Fluorescence correlation spectroscopy for the detection and study of single molecules in Biology

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Summary

The recent development of single molecule detection techniques has opened new horizons for the study of individual macromolecules under physiological conditions. Conformational subpopulations, internal dynamics and activity of single biomolecules, parameters that have so far been hidden in large ensemble averages, are now being unveiled. Herein, we review a particular attractive solution-based single molecule technique, fluorescence correlation spectroscopy (FCS). This time-averaging fluctuation analysis which is usually performed in Confocal setups combines maximum sensitivity with high statistical confidence. FCS has proven to be a very versatile and powerful tool for detection and temporal investigation of biomolecules at ultralow concentrations on surfaces, in solution, and in living cells. The introduction of dual-color cross-correlation and two-photon excitation in FCS experiments is currently increasing the number of promising applications of FCS to biological research. ***BioEssays*** **24:758–764, 2002.**

Introduction

In recent years, the development of novel techniques for detection and analysis of single molecules has opened up a new era of biological research. In contrast to ensemble methods, which only yield average values for physical and chemical properties and parameters, single-molecule experiments provide information on distributions and time trajectories that would otherwise be hidden by the statistical mean. Single-molecule experiments allow fluctuating systems to be studied under equilibrium conditions, and direct observation of individual steps or intermediates of (bio)chemical reactions that are difficult or impossible to synchronize at the ensemble level. Furthermore, single-molecule experiments can reveal individual static and dynamic behaviors in seemingly identical but, in fact, heterogeneous populations of (bio)molecules. Thus, identification, sorting and quantitative comparison of subpopulations is achievable at the single-molecule level.[1,2]

Currently, single molecules can be not only detected but even manipulated precisely.[3] Single-molecule detection and analysis is presently dominated by two different kinds of approaches: atomic force microscopy and optical methods.[4] Atomic force microscopy is based on the application of mechanical force and allows single molecule force measurements as well as single molecule imaging with extremely high spatial resolution but its applications are restricted to surfaces, as properly reviewed elsewhere.[5,6] In contrast, there are several optical techniques used for the observation of biomolecules inside the living cell, including FRAP (fluorescence recovery after photobleaching) and FRET (fluorescence resonance energy transfer), the latter with single-molecule resolution. More information about these techniques can be found in several excellent review articles.[2,7–11] The present review is devoted to the emergence and increasing use of FCS (fluorescence correlation spectroscopy) as a powerful single-molecule detection and analysis technique covering a wide spectrum of structural and functional studies in biological sciences.

Theoretical background

FCS is a time-averaging fluctuation analysis of small molecular ensembles, combining maximum sensitivity with high statistical confidence. Although it was introduced in the early seventies,[12,13] severe experimental difficulties precluded its practical development until the nineties and only in the last few years have its practical applications exponentially increased.[14] In contrast to classical relaxation techniques, FCS does not require external perturbations of the system as minute spontaneous fluorescence signal fluctuations (which always occur on a microscopic scale at ambient temperatures) are analyzed. Fluctuations in the fluorescence signal are induced by molecules entering and leaving the illuminated region by random diffusive motion or by reversible changes in the fluorescence capacity during a molecule's residence time,

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attributable to intermolecular or intramolecular reactions or by reversible changes in the microenvironment of a fluorophore.

A temporal analysis of average fluctuations δF in the recorded fluorescence signal F(t) is carried out by temporal autocorrelation of F(t), giving rise to a normalized fluctuation autocorrelation function $G(\tau)$:

$$G(\tau) = \langle \delta F(t)\ \delta F(t+\tau) \rangle / \langle F(t) \rangle^2 \qquad (1)$$

Equation (1) yields valuable information about the underlying molecular dynamics. In fact, as depicted in Fig. 1, the correlation function $G(\tau)$ exhibits at least three fundamental parameters: (i) the average number of simultaneously observed fluorescent particles, $G(0)^{-1}$, (ii) the molecular residence time, τ_d, restricted by mobility, and (iii) characteristic flickering times, τ_f, reflecting fast on/off dynamics, which also reduce the average number of fluorescent particles, accompanied by an increase in G(0).

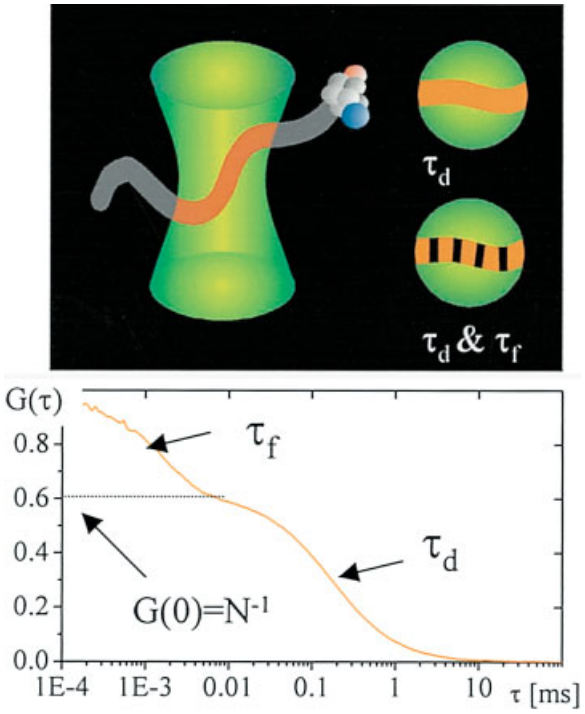


Figure 1. Principle of fluctuation analysis (top). Dye molecules (e.g., GFP) induce fluctuating fluorescence signals by random motion through the spot, or by internal dynamics inducing flickering on faster time scales. Representative autocorrelation curve $G(\tau)$ (bottom), describing the temporal decay function of fluctuations (time parameter τ). The characteristic decay times for molecular residence times in the volume (τ_d) and internal intensity fluctuations (τ_f) are indicated. The correlation amplitude G(0) refers to the inverse number of particles in the volume element.

FCS on a single molecule scale: the confocal detection scheme

Obviously fluctuation analysis is best performed if the system under observation is restricted to very small ensembles at the single-molecule level and if background is efficiently suppressed. This can be accomplished by a combination of very low sample concentrations with extremely small measurement volumes. A very simple and elegant setup fulfilling these requirements is the confocal detection scheme (Fig. 2) developed by Rigler et al.,[15,16] based on previous work carried out by Koppel et al.[17] A measurement volume even smaller than 10^{-15} litres is achieved by simply focussing a laser beam down to the resolution limit by an objective with high numerical aperture (NA > 0.9). Since fluorescence is only excited within the illuminated region, only molecules dwelling in this focal spot contribute to the measured signal. Photons emanating from there are projected onto a sensitive photodiode via a field aperture (pinhole) in the image plane, giving axial resolution. Reliable fluctuation analysis is possible by measuring at nanomolar or lower dye/fluorophore concentrations.

The autocorrelation analysis of conventional FCS is potentially applicable to every fast process that affects the fluorescence capacity of molecules (including proton or electron transfer reactions, oxygen or ion binding, or isomerization, among others), as well as association/dissociation reactions that induce marked changes in the diffusional mobility of labeled molecules. The range of FCS applications includes photophysical dynamics of fluorescent dyes or proteins, conformational dynamics, interactions of macromolecules and biochemical kinetics, among others.[18–33] Table 1 summarizes some of the biological experimental studies carried out so far using conventional FCS. The combination of confocal microscopy and FCS increases the respective range of applications by enabling single molecule detection and analysis in situ, including subcellular compartmentation of fluorophores, as shown in Fig. 3.

Dual-color cross-correlation and coincidence analysis

In the autocorrelation mode of FCS, analysis of molecular interactions (e.g. association/dissociation) can only be performed if the different diffusing species can be well-distinguished by their mobility on a single-molecule scale. In practice, this means that the diffusion times need to be sufficiently different; i.e. by a factor of at least two, generally requiring differences in their respective molecular weights by a factor of at least four to eight. Dual-color approaches can obviate these limitations while preserving the high sensitivity of FCS.

The concept of dual-color fluorescence cross-correlation spectroscopy (DC-FCCS) was firstly proposed by Eigen and Rigler in 1994.[34] DC-FCCS extends the standard FCS

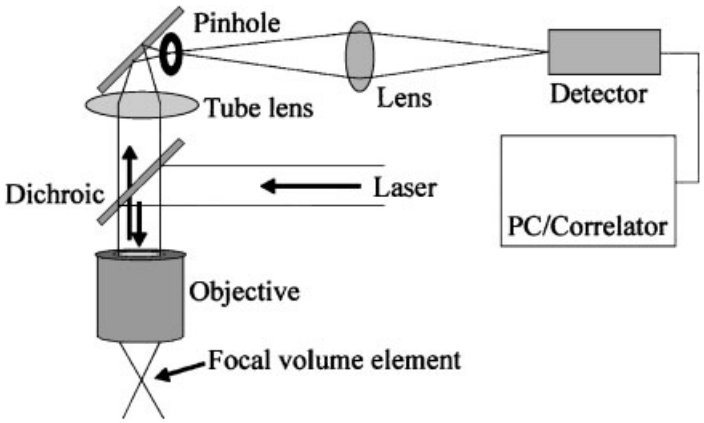


Figure 2. Confocal setup for FCS detection. The laser light is focused by an objective with high numerical aperture to the diffraction limit, the focal spot is imaged onto the detector via a pinhole or optical fiber of approx. 100 μm diameter. The pinhole, which has a variable diameter, is located in the image plane of the tube lens and can be adjusted in the x–y–z axes. This setup results in an open measurement volume of approx. 10^{-15} litres.

scheme by introducing two different fluorescent labels, which are each excited and detected by independent light sources and detectors, but share a common measurement volume. The instrumental setup (Fig. 4) and sample design are somewhat more elaborate, but this fact is compensated by a significant simplification in terms of data analysis.[35] The fundamental measurement parameter to basically all recent applications of DC-FCCS is the coincidence of distinguished labels on a single molecular entity and thus the amplitude of the cross-correlation function rather than its temporal evolution. Thus, in principle, all kinds of processes leading to conversions between single-colored and double-colored species can be investigated. Fig. 5 compares the analysis of a simple cleavage reaction by both single and dual-color FCS; the advantages of DC-FCCS are obvious.

The theoretical concept and first experimental demonstration of dual-color cross-correlation analysis was described by Schwille et al.[36] in a study of the annealing of two single-stranded oligonucleotides labeled with different fluorescent dyes. This concept has also been successfully applied to the study of DNA–protein interactions[37] and to the detection of amplified polymerase chain reactions products employing labeled primers for both strands.[38] Direct observation of enzymatic processes has been put forward by on-line measurements of the cleavage of a double-labeled oligonucleotide by *Eco*RI endonuclease and the enzymatic degradation of a peptide by subtilisin.[39,40]

The DC-FCCS concept has already yielded remarkable biotechnological applications. A rapid method for high-throughput screening and evolutionary biotechnology using the DC-FCCS scheme has been described.[41] Some modifications of this approach have led to the related technique of confocal fluorescence coincidence analysis that has been proposed as a novel approach to ultra high-throughput screening for drug discovery and evolutionary technology.[42]

It is noteworthy to mention that commercial variants of the original DC-FCCS setup[43] have recently become available (e.g. Zeiss ConfoCor microscope, Ref. 44). These systems offer significant advantages compared to home-built rigs, in particular with respect to the long-term stability of the optical setup and their user-friendliness in biological applications. In combination with confocal imaging and other complementary fluorescence techniques such as FRAP and FRET, we anticipate a strongly increasing use of FCS to solve biological problems in coming years.

Table 1. Some recent applications of standard fluorescence correlation spectroscopy to biological science research

Applications	Reference
1. Structural dynamics of fluorescent proteins *in vitro*	18,19
2. Molecular dynamics of fluorescent proteins *in vivo*	20–22
3. Biochemical kinetics	23,24
4. Macromolecule interactions	
• Nucleic acids/nucleic acids or proteins	25–27
• Proteins/proteins	28,29
5. Protein transport	29,30
6. Macromolecule/small molecule interactions	31
7. Single-molecule imaging of surface systems	32,33

Two-photon excitation FCS

Two-photon excitation of fluorescent molecules is accomplished if two photons of half the energy required for a transition to the excited state are simultaneously absorbed. This simultaneous ($\leq 10^{-15}$ s) absorption within the cross-section

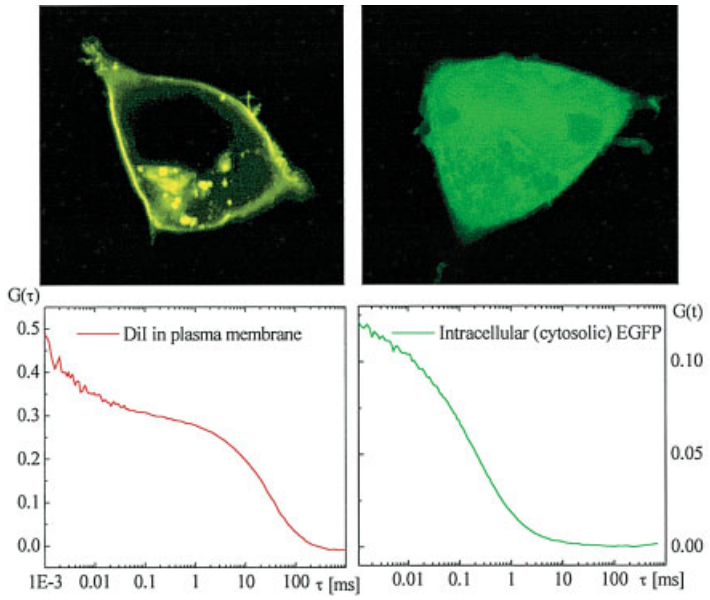


Figure 3. Confocal images and FCS diffusion measurements to investigate the local viscosity in different fluorescently labeled cellular compartments. Left, dil-C_{12} (a lipid analog) molecules staining and diffusing in the plasma membrane of mammalian cells. Right, cytosolic localization and analysis of enhanced GFP. The representative autocorrelation curves G(τ) (below) show different decay times, which demonstrates the slower movement of the probe in the membrane.

of the fluorophore (in the order of 10^{-16} cm^{-2}) requires high instantaneous photon flux densities (in the order of 10^{32} photons/cm^2). Pulsed infrared lasers are used to access common fluorescent probes in the visible or near-ultraviolet range, yielding a resolution comparable or even superior to that obtained by confocal pinholes. This feature, as well as the higher tolerance of infrared light by cells, makes two-photon excitation laser scanning microscopy an attractive alternative to confocal imaging.[45] Although two-photon excitation has been successfully applied in conventional FCS,[21,46] its employment for DC-FCCS is expected to significantly promote the use of FCS for cellular measurements in the near future. Two-photon excitation spectra of common fluorophores can differ considerably from their one-photon spectra without any difference in emission, giving simultaneous excitation of spectrally distinct dyes, as successfully demonstrated by Heinze et al.[47] The experimental setup is significantly simpler than the confocal DC-FCCS geometry (see Fig. 4) because only one laser line is now used for excitation and no pinholes are in principle

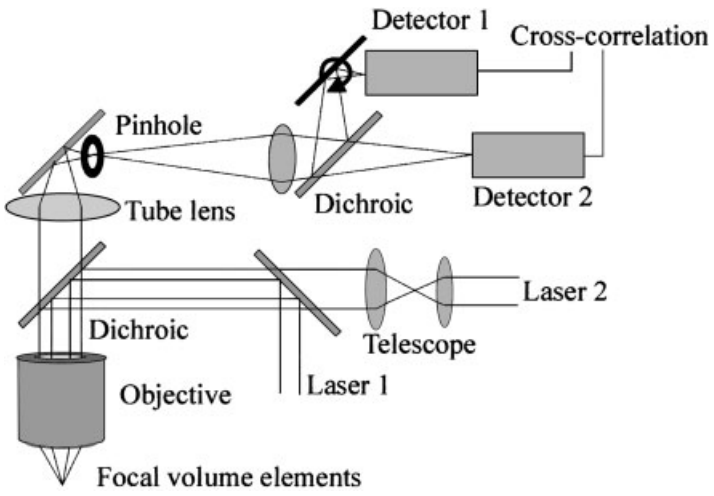


Figure 4. The dual-color cross-correlation FCS setup. Two parallel laser beams pass through a microscope objective in an epi-illumination arrangement, resulting in two superimposed focal spots in the sample, each in the sub-femtoliter range. The emitted fluorescence light is collected by the microscope objective, separated from the excitation light by a dichroic mirror, and focused onto a pisnhole by a lens. Fluorescence emission is separated by a dichroic mirror into two fractions of different colors and refocused on two avalanche photodiodes.

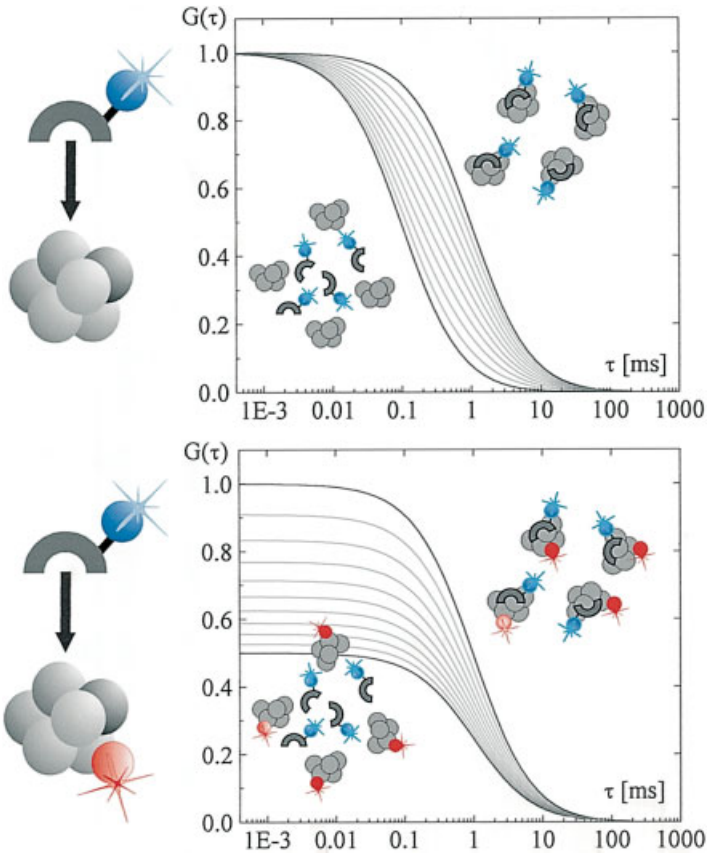


Figure 5. Comparison of single- and dual-color measurements for the analysis of the binding of two molecular species. When only one of the molecules is labeled, binding can be observed if it induces a change in the apparent molecular weight of the fluoresence emitting compound that can be detected as an increase in decay time τd of the autocorrelation curve. (top). When both molecules are labelled by different colors, binding gives rise to a great increase in the cross-correlation signal, independent on molecular weight, as depicted in the cross-correlation curves (bottom).

required in the detection pathway. In two-photon excitation DC-FCCS, the critical task is to find a system of suitable dyes that only exhibit minimal overlap of their emission spectra but also tolerate the same excitation intensities with comparably low photobleaching.[48]

Changes in molecular brightness and FCS

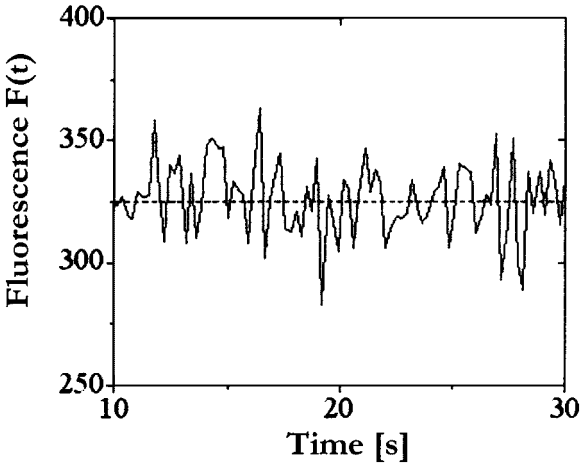
The need for a sizeable number of molecules to pass the focus limits the applicability of correlation analysis when measuring very rare targets. The impact of changing molecular-brightness parameters as an alternative means for FCS aggregation studies has been demonstrated.[49]When fluorescently labeled probe molecules aggregate, these aggregates can become highly fluorescent. As the unbound probe molecules are in excess (up to a factor of 10^6) compared to the sparse aggregates, the aggregates need to be separated from the unbound probe for accurate detection and quantification. When they reach the confocal detection volume, aggregates emit a fluorescent burst that can be orders of magnitude stronger than the signal of the free unbound probe. This fact allows aggregate and probe separation by their relative fluorescence intensities. An application of this principle to the detection of amyloid aggregates in the cerebrospinal fluid of patients with Alzheimer's disease has been reported.[50]-Based on this principle and using a dual-color scanning setup, an ultrasensitive quantitative detection method has been developed to detect pathological aggregates of prions at the femtomolar concentration range, which is sufficient to detect them in the cerebrospinal fluid in cases of the Creutzfeldt-Jakob disease.[51]

Concluding remarks

The extended availability of new generations of fluorescent dyes with high quantum yields and higher resistance to photobleaching, the generalization of use of green and other fluorescent proteins, the commercial availability of user-friendly FCS systems and the recent development of dual-color cross-correlation and coincidence schemes, as well as the expected impact of two-photon excitation DC-FCCS, warrant an extended "golden age" of FCS-based techniques with an ever-increasing number of specific applications in biological science research.

BOX 1: THE CORRELATION FUNCTION

In FCS, a small, geometrically defined light cavity is generated in the sample by confocal illumination. The fluctuations in the fluorescence signal within the illuminated small volume are recorded (see figure). From these measurements, the temporal average of fluorescence $\langle F(t)\rangle$ can be determined, and the fluorescence fluctuations δF, defined as the difference of the instantaneous fluorescence from the temporal average, $\delta F = F(t) - \langle F(t)\rangle$, can be calculated. The autocorrelation function (1) describes the normalized variance of the fluorescence fluctuations.



In FCCS, the fluorescence signals from two different fluorophores are recorded simultaneously, and the normalized fluctuation correlation function is given by

$$G_{ij}(\tau) = \langle \delta F_i(t)\ \delta F_j(t + \tau)\rangle \left[\langle F_i(t)\rangle \langle F_j(t)\rangle\right]$$

In which the fluctuations in the fluorescence signal of one fluorophore are correlated with those of the other fluorophore.

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