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Short Communication

Accessing Molecular Dynamics in Cells by Fluorescence Correlation Spectroscopy

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Fluorescence correlation spectroscopy (FCS) analyzes spontaneous fluctuations in the fluorescence emission of small molecular ensembles, thus providing information about a multitude of parameters, such as concentrations, molecular mobility and dynamics of fluorescently labeled molecules. Performed within diffraction-limited confocal volume elements, FCS provides an attractive alternative to photobleaching recovery methods for determining intracellular mobility parameters of very low quantities of fluorophores. Due to its high sensitivity sufficient for single molecule detection, the method is subject to certain artifact hazards that must be carefully controlled, such as photobleaching and intramolecular dynamics, which introduce fluorescence flickering. Furthermore, if molecular mobility is to be probed, nonspecific interactions of the labeling dye with cellular structures can introduce systematic errors. In cytosolic measurements, lipophilic dyes, such as certain rhodamines that bind to intracellular membranes, should be avoided. To study free diffusion, genetically encoded fluorescent labels such as green fluorescent protein (GFP) or DsRed are preferable since they are less likely to nonspecifically interact with cellular substructures.

Key words: Anomalous diffusion / DsRed / Green fluorescent protein / *In vivo* single molecule detection.

The great potential of fluorescence correlation spectroscopy FCS (Elson and Magde, 1974) to precisely determine local concentrations and molecular mobility parameters in cellular compartments has long been predicted, and initial experimental approaches to assess protein diffusion in membranes (Fahey *et al.*, 1977) were reported only a few years after the introduction of the technique (Magde *et al.*, 1972). However, due to experimental difficulties in restricting small enough volume elements to suppress cellular background and to record fluctuations in the fluorescence emission, the technique was put back for many years in favor of photobleaching recovery methods (FPR/FRAP) which tolerate and require higher dye concentrations (Axelrod *et al.*, 1976). Photobleaching recovery has proven to be a robust technique for determining diffusion coefficients of fluorescently labeled or intrinsically fluorescent proteins (GFP) in the membrane, cytosol, mitochondria, and the cell nucleus (Feder *et al.*, 1996; Swaminathan *et al.*, 1997; Partikian, 1998; Lukacs *et al.*, 2000) with a temporal resolution in the millisecond range. However, FCS utilizes equilibrium measurements of small ensembles rather than disturbance and subsequent relaxation of larger populations, and promises a far higher dynamic performance and sensitivity as required for the observation of very small molecules, molecules expressed at low concentrations or intramolecular dynamics in the submillisecond regime.

After the first FCS applications with single molecule resolution in confocal setups were reported (Rigler and Widengren, 1990; Rigler *et al.*, 1993; Eigen and Rigler, 1994), this technique experienced a rapid increase in popularity that recently has led to a revival of its use for intracellular measurements. Recent publications (Politz *et al.*, 1998; Brock *et al.*, 1998; Schwille *et al.*, 1999 a,b) report the precise determination of cytosolic, intranuclear and membrane diffusion of different biomolecules at nanomolar concentrations, with a temporal resolution from tens of nanoseconds up to seconds. For the first time, molecular mobility could be measured at a single molecule level in various cellular compartments with diffraction limited volume elements (down to approx. 0.5 µm in diameter). It has been demonstrated that translational diffusion and active transport can be distinguished (Köhler *et al.*, 2000), and differences in local viscosity can be studied by its alterations in the molecular mobility of known fluorophores. Applying the concept of anomalous subdiffusion (Schwille *et al.*, 1999a), the steric hindrance of diffusing molecules induced by local confinements or molecular interactions, in particular of membrane bound systems (Saxton, 1993) has been explored. Further more, the ability to simultaneously measure mobility parameters and association or dissociation kinetics, as demonstrated by various approaches *in vitro* (Kinjo and Rigler, 1995; Rauer *et al.*,1996; Schwille *et al.*,1997), suggests that FCS might in the near future even be a valuable tool for unraveling the mechanisms and pathways of signal transduction.

Although exciting perspectives have been opened up by intracellular FCS, the technique exhibits – due to its extreme sensitivity – a non-vanishing risk of measuring potential artifacts and thus producing erroneous data, and its obtainable signal quality is considerably reduced compared to measurements in water or buffer solution. The complex intracellular milieu includes various types of autofluorescent molecules, and is a rather hostile environment for experiments at a single molecule level. Therefore, careful calibration measurements as well as negative controls are strongly advised. Use of at least ten-fold higher probe concentrations (Schwille *et al.*, 1999a,b) allows for discrimination against correlating autofluorescent background, present in several cell lines (Brock *et al.*, 1998). Photobleaching of probe molecules, although a necessary requirement for FPR approaches, is a very prominent error source for FCS, because it artificially limits the observation time frame and yields wrongly increased molecular mobility parameters (Widengren and Rigler, 1997). To prevent photobleaching and cell damage effects due to light exposure, the intensity levels in cellular FCS applications should be kept far below the values commonly used in aqueous samples, thus reducing the signal/background values that could otherwise be as high as 1000 for certain fluorophores (Mets and Rigler, 1994).

In addition to systematic errors introduced by insufficient instrumental control, such as too high excitation power, there might be undesired features in the performance of chosen fluorescent probes that may be uncovered by use in FCS applications and could lead to misinterpretation of the measured dynamics. Two very common phenomena of dye-induced dynamics are the population of transient dark triplet states (Widengren *et al.*, 1995), and fluorescence fluctuations induced by environmental factors, such as pH or by the exciting laser light, as found in various mutants of GFP (Haupts *et al.*, 1998; Schwille *et al.*, 2000) or cyanine dyes (Widengren and Schwille, 2000). Additionally, unspecific probe interactions with cellular structures can be misleading in measurements of molecular mobility, an effect that should be particularly highlighted here. A selection of the most common error sources for FCS mobility analysis is schematically summarized in Figure 1.

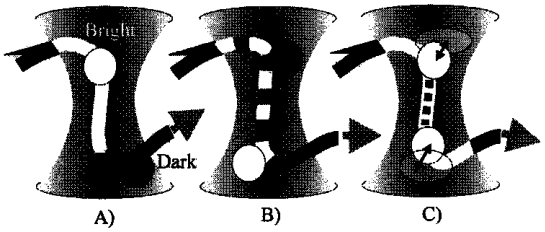

Fig. 1 Typical Error Sources in FCS Mobility Analysis.
Dynamic photobleaching, limiting the molecular observation period and thus falsely suggesting shorter residence times in the focal volume (A); on-off flickering of the fluorescence, *e. g.* due to intramolecular dynamics, which introduces additional characteristic time constants in the correlation curve (B), and nonspecific interactions of the probe with the environment, *e. g.* reversible binding to cellular structures resulting in anomalous subdiffusion (C).

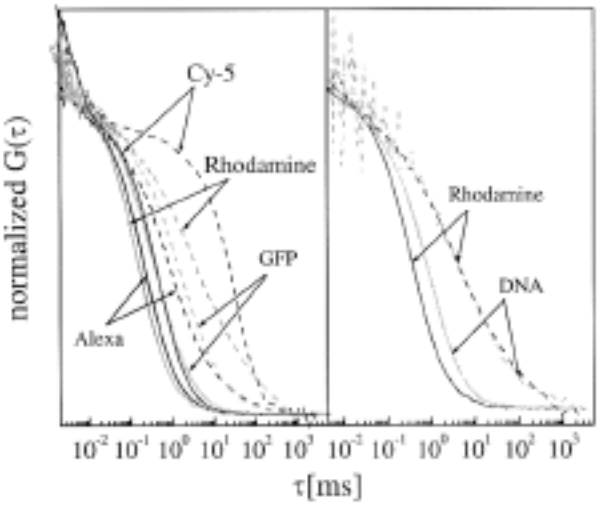

Fig. 2 Diffusion Measurements of Different Fluorescent Probes.
Left panel: correlation curves for dyes in buffer solution (solid) and loaded into HEK 293 (human embryonic kidney) cells by Influx Pinocytic Cell-loading Reagent (Molecular Probes) according to the recommended protocol (dashed). The cells were measured in PBS buffer on a custom-built FCS setup based on a Olympus IX-70 inverted microscope, underfilled back aperture of UPLAPO 60 × 1.2 objective and 100 µm pinhole diameter. For illumination, 488 nm (Ar-laser for rhodamine green, Alexa-488, rs-GFP) and 632 nm (He-Ne laser for Cy-5) were employed at 10 – 50 µW. The characteristic lateral and axial dimensions of the detection volume element are 0.5 µm and 2.0 µm, respectively. The data recording times were 10 × 10 s. It can easily be verified that the difference in diffusion times between buffer and cytosol varies strongly between the probes. While Alexa-488 and rs-GFP are slowed down only 3 – 4 times due to higher viscosity, rhodamine green and Cy-5 show dramatic deviations from their diffusion behavior in buffer. Anomalous 3D diffusion in cells is observed with $\alpha \approx 0.6$ for rhodamine and $\alpha \approx 0.85$ for Alexa-488. For rs-GFP $\alpha \approx 1$, for Cy-5, 2D diffusion with $\alpha \approx 0.9$ is recorded with a 60-fold increase in diffusion time. Right panel: comparison of free rhodamine green dye and rhodamine green labeled DNA in buffer (solid) and HEK 293 cells (dashed). Although the diffusion times vary in buffer by a factor of 2, no difference can be observed in cells. The diffusion is anomalous with $\alpha \approx 0.6$.

Due to the above mentioned restrictions in obtainable signal quality, the selection of proper labeling dyes for intracellular FCS is of crucial importance. Rhodamine derivatives, such as tetramethylrhodamine and rhodamine green, the more photostable Alexa dyes, as well as the far red dye Cy-5 are classical fluorophores frequently employed in high standard FCS experiments in the aqueous environment. However, use of these dyes in cells (Figure 2) may introduce artifacts due to their nonspecific lipophilic interactions with intracellular membranes (Figure 1C) yielding erroneously lower mobility parameters. Figure 2 (left) demonstrates that the free

rhodamine and cyanine dyes in particular should be avoided in intracellular mobility studies, because their diffusion behavior in the cytosol shows dramatic deviations from free diffusion in aqueous solutions. Comparisons with the cytosolically rather inert dyes, GFP and Alexa-488, indicate that the reduced rhodamine green and Cy-5 mobility cannot solely be attributed to increased intracellular viscosity. If the correlation curves in Figure 2 (left) are fitted with different models of free and restricted diffusion (summarized in Schwille *et al.*, 1999b), cytosolic rhodamine green diffusion can only be described as highly anomalous with $\alpha \approx 0.6$, or as a distribution of 2 or more diffusing species which differ by at least one order of magnitude in their respective diffusion coefficients. This indicates that a fraction of the dye is membrane – bound or at least shows a high affinity to cellular substructures. Similar observations were made with tetramethylrhodamine or rhodamine B (data not shown). Even more dramatic is the change in the intracellular behavior of Cy-5 which almost perfectly matches lipid diffusion in the cell membrane (Schwille *et al.*, 1999a,b) or diffusion of extremely large particles (three orders of magnitude larger in molecular weight than GFP) that the dye is bound to with hardly any contribution of free intracellular dye.

If the fluorophores are not employed as free dyes but as labels on larger biomolecules, such dye-induced artifacts may disappear, simply because the dye is shielded by the target or because stronger interactions between the labeled target and its specific binding sites dominate the dynamic behavior investigated by FCS. To test the difference of free rhodamine green dye and rhodamine green bound to a target, the intracellular diffusion of the dye itself was compared with that of a labeled small single-stranded DNA oligomer of 20 bases in length, as shown in Figure 2 (right). Surprisingly, no difference can be observed within cells although the DNA exhibits a two- to threefold slower diffusion than the free dye if measured in buffer. The diffusion of the DNA is still anomalous with $\alpha \approx 0.6$, which might indicate that the dye's affinity to membranes also plays a major role in the overall dynamics of the labeled DNA.

Based on various cytosolic mobility studies with different probes, intrinsically fluorescent genetic tags such as green fluorescent protein (GFP) are more favorable due to their lack of non-specific interactions. Free 3D diffusion with a diffusion coefficient 3–4 times smaller than in buffer solution was recorded for the mutants EGFP and S65T, regardless of the method of dye delivery to the cell – DNA expression or alternative conventional methods, such as cell loading reagents (Molecular Probes). Similar non-obstructed diffusion in the cytosol was found for the new red-shifted genetic tag DsRed (ClonTech, excited at 558, with maximum emission at 585 nm) which shows great potential to be used in multi-color approaches with blue or green GFP mutants. However, this fluorophore exhibits a remarkably strong flickering phenomenon (effect B in Figure 1), reflected by

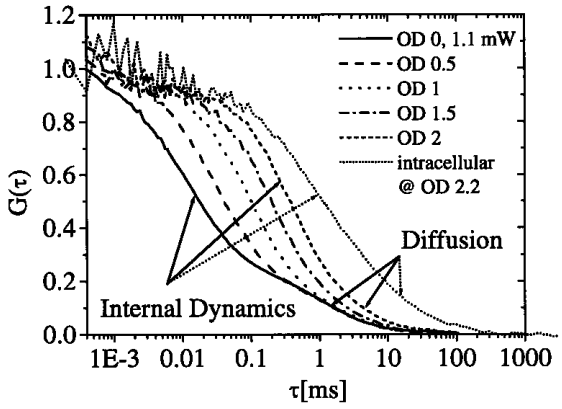

Fig. 3 Correlation Curves Recorded of DsRed, Illuminated at 543 nm (He-Ne Laser, at 1.1 mW and at Different Attenuation Levels up to 100-Fold) in Buffer and in Transfected HEK 293 Cells (Small Dots).
The fluorophore shows an intensity-dependent rapid flickering phenomenon at time scales of 10–100 µs with a nearly constant dark fraction. At the same time, the apparent diffusion time is reduced with increasing intensity due to photobleaching. Normal 3D diffusion, approximately 4 times slower than in buffer, can be observed in cells, however the fast flickering remains to be a dominant effect. OD: optical density.

an additional shoulder in the correlation curves in the time range of 100 µs which could be misinterpreted as diffusion of small molecules. Additionally, its photobleaching rate is quite high and could induce wrongly decreased diffusion times (effect A in Figure 1) even at relatively low intensities. Figure 3 shows correlation curves recorded from DsRed in buffer solution at different intensities compared to a representative intracellular measurement in a DsRed-expressing cell. Down to a certain power level, the diffusion time is severely affected by light intensity, and a strong flickering effect with a dark fraction of about 60% induces an additional shoulder in the 10–100 µs time range. The photophysics of this effect has been studied in detail (Malvezzi-Campeggi *et al.*, manuscript in preparation) and found to be analogous to the behavior of some yellow-shifted GFP mutants (YFP's; Schwille *et al.*, 2000).

In conclusion, FCS can be strongly recommended to cell biologists and biophysicists looking for quantitative and sensitive methods to assess not only local concentration but also molecular mobility, interactions and intrinsic dynamics of fluorescently labeled molecules. However, a careful selection of appropriate labels for each parameter to be determined is strongly advised. Lipophilic dyes such as certain rhodamine derivatives should better be avoided in cytosolic studies unless it is known that nonspecific interactions with cellular structures are shielded by the target. Genetically encoded fluorescent tags such as GFP and DsRed are favorable in many respects, but their intramolecular flickering effects, dependent on several external parameters, should be taken into account by proper calibration.

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Note Added in Proof

During the revision period of this manuscript, a detailed study regarding the photophysical properties of DsRed was published (Heikal *et al.*, Proc. Natl. Acad. Sci. USA *97*, 11996–12001), also highlighting the above mentioned light-driven blinking effect.

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