New and Notable

TIR-FCS: Staying on the Surface Can Sometimes Be Better

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Facing several decades of theoretical and experimental developments and improvements, fluorescence correlation spectroscopy (FCS) is a method that links the concepts and rationale of the early 70s' relaxation techniques to the steadily increasing pool of modern single molecule methods (Magde et al., 1972, Eigen and Rigler, 1994). With respect to fluctuation correlation analysis on fluorescently labeled molecules in laser-illuminated confocal setups, recent years have provided us with a multitude of interesting applications ranging from basic photophysical studies to cell biological research. Being sensitive to all processes that induce reversible changes in the emission behavior of fluorescent probes, FCS has developed into a routine technique to quantitatively analyze processes like diffusion, association, and dissociation, but also intramolecular dynamics. Although playing in the league of single-molecule techniques, it provides rather robust statistics on the measured parameters, due to the usually large averaging of many hundreds or thousands of single molecule transits through the observation volume during data recording.

The primary challenge for single molecule methods is the restriction of observation volumes to largely exclude signal contaminations by stray light or autofluorescent background. The most prominent approach for efficient background suppression in FCS has so far been the confocal setup, focusing the laser down to the resolution limit by a high numerical aperture objective, and axially confining the sample space by a pinhole in the image plane. According to theory, optical resolution in the axial direction scales with the inverse square of the numerical aperture, generally being at least threefold worse than the lateral resolution. For standard FCS setups, focal volume elements are usually of the order of 0.2–1 femtoliter (10^{-15}l) and ellipsoidal in shape with a height-to-diameter ratio of 3–5, dependent on the spherical correction of the objective lens and on the degree of overfilling of the back aperture. This ratio has always been considered a nuisance by FCS users, significant not only for detection, but also for illumination. In applications with limited dye resources, in particular within or on the surfaces of living cells, large illumination volumes can dramatically increase cumulative bleaching, inducing a rapid loss of fluorescent material and artifacts to the recorded correlation curves. This problem has, in recent years, been successfully dealt with by employing two-photon excitation. It can be shown that the obvious depth discrimination by the nonlinear excitation process indeed limits the amount of unwanted fluorescence excitation and thus improves signal quality and stability in long-term measurements (Schwille et al., 1999). However, two-photon excitation involves a massive technical complication of FCS setups as it requests rather expensive pulsed laser systems. Also, in spite of its obvious attractiveness for measurements inside cells and tissues, two-photon excitation does not improve experimental conditions for studies on intrinsically thin membranes.

In cellular applications, plasma membranes are probably the most obvious organelles to be investigated by FCS. Firstly, because the cell membrane is a clearly distinguishable macroscopic structure that is, by and large, free from spontaneous movements that interfere with the observation of fluctuating processes within. (In contrast, mitochondria or other small organelles show considerable positional fluctuations and thereby render quantitative FCS analysis particularly difficult.) Secondly, because many essential biochemical reactions take place at the cell membrane, like initial steps of receptor-induced signaling, that can be quantitatively studied by varying and controlling the amount of external ligand. Such conditions can hardly be achieved for association reactions inside the cell, where concentrations are difficult to determine, but even harder to change.

For studies on membranes, confocal but also two-photon FCS has the immanent problem that the membrane itself, with its thickness of, at maximum, 10 nm, covers only a vanishing axial fraction of the observation volume. As a consequence, the observation of membrane-bound molecules will always be compromised by the background of freely diffusing fluorescent molecules above and below the membrane of interest. Considering the rather low occupation density of many functional membrane proteins, and assuming that membrane-bound molecules are slower and more exposed to photobleaching during their transit through the illuminated area, it can, easily, be comprehended why very few quantitative FCS studies on receptor-ligand interactions have so far been reported (e.g., Rigler et al., 1999). The quantification is largely complicated by the excess of free ligand above the membrane, and elaborate fitting routines have to be applied to at least mathematically exclude the free ligand contributions when analyzing the ligand-receptor binding dynamics.

In this context, the contribution of Lieto et al. in this issue of the *Biophysical Journal* provides an elegant, although long-awaited, experimental solution by introducing a combination of FCS with total internal reflection (TIR) excitation to the study of receptor-ligand interactions at a

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model membrane. This nice experimental work is based on a large series of promising theoretical studies from the same group that raised the hopes of actually being able to study not only the diffusion behavior of membrane-bound molecules by FCS, but also the binding and unbinding process to and from the membrane by strictly limiting the observation depth. It is shown here that TIR can in fact achieve such an axial refinement of the measurement volume to close-to-membrane regions. If laser light is incident upon a medium of lesser refractive index under a critical angle, excitation will only occur in a thin surface layer of ~100 nm, the so-called evanescent field. In combination with a confocal pinhole for detection, unique resolution features can be obtained, and the separation of membrane-bound processes from excess ligand in the solution much improved.

Showing a clear distinction between characteristic autocorrelation decay times recorded from ligand added to membranes with and without receptor, and confirming the expected result that correlation amplitudes scale inversely with ligand concentration, the study by Lieto et al. suggests that TIR-FCS can indeed become the method of choice when looking at membrane-based dynamics, not only for studies on supported model bilayers but also on native cell membranes. In fact, fascinating work mainly from the cell biology community employing TIR imaging to track individual functional molecules on live cells (e.g. Sako et al., 2000) renders the combination of single molecule TIR imaging with FCS analysis highly desirable. However, several experimental challenges still remain to be met. E.g., Lieto et al. employ a rather ''classical'' TIR setup, where the evanescent field is induced by coupling an external laser through fused silica on top of which the model membrane is mounted. This geometry is perfectly suited for the system observed here but rather inappropriate to be used with cultured cells, where the simpler ''objective-type'' TIR would be more advisable. A second problem is the clear discrimination between binding processes and diffusion through the open measurement volume. FCS is extremely sensitive in resolving different timescales of fluorescence fluctuations, but blind to the actual mechanism behind them. In the present study, the receptor is assumed to be immobile and the correlation decay time solely attributed to ligand unbinding. However, timescales in the second regime can, in similar geometries, also be observed for translational membrane diffusion of some receptors (Schwille et al., 1999), and a clear distinction of processes may be more complicated.

Finally, one of the most crucial parameters to indicate the fidelity of FCS measurements, the fluorescence count-rate per single molecule, remains unmentioned in the present study, which makes it hard to speculate about the future potential of this approach in more complex systems. If it turns out that achievable molecular brightness values in TIR-FCS at least come close to those obtained in standard FCS measurements, dual-color cross-correlation applications (Schwille et al., 1997) might, in the end, be feasible, which would dramatically enhance the detection selectivity and broaden the spectrum of possible biological applications. If the technical challenges could be met appropriately, the combination of TIR with dual-color cross-correlation could be another valuable approach to solve the problem of detecting and quantifying weak molecular pairs in binding assays that request large concentrations of fluorescent molecules (Laurence and Weiss, 2003), comparable to—although not quite matching—the one that was recently envisioned in a study that combined FCS with zero-mode waveguides for axial but also lateral superresolution of the measurement volume (Levene et al., 2003).

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