Quantum yield determination by low-intensity Fluorescence Correlation Spectroscopy (liFCS)

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1 Introduction

Fluorescence correlation spectroscopy (FCS) is a method based on the correlation analysis of the temporal behaviour of fluorescence intensity fluctuations. It is commonly used to determine diffusion coefficients and concentrations of samples in the (sub)nano-molar concentration regime. Another quantity made accessible by FCS is the mean number of detected fluorescence photons per molecule and time, also referred to as molecular brightness (*MB*).

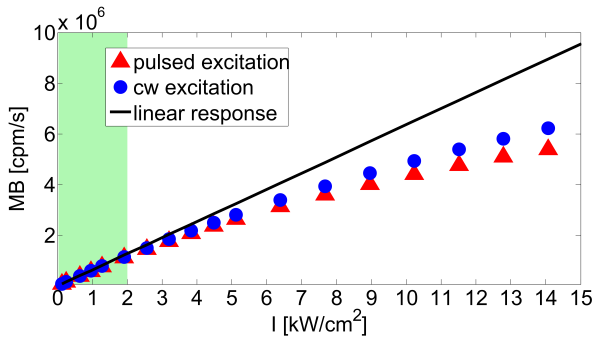

Figure 1: Theoretical prediction of the molecular brightness *MB* as a function of the excitation intensity *I* using photo-physical parameters known for Fluorescein (FL) in 0.1M NaOH; no setup-parameters taken into account; blue data points correspond to the case of continuous excitation, red data points to the case of pulsed excitation; in both cases, the data points coincide with a linear curve shape in the limit of low excitation intensities; this regime is shaded in green and corresponds to the range of excitation intensities used in liFCS

Low-intensity FCS (liFCS) exploits the advantages of FCS to determine accurate fluorescence quantum yield (*QY*) values at pico-to nanomolar sample concentrations [1]. The method is of particular interest for scientists specialized in single-molecule spectroscopy, as it allows to characterize samples under application-relevant conditions.
The determination of *QY*s by liFCS is easily implemented on the confocal fluorescence microscope MT200. Virtually all data analysis steps can be performed with the associated software Symphotime64. In this technical note, a protocol explaining all crucial measurement and analysis steps is given.

1.1 Theory

The working principle of liFCS is based on the fact that in the limit of low excitation intensities the *MB* shows a linear dependency on the impinging photon flux (see Fig. (1)). The type of excitation scheme, be it continuous or pulsed, makes no difference in this respect. The slope *m* of the linear curve is a product of the fluorescence quantum yield *QY*, the absorption cross-section σ, the transmission/detection efficiency of the setup *g* and the integrated molecule detection function *MDF*:

$$MB \approx QY \cdot g \cdot \sigma \cdot \int_V MDF(\vec{r}) \cdot I \qquad (1)$$

If the value of the integrated *MDF* is known, the *QY* can be calculated directly according to eq. (1). As this is typically not the case, an alternative approach is to measure the sample of interest (S) and a spectrally similar reference standard (R) with known *QY* under the same conditions. By taking the ratio of the slope *m* measured for S to the slope measured for R, the unknown QY_S can be determined according to eq. (2):

$$\boxed{QY_S \simeq \frac{g_R \cdot \epsilon_R(\lambda_{ex})}{g_S \cdot \epsilon_S(\lambda_{ex})} \cdot \frac{m_S}{m_R} \cdot QY_R} \qquad (2)$$

For convenience, the absorption cross-section σ can be replaced by the experimentally readily accessible molar absorption coefficient $\epsilon(\lambda_{ex})$ (see Fig. (4)).

2 Experimental Details

liFCS measurements were performed using the MicroTime 200 confocal microscope (PicoQuant, Berlin, Germany). Fifty microliter droplets of sample solutions were deposited on untreated cover slides. Laser light of wavelength $\lambda_{ex} = 487$ nm for continuous excitation (LDH-D-C 485, PicoQuant, Berlin, Germany) was used for irradiation. The maximal applied excitation power lied around 2.5 μW, which is equivalent to an excitation intensity of $\approx$ 2 $\frac{kW}{cm^2}$. The linearly polarized excitation light was focused by a water immersion objective (UplanSApo, 60x, NA 1.2, Olympus Deutschland, Hamburg, Germany) to a point approximately 10 μm above the glass surface. The objective correction collar was calibrated to achieve the maximum MB. After collection by the objective, the fluorescence emission passing through a dual-band dichroic mirror (XF2401, Omega Optical, Brattleboro, USA) was focused on a 30 μm pinhole. After being split by a 50/50 beam-splitter cube (Olympus Deutschland, Hamburg, Germany), and after passing the respective emission filter (FF01 530/55, Semrock, Rochester, USA), the emitted light was finally detected by single-photon avalanche diodes (τ-SPAD, PicoQuant, Berlin, Germany). The arrival time of each photon was recorded with a time-correlated single-photon counting module (PicoHarp300, PicoQuant, Berlin, Germany). The average measurement time per intensity step lied around 5 minutes. Correlation analysis of the intensity time-traces was performed using the Symphotime64 software (Picoquant, Berlin, Germany). Linear least-squares fitting was performed using Origin (OriginLab Corporation, Northampton, USA).

Absorption spectra were recorded in 1 cm path length (for the liFCS method) or 5 cm path length (for the steady-state method) quartz cuvettes (104F-QS, Hellma, Mühlheim, Germany) by using a double-beam UV-VIS spectrophotometer UV-2600 (Shimadzu, Kyoto, Japan). The fluorescence emission spectra were recorded with the spectrofluorometer QuantaMaster40 (PTI, Birmingham, USA) using 3 mm path length quartz cuvettes (105.253-QS, Hellma, Mühlheim, Germany) with a sample volume of $\sim$ 100 μl. All spectra were corrected for background intensities by substracting the spectra of pure solvent measured under identical conditions. Additionally, a correction function provided by the manufacturer was applied to the fluorescence spectra to account for the detection efficiency of the photomultiplier tube. To avoid possible distorsion of the spectra due to inner-filter effects, all sample solutions were diluted to optical densities below 0.01.

Fluorescein (FL) from a reference dye kit (Life Technologies, Carlsbad, USA) was delivered in dimethysulfoxide (DMSO) by the manufacturer, and dissolved in a 0.1 M sodium hydroxide (NaOH) solution for the measurements. Alexa Fluor 488 (AL488) (Life Technologies, Carlsbad, USA) functionalized with a succinimidyl ester group was dissolved in anhydrous DMSO (Sigma-Aldrich, St. Louis, USA) and subsequently diluted in phosphate buffered saline (PBS) (50 mM potassium phosphate, 150 mM NaCl, pH 7.2). For liFCS measurements, the sample concentrations were adjusted to result in an average number of 2-10 particles within the detection volume.

3 Step-by-step protocol

In the following, the determination of all parameters entering equation 2 is explained in detail in the referring subsections. All working steps are demonstrated using Fluorescein (FL) in 0.1M NaOH solution as the reference standard and Alexa Fluor 488 (AL488) in PBS as the sample of unknown *QY*. The identified *QY*-value is cross-checked with the result obtained by a commonly used steady-state optical method [2]. This method consists of measuring the integral fluorescence emission of reference and sample as a function of their optical densities (OD) (see Fig. (2)). A linear function is fitted to the obtained data sets and, based on the comparison of the slopes, the unknown *QY* can be calculated according to [2].

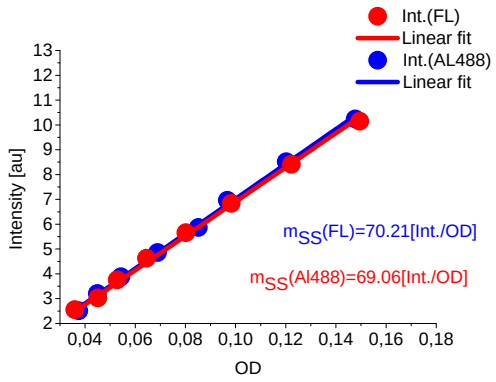

Figure 2: *QY* determination with the steady-state method; the integral fluorescence emission is measured as a function of the optical density (OD); the *QY* of AL488 is determined by comparing the two slopes; additionally, the refractive indices of the solvents have to be considered: *n*(PBS)=1.3355 and *n*(0.1M NaOH)=1.334

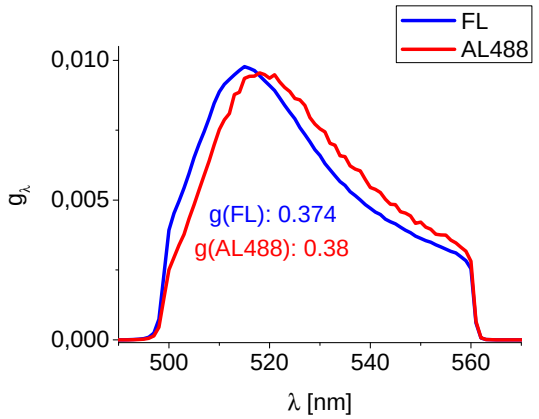

Figure 3: Wavelength dependent transmission/detection efficiency functions g_λ for FL and AL488 and the setup in use; optical elements taken into account are the major dichroic, the emission filters and the objective; further on, the detection efficiency of the single photon avalanche diodes is considered; the integrated transmission/detection functions *g* are given as red and blue numbers

3.1 Prerequisites

Before stepping through the protocol it should be remarked that the determination of *QY*s by liFCS relies on two requirements that have to be met. First, samples containing free fluorophores (reference or sample of interest) have to be homogeneous. Likewise, samples of fluorescently labeled biomolecules have to be homogeneous and, in any case, single-labeled. Secondly, reference and sample of interest have to be spectrally similar and dissolved in non-scattering media with similar refractive indices. If this condition is not met, the *MDF*s of reference and sample do not necessarily cancel out going from eq. (1) to eq. (2).

3.2 Determination of *g*

After background correction, the fluorescence spectra of FL and AL488 are area-normalized. The transmission spectra of all optical elements in the light path and the quantum efficiency spectra of the detectors are multiplied with each other. The computed function is multiplied with the normalized fluorescence spectra to generate a wavelength-dependent transmission/detection function *g(λ)*. Integration of *g(λ)* yields the parameter *g* (see figure 3).

3.3 Determination of $\epsilon(\lambda_{ex})$

The molar absorption coefficient $\epsilon(\lambda_{ex})$ at the excitation wavelength is determined by multiplying the value of the normalized absorption spectrum at λ_{ex} with the maximum absorption coefficient ϵ_{max} known from the literature (see figure 4).

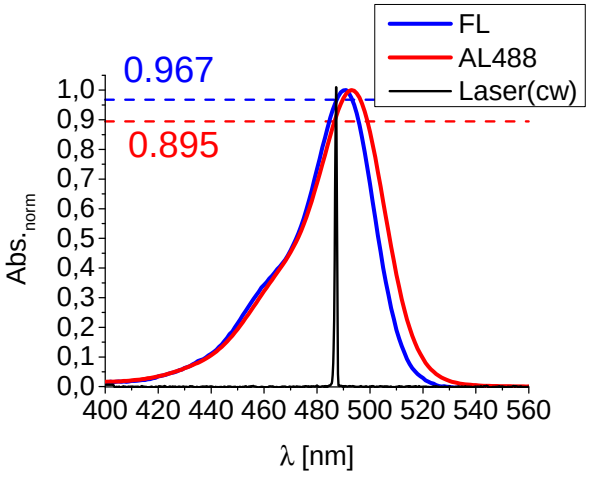

Figure 4: Normalized absorption spectra of FL and AL488 superimposed with laser spectrum; dotted lines indicate height of the point of intersection of laser and absorption spectra; the numbers are the corresponding scaling factors used to calculate $\epsilon(\lambda_{ex})$; the maximal absorption coefficients are literature values: ϵ_{max}(FL)=76,900$cm^{-1}M^{-1}$ and ϵ_{max}(AL488)=73,000$cm^{-1}M^{-1}$ [3] [4]

3.4 Determination of the slope *m*

The slope *m* is obtained by fitting a linear function to the obtained liFCS *MB*-values plotted as a function of the applied excitation intensity *I*. As a prerequisite, the excitation intensity or in practical terms the laser power regime appropriate for liFCS has to be determined. By analogy to Fig. (1), the experimentally determined *MB* is plotted as a function of increasing laser power. Then, the linear regime essential for liFCS is easily determined by visual inspection of the measured curve. Exemplarily, this is shown in Fig. (5) for AL488 in PBS.

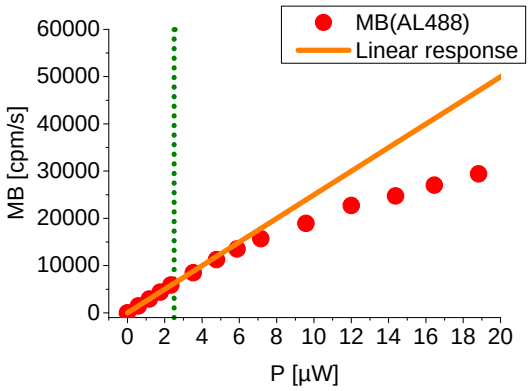

Figure 5: Experimentally determined *MB* of AL488 plotted as a function of the applied laser power (red); a linear curve (orange) is superimposed to simplify the determination of the linear regime essential for liFCS; the dotted green line indicates the estimated upper limit of the liFCS regime around a power of 2.5μW $\approx 2\frac{kW}{cm^2}$ [1]

Experimentally, the *MB*-values are determined by taking the ratio of the mean fluorescence count-rate $\overline{F}$ of a sample to the average number of fluorescent particles present in the detection volume $\langle N \rangle$. Using the Symphotime64-software, $\overline{F}$ is obtained by calculating the mean of the binned fluorescence intensity time trace (see figure 6).

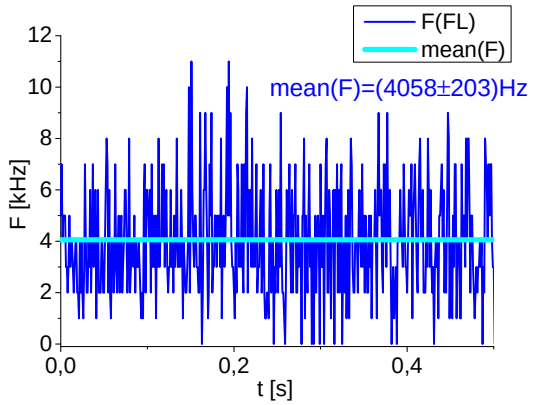

Figure 6: Binned fluorescence intensity time-trace of FL and calculated mean $\overline{F}$; the binning time equals 1ms

Since all liFCS measurements are performed at excitation intensities $\leq 2\frac{kW}{cm^2}$, the resulting signal-to-noise ratios are far from optimal. As a consequence, the influence of background should be taken into account:

$$\overline{F_c} = \overline{F} - \overline{BG} \tag{3}$$

Here, $\overline{BG}$ equals the mean of the count-rate generated by the pure solvent applying the same excitation intensity as used in the actual measurement.
$\langle N \rangle$ is obtained by calculating the liFCS-curve and fitting an appropriate model function to it (see sec. (5.2) and figure 7 for details).

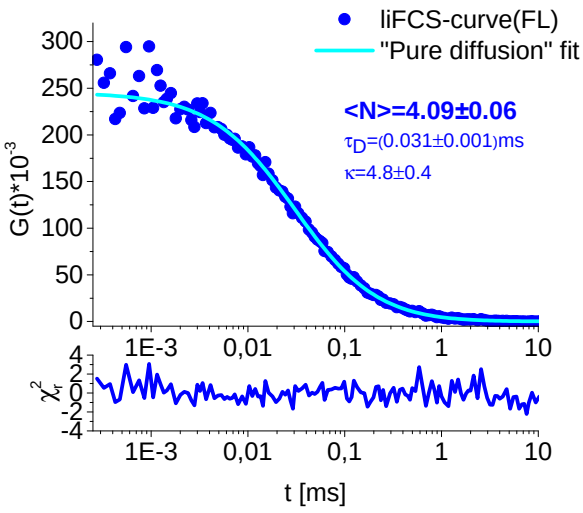

Figure 7: liFCS-curve of FL; the appropriate fitting model is the "pure diffusion" model in this case; $\langle N \rangle$ is the average number of particles diffusing in the effective volume; κ is a parameter describing the ratio of the axial and radial expansion of the effective volume; τ_D is the diffusion time of the molecules passing the effective volume

Again, taking into account the contribution of background yields a corrected value of ⟨N⟩ [5]:

$$\langle N_c \rangle = \langle N \rangle \cdot \frac{1}{(1 + \frac{\overline{BG}}{\overline{F_c}})^2} \tag{4}$$

The background corrected *MB* is then calculated according to:

$$\boxed{MB_c = \frac{\overline{F_c}}{\langle N_c \rangle}} \tag{5}$$

In Fig. (8), liFCS *MB*-values of FL and AL488 are plotted as a function of the applied excitation intensity. As mentioned before, the slope *m* is readily obtained by linear least-squares fitting.

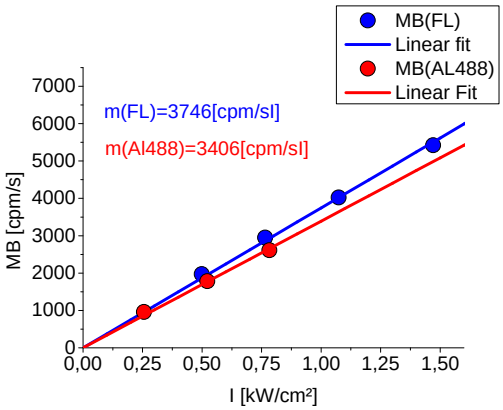

Figure 8: liFCS *MB*-values of FL and AL488 plotted as a function of the applied excitation intensity *I*; *I* was calculated by taking the ratio of twice the laser power to the minimal radial cross sectional area of the focused beam; in this case, the apparent difference in the slopes does not relate to distinct *QY*-values of AL488 and FL but mainly to differences in the absorption coefficients $\epsilon(\lambda_{ex})$

4 Results and conclusion

The parameters obtained as previously described are summarized in Table (1). The error of the absorption coefficients was assumed to be 1%. For the mean countrate $\overline{F}$, the standard deviation of three successive measurements was used while the error of ⟨N⟩ was determined by the fit of the liFCS-curve. The error limits of the slopes were taken from the linear least-squares minimization.
The *QY*-values of AL488 obtained with both the liFCS and the steady-state method coincide within the experimental errors. Both values are in agreement with the literature value known for AL488. Evidently, the *QY*-determination by liFCS yields accurate results.

Sample	**FL(R)**	**AL488(S)**
g	0.374	0.38
$\epsilon(\lambda_{ex})$ $[M^{-1}cm^{-1}]$	74362 ± 74	65335 ± 65
m [cpm/sl]	3794 ± 95	3406 ± 104
***QY*(liFCS)**	**0.92**	**0.93 ± 0.04**
***QY*(steady-state)**	**0.92**	**0.91 ± 0.03**
QY(literature)	0.92	0.92

Table 1: Overview of all parameters needed to determine the *QY* of AL488 using liFCS; the *QY*-values obtained by liFCS agree with both the steady-state and the nominal value

In comparison to the steady-state method, the *QY*-determination by liFCS allows to reduce the amount of needed sample by a factor of 100 [1]. This is particularly advantageous in the life-sciences where the available sample quantity can be limited due to low protein expression and/or labeling yields. Additionally, assuming a measurement time of five minutes per excitation intensity step, the *QY*-determination of a sample by liFCS takes approximately one hour only.

5 Troubleshooting

5.1 Remarks concerning the reference sample

The *QY* can depend on solvent properties like temperature, pH, viscosity etc.. Therefore, all reference measurements should be performed under the conditions prescribed by the manufacturer/literature. Additionally, fluorophores used as reference standard should be stored correctly. To our experience, the best choice is to use unfunctionalized fluorophores. If only functionalized dyes are available, they should be aliquotized as dried powder at $-80°$C, and dissolved in the measurement solution right before performing the experiment. If this is not an option, the -NHS and -COOH functionalized moieties appear to be photophysically stable for a few month when stored in anhydrous DMSO at $-20°$C . To test whether the reference dye in use is (still) reliable or not, its actual lifetime can be compared with the literature value to detect quenching effects due to wrong storage.

5.2 Pitfalls in the analysis of liFCS-curve

As was mentioned already, before fitting the liFCS curves one has to chose the appropriate model function carefully. It is a well-known fact that the photo-physical process of triplet formation is an excitation intensity dependent process. Therefore, the triplet fraction showing up in the liFCS curves is very small. Being rather insignificant in terms of the fit-quality, its value can fluctuate strongly when treated as a free parameter during the fitting procedure. As a consequence, the parameter $\langle N \rangle$ can be noticeably biased as well. To circumvent this problem we tested two strategies. First, we fitted the data using the "Triplet"-model provided by the Symphotime64-software and fixed the triplet fraction to its expected value [1]. Then, we fitted the data using the "Pure-Diffusion"-model. The difference in the outcome of the parameter $\langle N \rangle$ was below 1%. Consequently, even liFCS-curves of samples like FL that are showing rather severe triplet-formation can be fitted using the "Pure-Diffusion"-model.
On the other hand, carbocyanine dyes like Alexa647 are well-known to show photo-induced cis/trans-isomerization [6]. For this process, the fraction of molecules in the dark trans-state is not dependent on the applied excitation intensity. As a matter of fact, even for liFCS-curves a significant dark-state fraction shows up which is clearly visible by eye and well-separated from the diffusion-related part of the curve. As a consequence, these types of curves should be fitted with the "Triplet"-model provided by the Symphotime64-software.

References

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