Practical Course Biophysics:

Fluorescence Correlation Spectroscopy

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Chapter 1

Fluorescence Correlation Spectroscopy (FCS)

1.1 Introduction

Fluorescence Correlation Spectroscopy (FCS) is a well established technique that allows the measurement of diffusion coefficients and concentrations for fluorescing constructs. It is also possible to retrieve flow speeds and reaction rates. FCS is usually done on a confocal microscope and may be applied to any fluorescently labeled molecules in solution (usually water or buffer solution), on membranes and even inside living cells.

If the particle of interest is not fluorescing by itself (e.g. autofluorescent proteins), multiple labeling techniques are available. If proteins are observed one may either create fusion proteins with autofluorescing molecules (GFP, mRFP, ...) or covalently label the protein with a dye molecule (Alexa dyes, Atto dyes, Rhodamine-6G, Fluoresceine ...) or a quantum dot. If measurements should take place inside living cells fusion proteins come handy, as the cells produce them (stable/transient transfection) and they are mostly non-toxic. On the other hand autofluorescent proteins are rather large and may therefore influence the dynamic properties and reaction rates of the protein of interest. Chemical dyes may easily be attached to DNA and also proteins by linker systems (e.g. biotin/strepavidin) or reactive side groups of the protein (amine or thiol groups).

The next section will give an introduction into the principle of diffusion (which we observe with FCS in this practical) and the FCS method itself. The most important things that you should know after reading these pages are the basic principle of FCS, a feeling for the orders of magnitude of the variables involved and a rough idea of the setup we are going to use. To check your understanding of FCS, please also go through the questions in section A.

1.2 Theoretical Background

1.2.1 Diffusion

As already mentioned FCS may be used to measure dynamical properties of particles. One of the most important properties is the diffusion coefficient $D$, as it contains informations about the size and weight of the moving particle.

The diffusion coefficient $D$ (units $\mu m^2/s$) describes how far a particle may reach due to its diffusive motion. Diffusion can also be described as a random walk in the solution. While a protein moves in water the water molecules collide with the protein and it thus receives a series of small kicks in random directions. This leads to a random trajectory $\vec{r}(t)$ of the individual molecule (for
simulation examples, see Fig. 1.1). The larger the diffusion coefficient is, the faster the particle moves. The trajectory may be characterized by its so called mean squared displacement (MSD) \( \langle r^2 \rangle \), which describes the area that the particle covers in a certain time\(^1\). It is linear in time and the proportionality is given by the diffusion coefficient:

\[
\langle r^2 \rangle = 6 \cdot D \cdot \tau.
\]  

(1.2.1)

We will now show the meaning of this relation: We start with a protein that has a diffusion coefficient of about \( D = 50 \, \mu\text{m}^2/\text{s} \) inside the cytoplasm. So to have a good probability of reaching each position inside the cell (diameter around 20\,\mu m) we will have to let it diffuse for about \( \tau \approx (10 \, \mu\text{m})^2/(6 \cdot 50 \, \mu\text{m}^2\,\text{s}) \approx 0.3 \, \text{s} \). This rough estimate already shows that diffusion is sufficiently fast to serve as a transport process inside cells (e.g. in signal transduction).

![Fig. 1.1: Four trajectories of random walks with different diffusion coefficients that increase (red, green, blue, magenta) by a factor of five between two subsequent walks. The circles show the root mean squared displacement \( \sqrt{\langle r^2(t) \rangle} \) of the particle from its origin, a measure of the area, the particle covers during the simulation time.](image)

Until the beginning of the 20\(^{th} \) century, a macroscopic diffusion law (Fick’s law) was known that describes the effects of diffusion (equilibration of concentration gradients), but it could not be derived from microscopic observations (brownian motion). Albert Einstein succeeded in this task. He also derived a relation for the diffusion coefficient, which connects it with the properties of the particles and the solution they move in. This Stokes-Einstein equation is:

\[
D = \frac{k_B T}{6 \pi \eta R_h}
\]

(1.2.2)

where \( k_B = 1.3806504 \cdot 10^{-23} \, \text{J/K} \) is Boltzman’s constant, \( T \) is the absolute temperature and \( \eta \) is the solutions viscosity (see appendix C.1). The particles are describes by their hydrodynamic radius \( R_h \), which is the radius of an equivalent sphere that has the same diffusion coefficient as the given molecule. If the molecule is spherical, \( R_h \) and the real radius of the molecule will be nearly

\(^1\)To be more exact, the MSD equals the variance \( \langle r^2 \rangle \) of the trajectory
equal. For molecules with different shape (like e.g. the rod-shaped EGFP), there will be deviations. The hydrodynamic radius will also usually be a bit larger than the real radius of a sphere, as it also takes into account effects like the solvent molecules that are dragged along with the molecule. Note that the temperature dependence of $D$ is not linear, as also the viscosity $\eta$ shows a strong temperature dependence. If we assume that the volume $V_p$ of a particle scales linearly with its mass $m$, we get that $V_p = \frac{4}{3}\pi R^3_h \propto m$. From this we can easily derive the mass-dependence of the diffusion coefficient:

$$D \propto \frac{1}{\sqrt{m}}.$$

### 1.2.2 Particle number fluctuation

In the last section we have seen that particles move with different speeds. As their movement is not directed, its speed is described by the diffusion coefficient $D$. We will now discuss how the diffusion coefficient can be measured experimentally. In this practical course we will use the method called Fluorescence Correlation Spectroscopy (FCS), which derives the properties of the sample from fluctuations caused by the moving particles. As the name already states, the measured signal is the fluorescence intensity $I(t)$ emitted by the observed particles. As we have already seen in section ?? each particle (if excited to the same level) adds the same amount of fluorescence to the measured signal, so the overall intensity is proportional to the number of particles $N$ we observe. For the further analysis, we will therefore look at the particle number $N(t)$. In FCS we split up the overall particle number $N(t)$ over time, into an average value $\langle N \rangle$ and (small) fluctuations $\delta N(t)$ around $\langle N \rangle$ (see Fig. 1.2, right):

$$N(t) = \langle N \rangle + \delta N(t).$$

In FCS, only the fluctuations $\delta N(t)$ are analyzed, the average $\langle N \rangle$ is mostly left out during the data processing. These intensity fluctuations are caused by particles leaving and entering the observation volume $V_{\text{obs}}$. If we know how long a particle stays inside the observation volume, we can also determine its diffusion coefficient, which corresponds to its speed. So if we observe particles with a large $D$, they will often enter and leave the observation volume in a fixed observation time, and thus show quick fluctuations $\delta N(t)$. On the other hand these fluctuations will be slower for small diffusion coefficients. The correlation analysis performed in FCS extracts the characteristic timescale of these fluctuations which may subsequently be converted into a diffusion coefficient. Figure 1.2 depicts these findings.

![Fig. 1.2: Particle number $N(t) = \langle N \rangle + \delta N(t)$ in an observation volume for two groups of particles (red, blue) with different diffusion coefficients ($D_{\text{blue}} > D_{\text{red}}$). Observe the different timescales of the fluctuations $\delta N(t)$.](image-url)
As we want to extract information about the movement of single particles, we have to be able to detect the fluctuations $\delta N_{\text{single}} = 1$ caused by a single particle entering or leaving the focus. It is easy to detect this fluctuation caused by one particle if there are only few particles (small $N$) in the observation volume. If the average particle number $N$ increases, it becomes more and more difficult to see the fluctuation of one particle and therefore extract valid information about the movement of a single particle. Figure 1.3 shows two examples for this and gives a table with the relative fluctuations $\delta N_{\text{single}}/N = 1/N$ for different average particle numbers in the detection volume. For $N = 100$ the relative fluctuation have already dropped to only 1%. For particle numbers above this value they are hardly detectable.

![Fig. 1.3: Relative particle number fluctuations $\delta N_{\text{single}}/N$ of one particle entering or leaving a volume with $N$ particles on average.](image)

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Using these results we can estimate the typical volume $V_{\text{sample}}$ and concentration $c_{\text{sample}}$ of the sample, that we will have to use. In a typical fluorimeter the volume of the cuvette is $V_{\text{sample}} = 100 \mu l$ and the typical sample concentration is $c_{\text{sample}} = 100 \text{nM}$ (i.e. $\langle N \rangle = 100 \cdot 10^{-9} \cdot 6.022 \cdot 10^{23} \cdot 100 \cdot 10^{-6} \approx 6 \cdot 10^{12}$ particles in the cuvette). Here the relative fluctuations are only about $4 \cdot 10^{-7} = 0.00004\%$ which is way too low to give valid results. To come past these problems we could either reduce the sample concentration, or the observation volume. If we stick to our cuvette, we would have to go concentrations of $10^{-22} \text{M}$ which is impractically low (for this value we would be left with about 20 molecules in the cuvette). So we will have to reduce the sample volume. If we could go as low as $V_{\text{sample}} = 1 \text{ fl} = 10^{-15} \text{ l} = 1 \mu \text{ m}^3$ we get around 10 particles in this volume for a reasonable concentration of $c_{\text{sample}} = 18 \text{nM}$.

So far we have seen that we can extract information about the motion of particles in solution, if we observe their particle number fluctuations, while particles move in and out of a small volume. As the relative fluctuations get smaller with increasing particle number we have to use small particle numbers. We have estimated that the volume has to be around $V_{\text{sample}} = 1 \text{ fl}$ if we still want to work at reasonable concentration in the nano-molar range. Such volumes may be realized, if we use a confocal microscope, which is a common instrument for biological imaging. The next section will briefly explain the setup of such an instrument.

### 1.2.3 Confocal microscopy

In FCS the prerequisite of small system volumes is usually achieved by using a confocal microscope with an objective of high numerical aperture (NA > 0.9). The focal volume obtained by this setup is on the order of $1 \text{ fl} = 10^{-15} \text{ l} = 1 \mu \text{ m}^3$. Figure 1.4 shows the optical setup of a confocal microscope. Incident excitation light (blue) is focused by an objective lens into the sample. The size $d$ of the focus may be approximated by Abbe’s law, which states that

$$d = \frac{\lambda}{2 \cdot n \cdot \text{NA}}$$  \hspace{1cm} (1.2.4)
where $\lambda$ is the wavelength of the light, $n$ is the refractive index of the sample solution and NA is the numerical aperture of the microscope lens. The emitted fluorescence is imaged onto a pinhole which filters out-of-focus light (green) and afterwards onto a fast detector (like a photo-multiplier tube, PMT, or in our case an avalanche photodiode, APD). APDs allow for fast detection of single photons, so timescales from the sub-microsecond to the second range are observable. The APDs produce an electrical pulse for each single photon they detect (around every second photon is actually detected) and these pulse can be counted and processed by a computer. One dichroic mirror is used to separate between excitation and fluorescence light. A second dichroic mirror (together with a set of matching band-pass absorption filters) may be used to split the emitted light into several detection channels, so it is possible to distinguish between several dyes. As illumination light source we will use an Argon-Krypton ion laser which emits on three main laser lines (488 nm, 568 nm and 647 nm). We use a laser not because of the coherence properties of its light, but due to its stability and high power.

![Fig. 1.4: Principle of a confocal microscope](image)

By using a laser-scanner that deflects the laser beam in two directions directly in front of the objective lens, it is possible to convert the confocal microscope into a laser-scanning confocal microscope (LSCM) which may also be used to do fluorescence imaging of the sample. You will use this feature of our setup to image fluorescently labeled HeLa cells during the practical course. With such a LSCM it is also possible to freely select the location for the FCS measurement inside the sample.

A description of the microscope, which you will use during the practical course, can be found in [1, 2, 6].

### 1.3 Autocorrelation analysis

As of now you should have gained a basic understanding of how we can extract information about the particle motion from their number fluctuations in a confocal volume. This section will explain in more detail what an FCS measurement looks like and how we can extract these informations from it. As we have seen in section 1.2.2, we want to measure the particle number
fluctuations $N(t) = \langle N \rangle + \delta N(t)$ of fluorescing molecules entering and leaving the focus of a confocal microscope. As we cannot directly count the number of particles, we measure the fluorescence intensity $I(t)$ emitted by the particles currently inside the focus and analyse this. While a particle is inside the focus, it is excited by the laser of the microscope and therefore constantly cycles between its ground and excited state (it stays around $1\ldots10\text{ns}$ in the excited state, the fluorescence lifetime, see section ??). In each cycle a fluorescence photon is emitted in a random spatial direction. A fraction of these photons is then collected by the objective lens and subsequently detected on an APD. The output of the APD, we will call $I(t)$. Therefore the intensity $I(t)$ fluctuates in the same way, as the particle number $N(t)$ and every property we derived for $N(t)$ is also valid for $I(t)$. We can also split it into a constant offset $\langle I \rangle$ and the fluctuations $\delta I(t)$ (see e.g. Fig. 1.5, left panels):

$$I(t) = \langle I \rangle + \delta I(t)$$

From this measured signal, we now have to extract how fast it fluctuates, in order to gain information on how fast the particles move in the sample (see section 1.2.2 and especially 1.2). To do this, we use a mathematical tool, called autocorrelation analysis (therefore also the name fluorescence correlation spectroscopy, FCS). In FCS a special computer card calculates the autocorrelation function $g(\tau)$ from the intensity signal $I(t)$ measured in the microscope. The autocorrelation function is mathematically defined as:

$$g(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle_t}{\langle I(t) \rangle_t^2},$$

(1.3.1)

where $\langle \ldots \rangle_t$ denotes a time average over the time variable $t$:

$$\langle I(t) \rangle_t = \frac{1}{T} \int_1^T I(t) dt$$

It measures the self-similarity of the fluctuations $\delta I(t)$, if compared to itself, a time $\tau$ (lag time) later. If we look at the fluctuations $\delta I(t)$ and $\delta I(t + \tau)$ with a long lag time $\tau$, we do not detect any self-similarity in the signal, as the fluctuations at time $t + \tau$ are caused by different particles than at time $t$, which are independent of each other. So the autocorrelation $g(\tau)$ should be small (around 0). In contrast, if the lag time $\tau$ is so small, that the same particles create the fluctuations at time $t$ and $t + \tau$ (so smaller than the average retention time of the particles in the focus), we will have some degree of self-similarity between $\delta I(t)$ and $\delta I(t + \tau)$ (high autocorrelation $g(\tau > 0)$).

So we have seen that the autocorrelation function allows us to extract the non-random information (the fact that a particle needs a certain time to pass the focus) from the random intensity fluctuations $I(t)$. Figure 1.5 shows two examples of autocorrelation functions (right) as they could appear in FCS. They were calculated from simulated particles, passing the focus of a virtual confocal microscope. It can be seen that the decay time $\tau_{\text{decay}}$ (width at half maximum of $g(\tau)$) of the autocorrelation functions $g(\tau)$ scales with the timescale of the particle number variations (blue), so the desired information is extract from the noisy measurement signal $I(t)$ (red).

If you are more interested in how the autocorrelation function is calculated, you may have a look at appendix D.

### 1.3.1 The autocorrelation function of diffusing particles
As already mentioned each FCS measurement yields an autocorrelation curve $g_{\text{(measured)}}(\tau)$ which we have to further evaluate. To obtain the desired values – diffusion coefficient $D$ and particle number $N$ – we have to fit theoretical models $g(\tau)$ to the measured autocorrelation function $g_{\text{(measured)}}(\tau)$. These models may be derived by plugging into the diffusion law into (1.3.1) and doing all the integrals. For a solution of a single species with a diffusion coefficient $D$ and $N$ particles in a confocal focus (on average), we get:

$$g(\tau) = \frac{1}{N} \cdot \left(1 + \frac{4D\tau}{w_{xy}^2}\right)^{-1} \cdot \left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2} \quad (1.3.2)$$

Fig. 1.6: Approximated Focus of a confocal microscope

where $w_{xy}$ is the width in the xy-plane and $z_0$ the length (z-direction) of the measurement volume (confocal focus). The focus is estimated to have the shape of an ellipsoid with two equal axes $w_{xy}$ and a length $z_0$ (see Fig. 1.6). Usually (1.3.2) is rewritten in terms of the average retention time or diffusion time $\tau_D = \frac{w_{xy}^2}{4D}$ of a particle in the focus:

$$g(\tau) = \frac{1}{N} \cdot \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \cdot \left(1 + \frac{\tau}{\gamma^2\tau_D}\right)^{-1/2} \quad (1.3.3)$$

$$D = \frac{w_{xy}^2}{4\tau_D} \quad (1.3.4)$$

$$\gamma = \frac{z_0}{w_{xy}} \quad (1.3.5)$$

where $\gamma$ is called structure factor or axial ratio of the focus (see Fig. 1.6).

Some example curves, according to (1.3.3) are plotted in Fig. 1.7. Observe how the decay of the curves shifts to the right for increasing diffusion times $\tau_D$ and how the amplitude is decreased.

![Fig. 1.7: Examples of Autocorrelation functions $g(\tau)$ calculated from a noisy signal $I(t)$ with a slow (blue) and a fast (gaussian noise) varying component.](image-url)
for increasing particle numbers $N$. In fact the amplitude approaches the value $g(0) = 1/N$, so one can read the inverse particle number directly from the extrapolated zero-lag correlation. This again shows that the correlation gets less and less pronounced (low amplitude) for large particle numbers $N$. Also the diffusion time can directly be estimated from the graphs, by reading the width of the correlation function at half its amplitude $g(0)$.

![Autocorrelation function for simple diffusion](image)

**Fig. 1.7: Autocorrelation function for simple diffusion.** Left: $N = 10$ particles and increasing diffusion time, Right: increasing particle number with diffusion times $\tau_D = 100\,\mu s$

In order to calculate the diffusion coefficient $D$ we have to know the lateral size of the focus $w_{xy}$ and the structure factor $\gamma$. The latter one has been determined for our instrument from an image stack of immobile particles to be $\gamma \approx 6$. This value is not prone to large deviations due to slight misalignment, so you can simply use the given value for the data evaluation. The size of the focus $w_{xy}$ is determined by a calibration measurement of a solution with known diffusion coefficient (in our lab usually Alexa-488), which has to be redone on a day-to-day basis, or when parts of the setup change (sample chamber...). Knowing the size of the focus, we can also estimate its volume $V_{\text{eff}}$ and therefore the concentration $c$ of the solution:

$$V_{\text{eff}} = \frac{\pi}{2} w_{xy}^2 z_0 = \frac{\pi}{2} \cdot \gamma w_{xy}^3$$

$$c = \frac{N}{V_{\text{eff}}}$$

### 1.3.2 The autocorrelation function for multiple diffusing species

The autocorrelation function (1.3.3) can be extended to a case where $k$ species with different diffusion times $\tau_{D,i}$ are present:

$$g(\tau) = \frac{1}{N} \cdot \sum_{i=1}^{k} \rho_i \cdot \left(1 + \frac{\tau}{\tau_{D,i}}\right)^{-1} \cdot \left(1 + \frac{\tau}{\gamma^2 \tau_{D,i}}\right)^{-1/2}$$

$$\sum_{i=1}^{k} \rho_i = 1, \quad \text{and} \quad 0 \leq \rho_i \leq 1$$

$N$ is the overall particle number in the focus and the $\rho_i$ characterize the fraction of particles of species $i$. If all species have fluorophores of the same quantum yield $\phi_i \equiv \phi$ (see section ??), $\rho_i$ in (1.3.8) directly equals the fraction of particles of the $i$-th species. So $N_i = \rho_i \cdot N$ particles of species
are present in the focus. Figure 1.8 shows some example curves for two species with different diffusion times. Note that it is not possible to distinguish between different species with nearly the same diffusion coefficient (e.g. two different proteins of roughly the same size and mass, see red curve on right-hand side).

Fig. 1.8: Example autocorrelation functions $g(\tau)$ for two components with different diffusion times $\tau_D^i$ and fractions $\phi_i$. The black dashed line is the autocorrelation function for a single component diffusion with $\tau_D = 100 \mu s$. The particle number was $N = 10$ for all curves.

If the quantum yields $\phi_i$ are different, we have to account for this and the corrected fraction $\rho_i^{\text{corrected}}$ of species $i$ is:

$$\rho_i^{\text{corrected}} = \rho_i \cdot \left( \sum_{j=1}^{k} \rho_j \phi_j \right)^2 \phi_i^2.$$

1.3.3 Effect of triplet dynamics

So far we have discussed how moving particles influence the autocorrelation function, but fluctuations in the fluorescence intensity $I(t)$ and therefore additional components in the autocorrelation function may also be created by "switching" dyes on and off, or by altering their quantum efficiency $\phi_i$ (which basically has the same effect). There are several possible mechanisms that lead to the described behavior. The most important of these – at least under practical considerations – are "triplet" processes. Here the dye changes from its excited state $S_1$ into a long-living (several microseconds) dark triplet state $T$ (see Fig. 1.9). From there it relaxes down to the ground state after an average lifetime $\tau_T$. This leads to a blinking (switching off of the dye) on a characteristic timescale $\tau_T$. 
Triplet dynamics leads to an additional factor in the correlation function. If it is described by simple on-off dynamics (which is a good approximation), this additional factor is exponential:

\[
g^{(\text{triplet})}(\tau) = \frac{1 - \Theta_T + \Theta_T \cdot e^{-\tau/\tau_T}}{1 - \Theta_T} \cdot g(\tau)
\]

(1.3.10)

where \(g(\tau)\) is one of the correlation functions from sections 1.3.1 to F.2 and \(\Theta_T\) is the fraction of the particles in the focus that are in the triplet state T. When explicitly plugging in the diffusion function (1.3.3), we get:

\[
g^{(\text{triplet})}(\tau) = \frac{1}{N} \cdot \frac{1 - \Theta_T + \Theta_T \cdot e^{-\tau/\tau_T}}{1 - \Theta_T} \cdot \left(1 + \frac{4D\tau}{\xi_0^2}\right)^{-1} \cdot \left(1 + \frac{4D\tau}{\gamma^2\tau_D}\right)^{-1/2}
\]

(1.3.11)

Figure 1.10 shows some examples of the resulting correlation curves.
Fig. 1.10: Example autocorrelation curves with triplet dynamics. The curves contain a diffusing component with $\tau_D = 100\,\mu s$ and a triplet component with $\tau_T = 3\,\mu s$ and fractions of 0%, 20% ... 80%.
Appendix A

Preparatory tasks

1. In Fig. A.1 below you see an eppendorf tube containing a rather concentrated solution of EGFP. You find the absorption and emission spectrum in section C.5.3. Explain the yellow-green color of the solution and the yellow color of the transmitted daylight.

2. Why do we use autofluorescent proteins for labeling when measuring in cells? Which other labeling strategies are available?

3. Which factors influence the diffusion coefficient of a protein in buffer solution? Which factors play a role inside a cell?

4. Compare two proteins of masses 27kDa and 54kDa. By how much does their diffusion coefficient in the same buffer solution differ?

5. Estimate the diffusion coefficient $D_{\text{water}}(37^\circ \text{C})$ for Alexa-488 in water at 37°C and $D_{\text{sucrose}}(22.5^\circ \text{C})$ in a sucrose solution with viscosity $\eta = 2 \text{ mPa} \cdot \text{s}$ at 22.5°C, if the diffusion coefficient $D_{\text{water}}(22.5^\circ \text{C}) = 435 \mu \text{m}^2/\text{s}$ is known! The viscosity of water at 22.5°C is $\eta_{\text{water}}(22.5^\circ \text{C}) = 0.9436 \text{ mPa} \cdot \text{s}$ and at 37°C it is $\eta_{\text{water}}(37^\circ \text{C}) = 0.69 \text{ mPa} \cdot \text{s}$ hint: Use (1.2.2).

6. What is the approximate size of a focal volume at $\lambda = 488 \text{ nm}$ in water (refractive index $n = 1.33$) if an objective with NA = 0.5 and NA = 1 is used?

7. For a confocal volume with width $w_{xy} = 250 \text{ nm}$ and aspect ratio $\gamma = 6$, which concentration do you have to choose to achieve 1, 10 and 50 particles in the focus on average.

8. Estimate the retention time of particles with diffusion coefficients of $D = 10 \mu \text{m}^2/\text{s}$, $D = 50 \mu \text{m}^2/\text{s}$ and $D = 500 \mu \text{m}^2/\text{s}$ inside the above described laser focus ($w_{xy} = 250 \text{ nm}$, $\gamma = 6$) at a temperature of 37°C.

9. Look at the two FCS autocorrelation curves in Fig. A.2 (No photophysical effects are incorporated!). Estimate for each graph how many particles $N$ have been observed, how many different components there were, what their diffusion time $\tau_D$ was and possibly the number of particles in each species! Also estimate the diffusion coefficients $D$ of the different particles ($w_{xy} = 250 \text{ nm}$). Assume equal quantum yields for all species.
Fig. A.1: EGFP solution illuminated by day light

Fig. A.2: Example FCS autocorrelation curves. No photophysical effects are incorporated!
Appendix B

 Tasks during practical course

• fluorescence correlation spectroscopy (FCS):
  – Calibration measurement with 20nM Alexa-488 solution (every day and for every type of sample chamber!)
  – Dilution series with Alexa-488, using 500nM, 200nM, 50nM, 20nM, 2nM, 0.2nM solutions. Determine the dependence of \( N \) and the diffusion coefficient \( D \) on the sample concentration. What do you observe? What is a good sample concentration to perform FCS? Does the amount of light in the lab change the results? Measure also the background intensity (measured intensity without sample).
  – Determination of diffusion coefficient \( D \) and hydrodynamic radius \( R_h \) of different samples in solution (e.g.: EGFP-monomers, EGFP-tetramers, quantum dots, latex beads, labeled DNA, ...). Also look at the molecular brightness (average countrate divided by the number of particles) for different dyes and for the same dye free in solution and bound to a target molecule (e.g. Alexa-488, bound to DNA).
  – Measure the diffusion of Alexa-488 in 20% sucrose solution. What changes? How can you correct for artifacts? See also appendix C.3.2.
  – Measurement of a mixture of two fluorescing molecules with different molecular weights. Can you recover the expected diffusion coefficients and particle number fractions \( \rho_i \)?
  – FCS measurement of EGFP mono- and tetramers (1xEGFP, 4xEGFP) in HeLa cells. Which fit model describes your data best? Which would you choose? How do the results differ for different positions in the cells and mono- and tetramers? Compare your results to the measurements of 1xEGFP/4xEGFP in solution!
Appendix C

Useful Data

C.1 Constants

- Boltzman’s constant: \( k_B = 1.38 \cdot 10^{-23} \text{ J/K} \)
- Avogadro’s number: \( N_A = 6.022 \cdot 10^{23} \text{ mol}^{-1} \)

C.2 Unit conversions

- 1 Pa = 1 kg/(m \cdot s^2) = 1 N/m^2
- 1 J = 1 kg \cdot m^2/s^2
- 1 l = 1 dm^3, 1 fl = 10^{-15} l = 1 \mu m^3
C.3 Material properties

C.3.1 Water

- refractive index ($\nu = 20^\circ\text{C}, \lambda = 589.29\text{nm}$): $n = 1.3330$

![Viscosity of water graph](image_url)

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Fig. C.1: Viscosity of water
**C.3.2 Sucrose Solution**

- Sucrose, molecular formula: $C_{12}H_{22}O_{11}$
- Sucrose, molar mass: 342.30 g/mol

This table shows the refractive index $n$ and the dynamic viscosity $\eta$ for a sucrose solution. All data was taken from [5, 7].

<table>
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<tr>
<th>mass %</th>
<th>$n$</th>
<th>$\eta(20^\circ\text{C})$ [mPa·s]</th>
<th>$\eta(30^\circ\text{C})$</th>
<th>$\eta(40^\circ\text{C})$</th>
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**Fig. C.2: Viscosity $\eta$ and refractive index $n$ of sucrose solution at 20°C**
Fig. C.3: The electromagnetic spectrum, together with the emission and absorption maximum of some important fluorescence dyes, as well as often used laser lines.
C.5 Fluorophore data

C.5.1 Alexa-488

- max. excitation wavelength: $\lambda_{\text{ex,max}} = 494\text{nm}$
- max. emission wavelength: $\lambda_{\text{em,max}} = 517\text{nm}$
- molecular weight: 643.41 Da
- molar extinction: $\varepsilon(493\text{nm}) = 73000\text{M}^{-1}\text{cm}^{-1}$
- fluorescence lifetime (20°C, pH = 7.4): $\tau_{\text{fl}} = 4.1\text{ns}$
- quantum yield (50mM potassium phosphate, 150mM NaCl pH=7.2 at 22°C): $\phi_{\text{fl}} = 92\%$
- diffusion coefficients in water:
  - $D(22.5\text{°C}) = 435\mu\text{m}^2/\text{s}$ [4], $D(25\text{°C}) = 465\mu\text{m}^2/\text{s}$, $D(37\text{°C}) = 624\mu\text{m}^2/\text{s}$

If not state otherwise data was taken from invitrogen.
C.5.2 Alexa-594

- max. excitation wavelength: $\lambda_{ex,max} = 590\text{nm}$
- max. emission wavelength: $\lambda_{em,max} = 617\text{nm}$
- molecular weight: 820Da
- molar extinction: $\varepsilon(493\text{nm}) = 90000\text{M}^{-1}\text{cm}^{-1}$
- quantum yield: $\phi_{fl} = 66\%$

If not state otherwise data was taken from invitrogen.
C.5.3 Enhanced green fluorescing protein (EGFP)

- max. excitation wavelength: $\lambda_{\text{ex, max}} = 489\text{ nm}$
- max. emission wavelength: $\lambda_{\text{em, max}} = 508\text{ nm}$
- molecular weight: $\approx 26.9\text{ kDa}$
- molar extinction: $\epsilon(489\text{ nm}) = 55000\text{ M}^{-1}\text{cm}^{-1}$
- quantum yield: $\phi_{\|} = 60\%$
- diffusion coefficient in 100 mM phosphate-citrate buffer (pH=7.5): $D(22.5^\circ\text{C}) = 95\text{ }\mu \text{m}^2/\text{s}$ [4], $D(25^\circ\text{C}) = 102\text{ }\mu \text{m}^2/\text{s}$, $D(37^\circ\text{C}) = 136\text{ }\mu \text{m}^2/\text{s}$

If not state otherwise data was taken from [3].
C.5.4 Rhodamine 6G

![Rhodamine 6G molecular structure](image)

Note, the given spectrum was taken in ethanol.

- max. excitation wavelength: $\lambda_{\text{ex, max}} = 529.75$ nm
- max. emission wavelength: $\lambda_{\text{em, max}} = 555$ nm
- molecular weight: 479.02 Da
- molar extinction: $\varepsilon(529.75 \text{ nm}) = 116000 \text{ M}^{-1}\text{cm}^{-1}$
- quantum yield: $\phi_{\text{f}}(300...600\text{ nm, EtOH}) = 0.9\%$
- diffusion coefficient in water: $D(22.5^\circ\text{C}) = 426 \mu\text{m}^2/\text{s} [4]$

Appendix D

Mathematical definition of the autocorrelation function

This appendix gives a pictorial description of how autocorrelation works.

\[ g(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} = \frac{T \cdot \int_{0}^{T} \delta I(t) \cdot \delta I(t + \tau) \, dt}{\left( \int_{0}^{T} I(t) \, dt \right)^2}. \]

Figure D.1 illustrates the process of calculating the autocorrelation function \( g(\tau) \). First have a look at Fig. D.1(A). Here the signal (red curves) is a simple pulse between 0 and 1. When we shift (blue curve) and multiply, we get a signal (green curve) which is also a pulse, but with a width, that is decreasing with the shift \( \tau \). If we then integrate over this green signal (calculate the area below the curve), we get the values depicted as curve in the right plot, which is already the autocorrelation function. It can easily be seen that the width of the triangle in \( g(\tau) \) corresponds to the width of the pulse. Figure D.1(B) shows the same process, but for a train of pulses between 0 and 1. If the shift \( \tau \) is approaching the inter pulse time \( \Delta t \), the area below the multiplied signals \( I(t) \cdot I(t + \tau) \) is increasing again and as a result we obtain an autocorrelation curve with a series of triangles. Figure D.1(C) finally shows a more relevant example: The left graph shows a signal (red) which consists of pulses (blue) with random length and an overlayed gaussian noise. The right graph displays the autocorrelation function of this signal on a semi-logarithmic scale. Also here we observe that the width \( \tau_{\text{decay}} \) of the decrease in correlation for small lags corresponds with the average length of the pulses (compare upper and lower curves).
Fig. D.1: Examples of autocorrelation functions and the process of their calculation (A,B). (C) shows the time trace $I(t)$ and the autocorrelation function $g(\tau)$ of a process where random length pulses are overlayed with Gaussian random noise.
Appendix E

Data Fitting

For FCS evaluations we will use a method called **Least-Squares Fitting** during this practical course. As we have seen in section 1.3 there are a lot of known models for FCS data. So if we knew all the properties of a sample we could predict how the autocorrelation curve should look. Here we have the opposite problem: We have a measurement of the autocorrelation curve and want to estimate the parameters that best describe our measurement (e.g. the diffusion coefficient $D$ or the particle number $N$).

First of all we have to define what we mean by "best describes the measurements": From the FCS experiment we get a set of data pairs $(\tilde{\tau}_i, \tilde{g}_i), i = 1, 2, 3, \ldots$ that form the measured autocorrelation curve, i.e. for some lag times $\tau_1, \tau_2, \tau_3, \ldots$, we get the value of the correlation curve $\tilde{g}_1, \tilde{g}_2, \tilde{g}_3, \ldots$ (see Fig. E.1(a) for an example). If we now assume that some model $g(\tau; N, \tau_D)$ (e.g. from equation (1.3.2) with a fixed value for $\gamma$) describes the measurement, we can evaluate this model function for a given parameter set $(N, \tau_D)$ for every $\tilde{\tau}_i$. This gives us a set of estimates $g^{(m)}_i = g(\tilde{\tau}_i; N, \tau_D)$ that we can compare to the measurements (see Fig. E.1(b)). To determine how far the model lies away from the measurement, we can simply calculate the squared distance $(\tilde{g}_i - g^{(m)}_i)^2$ and sum over all these values:

$$
\chi^2(N, \tau_D) = \sum_{i=1}^{K} (\tilde{g}_i - g^{(m)}_i)^2 = \sum_{i=1}^{K} (\tilde{g}_i - g(\tilde{\tau}_i; N, \tau_D))^2
$$

(E.0.1)

We use the square of the distance, so negative and positive distances do not cancel each other out. This function now only depends on the values for the two parameters $N$ and $\tau_D$ and we are left with the problem to find the parameter combination $(\hat{N}, \hat{\tau}_D)$ that minimizes $\chi^2(N, \tau_D)$, i.e. best describes the data. To do so, we can look at the surface formed by evaluating $\chi^2(N, \tau_D)$ for different combinations of parameters (see Fig. E.1(c)). Then we have to find the deepest valley in this "search landscape". This is the same problem, as finding a protein configuration/structure with minimum energy, in protein folding simulations/structure prediction. But in that case the parameter space is much larger (not only $N$ and $\tau_D$, but all the atoms positions in space).
Fig. E.1: (a) Measurement result of a FCS experiment. (b) Data, as in (a) together with best fit model function. (c) Search landscape for the problem from (a) and (b)

One simple method to find this deepest valley, i.e. the best fit parameters is called "steepest descent": If we start at a point near the valley we only have to walk down the steepest slope and this way will end up at the lowest point of the surface (see Fig. E.1(c) ). In the practical course we will use a slightly more elaborate method, called Levenberg-Marquardt (LM) fit, which only relies for its first steps on the steepest descent scheme. Later it uses a different method to find the minimum quicker and more accurate. This method usually works well if there are no local minima in which we may be stuck and can not leave, as we would have to cross a high barrier. For those cases we can also use stochastic fitting routines (like e.g. simulated annealing) which are not rigorously bound to the steepest descent rule, but which may (with a certain probability) also walk "up the hill". This makes them generally slower and less accurate, but they are better at coping with wavy search landscapes. A good choice is therefore to combine both methods by first using one run of a stochastic optimizer and the a second LM run to refine the results.
Appendix F

Anomalous Diffusion

F.1 Introduction

When looking at diffusion in cells the situation is a bit more complex than described above for the simple case of diffusion in liquids. Cells are much more complicated objects than solutions. They are compartmentalized by membranes and the space in between is filled with different sized organelles, vesicles, (macro-)molecules and so forth. The cellular nucleus contains chromatin which fills about 10-20% of the available space, together with the protein machinery for the DNA transcription. This already shows that diffusion may no longer be described by only small water molecules bumping into the observed particle, but there is now a whole distribution of smaller and larger sized particles which collide with each other (crowding). In addition the observed particle may also bind to certain structures in the cell (membranes, vesicles, DNA, ...), or it may be transported actively along e.g. the cytoskeleton. Inside the nucleus the space is compartmentalized by the chromatin network which also hinders the diffusion. For this special case of hindered Brownian motion, the diffusion may be described by a slightly modified mean squared displacement (compare equation (1.2.1) and Fig. F.1(A)):

\[ \langle r^2 \rangle = \Gamma \cdot \tau^\alpha, \quad 0 < \alpha < 1 \]  

(F.1.1)

where \(\alpha\) is called anomaly parameter (for \(\alpha = 1\) (F.1.1) resembles (1.2.1)) and \(\Gamma\) is the proportionality constant. In this model the particles do not reach as far as normally diffusing particles in the same time \(\tau\), as they may get stuck in cages or dead ends.

Figure F.1(B) once again summarizes the effects described above, which will influence your diffusion measurements inside living cells.
Fig. F.1: (A) effect of hindered diffusion on the mean squared displacement (B) Different particle motion types inside living cells.

F.2 The autocorrelation function of anomalous diffusion

In section 1.2.1 we also discussed anomalous diffusion due to hindered diffusion in the cellular nucleus. This type of motion shows up in a deviation of the autocorrelation curve from equation (1.3.3), which may be described in two more or less equally valid ways. Either one uses the description of anomalous diffusion with a changed mean squared displacement. This then leads to a modified correlation function:

\[ g(\tau) = \frac{1}{N} \left( 1 + \left( \frac{\tau}{\tau_D} \right)^{\alpha} \right)^{-1} \left( 1 + \frac{1}{\gamma^2} \cdot \left( \frac{\tau}{\tau_D} \right)^{\alpha} \right)^{-1/2} \tag{F.2.1} \]

where \( \alpha \) is again the anomality parameter and (F.2.1) reduces to equation (1.3.3), if we set \( \alpha = 1 \). See Fig. F.2 for example plots.

Fig. F.2: Example autocorrelation functions for anomalous diffusion (colored curves). The red curve (\( \alpha = 1 \)) equals the model for normal diffusion. The black dashed curve was plotted from a two-component model with parameters \( \tau_D1 = 25 \mu s, \tau_D2 = 250 \mu s, N = 10, \phi_1 = 0.4, \phi_2 = 0.6, \gamma = 6 \) (the same as for the anomalous diffusion model plots).
The second possibility to describe the effect of particles that are hindered by caves, dead ends and other obstacles is to use two different diffusing components, as described in the last section. Then, one component describes the free diffusion and the second describes the slower fraction of particles that stuck up somewhere. Figure F.2 shows also how a two-component model (black dashed line) may be used to create nearly the same FCS autocorrelation function, as with anomalous diffusion (here for $\alpha = 0.8$).

Note that both models are only approximations. Therefore a real decision, which one is the right model is not possible, as both interpretations tell you something about the system under observation.
References


