Fluorescence Correlation Spectroscopy

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1 Goals

To learn about the theoretical concept and the experimental implementation of confocal Fluorescence Correlation Spectroscopy (FCS) on a system of freely diffusing fluorescently labeled polystyrene (fPS) nanoparticles in aqueous solution. The basic measurement parameters of FCS such as concentration, molecular diffusion coefficient and kinetic rate constants are introduced and measured on small molecular ensembles consisting of several particles.

2 Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) belongs to the family of fluctuation spectroscopy techniques. These methods provide information on dynamic properties of a system under investigation based on the analysis of fluctuations. Experimentally, FCS relies on the detection of fluorescence photons sent out by tiny amounts of fluorescing molecules in a small probe volume. The number of detected fluorescence photons per time unit is however a fluctuating quantity for a number of reasons, such as intrinsic photophysics of dye, the Brownian motion, conformational changes of molecules or due to chemical reactions. All these fluctuations obey typical timescales, which can be extracted from a timetrace of the fluorescence signal by calculating its autocorrelation function.

Fluorescence correlation spectroscopy has been developed in the early seventies as a special case of relaxation analysis. Classical relaxation methods induce certain kinds of external perturbations such as temperature or pressure jumps to a reaction system, and gain information about involved kinetic parameters from the way the system returns back to equilibrium. The concept of FCS with respect to these classical techniques is to take advantage of the minute spontaneous fluctuations of physical parameters that are somehow reflected by the fluorescence emission of the molecules. Such fluctuations are incessantly occurring at ambient temperatures and are generally represented as (unwanted) noise patterns of the measured signal, in our case fluorescence. The fluctuations can be quantified in their strength and duration by temporally autocorrelating the recorded intensity signal,

a mathematical procedure that gave the technique its name. Autocorrelation analysis provides a measure for the self-similarity of a time series signal and therefore describes the persistence of information carried by it. Essential information about processes governing molecular dynamics can thus be derived from the temporal pattern by which fluorescence fluctuations arise and decay.

At its first introduction by Madge, Elson and Webb in 1972, FCS was applied to measure diffusion and chemical dynamics of DNA-drug intercalation. This pioneering study was then followed by a number of other publications by many different groups describing, e.g., attempts to determine particle concentration, translational and rotational mobility in two or three dimensions, even in the cellular environment or in flow systems. Nevertheless, these early measurements suffered from poor signal-to-noise ratios, mainly because of low detection efficiency, large ensemble numbers and insufficient background suppression.

Imagine trying to find out if a friend had entered or left an overcrowded shopping center without a call to the information desk. Unless you are holding hands, this is next to impossible. And now imagine watching your friend in your living room. In the latter case you simply can't fail to notice him (or her) coming or leaving. This is the basic concept of FCS: Make the number of observed molecules low enough so that each of them contributes substantially to the measured signal. Then and only then, one can truly perform analyses of spontaneous, non-coordinated fluctuations.

It is obvious that FCS can only function properly if one somehow manages to reduce the concentrations and observation volumes such that only few molecules are simultaneously detected, and at the same time increase the fluorescence photon yield per single molecule. A major improvement could be made by using efficient fluorescent dyes to label the molecules of interest, strong and stable light sources like lasers, and ultrasensitive detectors, e.g. avalanche photodiodes with single-photon sensitivity. The final breakthrough was achieved in Stockholm by Rigler and his coworkers by combining the FCS technique with confocal detection. Here, the incoming laser light is strongly focused by a high numerical aperture objective (ideally NA>0.9) to a diffraction limited spot. Only the few fluorophores within the illuminated region are excited. In order to limit the detection volume also in axial direction, a pinhole is introduced in the image plane, which blocks all light not coming from the focal region.

To date, most FCS measurements are performed on fluorescently labeled biomolecules diffusing in aqueous buffer solution. Because of the most elegant way of limiting the detection volume to less than one femtoliter, i.e. approximately the volume of an E.coli bacterial cell, concentrations in the nanomolar range are optimal for FCS measurements. Under these circumstances, the signal fluctuations induced by molecules diffusing into or out of the focal volume are large enough to yield good signal-to-noise ratios. During the time a particle spends in the focus, chemical or photophysical reactions or conformational changes may alter the emission characteristics of the fluorophore and give rise to additional fluctuations in the detected signal.

3 Theoretical Foundation

The fluorescence signal from a volume of a sample can be mathematically described as

$$F(t) =< F(t) > +\delta F(t) \tag{1}$$

where $< F(t) >$ denotes the average fluorescence intensity and $\delta F(t)$ describes the fluctuations of the signal around this mean value. The mean value of the fluctuations itself vanishes then of course.

$$< \delta F(t) >= 0 \tag{2}$$

Using this equation (eq. 1) for the fluorescence signal, one can write down the fluorescence autocorrelation as

$$g(\tau) =< F(t+\tau)F(t) > \tag{3}$$

which finally leads together with 2 to

$$g(\tau) =< F(t) >^2 + < \delta F(t+\tau)\delta F(t) > \tag{4}$$

In FCS the autocorrelation function is generally used in a slightly different form, to some part to insure, that the correlation function decays to zero if the time lag τ gets very large. This form is:

$$G(\tau) = \frac{g(\tau)}{< F(t) >^2} - 1 = \frac{< \delta F(t+\tau)\delta F(t) >}{< F(t) >^2} \tag{5}$$

A more detailed description of the fluorescence signal $F(t)$ now requires consideration of some experimental details. Since FCS is carried out in a confocal microscope, the fluorescence signal will be proportional to the product

$$\Omega(\mathbf{r}) = I(\mathbf{r})S(\mathbf{r}) \tag{6}$$

of the spatial intensity profiles of the exciting laser beam and the fluorescence detection probability denotes as $I(\mathbf{r})$ and $S(\mathbf{r})$. The detected fluorescence intensity at a time t is then the sum of fluorescence contributions of all points in the detection volume

$$F(t) = \phi\epsilon Q \int \Omega(\mathbf{r})c(\mathbf{r},t)d\mathbf{r} \tag{7}$$

where $c(\mathbf{r},\mathbf{t})$ is the concentration of fluorescent molecules, ϕ the efficiency of the detector, ϵ the molar extinction coefficient of the fluorophore and Q the fluorescence quantum yield of the fluorophore.

The concentration of fluorescing particles or molecules can then be further expressed by

$$c(\mathbf{r},t) = < c > + \delta c(\mathbf{r},t) \tag{8}$$

which finally leads to

$$F(t) = \phi \epsilon Q \int \Omega(\mathbf{r})(< c > + \delta c(\mathbf{r},t)) d\mathbf{r} \tag{9}$$

for the fluorescence intensity detected.

The autocorrelation function (eq. 5) can then be written as:

$$G(\tau) = \frac{\int\int \Omega(\mathbf{r}) < \delta c(\mathbf{r},\tau)\delta c(\mathbf{r}',0) > \Omega(\mathbf{r}') d\mathbf{r} d\mathbf{r}'}{< c >^2 \left(\int \Omega(\mathbf{r}) d\mathbf{r}\right)^2} \tag{10}$$

The latter formula can be used as a starting point for any kind of derivation concerning different origins of fluctuations in FCS. In the following, we will just consider the diffusion of particles or molecules being the source of the fluorescence intensity fluctuation in the detection volume of a confocal microscope. Diffusion is in general described by the diffusion equation (eq. 11)

$$\frac{\partial}{\partial t}\delta c(\mathbf{r},t) = D\nabla^2 \delta c(\mathbf{r},t) \tag{11}$$

Despite the fact, that the sample does not contain any macroscopic concentration gradient of the particles or molecules, the diffusion equation is still valid on a microscopic scale, where it describes a local concentration gradient or a local concentration fluctuation. The solution of (11) can be written in terms of the so called Green's function $\mathcal{G}(\mathbf{r},t|\rho,0)$ as

$$\delta c(\mathbf{r},t) = \int \delta c(\rho,0)\mathcal{G}(\mathbf{r},t|\rho,0) d\rho. \tag{12}$$

This will lead to the following expression for the local concentration fluctuations

$$< \delta c(\mathbf{r},\tau)\delta c(\mathbf{r}',0) > = < c > \mathcal{G}(\mathbf{r},\tau|\mathbf{r}',0) \tag{13}$$

and the autocorrelation function

$$G(\tau) = \frac{\int\int \Omega(\mathbf{r}) G(\mathbf{r},\tau|\mathbf{r}',0)\Omega(\mathbf{r}') d\mathbf{r} d\mathbf{r}'}{< c > (\int \Omega(\mathbf{r}) d\mathbf{r})^2} \tag{14}$$

Assuming now free diffusion, one can write down the Green's function (eq. 15).

$$\mathcal{G}(\mathbf{r},\tau|\rho,0) = \frac{1}{(4\pi D\tau)^{k/2}} \exp\left(-\frac{(r-\rho)^2}{4D\tau}\right) \tag{15}$$

To calculate the final form of the correlation function, one further has to consider the shape of the confocal detection efficiency profile, which is in first approximation assumed to have a three-dimensional Gaussian shape

$$\Omega(\mathbf{r}) = \Omega_0 \exp\left(-2\frac{x^2+y^2}{r_0^2}\right) \exp\left(-2\frac{z^2}{z_0^2}\right) \tag{16}$$

This leads to the final result (eq. 17)

$$G(\tau) = \frac{1}{< N >}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\left(\frac{r_0}{z_0}\right)^2\frac{\tau}{\tau_D}\right)^{-1/2} \tag{17}$$

with

$$\tau_D = \frac{r_0^2}{4D} \tag{18}$$

as the diffusion time, which determines the timescale on which the correlation function $G(\tau)$ drops to zero. The quantity

$$< N >= \pi^{3/2} r_0^2 z_0 < c > \tag{19}$$

corresponds to the mean number of molecules/particles in the detection volume. Using equation (17), one is now able to extract the diffusion constant of particles or even single molecules from a FCS measurement by fitting the obtained fluorescence intensity autocorrelation function with equation (17). Note that the fitting function contains information on the size of the focal volume with the values r_0 and z_0. These are the spatial extensions of the focus in the focal plane and along the optical axis. These sizes are a priori not known and either have to be determined by measuring the point spread function of a confocal microscope or by doing a reference FCS measurement on a standard sample with known diffusion time τ_D

4 Practical Work

4.1 Setup

All FCS-measreuments will be carried out on a home-built confocal microscope setup. Figure 4.1 shows the principal scheme of the setup.

The setup uses a blue pulsed laser diode ($470\,nm$) or an Argon laser ($\lambda_{exc} = 488\,nm$ or $532\,nm$) as excitation source (excitation), which is coupled into the setup via a dichroic mirror. This mirror reflects the excitation wavelength but transmits the detection wavelength ($\lambda_{det} > 560\,nm$). The excitation light is then focused by a microscope objective Olympus 100x/1.4 NA onto the sample, which is mounted on a piezo stage. The fluorescence excited by the laser is collected with the same microscope objective, then passes

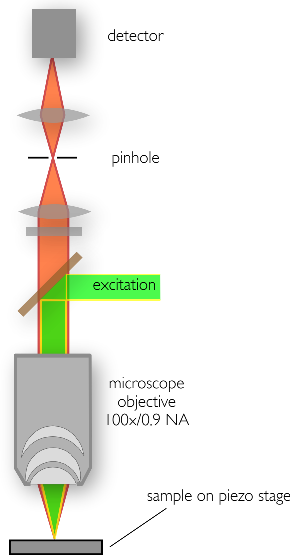

Figure 1: Scheme of the confocal microscopy setup used for the FCS measurements within this experiment.

the dichroic mirror, is cleaned from residual excitation light by a long pass interference filter and focused onto a pinhole by an achromatic lens. Light passing through the pinhole (50 μm diameter) is focused on an Avalanche photon counting module with another lens. The Avalanche module houses a photodiode, which is highly sensitive (do never expose to light!) and converts about 70 % of all arriving photons into a short TTL pulse. The number of pulses and their arrival time is then counted by a multichannel single photon counting card (Becker & Hickl DPC 230). The photon counting card is controlled with a computer and capable of calculating the required intensity autocorrelation functions directly.

4.2 Materials and Sample Preparation

4.2.1 Materials

Measurements will be carried out on polystyrene particles (Duke Scientific) which are stained with the fluorescent dye Nile Red or Rhodamine B. Solutions of particles with a diameter of 28 nm, 48 nm, 100 nm and 460 nm (latter ones stained with Rhodamine B) are available. To obtain a suitable sample, all particle solutions have to be diluted with pure water to a concentration of 10 nM. Further to measure the concentration dependence of the FCS signal, a concentration series with 5 different concentration values ranging from 1 nM to 100 nM shall be prepared. Note the concentration on the sample bottles.

Further, the calibration measurements to determine the size of the focal volume shall be carried out on Rhodamine 6G. Rhodamine 6G has a molar extinction coefficient of 116,000 $M^{-1}cm^{-1}$ at 529.75nm in ethanol.

4.2.2 Sample Preparation

To avoid the evaporation of water from the samples, which will lead to a concentration change, small water droplets on a glass cover slide can be covered with an immersion oil droplet.

4.3 Experimental Tasks

- Determine the aspect ratio of the point spread function of the confocal microscope for the use in equation (17) by measuring a standard sample (Rhodamine 6G in water) and by imaging a 28 nm polystyrene bead in the scanning mode of the confocal microscope.

  a) Prepare a solution of Rhodamine 6G. Determine its concentration by measuring the absorbance of the solution with an absorption spectrometer. Dilute the solution to 1nM.

  b) Distribute fluorescent polystyrene beads (28 nm diameter) on a cleaned glass cover slide by spin coating a drop of the bead solution. Measure the point spread function of the confocal microscope by scanning over one single particle in the xy and zy plane.

- Measure the size dependence of the diffusion constant of dye labeled polystyrene particles in aqueous solution with the help of fluorescence correlation spectroscopy. Compare the results to the predictions of the Stokes-Einstein relation.

- Determine the concentration of 5 different solutions of dye labeled polystyrene particles.

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