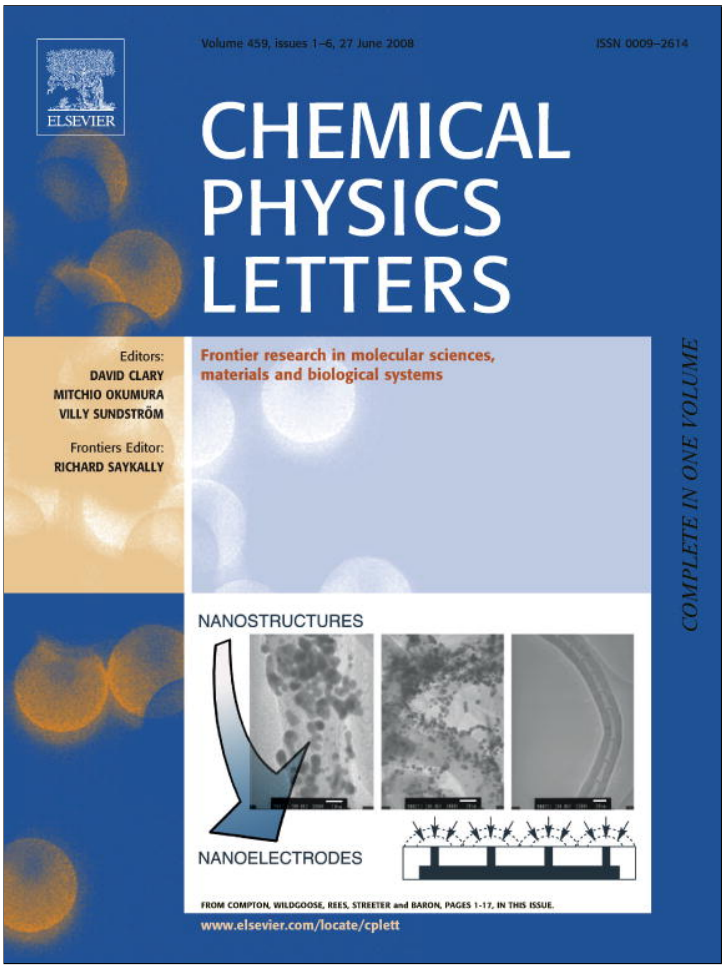

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Comparison of optical saturation effects in conventional and dual-focus fluorescence correlation spectroscopy

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ABSTRACT

We present diffusion measurements of the fluorescent dye Cy5 in aqueous solutions using conventional fluorescence correlation spectroscopy (FCS) and the recently introduced dual-focus fluorescence correlation spectroscopy (2fFCS). We study the sensitivity of both methods with respect to excitation intensity. Due to the light-driven transitions of Cy5 between fluorescent and non-fluorescent states, conventional FCS shows a strong dependence of the apparent diffusion coefficient on excitation intensity, whereas 2fFCS is virtually free from that artifact. Moreover, 2fFCS allows us to obtain the first precisely measured absolute value for the diffusion coefficient of Cy5.

1. Introduction

Fluorescence correlation spectroscopy (FCS) is a powerful technique for measuring the diffusion, concentration and fast dynamical processes with single-molecule sensitivity. It was originally introduced by Elson, Magde and Webb in the early seventies [1–3] and has become a very popular technique over the last two decades. This was facilitated by the development of high-quality objectives having large numerical aperture, the wide distribution of affordable laser sources, and the introduction of solid-state single-photon detectors with better than 50% quantum efficiency. Today, FCS has become an important spectroscopic technique that is used in numerous biophysical and physico-chemical studies. Excellent introductions and overviews to FCS can be found in Refs. [4–9].

However, conventional FCS using a standard confocal microscope is troubled by its enormous sensitivity to smallest changes in experimental conditions such as refractive index mismatch or laser beam parameters (for a critical review see Refs. [10,11]). A particularly disturbing effect in FCS measurements is the dependence of the autocorrelation function (ACF) on excitation intensity due to optical saturation [12–14]. Optical saturation occurs when the excitation intensity becomes so large that a molecule spends more and more time in a non-excitable state, so that increasing the excitation intensity does not lead to a proportional increase in emitted fluorescence intensity. The most common sources of optical saturation are (i) excited state saturation, i.e. the molecule is still in the excited state when the next photon arrives; (ii) triplet state saturation, i.e. the molecule undergoes intersystem-crossing from the excited to the triplet state so that it can no longer become excited until it returns back to the ground-state; (iii) other photo-induced transitions into a non-fluorescing state, such as the photo-induced *cis-trans*-isomerization in cyanine dyes, or the optically induced dark states in quantum dots. The exact relation between fluorescence emission intensity and excitation intensity can be very complex and even dependent on the excitation mode (pulsed or continuous wave) [14].

Recently, we introduced a modification to conventional FCS which is called dual-focus FCS or 2fFCS [15]. In 2fFCS, one introduces an external ruler into the measurement by generating two overlapping laser foci of precisely known and fixed distance. The presence of the external ruler allows for determining absolute values of the diffusion coefficient with an accuracy of a few percent. Moreover, although the exact shape of each focus still depends on the experimental details such as sample refractive index or cover slide thickness, they will not change the center distance between the foci and thus the external ruler used for obtaining quantitative values of the diffusion coefficient. As was shown in Ref. [15], this ruler is also not influenced by optical saturation due to ground-state depletion (type (i) of optical saturation discussed above). However, it has to be shown that the method works also well for dyes showing extensive optical saturation due to strong intersystem crossing (triplet state dynamics) or light-driven *cis-trans* isomerization between a fluorescent trans- and a rather non-fluorescent cis-state. The popular cyanine dye Cy5 seems to be an ideal candidate to validate the robustness of 2fFCS against these forms of optical saturation (ii + iii), because it exhibits strong light-driven transitions between fluorescent and non-fluorescent

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states [16] with a very complex underlying photophysics [17,18]. This is the main issue of the present Letter. Moreover, we present the first precisely measured absolute value of the diffusion coefficient of Cy5 in aqueous solution.

2. Experimental details

2.1. Materials

Cy5 in form of NHS ester was purchased from Invitrogen, Karlsruhe, Germany. The dye was diluted in bi-distilled water to 0.2 nM concentration. Lab-Tek II chambered coverglass system was purchased from Nalge Nunc International. The sample temperature was measured with a HH500 digital thermometer purchased from Omega Engineering, Stamford, CT, USA.

2.2. Measurement set-up

The used 2fFCS set-up is schematically shown in Fig. 1. It is based on an inverse time-resolved epi-fluorescence microscope (MicroTime 200, PicoQuant, Berlin, Germany) containing two pulsed excitation lasers at 637 nm (LDH-P-635, PicoQuant, Berlin, Germany). The two lasers have orthogonal linear polarization and are combined by a polarizing beam splitter cube. The combined beam is optically shaped by sending it through a polarization-preserving single mode fiber. The resulting beam consists of a train of light pulses with temporally alternating polarization. Before entering the back aperture of the water immersion objective (UPLAPO 60x W, 1.2 N.A., Olympus Europa, Hamburg, Germany), the light is passed through a Nomarski prism (U-DICTHC, Olympus Europa, Hamburg, Germany) which is usually used for differential interference contrast (DIC) microscopy. This prism deflects the laser pulses into two slightly different directions depending on their polarization. After focusing through the objective, one thus obtains two overlapping foci with fixed lateral distance determined solely by the properties of the Nomarski prism. Fluorescence is collected by the same objective, separated from the excitation light by a dichroic mirror (Q 660 LP, Chroma Technology, Rockingham, VT, USA), focused through a single pinhole of 150 μm diameter, re-collimated, split by a polarizing beam splitter cube, and refocused onto two single photon avalanche diodes (SPAD, AQR13, Perkin Elmer, Wellesley, MA, USA).

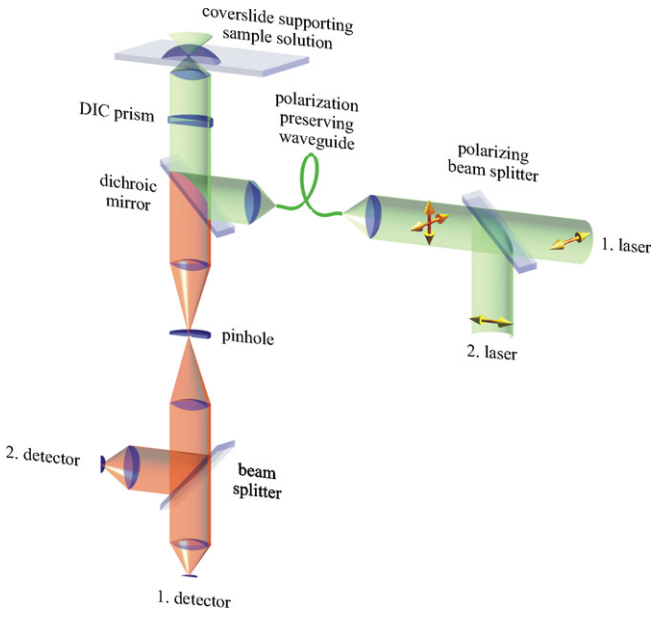

Fig. 1. Schematic of the 2fFCS set-up. For details see main text.

A dedicated single-photon counting electronics (PicoHarp 300, PicoQuant Company, Berlin, Germany) is used to record the detected photons with a temporal resolution of 4 ps. By evaluating the arrival times of the photons on a nanosecond time scale, the detected photons can be unequivocally associated with its corresponding excitation pulse and thus with the corresponding focus (principle of pulsed interleaved excitation or PIE [19]). Thus, it is possible to calculate the ACFs for each focus separately, as well as the cross-correlation function (CCF) between photons emerging from both foci. Calculation of the ACFs and CCF was performed by using the algorithm published in Ref. [20]. Only photons from the two different detectors are correlated to prevent distortions of the resulting ACF by SPAD after-pulsing [21].

The calculated correlation functions were fitted using the model presented in Ref. [15]. This model is based on the assumption that the molecule detection function (MDF) $U(\vec{r})$, which describes the position dependent efficiency to excite and detect a fluorescence photon from a molecule at position $\vec{r}$, can be sufficiently well approximated by a combination of a Gauss–Lorentz excitation intensity profile and a simple pinhole function, resulting in the explicit functional form

$$U(\vec{r}) = \frac{\kappa(z)}{w^2(z)} \exp\left[-\frac{2}{w^2(z)}(x^2+y^2)\right] \tag{1}$$

where x, y, and z are Cartesian coordinates with the z-axis along the optical axis, and the functions $w(z)$ and $\kappa(z)$ are given by

$$w(z) = w_0\sqrt{1+\left(\frac{\lambda_{ex}z}{\pi w_0^2 n}\right)^2} \tag{2}$$

and

$$\kappa(z) = 1 - \exp\left(-\frac{2a^2}{R_0^2 + (\lambda_{em}z/\pi R_0 n)^2}\right) \tag{3}$$

where λ_{ex} and λ_{em} are the excitation and center emission wavelengths, respectively, n is the sample refractive index, a is the confocal pinhole radius, and w_0 and R_0 are two free fit parameters. Using this MDF, the diffusion related model CCF is given by

$$g(t) = g_\infty(\delta) + 2\epsilon_1\epsilon_2 c\sqrt{\frac{\pi}{Dt}}\int_{-\infty}^{\infty}dz_1\int_{-\infty}^{\infty}dz_2\frac{\kappa(z_1)\kappa(z_2)}{8Dt + w^2(z_1) + w^2(z_2)} \cdot\cdot \exp\left[-\frac{(z_2-z_1)^2}{4Dt} - \frac{2\delta^2}{8Dt + w^2(z_1) + w^2(z_2)}\right] \tag{4}$$

which has to be evaluated numerically. Here, δ is the lateral distance between the foci, ϵ_1 and ϵ_2 are two factors proportional to the overall excitation intensity and detection efficiency in each laser focus, c is the concentration of the fluorescent molecules, and D is their diffusion coefficient. For calculating the ACF of each focus, one has to set $\delta = 0$ and to replace $\epsilon_1\epsilon_2$ by either ϵ_1^2 or ϵ_2^2, respectively. If a dye shows fast photophysical relaxation on the microsecond time scale (such as that of Cy5), an additional exponential function is added to the correlation functions [22]. When fitting experimentally measured data, one fits the two ACFs (which are identical in shape) and the CCF simultaneously, having as fit parameters $\epsilon_1\sqrt{c}$, $\epsilon_2\sqrt{c}$, w_0, R_0, D, and, potentially, a photophysical relaxation time. It should be emphasized that the model MDF has not to be an *exact* representation of the actual MDF as long as it yields a reasonable fit quality of the experimentally measured curves. The important parameter defining the absolute accuracy of the resulting values of the diffusion coefficient is the lateral distance δ between the foci. This distance is set by the optical properties of the Nomarski prism and can be different (on a nanometer scale) for different prisms even of the same type from the same provider. We

determined the interfocal distance to be equal to 403 nm by comparing the diffusion of fluorescently labeled polymer beads (TetraSpeck 100 multi-fluorescent latex beads, Invitrogen, Karlsruhe, Germany) as measured with dynamic light scattering and with our 2fFCS system [23]. All measurements were done at room temperature, which was measured with a standard thermometer.

3. Results and discussion

We measured FCS curves using the 2fFCS for different values of total excitation power per laser between 5 and 40 μW. A typical measurement result is shown in Fig. 2 displaying the ACFs for each focus as well as the CCF between photons from different foci for a total power of 20 μW per laser. The figure shows also fits of model Eq. (4) to the measurements. As mentioned above, for taking into account the fast correlation decay due to the Cy5 photophysics on the microsecond time scale, an additional exponential term was included into the fitting. The lower panel of the figure shows the residuals between fitted and measured curves, demonstrating the excellent quality of the fit.

One result of the fitting is the absolute value of the diffusion coefficient. For comparing 2fFCS with conventional single-focus FCS, we performed FCS measurements with the same experimental system but using only one of both lasers. The resulting ACFs were fitted using again Eq. (4) but with $\delta = 0$. For evaluating the ACFs of conventional FCS, the standard model approach assuming a three-dimensional Gaussian distribution for the MDF would have been sufficed as well, because conventional FCS is not an absolute method for determining diffusion coefficients: it has usually to be calibrated against a reference standard with known diffusion coefficient. However, it should be mentioned that for diffusion measurements in planar systems, FCS can yield absolute values

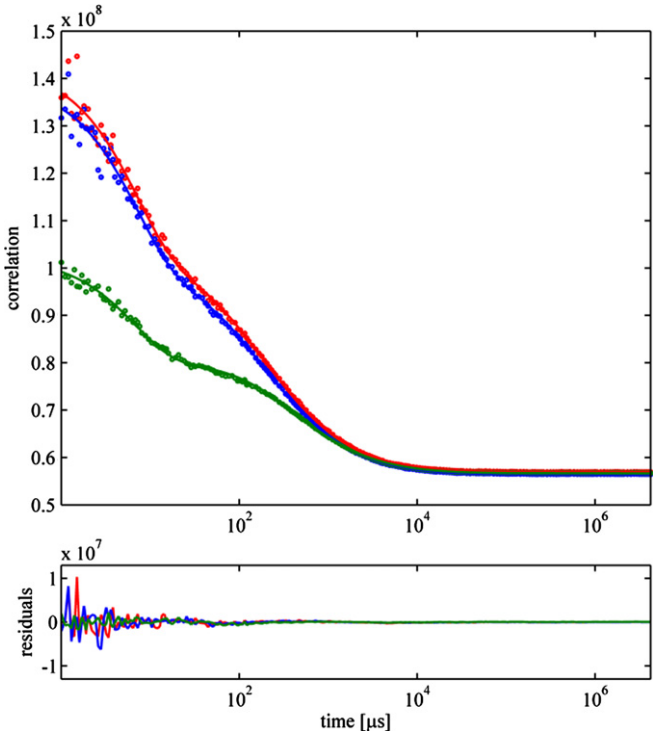

Fig. 2. Typical 2fFCS measurement result: auto- and cross-correlation functions measured for Cy5 with ~20 μW total laser power per excitation focus. Although both ACF curves have the same shape, their amplitudes are slightly different due to a minute power difference between both lasers.

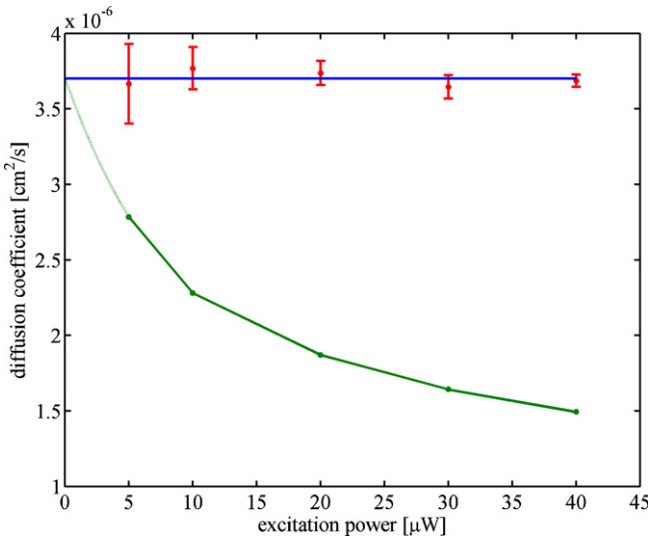

Fig. 3. Determined diffusion coefficient as a function of total laser excitation power per focus. Points with error bars are the results of 2fFCS, using 10 measurements for each point to determine a standard deviation of the diffusion coefficient. Solid horizontal line shows the average value of all 2fFCS measurements. Lower intensity-dependent curve refers to the results of conventional FCS, using the extrapolated zero-intensity value as reference. Dotted line is an extrapolation of the determined power dependence toward zero power.

of diffusion coefficients by performing measurements at different positions of the laser focus with respect to the sample plane (z-scan method) [24,25]. Unfortunately, this method is not easily applicable to solution measurements. In the present Letter, we use as reference the diffusion coefficient of Cy5 as determined with our 2fFCS method. The final dependence of the determined values of the diffusion coefficient as a function of total excitation power per focus is depicted in Fig. 3. As one can see, the values as determined with 2fFCS are insensitive to the excitation power within the range of employed values, giving an average absolute value of the diffusion coefficient of $D_{25\,^\circ C}(\mathrm{Cy5}) = (3.7 \pm 0.15) \times 10^{-6}\mathrm{cm}^2/\mathrm{s}$ (this value was derived from the experimental values by recalculating it to a temperature of 25 °C using the Stokes–Einstein equation and the known temperature dependence of the viscosity of water).

In contrast, the values obtained with conventional FCS are strongly dependent on excitation power. For better comparison with 2fFCS, we extrapolated this dependence toward zero excitation power (dotted line) and used the obtained zero-intensity value as reference point for all conventional FCS measurements. The obtained dependence of the apparent diffusion coefficient on excitation power is in perfect qualitative agreement with theoretical estimates, see Fig. 8 in Ref. [11], showing the counterintuitive effect that the sensitivity of the determined diffusion coefficient on excitation power becomes the larger the smaller the excitation power is. The found result strongly emphasizes the unreliability of conventional FCS in determining an absolute value of a diffusion coefficient, especially if the dye exhibits strong light-driven transitions between differently fluorescing states. Fortunately, 2fFCS is basically insensitive against optically saturation, moreover yielding absolute values of the diffusion coefficient without the necessity to calibrate against a known standard.

Acknowledgements

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