suffix is automatically appended to the name. Default value is 1.
Comment	The comment add to the module LAS X FLIM/FCS



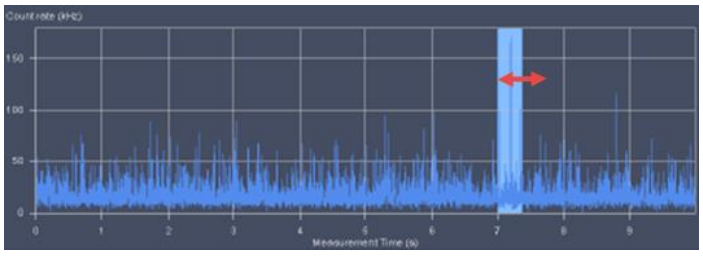
Graphical user interface for the evaluation of FCS experiments

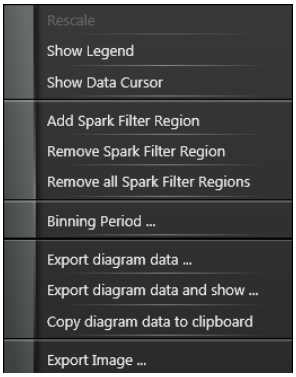
13-①Setting for correlation

Clicking the Correlation, open the dialog

Spark Filter

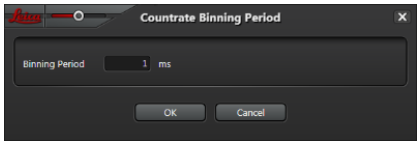
In this dialog, you can remove regions from the intensity distribution diagram in order to exclude them from the correlation calculation. When check box is enabled, bars are displayed in the diagram, which can be manually dragged with tho mouse to the ares you want to remove. You can change the width of the bar and both add new bars or remove them from the contest menu.



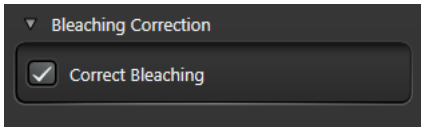


Right click on the diagram, open the contest menu (Next page).

Add Spark Filter Region	Inserts filters in order to remove regions from the intensity duration diagram and exclude them from the correlation calculation
Remove Spark Filter Region	Delete a previously selected filter
Remove all Spark Filter	Delete all existing filters
Binning Period	Open the Contrate Binning Period dialog where you can set the support points for the photon counting



In this dialog, you can configure the support positions for photon counting



Bleaching Correction

Photon bleaching occurs with many fluorochromes and is observed as a fluorescence decay curve over time. This decay is also monitored in the case of autocorrelation. You can use this dialog the Correct Bleaching checkbox, the software detects the region where a fluorescence decay takes place and corrects it.

Lifetime Filter, Time Gate Filter: Ref. page 25-27

Correlation Assignment: In this process, the first 4 FCS detectors that have been activated in the beam path setting are each assigned an FCS correlation channel. Thus, correlation curve is generated for each correlation channel.

FCS（Auto-Correlation）

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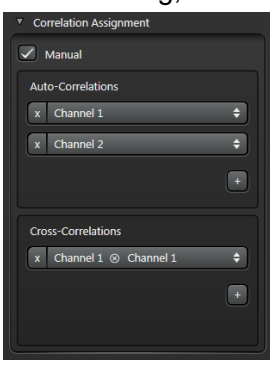
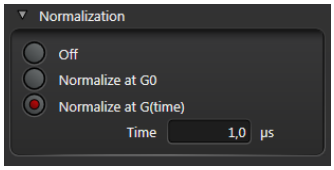
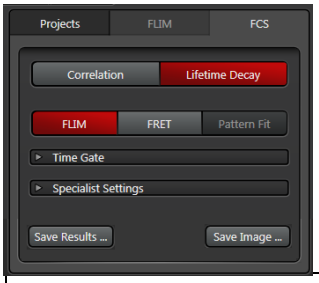


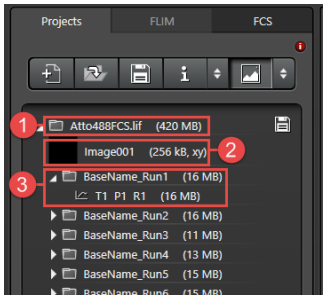


Normalization ：In this dialog, you can enable the normalization of the correlation function

Off	No standardization of the correction
Normalize at G0	The corrective function is standardized to the value G（tau）=0
Normalize at G（time）	The corrective function is standardized to the value G（tau）=1 µs The default value 1 µs can be changed in the Time field



FLIM/FRET/Pattern Fit	（Ref. FLIM manual）
Time Gate	（Ref. FLIM manual）
Special Settings	（Ref. FLIM manual）
Save Results	The current status of all analysis results is stored, including all settings.
Save Image (Only for FLCS)	



1: Higher level directory in the which all data is stored.

2: Frame or image series used as a reference for setting the FCS measuring point

3: With each click on the Run FCS, a new FCS measurement and a new folder are generated. The BaseName_Run number specified in the Run suffix automatically increases by increments of 1.

T： Trace

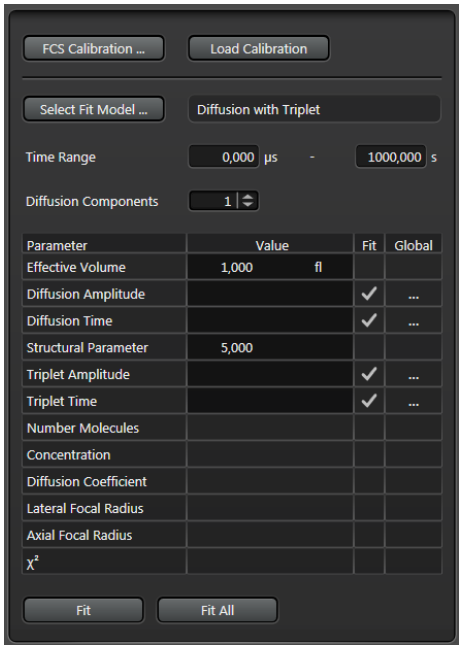
P： Point

R： Run

13-②After the FCS measurement, you can switch between the following displays

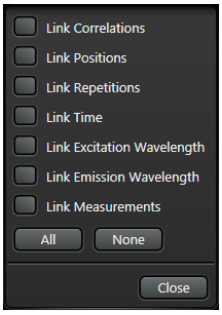
Image	Display the reference frame with the defined FCS measuring points
Counts	Representation of the counted photons in the FCS volumes over time

13-③Curve fitting setting



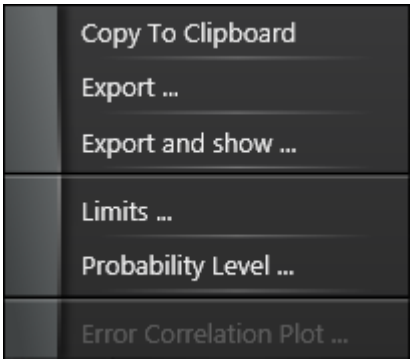
FCS Calibration	Open a dialog for the calibration of the FCS calibration volume（Ref. page 16）
Load Calibration	If the results of the current calibration in the table have been overwritten, you can reload them by clicking this button
Select Fit Model	Open a dialog in which you can select a calculation model for the curve fitting
Pure Diffusion	Only diffusion contributions are present (no triplet states)
Diffusion with Triplet	There is possibility that molecules from the observation volume transition to non-fluorescent status (dark states, triplet states)
Triplet Extended 3D	There is possibility that movement restrictions in the 3D volume or obstructed diffusion arise
Triplet Extended 2D	There is possibility that movement restrictions arise in a membrane. For STED-FCS
Protonation	If protonation reactions are to be expected
Conformational	For conformational change
Time Range	This specifies the time gate in which the curve fitting is to take place

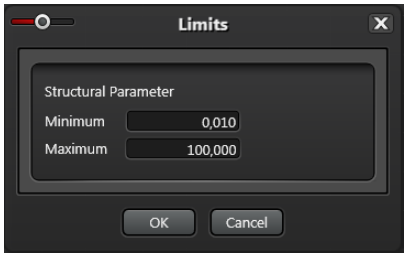
Some parameters ca either be set to a user-defined value(Value) or adjusted (Fit) or globally (Global) linked. Clicking under Global in the field … of the respective parameter open the following context menu in which you can select for which parameters the same values are to be used.

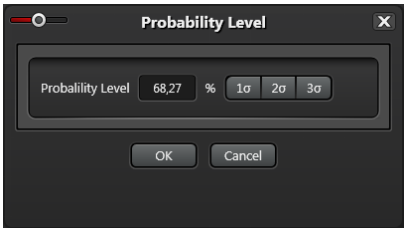


Diffusion Components	Enter the number of diffusion components
Fit	Clicking this button calculates the curve fitting listed above for the curve selected in the navigation table
Fit All	Clicking this button calculates the curve fitting listed above for all curves of the navigation table

You can click the right mouse button to open these context menus that provides functions.

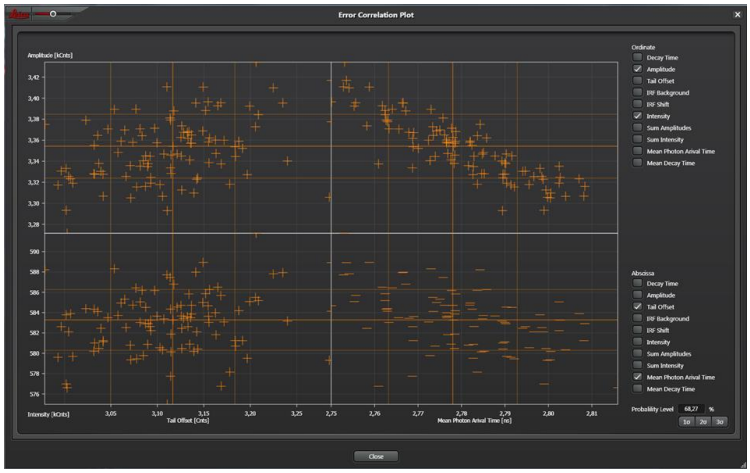






Copy to Clipboard	You can paste the content (parameters and values) into the Excel table
Export	You can export the parameter table
Export and show	You can export the parameter table and open the Excel table
Limits	Open the limit dialog, in which you can configure upper and lower limits for the respective parameters
Probability Level…	Open the Probability Level dialog, in which you can define the rules for specifying the accuracy of the respective parameter after the fit（σ1:68.27%、σ2:95.45%、σ3:99.73%）

Parameter Plot



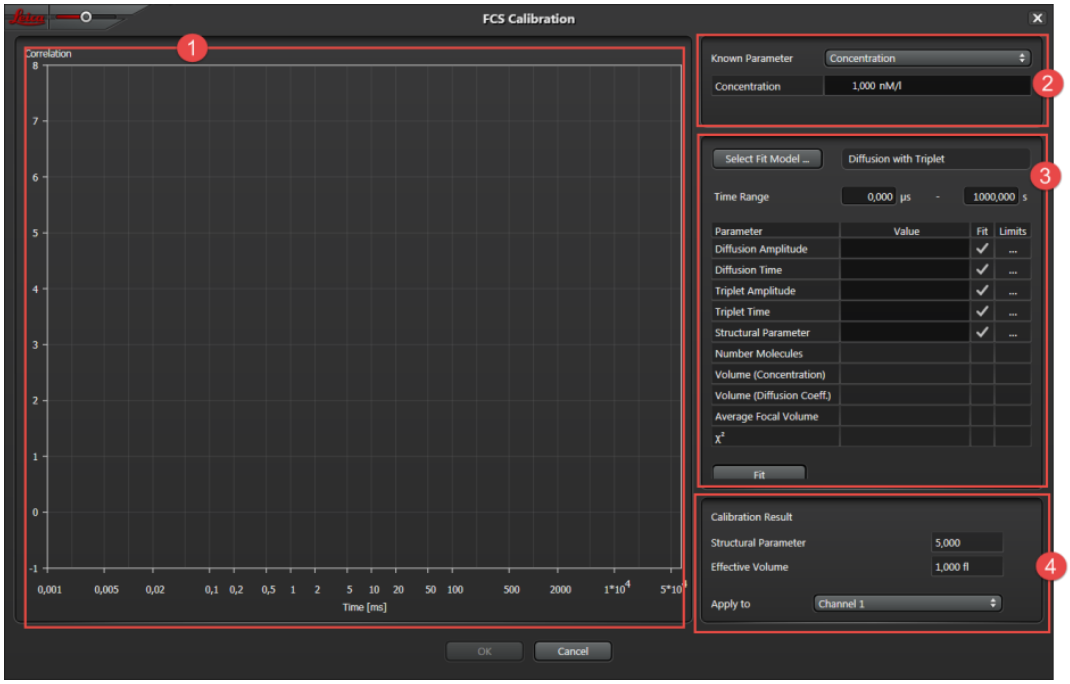
Error Correlation plot…	Display the data of a bootstrap analysis for the fit parameters in graphic form
Ordinate	Select the parameters for display on the Y-axis
Abscissa	Select the parameters for display on the X-axis
Probability Level	You can enter a self-defined percentage for the confidence interval, you can also define a multiple of the standard deviation（σ1:68.27%、σ2:95.45%、σ3:99.73%）

FCS Calibration

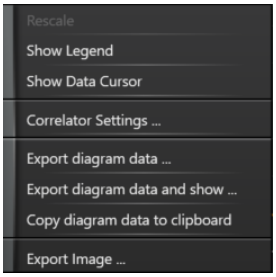
The calibration is a part of the optimization of the FCS measurement conditions.

The calibration routine uses a solution with known parameters which must be inserted in the stand before each calibration.

(Ref. page 13, FCS Calibration)



①The test measurement correlation curve is displayed here



Rescale	Zoom in and out on the curve using the mouse wheel
Show Legend	Show the legend in the diagram
Show Data Cursor	Show the value for time and intensity for each point
Show Data Cursor	Show the value for time and intensity for each point
Export diagram data…	Export the diagram data to the excel table
Export diagram data and show	Export the diagram data to the excel table and open the excel table
Copy diagram data clipboard	Store the diagram data in the clipboard
Export Image	Store the diagram as TIFF, JPEG, PNG, BMP, GIF

②You can enter the known constant for the calibration here.　You can choose between Diffusion Coefficient, Concentration or Diffusion Coefficient and Concentration

③Setting parameters for the calibration

Select Fit Model	Open a dialog in which you can select a calculation model for the curve fitting
Pure Diffusion	Only diffusion contributions are present (no triplet states)
Diffusion with Triplet	There is possibility that molecules from the observation volume transition to non-fluorescent status (dark states, triplet states)
Time Range	This specifies the time gate in which the curve fitting is to take place
Diffusion Amplitude	Intensity
Diffusion time	The time molecule spend within the observation volume
Triplet Time	Time molecules spend in dark state
Structural Parameter	z/w ratio of observation volume
Volume (Concentration)	Calculated volume based on the Concentration
Volume (Diffusion coeff.)	Calculated volume based on the Diffusion coefficient
Average Focal Volume	Average volume of Volume (Concentration) and Volume (Diffusion coefficient)
χ^2	Fit error or the fit precision for pixel
Fit	By clicking Fit, the calibration with the above listed setting is completed

④Result of the calibration

Structural Parameter	Ratio of the axial and radial expansion of the focal volume
Effective Volume	Calculated effective observation volume, measured in （fl、10^{-15} l）
Apply to	You select which channel you would like to assign the calibration value to. The results of the calibration are displayed in the parameter table under Fit Setting The calibration values are applied for each following measurement.

Fluorophore	λ_{Em} maximum in nm	Diffusion coefficient in water **at 25°C (298.15 K)** in 10^{-6} cm^2s^{-1}	Methods and references
Atto655-maleimid	686	4.07 ± 0.10 4.06 ± 0.09 4.09 ± 0.07	2fFCS [1], PFG-NMR [1] 2fFCS [4, 8] pmFCS [4]
Atto655-carboxylic acid	685	4.26 ± 0.08	2fFCS [1,3], PFG-NMR [1,3]
Atto655-NHS esther	685	4.25 ± 0.06	2fFCS [8]
Cy5	670	3.6 ± 0.1	2fFCS [8]
Alexa 647	665	3.3 ± 0.1	2fFCS [8]
Alexa 633	647	3.4 ± 0.1	2fFCS [8]
Rhodamine 6G	550	4.14 ± 0.05 4.3 ± 0.4 4.14 ± 0.01	2fFCS [1, 8] PFG-NMR [6] PB/CF [7]
Rhodamine B	560	4.5 ± 0.4 4.27 ± 0.04	PFG-NMR [6] PB/CF [7]
Rhodamine 123	530	4.6 ± 0.4	PFG-NMR [6]
Rhodamine 110	535	4.7 ± 0.4	PFG-NMR [6]
Fluorescein	520	4.25 ± 0.01	PB/CF [7]
Oregon Green 488	550	4.11 ± 0.06 4.10 ± 0.08	2fFCS [1] 2fFCS [8]
Atto488-carboxylic acid	523	4.0 ± 0.1	2fFCS [5]
TetraSpeck Beads, 0.1µm diameter	430 515 580 680	0.044 ± 0.07	2fFCS [2], DLS [2]

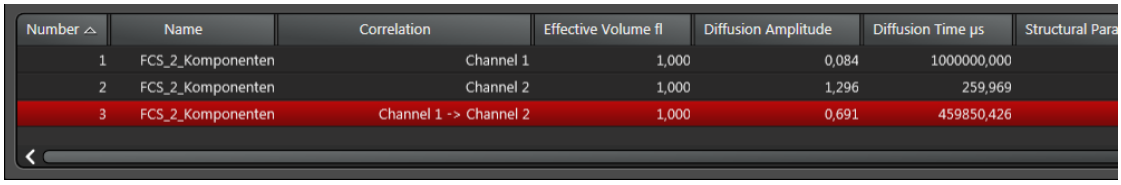
Abbreviations of measurement methods:

2fFCS, Dual Focus Fluorescence Correlation Spectroscopy; PFG-NMR, Pulsed Field Gradient Nuclear Magnetic Resonance; pmFCS, Polarization-Modulation Fluorescence Correlation Spectroscopy; PB/CF, Plug Broadening/Capillary Flow; DLS, Dynamic Light Scattering.

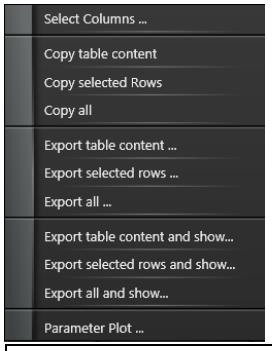
13-④Representation of the auto-correlation curve or cross-correlation curve in real curve in real time during FCS tests and the FCS measurement

13-⑤Result table

After the measurement, one row is created for each channel



Number	Name	Correlation	Effective Volume fl	Diffusion Amplitude	Diffusion Time µs	Structural Para
1	FCS_2_Komponenten	Channel 1	1,000	0,084	1000000,000	
2	FCS_2_Komponenten	Channel 2	1,000	1,296	259,969	
3	FCS_2_Komponenten	Channel 1 -> Channel 2	1,000	0,691	459850,426	

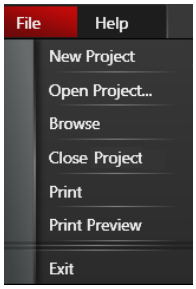


Right click on the table open the dialog

Select Columns	Open a new dialog in which you can select which columns (parameters) are shown in the result table
Copy table content	Copies the entire table contents to the clipboard
Copy selected Rows	Copies the content of selected rows to the clipboard
Copy all	Copies the values of all parameters to the clipboard, including columns not selected under select columns…
Export table content	The table contents can be exported in Excel or CSV format
Export selected rows	Selected contents can be exported in Excel or CSV format
Export all	Entire table contents can be exported in Excel or CSV format
Export table content and show…	The table contents can be exported and show this in Excel
Export selected rows and show	The table contents can be exported and show this in Excel
Export all and show…	Entire table contents can be exported and show this in Excel
Parameter Plot…	You can carry out the data analysis for multiple data records and graphically display it for selected parameters (Ref. page 23)

13-⑥ For information on how to save the analysis results

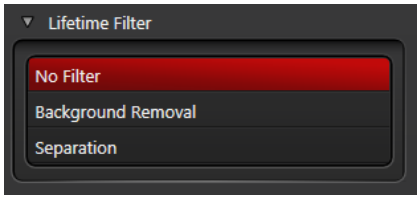
13-⑦ Menu for general setting



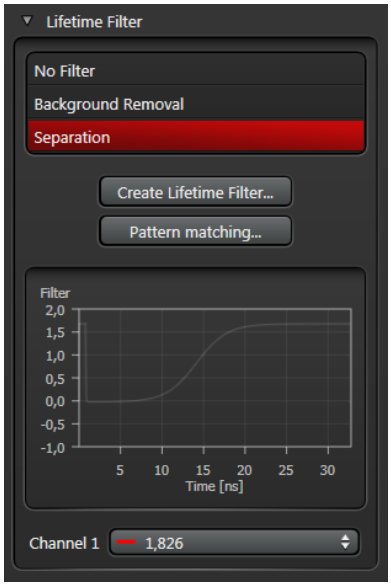
New Project	Create a new project/experiment
Open Project	Open a previously saved experiment in the .lif, .lei or tif format
Browse	Open the project browser
Close	Close the selected experiments in the project directory
Print	Open a printer dialog setting
Print Preview	Show a preview of the printout
Exit	Close the LAS X FLIM/FCS

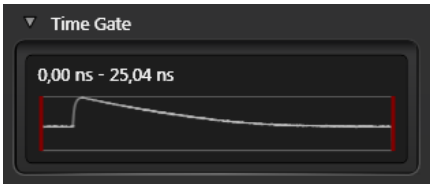
Lifetime Filter：FLCS can separate contribution from background, scattered light.

*For FLCS, the recommended frequency is 40 MHz



No Filter	No filter is applied
Background Removal	Applied a filter that excludes background contribution from the correlation calculation
Separation	This option can be used to remedy crosstalk for FCS data records with a mixture of multiple fluorochromes. In this dialog, you can create 2 different photon filters in order to split and correlate the raw data in various channels. Create Lifetime Filter Pattern matching

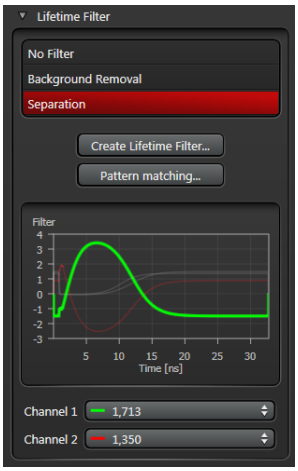




Create Lifetime Filter

You can create a filter, that based on the fluorescent lifetime of photons, achieves separation in the individual fluorescent components. Filter can be selected for FCS and FCCS.

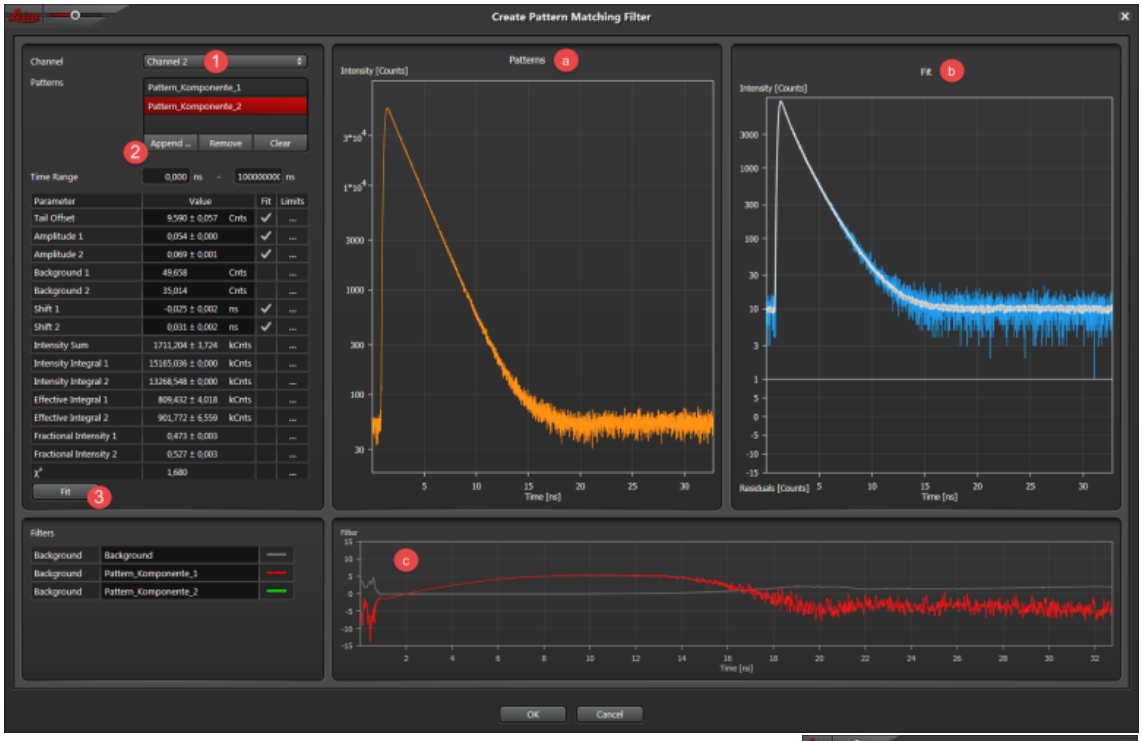
1. ①Select the channel whose fluorochrome you would like to remove under Channel
2. ②Click Select Fit Model
3. ③Under Time Range, select the time gate around the point in time of the laser pulse in which the curve is to take place
4. ④ Under Exponential Components, set the number of fluorescent lifetime components
5. ⑤Apply the preset parameters. (Ref. page 16)
6. ⑥Click Fit to fit the decay curve. The calculation is carried out and the result are shown in Fit diagram ⓐ.



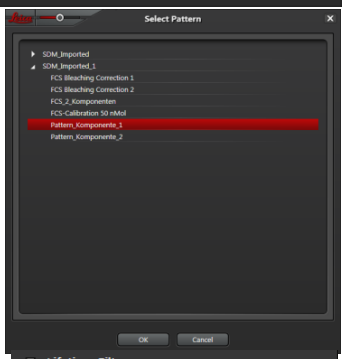
7. ⑦Repeat this step. The software calculation the suitable filters as a result and display them graphically in the dialog field ⓑ.
8. Click OK. The dialog window is close

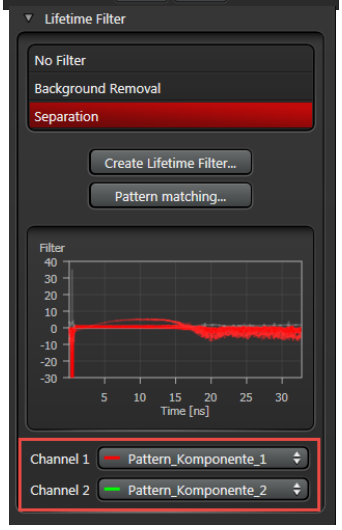
Create Pattern Matching Filter

To be able to separate the fluorochromes in the FCCS, you can create filters in this dialog that are based on acquired reference data records with pure fluorochromes.



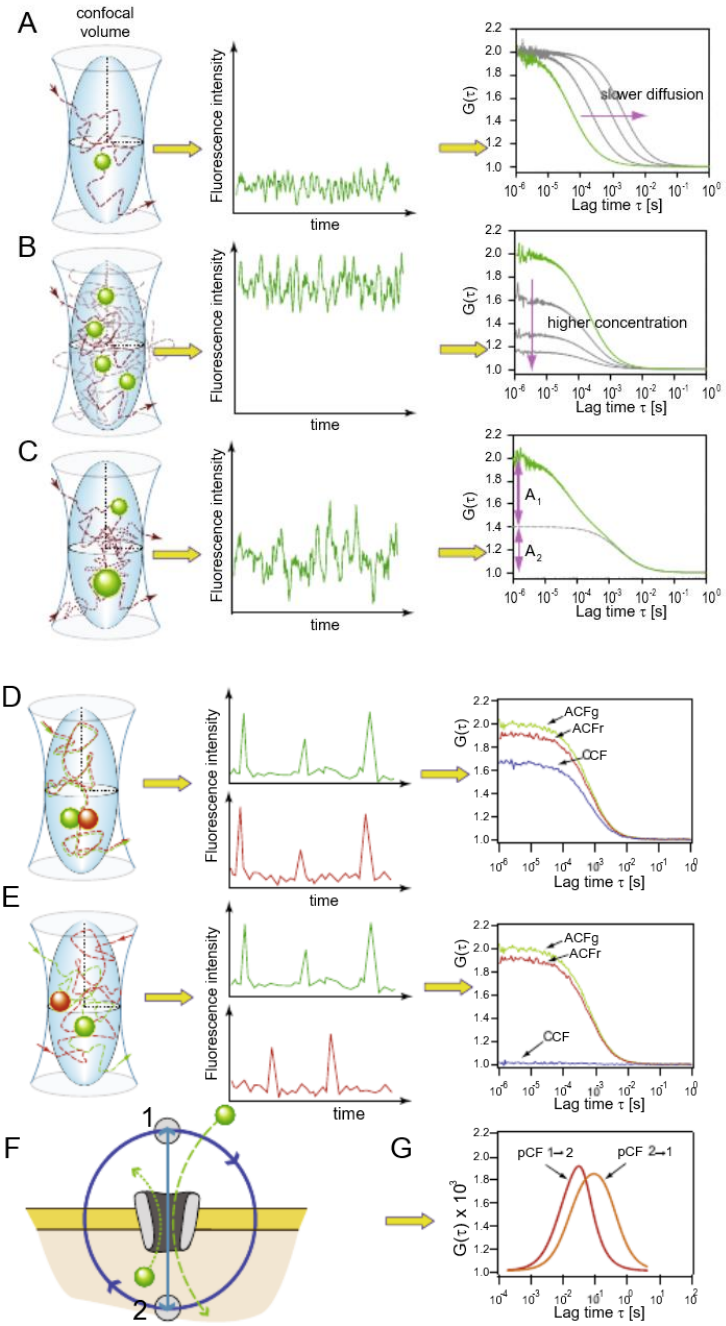
1. ①Select the channel whose fluorochrome you would like to remove under channel
2. ②In the patterns dialog, click the Append in order to load the reference data with a pure fluorochrome (Pattern)
3. The Select Pattern dialog opens
4. The reference data record is displayed in the Patterns dialog and its curve on the right side ⓐ
5. ③Click Fit to fit the reference data record curve. The calculation is carried out the results are shown in the Fit dialog as a diagram.
6. Repeat this step accordingly for the suitable filters as a result and display them graphically in the lower dialog field ⓒ.
7. Click OK. The dialog window is closed





Principal of FCS, FCCS, pCF

FCS(A-C), FCCS(D, E), pCF(F, G)



Effect of the molecular size

The larger molecule, the slower diffusion

Effect of the concentration

The higher concentration, the lower correlation

Effect of mixture

Cross-correlated

Non Coross-correlated

1 -> 2 faster than 2 -> 1

Ref: R. Macháň, T. Wohland / FEBS Letters 588 (2014) 3571–3584

Statistical filter function

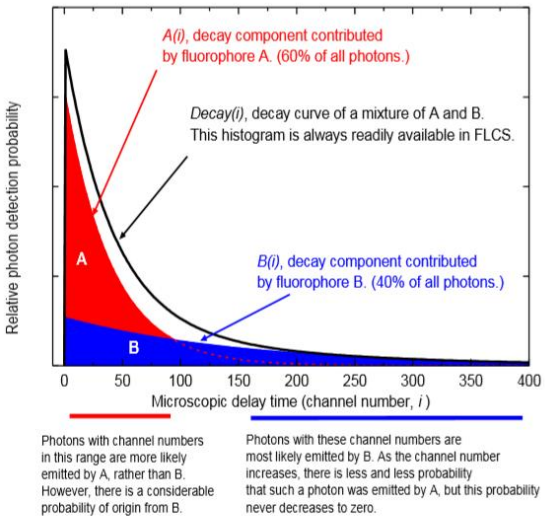


Fig. 2: Pulsed excitation: Note that the vast majority of photons has a very ambiguous origin. However, the relative probability of origin changes during the decay and can be determined for each single photon, looking up its channel number.

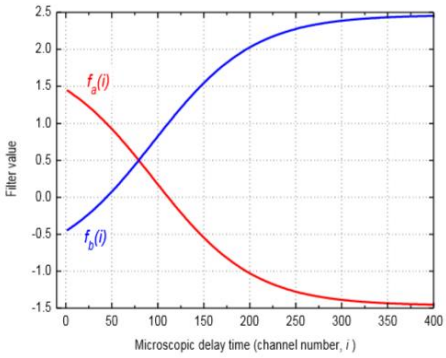
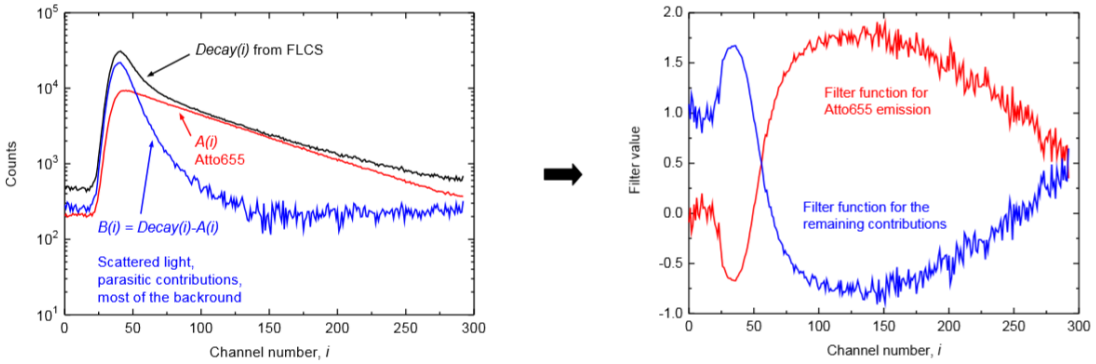


Fig. 3: Example of statistical filter functions

Note that the filter value entering the calculation is not an integer and can be even negative! However, the sum of the two filter values is always exactly 1. Applying the $f_a(i)$ filter during software correlation of *all* photon records one obtains the ACF of compound A. The same holds for $f_b(i)$ and compound B.



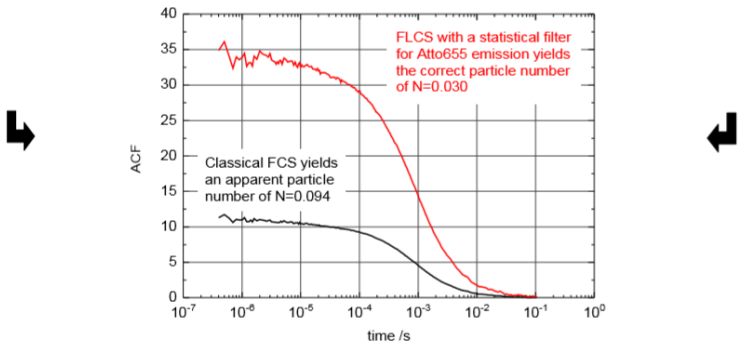


Fig. 4.: FLCS can be used to remove the influence of scattered light. The example shows results from measurements with Atto655.

Ref: PicoQuant Application Note, FLCS – Fluorescence Lifetime Correlation Spectroscopy

Diffusion coefficient D given by

$$\mathrm{D} = \frac{R\mathrm{T}}{6\pi\eta r N_A}$$

Universal gas constant R, the temperature T, the particle radius r, viscosity η, Avogadro constant N_A