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Statistical Analysis of Diffusion Coefficient Determination by Fluorescence Correlation Spectroscopy

Jörg Enderlein,[1,2] Ingo Gregor,[1] Digambara Patra,[1] and Jörg Fitter[1]

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Fluorescence correlation spectroscopy (FCS) has become an important and widely used technique for many applications in physics, chemistry, and biology. The parameter most frequently addressed by FCS is the diffusion of molecules in solution. Due to the highly non-linear connection between the diffusion coefficient and a measured autocorrelation function, it is extremely difficult to analyse the accuracy of the diffusion-coefficient determination in a FCS experiment. Here, we present a simplified analysis based on some general maximum-likelihood considerations, and numerical result are given for the dependence of the accuracy of the diffusion-coefficient determination on sample concentration, brightness, and measurement time. Optimal concentration values for performing FCS are found.

KEY WORDS: Fluorescence correlation spectroscopy; diffusion coefficient; statistical accuracy.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) is a relatively old technique, originally introduced by Elson, Magde and Webb in the early seventies [1–3]. However, it took nearly two decades until the technique has seen a renaissance in single molecule spectroscopy (SMS) after the development of new lasers with high beam quality and temporal stability, low-noise single-photon detectors, and high-quality microscope objectives with nearly perfect imaging quality at high numerical aperture. Achieving values of the detection volume within the range of a few μm^3 made the technique applicable for samples at reasonably high concentrations (nM) and enabled sufficiently short measurement times (minutes). An excellent and extensive description of FCS can be found in [4,5]. In FCS, the detected fluorescence intensity is correlated with a time-shifted replica of itself at different values of time shift (lag time). The result is the so-called autocorrelation function (ACF), i.e. the second-order correlation function of the fluorescence intensity signal. The physical meaning of the ACF is that it is proportional to the probability to detect a photon at some later time (lag time) if there was a detection event at time zero. This probability is composed of two basically different terms: The two photons detected at time zero and some later lag time can originate from uncorrelated background or from different fluorescing molecules and therefore do not have any physical correlation (provided there is no interaction of the different fluorescing molecules). These events will contribute to a constant offset of the ACF that is completely independent on lag time t. Alternatively, the two photons originate from one and the same molecule and therefore are physically correlated, leading to a time-dependent component of the ACF. Thus, the temporal behaviour of the ACF is solely determined by the correlated contributions of individual molecules. In this sense, FCS is a true SMS technique, although the analysis is not explicitly identifying single molecule detection events.

On different time scales, the temporal behaviour of the autocorrelation function is determined by different properties of the fluorescing molecules: On a nanosecond time-scale, photon antibunching can be observed,

[1] Institute for Biological Information Processing 1 & 2, Forschungszentrum Jülich, D-52425 Jülich, Germany.
[2] To whom correspondence should be addressed. E-mail: j.enderlein@fz-juelich.de

reflecting the fact that directly after the emission of a photon the molecule needs to get re-excited for being able to emit the next photon, leading to a steep decrease of the ACF towards short times. On a microsecond time scale, the ACF is dominated by triplet state dynamics. If excitation and/or detection are done through polarization filters, the autocorrelation will also show contributions from rotational diffusion dynamics of the molecules. On a millisecond to second time-scale, the ACF shows a typical decay due to the lateral diffusion of the molecules out of the detection region. Diffusion is also the process most frequently addressed by FCS measurements. FCS has been extensively used in numerous studies, of which only few are cited here. FCS was used for studying diffusion of molecules in homogeneous and heterogeneous environment [6–8], intermolecular binding and reaction kinetics [9–15], single molecule photophysics [16–24], and conformational dynamics [25]. For recent reviews see [26,27] and the book [28].

In spite of the importance of the subject, there are only few papers which have dealt with matters of statistical accuracy in fluorescence fluctuation experiments. The first fundamental study of the subject, shortly after the report of the first experiments by Magde *et al*. [1], was presented by Koppel [29]. Koppel's calculations assumed high fluorophore concentrations, corresponding to the experimental conditions of the time. Qian [30] extended Koppel's earlier work by considering a two-dimensional Gaussian sample profile instead of the uniform spot of Koppel's theory, and also considering low fluorophore concentrations. Kask *et al*. [31] presented a more general theory of the signal-to-noise-ratio of a FCS measurement, applicable to an arbitrary profile of the molecule detection function. Meseth *et al*. [32] studied the resolution of a FCS measurement when more than a single fluorescing species is present. Finally, Wohland *et al*. [33] gave a detailed analysis of the standard deviation of a FCS measurement derived from Monte-Carlo simulations. In all these papers, the emphasis was on deriving some general measure of the standard deviation or signal-to-noise-ratio of a FCS measurement.

The present paper focuses on a more specific question: The accuracy of determining a diffusion coefficient by FCS. The main reason why this question was not studied in detail before is the highly non-linear relationship between the diffusion coefficient as extracted from a FCS measurement and the original fluorescence intensity fluctuations. This fact prevents an analytical analysis of the accuracy of this determination. However, by considering the much simplified question of how accurate a choice can be made between only *two* possible diffusion coefficients, it is possible to derive analytical estimates for the involved error. This analysis can then be extended to obtain some general estimates of the accuracy of determining diffusion coefficients in dependence on fluorophore concentration, fluorescence brightness, and measurement time.

THEORY

In an ideal FCS measurement, one records the number n_j of detected fluorescence photons in consecutive time bins, $1 \le j \le T$, where T is the maximum number of time bins (defining the measurement time), and calculates the ACF g_t at different lag times t as

$$g_t = \frac{1}{T-t}\sum_{j=1}^{T-t} n_j n_{j+t}. \tag{1}$$

The standard approach of extracting a value for the diffusion coefficient from such an ACF is to fit, by a non-linear fitting routine like the Nelder–Mead simplex algorithm, the infinite-time limit ($T \to \infty$) of a model ACF against the measured curve g_t using the diffusion coefficient D and the concentration c as the fit parameters. For simplicity reasons, let us follow the standard assumption of a three-dimensional Gaussian shape of the molecule detection function (MDF) $U(\mathbf{r})$, i.e. let us assume that the probability to detect a photon when a molecule is located at position $\mathbf{r}$ is proportional to

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

where a and b are two characteristic parameters describing the MDF, κ is a factor accounting for the overall light detection efficiency of the measurement system as well as the molecules' absorption cross-section and fluorescence quantum yield (fluorescence brightness), and x, y and z are Cartesian coordinates, with z along the optical axis. The infinite-time limit of the ACF is then given by [4,5]

$$\bar{g}_t = \frac{\pi^{3/2}\kappa^2 a^2 b}{8}\left[\frac{c}{(1+4Dt/a^2)\sqrt{1+4Dt/b^2}} + c^2\pi^{3/2}a^2 b\right], \tag{3}$$

with c being the fluorophore concentration in the sample solution. This curve serves as the fit function that is fitted against the measured g_t, yielding values for the diffusion coefficient D and concentration c. It is obvious that, in such a procedure, the connection between the originally measured fluorescence intensities, n_j, and the finally obtained value of the diffusion coefficient, D, is highly non-linear, which makes an exact statistical analysis of the accuracy of determining D in dependence on measurement time, sample concentration or molecule brightness (i.e. the value of κ) extremely difficult.

To simplify matters, we will proceed in a different way. Firstly, instead of fitting an unknown diffusion coefficient, one assumes that one has only to choose between two possible diffusion coefficients, D_0 and D, and one asks whether a measured ACF corresponds more to a model ACF based on the value D_0 or one based on D. This can be done by calculating a likelihood value M according to

$$M = \sum_{k=1}^{N} m_k\, g_k = \sum_{k=1}^{N} m_k \frac{1}{T-k} \sum_{j=1}^{T-k} n_j\, n_{j+k} \qquad (4)$$

where N is the maximum lag time considered, and m_k is a suitably chosen function so that M is positive if the measured ACF g_k resembles more the model ACF based on diffusion coefficient D_0, and negative if g_k resembles more the model ACF based on diffusion coefficient D. A suitable candidate for m_k is given by

$$m_j = \ln\left[\frac{\bar{g}_j(D_0)}{\bar{g}_j(D)}\right] \qquad (5)$$

which would be the mathematically optimal choice (a maximum-likelihood estimator) if the values of the ACF follow a Poisson statistics with mean values $\bar{g}_j$. Of course, this is far from being the case (the $\bar{g}_j$ are not mean values of stochastic variables with Poisson statistics but the values of the ACF at infinite measurement time) – nonetheless Eq. (5) yields still a good function for making a choice between two possible diffusion coefficients (see also Results and Discussion section).

Thus, for making a decision between D_0 and D, one measures the fluorescence intensities n_j, calculates the ACF g_t, then the likelihood value M, and if M is larger than zero, one chooses D_0 as the correct diffusion coefficient, otherwise D. If the true diffusion coefficient of the sample is D_0, then the frequency of choosing D instead of D_0 corresponds to the error $\mathrm{err}(D|D_0)$ of determining the wrong diffusion coefficient in this two-value decision procedure. By mapping this error $\mathrm{err}(D|D_0)$ for all possible values of D at fixed D_0, one obtains some analogue of a probability distribution of assigning the wrong diffusion coefficient to a measured ACF originating from a sample with diffusion coefficient D_0. The width of this distribution is a measure of the accuracy with which a diffusion coefficient can be determined. Although this kind of reasoning does not give the direct probability distribution for the diffusion coefficient, it has the enormous advantage to being amenable to an exact statistical analysis: because there is a polynomial connection (second order) between M and the n_j, the moments of M can be calculated exactly, on the basis of the Poisson statistics of the n_j (Poisson photon detection statistics). This allows an exact calculation of the distribution $\mathrm{err}(D|D_0)$, the width of which will be taken as a measure of the accuracy to determine a correct value of the diffusion coefficient.

In a first step, the first and second moments of the probability distribution of the likelihood value M are determined. For doing that, M is rewritten as a quadratic form of the variables n_j:

$$M = \frac{1}{2} \sum_{j,k} n_j \alpha_{jk} n_k, \qquad (6)$$

with the matrix α defined by

$$\alpha_{jk} \equiv \alpha_{|j-k|} = \frac{m_{|j-k|}}{T-|j-k|} \text{ for } 1 \le |j-k| \le N, \quad \text{else } \alpha_{jk} = 0. \qquad (7)$$

Calculation of the first and second moments of the M-distribution involves averaging over squares and double squares of the variables n_j, and assuming that the photon counts per time bin follow a Poisson statistics, these averages are given by

$$\langle n_j n_k \rangle = \left\langle \lim_{\{\varepsilon \downarrow 1\}} \left(\varepsilon_j \frac{\partial}{\partial \varepsilon_j}\right)\left(\varepsilon_k \frac{\partial}{\partial \varepsilon_k}\right) \exp\left[\sum_p (\varepsilon_p - 1) I_p\right]\right\rangle = \langle I_j I_k \rangle + \delta_{jk} \langle I_j \rangle \qquad (8)$$

and

$$\langle n_j n_k n_r n_s \rangle = \left\langle \lim_{\{\varepsilon \downarrow 1\}} \left(\varepsilon_j \frac{\partial}{\partial \varepsilon_j}\right)\left(\varepsilon_k \frac{\partial}{\partial \varepsilon_k}\right)\left(\varepsilon_r \frac{\partial}{\partial \varepsilon_r}\right) \times \left(\varepsilon_s \frac{\partial}{\partial \varepsilon_s}\right) \exp\left[\sum_p (\varepsilon_p - 1) I_p\right]\right\rangle \qquad (9)$$

where the I_j are mean intensity values per time bin, and the brackets on the r.h.s. denote averaging over all possible positions and paths of the diffusing molecules in the sample. Taking into account that $\alpha_{jk} = 0$ when $j = k$, the first moment of the M-distribution is thus given by

$$\langle M \rangle = \frac{1}{2} \sum_{j,k} \alpha_{|j-k|} \langle I_j I_k \rangle. \qquad (10)$$

Analogously, but more tediously, one finds for the second moment

$$\langle M^2 \rangle = \frac{1}{4} \sum_{j,k,r,s} \langle \alpha_{jk} \alpha_{rs} n_j n_k n_r n_s \rangle = \frac{1}{4} \left[\sum_{j,k} 2\alpha_{|j-k|}^2 \langle I_j I_k \rangle + 4 \sum_{j,k,r} \alpha_{|j-k|} \alpha_{|j-r|} \langle I_j I_k I_r \rangle + \sum_{j,k,r,s} \alpha_{|j-k|} \alpha_{|r-s|} \langle I_j I_k I_r I_s \rangle \right]. \qquad (11)$$

Of main interest is the case where the measurement time is much larger than the maximum lag time of the autocorrelation curve, i.e. where $T \gg N$. In that case, the above expressions can be simplified, retaining only terms in the leading order of T^{-1}:

$$\sum_{j,k} \alpha^{\mu}_{|j-k|} \langle I_j I_k \rangle \approx \frac{2}{T^{\mu}} \sum_{j<k} m^{\mu}_{k-j} \langle I_j I_k \rangle = \frac{2}{T^{\mu-1}} \sum_{u>0} m^{\mu}_u \langle I_0 I_u \rangle$$

$$4 \sum_{j,k,r} \alpha_{|j-k|}\alpha_{|j-r|} \langle I_j I_k I_r \rangle \approx \frac{8}{T^2} \sum_{j \le k \le r} (m_{|j-k|} m_{|j-r|} + m_{|j-k|} m_{|k-r|} + m_{|j-r|} m_{|k-r|}) \langle I_j I_k I_r \rangle \approx \frac{8}{T} \sum_{u,v \ge 0} [(m_u + m_v) m_{u+v} + m_u m_v] \langle I_0 I_u I_{u+v} \rangle$$

$$\sum_{j,k,r,s} \alpha_{|j-k|}\alpha_{|r-s|} \langle I_j I_k I_r I_s \rangle \approx \frac{4}{T^2} \sum_{j \le k \le r \le s} (m_{k-j} m_{s-r} + m_{r-j} m_{s-k} + m_{s-j} m_{r-k}) \langle I_j I_k I_r I_s \rangle \approx \frac{4}{T} \sum_{u,w,v \ge 0} (m_u m_w + m_{u+v} m_{v+w} + m_{u+v+w} m_v) \times \langle I_0 I_u I_{u+v} I_{u+v+w} \rangle$$

so that we obtain the final expressions for the mean and mean square of M as

$$\langle M \rangle \approx \sum_{u>0} m_u \langle I_0 I_u \rangle$$

and

$$\langle M^2 \rangle \approx \frac{1}{T} \left[\sum_{u \ge 1} m_u^2 \langle I_0 I_u \rangle + 2 \sum_{u,v \ge 1} [(m_u + m_v) m_{u+v} + m_u m_v] \langle I_0 I_u I_{u+v} \rangle + \sum_{u,v,w \ge 1} (m_u m_w + m_{u+v} m_{v+w} + m_{u+v+w} m_v) \times \langle I_0 I_u I_{u+v} I_{u+v+w} \rangle \right]$$

In a second step, the obtained first and second moments of the M-distribution are used to approximate it by a Gaussian distribution with mean $\langle M \rangle$ and mean square deviation $\delta M = \langle M^2 \rangle - \langle M \rangle^2$,

$$P(M, D|D_0) \approx \frac{1}{\sqrt{2\pi \langle \delta M^2 \rangle}} \exp\left[-\frac{(M - \langle M \rangle)^2}{2 \langle \delta M^2 \rangle} \right],$$

allowing immediately the calculation of the error $\mathrm{err}(D|D_0)$ as

$$\mathrm{err}(D|D_0) \approx \int_{-\infty}^{0} dM \frac{1}{\sqrt{2\pi \langle \delta M^2 \rangle}} \exp\left[-\frac{(M - \langle M \rangle)^2}{2 \langle \delta M^2 \rangle} \right] \approx 1 - \mathrm{erf}\left(\frac{\langle M \rangle}{\sqrt{2 \langle \delta M^2 \rangle}} \right) \tag{12}$$

The dependence of this expression on D and D_0 is implicit via the dependence of the M-defining matrix α_{jk} on D and D_0.

It remains to calculate the averages of the products of the mean intensities I_u. Consider the simple average $\langle I_u \rangle$. The mean detected fluorescence intensity is the sum of the mean fluorescence intensities of all molecules in the sample. The probability density to find the first molecule at position $\mathbf{r}_1$, the second at $\mathbf{r}_2$, etc. is given by the product $\prod_n (V^{-1} d\mathbf{r}_n)$, where V is the total sample volume. The average $\langle I_u \rangle$ is calculated by averaging over all possible molecule positions, i.e.

$$\langle I_u \rangle \equiv \langle I \rangle = \left(\prod_n \frac{1}{V} \int_V d\mathbf{r}_n \right) \left(\sum_n U(\mathbf{r}_n) \right) = c \int_V d\mathbf{r}\, U(\mathbf{r}) = \left(\frac{\pi}{2} \right)^{3/2} c\kappa a^2 b.$$

In a similar way, averages of the higher-order products of the intensities are derived, additionally taking into account that the probability to find the same molecule at position $\mathbf{r}_2$ at time t_2 when it was at position $\mathbf{r}_1$ at time t_1 is given by Green's function $G(\mathbf{r}_2 - \mathbf{r}_1,\ t_2 - t_1)$ of the diffusion equation,

$$G(\mathbf{r}_2 - \mathbf{r}_1, t_2 - t_1) = \frac{1}{[4\pi D(t_2 - t_1)]^{3/2}} \exp\left[-\frac{(\mathbf{r}_2 - \mathbf{r}_1)^2}{4D(t_2 - t_1)} \right].$$

Using this function, the so-called one-particle k-point correlators can be introduced

$$Z_k(t_1 \ldots, t_k) = \left(\prod_{j=0}^{k} \int d\mathbf{r}_j \right) \left(\prod_{j=1}^{k} U(\mathbf{r}_j)\, G(\mathbf{r}_j - \mathbf{r}_{j-1}, t_j) \right) U(\mathbf{r}_0),$$

through which the higher-order product averages are expressed as

$$\langle I_0 I_u \rangle = \kappa^2 \left[c Z_1(u) + c^2 Z_0^2 \right],$$

$$\langle I_0 I_u I_{u+v} \rangle = \kappa^3 \left\{ c Z_2(u, v) + c^2 Z_0 [Z_1(u) + Z_1(v) + Z_1(u+v)] + c^3 Z_0^3 \right\},$$

$$\langle I_0 I_u I_{u+v} I_{u+v+w} \rangle = \kappa^4 \left\{ c Z_3(u, v, w) + c^2 Z_0 [Z_2(u, v) + Z_2(u, v+w)] \right.$$

$$
\begin{aligned}
&+ Z_2(u+v,w) + Z_2(v,w)] \\
&+ c^2[Z_1(u)Z_1(w) + Z_1(u+v) \\
&\times Z_1(v+w) + Z_1(u+v+w) \\
&\times Z_1(v)] + c^3 Z_0^2[Z_1(u) + Z_1(v) \\
&+ Z_1(w) + Z_1(u+v) + Z_1(v+w) \\
&+ Z_1(u+v+w)] + c^4 Z_0^4\}.
\end{aligned}
$$

By inserting the explicit functional form of the MDF $U(\mathbf{r})$ into the integrals defining the correlators Z_k, these are calculated to be

$$Z_k(t_1, \ldots, t_k) = \zeta_k^2(a, t_1, \ldots, t_k)\zeta_k(b, t_1, \ldots, t_k)$$

with the ζ_k defined by

$$\zeta_0(\xi) = \sqrt{\frac{\pi}{2}}\xi,$$

$$\zeta_1(\xi, t) = \frac{\sqrt{\pi}}{2}\frac{\xi}{\sqrt{1+4Dt/\xi^2}},$$

$$\zeta_2(\xi, t_1, t_2) = \sqrt{\frac{\pi}{2}}\frac{\xi}{\sqrt{3+16\,D(t_1+t_2)/\xi^2 + 64\,D^2 t_1 t_2/\xi^4}},$$

$$
\begin{aligned}
&\zeta_3(\xi, t_1, t_2, t_3) \\
&= \sqrt{\frac{\pi}{8}}\left(\xi \Big/ \sqrt{\begin{array}{l}1 + 2\,D(3t_1 + 4t_2 + 3t_3)/\xi^2 + 64\,D^2 \\ \times (t_1t_2 + t_1t_3 + t_2t_3)/\xi^4 + 128\,D^3 t_1 t_2 t_3/\xi^6\end{array}}\right).
\end{aligned}
$$

RESULTS AND DISCUSSION

Although the derived expressions seem to be rather complex, some general conclusions can be drawn by analyzing the dependence of $\mathrm{err}(D/D_0)$ on measurement time T, molecule brightness κ, and concentration c. The dependency on measurement time is most easily analyzed. Careful inspection of the found expression for $\delta M = \langle M^2\rangle - \langle M\rangle^2$ reveals that it decays as T^{-1}. This is due to the fact that in the expression of $\langle M^2\rangle$, only the sum involving the term $m_u m_w \langle I_0 I_u I_{u+v} I_{u+v+w}\rangle$ yields a contribution independent on T which is exactly cancelled by $\langle M\rangle^2$ when calculating δM. Thus, the error $\mathrm{err}(D/D_0)$ falls off as $1 - \mathrm{erf}\,(\mathrm{const} \times T)$. The dependence of $\mathrm{err}(D/D_0)$ on concentration c and brightness κ is more involved. Inspection of the expressions found for the $\langle I\rangle$, $\langle I_0 I_u\rangle$ etc. shows that $\langle M\rangle$ and δM depend on c and κ in a complex polynomial way (up to fourth order in each variable). As a numerical example, let us consider a sample containing fluorescent molecules with diffusion coefficient of 10^{-5} cm^2/s. The characteristic dimensions of the 3D-Gaussian detection volume are set to be $a = 0.5\,\mu$m and $b = 2\,\mu$m. Figure 1 shows different curves of $\mathrm{err}(D/D_0)$ for increasing values of measurement time T at fixed concentration $c = 1/\mu\mathrm{m}^3$ (1.66×10^{-9} M) and brightness $\kappa = 10^3$ photons/s. It should be noted that the distributions shown in Fig. 1 are nearly symmetric around the value D_0 on a logarithmic scale of D, i.e. it is a function of $\log D/D_0$ only. This shows that only the ratio between two diffusion coefficients is important for the accuracy of distinguishing between them. It is reasonable to define the "width" of the distributions $\mathrm{err}(D/D_0)$ as $w = 0.5(D_{\max}/D_0 + D_0/D_{\min})$, where $D_{\min}$ and $D_{\max}$ are the two values of D where the distribution $\mathrm{err}(D/D_0)$ is equal to some constant value, e.g. 0.1 (10% error, see dotted line in Fig. 1). This width can be taken as a measure of the relative accuracy of determining the diffusion coefficient from a measured ACF. Figure 2 shows the dependence of w on measurement time for four different values of brightness κ at fixed concentration $c = 1/\mu\mathrm{m}^3$. As can be seen, after a fast fall-off of w at small measurement times, it approaches its minimum possible value $w = 1$ asymptotically slowly with increasing measurement time. Even increasing κ only slightly improves that situation: The curve for κ=10^4 photons/s is already indistinguishable from the asymptotic curve for $\kappa \to \infty$.

To visualize the dependency of the accuracy of the diffusion-coefficient determination on all three parameters c, κ and T in a most compact way, the following particular question will be studied: What is the minimum measurement time T at given concentration c and brightness κ for which the error to make the wrong decision between the correct value of the diffusion coefficient $D_0 = 10^3\,\mu\mathrm{m}^2$/s and its half value $D = D_0/2 = 500\,\mu\mathrm{m}^2$/s is less than 10%, i.e. $\mathrm{err}(D/2|D_0) \le 0.1$ (see broken line in Fig. 1)? The choice $D = D_0/2$ and $\mathrm{err} = 0.1$ is completely arbitrary and any other value could also be considered. However, the principal dependence of the measurement time for achieving a given accuracy on concentration and brightness will be similar. Figure 3 shows different curves which correspond, from top to bottom, to increasing values of brightness κ as indicated in the legend right to the figure. A nearly identical figure results if one chooses for D a doubled value of D_0 instead of its half, due to the fact that $\mathrm{err}(D/D_0)$ is approximately a function of $\log D/D_0$ only. In Fig. 3, curves are shown only down to a measurement time value of 100 s (dotted line): below that value, the basic assumption that the measurement time is much larger than the maximum lag time is no longer valid. The most striking feature in Fig. 3 is the strong convergence of all curves to a limiting envelope towards

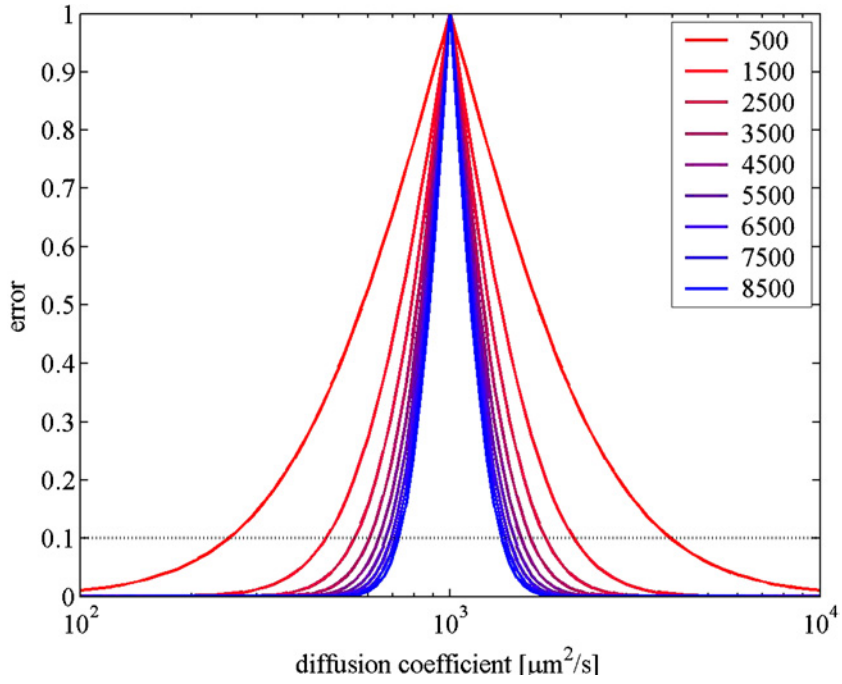

Fig. 1. Error distribution for distinguishing between the correct diffusion coefficient $D_0 = 10^3\ \mu\text{m}^2/\text{s}$ and a wrong value D (horizontal axis) for sample concentration of $c = 1/\mu\text{m}^3$ and brightness of $\kappa = 10^3$ photons/s. Different curves correspond to different measurement times in seconds (see legend): The larger the measurement time, the narrower the error function $\text{err}(D|D_0)$.

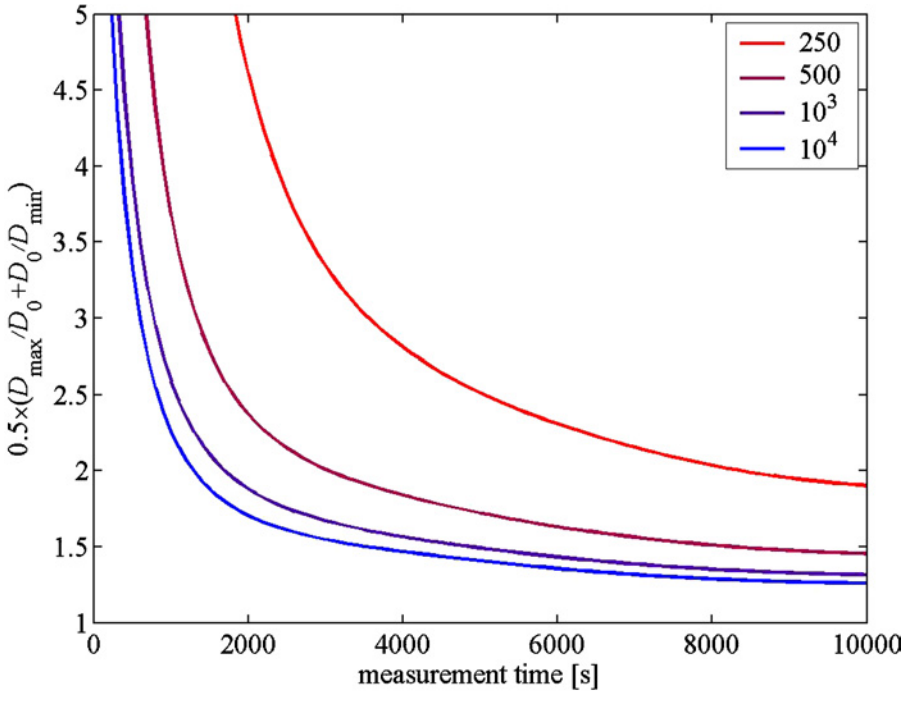

Fig. 2. Dependence of $w = 0.5(D_{\text{max}}/D_0 + D_0/D_{\text{min}})$ on measurement time T, where D_{min} and D_{max} are the two values of D where $\text{err}(D|D_0) = 0.1$ (see also previous figure). Shown are four curves, from top to bottom, for increasing values of κ as indicated by the legend where numbers are given in photons/s. Sample concentration is $1/\mu\text{m}^3$.

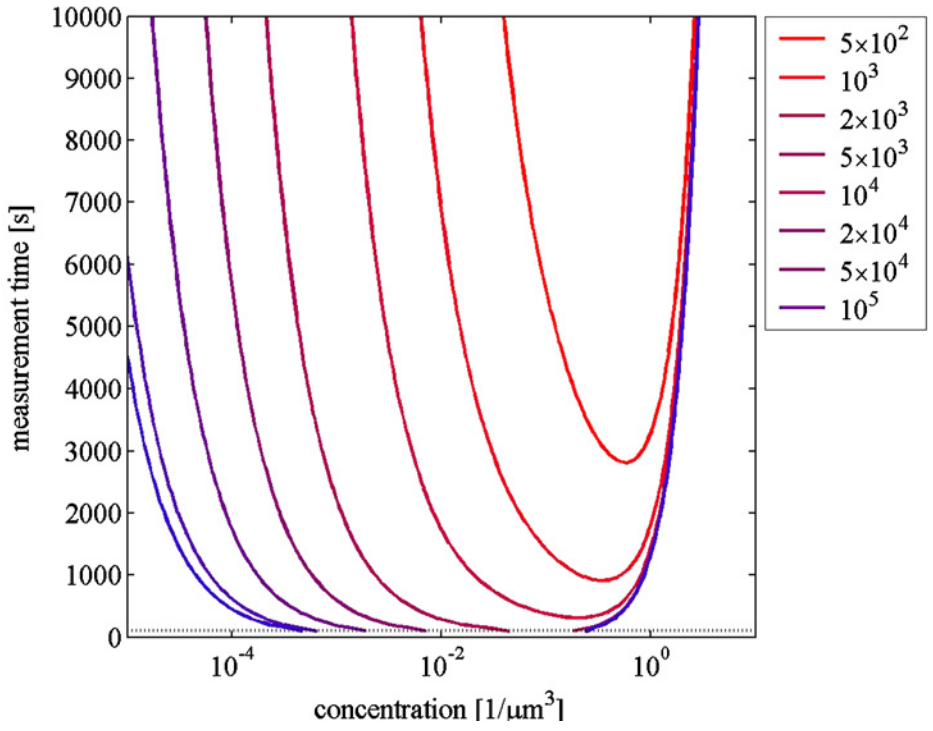

Fig. 3. Dependence of measurement time on concentration for different values of molecule brightness as indicated in the legend where numbers are given in photons/s. Shown are contour lines where $\mathrm{err}(D/2|D_0) = 0.1$; D_0 is set to $10^3\ \mu\mathrm{m}^2/\mathrm{s}$, the long and short axis of the 3D-Gaussian detection volume are $a = 0.5\ \mu\mathrm{m}$ and $b = 2\ \mu\mathrm{m}$. Curves are shown only for measurement time values T larger than 100 s (dotted line), where the assumption applies that the measurement time is much larger than the maximum lag time.

high concentration values, which quickly goes beyond any upper limit. Increasing the fluorescence brightness of the molecules does not improve much the accuracy when measuring at high concentrations. In contrast, towards low concentration values, increasing the brightness can dramatically enhance the accuracy. This can be understood recalling that only photons originating from one and the same molecule contribute to the non-uniform part of the ACF. With increasing brightness, the ACF can be efficiently sampled over an ever increasing volume, because even molecules far away from the center of the detection volume have a chance to contribute to the ACF. However, this does not apply for high concentrations, where at every time already more than a single molecule is present in the detection volume. With increasing concentration, the amplitude of the non-uniform part of the ACF, which is generated solely by photon pairs coming from one and the same molecules and is proportional to concentration c, is becoming increasingly smaller with respect to the uniform part of the ACF, which is caused by photon pairs coming from different molecules and is thus proportional to the square of the concentration c^2. Because the determination of the diffusion coefficient lives exclusively on the non-uniform part of the ACF, its accuracy deteriorates quickly when c exceeds some optimum value: beyond that value the ACF becomes increasingly dominated by its uniform part which rises proportional to c^2. Although it was well known, from the very beginning of FCS, that there has to be an optimum concentration for FCS measurements, Fig. 3 presents the first published calculation of this value, showing also its shift towards smaller concentration values with increasing brightness κ. A signal-to-noise analysis as presented in [31] does not yield explicit values for that optimum concentration: The signal-to-noise ratio enhances with increasing concentration reaching a plateau at high concentration (cf. Fig. 1 in [31]), giving no information about the quality of a FCS measurement in the sense of how accurate a diffusion coefficient can be extracted from a measured ACF.

Although Fig. 3 was calculated for the special case of correctly identifying D_0 against $D = D_0/2$ with 10% error rate, qualitatively similar figures occur if one chooses some other value for D, or asks for a different error threshold. Choosing different values for these parameters will mainly rescale the vertical axis of measurement time, but will not change significantly the dependence on

concentration. The main features of an upper concentration limit where the accuracy of the diffusion-coefficient determination breaks rapidly down, and of the strong enhancement of accuracy with increasing brightness values at small concentration values, will not change.

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