Introduction

Fluorescence Correlation Spectroscopy (FCS) and related techniques are becoming widely used to measure molecular dynamics in a broad variety of specimens. These techniques provide important quantitative information on dynamics - such as diffusion coefficients, local concentration, binding reactions under equilibrium conditions - with high spatial and temporal resolutions. Also, they are minimal invasive since they work at very low concentrations of fluorescent molecules and do not require the use of high power lasers as Fluorescence Recovery After Photobleaching (FRAP) does, making them very attractive to studies in living specimens.

Principles

FCS measures the fluctuations of fluorescence intensity due to the transit of fluorescent molecules through the focal volume of a confocal or multiphoton microscope.

In the simplest case, these fluctuations will be given by passive diffusion of the molecules. Changes on the mobility properties such as those occurring during binding will affect the transit time through the excitation volume and thus the intensity fluctuations.

Figure 1 shows the fluctuations on intensity obtained at nanomolar and micromolar concentrations of a fluorescent protein. As can be observed, the fluctuations are more visible at low concentrations since just a few molecules are present in the excitation volume and the fluctuations due to the entrance or exit of a single molecule will modify significantly the average intensity value. Otherwise the number of fluorescent molecules in the excitation volume will be large and the intensity variation due to the transit of a single molecule small.
Figure 1: Intensity traces and relative fluctuations measured at micro (Panels A and C) and nanomolar (Panels B and D) concentrations.

The fluctuations in the intensity are quantified by calculating the normalized autocorrelation function of the intensity signal (G):

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I \rangle^2}$$

where $\delta I(t) = I(t) - \langle I \rangle$ is the fluctuation on intensity a time $t$ and $\tau$ is a lag time. The brackets represent the time average.

Figure 2 shows a graphical representation of the calculation of the autocorrelation function ($G(\tau)$). Qualitatively, $G(\tau)$ is calculated by comparing the measured data with a time-shifted version (the lag time $\tau$) of itself. If there is no time-shift, both data traces are identical i.e.
the correlation is high. If the shift is large, the two traces are very different and the correlation is low.

Figure 2: Calculation of the autocorrelation function.

This interactive tutorial explores how different parameters affect the intensity fluctuations and the autocorrelation function. The tutorial initializes with molecules randomly moving in the confocal volume. The right size of the figure shows the autocorrelation function calculated as described above. The particle size is controlled by the molecular weight slider. Clicking on the association, flux and confinement buttons shows the effect of these processes in the autocorrelation function. Use the sliders protein concentration and oligomer size to examine the effects of these parameters on the autocorrelation function.
The autocorrelation function

In a system where the molecules are randomly diffusing in three dimensions, the autocorrelation function will be given by:

\[
G(\tau) = \frac{\gamma}{N} \left[ \frac{1}{1 + (\tau/\tau_D)^2} \right] \left[ \frac{1}{\left(1 + (wo/wz)^2(\tau/\tau_D)\right)} \right]
\]  (2)

where \(wo\) and \(wz\) are the radial and axial waists of the observation volume, \(\gamma\) is a geometrical constant, \(N\) is the average number of molecules in this volume and \(\tau_D\) is a characteristic time where the autocorrelation function has decayed to one-half of the initial value.

If the molecules are randomly diffusing in the volume \(\tau_D\) is given by:

\[
\tau_D = \frac{wo^2}{nD}
\]  (3)

where \(D\) is the diffusion coefficient of the molecule and \(n\) is an index equal to 4 or 8 for 1-photon or 2-photon excitation, respectively.

This relation show that it is possible to obtain the diffusion coefficient of a given molecule by FCS measurements. This coefficient can be related to the hydrodynamic radius of the diffusing molecule \(r\) using the Stokes-Einstein relation:
\[ D = \frac{kT}{6\pi \eta r} \]  \hspace{1cm} (4)

where T is the absolute temperature, \( k \) is the Boltzmann’s constant divided by Avogadro’s number, \( \eta \) is the microviscosity in the observation volume.

Since \( D \) depends on the particle size, FCS measurements give information about processes that affect this size such as aggregation and binding. However, it is important to notice that processes that scarcely affect the particle radius and therefore the diffusion coefficient, such as binding to a small ligand, are difficult to detect by a simple FCS experiment.

**Fluorescence cross-correlation spectroscopy**

Cross-correlation experiments are an alternative to one color FCS experiments when measuring association reactions that involves a small change on the diffusion properties of the molecule.

In these experiments, the two interacting species are labeled with different fluorescent dyes with non-overlapping fluorescence spectra. The experiment is run as a FCS experiment but the fluorescence is split in two beams that are collected in two detectors using emission filters to selectively register the fluorescence coming from one of the species in each detector.

Figure 3 shows the intensity signals registered at the detectors for non-interacting and interacting particles.

**Figure 3 ANIMATION**

The figure 3 shows that the transit through the excitation volume of associated species produces simultaneous fluctuations in both channels and isolated species only produce fluctuations in one of the channels. To quantify this process, the crosscorrelation function is calculated as follows:
Crosscorrelation function is then analyzed as a regular autocorrelation function to obtain the information of the motion of the associated species.

**Scanning FCS**

In complex samples as cells, processes do not occur everywhere in the sample but are heterogeneous in space. Thus, it is sometimes important to study the dynamics of a given molecule in selected regions of the sample. When doing a conventional FCS experiment, fluctuations are registered in a single point of the sample. Scanning FCS techniques overcome this limitation by registering fluctuations in multiple points of the sample. Fluctuations in each of the scanned point are then analyzed as a single-point FCS experiment.

Commercial laser scanning microscopes (LSM) normally allows line or raster scan modalities enabling data acquisition from either a line or a whole area of the sample.

![Figure 4: Line-FCS experiment in a sample.](image)

An important limitation of scanning-FCS experiments is that the time resolution of the experiment is limited by the speed in which the laser is scanned. In most LSM, while fluorescence for a single point can be collected as fast as microseconds, a single line requires milliseconds and a small area needs seconds.