

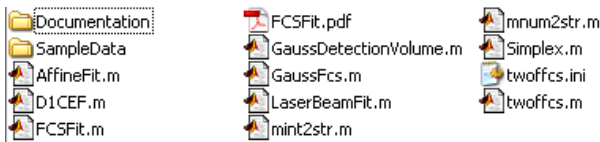


Analysis of 2fFCS data using SymPhoTime and MatLab

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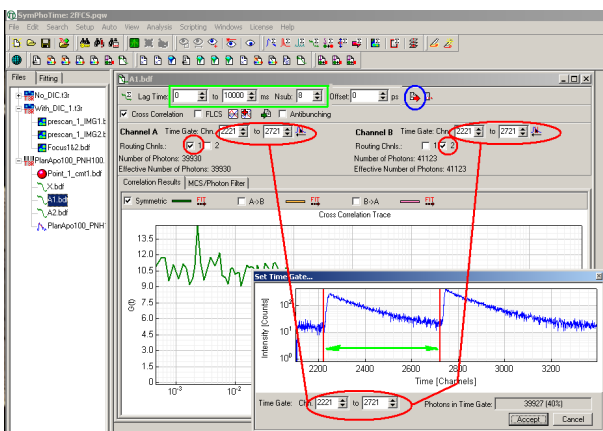
Prerequisites

- Valid dual focus FCS data in any TTTR format (.t3r, .pt3, .pt2, .ht3, .ht2). At least several tens of millions of events (photons) recorded from each foci. It is strongly recommended to use a dual detector setup, so that cross correlation is possible not only between the foci but also between the routing channels (i.e. detectors). The latter is important in order to eliminate the detector afterpulsing effects. Use FLCS filtering by background subtraction if you have a single detector data.
- SymPhoTime ver. 5.1.3 and MatLab ver. 7 or newer.
- A package of MatLab scripts (m-files) collected in a single directory (folder):



Step 1

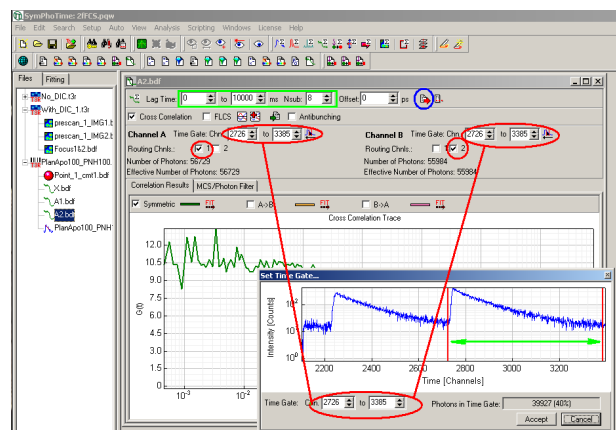
Calculate the autocorrelation function of the first focus.



Set up the *Time Gate* of the first focus correctly and consistently (red ovals). If you have used two detectors, cross-correlate their signal, that means associate *Routing Chnl 1* with data **Channel A** and *Routing Chnl 2* with data **Channel B** (red circles). Select reasonable *Lag Time* limits and sampling values (*Nsub*, green rectangle). After the calculation, export the results as an ASCII file (blue). The file name is arbitrary but must be unique with an extension .dat, for example A1.dat

Step 2

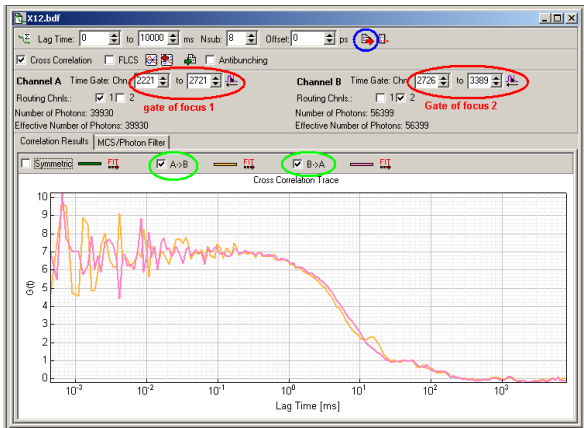
Calculate the autocorrelation function of the second focus.



The best is to create a copy of the bdf result file created by the previous step. (Select *Edit*, then *Duplicate* from the SPT main menu.) Rename this new copy and open it again. Set the appropriate new *Time Gate* correctly and consistently (red ovals). **All the other settings must remain the same as in Step1.** After the calculation, export the results as ASCII file (blue). The file name is arbitrary but must be unique with an extension .dat, for example A2.dat

Step 3

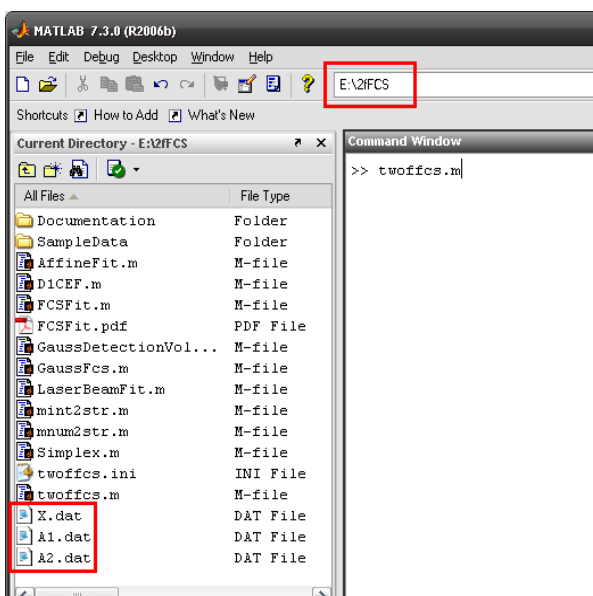
Calculate the cross-correlation of the foci.



Again, the best is to create a copy of one of the previous windows. Rename this copy and open it again. Change the time gates correctly and consistently (red). Note the different *Time Gates* for data **Channel A** and data **Channel B**. All the other settings must remain the same as in Step1 and Step2. We will use both the cross-correlation of focus1→focus2 and the cross-correlation of focus2→focus1. Both are calculated automatically by SymPhoTime. By default, only their sum (called Symmetric) is displayed. Modify the FCS dialog (green ovals) if you want to see them. Displaying these curves has no effect on the exported data, all three calculated curves and their common time axis is will be exported in a single ASCII file (blue). The file name is arbitrary, but must be unique with an extension `.dat`, for example `X.dat`.

Step 4

Prepare the exported ASCII files for fitting with MatLab scripts. It has some advantage, if you copy the ASCII files into the directory where the scripts are. Start the MatLab. (Ver.7 or newer is necessary.) Set the *Current Directory* in MatLab to



the folder containing the scripts and the ASCII data. In MatLab command line, type `twoffcs`. This starts the script `twoffcs.m`, which is a tool for assembling the necessary input data set. It also collects the required input parameters for fitting.



The first three input dialogs ask for the ASCII input data file names. The fourth one collects the following set of parameters related to the experiment:

These parameters are constants determined by the optical setup (MicroTime 200); all must be entered and must have meaningful values. The script will remember (save) the last set of values, so next time you can proceed faster just by clicking on OK.

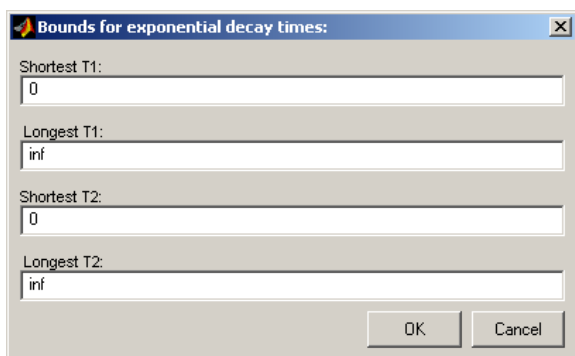
Note that the optical *Magnification* equals to the value written on the objective only when the proper tube lens is used. This is the case of MicroTime 200 microscopes, but may be different on other, home built or modified set-ups. A very important parameter is the lateral distance between the two foci. If you do not know the exact value for your setup, enter here a reasonable estimate (for example 400 nm) to start with. Note that the fitted diffusion constant is a very sensitive function of this parameter. (See **Calibration** further below.)

The next input dialog asks for initial estimates of the parameters to be optimized:

w_0 and R_0 are related to the shape of the confocal volume, more precisely speaking to the model function used to describe the molecule detection function (MDF). One of the cornerstones of 2fFCS is that it uses a very realistic description of the real confocal volume instead of the common Gaussian approximation. The shape of the confocal volume is obtained by optimization of these two parameters. The mathematical form of the MDF and further details can be found in the Documentation sub-folder.

Up to two diffusion constants (D_1 and D_2) can be optimized simultaneously, corresponding to the "model of two independent diffusing particles". At least D_1 must be specified. As an option, up to two exponentials can be included in the model, e.g. in order to approximate the effect of photophysical processes such as transition to triplet state or isomerization. However, it is strongly recommended to use the simplest possible model for fitting. Estimates of D_2 or T_2 are accepted only when there are already a valid values entered for D_1 and T_1 .

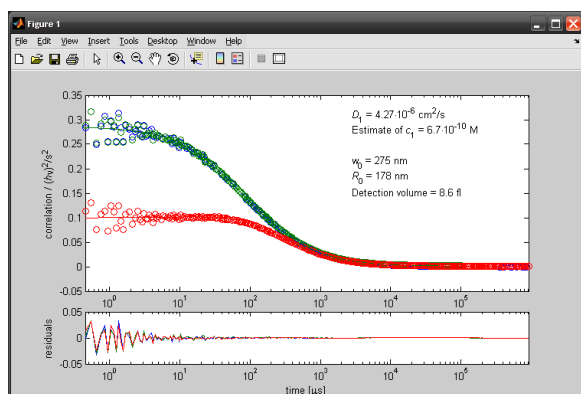
Only if T_1 , or both T_1 and T_2 are specified, there is an additional input dialog before starting the fitting:



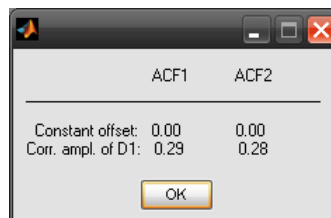
Here you can specify bounds for the exponential relaxation times. This is useful for example when a known triplet decay time is to be fixed in the model.

Step 5

The data set and collected parameters (plus some more) are automatically passed to the main fitting routine, **FCSFit.m**. You can watch the progress during the optimization. After a convergence (or latest after having performed 400 iterations) the script terminates and provides two graphical outputs:



In the inset of the main graph, D_1 (and also D_2 , T_1 and T_2 , if they were included in the model) is displayed as the major result. w_0 and R_0 are results of optimization too, and used to calculate the detection volume. The concentration value is just an estimate, based on the detection volume and the (average) correlation amplitude(s) of diffusional component(s). Note that we have two autocorrelation curves, thus two sets of optimized amplitudes and offset. These are displayed in the second output window:



Note

FCSFit.m calculates and returns back to **twoffcs.m** many more output values. After the calculations, these are available in the current MatLab workspace as new variables and arrays. The complete list of these output parameters (typed in MatLab style) is:

```
dc    % fitted values of diffusion
      % coefficient(s) in cm2/s

v     % estimate of detection volume in
      % micrometer3, that means
      % femtoliters.

conc  % estimate of concentration in M =>
      % does not work for FCS curves from
      % SPT

w0    % fitted value of w0 parameter
      % (guess value was p(1))

r0    % fitted value of R0 (guess value
      % was p(2))

triplet % fitted values of exponential
      % decay time(s) of fast photophysical
      % process(es)

c     % amplitudes of different
      % components contributing to
      % correlation

velo  % fitted values of velocity vector

err   % fit error, defined as sum((y
      % z)^2), where y are the data and z
      % the fitted curve

z     % fitted curves
```

Experienced MatLab users may want to process further (e.g. to display) these values by modifying the **twoffcs.m**. A full description of all possible input and output parameters of **FCSFit.m** is available in its associated documentation, **FCSFit.pdf** that can be found in the Documentation sub-folder.

Calibration:

How to determine the exact lateral distance between two foci

The major result of the fitting process, the absolute diffusion constant is very sensitive to this parameter. It is possible to obtain a good estimate by dual-focus imaging of a sub-resolution size fluorescent particle, for example using the 100 nm diameter TetraSpeck bead slide delivered with the MicroTime 200. However, the most accurate way to measure the exact distance is to calibrate your set-up. Perform a 2fFCS measurement of a standard sample (see below) with a known absolute diffusion coefficient. In order to determine the distance between foci with sufficient precision, a high quality data set is required (long measurement, good signal to noise ratio, large TTTR file).

Run several fitting sessions in MatLab and systematically vary the distance parameter, until the fitted diffusion coefficient equals to the published value. This focal distance is the correct one for your optical configuration (excitation wavelength, DIC-prism, microscope objective).

Properties of a few reference compounds suitable for calibration purposes are summarized in the following table:

Fluorophore	λ_{Em} maximum in nm	Diffusion coefficient in water at 25°C (298.15 K) in $10^{-6} \text{ cm}^2\text{s}^{-1}$	Hydrodynamic radius in Å
Atto655-maleimid	686	4.1 ± 0.1	6.0 ± 0.1
Atto655-carboxylic acid	685	4.3 ± 0.1	5.7 ± 0.1
Atto655-NHS ester	685	4.3 ± 0.1	5.7 ± 0.1
Cy5	670	3.6 ± 0.1	6.8 ± 0.1
Alexa 647	665	3.3 ± 0.1	7.4 ± 0.1
Alexa 633	647	3.4 ± 0.1	7.2 ± 0.1
Rhodamine 6G	550	4.1 ± 0.1	6.0 ± 0.1
Oregon Green 488	550	4.1 ± 0.1	6.0 ± 0.1
Atto488-carboxylic acid	523	4.0 ± 0.1	6.1 ± 0.1
TetraSpeck Beads, 0.1µm diameter	430 515 580 680	0.044 ± 0.07	556 ± 6

Source:

Müller C. B., Loman A., Pacheco V., Koberling F., Willbold D., Richtering W., Enderlein J.:
Precise measurement of diffusion by multi-color dual-focus fluorescence correlation spectroscopy
EPL, 83 (2008) 46001, <http://dx.doi.org/10.1209/0295-5075/83/46001>

Müller C. B., Weiß K., Richtering W., Loman A., Enderlein J.:
Calibrating differential interference contrast microscopy with dual-focus fluorescence correlation spectroscopy
Optics Express, 16 (2008) 4322, <http://dx.doi.org/10.1364/OE.16.004322>

Dertinger T., Pacheco V., von der Hocht I., Hartmann R., Gregor I., Enderlein J.:
Two-focus fluorescence correlation spectroscopy: A new tool for accurate and absolute diffusion measurements
ChemPhysChem, 8 (2007) 433, <http://dx.doi.org/10.1002/cphc.200600638>

Korlann Y., Dertinger T., Michalet X., Weiss S., Enderlein J.:
Measuring diffusion with polarization-modulation dual-focus fluorescence correlation spectroscopy
Optics Express, 16 (2008) 4609, <http://dx.doi.org/10.1364/OE.16.014609>

Dertinger T., Ewers B.:
Unpublished results PicoQuant GmbH (2008)

Loman A., Müller C. B., Koberling F., Richtering W., Enderlein J.:
Absolute and precise measurements of the diffusion of small fluorescent dye molecules across the visible spectrum.
Poster, 14th International Workshop on Single Molecule Spectroscopy and Ultrasensitive Analysis in Life Sciences, September 17-19, 2008, Berlin, Germany

Note that the **diffusion coefficient is temperature dependent** (Stokes-Einstein relationship), and the **viscosity of water is also a function of temperature**:

$$D(T) = \frac{kT}{6\pi\eta(T)r}$$

where D is the diffusion coefficient, k is the Boltzmann constant ($1.3807 \cdot 10^{-23} \text{ J} \cdot \text{K}^{-1}$), r is the hydrodynamic radius and $\eta(T)$ is the temperature dependent viscosity.

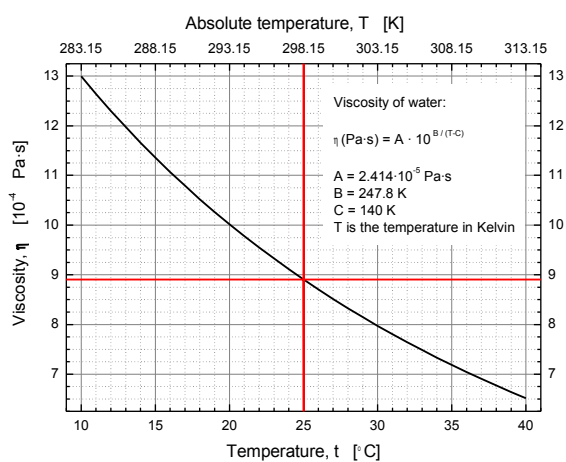
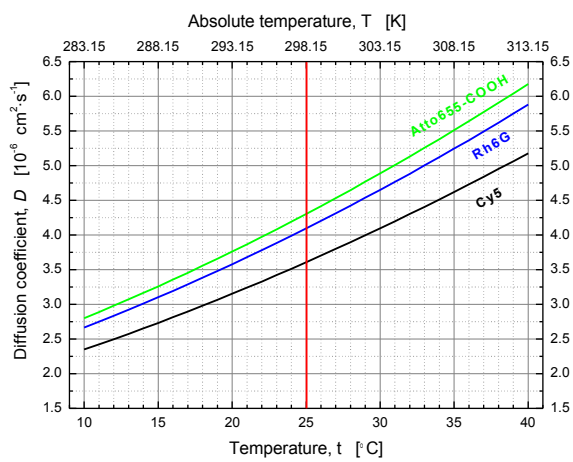
If the calibration experiment is not performed at

$t = 25^\circ\text{C}$, you need to recalculate the tabulated values using this equation:

$$D(T) = D(25^\circ\text{C}) \cdot \frac{T}{298.15 \text{ K}} \cdot \frac{8.9 \cdot 10^{-4} \text{ Pa} \cdot \text{s}}{\eta(T)}$$

$$= D(25^\circ\text{C}) \cdot \frac{t + 273.15}{\eta(t)} \cdot 2.985 \cdot 10^{-6} \text{ Pa} \cdot \text{s} \cdot \text{K}^{-1}$$

The temperature dependence is strong and unfortunately often forgotten when comparing results. The effect is illustrated below for three popular fluorophores and the viscosity of water:



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