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 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. Sometimes, however, as in multiparameter analysis, statistical correlations between different quantities are obtained, and this relates directly to single-molecule methods, because these correlations are 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 plot a histogram of the signal intensity, i.e. the number of counts during a given time interval, for example a few tens of microseconds for intense signals. For a single emitter with constant rate, the distribution is Poissonian. In the case of diffusing molecules, the distribution of the number of molecules 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. Each fluorescent molecule crossing the laser focus gives a burst of light, whose intensity is determined mainly by the flow velocity

(if the flow is faster than diffusion over the focus). 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 has been devised in the 1970's to analyze thermodynamic fluctuations of fluids via light scattering. Quasi-elastic light scattering can be measured in the spectral domain (first-order correlation of the field), or in the intensity domain (second-order correlation). It was then realized that one of the fluctuating quantities could be the number of emitting molecules in the focal volume, 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. The method is very powerful and 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 limited by the photon count rate and the detector dead time (but this limitation can be circumvented by cross-correlating the signals of two detectors) on the short-time side, 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, noise is considerably reduced, enabling weak and subtle effects to be detected,
- FCS is much less sensitive to concentration requirements than single-molecule measurements. It also works for numbers of molecules in the focus 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, FCS is also less sensitive to photobleaching. The illumination doses received by each molecule are much lower than in other single-molecule observations.

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 volume sampled by a diffusing molecule decreases as a power 1.5 of time. Because the initial position cannot be known more accurately than the point-spread function, the fluctuation decay is cutoff for short times by the diffusion time

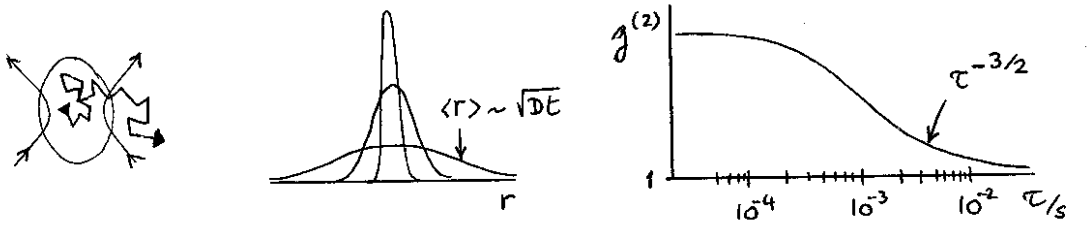

Figure 4.1 : Translational diffusion of a molecule in a laser focus (left). The center image shows the spread of the probability density as a function of time. The correlation function (right) decays with time as the probability density of the molecule, i.e. as the power 1.5 of time for long times. Note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

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 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.

Einstein related the diffusion coefficient to the local viscosity η around the molecule and its diameter R (if the molecule is a sphere). The Stokes friction force for a 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 time step τ 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 diffusion coefficient D :

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

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.

4.2.2 Rotational diffusion

A molecule absorbs and fluoresces as a dipole (not necessarily with the same direction(s) for both processes). Therefore, rotation 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 is multi-exponential, involving spherical harmonics and Legendre polynomials. In the case of a spherical rotor, the diffusion is single-exponential.

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:

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

Therefore, the correlation function will decay exponentially (Note the difference with the translational case: because the space of available angles is finite, the equilibrium is reached more rapidly).

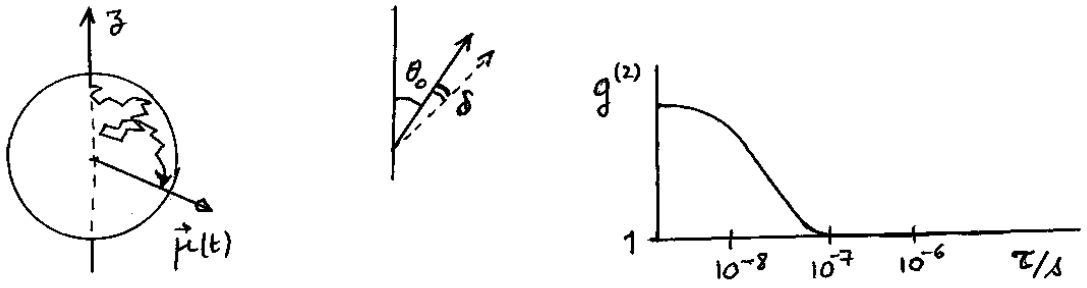

Figure 4.2 : Rotational diffusion of a dipole moment (left) as a random walk on a sphere. The center picture shows the average moment of a bunch of molecules, and a step of size δ of one of them. Upon averaging, the squared projection decreases exponentially with time, leading to the correlation on the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

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:

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

The hydrodynamic volume of the object is *defined* by the above relation. For a sphere, $V_H = 8\pi R^3$. For small dye molecules, 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. This can be detected in steady state or in pulsed experiments by measuring fluorescence polarization, an 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 more often 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 :

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

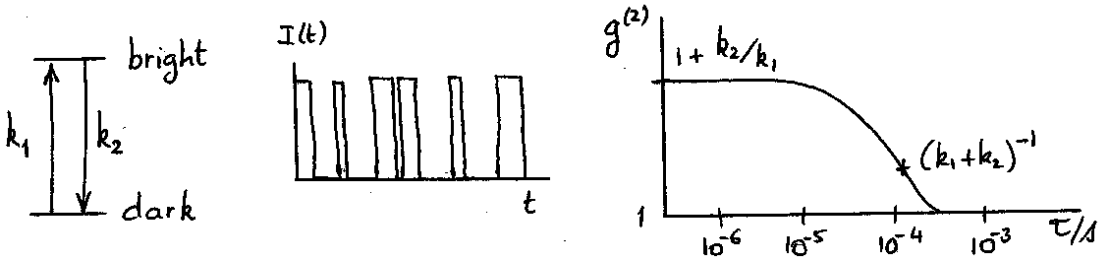

Figure 4.3 : Random jumps between a bright and a dark state, leading to a random telegraph signal for a single molecule. The corresponding correlation (right) decays exponentially with the sum of the jump rates. The contrast increases when the duration of the bright periods decreases. The times given 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. In the case where the fluorescence intensity can be written as a product of independently fluctuating quantities (for example translational diffusion, rotational diffusion, and chemical fluctuations), the correlation function writes as a product of averages, i.e. as a product of the functions pertaining to each type of fluctuations 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 on a logarithmic scale of the correlation, 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 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 (this effect is called optical sectioning). Photobleaching is suppressed altogether for out-of-focus molecules, although it is often enhanced for the molecules in the focus.

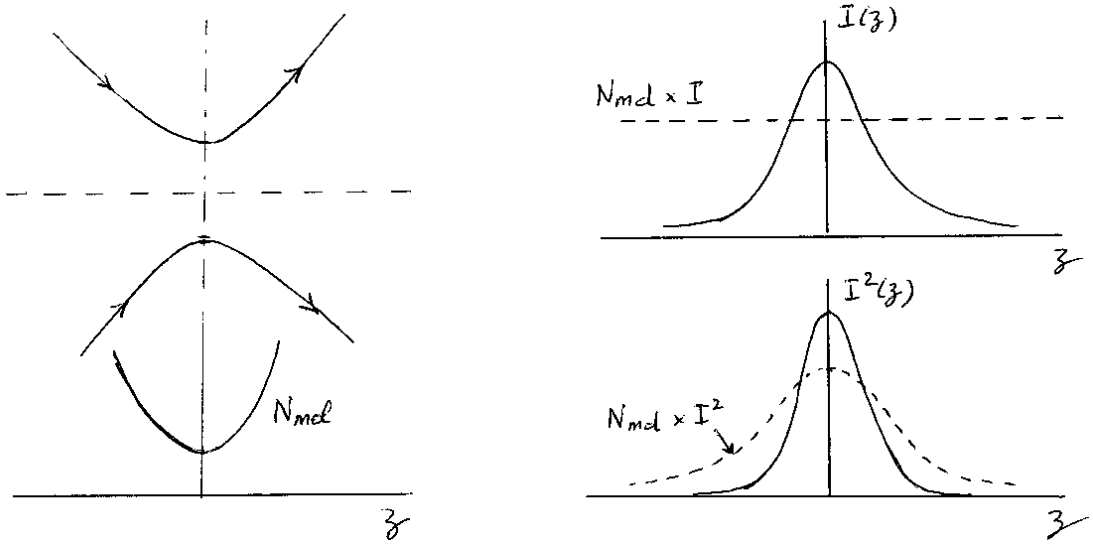

Figure 4.4 : Intensity profile around the waist of a Gaussian beam (upper left), with a line density of molecules along z increasing quadratically on both sides of the focus. The intensity along the axis varies as a Lorentzian (upper right), which leads to strong background for one-photon excited fluorescence. This background is reduced by the pinhole in confocal microscopes. For two-photon

excitation, on the contrary (lower right), the intensity decreases much faster, leading to a localized excitation spot (dashed curve). The pinhole is no longer necessary.

- 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 100 nm in diameter, such as organelles in cells.

4.4. Multiparameter analysis

Fluorescence gives rise to several different observables : intensity, lifetime, spectrum, polarization, FRET (see 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 suitable 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: 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.*

Exercise 4.2: 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).