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Path integral approach to fluorescence correlation experiments

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Abstract

Fluorescence correlation spectroscopy is a powerful tool for studying physical properties of fluorescing molecules. By using a path integral approach, an approximation of the fluorescence autocorrelation function for the case of significant photobleaching of the studied molecules is derived. The found analytical result is compared with the calculation of a Monte Carlo simulation.

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1. Introduction

The experimental technique of fluorescence correlation spectroscopy (FCS) is a powerful tool for obtaining information about physical properties of fluorescing molecules. The idea behind the method is to measure the fluorescence intensity fluctuations of molecules floating in and out of a focused laser beam. The autocorrelation function of this fluctuating signal contains information about lateral and rotational diffusivity or photophysical properties such as depolarization rates and triplet lifetime of the fluorescing molecular species. For the example of lateral diffusion, the autocorrelation of the fluorescence intensity becomes shorter as the molecules diffuse faster in and out of the detection region.

There exist numerous publications covering the various experimental applications of FCS as well as the theoretical evaluation and modeling of FCS data, see e.g. Refs. [1–3]. Usually in studying FCS data, the effect of photobleaching of the fluorescing molecules is neglected. This is acceptable as long as sufficiently low laser intensities and sufficiently photostable fluorescent dyes are used. But with the advancement of refined experimental FCS systems, which work with very small detection volumes (ca. 0.4 fl) at true single molecule level, one is interested in gathering as many photons per molecule as possible [4,5]. Thus, it is of interest to include photobleaching effects. This is also the case if one wishes to study molecular systems with much lower photostability than that of standard fluorescing dyes. The present paper will give an approximate theoretical

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evaluation of the autocorrelation function for FCS experiments, where photobleaching effects are no longer neglected.

In a FCS measurement, the measured signal is the time dependent fluorescence intensity $I(t)$, and the autocorrelation function of its fluctuations is defined by

$$A(\tau) = \langle [I(t) - \langle I \rangle] [I(t + \tau) - \langle I \rangle] \rangle = \langle I(t)I(t + \tau) \rangle - \langle I \rangle^2, \quad (1)$$

where $\langle \rangle$ denotes the time average over t .

The main problem considered here is the theoretical estimation of this function based on knowledge of the significant physical parameters of the system, namely, the laser beam geometry and intensity, the detection optics characteristics, the diffusion constant, and the photostability of the fluorescent molecules. The theory of FCS is well established for the case of negligible photodestruction of the studied molecules. But the situation becomes very complicated if this is no longer the case. To understand this in more detail, we need to recall the standard way of finding the autocorrelation function (see also Ref. [6]). The measured fluorescence intensity $I(t)$ is given by the integral

$$I(t) = \int_{\Omega} d\mathbf{r} \varepsilon(\mathbf{r}) c(\mathbf{r}, t), \quad (2)$$

where $\varepsilon(\mathbf{r})$ is the probability that, for a given molecule at position \mathbf{r} , a fluorescence photon is excited and detected by the measurement system, $c(\mathbf{r}, t)$ is the local concentration of fluorescing molecules, and the integration extends over the whole detection volume Ω . Therefore, the autocorrelation function of the fluorescence intensity fluctuations is given by

$$A(\tau) = \int_{\Omega} d\mathbf{r} \int_{\Omega} d\mathbf{r}' \varepsilon(\mathbf{r}) \varepsilon(\mathbf{r}') \langle \delta c(\mathbf{r}, t) \delta c(\mathbf{r}', t + \tau) \rangle \quad (3)$$

and its calculation reduces to the determination of the autocorrelation of the local concentration fluctuations $\delta c(\mathbf{r}, t) = c(\mathbf{r}, t) - \langle c(\mathbf{r}, t) \rangle$. However, the local concentration fluctuations $\delta c(\mathbf{r}, t)$ themselves are governed by the equation

$$\frac{\partial \delta c(\mathbf{r}, t)}{\partial t} = -V(\mathbf{r}) \delta c(\mathbf{r}, t) + D \Delta \delta c(\mathbf{r}, t). \quad (4)$$

The first term on the right hand side describes the photodestruction of the fluorescent molecules in the laser beam; where $V(\mathbf{r})$ is the product of photobleaching quantum yield, absorption cross section, and coordinate dependent laser light intensity. The second term describes the diffusion of the molecules, having the diffusion constant D . If the photobleaching is negligible, the solution of the resulting diffusion equation can be explicitly written down. In Fourier space it reads

$$\delta \tilde{c}(\mathbf{k}, t) = \delta \tilde{c}_0(\mathbf{k}) \exp(-Dk^2 t), \quad (5)$$

where \mathbf{k} denotes the wave vector in Fourier space. $\delta \tilde{c}(\mathbf{k}, t)$ is the Fourier amplitude of the local concentration intensity fluctuation, and $\delta \tilde{c}_0(\mathbf{k})$ denotes the initial amplitude value at $t = 0$. Inserting this result into Eq. (3) one obtains the desired autocorrelation function. But before arriving at an explicit expression for $A(\tau)$, one has to know the form of the function $\varepsilon(\mathbf{r})$. Usually, it is modeled by a three-dimensional Gaussian profile, namely

$$\varepsilon(\mathbf{r}) = \varepsilon_0 \exp\left(-\frac{x^2 + y^2}{a^2} - \frac{z^2}{c^2}\right). \quad (6)$$

Taking into account that for a random distribution of fluorescing molecules one has

$$\langle \delta \tilde{c}_0(\mathbf{k}_1) \delta \tilde{c}_0(\mathbf{k}_2) \rangle = \text{const} \times \delta(\mathbf{k}_1 + \mathbf{k}_2),$$

the final result for $A(\tau)$ reads

$$\frac{A(\tau)}{A(0)} = \frac{a^2 c}{(a^2 + 2Dt)\sqrt{c^2 + 2Dt}}. \tag{7}$$

Unfortunately, this simple equation is no longer valid if the photobleaching term in Eq. (4) becomes significant. One could try to find an approximate solution of Eq. (4) by means of a perturbation series. However, an alternative approach was chosen which is described in the next section.

2. Path integral approach

The autocorrelation function can be elegantly transformed into a path integral representation [7]. The advantage of this approach lies in the ease with which one can obtain perturbation series expansions from such a representation.

At the first step, the pure diffusion of one single molecule is considered. To obtain the transition probability $K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a)$ that the molecule will be at position \mathbf{r}_b at time t_b if the molecule was at position \mathbf{r}_a at time t_a , one has to calculate, and sum up, the probability for such a transition for *every* possible path the molecule could take. Each path will be thought to consist of a large number N of small transitions of duration $\delta t = (t_b - t_a)/N$. The probability that the molecule will make a small movement of $\delta \mathbf{r}$ during δt is given by

$$\frac{1}{(4\pi D\delta t)^{3/2}} \exp\left(-\frac{\delta \mathbf{r}^2}{4D\delta t}\right).$$

This is simply the solution of the diffusion equation with appropriate initial conditions.

Next, the photodestruction of the molecule can be taken into account by noting that at every position \mathbf{r} of the molecule the probability of not becoming bleached is given by $1 - \delta t V(\mathbf{r}) \approx \exp[-\delta t V(\mathbf{r})]$. Thus, the complete transition probability $K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a)$ reads

$$K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a) = \left(\prod_{k=1}^{N-1} \int_{\Omega} \frac{d^3 \mathbf{r}_k}{(4\pi D\delta t)^{3/2}} \right) \exp\left[-\sum_{k=0}^{N-1} \left(\frac{[\mathbf{r}_{k+1} - \mathbf{r}_k]^2}{4D} + V(\mathbf{r}_k) \right) \delta t \right],$$

with $\mathbf{r}_0 = \mathbf{r}_a$ and $\mathbf{r}_N = \mathbf{r}_b$.

Taking the limit $N \rightarrow \infty$ and $\delta t \rightarrow 0$, one arrives at the desired path integral representation

$$K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a) = \int_{(\mathbf{r}_a, t_a) \sim (\mathbf{r}_b, t_b)} \mathcal{D}\mathbf{r}(\tau) \exp\left[-\int d\tau \left(\frac{\dot{\mathbf{r}}(\tau)^2}{4D} + V[\mathbf{r}(\tau)] \right)\right], \tag{8}$$

where $\int_{(\mathbf{r}_a, t_a) \sim (\mathbf{r}_b, t_b)} \mathcal{D}\mathbf{r}(\tau)$ denotes the integration over all possible paths (with an appropriate integration measure) connecting \mathbf{r}_a and \mathbf{r}_b within time $t_b - t_a$.

To obtain from the transition probability $K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a)$ the autocorrelation function $A(t)$ two additional functions are needed. First, the laser illumination induces a non-uniform probability distribution $p_0(\mathbf{r})$ of finding a molecule at position \mathbf{r} (due to photobleaching, the probability of finding a molecule in regions with higher laser light intensity is lower than in regions of lower intensity). One could assume that the experiment begins only after a sufficiently long time of laser light illumination, so that a *stationary* profile $p_0(\mathbf{r})$ has developed. Second, one has to know the probability $\varepsilon(\mathbf{r})$ of exciting and detecting a photon when the molecule is at position \mathbf{r} . It is assumed that this function is given by Eq. (6). With both these functions at hand the autocorrelation function takes the form

$$A(t) = \int d\mathbf{r}_b \int d\mathbf{r}_a \varepsilon(\mathbf{r}_b) K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a) \varepsilon(\mathbf{r}_a) p_0(\mathbf{r}_a). \tag{9}$$

Since the fluorescence signals coming from different molecules are statistically independent, Eq. (9) is valid for any number of molecules within the detection volume – this is true despite the fact that Eq. (9) was derived by considering only a single molecule.

As an example, the standard autocorrelation function Eq. (7), will be recalculated (see also Ref. [7]). The time dependent path $\mathbf{r}(t)$ can be written as a harmonic series expansion

$$\mathbf{r}(t) = \frac{\mathbf{r}_b - \mathbf{r}_a}{t_b - t_a} (t - t_a) + \sum_{m=1}^{\infty} \mathbf{r}_m \sin[\omega_m(t - t_a)],$$

with frequencies $\omega_m = \pi m / (t_b - t_a)$. Inserting this into Eq. (8) one gets ($V(\mathbf{r}) = 0$)

$$K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a) = \exp\left(-\frac{(\mathbf{r}_b - \mathbf{r}_a)^2}{4D(t_b - t_a)}\right) f(t_b, t_a),$$

where $f(t_b, t_a)$ is some function only dependent on t_b and t_a . Since the diffusion of the molecule is a probability conserving process, this function can be easily calculated as the normalising factor of $K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a)$. So one obtains

$$K(t_b, t_a, \mathbf{r}_b, \mathbf{r}_a) = \frac{1}{[4\pi D(t_b - t_a)]^{3/2}} \exp\left(-\frac{(\mathbf{r}_b - \mathbf{r}_a)^2}{4D(t_b - t_a)}\right). \quad (10)$$

As expected, this is simply the solution of the pure diffusion equation with the initial condition $K(t_a, t_a, \mathbf{r}_b, \mathbf{r}_a) = \delta(\mathbf{r}_b - \mathbf{r}_a)$. By inserting this result into Eq. (9) and assuming a uniform distribution function $p_0(\mathbf{r}) = \text{const}$ one arrives again at the classical formula (7).

The path integral representation of the transition function is now especially suitable for deriving an approximation series for the case of non-negligible $V(\mathbf{r})$. For developing such a series, the following average $\langle \rangle$ for an arbitrary functional $\mathcal{F}[\mathbf{r}(t)]$ is defined as

$$\langle \mathcal{F} \rangle = \frac{\int_{\text{any path}} \mathcal{D}\mathbf{r}(t) \mathcal{F}[\mathbf{r}(t)] \varepsilon(\mathbf{r}_b) \exp(-\int_0^t d\tau \dot{\mathbf{r}}^2/4D) \varepsilon(\mathbf{r}_a) p_0(\mathbf{r}_a)}{\int_{\text{any path}} \mathcal{D}\mathbf{r}(t) \varepsilon(\mathbf{r}_b) \exp(-\int_0^t d\tau \dot{\mathbf{r}}^2/4D) \varepsilon(\mathbf{r}_a) p_0(\mathbf{r}_a)}, \quad (11)$$

where the path integration now extends over any path with an arbitrary initial and final position. Following this definition, the autocorrelation function Eq. (9) acquires the simple form

$$A(t) = A_0(t) \langle \exp(-\mathcal{V}[\mathbf{r}(t)]) \rangle, \quad (12)$$

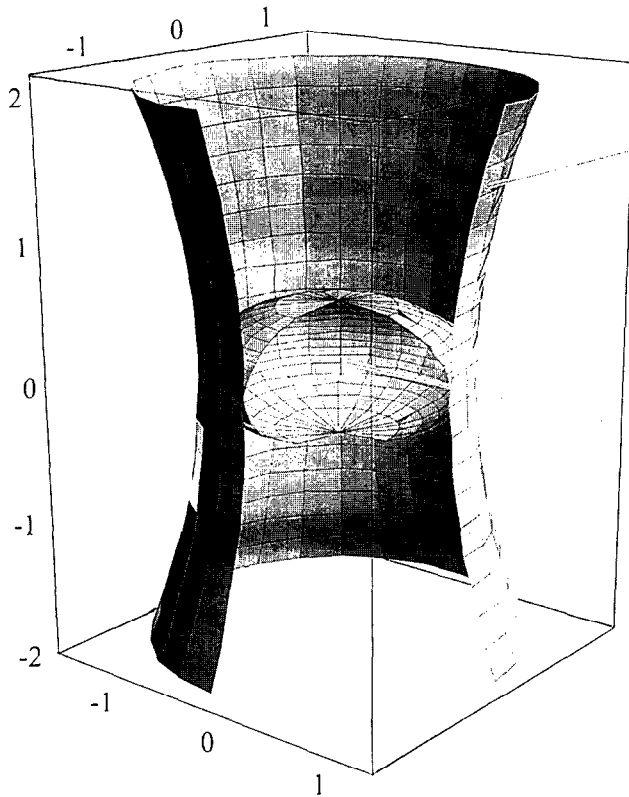
where the abbreviation $\mathcal{V}[\mathbf{r}(t)] = \int_0^t d\tau V(\mathbf{r}(t))$ was used, and $A_0(t)$ is the autocorrelation function, Eq. (7), in the absence of photobleaching. Now, the average of the exponential function can be transformed into an exponent of a cumulant expansion, yielding the expression (for the first three orders of \mathcal{V})

$$\langle \exp(-\mathcal{V}) \rangle = \exp\left(-\langle \mathcal{V} \rangle + \frac{1}{2!} \langle (\mathcal{V} - \langle \mathcal{V} \rangle)^2 \rangle - \frac{1}{3!} \langle (\mathcal{V} - \langle \mathcal{V} \rangle)^3 \rangle + \dots\right). \quad (13)$$

3. First order approximation

In the first order approximation, only the term $\langle \mathcal{V} \rangle$ is conserved in the cumulant expansion of Eq. (13). Thus, the problem reduces to finding the average of \mathcal{V} . This average can be written as

$$\langle \mathcal{V} \rangle = \frac{\int_0^t d\tau \int_{\Omega} d\mathbf{r}_a \int_{\Omega} d\mathbf{r}_b \int_{\Omega} d\boldsymbol{\rho} \varepsilon(\mathbf{r}_b) \Gamma_0(t - \tau, \mathbf{r}_b - \boldsymbol{\rho}) V(\boldsymbol{\rho}) \Gamma_0(\tau, \boldsymbol{\rho} - \mathbf{r}_a) \varepsilon(\mathbf{r}_a) p_0(\mathbf{r}_a)}{\int_{\Omega} d\mathbf{r}_a \int_{\Omega} d\mathbf{r}_b \varepsilon(\mathbf{r}_b) \Gamma_0(t, \mathbf{r}_b - \mathbf{r}_a) \varepsilon(\mathbf{r}_a) p_0(\mathbf{r}_a)}, \quad (14)$$



Isosurface of Laser
Beam Intensity

Effective Detection
Volume

Fig. 1. Geometry of the laser beam profile and the emission/detection probability in an epifluorescence setup for FCS.

where the abbreviation

$$\Gamma_0(t, \mathbf{r}) = \frac{1}{(4\pi Dt)^{3/2}} \exp\left(-\frac{\mathbf{r}^2}{4Dt}\right)$$

was used. The function $\Gamma_0(t, \mathbf{r})$ is nothing but the transition function $K(t, 0, \mathbf{r}, 0)$ in the absence of photobleaching (“free propagator”).

The photobleaching function $V(\mathbf{r})$ is proportional to the laser light intensity. For a confocal epifluorescence microscope, the laser intensity is given by a Gaussian laser beam profile (the z -axis is assumed to be the optical axis of the microscope) so that

$$V(\mathbf{r}) \propto \frac{2\Phi_b \sigma P_0}{\pi w^2} \exp\left(-\frac{x^2 + y^2}{\frac{1}{2}w^2}\right),$$

with $w = w_0 \sqrt{1 + (\lambda z / \pi w_0^2)^2}$ and w_0 being the waist diameter of the beam, λ the wavelength of the light, Φ_b the photobleaching quantum yield, σ the absorption cross section, and P_0 the total laser beam intensity. In Fig. 1, a typical isosurface of such a laser beam intensity and an isosurface of the excitation/detection function $\varepsilon(\mathbf{r})$ are shown.

For the sake of obtaining an explicit analytical result, the z -dependence of the laser profile will be neglected in the following calculations ($w \approx w_0$). This is acceptable for a sufficiently small beam divergence, $\lambda / \pi w_0$, and a small detection region. As a further simplification, it is assumed that $p_0(\mathbf{r}) = \text{const}$, even for

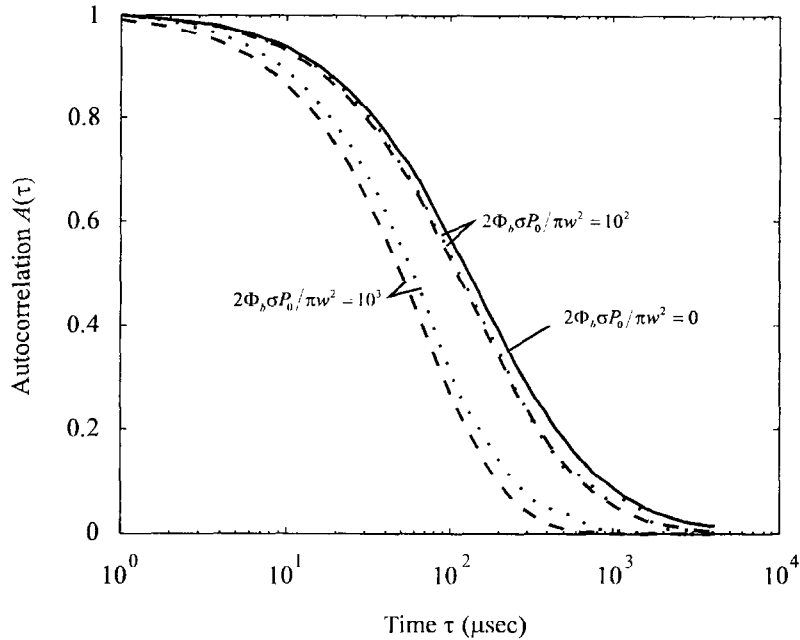


Fig. 2. Approximate autocorrelation functions (dashed line) and the corresponding MCS results (dotted line) for $2\Phi_b\sigma P_0/\pi w^2 = 10^2$ and 10^3 . For comparison, the exact autocorrelation function for the case of the absence of photobleaching is shown (solid line).

non-vanishing photobleaching of the molecules. Thus, the integrations in Eq. (14) can be performed, explicitly yielding the final result

$$\langle \mathcal{V} \rangle = \frac{2\Phi_b\sigma P_0}{\pi w_0^2} \frac{w_0^2}{2D} \sqrt{\frac{a^2 + 2Dt}{a^2 + w_0^2 + 2Dt}} \operatorname{Arctanh} \left(\frac{2Dt}{\sqrt{(a^2 + 2Dt)(a^2 + w_0^2 + 2Dt)}} \right). \quad (15)$$

Inserting this expression into Eq. (12) yields the first order approximation of the autocorrelation function.

To compare the quality of the acquired approximation with the exact autocorrelation, a Monte Carlo simulation (MCS) of the FCS experiment was performed. The following numerical parameter values were chosen for the calculations: $a = 2 \mu\text{m}$, $b = 1 \mu\text{m}$, $D = 10^3 \mu\text{m}^2/\text{s}$, $w_0 = 4 \mu\text{m}$, and the prefactor $2\Phi_b\sigma P_0/\pi w_0^2$ was chosen to be equal to 0, 10^2 and 10^3 respectively. For $2\Phi_b\sigma P_0/\pi w_0^2 = 0$, the result of the simulation was in perfect agreement with the exact result of Eq. (7). The results for $2\Phi_b\sigma P_0/\pi w_0^2 = 10^2$ and 10^3 are shown in Fig. 2. It should be mentioned that for small values of $A(\tau)$ the result of the MCS becomes more inaccurate because of the decreased number of sampled events.

Taking into account the simplicity of the approximation, the achieved accuracy is rather amazing. Unfortunately, for higher orders of \mathcal{V} , or for more complicated functions of $V(\mathbf{r})$ or $p_0(\mathbf{r})$, the corresponding averages in Eq. (13) cannot be given in a closed form. However, it should be possible to calculate them numerically. In any case, the high quality of the simple first order approximation shows that the path integral approach is an excellent tool for calculating the autocorrelation of non-trivial FCS experiments.

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