

Fluorescence Lifetime Correlation Spectroscopy using the SymPhoTime Software: FLCS Tutorial



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Introduction

FLCS is an elegant and very powerful extension of conventional FCS. It can be regarded as a fusion of Time-Correlated Single Photon Counting and Fluorescence Correlation Spectroscopy. The key feature of this method is the possibility to separate the autocorrelation function of various signal components.

FCS is based on recording intensity fluctuations detected by sensitive photon counting detectors. Unfortunately, the fluorescence of molecules diffusing through the confocal volume – our main interest – is not the only signal source. There are unavoidable dark counts and afterpulses coming from the detectors. Raman and Rayleigh scattered excitation photons, as well as fluorescence from impurities are detected too. All these components contribute to intensity fluctuations and influence the result. The shape of auto- or cross-correlation function (ACF or XCF hereafter) becomes complicated or distorted. Attempts to include these effects into the analysis lead to complicated mathematical expressions and multiparameter fitting formulae. Due to mutual influences of adjustable model parameters, resolving various contributions to the ACF is generally difficult and often impossible. Instead of fitting a multiple parameter model to a complicated correlation function, FLCS calculates a separate ACF for each signal component. Because these are identified by their temporal behavior on the picosecond to nanosecond time scale (TCSPC), the FLCS experiment must be performed using pulsed laser excitation instead of a conventional CW illumination. The second basic requirement is the so-called Time-Tagged Time-Resolved photon counting detection. In principle, all MicroTime 200 microscope users as well as FCS capable PicoQuant LSM Upgrade Kit owners are qualified to perform FLCS.

This application note describes three examples demonstrating how FLCS solves many of the inherent problems of classical FCS. Data analysis was performed with SymPhoTime version 4.7.2 and the text uses the terminology of this software. It is assumed that the reader already understands the basic principles of FLCS.

For a primer on FLCS please refer to the following publications:

[1] Böhmer M., Wahl M., Rahn H.-J., Erdmann R., Enderlein J.:

Time-resolved fluorescence correlation spectroscopy
Chemical Physics Letters, Vol.353, p.439-445 (2002)
(This is the seminal paper presenting the idea.)

[2] Enderlein J., Gregor I.:

Using fluorescence lifetime for discriminating detector after-pulsing in fluorescence-correlation spectroscopy
Review of Scientific Instruments, Vol.76, 033102 (2005)
(Another possible application of FLCS.)

[3] Benda A., Hof. M., Wahl M., Patting M., Erdmann R., Kapusta P.:

TCSPC upgrade of a confocal FCS microscope
Review of Scientific Instruments, Vol.76, 033106 (2005)
(An example of hardware implementation and application examples including cross-correlation.)

[4] Kapusta P., Wahl M., Benda A., Hof M., Enderlein J.:

Fluorescence lifetime correlation spectroscopy
Journal of Fluorescence, Vol.17, p.43-48 (2007)
(Pictorial explanation of the basic principles)

[5] Gregor I., Enderlein J.:

Time-resolved methods in biophysics. 3. Fluorescence lifetime correlation spectroscopy
Photochemical&Photobiological Sciences, Vol.6, p.13-18 (2007)
(A thorough introduction to the method.)

Purging detector dark counts and afterpulsing artefacts

Sample: 5 nM Atto655 in aqueous buffer at 20°C.

Instrument: MicroTime 200 with LDH-P-C-640B laser diode pulsing at 20 MHz as an excitation source.

For demonstration purposes, we have split the collected fluorescence with a 50/50 beam splitter and detected it with two routed SPAD detectors. This is not required by FLCS, but will allow us to compare the result with the outcome of a cross-correlation analysis. The following FLCS procedure can be applied to whichever selected routing channel. We will treat the sum of two routing channels as a single intensity record, which is equivalent to the simplest single detector FCS setup.

FLCS analysis starts with a preliminary inspection of the overall TCSPC histogram of the measurement. Atto655 has a monoexponential decay and there is no sign of a scattering contribution (spike at the beginning) in this decay curve [see Fig.1]. This is expected, as the solution is fairly concentrated and the detected signal should be dominated by the Atto655 fluorescence. There is, however, a considerable background level under the decay curve caused by unavoidable dark counts and afterpulsing. Dark counts due to thermal noise are entirely uncorrelated, random events with even distribution between the histogram time channels. Detector afterpulses are triggered by true photon or dark counts. Afterpulses are correlated events on a longer

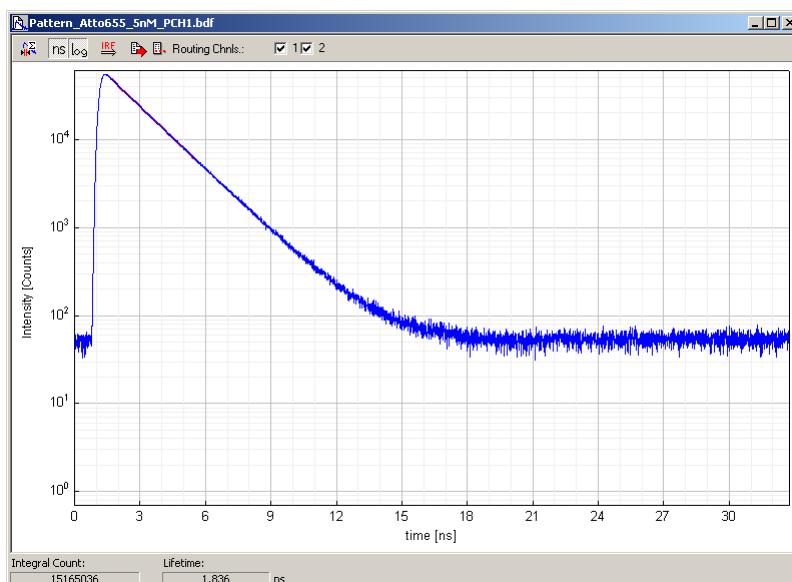


Fig. 1: Single exponential decay of Atto655 with a considerable background

(100 ns to microseconds) time scale. The 20 MHz laser repetition rate limits the histogram time span to a maximum of 50 ns. Longer delay times are wrapped around. For example, an afterpulse occurring 520 ns after a true count would be sorted into a time channel which is offset by $520 \text{ ns} - 10 * 50 \text{ ns} = 20 \text{ ns}$ from the time channel corresponding to the triggering event. As a result, afterpulses appear completely uncorrelated on the TCSPC time scale. They are also randomly distributed among the histogram channels.

A fundamental assumption of FLCS is that the identified signal components have characteristic, constant TCSPC patterns, and the momentary TCSPC histogram of whichever short signal period (e.g. a photon burst) is a linear combination of these and only these patterns.

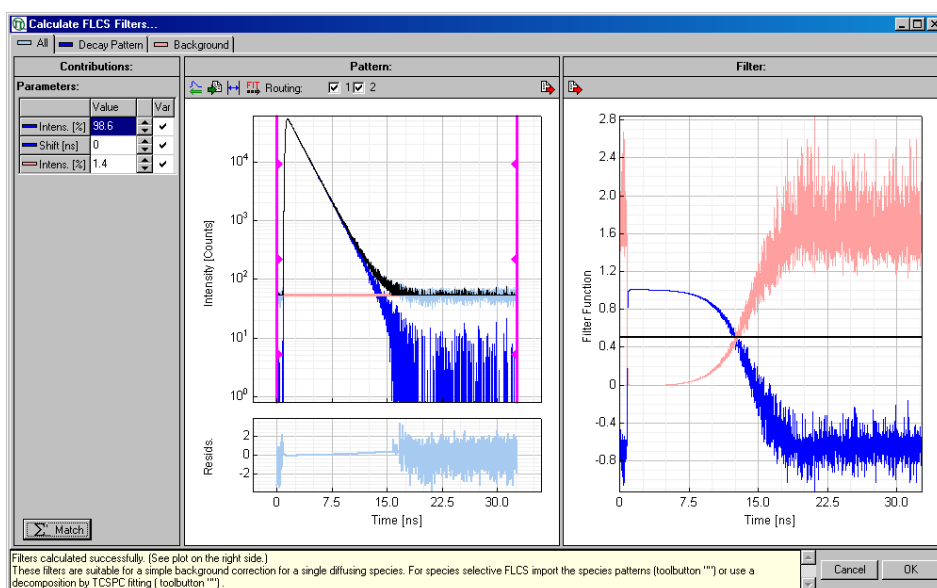


Fig. 2: Simple decomposition using background line subtraction

In this example we have only two patterns. The flat background line can be easily distinguished and separated from the typical single exponential decay of Atto655 fluorescence [see Fig. 2]. SymPhoTime automatically determines the average background value (horizontal line) and subtracts it from the total histogram (black curve). The resulting component histogram (blue) is the pure Atto655 fluorescence pattern.

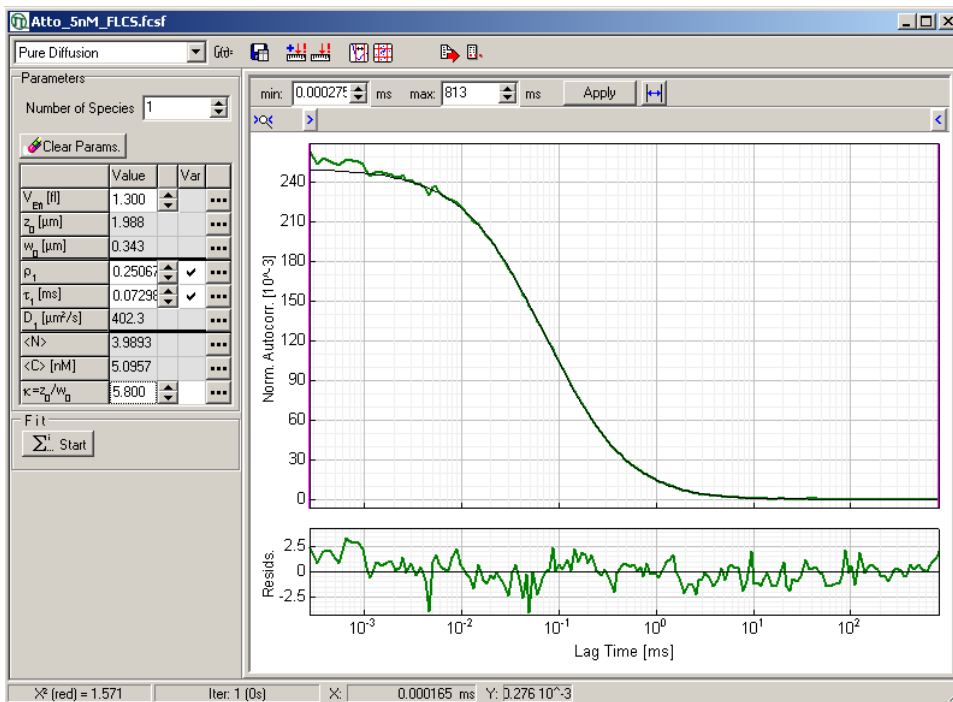


Fig. 3: FLCS filtered ACF of Atto655 and a single particle diffusion model fitted to it.

Decomposition reveals that almost 99% of all counts are due to photons emitted with Atto655 decay pattern. Filter functions corresponding to pure fluorescence and the background are displayed at the right hand side. Using the appropriate filter we obtain a separated ACF of pure Atto655 fluorescence. Because Atto655 has uncomplicated photophysics [2], the simplest FCS model (single particle diffusion) can be fitted to the FLCS result [see Fig. 3].

Up to now, we have intentionally neglected the detector routing information. Sorting the TTR records according to their routing bits, we are able to cross-correlate the signal of the two detector channels. In order to prove that FLCS gives the correct result even with a single detector setup, we now calculate and fit the XCF of the same measurement [see Fig. 4].

Both FLCS and two detector cross-correlation yield practically identical results for Atto655 at this concentration. Support plane error analysis performed with SymPho-

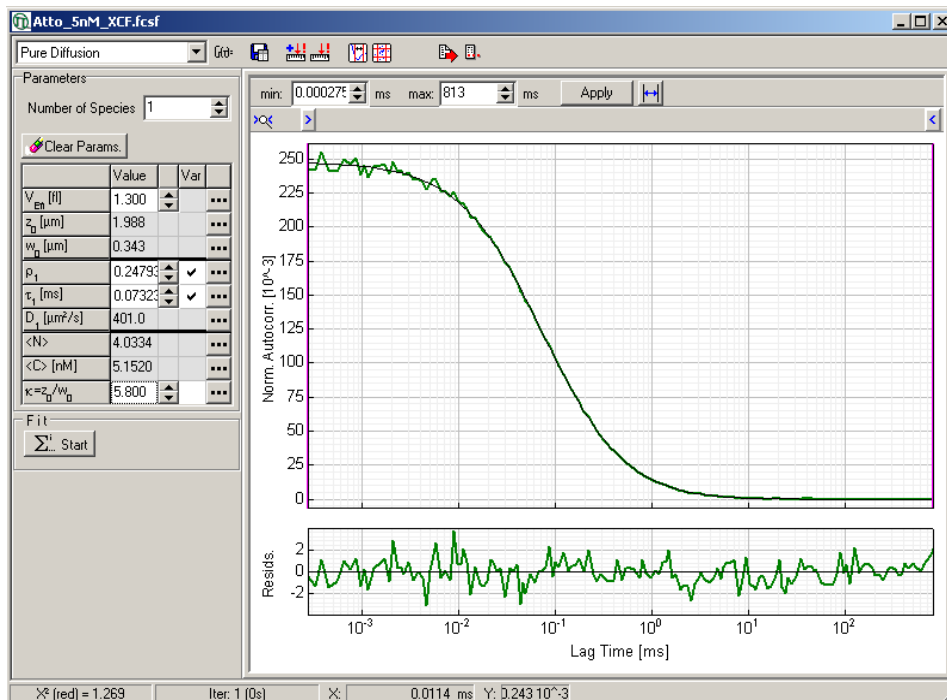


Fig. 4: Two detector XCF of Atto655 solution and a single particle diffusion model fitted to it.

Time reveals that the recovered τ_1 -values in Fig. 3 and Fig. 4 are equal within parameter precision, but the FLCS amplitude $\rho=0.251\pm 0.001$ is slightly higher than $\rho=0.248\pm 0.001$ obtained by XCF. This is another advantage of FLCS over two detector XCF. The former also eliminates the damping effect of uncorrelated background. This influence is hardly visible here, but becomes significant at lower signal to background ratios.

Finally, let us calculate the ACF with a conventional FCS approach [see Fig. 5] Detector afterpulsing causes a fast initial decay and hampers

the analysis. Note that dark counts and afterpulsing together contributed only 1.4% of the total detected counts in this experiment!

Summary

Afterpulsing severely distorts the outcome of a standard FCS measurement. An expensive work-around is to use a two detector setup and cross-correlation. FLCS solves the problem effortlessly even in a single detector setup.

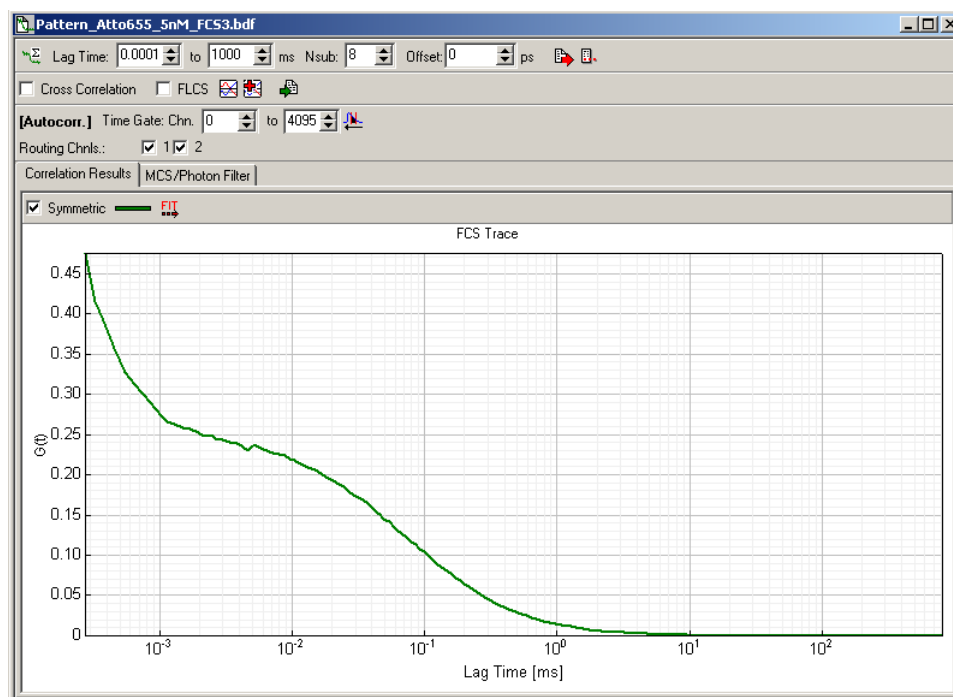


Fig. 5: ACF of Atto655 obtained by conventional FCS approach.

Correcting for scattered excitation light

Sample: 15 pM Atto655 solution in aqueous buffer at 25°C temperature.

Instrument: MicroTime 200 with LDH-P-C-640B laser pulsing at 40 MHz as an excitation source.

Just like in the previous example, the collected fluorescence has been split by a 50/50 beam splitter and detected with two routed SPAD detectors. We ignore the routing information, thus treating the sum of two routing channels as a single intensity record. This is equivalent to the simplest single detector FCS setup.

Again, we start with a preliminary inspection of the overall TCSPC histogram. In order to fulfill the basic requirements of FLCS, we have to identify all TCSPC patterns [see Fig 6].

This is a very diluted solution, therefore a clearly visible scattering spike appears at the beginning. The relative contribution of the background (due to dark counts and afterpulsing) is very large, almost 300 counts in every time channel. A simple background subtraction alone would not lead to correct results, since it cannot account for the scattering. We have to quantitatively separate the scattered excitation light and the background from the Atto655 fluorescence.

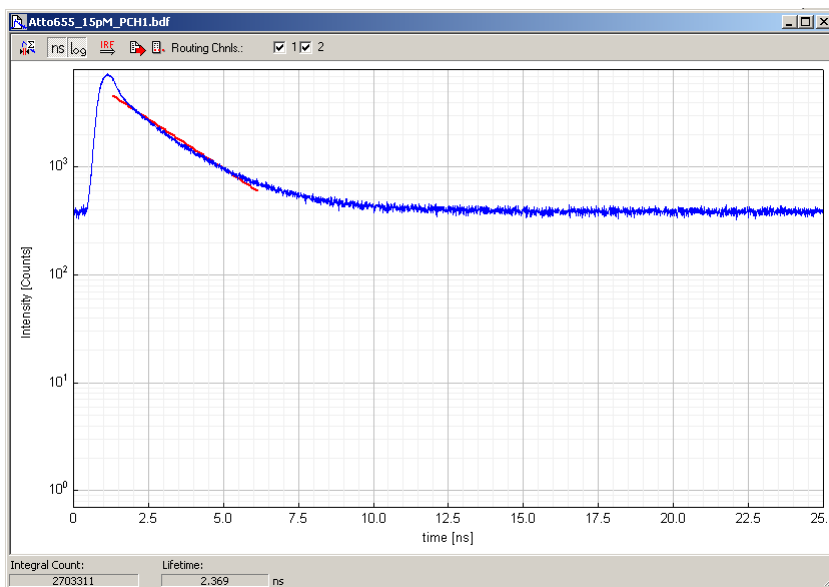


Fig. 6: Decay curve of Atto655 influenced by scattered photons and high background.

A background subtracted TCSPC histogram of a 30-times more concentrated Atto655 solution measured under identical conditions is a good approximation of the pure Atto655 pattern, because the scattering contribution is negligible there.

A pure scattering pattern can be obtained by measuring a pure buffer solution without an emission filter (in fact, this is a standard IRF measurement). We decompose the multicomponent decay curve using these two experimentally obtained patterns together with the flat background pattern which is included automatically [see Fig. 7].

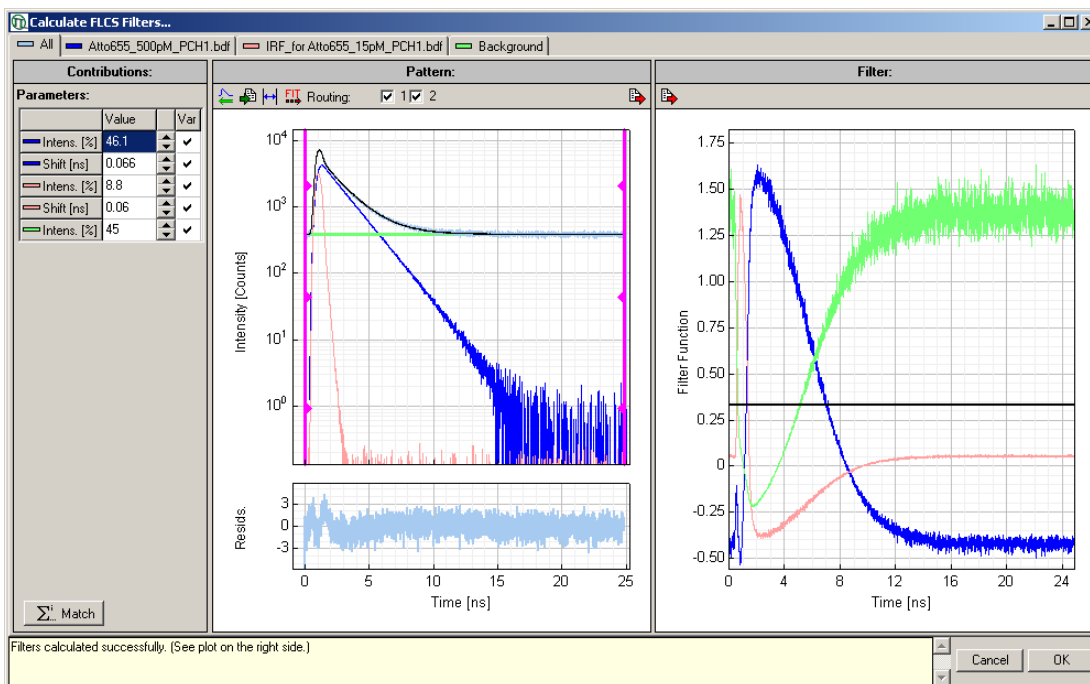


Fig. 7: Decomposition into three patterns: scattering, single exponential decay and flat background

Although not perfect, the decomposition result is sufficient. (Most of the residuals are within ± 3 standard deviations.) Note that ca. 9% of the counts are actually scattered excitation photons; pure Atto655 fluorescence accounts for only 46% of the detected intensity. Using the Atto655 filter function (blue curve in Fig. 7, right hand side), we obtain the ACF shown in Fig. 8.

Our main interest is now the correct value of the correlation amplitude. $G(t \rightarrow 0)$ approaches 85, corresponding to an average particle number of $N = 0.012$ in the confocal volume, as expected for

this Atto655 concentration. (We have found $N = 4$ for a 5 nM solution in the previous example.) The significance of this becomes obvious when compared to the standard FCS result. In order to avoid the afterpulsing artifacts we perform a two detector cross-correlation. The result can be seen in Fig. 9.

It is not surprising that the correlation amplitude, $G(t \rightarrow 0)$ is now much smaller. By FCS fitting we found that the diffusion parameters are practically the same, but the concentration calculated from the apparent particle number and the same confocal volume size is wrong (70 pM).

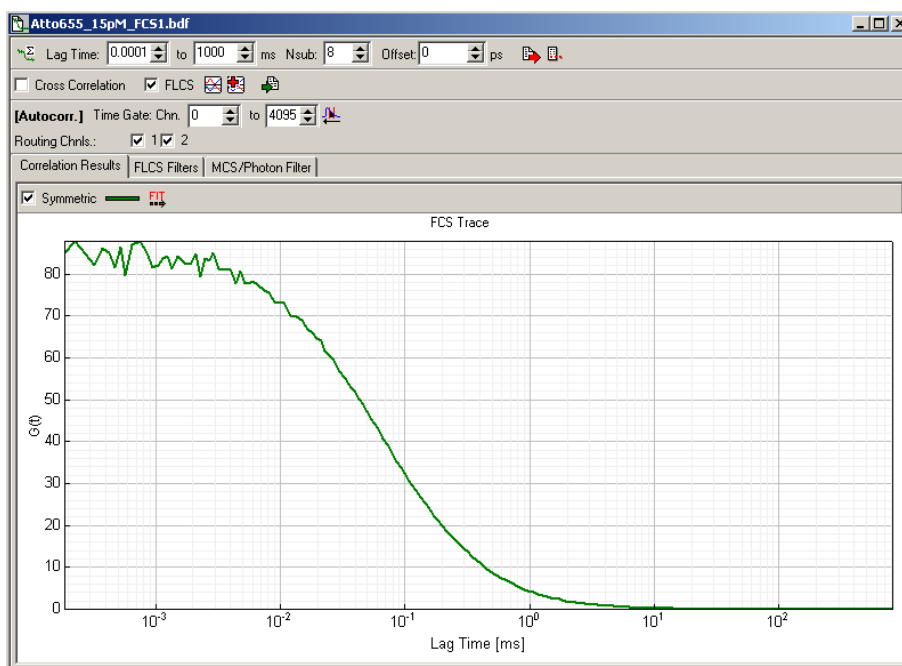


Fig. 8: FLCS filtered ACF of a 15 pM Atto655 solution. $G(t \rightarrow 0)$ approaches 85.

Summary

FCS fails to recover the correct particle number when there is a significant contribution of uncorrelated (on FCS time scale) background. Thanks to their characteristic, easily obtainable TCSPC patterns, FLCS provides a straightforward and robust tool to discriminate these unwanted components.

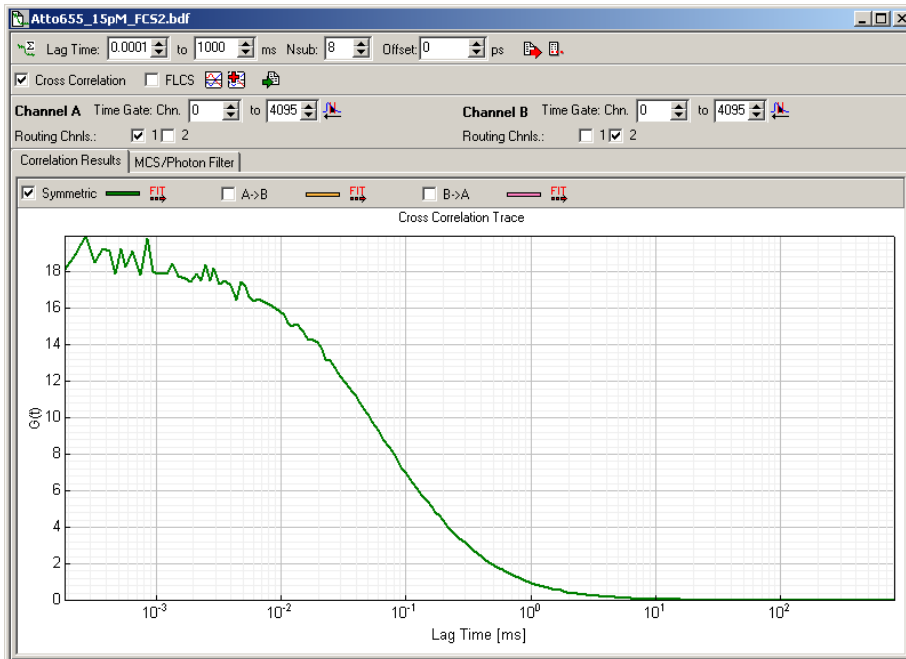


Fig. 9: Conventional XCF of the same, 15 pM Atto655 solution. $G(t \rightarrow 0)$ approaches only 19.

Separating the contribution of two fluorophores

Sample: Equimolar solution of Atto655 and Cy5 in aqueous buffer at 25°C, prepared by mixing equal amounts of 1 nM solutions.

Instrument: MicroTime200 system equipped with two SPAD detectors, 50/50 beam splitter, LDH-P-C-640B laser diode pulsing at 20 MHz.

This time we start with conventional FCS cross-

correlation calculation. Although the resulting XCF (corresponding to the ACF of the mixture) is free of afterpulsing artefacts, it is still complicated due to the photophysics of Cy5 (light driven transition between fluorescent and non-fluorescent state) and the presence of two independently diffusing components. Because the diffusion coefficients of these dyes are similar, it is impossible to resolve their contributions by FCS model fitting. Indeed, already a model with a single diffusing particle with triplet term describes the XCF in Fig. 10.

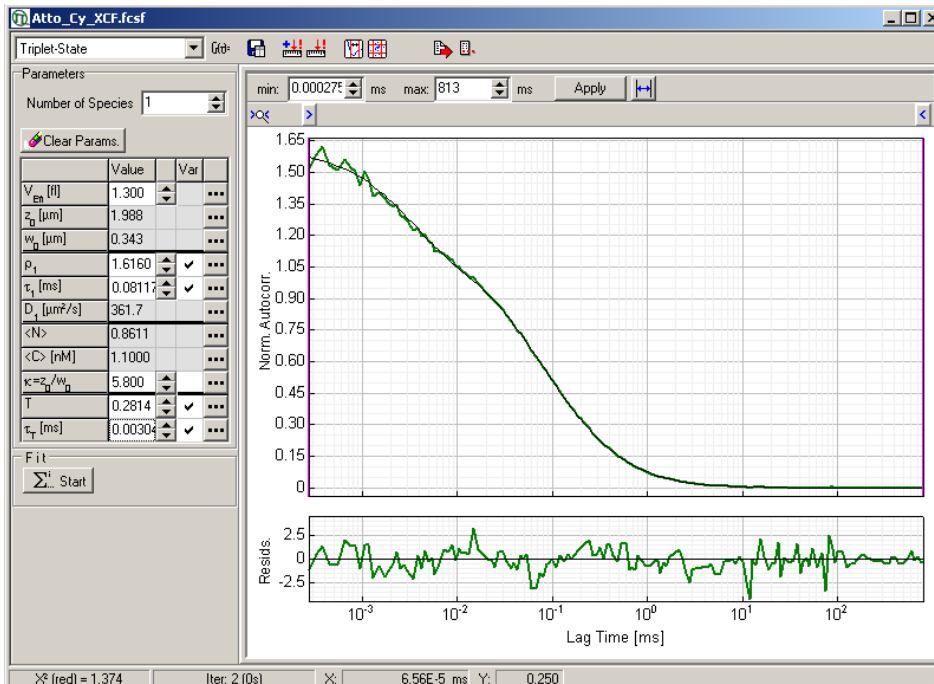


Fig. 10: Two-detector XCF of a mixture of Cy5 and Atto655. It is not possible to resolve two slightly different diffusion times.

FLCS is a much more powerful tool. We need to know, however, the decay patterns of the dyes. These patterns can be easily obtained by e.g. reference measurements of pure Cy5 and Atto655 stock solutions. The decay decomposition is nearly perfect. [Fig. 11.]

Selecting the corresponding filter function for correlation calculation we obtain the separated ACFs of the components.

Fig. 12 shows the separated ACF of Atto655 and the fit of the single particle diffusion model (note: there is no triplet term necessary).

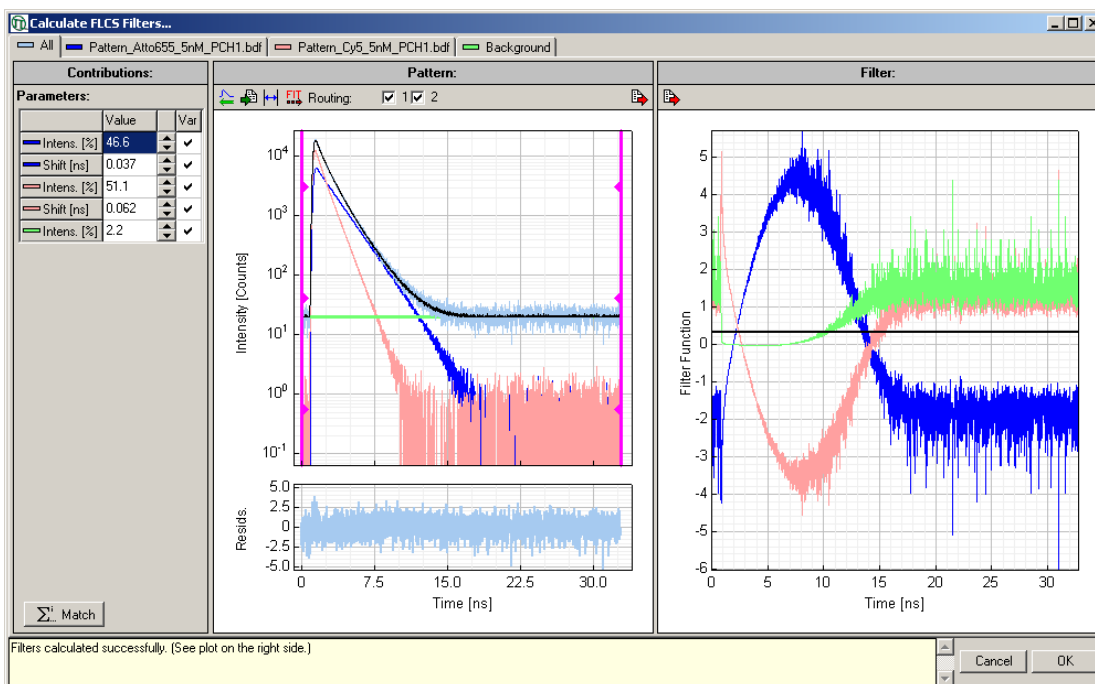


Fig. 11: Decomposition of the mixture decay into two decay patterns plus the background.

The separated ACF of Cy5 and the fit of the single particle diffusion with a triplet-term are shown in Fig. 13.

The expected ratio of particle numbers is 1 for an equimolar solution. We have got $\langle N \rangle_{\text{Atto655}} / \langle N \rangle_{\text{Cy5}} = 1.06$. Moreover, the recovered diffusion coefficients agree well ($\pm 10\%$) with those obtained for pure stock solutions at the same temperature.

Summary

FLCS makes possible to simultaneously monitor the concentration and diffusion speed of two dyes with completely overlapping emission spectra, with very similar diffusion coefficients and using only a single detector.

Concluding remarks

The above application examples demonstrate the robustness of FLCS in comparison to conventional FCS. Efficient use of the TCSPC information allows to overcome most of the inherent limitations of FCS. Common errors due to detector afterpulsing and/or scattered light can be avoided by the simplest FLCS methods. The

last example shows a complete separation of two auto-correlation functions using only a single excitation wavelength and a single detector. Such simplicity is in striking contrast to the complexity of advanced FCS setups (e.g. dual-color FCS, alternating laser excitation, etc.) heretofore employed to solve the same problem. Multicolor excitation/detection schemes always suffer from problems related to non-ideal confocal volume overlap or spectral cross-talk. FLCS is limited only by the similarity of the TCSPC patterns.

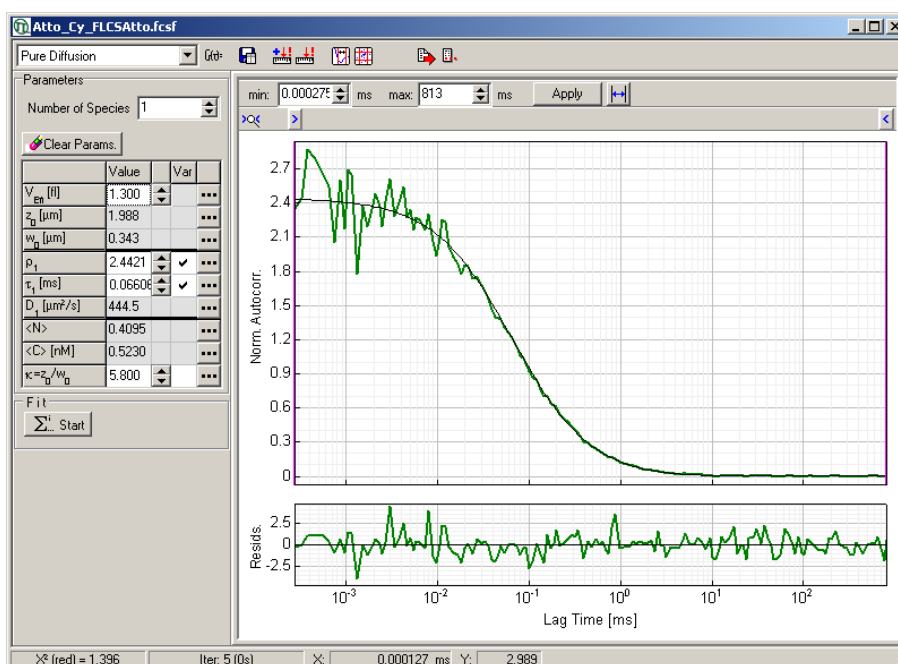


Fig. 12: Separated ACF of Atto655 obtained by FLCS filtering and the single particle diffusion model fitted to it. Note, there is no triplet-like contribution.

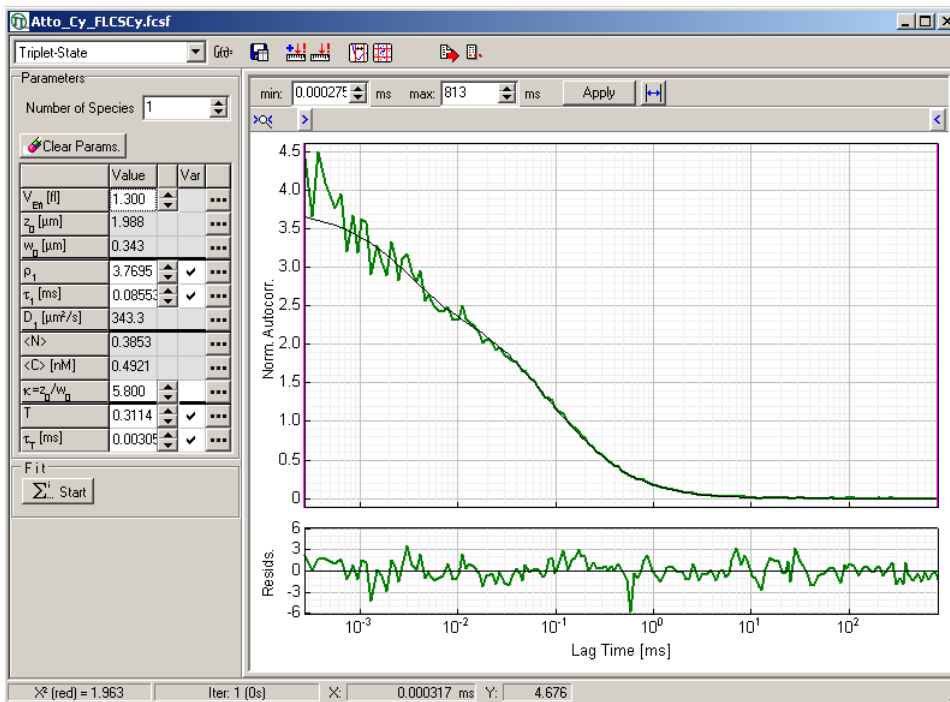


Fig. 13: Separated ACF of Cy5 obtained by FLCS filtering and the single particle diffusion model with a triplet-term fitted to it.

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