Application Note

FLCS – Fluorescence Lifetime Correlation Spectroscopy

Peter Kapusta, Michael Wahl, PicoQuant GmbH
Aleš Benda, Martin Hof, J. Heyrovsky Institute of Physical Chemistry, Prague, CZ
Jörg Enderlein, Forschungszentrum Jülich, Germany

Introduction

The fusion of Time-Correlated Single Photon Counting and Fluorescence Correlation Spectroscopy, called Fluorescence Lifetime Correlation Spectroscopy (FLCS), is a method that uses pico-second time-resolved fluorescence detection for *separating* different FCS-contributions. The main emphasis is on the word *separating*. FLCS does not involve fitting of a complicated model equation to a complicated autocorrelation function. Instead, a *separated* autocorrelation function is calculated for each expected intensity component, emitted for example by various species in the sample. The on-ly assumption is that various emissions have diffe-rent TCSPC histograms (i.e. different fluorescence lifetimes), which is practically always the case. The core of the method is a statistical separation of dif-ferent intensity contributions, performed on a single photon level.

An essential requirement for FLCS is a sub-nano-second pulsed excitation instead of conventional continuous wave (CW) illumination. The second keystone is the ability to simultaneously measure the fluorescence photon arrival time on two diffe-rent time scales: relative to the excitation pulse with picosecond resolution and relative to the start of the experiment with nanosecond precision.

The experimental and analysis technique outlined here seriously pushes the limits of FCS. The idea is already four years old [1], but received only little attention until now, though two recent articles [2, 3] present exciting new developments made possible by utilizing this method. This poster explains the basic principles of FLCS.

The principle

FLCS is best understood in comparison to classical FCS with CW excitation and single channel detec-tion. The autocorrelation function (ACF) is calcu-lated from the various arrival times of detec-ted fluorescence photons, either by a hardware autocorrelator or by software processing of the record of arrival times. If the sample contains, for example, two kinds of diffusing fluorophores, both contribute to the detected intensity fluctuations and the resulting ACF is a complicated superposition, not a linear combination. Taking into account the ubiquitous triplet-populations, scattered excitation light, impurities, etc., the analysis of such an ACF is complex owing to the number of adjustable parameters in the model equation used for fitting. *Resolving* an ACF is difficult. It is well known, for example, that the diffusion times of two species must differ at least by a factor of 1.6 in order to distinguish them [4].
In FLCS data acquisition, the excitation is pulsed and two independent timings are performed and recorded for every detected photon.

1) The *macroscopic* arrival time, measured relative to the beginning of the experiment, contains the information related to, e.g., diffusion and is used just like in a classical FCS.
2) The *microscopic* delay time (in TCSPC traditionally called *channel number*) measured relative to the onset of the excitation pulse, re-presents a new quality which is essential for determining the contribution of the photon to the ACF.

These two timings are evaluated simultaneously when calculating the ACF. However, the calculation itself is not straightforward. One has to separate the various contributions to the detected intensity trace first.

As an example, let us consider a sample with two fluorescent components, A and B, which have different decay behaviour. Let us assume that, say, 60% of all photons captured during the FCS ex-periment were emitted by compound A and the rest by B.

In case of CW excitation (see Fig.1), when a single photon is detected there is always a 60% probability that this was emitted by A. This *probability of origin* is constant in time, because there is no defined moment of excitation. FLCS is based on a simple fact that with pulsed excitation the *probability of origin* of a detected photon is changing in time, because A and B are decaying differently (see Fig.2). The measured *microscopic* delay time of a photon (expressed as a *channel number*, i) allows for a statistical evaluation of its origin. Roughly speaking, if the *microscopic* delay of a photon is long, then it was most probably emitted by the slower decaying fluorophore, though there is a finite probability that it came from the faster decaying one. Note that the TCSPC histogram of the mixture of A and B, *Decay(i)*, is readily available in FLCS.

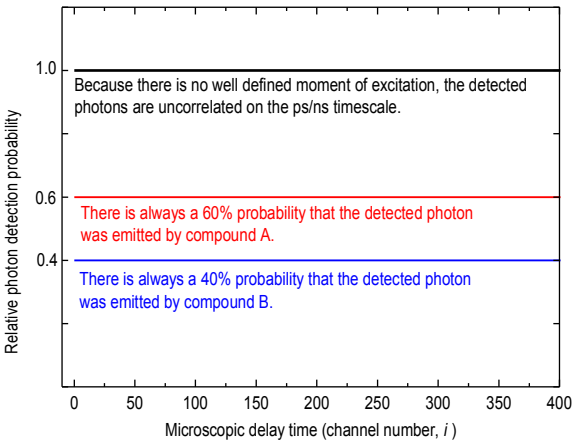

Fig. 1: CW excitation: Note that all photons are equal. On this time scale, the probability of their origin is constant.

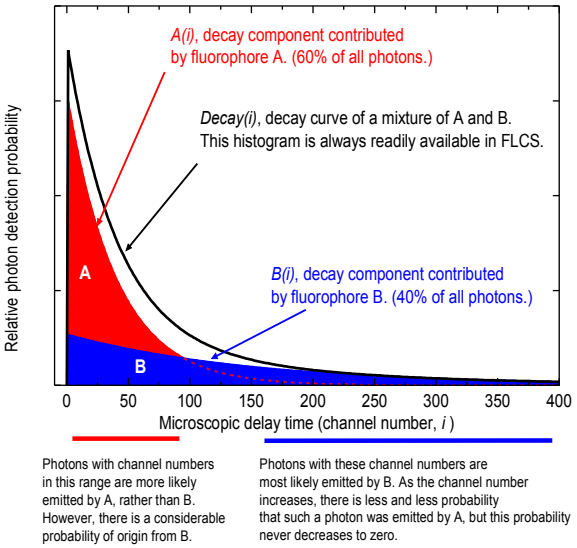

Fig. 2: Pulsed excitation: Note that the vast majority of photons has a very ambiguous origin. However, the relative probability of origin changes during the decay and can be determined for each single photon, looking up its channel number.

Now it remains to explain how to use the *probability of origin* of a photon for the calculation of the ACF of a selected component.
The shape of *A(i)* and *B(i)* histograms can be obtained from independent TCSPC measurements, or from the analysis (e.g. fitting) of *Decay(i)*. Let us normalize *A(i)* and *B(i)* to a unit area, $a(i)=A(i)/\Sigma A(i)$, $b(i)=B(i)/\Sigma B(i)$, that is $\Sigma a(i)=\Sigma b(i)=1$. We will refer to these area-normalized curves as to *decay patterns*. It is obvious that the decay curve of a mixture, *Decay(i)*, can be expressed as a linear combination, $Decay(i)=w_a \cdot a(i)+w_b \cdot b(i)$, where w_a and w_b are the amplitudes of the photon count contribution (in number of photons) of compounds A and B, respectively.

Let us now define two functions, $f_a(i)$ and $f_b(i)$ with the following behaviour:

$$\langle \Sigma\, f_a(i) \cdot Decay(i) \rangle = w_a$$
$$\langle \Sigma\, f_b(i) \cdot Decay(i) \rangle = w_b$$

The brackets $\langle \ldots \rangle$ denote averaging over infinite number of measurements. Further requirement for $f_a(i)$ and $f_b(i)$ functions is that they must simultaneously minimize the relative errors:

$$\langle (\Sigma\, f_a(i) \cdot Decay(i) - w_a)^2 \rangle$$
$$\langle (\Sigma\, f_b(i) \cdot Decay(i) - w_b)^2 \rangle$$

One can see from the functional form of the above expressions, that $f_a(i)$ and $f_b(i)$ act like statistical filters, or weighting schemes, applied to the content of each TCSPC histogram channel. These func-tions can be numerically calculated from *Decay(i)* and from the *decay patterns*. The relevant matrix manipulation is described in reference [1]. The key point is that using $f_a(i)$ and $f_b(i)$ makes possible to statistically separate the intensity contributions of A and B, photon by photon. The *sign and magnitude of a single photon contribution* to an intensity trace can be determined from the photon's *channel number* through the corresponding filter function.

Having obtained $f_a(i)$ and $f_b(i)$, one can start a software correlation, for example as described in reference [5]. In classical FCS, every photon con-tributes equally and the calculation involves only zeros and ones. In FLCS, there is one more step involved: One has to look up the filter value corresponding to the photon's *channel number*. It is valuable to have a look at the shape of $f_a(i)$ and $f_b(i)$ calculated for the above hypothetical example (See Fig. 3).

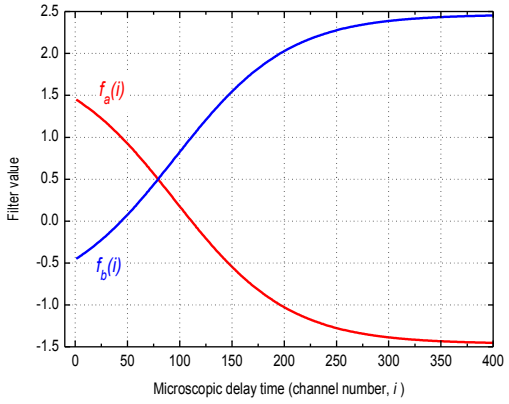

Fig. 3: Example of statistical filter functions

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.

Remarkable features of FLCS

- The method represents a quasi-multichannel detection scheme. FLCS is a lifetime analogy of a multicolor FCS measurement, with several in-herent advantages (see below).
- FLCS goes far beyond the capabilities of simple time-gating [6]. It separates the ACFs of diffe-rent signal components (e.g. emitters) quantita-tively, because it uses all photons. It is possible to separate even more contributions, provided that their decay patterns are sufficiently diffe-rent. In general, no assumptions on the functio-nal form of the decay patterns are involved.
- Owing to the separation principle based on TCSPC decay behaviour, distinct ACFs of two compounds can be obtained even if they have equal diffusion times. FLCS works equally well for emitters with completely overlapping fluores-cence spectra.
- No assumptions are made on the resulting ACFs. One can continue the analysis with the u-sual fitting of standard FCS models.
- Once the intensity contributions are separated, cross-correlation is straightforward. [1,2] Note that the signal containing the various contribu-tions is recorded by a single detector, hence the separately calculated ACFs correspond to the same detection volume, which can be inter-preted as two (or more) perfectly overlapped excitation/detection volumes.
- FLCS is easy to imple-ment. It is not necessary to change the optical hardware of a contem-porary FCS setup whatsoever, because it contains almost all the required components. Affordable diode lasers (e.g., the PicoQuant PDL 800-B, PDL 808 *"Sepia"* or PDL 828 *"Sepia II"* driven laser heads) are well suited for pulsed excitation. Of course, existing picosecond pulsed excitation systems can be used as well. There are also commercially available solutions for the second keystone, the simultaneous mea-surement of the photon arrival time on two dif-ferent time scales. (For example PicoQuant's TimeHarp200 PC board or the PicoHarp300 TCSPC system.)

Measurement examples

- Quasi-multichannel detection

  The ability of FLCS to separate the ACFs of two components (for example for simultaneous tracking of concentrations) was demonstrated in references [1] and [2]. When appropriate for the sample, a logical step further is the cross-cor-relation analysis of the separated intensities, as described in [2]. Furthermore, FLCS is a very versatile method to purge the ACF from various unavoidable experimental artefacts.[3]

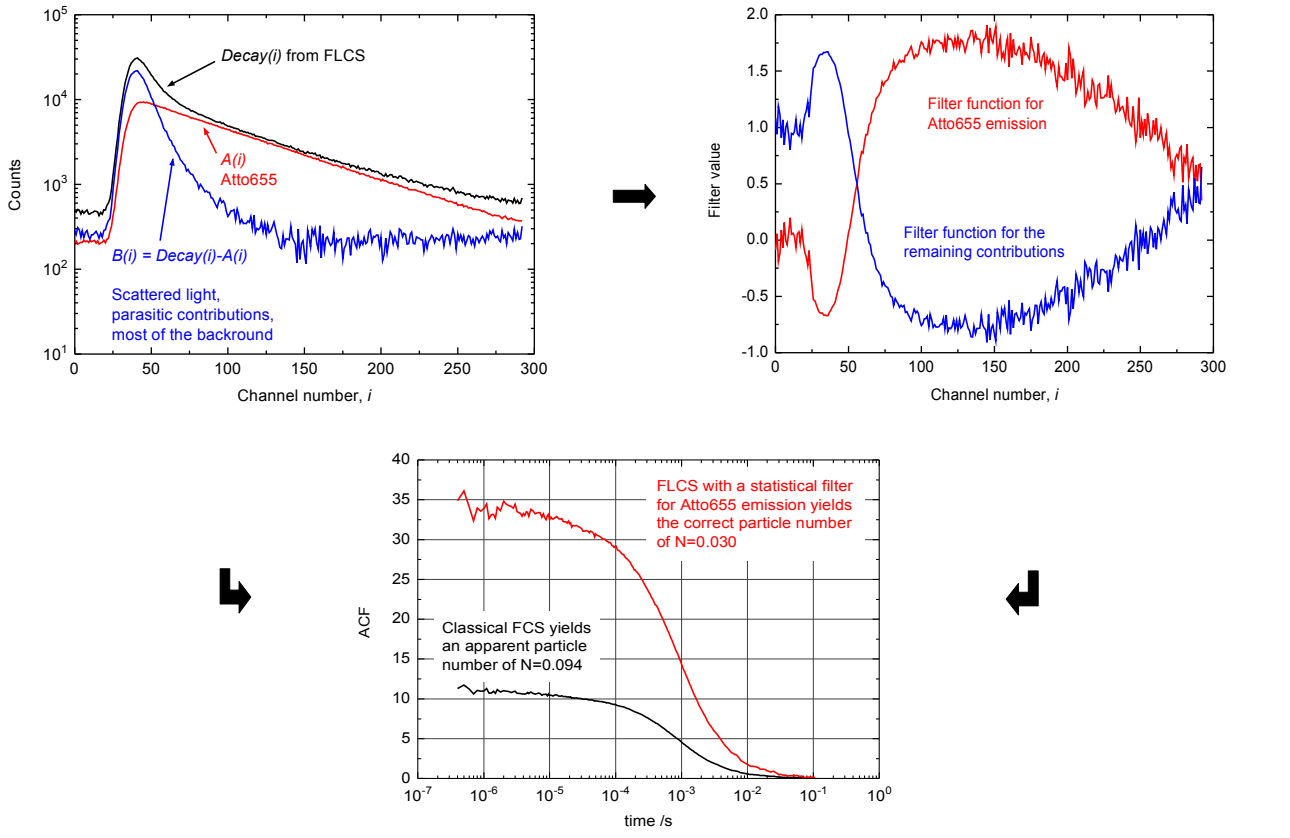

Fig. 4.: FLCS can be used to remove the influence of scattered light. The example shows results from measurements with Atto655.

- Suppressing of scattered light and various para sitic contributions

  At very low concentrations, typical for FCS, a considerable portion of the detected intensity is Rayleigh and Raman scattered excitation light. In this example the sample was a 10 picomolar solution of Atto655 in ethylene glycol. Analysis of the TCSPC histogram reveals that only 60% of the photons are emitted by Atto655 mole-cules. The decay pattern of pure Atto655 was obtained in a separate measurement of a nanomolar solution, where the amount of parasitic con-tributions can be neglected. Using the appro-priately scaled Atto655 pattern, first the "decay curve" of the remaining contribution was deter-mined, and then appropriate statistical filters were calculated (See Fig. 4)

Note the difference of ACF amplitudes. FLCS yields the correct particle number (i.e. concen-tration). The effect of the presence of scattered photons is strongly non-linear. Their contribution to the total detected intensity is only 40%, but the ap-parent particle number is increased by a factor of more than 3.

Detector afterpulsing is a very common and often overlooked instrumental artefact that leads to a dis-tortion of the ACF at short lag times. A fast initial decay of an ACF can be easily misinterpreted as a triplet contribution. At high excitation repetition ra-tes (in this example: 80 MHz), the spurious after-pulsing events are evenly distributed among the histogram channels, therefore appear as an increa-sed background. [3] This simple temporal be-haviour makes it easy to obtain the two patterns and the corresponding statistical filters (See Fig. 5). Of course, only the filter corresponding to the "pure" decay is suitable for correlation analysis.

The ACF obtained with afterpulsing suppression by means of FLCS can be fitted with a simple diffusion model, unlike the conventionally calculated ACF. The sample was a 100 picomolar water solution of Atto655.

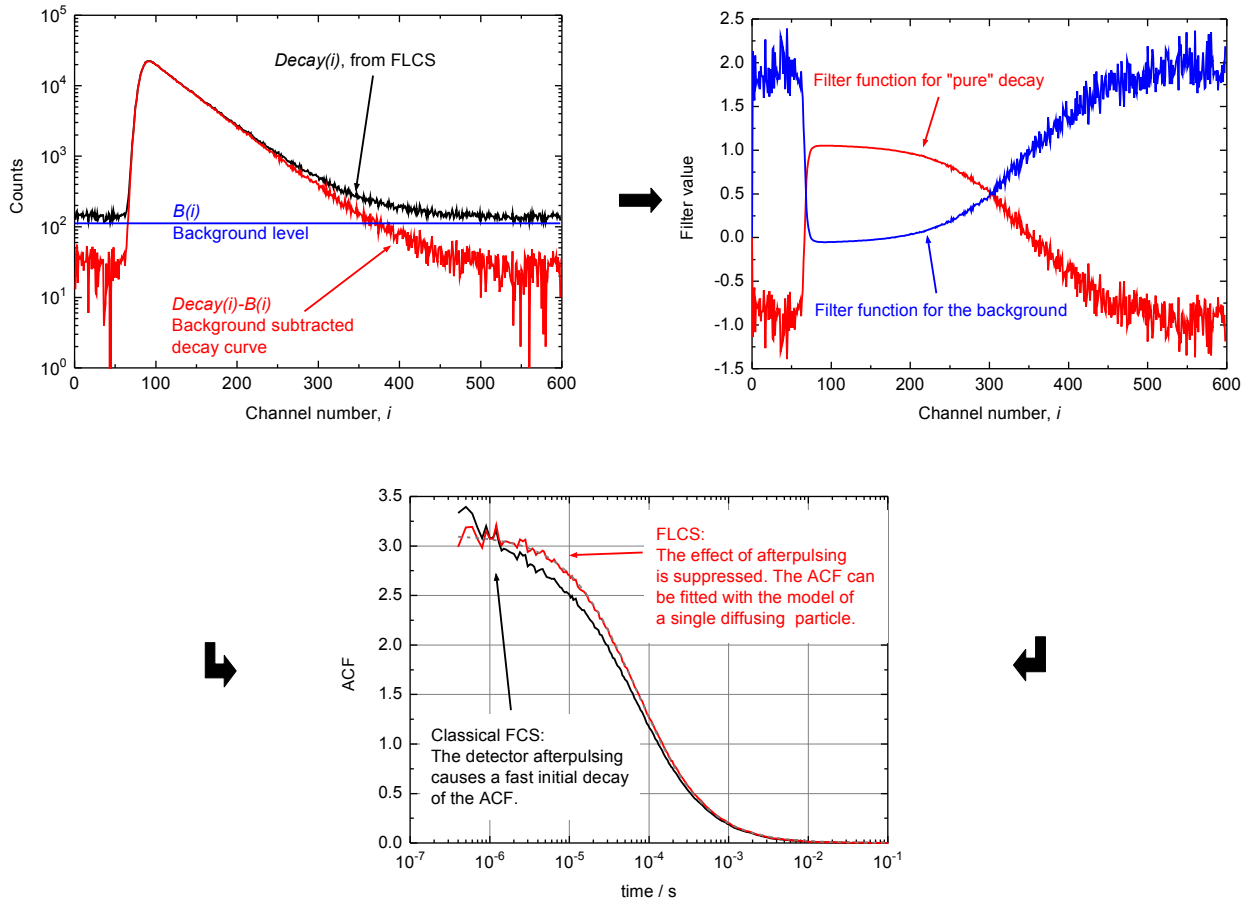

Fig. 5: Example of FLCS used to remove influence of afterpulsing from detectors

References

[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)

[2] 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)

[3] Enderlein J., Gregor I., Using fluorescence lifetime for discriminating detector afterpulsing in fluorescence-correlation spectroscopy, Review of Scientific Instruments, Vol.76, 033102 (2005)

[4] Meseth U., Wohland Th., Rigler R., Vogel H., Resolution of fluorescence correlation measurements, Biophysical Journal, Vol.76, p.1619-1631 (1999)

[5] Wahl M., Gregor I., Patting M., Enderlein J., Fast calculation of fluorescence correlation data with asynchronous time-correlated single-photon counting, Optics Express, Vol.11, p.3583-391 (2003)

[6] Lamb D. C., Schenk A., Röcker C., Scalfi-Happ C., Nienhaus G. U., Sensitivity enhancement in fluorescence correlation spectroscopy of multiple species using time-gated detection, Biophysical Journal, Vol.79, p.1129-1138 (2000)

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
D-12489 Berlin
Germany

Phone +49-(0)30-6392-6560
Fax +49-(0)30-6392-6561
Email info@picoquant.com
WWW http://www.picoquant.com