4. Fluid solutions

We discuss the case of fluorescent molecules diffusing in a fluid environment, for example a drop of solution, a thin liquid film or a Langmuir film, a membrane, a capillary channel, or a cell. The most important feature of such experiments is that molecules come and go in the focal volume, and that statistics over large numbers of molecules are both necessary and possible. Usually, there are not enough photons emitted for a detailed study of each single diffusing molecule, therefore one has to accumulate data collected over large numbers of molecules. Microscopy and spectroscopy methods in fluid solutions are strongly related to single-molecule methods, because usually only one molecule is detected at a given time (although fluorescence correlation also works for small numbers of them simultaneously present in the detection volume), but they are not truly single-molecule methods because it is in general impossible to focus on individuals and to compare different individuals. In this sense, these are ensemble methods, giving statistical histograms. However, if different quantities can be measured on the same individual events, i.e., on the same molecules, statistical correlations between these different quantities can be obtained. This so-called multiparameter analysis, pioneered by Claus Seidel's group, relates directly to single-molecule methods, because such correlations are uniquely obtained on a molecule-per-molecule basis.

4.1. Photon counting histograms, burst analysis

A first way to analyze the fluorescence signal statistically is to record a histogram of the signal intensities in a time trace, i.e. the number of counts recorded during a given time interval, or bin, for example a few tens of microseconds for intense signals. For a single emitter with constant rate, the distribution of counts in the time bins is Poissonian. In the case of diffusing molecules, the distribution of the number of molecules in the focus is also Poissonian, and the observed histogram is a convolution of these two distributions. Diffusion during the emission broadens the distributions even more. Note that this method does not give the time-dependence of the fluctuations, unless the time window is varied.
A variant of this method is the analysis of burst sizes. This is particularly useful in the case of a solution flowing in a capillary or microfluidics channel, where the dwell

times of the molecules within the focus are well controlled. Each fluorescent molecule crossing the laser focus gives a burst of light. If the flow motion is faster than free diffusion across the focus, the burst intensity is determined mainly by the flow velocity. Burst size analysis is used in fluorescence biomedical assays.

4.2. Fluorescence correlation spectroscopy (FCS)

We have already discussed the principle of the method, which is to keep track of the intensity fluctuations of a fluorescence signal. The correlation method was applied in the 1970's to study the thermodynamic fluctuations of fluids via light scattering. Quasi-elastic light scattering can be measured in the spectral domain (through the first-order correlation of the field), or in the intensity domain (by second-order correlation of the intensity). It was then realized that this method could be applied to fluorescent liquids. However, because fluorescence is an incoherent process, the total emission is the sum of emissions from all emitting molecules in the (con)focal volume. The ensuing fluctuations can be measured, giving rise to fluorescence correlation spectroscopy. FCS really took off in the early 1990's with the large increase in detection efficiency thanks to APD's, microscope optics, and better filters. This very useful and powerful method is now widely used for the following reasons:
- it gives access to a wide range of times, often more than 8 orders of magnitude! This range is only limited by the photon count rate and the detector dead time on the short-time side (but this limitation can be circumvented by cross-correlating the signals of two detectors), and only by the experiment's duration on the long-time side.
- because the correlation data are averaged over a long integration time which can reach minutes or hours, statistical noise can be considerably reduced, enabling detection of weak and subtle effects;
- concentration requirements in FCS are much less stringent than in single-molecule measurements. FCS also works for numbers of molecules in the focus significantly larger than 1 (up to 100 or 1000), at the expense of a lower contrast (only partly offset by the higher signal). Because the number of molecules used is high and molecules are renewed constantly, FCS is also less sensitive to photobleaching. The illumination doses received by *each molecule* are usually much lower than in other single-molecule observations.

- FCS is a direct statistical method that does not require thresholds or other arbitrary parameters. It is largely insensitive to experimental conditions: i) the correlation function does not depend on detection yield (it cancels in the normalization); ii) a constant background will change the contrast of the correlation function only, not its time dependence.

All sources of fluctuations of the fluorescence can give rise to a signature in the correlation function. We examine the main causes of fluctuations hereafter.

4.2.1 Translational diffusion

A molecule crossing the laser focus can be seen as a concentration fluctuation, which will relax according to Brownian diffusion. From elementary diffusion theory, we know that the mean square displacement of a diffusing molecule scales linearly with time and that the volume sampled scales as time to the power 1.5.

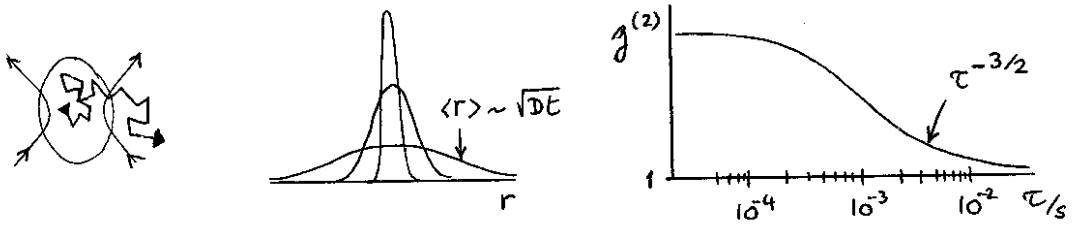

Figure 4.1 : Translational diffusion of a molecule in a laser focus (left). The center image shows the spread of the probability density as a function of time. The correlation function (right) decays with time as the probability density of the molecule, i.e. as the power 1.5 of time for long times in 3D space. Note the logarithmic timescale, very often used in correlation spectroscopy to visualize the broad range of times accessible.

Because the initial position cannot be known more accurately than the point-spread function, the fluctuation decay is cut off for short times by the diffusion time within the focus, $\tau_d = r^2 / 4D$, where r is the size of the focus and D is the diffusion coefficient. A typical value of D for a fluorescent dye in water is $10^{-9}\,\mathrm{m^2 s^{-1}}$ ($1\ \mu\mathrm{m^2/ms}$, corresponding to less than a millisecond in a laser's focal volume). A more rigorous treatment is based on a Fourier transform of the diffusion equation, assuming the fluorescence efficiency to vary spatially as a Gaussian (this is valid in the transverse plane for a Gaussian laser beam, but only approximately so in the axial direction). This calculation gives for the correlation function :

$$g^{(2)}(\tau) = 1 + \frac{1}{N}\left(1 + \frac{4D\tau}{{\omega_t}^2}\right)^{-1}\left(1 + \frac{4D\tau}{{\omega_a}^2}\right)^{-1/2}$$

where ω_t and ω_a are the transverse and axial beam waists, and N is the average number of molecules in the excitation volume. Note that statistical fluctuations normally scale as $1/\sqrt{N}$, but because these fluctuations contribute the correlation function through their square, we obtain a $1/N$ scaling.

Einstein related the diffusion coefficient to the local viscosity η around the molecule and its diameter R (if the molecule is assimilated to a sphere). The Stokes drag force on such a sphere for velocity v is:

$$F = 6\pi\eta R v .$$

The mean free path L of the Brownian random walk is related to the average velocity v and to the time step τ of the walk by :

$$L = v\tau .$$

Writing that the typical energy dissipated or absorbed in a step of the random walk is the thermal (Boltzmann) energy,

$$k_B T = FL ,$$

we deduce the Stokes-Einstein relation for the diffusion coefficient D :

$$D = \frac{L^2}{\tau} = \frac{k_B T}{6\pi\eta R} .$$

Although we obtained it from qualitative arguments, this relation is exact. The diffusion coefficient is inversely proportional to viscosity and to particle *radius*. Big molecules (proteins, for example) diffuse slower than small ones, but only by a factor scaling as the cubic root of their mass or volume.

Quite generally, note that viscous damping of the particle's velocity must always be associated to a heating mechanism. This heating ensures equipartition of the energy, and is related to friction by the fluctuation-dissipation theorem.

4.2.2 Rotational diffusion

A molecule absorbs and fluoresces as a dipole (albeit this dipole may differ in direction for the different transitions in those processes). Therefore, rotational diffusion of the molecule has an influence on the absorption of laser light (which is transverse to propagation and thus anisotropic, even in the case of an unpolarized laser beam) and on the detection of fluorescence if there is an analyzer in the detection path. Angular diffusion in three dimensions is a complex process, which obeys a diffusion equation similar to the Schrödinger equation of a rotor, but with an imaginary time. The solution involves spherical harmonics, each harmonic relaxing with time $\ell(\ell+1)\tau_r$, τ_r being the shortest rotational relaxation time $1/D_r$ and ℓ an integer characterizing the angular momentum value probed in the measurement. An arbitrary object will in general present three principal rotation axes with different rates of diffusion around those axes. In the case of a spherical rotor, however, these three axes are degenerate. Moreover, for a simple observable such as the intensity of polarized fluorescence from a linear dipole moment, the rotational diffusion of a sphere relaxes through a single exponential with time $\tau_r = 1/6\Theta$ corresponding to $\ell = 2$.

Here, we discuss a simple one-dimensional version. Let us consider a dipole moment performing a random walk in rotation around a fixed axis perpendicular to the dipole. Starting from an angle θ_0 with the excitation polarization (supposed to be linear), a random walk will add a small angle δ after diffusion time τ, ($\delta^2 = \Theta\tau$, where Θ is the rotational diffusion coefficient). The fluorescence intensity is proportional to the squared projection of the dipole moment on the laser polarization (provided no analysis is made upon detection). Calculating this averaged square, we find that it relaxes exponentially to the isotropic distribution:

$\left\langle \cos^2\theta(\tau)\right\rangle - 1/2 = \left(\cos^2\theta_0 - 1/2\right)e^{-2\Theta\tau}$.

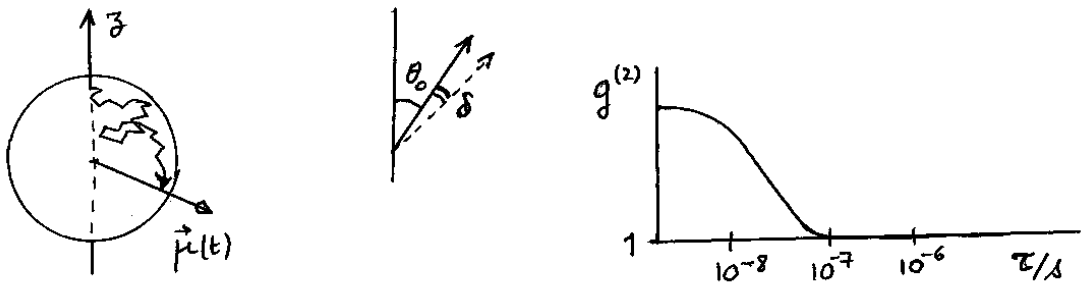

Figure 4.2 : Rotational diffusion of a dipole moment (left) as a random walk on a sphere. The center picture shows the average moment of a bunch of molecules, and a step of size δ of one of them. Upon averaging, the squared projection decreases exponentially with time, leading to the correlation on the right.

Therefore, the correlation function will decay exponentially. Note the difference with the case of translational diffusion: because the space of available angles is finite, the equilibrium is reached exponentially, much more rapidly than in the translational case. A reasoning similar to the one for translational diffusion gives the rotational diffusion constant as a function of viscosity and of the hydrodynamic volume V_H of the diffusion object, by the Debye-Stokes-Einstein relation:

$$\Theta = \frac{k_B T}{\eta V_H}.$$

We *define* the hydrodynamic volume of the object by the above relation. For a sphere, hydrodynamics calculations show that $V_H = 8\pi R^3$. For small dye molecules in water or usual solvents, rotational diffusion times are on the order of nanoseconds, comparable to fluorescence lifetimes. This means that the orientation of a molecule may vary considerably during fluorescence. The effect of rotational diffusion can be detected in steady state or in pulsed experiments by measuring fluorescence polarization, providing an average or time-dependent observable called *fluorescence anisotropy.*

4.2.3 Dark state (triplet)

We now suppose that the fluorescence intensity may vary because the molecule can go to a different state, usually a dark one in which fluorescence is suppressed. Chemical or physical changes in the molecule or its environment can lead to changes

in the absorption, or to quenching of the fluorescence. The resulting fluorescence fluctuations are due to random passages in the dark state, so that the signal of each single molecule resembles a random telegraph (Fig. 4.3). The kinetic rate equations lead to an exponential decay of the correlation function :

$$g^{(2)}(\tau) = 1 + \frac{k_2}{k_1} e^{-(k_1 + k_2)\tau}$$

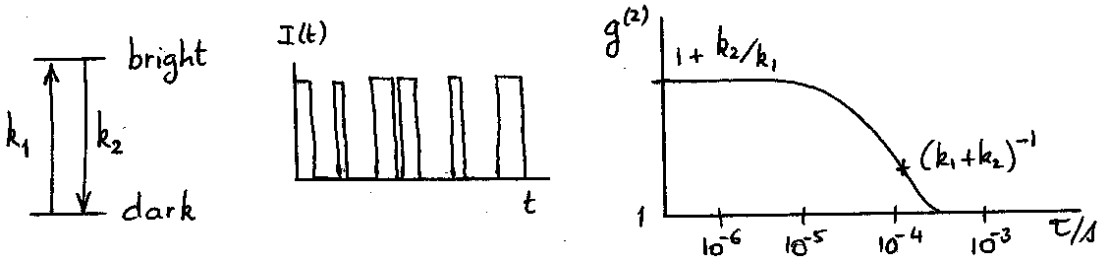

Figure 4.3 : Random jumps between a bright and a dark state, leading to a random telegraph signal for a single molecule. The corresponding correlation (right) decays exponentially with the sum of the jump rates. The contrast increases when the duration of the bright periods decreases. The times given are typical for intersystem crossing transitions to and from a triplet state.

In general, a molecule in a fluid solution is subject to all of these effects, and the correlation function can be quite complex. Figure 4.4 shows an example of a correlation function for a dye solution in water, showing antibunching at nanosecond times, triplet blinking at microsecond times and translational diffusion at millisecond times. For this small molecule, rotational diffusion is too fast to be distinguished from antibunching appearing around the fluorescence lifetime.

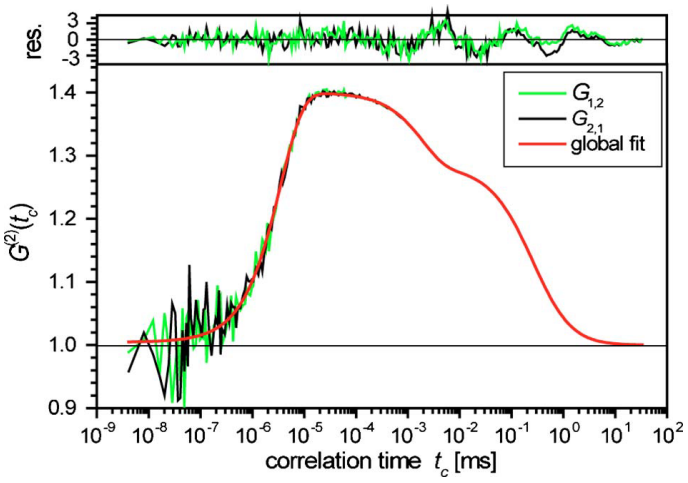

Figure 4.4 : Example of a fluorescence intensity correlation function for rhodamine 110 in water showing fluctuation phenomena at different time scales (work from Seidel's lab: Felekyan et al., Rev. Sci. Instrum. **76** (2005) 083104*).*

In many cases, the fluorescence intensity can be written as a product of *independently* fluctuating quantities. This is the case for translational diffusion, rotational diffusion, and chemical fluctuations. However, the triplet photophysics depends on the local excitation intensity seen by the molecule, and will therefore correlate with fluctuations due to translational diffusion in the excitation spot. If two different fluctuation processes are uncorrelated, and if the intensity is the product of two fluctuating functions, its correlation function writes as a product of correlation functions, i.e. as the product of the two functions pertaining to each type of fluctuation alone:

$$I(t) = f(t) \times g(t)$$

$$\langle I(t)I(t+\tau)\rangle = \langle f(t)f(t+\tau)\rangle \times \langle g(t)g(t+\tau)\rangle,$$

which means that each type of fluctuations can be recognized and identified on a logarithmic scale of the correlation times, independently of the other processes.

4.3. Variants of FCS

The correlation function is a general method, which provides time-resolved information from a fluctuating signal. It can obviously be applied to other signals than fluorescence intensity, for example fluorescence lifetimes, but also to non-optical signals such as currents in ion channels, for instance. Hereafter, we mention a few extensions of FCS.

- Two different signals can be correlated with one another, for example two intensities. This is cross-correlation. As an example, we briefly discuss dual-color FCS because of its importance in molecular biology. The fluorescence signals of two different dyes are separated by a dichroic beam splitter and measured by two detectors. If the two dyes are linked (for example due to protein-protein interaction), the translational diffusion of the complex will appear in the cross-correlation. If the molecules do not interact, no correlation appears (the fluctuations are independent).
- Fluorescence can be generated by two-photon or three-photon pulsed excitation. The advantages of this scheme are that there is less scattering of the longer excitation wavelengths, and that the focal volume is limited by the nonlinearity, therefore no pinhole is needed in the detection to obtain a slice of the sample (see Fig. 4.5; this effect, called optical sectioning, was mentioned earlier). Photobleaching is suppressed

altogether for out-of-focus molecules, although it is often enhanced for the molecules in the focus.

- Correlation can also be applied to any optical signal, linear or nonlinear. A good example is Coherent Anti-Stokes Raman Scattering (CARS). Being a coherent process, CARS would be very difficult to observe with a single molecule. However, it can be observed with small particles down to some tens of nm in diameter, such as organelles in cells.

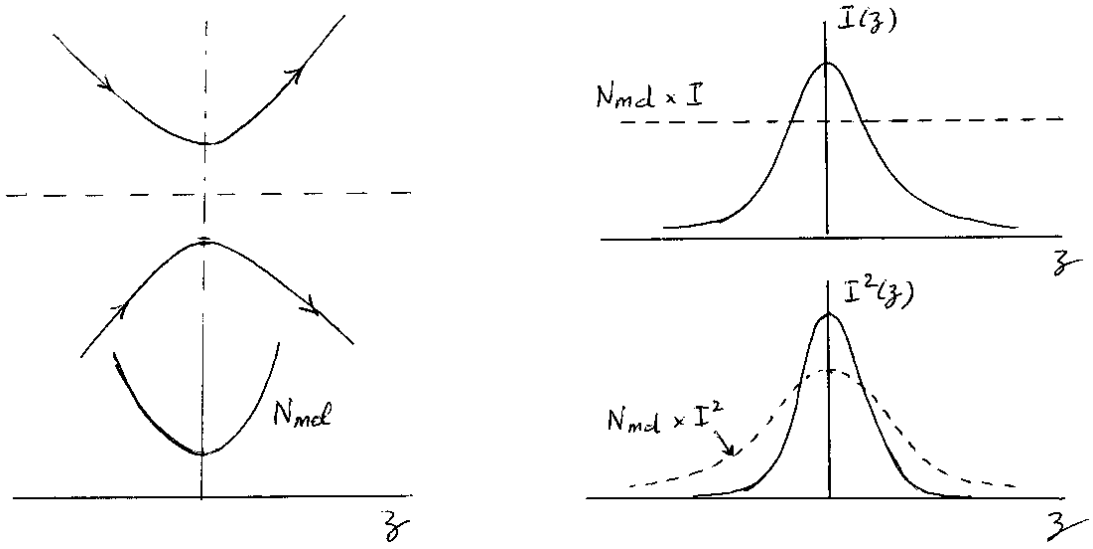

Figure 4.5 : Intensity profile around the waist of a Gaussian beam (upper left), with a line density of molecules along z increasing quadratically on both sides of the focus. The intensity along the axis varies as a Lorentzian $1/\left(a^2+z^2\right)$ *(upper right), which leads to strong background for one-photon excited fluorescence. This background is reduced by the pinhole in confocal microscopes. For two-photon excitation, on the contrary (lower right), the fluorescence intensity decreases as* $1/\left(a^2+z^2\right)^2$*, leading to a localized excitation spot (dashed curve). The pinhole is no longer necessary.*

4.4. Multiparameter analysis

Fluorescence gives rise to several different observables : intensity, lifetime, spectrum, polarization, FRET (see a later lecture), etc.. In order to fully exploit the fluorescence signal of a single molecule, one ideally would like to measure all of them with a time resolution as high as possible. Because the number of fluorescence photons per unit time is limited, one has to choose the more relevant parameters, and measure these

with the highest available time resolution. Determination of a fluorescence lifetime with an accuracy of a few % requires at least a thousand photons. A polarization measurement (or a crude measurement of the shift of a fluorescence spectrum) can be done with two detectors and a polarizing beam-splitter (or a dichroic beam splitter) and requires at least a few tens of photons. Once two (or more) quantities are measured for a population of molecules which have crossed the excitation volume, the quantities can be cross-correlated. The correlation may reveal different conformations of proteins, protein-protein or protein-DNA complexes (see the work of C. Seidel and collaborators).

Exercise 4.1: Consider an uncorrelated random stream of photons of n *counts per second. We are interested by coincidences within a short time* τ *. What is the number of coincidences measured during some long integration time* T *?*
This signal is provided by a single molecule, which on average will provide only N *fluorescence photons before bleaching. Which experiment will provide the best correlation signal on time* τ *:*
i) a long experiment with weak excitation intensity?
ii) or a short experiment with high excitation intensity?

Exercise 4.2: A laser source presents a weak oscillating noise corresponding to an intensity of the form $f(t) = 1 + \varepsilon \cos \omega t$ *, where* ε *is small.*
i) Calculate the correlation function of this intensity by integration over some long interval T *. Remove the oscillating terms due to the boundary condition of integration by assuming a weak damping constant.*
ii) This source is now used to excite fluorescence in a sample that, with a perfect source, would give correlation $g_0^{(2)}(\tau)$ *. What is the measured correlation function* $g^{(2)}(\tau)$ *, assuming the processes in the sample are not correlated with the source noise?*
iii) Sketch this correlation function on a log scale of time for a translational diffusion process.

Exercise 4.3: As a simple model of rotational diffusion, consider a rotator around a single axis, with position defined by angle θ. *The rotor performs a random walk with small angle steps* $\Delta\theta$ *done randomly after time step* Δt. *Show that the average of* $\cos^2\theta(t)$ *decays exponentially with time, and find the time constant, related to the rotational diffusion constant.*

Hint: relate the average of $\cos^2\theta(t+\Delta t)$ *to its value at time t, and expand to second order in* Δt. *Use the definition of the rotational diffusion constant.*

Exercise 4.4: Show that the correlation of a random telegraph intensity signal, switching between intensities I_1 *and* I_2 *with rates* k_1 *and* k_2 *for leaving state (1) or (2) respectively is given by:*

$$g^{(2)}(\tau) = 1 + \frac{k_1 k_2 (I_1 - I_2)^2}{(k_2 I_1 + k_1 I_2)^2} \exp\left[-(k_1 + k_2)\tau\right]$$

Hint: start from state (1) or (2) at time zero and calculate the time-dependent probability to be in state (1) or (2) at a later time (4 possible cases).
Alternatively, you might decompose the signal into a background I_1 *and a signal* $I_2 - I_1$ *and apply the relation obtained in Exercise 3.3.*