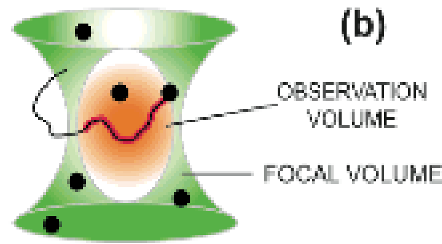
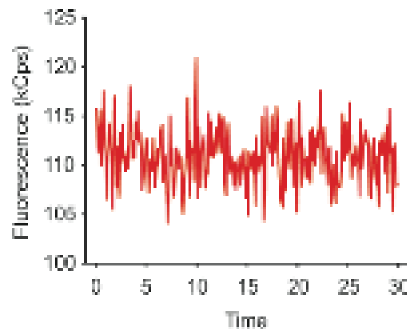
Autocorrelation Functions and Determination of Diffusion Coefficients
by Fluorescence Correlation Spectroscopy

Time-dependent fluctuations in a signal that may seem at first sight to be 'random' may not in fact be entirely random. To reveal the information hidden in the signal, an appropriate mathematical analysis of the fluctuations is needed. For fluorescence correlation spectroscopy (FCS), the signal is the intensity of fluorescence recorded from a tiny region of solution (the observation volume) as fluorescent molecules 'wander' (diffuse) randomly into and out of this region:

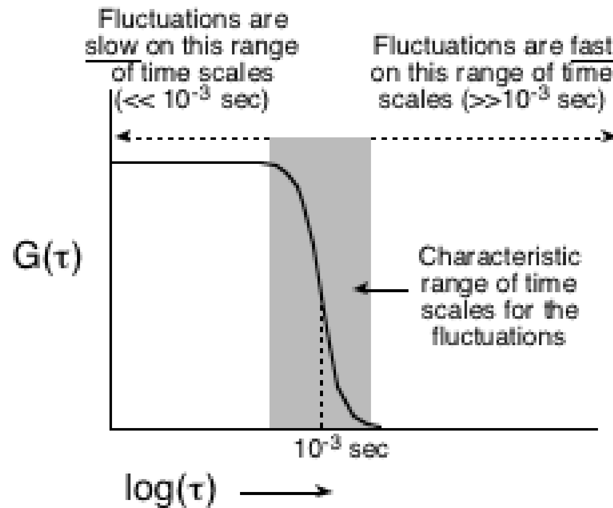


The diffusion of fluorescent-labeled molecules into and out of this region will result in fluctuations in the intensity of fluorescence recorded from the observation volume, F(t) (F(t) is proportional to the number of molecules present within the observation volume at any given time):



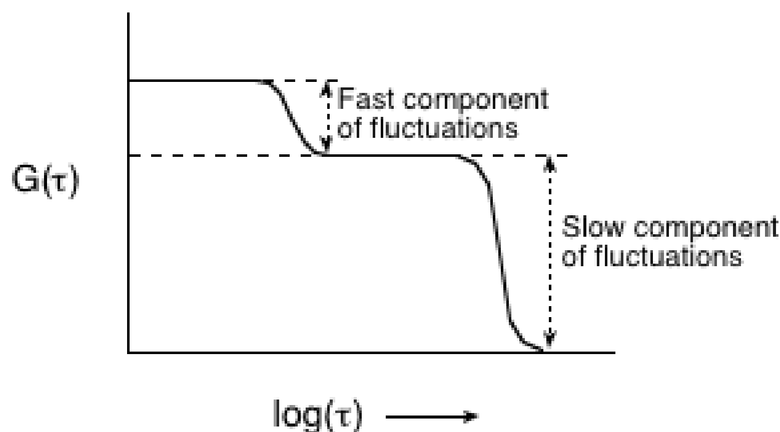
By computer analysis of this fluctuating signal F(t) over a time period that is much longer than the time scale of the fluctuations (so that we average over many such fluctuations) we can determine the autocorrelation function, $G(\tau)$, from which as discussed later we can determine the diffusion coefficient, D for the fluorescent species. However, we first need to examine in a bit more detail what the autocorrelation function represents in an FCS experiment.

$G(\tau)$ measures how the average correlation between F(t) at two time points t and $(t + \tau)$ falls off as the time interval τ between the two points increases; the poorer is the correlation, the lower is the value of $G(\tau)$. In essence, a plot of $G(\tau)$ as a function of τ (usually plotted vs. log τ) gives us quantitative information about the time scale(s) of the fluctuations in the signal intensity F(t):



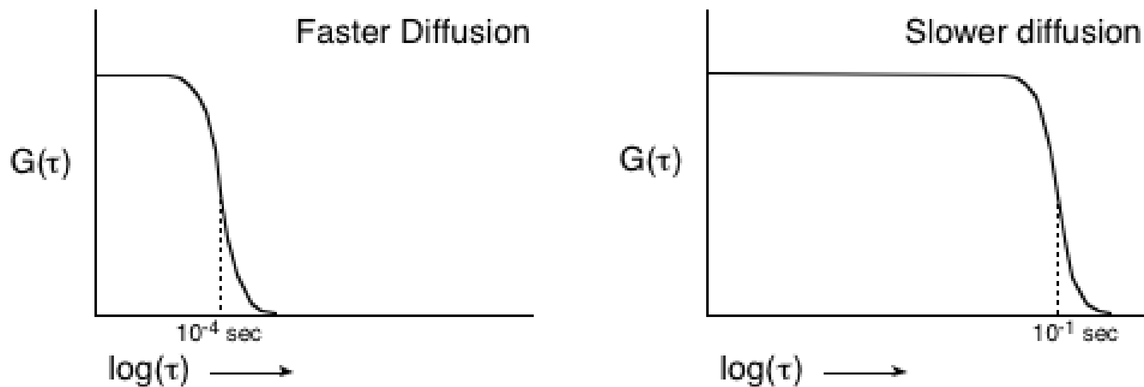
The more the signal fluctuates on average between times t and (t + τ), the poorer the average correlation will be between F(t) and F(t + τ), and the more closely G(τ) will approach its minimum value of zero. Therefore, for values of τ that are much <u>longer</u> than the average time scale of the fluctuations, G(τ) will fall to zero as shown at the right of the above plot. Conversely, for values of τ that are much <u>shorter</u> than the time scale of the fluctuations in F(t), so that on average the signal intensity does not fluctuate (change) significantly between times t and (t + τ), the correlation between F(t) and F(t + τ) will be very high, and G(τ) will approach its maximum value, as shown at the left of the above plot. For a 'simple' fluctuating signal (in which the fluctuations have one underlying physical basis and so occur over a single characteristic range of time scales), one can easily identify the time scale of the fluctuations by identifying the range of τ-values over which G(τ) changes rapidly as a function of τ (shown by the shaded box above).

An autocorrelation function can also have multiple sigmoid components, indicating that there are multiple types of fluctuations in the signal, occurring on different time scales:



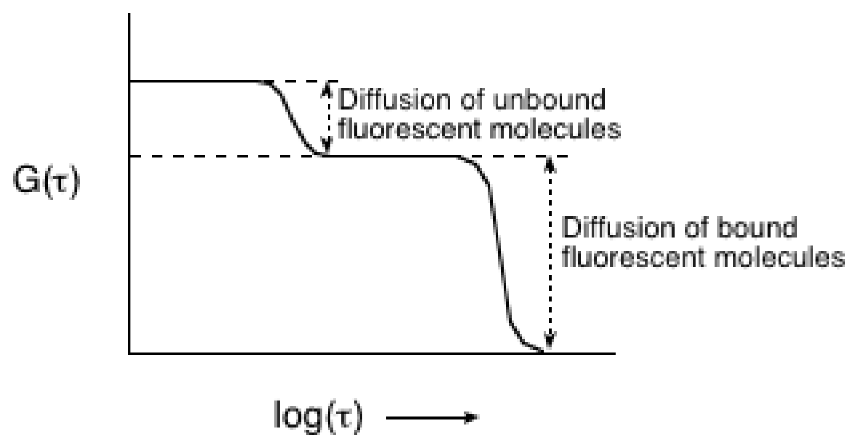
Let's now relate this to diffusion of fluorescent particles in an FCS experiment. As discussed above, random diffusion of the particles into and out of the observation volume causes the fluorescence detected from this small region of the sample, F(t), to fluctuate.

The faster the particles diffuse (i.e., the higher is their diffusion coefficent D), the shorter on average will be the time that a given particle remains within the observation volume once it ‘wanders’ into this region of the sample, and the faster the fluorescence signal F(t) will fluctuate. Conversely, the slower the particle diffuses, the longer on average will be the time that it spends ‘wandering’ through the detection volume once it enters this region, and the more slowly F(t) will fluctuate. This is directly reflected in the autocorrelation function that is calculated in an FCS experiment:



You might expect that the average time for a particle to ‘wander’ through the observation volume would also depend on the exact size and shape of this region, and in fact it does. However, it is a straightforward matter to calibrate the FCS apparatus to take account of these properties of the instrument’s observation volume, so that the FCS data (specifically, the calculated autocorrelation function $G(\tau)$), can be used to calculate D directly. Not surprisingly, the larger is the value of D, the smaller is the τ-value at which $G(\tau)$ falls to a half-maximal value.

An interesting application of FCS is to monitor binding of fluorescent molecules to larger particles (e.g., a small fluorescent molecule to a protein, a fluorescent protein to lipid vesicles, etc.). In this case the fluorescent species can exist in two states, one free and one bound to the larger particle. The free molecules of the fluorescent species will then diffuse faster than the bound molecules of this species (the bound molecules diffuse at the relatively slow rate that the larger particles do - remember that large particles typically diffuse faster than smaller ones). The effects of such binding on $G(\tau)$ are simplest to understand in the case where the average lifetime for a ((fluorescent molecule)•(larger particle)) complex is longer than the average time required for such a complex to diffuse out of the observation volume once it has diffused into this region. The autocorrelation function will then show two ‘humps,’ with inflection points at two very different τ-values (a shorter one for the free fluorescent species and a longer one for the bound species):



By appropriate curve fitting, the experimenter can calculate the relative amounts of the free and bound species. (This can also be done, in a somewhat different way, when the average lifetime of the complex is not longer than the average time for the larger particles to diffuse through the observation volume.) One can use this information to determine the affinity constant for the binding process, without having to separate the free and bound molecules of the fluorescent species. This can often be a major advantage, for example when the binding affinity is relatively weak.