

## Derivation of expressions for the FCS correlation function

(Supplementary material for the F-Praktikum)

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### 1. Introduction

Fluorescence correlation spectroscopy (also known as fluorescence fluctuation spectroscopy) is a technique that belongs to the family of fluctuation spectroscopy methods. These methods provide information of dynamic properties of a system under investigation based on the analysis of fluctuations of its parameters. Another optics-based fluctuation spectroscopy technique extensively used in chemical and biological physics is dynamic light scattering [1]. Experimentally, information about the system is obtained by monitoring a time dependence of relevant fluctuating physical quantities and estimating statistical properties of their fluctuations.

In fluorescence correlation spectroscopy (FCS), the fluctuating physical quantity of interest is the intensity of fluorescence emitted by fluorescent probe molecules in a small observation volume. These fluorescent molecules are, as a rule, located in a fluid medium which is typically kept under conditions of thermodynamic equilibrium. In the standard implementation of the technique, this volume is produced by tightly focusing a laser light beam by a microscope objective and then confocally detecting fluorescence from the illuminated focal spot with the use of a pinhole. Brownian motion of fluorescent particles across the detection volume and/or chemical reactions leading to change in the fluorescence intensity of individual particles will result in temporal fluctuations of the fluorescence signal collected from the observation volume.

### 2. Fluctuating fluorescence intensity and its autocorrelation

In an FCS experiment, we are interested in estimating the two-time autocorrelation function of fluctuating fluorescence intensity:

$$g(t_1, t_2) = \langle F(t_1) F(t_2) \rangle \quad (1)$$

where  $\langle \cdot \rangle$  denotes ensemble average. The autocorrelation function gives the measure of interdependence of values of the random signal at two instants separated by some time interval [2, 3].

We assume that the system under investigation is in the equilibrium state, and therefore the detected fluorescence signal  $F(t)$  is a stationary random process (see, e.g., [2, 3]), which means that it can be presented as zero-mean fluctuations around a constant mean value:

$$\begin{aligned} F(t) &= \langle F \rangle + \delta F(t) \\ \langle \delta F(t) \rangle &= 0 \end{aligned} \quad (2)$$

and that its correlation function depends only on the difference of the time arguments:

$$g(t_1, t_2) = g(|t_1 - t_2|) \quad (3)$$

Thus, we have

$$g(\tau) = \langle F(t+\tau)F(t) \rangle, \quad \tau \geq 0 \quad (4)$$

which, taking into account Eq. (2), can be written as

$$g(\tau) = \langle F \rangle^2 + \langle \delta F(t+\tau)\delta F(t) \rangle \quad (5)$$

where the quantity  $\langle \delta F(t+\tau)\delta F(t) \rangle$  is known as autocovariance.

Notice that when dealing with experimental or simulated data, the ergodic theorem is applied, and based on the assumption of stationarity, ensemble averaging is replaced by time-averaging (see, e.g., [3]), so that

$$\langle \delta F(t+\tau)\delta F(t) \rangle = \lim_{T \rightarrow \infty} \frac{1}{T} \int_0^T \delta F(t+\tau)\delta F(t) dt \quad (6)$$

As an example, let us consider a realization of a stationary zero-mean Gaussian random process  $F(t) = \delta F(t)$  with an exponentially decaying autocorrelation (Fig. 1). From the Figure we see that although the fluctuations in the signal look random (upper panel), the process obviously possesses some finite memory. Indeed, a careful examination of this random trace shows that if at a certain time instant  $t$  the signal is positive (negative), then with a high probability it will preserve its sign within some time interval around  $t$ . This memory, however, has a finite duration, and at time intervals longer than some characteristic time, the values of the signal become independent of each other. (In other words, one may say the signal in Fig. 1 has a ‘spiky’ structure, and, roughly speaking, the concept of the memory in this case just reflects the fact that the spikes in the signal are characterized by some nonzero duration.)

To illustrate the above discussion, let us calculate an estimate of the covariance function of the process. For that, according to (6), we need to calculate the product  $\delta F(t+\tau)\delta F(t)$  for a set of different fixed lag times  $\tau$  and average the result over  $t$ . It is obvious that for  $\tau = 0$  the product will be nonnegative, and therefore the covariance for the zero lag time will be positive. With increasing lag time  $\tau$ , certain parts of the product  $\delta F(t+\tau)\delta F(t)$  will remain positive

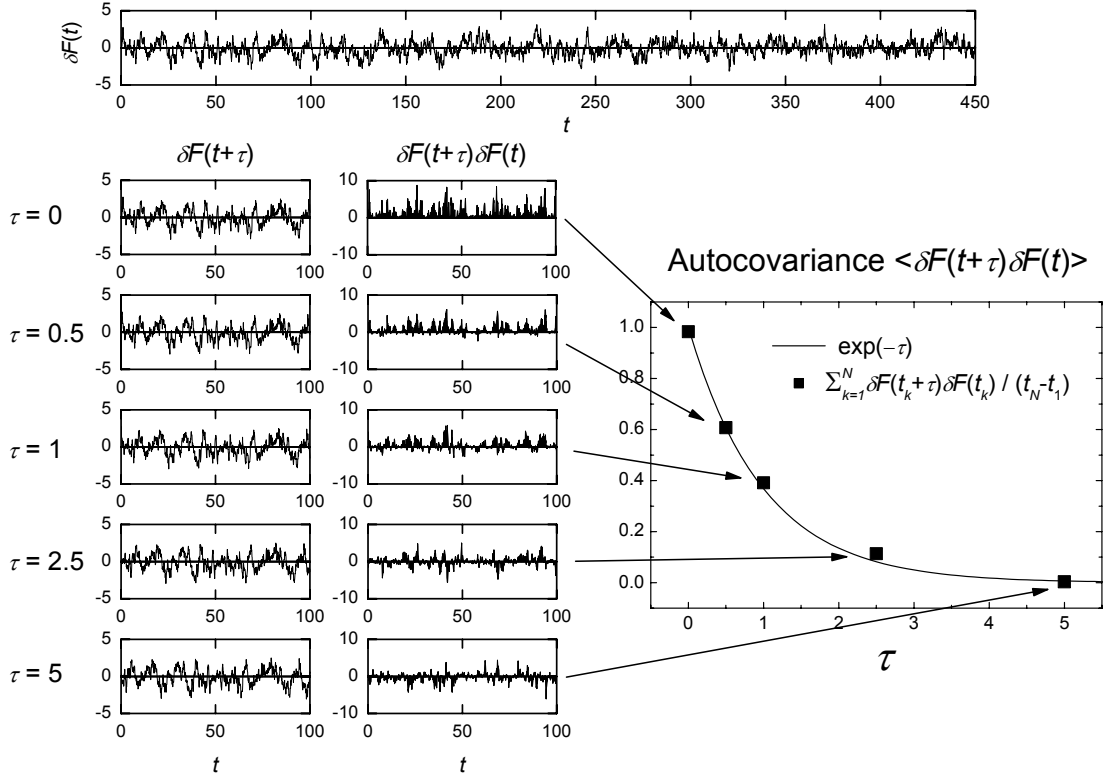


Fig. 1. Realization of a stationary zero-mean exponentially correlated Gaussian process (upper panel) and illustration to calculation of its autocovariance function for five different lag times  $\tau$ .

due to the memory of the process (finite duration of random spikes). At the same time, some intervals where the product  $\delta F(t+\tau)\delta F(t)$  is negative, will appear. As a result, the calculated covariance value will decrease. Finally, when lag times  $\tau$  become large compared to the characteristic memory time of the system, the signal values separated by  $\tau$  become statistically independent, and the product  $\delta F(t+\tau)\delta F(t)$  will have positive and negative values with equal probability, giving, as a result, zero covariance.

In fluorescence correlation spectroscopy for reasons which will become clear later, the quantity studied in experiments is the *normalized* correlation function of fluorescence intensity fluctuations<sup>\*)</sup>:

$$G(\tau) = \frac{g(\tau)}{\langle F \rangle^2} - 1 = \frac{\langle \delta F(t+\tau)\delta F(t) \rangle}{\langle F \rangle^2} \quad (7)$$

<sup>\*)</sup> In this case, the fluctuating quantity is the fluorescence intensity; therefore, its mean is strictly positive:  $\langle F \rangle > 0$ , and expression (7) always has sense.

### 3. General consideration of the FCS experiment

Tight focusing of the exciting laser light beam and the confocal detection by means of pinhole-based rejection of the out-of-focus contributions determine the so-called fluorescence detection efficiency profile

$$\Omega(\mathbf{r}) = I(\mathbf{r})S(\mathbf{r}) \quad (8)$$

which is proportional to the product of the spatial intensity profiles of the exciting laser beam and fluorescence detection probability denoted here as  $I(\mathbf{r})$  and  $S(\mathbf{r})$ , respectively.

Fluorescence emission is incoherent due to a random delay between the fluorescence photon emission and absorption of the quantum of the exciting light. This takes place as a result of a finite lifetime of the excited state of the fluorescing species. Therefore, the fluorescence intensity detected at a time instant  $t$  by the detector is determined by the sum of fluorescence contributions from different points in the detection volume:

$$F(t) = \kappa \varepsilon Q \int \Omega(\mathbf{r}) c(\mathbf{r}, t) d\mathbf{r} \quad (9)$$

where  $c(\mathbf{r}, t)$  is the concentration of fluorescent molecules,  $\kappa$  is the efficiency of the fluorescence detector (including the detector quantum efficiency, its sensitivity to the particular wavelength range, and losses in the optical system),  $\varepsilon$  is the molar extinction coefficient of the fluorophore at the wavelength of the exciting laser radiation, and  $Q$  is the fluorescence quantum yield of the fluorophore.

In the equilibrium state, the concentration of fluorescent molecules undergoes fluctuations around its constant mean value:

$$c(\mathbf{r}, t) = \langle c \rangle + \delta c(\mathbf{r}, t) \quad (10)$$

Therefore

$$F(t) = \kappa \varepsilon Q \int \Omega(\mathbf{r}) (\langle c \rangle + \delta c(\mathbf{r}, t)) d\mathbf{r} \quad (11)$$

Then Eq. (7) can be written as follows:

$$G(\tau) = \frac{\int \int \Omega(\mathbf{r}) \langle \delta c(\mathbf{r}, \tau) \delta c(\mathbf{r}', 0) \rangle \Omega(\mathbf{r}') d\mathbf{r} d\mathbf{r}'}{\langle c \rangle^2 \left( \int \Omega(\mathbf{r}) d\mathbf{r} \right)^2} \quad (12)$$

Equation (12) can be called the main equation of the theory of the FCS, since typically this is the starting point of derivations of formulas related to a particular behavior and structure of the system under investigation.

#### 4. FCS on diffusing fluorescent particles.

##### 4.1 General consideration

In the simplest experimental situation, fluctuations of the concentration of fluorescing particles in the detection volume take place solely due to the diffusional motion of the particles, and therefore concentration fluctuations satisfy the diffusion equation

$$\frac{\partial}{\partial t} \delta c(\mathbf{r}, t) = D \nabla^2 \delta c(\mathbf{r}, t) \quad (13)$$

supplemented by a set of boundary conditions corresponding to the particular geometry of the sample

$$\Gamma_{\mathbf{r},t}(\delta c) = 0 \quad \text{for } \mathbf{r} \in S \quad (14)$$

Here  $S$  is the geometric boundary of the sample, and  $\Gamma$  is an operator describing the boundary conditions (for example, in case of absorbing boundaries the boundary condition is  $\delta c|_{\mathbf{r} \in S} = 0$ , and in case of reflective boundaries one should set the derivative with respect to the (external) normal of  $S$  to zero:  $\partial_N(\delta c)|_{\mathbf{r} \in S} = 0$ ).

The solution of Eq. (13) is expressed as follows [4]:

$$\delta c(\mathbf{r}, t) = \int \delta c(\mathbf{p}, 0) \mathcal{G}(\mathbf{r}, t | \mathbf{p}, 0) d\mathbf{p} \quad (15)$$

where  $\mathcal{G}(\mathbf{r}, t | \mathbf{p}, 0)$  is the Green function of Eq. (13), i.e., a solution of this partial differential equation satisfying the special initial condition

$$\mathcal{G} = \delta(\mathbf{r} - \mathbf{r}') \quad \text{for } t = 0 \quad (16)$$

Notice that the particular form of the Green function of the diffusion equation Eq. (13) depends on the boundary conditions in the system under investigation.

Thus, the correlation function of concentration fluctuations takes the following form:

$$\langle \delta c(\mathbf{r}, \tau) \delta c(\mathbf{r}', 0) \rangle = \int \langle \delta c(\mathbf{p}, 0) \delta c(\mathbf{r}', 0) \rangle \mathcal{G}(\mathbf{r}, \tau | \mathbf{p}, 0) d\mathbf{p} \quad (17)$$

In a sample with a low concentration of the fluorescent particles maintained at a state far from phase transitions, the equilibrium concentration fluctuations of the fluorescent admixture are spatially uncorrelated, so that

$$\langle \delta c(\mathbf{p}, 0) \delta c(\mathbf{r}', 0) \rangle = \langle \delta c^2 \rangle \delta(\mathbf{p} - \mathbf{r}') \quad (18)$$

Additionally, if we assume that the concentration of diffusing fluorescent particles is low and they do not affect motion of each other, then the number of particles in any finite volume is a Poisson variable, and therefore

$$\langle \delta c^2 \rangle = \langle c \rangle \quad (19)$$

Thus, by combining Eqs. (17)-(19), we obtain

$$\langle \delta c(\mathbf{r}, \tau) \delta c(\mathbf{r}', 0) \rangle = \langle c \rangle \mathcal{G}(\mathbf{r}, \tau | \mathbf{r}', 0) \quad (20)$$

Then the correlation function of fluorescence fluctuations Eq. (12) takes the following form:

$$G(\tau) = \frac{\iint \Omega(\mathbf{r}) \mathcal{G}(\mathbf{r}, \tau | \mathbf{r}', 0) \Omega(\mathbf{r}') d\mathbf{r} d\mathbf{r}'}{\langle c \rangle \left( \int \Omega(\mathbf{r}) d\mathbf{r} \right)^2} \quad (21)$$

Some properties of the correlation function can be established without specifying the particular form of the Green function. For example, it is easy to show that

$$G(0) = \langle N \rangle^{-1} \quad (22)$$

where

$$\langle N \rangle = \langle c \rangle \frac{\left( \int \Omega(\mathbf{r}) d\mathbf{r} \right)^2}{\int \Omega^2(\mathbf{r}) d\mathbf{r}} \quad (23)$$

is the effective mean number of molecules which are simultaneously present in the observation volume. Additionally, in the case of immovable boundaries

$$G(\tau) \xrightarrow{\tau \rightarrow \infty} 0 \quad (24)$$

#### 4.2 Free diffusion

If we assume that the sample is homogeneous and uniform, and the focal volume is orders of magnitude smaller than the size of the sample, then diffusion can be considered as taking place in a system with infinitely remote boundaries. Thus, the problem is reduced to that of free diffusion, and in a space with dimensionality  $k$  ( $k = 1, 2, 3$ ) the Green function takes the following form (see, e.g., [2, 4]):

$$\mathcal{G}(\mathbf{r}, t | \mathbf{p}, 0) = \frac{1}{(4\pi Dt)^{k/2}} \exp\left(-\frac{(\mathbf{r} - \mathbf{p})^2}{4Dt}\right) \quad (25)$$

Notice that in this particular case the system is spatially homogeneous and the Green function depends only on the difference of spatial arguments – this fact can substantially simplify subsequent calculations. However, it should be kept in mind that this nice property disappears upon breakdown of translational invariance in the system, e.g., when surfaces and interfaces bounding diffusing species cannot be taken as infinitely remote.

Under typical conditions of the FCS experiment (Gaussian laser beam profile, confocal detection) the fluorescence detection efficiency profile can be approximated by a three-dimensional Gaussian distribution:

$$\Omega(\mathbf{r}) = \Omega_0 \exp\left(-2 \frac{x^2 + y^2}{r_0^2}\right) \exp\left(-2 \frac{z^2}{z_0^2}\right) \quad (26)$$

Substitution of Eqs. (25) and (26) into Eq. (21) and calculation of the resulting Gaussian integrals leads to the following result:

$$G(\tau) = \frac{1}{\langle N \rangle} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \left(\frac{r_0}{z_0}\right)^2 \frac{\tau}{\tau_D}\right)^{-1/2} \quad (27)$$

Here

$$\tau_D = \frac{r_0^2}{4D} \quad (28)$$

is the so-called diffusion time of fluorescence species characterizing the typical scale on which the correlation function of fluorescence fluctuations substantially decreases its amplitude. Notice that the diffusion time corresponds to the typical time scale on which the molecule diffuses through the detection volume. The quantity

$$\langle N \rangle = \pi^{3/2} r_0^2 z_0 \langle c \rangle \quad (29)$$

is the effective mean number of molecules in the detection volume.

#### 4.3 Diffusion + chemical reaction

In a more general case that additionally includes a chemical reaction of diffusing species, one should solve a set of coupled reaction-diffusion equations describing diffusional and reaction dynamics of each of the  $n$  species being present in the system [1, 5]:

$$\frac{\partial}{\partial t} c_j(\mathbf{r}, t) = D_j \nabla^2 c_j(\mathbf{r}, t) + \sum_{k=1}^n T_{jk} c_k(\mathbf{r}, t) \quad (30)$$

where  $T$  is a matrix of kinetic coefficients.

Unfortunately, even in the case of two interacting species, Eq. (30) generally has no closed-form solution. Still, analytical solutions can be obtained in some particular cases [1, 5].

For example, the protonation/deprotonation reaction of the chromophore of the green fluorescent protein (GFP) can be approximately treated as a reversible pseudo-first order reaction:



where  $A^-$  and  $AH$  denote the fluorescent (bright) deprotonated and non-fluorescent (dark) protonated states of the chromophore. In the particular experiment, the deprotonated chromophore of the GFP efficiently absorbs light at the wavelength of 488 nm used for excitation and subsequently reemits part of it as fluorescence, whereas upon protonation, the

absorption spectrum of the chromophore shifts substantially toward shorter wavelengths with respect to the excitation wavelength, so that the protonated chromophore is not excitable anymore, and therefore in this particular experiment the protonated state can be treated as dark.

In this system the diffusion coefficients of both forms can be considered as equal, and we arrive at the following set of equations for concentrations of bright ( $c_b$ ) and dark ( $c_d$ ) states with the pseudo-first-order protonation (bright-to-dark state conversion) rate constant  $k_{bd}$  and first-order deprotonation (dark-to-bright state conversion) rate constant  $k_{db}$ :

$$\begin{aligned}\frac{\partial}{\partial t} c_b(\mathbf{r}, t) &= D \nabla^2 c_b(\mathbf{r}, t) - k_{bd} c_b(\mathbf{r}, t) + k_{db} c_d(\mathbf{r}, t) \\ \frac{\partial}{\partial t} c_d(\mathbf{r}, t) &= D \nabla^2 c_d(\mathbf{r}, t) + k_{bd} c_b(\mathbf{r}, t) - k_{db} c_d(\mathbf{r}, t)\end{aligned}\tag{32}$$

System of equations (32) can be solved analytically in the case of free diffusion. For details on derivation of the solution of Eq. (32) the reader is referred to [1, 4, 5]. By substituting the resulting Green function for the bright species in Eq. (21) and taking into account the shape of the fluorescence detection efficiency profile Eq. (26) one obtains the following expression for the correlation function of fluorescence fluctuations:

$$G(\tau) = \frac{1}{\langle N \rangle} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \left( \frac{r_0}{z_0} \right)^2 \frac{\tau}{\tau_D} \right)^{-1/2} \left( 1 + \frac{f_D}{1 - f_D} \exp(-\lambda \tau) \right)\tag{33}$$

where

$$f_D = \frac{k_{bd}}{k_{bd} + k_{db}}\tag{34}$$

is the fraction of GFP fluorophores in the dark state and

$$\lambda = k_{bd} + k_{db}\tag{35}$$

is the apparent reaction rate constant.



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