



# Time-Gated Fluorescence Correlation Spectroscopy for Improved Concentration Determinations

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## Introduction

Fluorescence Correlation Spectroscopy (FCS) is a very popular method to determine the diffusion coefficient of molecules in solution. However, the information contained in a FCS curve can be further exploited: As the mean number of molecules  $\langle N \rangle$  in the confocal detection volume  $V_{\text{eff}}$  is inversely proportional to the amplitude of the correlation function at very short correlation times  $G(\tau \approx 0)$ , FCS also allows to measure the concentration of the fluorescing molecules in the sample [1,2]. This is described by equation 1, if excitation into triplet states of the fluorophore and the background signal can be neglected.

$$G(\tau \approx 0) = \frac{1}{\langle N \rangle} = \frac{1}{\langle C \rangle V_{\text{eff}}} \quad [\text{Eq. 1}]$$

Typical concentrations that can be investigated with this method range from  $10^{-8}$  M to  $10^{-11}$  M (M = mol/liter). Concentrations above  $10^{-8}$  M lead to very low correlation amplitudes while lower concentrations as  $10^{-11}$  M require very long measurement times.

In most practical application, however, the evaluation of [Eq. 1] is not straightforward, as even the most adequate optical filters today cannot suppress the background signal completely. Therefore, in order to calculate a correct concentration from a FCS measurement, the background signal has to be considered.

This application note describes a method, which allows to eliminate background signal originating from elastic and Raman scattering using a time gated photon analysis. The method is applicable in

cases, where a pulsed excitation source is used and the temporal decay of the fluorescence is substantially longer than the temporal resolution of the system. The demonstrated measurement and analysis steps can be performed using an up-graded Laser Scanning Microscope (LSM) for FCS and lifetime measurements [3] or the time-resolved confocal microscope MicroTime 200 [4].

## Principle of time-gated FCS

FCS is usually performed using cw-laser sources for the excitation. However, as this is not a prerequisite for this method, pulsed laser excitation sources can also be applied. In this case the background originating from scattered excitation light has a characteristic temporal shape, as it essentially follows the temporal pattern of the excitation pulse. The fluorescence signal on the other hand is typically substantially longer than the excitation pulse. If this condition is met, then the complete detected signal can be described as a superposition of these two signals. In practice, however, one cannot measure the pulse shape directly, as such a measurement is influenced by the timing uncertainties of the detector and the timing electronics. Instead, the complete temporal resolution of the system, the so called Instrument Response Function (IRF) is measured and used to characterize the laser shape (see Fig. 1).

The difference in temporal patterns now permits the separation of signal and background by means of time-gating. This is particularly convenient if the photons are collected by means of Time-Correlated Photon Counting (TCSPC) electronics that give full access to the timing information, typically

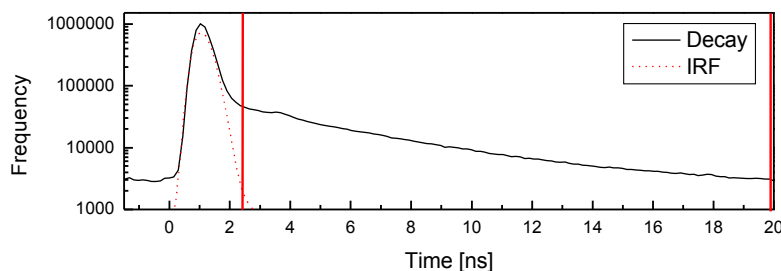


Fig. 1: Time-Correlated Single Photon Counting Histogram (TCSPC) showing a pronounced peak at short times due to scattering and a long tail due to the main fluorescence. Taking into account only photons with arrival times between the red lines selects photons originating from fluorescence. The measured Instrument Response Function (IRF) is depicted in red showing that the peak at early times originates from very fast scattering processes like elastic- and Raman scattering.

with picosecond resolution. The time-gate is chosen to start at the point where the amplitude of the excitation pulse has decreased substantially, but fluorescence is still at a high level. Only photons within the temporal limits of the time-gate are used for the FCS calculation, which allows concentration measurements free of artefacts caused by elastic and Raman scattering.

## Experimental details

The experiment was performed on a MicroTime 200 confocal microscope [3]. The sample consists of a  $3 \times 10^{-10}$  M aqueous solution of Atto 488 (www.atto-tec.com). The dye was excited with a LDH-P-C-470 pulsed picosecond diode laser operated at 40 MHz. Narrow band clean-up filters ensured that no parasitic light reached the sample. The fluorescence was detected confocally after a 500 nm longpass filter blocking the excitation wavelength with an optical density of 6 orders of magnitude. In order to suppress influences from afterpulsing, which is typically observed with Single Photon Avalanche Diodes (SPAD), the fluorescence light was split with a 50/50 beam splitter cube onto two SPADs (SPCM-AQR-14, Perkin Elmer Inc.) and cross correlation analysis was applied. The high numerical aperture apochromatic water immersion objective (60x, NA 1.2, Olympus) along with the 50  $\mu$ m confocal pinhole resulted in a confocal detection volume of 0.5 femtoliter. The confocal detection volume was calculated using TetraSpec beads (Molecular Probes) with a diameter of 100 nm immobilized on a cover glass and

the measured point spread function was approximated with a three dimensional Gaussian function. The fluorescence was detected applying Time-Correlated Single Photon Counting (TCSPC) with the TimeHarp 200 PCI-board. The data was stored in the Time-Tagged Time-Resolved Mode (TTTR), which allows to record every detected photon with its individual timing and detection channel information [5]. The presented measurement was performed 10  $\mu$ m deep in the solution with a total acquisition time of 10 minutes.

## Results

The MicroTime 200 software offers several possibilities to display and analyze the acquired TTTR raw data. For a first visualisation the single photon data of the two detector channels was summed up in bins of 1 ms, resulting in the time trace shown in Fig. 2.

The time trace exhibits photon bursts originating from single fluorophores diffusing through the laser focus but also a distinct background signal contribution. Applying the time-gate as shown in Fig. 1, results in a time trace with strongly reduced background as can be seen in Fig. 3 - the signal to noise ratio is increased by more than a factor of three. The calculated cross correlation from the raw data yields the curve shown in Fig. 4 - the correlation amplitude at short lag times corresponds to  $G(0) \approx 0.8$ . In contrast, by applying the time-gate, the value of the cross correlation at short lag times increases to  $G(0) \approx 7$  (see Fig. 5). This value is around nine times higher compared to the value

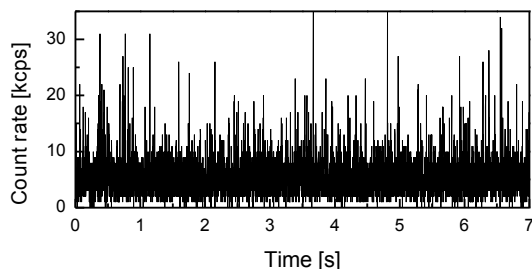


Fig. 2: Time trace of Atto 488 molecules diffusing through the confocal detection volume. No time gating was applied.

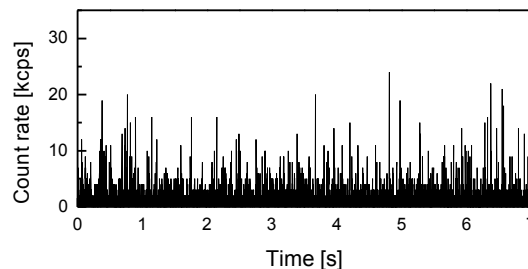


Fig. 3: Time trace of Atto 488 molecules using the time gate displayed in Fig. 1. A significant background reduction is visible. The signal to noise ratio increased by more than 3 times.

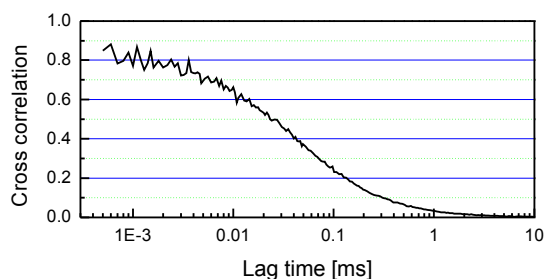


Fig. 4: FCS cross correlation of all photon events results in a  $G(0)$  value around  $G(0) \approx 0.8$

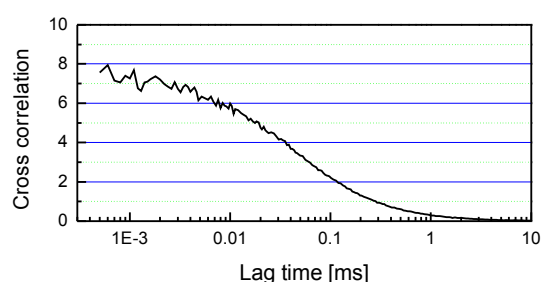


Fig. 5: Applying time gating increases the  $G(0)$  value of the cross correlation to about  $G(0) \approx 7.2$

without time-gating. Using equation (1), the value of  $G(0) \approx 7$  leads to a concentration of 0.27 molecules per femtoliter or  $4.5 \times 10^{-10}$  M, which corresponds nicely to the predicted concentration of the solution of  $(3 \pm 2) \times 10^{-10}$  M, that was independently determined by absorption measurements and following dilution steps.

## Discussion

With time-gated FCS even very low concentrations of the dye Atto 488 could be measured in the presence of a high background level. Essentially, the background level present during the data collection changes only the amplitude of the correlation curve as predicted by theory [6]. Multiplying the graph of Fig. 4 with a factor of 8.85 leads to a curve very similar to the graph in Fig. 5 as can be seen in Fig. 6:

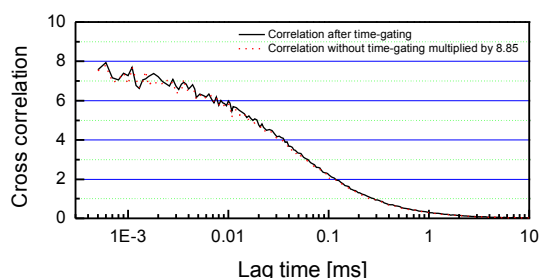


Fig. 6: Comparison of the correlation curve after time-gating with the original correlation curve after multiplication with a factor of 8.85.

In principle, this factor can also be calculated using the mean count rate for both detectors originating from the fluorescence and the mean count rate for the background, which can e.g. be measured in a separate experiment investigating only the liquid used for the dilution of the sample. However, separate experiments with the pure solvent are often not possible (e.g. in living cells).

The correlation at short lag times  $G(0)$  is not independent from the setting of the time-gate. The correlation between the start position of the time gate and the  $G(0)$  value is shown in Fig. 7.

As can be seen, a maximum is reached after 2.3 ns, indicating the best signal to noise ratio of the collected fluorescence data. At later times the signal to noise ratio and in consequence the  $G(0)$  value are decreasing, since photons originating

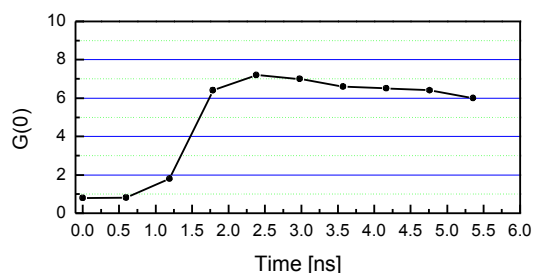


Fig. 7: Correlation of the  $G(0)$  value of the fluorescence correlation curve as a function of the start of the time gate.

from fluorescence are gated out in a greater amount with respect to photons stemming from the background. As a rule of thumb, one can state that the starting point of the time gate should be set at a time where the IRF has decreased considerably – at least around three orders of magnitude.

However, there are some experimental conditions in which the background signal is not entirely due to scattering, but can also include e.g. autofluorescence with a long fluorescence lifetime. In those cases time-gating will not allow to discriminate the signal completely from the background. Alternatively, the method of Fluorescence Lifetime Correlation Spectroscopy (FLCS) as developed by Jörg Enderlein can be employed and allows to separate the FCS curve of the desired fluorescence from the underlying background, provided that both have sufficiently different decay times [7, 8].

## Conclusion

The presented results show how time-gated FCS can be used to remove background signal originating from elastic scattering and Raman contributions, provided that the temporal patterns of both signals are substantially different. This procedure allows a quantitative concentration measurement with FCS even in the presence of background. The procedure is easy and fast applicable and relies on pulsed excitation sources and TCSPC for data acquisition.

## Further Applications of time-resolved FCS:

Using the information obtained by the TCSPC histogram even more powerful evaluations can be done, e.g. to separate two FCS curves of different dyes in a mixture [7, 8]. Benda et. al. used for this experiment a Zeiss confocal microscope with the PicoQuant LSM upgrade kit for confocal scanning microscopes [8]. Another application allows to correct for afterpulsing of photomultipliers (PMTs) and avalanche single photon diodes (SPADs) [9] without the need to do a cross correlation, which requires at least two detectors.

## Other general applications for FCS measurements include:

- Determination of diffusion coefficients in liquids or biological samples like living cells
- Measurement of concentrations
- Molecular brightness measurements
- Determination of the population of triplet states
- Confocal volume measurements using test samples with known concentration and diffusion coefficients
- Observation of kinetic parameters of chemical or photophysical reactions and conformational changes

## Further reading

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