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Precise measurement of diffusion by multi-color dual-focus fluorescence correlation spectroscopy

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Abstract – Dual-focus fluorescence correlation spectroscopy is a method for precisely measuring the diffusion coefficient of fluorescing molecules close to the infinite dilution limit in a reference-free and absolute manner. We apply the method to determine the diffusion coefficients of three fluorescent dyes across the visible spectrum. These values can be used as absolute reference standards for fluorescence correlation spectroscopy. In particular, it is found that the diffusion coefficient of the widely used reference dye Rhodamine 6G is by 37% larger than the value used in most publications on fluorescence correlation spectroscopy over the last three decades.

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Introduction. – Thermal, or Brownian, diffusion of single molecules in solution is one of the best studied processes in statistical physics and is usually considered to be the master example of a stochastic process [1]. Its study was at the beginning of the modern age of statistical physics [2] and has attracted considerable attention in the theoretical literature [3,4]. Moreover, the famous Stokes–Einstein relation [5] that couples temperature, solvent viscosity, and the hydrodynamic radius of diffusing molecules, allows for determining the latter and thus for estimating the molecular size. This ability to size molecules via their diffusion has wide potential applications such as monitoring conformational changes in biomolecules or binding between different molecules.

Surprisingly, up to this day it is rather difficult to measure molecular diffusion with high precision if their concentration is close to the important limit of infinite dilution (pico- to nano-molar) where intermolecular interaction between molecules can be neglected. There are several powerful ensemble techniques available for precise diffusion measurement such as dynamic light scattering (DLS) [6] or pulsed-field gradient nuclear magnetic resonance (pfNMR) [7]. However, DLS requires a large electric polarizibility of the diffusing molecules, and both DLS and pfNMR need rather high solute concentrations to achieve high accuracy within a reasonable measurement time.

More than three decades ago, Magde, Elson and Webb invented fluorescence correlation spectroscopy (FCS) for measuring diffusion coefficients of fluorescent molecules [8]. In FCS, fluorescence intensity fluctuations detected out of a small detection volume (usually on the order of one femtoliter or less) are recorded, and the resulting signal is autocorrelated yielding the second-order or autocorrelation function (ACF) of the fluctuating signal. If the average number of molecules within the detection volume is small enough, the fluctuations are dominated by the random diffusion of the molecules out of that volume, and the ACF shows a prominent decay which is directly related to the diffusion coefficient. However, for obtaining a precise value of the diffusion coefficient from an FCS measurement, one needs to know the molecule detection function (MDF), which describes the position-dependent efficiency to excite and detect a fluorescence photon from a single molecule. Unfortunately, the exact shape of the MDF in a confocal microscope depends on many rather imprecisely known parameters [9], so that the standard approach is to compare a measured ACF against another ACF measured on reference molecules with known diffusion coefficient. But even then, one of the most disturbing observations

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in recent years was that an ACF depends on excitation intensity due to optical saturation of fluorescence, and is thus dependent on the photophysical characteristics of each molecular species [10,11]. This makes even comparative measurements problematic, moreover when taking into account that the photophysical properties of even the same molecule can change when it is, for example, chemically bound to another molecule.

Materials and methods. – Recently, we introduced a modification to standard FCS which introduces an *external length scale* into the measurement. It is based on creating two laterally shifted but overlapping foci with well-known and fixed distance (dual-focus FCS or 2fFCS) [12]. By measuring the ACF for each focus separately as well as the cross-correlation function (CCF) of the fluorescence between both foci and analyzing the delay of the CCF decay in comparison to the ACF decay (which is due to the extra distance between both foci), one can calculate the diffusion coefficient of the fluorescent molecules absolutely and without reference. The important point is that, although optical aberrations or saturation effects may distort the shape of the MDF of each focus, the distance between them and thus the intrinsic ruler used for the diffusion coefficient calculation is not changed by these effects.

In ref. [12], two independent lasers were used for creating two separate foci. Here, we demonstrate that *any* confocal fluorescence microscope with pulsed excitation and the capability of time-correlated single-photon counting can be modified for being used in 2fFCS. The additional Nomarski prism is a standard component which is normally used in DIC-microscopy and can be purchased for most commercial microscopes. A schematic of the experimental set-up is shown in fig. 1. It is is based on an inverse time-resolved epi-fluorescence microscope (MicroTime 200, PicoQuant, Berlin, Germany) with three different pulsed excitation lasers at 470 nm, 532 nm, and 637 nm (LDH-P-C-470B, PicoTA 530N, and LDH-P-635, respectively, all from PicoQuant, Berlin, Germany). Each laser beam was split into two beams by a polarizing beam splitter and a zeroth-order half-wave plate. The light of one of both beams was time-delayed by half of the laser repetition period (50 ns) using an optical fiber delay of corresponding length. After the fibers, both beams are collimated and reunited by a polarizing beam splitter (Ealing Catalogue, St. Asaph, UK). The different wavelength beams are combined with dichroic mirrors, and are then optically cleaned and re-collimated by another polarization-preserving single-mode fiber. The resulting single light beam contains a train of light pulses with temporally alternating polarization at each wavelength. Before entering the back aperture of the water immersion objective (UPLAPO 60× 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

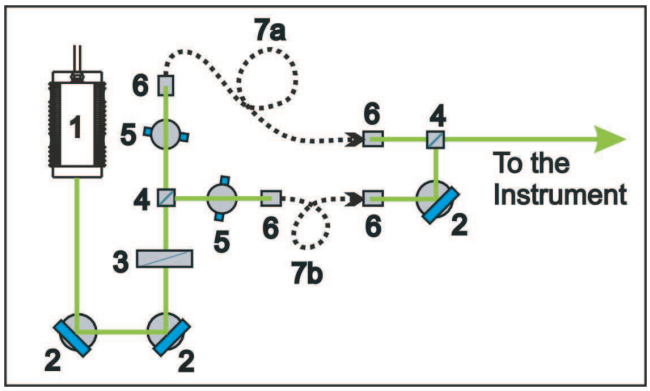

Fig. 1: Single laser set-up for dual-focus experiments, equipped with: 1) laser head, 2) mirror, 3) adjustable zeroth-order half-wave plate, 4) polarizing cube, 5) beam displacer, 6) fiber coupler, 7a) long single-mode fiber, 7b) short single-mode fiber.

for differential interference contrast (DIC) microscopy. This prism deflects the laser pulses into two slightly different directions depending on pulse 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 triple band dichroic mirror (z470/532/638rpc, AHF-Analysentechnik, Tübingen, Germany), focused through a single pinhole of 200 μm diameter, re-collimated and split by a non-polarizing beam splitter cube, and refocused onto two single-photon avalanche diodes (SPAD, PDM series, Micro Photon Devices, Bolzano, Italy). Measurements were performed with a total excitation power of ca. 10 μW, and it was checked that a slight increase in excitation power did not change the final values of determined diffusion coefficients.

When calculating correlation functions, only photons from the two different detectors are correlated to prevent distortions of the resulting ACF by SPAD after-pulsing. 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 [13]). Thus, it is possible to calculate the ACFs for each focus separately, as well as the CCF between photons emerging from both foci. Calculation of all ACFs and CCF was performed by using the algorithm published in ref. [14]. The sample temperature was controlled by a homemade temperature regulator with absolute accuracy of ±0.1 K [15,16]. All values of diffusion coefficients that will be reported in this letter are given for a temperature of 25 °C. When they had been measured at a different temperature, they were re-calculated for 25 °C by using the well-known

Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta R_h}, \tag{1}$$

connecting the diffusion coefficient D with temperature T, solvent viscosity η, and hydrodynamic radius R_h, with k_B denoting the Boltzmann constant. The dependence of the viscosity of water on temperature was taken from ref. [17]. Sample solutions were sealed into closed sample cells to prevent solvent evaporation [16].

An important feature of 2fFCS is to use an appropriate model for the MDF. We found that the usual assumption that the MDF has the shape of a three-dimensional Gaussian distribution is inadequate in the following sense: even when referencing FCS measurements against a known standard (and this is, to some extent, also done with 2fFCS when calibrating the interfocal distance with the diffusion of fluorescent beads with known size), one has still systematic errors when experimental conditions slightly differ from measurement to measurement, such as refractive index of the solution or optical saturation properties of the dye [9,12,18]. 2fFCS is mostly insensitive to these variations but only when using a MDF model function which is a better description of the optical reality than the standard three-dimensional Gaussian approximation. As was shown in ref. [12], a combination of a Gauss-Lorentz excitation intensity profile and a simple pinhole function is an excellent description for the MDF $U(\vec{r})$ as a function of position $\vec{r}$ under ideal optical conditions, and yields still correct results even when the MDF is strongly affected by refractive-index mismatch or optical saturation [12,18]. The explicit functional form of this MDF is given by

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

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

$$w(z) = w_0\sqrt{1+\left(\frac{\lambda_{ex} z}{\pi w_0^2 n}\right)^2} \tag{3}$$

and

$$\kappa(z) = 1 - \exp\left(-\frac{2a^2}{R_0^2 + (\lambda_{em} z/\pi R_0 n)^2}\right), \tag{4}$$

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

$$\begin{aligned} g(t) = {} & g_\infty(\delta) + 2\epsilon_1\epsilon_2 c\sqrt{\frac{\pi}{Dt}} \\ & \times \int_{-\infty}^{\infty} \mathrm{d}z_1 \int_{-\infty}^{\infty} \mathrm{d}z_2 \, \frac{\kappa(z_1)\kappa(z_2)}{8Dt + w^2(z_1) + w^2(z_2)} \\ & \times \exp\left[-\frac{(z_2-z_1)^2}{4Dt} - \frac{2\delta^2}{8Dt + w^2(z_1) + w^2(z_2)}\right] \end{aligned} \tag{5}$$

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, c is the concentration of the fluorescent molecules, and D is the 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, an additional exponential function is added to the correlation functions [19]. When fitting experimentally measured data, one fits the 2 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 is important to note that, although the absolute values of the ACF and CCF do not have any effect on the fit results for the diffusion coefficient, the ratio of the ACF-to-CCF amplitude does: It is a direct measure of the focus overlap. Thus, rescaling all correlation curves by some common factor has no impact on the fitted value of the diffusion coefficient, as long as the relative amplitude ratio is not changed.

It should be emphasized that the model MDF does not need to be an *exact* representation of the actual MDF as long as it yields a reasonable fit quality of the experimentally measured curves. We have checked by direct wave-optical calculation of the MDF under various conditions and numerical computation of the resulting ACF and CCF curves (similar to the study presented in ref. [9]) that ellipticity of the foci and even the slight asymmetric clipping of the MDF by the concentric pinhole will not lead to wrong values of the diffusion coefficient when using the above model MDF for fitting. Detailed results of these model calculations will be presented elsewhere. The important parameter determining the absolute accuracy of the resulting values of the diffusion coefficient is the lateral distance δ between the foci. This distance is determined 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. Thus, it is necessary to calibrate the system once by determining this value. We have done that by measuring the diffusion of fluorescently labeled polymer beads (TetraSpeck 100 multi-fluorescent latex beads, Invitrogen, Karlsruhe, Germany) with DLS as well as with our 2fFCS system [20]. The wavelength-dependent shear distances of the Nomarski prism were determined using the diffusion of fluorescently labeled beads of known diameter, as described in ref. [20]. They have been determined as 370 nm at $\lambda_{ex} = 470$ nm, 389 nm at $\lambda_{ex} = 532$ nm and 395 nm at $\lambda_{ex} = 637$ nm with an accuracy of ± 1.5 nm. As was shown in ref. [21], the finite size of the particles ($\sim$100 nm diameter) can still be ignored in comparison to the detection volume, and they can be considered as point-like objects when evaluating FCS data without introducing additional errors.

A crucial assumption of 2fFCS is the perfect and crossed polarization of the excitation light and its alignment

with respect to the Nomarski prism. We checked, by using a Glan-Thomson polarizer, that the polarization ratio of both laser pulse trains close to the DIC prism position is better than 1 : 100, and estimated with model calculations that the resulting mixing of auto- and cross-correlation due to the residual depolarized light leads to errors of the less than one percent in the determined diffusion coefficient. Perfect alignment of polarization axes and the DIC prism was achieved by slightly turning the polarization axis of the excitation light with respect to the DIC prism until maximum amplitude contrast between auto-and cross-correlation was achieved. Finally, all reported errors of the diffusion coefficient values below are standard deviations derived from at least twenty measurements.

Results and discussion. – Using our 2fFCS system, we determined the absolute diffusion coefficients of three widely used fluorescent dyes (Atto655-maleimid, Rhodamine 6G, and Oregon Green® 488) in aqueous solutions (solvent LichroSolv water for chromatography, No. 115333, Merck KGaA, Darmstadt, Germany) at three different excitation wavelengths (470 nm, 532 nm, 637 nm) across the visible spectrum. In all cases, it was checked that the obtained diffusion coefficient was independent of excitation intensity, in stark contrast to typical single-focus FCS measurements. Only when the excitation power per focus was exceeding ca. 50 μW, photobleaching started to accelerate the apparent diffusion coefficient.

For the dye Atto655-maleimid (AttoTec, Siegen, Germany), a diffusion coefficient in water $D_{25\,^\circ\mathrm{C}}$(Atto655-maleimid) $= (4.07 \pm 0.1) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$ was found. We determined the diffusion coefficient of the same dye in deuterized methanol (methanol4d) with pfNMR. In both cases we find, via the Stokes-Einstein relation, that the hydrodynamic radius of the dye is identical (within our measurement errors) in both solvents. This is a non-trivial result in itself, because it would be possible that the hydrodynamic radius in different solvents is not only dependent on macroscopic viscosity but also on specific microscopic interactions such as the formation of hydrogen bonds. We found a similar equality between 2fFCS and pfNMR measurements for the free acid form of the dye ($D_{25\,^\circ\mathrm{C}}$(Atto655-carboxylicacid) $= (4.26 \pm 0.08) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$). Thus, 2fFCS is sensitive enough to be able to resolve side group variations leading to diffusion coefficient differences of only 4%.

Rhodamine 6G (Rh6G) was used over three decades as calibration standard for FCS experiments with a reported diffusion coefficient of $D_{22\,^\circ\mathrm{C}}(\mathrm{Rh6G}) = (2.8 \pm 0.7) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$ in a buffer solution of 10^{-4} M Na ethylenediaminetetraacetic acid, 10^{-1} M NaCl, 10^{-2} M tris-(hydroxymethyl)-aminomethane, with *p*H 8.0 [22]. In most publications, the effect of the buffer solution is neglected and that value is used directly for aqueous solutions of Rh6G, where it translates into a value of $D_{25\,^\circ\mathrm{C}}(\mathrm{Rh6G}) = (3.0 \pm 0.8) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$ at 25 °C when using the known dependence of water viscosity on temperature.

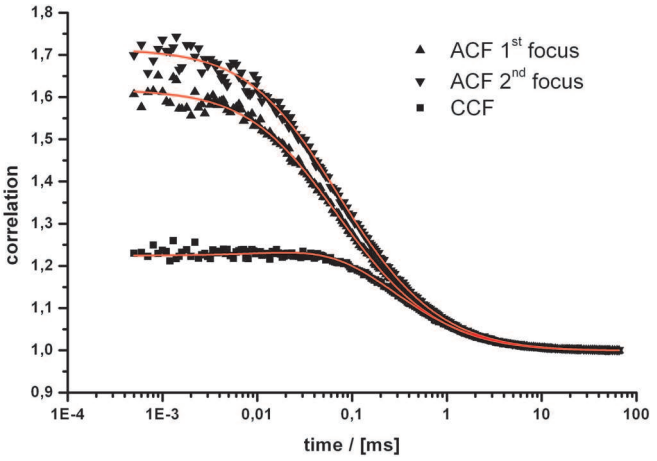

Fig. 2: 2fFCS measurement on an aqueous solution of Rhodamine 6G. The ACF for the first focus (ACF 1st focus), second focus (ACF 2nd focus), and the CCF between both foci are shown. All correlation curves are shown as normalized to the value at infinite lag time. The slight difference in the ACF amplitudes is *not* due to different excitation volumes in both foci but due to slightly different scattering background for both excitation polarizations and thus foci. Laser excitation power was $= 10.2\,\mu$W. Markers indicate experimental values, solid lines are global fits using eq. (5) with fit-parameters: $w_0 = 245$ nm, $R_0 = 150$ nm, the triplet state relaxation was not applied to the fit, and diffusion coefficient $D_{25\,^\circ\mathrm{C}}(\mathrm{Rh6G}) = 4.14 \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$.

We performed measurements on aqueous solutions of Rh6G at 25 °C. Rh6G was obtained from two different suppliers because it is known that the grade of purity is quite different for commercially available Rh6G. For Rhodamine 6G (No. 83697, Sigma-Aldrich, Seelze, Germany), a diffusion coefficient $D_{25\,^\circ\mathrm{C}}(\mathrm{Rh6G}) = (3.89 \pm 0.3) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$ was found. A HPLC measurement with an eluent of 85%/15% MeOH/H_2O on a RP17 column with a UV/VIS detection at 532 nm shows a purity grade of less than 55% Rh6G, but the remainder also absorbs at 532 nm and shows fluorescence. Measuring this mixture of multiple species having slightly different hydrodynamic radii with FCS leads to compound correlation functions. When evaluating these correlation functions with a model for only one single species, one observes a large fit variation from measurement to measurement.

For a pure sample of Rhodamine 6G (No. R634, Invitrogen, Karlsruhe, Germany), a diffusion coefficient of $D_{25\,^\circ\mathrm{C}}(\mathrm{Rh6G}) = (4.14 \pm 0.05) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$ was found, exemplary autocorrelation functions of Rh6G are shown in fig. 2. HPLC shows a purity grade better than 95% and therefore the sample can be recommended for calibration. The diffusion coefficient corresponds to a hydrodynamic radius of 5.89 Å and is in perfect agreement with a recently published measurement using plug broadening

in capillary flow [23]. It is important to note that the found value of the diffusion coefficient is by 37% larger than the usually used value for referencing standard FCS measurements.

Finally, the diffusion coefficient of the dye $2',7'$-difluorofluorescein (Oregon Green® 488) (No. D6145, Invitrogen, Karlsruhe, Germany) was found to be equal to $D_{25\,^\circ\mathrm{C}}(\text{Oregon Green}^{®}\ 488) = (4.11 \pm 0.06) \times 10^{-6}\mathrm{cm}^2/\mathrm{s}$, corresponding to a hydrodynamic radius of the molecule of 5.95 Å.

Conclusion. – In summary, we determined precise absolute values of diffusion coefficients and hydrodynamic radii for three dyes excitable between 470 nm and 640 nm, a spectral region where most of the FCS measurements are performed. These values can serve as diffusion standards for precisely calibrating diffusion measurements using fluorescent markers across the visible spectrum.

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