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# Fluorescence lifetime correlation spectroscopy: Basics and applications

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ABSTRACT

This chapter presents a concise introduction into the method of Fluorescence Lifetime Correlation Spectroscopy (FLCS). This is an extension of Fluorescence Correlation Spectroscopy (FCS) that analyses fluorescence intensity fluctuations from small detection volumes in samples of ultra-low concentration. FCS has been widely used for investigating diffusion, conformational changes, molecular binding/unbinding equilibria, or chemical reaction kinetics, at single molecule sensitivity. In FCS, this is done by calculating intensity correlation curves for the measured intensity fluctuations. FLCS extends this idea by calculating *fluorescence-lifetime specific* intensity correlation curves. Thus, FLCS is the method of choice for all studies where a parameter of interest (conformational state, spatial position, molecular environmental condition) is connected with a change in the fluorescence lifetime. After presenting the theoretical and experimental basis of FLCS, the chapter gives an overview of its various applications.



**Contents**



## 1. Introduction

Fluorescence Correlation Spectroscopy or FCS was first invented by Magde, Elson and Webb in the seventies of the last century [1–3]. Since then, it has become an indispensable tool for many applications in biology, biophysics, chemistry or physics [4–7]. In FCS, one excites and detects fluorescence emission of fluorescent or fluorescently labeled molecules out of a tiny detection volume (typically femtoliters), and applies a correlation analysis to the recorded fluorescence intensity fluctuations. Any process that influences the measured fluorescence intensity (such as changes of emitter position due to diffusion, photophysical transitions, chemical reactions, or conformational changes) will show up as a prominent correlation decay in the correlation curve. For example, molecules that diffuse in and out of the detection volume will generate a stochastically fluctuating fluorescence signal, the correlation time of which is directly connected with their diffusion constant. Similarly, molecules that can switch into a non-fluorescent triplet state (intersystem crossing) will show a partial fluorescence correlation decay on the time scale of the intersystem crossing and triplet-to-ground state relaxation.

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Most of the applications of FCS are concerned with diffusion measurements. However, FCS can also be used to measure photophysical processes, molecular interactions, active transport etc. A special but powerful variant of FCS is Fluorescence Cross-Correlation Spectroscopy (FCCS) which measures the cross-correlation of fluctuating fluorescence signals between spectrally different detection channels. This technique has been widely used to monitor the co-diffusion of different molecular species which are labeled with fluorescent dyes of different color [7–9].

However, cross-correlations cannot only be performed between fluorescent signals coming from different spectral detection windows, but also between fluorescent signals having different fluorescence lifetime signatures. This is the core idea of Fluorescence Lifetime Correlation Spectroscopy or FLCS, and was first demonstrated in [10]. Similar to dual-color FCCS, FLCS calculates auto- and cross-correlation curves, but not in a spectrum-specific manner, but in a fluorescence-lifetime specific manner. The fundamental experimental requirement for FLCS is the ability to measure not only intensity fluctuations on a "long" time scale (microseconds to seconds), but to simultaneously monitor also the fluorescence decay on nanosecond time-scales. This is usually done by measuring fluorescence decay curves with Time-Correlated Single-Photon Counting (TCSPC) [11,12], using pulsed excitation with high repetition rate (~10–100 MHz) and short (~100 fs–100 ps) laser pulses.

After the first publication of the concept of FLCS [10] (at that time called time-resolved FCS), it did not attract much attention, probably due to the exploding number of successful applications of conventional FCS at the time. FLCS was again picked up in 2005 [13], when it was used to efficiently eliminate effects of detector afterpulsing in FCS. In the same year, Benda et al. presented an upgraded lifetime-measuring confocal FCS system [14] that is ideally suited for performing FLCS. The term "FLCS" was then, for the first time, used in the following year in Refs. [15–17].

As already mentioned, FLCS is a cross-correlation spectroscopy technique that uses *fluorescence lifetimes* for calculating auto- and cross-correlations similar to conventional dual-color FCCS, which uses emission colors for discriminating between different fluorophores or fluorescent states. The fascinating property of FLCS is that one can distinguish fluorescence correlations of two or more emitting species that emit in the same spectral range, solely on the basis of their fluorescence lifetimes. In particular, the technique relies on the calculation of *separate* Auto-Correlation Functions (ACFs) for each emitting species by weighing the recorded photons with a filter function based on their emission delay with respect to the exciting laser pulses. These filter functions act as statistical filters which are calculated from "pure" decay patterns (TCSPC histograms) of each component. As such, FLCS can be applied in a similar way as is done with dual-color FCCS. However, its range of applications can be much wider: Recently, a conjunction of FLCS with STED has lead to a significant improvement in resolution, background suppression, and thus contrast in super-resolution fluorescence microscopy. A comprehensive introduction into FLCS and it's various applications until 2012 is nicely summarized in the review by Kapusta et al. [18]. Here, we provide an updated introduction into and review of FLCS which includes also its most recent applications and developments.

## 2. Principle of FLCS

An excellent introduction into the theoretical basis of FLCS has been given in [16], and we will briefly recapitulate it here. Let us consider a sample consisting of $m$ different species emitting fluorescence with different decay patterns such that the number of photons in the $i$th TCSPC channel $(i = 1, \ldots, L)$ at measurement time $t$ is

$$I_i(t) = \sum_{\alpha=1}^{n} c_\alpha(t)\, p_{\alpha i} \tag{1}$$

where $p_{\alpha i}$ is the discrete probability distribution function for detecting a photon in TCSPC-channel $i$ if the fluorescence comes from species $\alpha$, and $c_\alpha(t)$ is the momentary fluorescence intensity corresponding to species $\alpha$ at time $t$. We are interested in calculating fluorescence-decay specific two-photon auto- and cross-correlation functions defined by

$$g_{\alpha\beta}(t) = \langle c_\alpha(t_0)\, c_\beta(t + t_0)\rangle_{t_0} \tag{2}$$

where $\alpha$ and $\beta$ can take values from 1 to $m$, and the angular brackets denote averaging over $t_0$. For that purpose, one has to extract the values $c_\alpha(t)$ from the measured photon stream. This is performed by calculating statistical *filter functions* $f_{\alpha i}$ such that they satisfy the relations

$$\sum_i f_{\alpha i}\langle I_i(t)\rangle = \langle c_\alpha(t)\rangle \tag{3}$$

and minimize the mean square errors

$$\left\langle \left( \sum_i f_{\alpha i} I_i(t) - \langle c_\alpha(t)\rangle \right)^2 \right\rangle \tag{4}$$

where angular brackets denote time averaging, and the summations are performed over all TCSPC channels $i$. Using the fact that the photon detection in each TCSPC channel follows Poissonian statistics, these filter functions can be calculated using a weighted quasi-inverse matrix operation [19] as

$$\mathbf{f} = \left[\hat{\mathbf{M}} \cdot \mathrm{diag}\langle I\rangle^{-1} \cdot \hat{\mathbf{M}}^T\right]^{-1} \cdot \hat{\mathbf{M}} \cdot \mathrm{diag}\langle I\rangle^{-1}. \tag{5}$$

Here, $\mathbf{f}$ is a matrix with elements $f_{\alpha i}$, $\widehat{\mathbf{M}}$ a matrix with elements $p_{\alpha i}$, and $\mathrm{diag}\langle I\rangle^{-1}$ is an $L \times L$-dimensional matrix with diagonal elements $\langle I_j\rangle^{-1}$. A big $T$ superscript denotes matrix transposition. An important point that should be emphasized here is that these filter functions form a dual orthogonal basis to the decay patterns. This means that element-wise multiplication and summation of these filter functions with the fluorescence decay patterns yields an identity matrix. Using these filters, the second order auto- and cross-correlation function from Eq. (2) can now be expressed as

$$g_{\alpha\beta}(t) = \sum_{j=1}^{L}\sum_{k=1}^{L} f_{\alpha j} f_{\beta k}\langle I_j(t + t_0) I_k(t_0)\rangle_{t_0} \tag{6}$$

For $\alpha = \beta$, we obtain lifetime auto-correlations which represent the intensity fluctuations of each individual lifetime species, and for $\alpha \neq \beta$, we obtain the cross-correlations of $\alpha$ versus $\beta$, which indicate the probability of detecting a second photon from species $\beta$ at time $t$ after detecting a photon from species $\alpha$ at time zero. Thus, for a sample comprising of two molecular species with two distinct fluorescence decay patterns, one obtains two lifetime auto-correlation curves, one for each pattern, and two cross-correlation curves for correlations between the patterns. Generally, the cross-correlations are not symmetric, $g_{\alpha\beta} \neq g_{\beta\alpha}$. Equality occurs only under strict conditions such as pure co-diffusion, but if one considers e.g. transitions between two states that are part of an interconnected multi-state system containing more than only these two states, the observed cross-correlations between the two states may be asymmetric.

It is important to emphasize that throughout the above described mathematics, we did not put any restriction on the nature of $p_{\alpha i}$. Therefore, one can apply FLCS to fluorescent samples exhibiting any form of fluorescence decay kinetics (single-exponential, multi-exponential, etc.). A straightforward extension of the above concept is to include an additional component with