MINIREVIEW

A New Dimension for the Development of Fluorescence-Based Assays in Solution: From Physical Principles of FCS Detection to Biological Applications

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Ultrasensitive detection methods such as laser-induced fluorescence represent the current state-of-the-art in analytics. Single-molecule detection in solution has received a remarkable amount of attention in the last few years because of its applicability to life sciences. Studies have been performed on the fundamentals of the detection processes themselves and on some biological systems. Fluorescence correlation spectroscopy (FCS) is the link for ultrasensitive multicomponent analysis, showing possibilities for experiments on molecular interactions. Based on the theoretical background of FCS, this article gives full explanation of FCS and an update of highlights in experimental biology and medicine studied by FCS. We focus on a repertoire of diverse immunoglobulin specificities, a ribosome display system, single-molecule DNA sequencing, and a mutant enzyme generated by random mutagenesis of amino acids. We describe the usefulness and the enormous potential of the methodology. Further, this contribution clearly indicates that FCS is a valuable tool for solution-phase single-molecule (SPSM) experiments in immunobiology and medicine. In experiments with the Goodpasture autoantibody, we worked out conditions for the design of experiments on a complex single molecule in solution. The possibility to use SPSM-FCS as a quantitation methodology opens up other important applications beyond the scope of this article. Original results extending the published studies are presented for the rational foundation of SPSM-FCS. In this original contribution, we deal with experimental systems for biology and medicine where the number of molecules in solution is very small. This article is mandatory for gaining confidence in the interpretation of experimental SPSM-FCS results on the selfsame, individual single molecule in solution. [Exp Biol Med Vol. 227(5):291–300, 2002]

Key words: ultrasensitive detection of molecular interactions; confocal microscopy; conventional fluorescence correlation spectroscopy (FCS); conventional two-color cross-correlation FCS; single (solution)-phase single-molecule (SPSM) FCS; single molecules in solution; the selfsame single molecule in solution; immunofluorescence-based assays of individual single molecules; molecular diagnostics of individual molecules

A few hundred different cell types can be found in the human body. Wide variations and individuality within each type of cell are evident. For example, B cells of the immune system are clonal with the potential to produce about 10 million different antibodies. Their principal variability is even higher. The individuality of the antibodies is caused by the individuality of the DNA molecule. However, experimental immunobiology has not yet investigated individual properties. By contrast, it is assumed that bulk parameters represent those of the individual elements. The bulk parameters would give only sufficient information on the individual level if statistical laws such as Gaussian distribution could describe the variability.

In the last decade, innovative technical developments in different fields of optical spectroscopy and microscopy have increasingly been focusing on the detection of single ions, atoms, molecules, and supramolecular complexes (1). Until recently, only a few specialists had the opportunity to perform investigations on single molecules. The spatial, temporal, and spectral isolation of single molecules is still the main difficulties. In principle, single-molecule detection is performed by immobilized molecules or by measuring molecules in solution-phase one by one. Immobilization of a molecule allows its continued investigation provided the molecule survives the interaction with the optical detection

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system. In this way, slow phenomena can be studied. Single-molecule detection in solution is a laser-based, ultrasensitive detection by which individual molecules can be studied one by one. Since the first report of the detection of individual single-chromophore molecules in bulk aqueous solution (2, 3), there have been several achievements in experimental capabilities (4). These achievements have resulted in time-gated detection techniques, confocal microscopy with detection volumes in the femtoliter region, two-photon excited fluorescence, near-infrared fluorescence, and diode laser excitation in the red region (1). Confocal fluorescence correlation spectroscopy (FCS) is an exploding field for studying kinetics, dynamics, and conformational interactions (5). How this solution-phase, optical methodology can currently be used in ultrasensitive, immunological, and medical diagnostics will affect the change in molecular and immunological research on bulk parameters for years to come (5, 6, 38).

FCS is a solution-phase, optical methodology that is used to detect the random Brownian motion of fluorescent-labeled molecules. It monitors the time-dependent fluctuations in the fluorescence intensity from a very tiny subvolume of the solution of about 0.2 fl (0.2×10^{-15} liters), defined by the focal region of an excitation laser beam. FCS is an extremely important new methodology, as evidenced by a Med Search that listed over 1000 references on this topic. For example, there are recent papers on the FCS calculations of Brownian motion and the factors that influence the observed results (7). In addition, effects of the local environment on dye probes were investigated (8). In such sensitive methods as FCS, the key issue is the signal-to-noise ratio. In a recent study, an algorithm was presented that considers the essential features of calculating standard deviations in FCS (9). Multiple other studies were recently published presenting novel applications of FCS to confocal microscopy (10–12). Földes-Papp and Kinjo gave a comprehensive survey of DNA analytics performed by FCS (13).

In this contribution, we provide full explanation of FCS, including its conceptional basis with a few analytical (mathematical) expressions, which are necessary for gaining the relevant information from the data. For the reader who is not experienced with FCS, insights into the theoretical background are important for understanding the usefulness and the enormous potential of the methodology in experimental biology and medicine. Thus, we shall step from the conceptional viewpoint of FCS to some selected highlights of experimental biology and medicine that have been studied by FCS. We shall show molecular biological, immunological, and biophysical properties that can be analyzed in detail by FCS. As a quantitation method, FCS has opened up other important applications beyond the scope of this overview. The last two sections of the article extending the published studies have intended as an addition to a novel concept, which we call solution-phase single-molecule (SPSM) FCS (5). It is mandatory for gaining confidence in the interpretation of experimental FCS results on a complex single molecule in solution. The last two sections (SPSM-FCS and Measuring the Selframe Single Fluorescent Molecule by SPSM-FCS) first report theoretical results (37) on which some key papers (5, 6, 38) are based. However, before starting the explanation of modern FCS in view of its usefulness and broad applicability in experimental biology and medicine, the up-to-date FCS technology (FCS instrument) needs to be introduced.

Modern Technology

Nowadays, the commercially available instrument Confocor 2 (Zeiss, Jena, Germany) is used for performing biological experiments (Fig. 1). Confocor 2 is a PC-controlled fluorescence correlation spectrometer consisting of excitation lasers, an epifluorescence microscope, focusing and imaging optics, avalanche photodiodes (EG&G, MA) operating in the photon counting mode, electronics for controlling and steering the device, and computer interfacing for controlling the experiment, collecting, and analyzing the data. The instrument operates at five different excitation wavelengths: 458, 488, 514 (argon-ion laser), 543 (helium-neon laser 1), and 633 nm (helium-neon laser 2). Essential components of the optical setup are the excitation and the detection branches (Fig. 1). The excitation branch of the

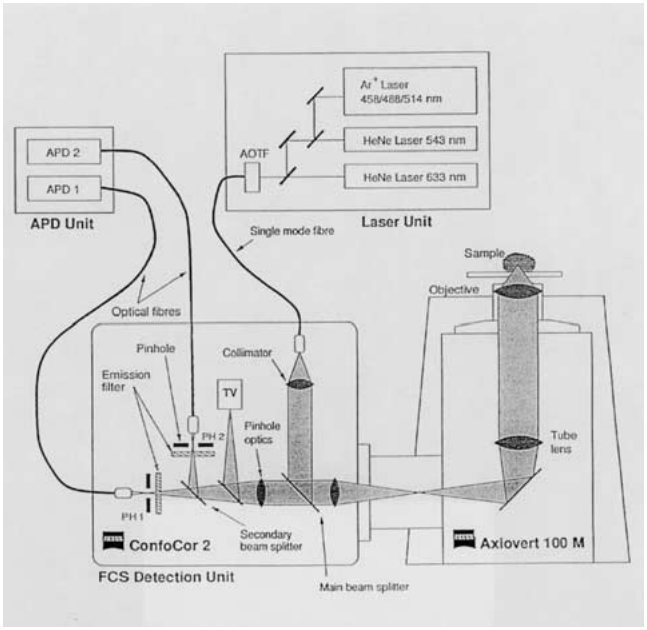

Figure 1. The optical setup of the modern FCS device ConfoCor2. An argon-ion laser (458, 488, or 514 nm) and two helium-neon lasers (543 and 633 nm) can be used for the excitation of the sample molecules by appropriate excitation filters. The main (dichroic) beam splitter reflects the excitation light toward the objective and transmits the fluorescence light originating from the sample. The laser beams are focused with epi-illumination optics to small spots in the sample solution. It is contained by a chambered cover glass. The solution is on the stage of an inverted microscope (Axiovert 100). The emitted fluorescence light is directed to pinholes and monitored by photodiodes. AOTF, acusto optical tunable filter; APD, avalanche photo diodes.

Confocor 2 LSM510 consists of fibers, beam collimation objective, excitation filter, e.g., main dichroic beam splitter, microscope objective, and cover glass. The detection branch consists of cover glass, microscope objective, e.g., main dichroic beam splitter, pinhole, additional lenses, e.g., second dichroic beam splitter, emission filter, focusing lenses, and silicon avalanche photodiodes. The fluorescence signal is measured in real time by a software correlator. The instrument settings of the Confocor 2 can be checked, changed, and properly adjusted via the interactive communication with the personal computer running under Windows NT (Microsoft, Redmond, WA). The structure of the program package is determined by the main functions such as choice of laser lines and their respective intensities, choice of filters, mirrors, beam splitters, pinhole settings and pinhole adjustment, as well as the measurement parameters and preset parameters for the data evaluation method. The Confocor 2 is a unique machine, also allowing the combination of FCS with laser scanning microscopy (LSM).

FCS has proved to be extremely successful in the measurements of molecular diffusion processes, chemical kinetics, and dynamics because of the introduction of the confocal detection volume (14–16) as shown in Figure 2.

Detection of Brownian Motion by FCS

Molecules (dyes, enzymes, immune complexes, etc.) are in a state of motion because of their temperature. There is a fundamental relationship between molecular motion and temperature. A particle of mass m and velocity v_x on the x coordinate has a kinetic energy associated with the motion of

$$\left\langle \frac{m \cdot v_x^2}{2} \right\rangle = \frac{k \cdot \Gamma}{2}, \qquad [1]$$

where k is Boltzmann's constant, Γ is the absolute temperature, and $< >$ denotes an average over time or over an ensemble of similar particles. Albert Einstein showed in 1905 that this is true for particles of any size (17, 18). We refer to this phenomenon as Brownian motion. The quantity $m \cdot v_x^2/2$ fluctuates, but to detect the fluctuations, one has to reduce the number of molecules. FCS measures the Brownian motion of fluorescent molecules. A fluorescent molecule passes through a laser focus by Brownian motion (Fig. 3). It is repeatedly cycled between the ground electronic state and the first excited electronic state. Thereby, the molecule emits photons, producing photon bursts. For the detection of the emitted photon bursts, it is mandatory to minimize the background noise due to scattering and luminescence. Because the background noise is directly proportional to the size of the detection volume, FCS utilizing subfemtoliterized probe regions (14–16) is ideally suited for the measurement of Brownian motion of fluorescent particles (Fig. 3).

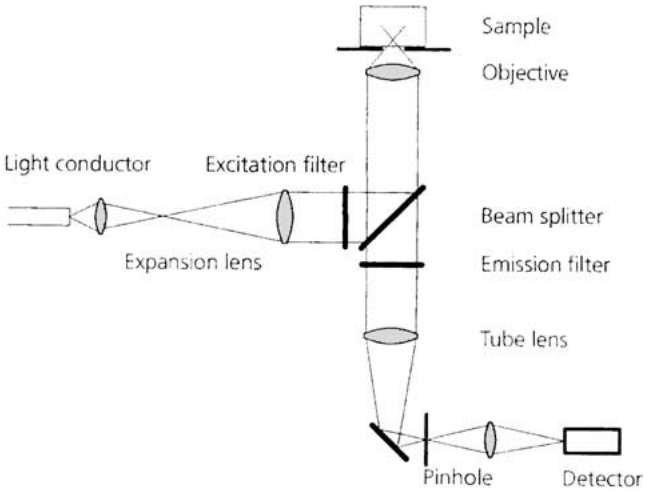

Figure 2. Main components (confocal arrangement) of the efficiency of light collection in FCS resulting in a femtoliterized detection volume within the sample first introduced in refs. (14, 15, 16). The detection branch of the optical system (emission-measuring system) is focused in the object plane. Fluorescence emissions are collected by the objective (numerical aperture 1.2) and passed through a beam splitter (dichroic mirror). The transmitted light is focused by a lens on the pinhole. Since the excitation and emission optical pathways are overlapping (confocal arrangement), out-of-focus emissions and scattered light are efficiently blocked by the pinhole. A pinhole size of 30, 50, or 70 nm is commonly used for the measurements. The emissions are focused by a lens and measured by an actively quenched avalanche photodiode (photon counting mode, SPCM 131-AQ, EG&G). The electronic output signals are autocorrelated and crosscorrelated, respectively.

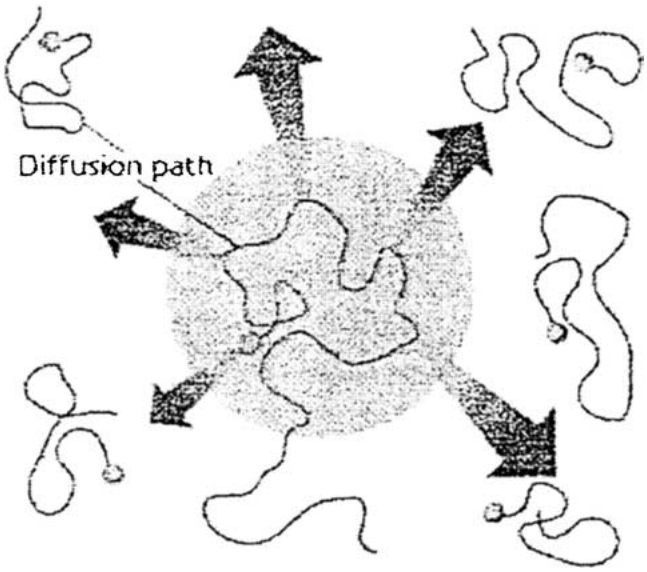

Figure 3. Probe region (volume of detection) in the x,y-plane. The focused laser beam is shown as an open cylinder. The axial distance (radius, ω) of the blue Gaussian beam profile was determined to be 0.177 μm. FCS detects the Brownian motion of fluorescent molecules. The Brownian motion is measured as fluctuations of the emitted fluorescence intensity. This is the principal difference compared with detection of mean fluorescence intensities (static fluorescence). Measurements can be performed in small volumes (down to 1 μl) within a short experimental time (1 to 60 s). During this time period the fluorescence intensity of molecules in the small volume element (0.2 femtoliter) is recorded and correlated in time. By using such a small measurement volume, background fluorescence can be minimized since contaminating fluorescent molecules will only rarely enter the measurement volume.

The FCS Experiment

To get a molecule to fluorescence you have to excite it. The excitation and emission spectra of the fluorophores must be compatible with the excitation wavelengths and the spectral separation of fluorescence emissions of the fluorescence correlation spectrometer used for the measurements. We are taking as labeling agents reporter dyes such as rhodamine green (excitation maximum at 504 nm, emission maximum at 532 nm), tetramethylrhodamine (excitation maximum at 546 nm, emission maximum at 576 nm), and Cy5 (excitation maximum at 650 nm, emission maximum at 670 nm). How does FCS work? A fluorescent-labeled molecule in solution emits photons as long as it moves through a laser focus (spot). The number of (electronic) detector pulses, originated from detected photons and recorded during a time interval T, corresponds to measured fluorescence intensity. A biochemical or biological system is fluctuating (see Equation [1]). The force of fluctuations is related to the Boltzmann energy ($k \cdot \Gamma$). The number of emitted photons per time depends on the number of molecules (bulk concentration), the diffusion time (mass of molecules), the quantum yield (fluorescence efficiency and property of dye), and the focus size (instrumental parameter). Having the relationship, we can directly read out the absolute mean number of molecules N in the detection volume (19, 20) of conventional FCS without any calibration or internal standard by the experimental and theoretical auto-correlation curve

$$G(\tau) = \frac{1}{N} \cdot \left[\frac{1}{\left(1 + \frac{\tau}{\tau_D}\right) \cdot \sqrt{1 + \left(\frac{\omega}{z}\right)^2 \cdot \frac{\tau}{\tau_D}}} \right] + 1, \qquad [2]$$

where τ_D is the characteristic translational diffusion time of the fluorescent molecules, $\omega_{x,y}$ is the radius (waist), and ω_z is the half-length of the elliptically shaped confocal volume element (see Fig. 4). Equation [2] can easily be expanded to three components (molecule species) occupying the volume element with the fractions y and z of fluorescent component 2 and 3 (see Fig. 5 for multicomponent analysis of two different species).

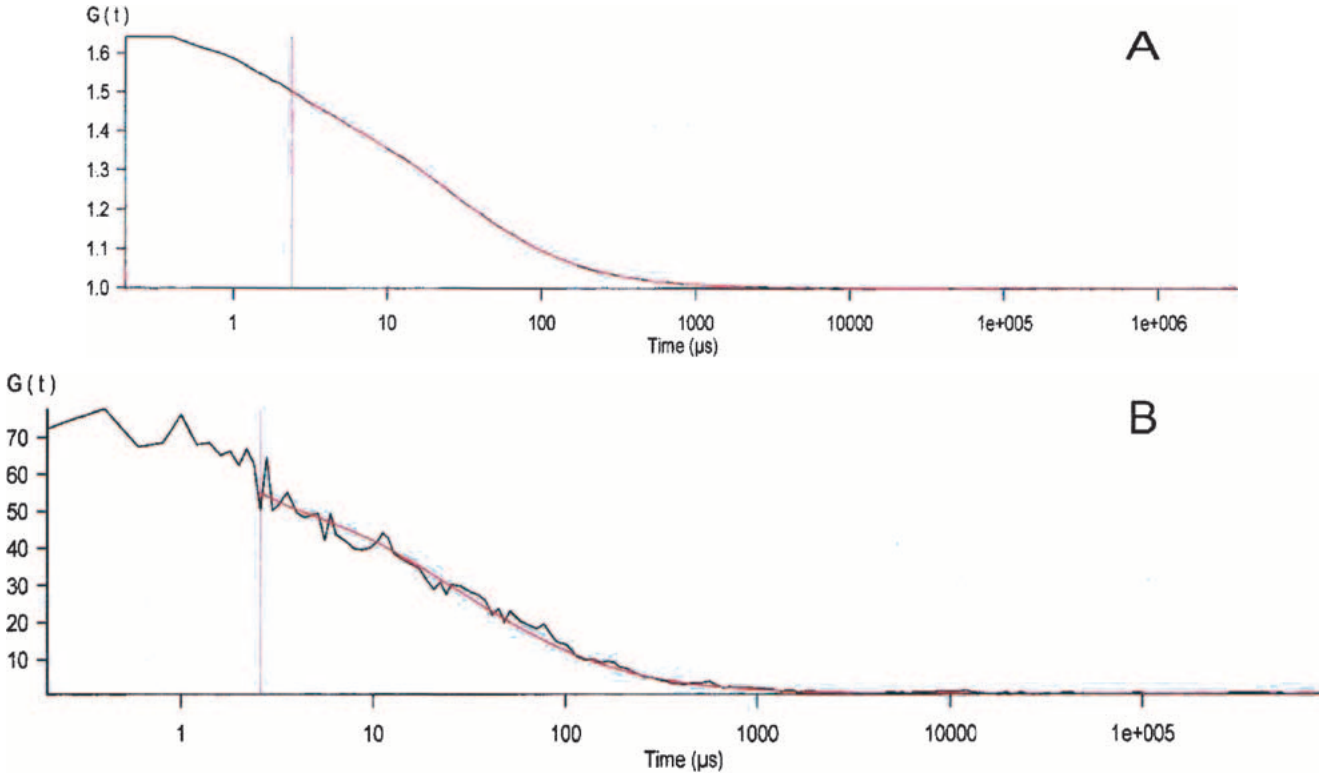

Figure 4. Results of the experimental auto-correlation function (black) for rhodamine green molecules in solution and Gaussian curve fit (red). The fit was performed with the Eq. [2] describing the three-dimensional Brownian motion of the particles (19, 20). Excitation was at 488 nm, and emission was measured at 535 nm (peak intensity value). The measurement time was 60 seconds. Calculating the auto-correlation function (red curve) reveals similar features over time of the fluctuating signal. The absolute particle number N of rhodamine green molecules can be directly obtained from the amplitude of the diffusion part of the correlation function indicated by a line (inverse amplitude). N is the average number of fluorescent molecules observed in the volume of detection. Other physical processes than diffusion are not considered here. **A:** Passage of 2 rhodamine green molecules on average. To correctly interpret the sensitivity level, it is necessary to measure, under the same excitation intensity, a highly concentrated solution of rhodamine-green. **B:** Passage of 0.018 rhodamine green molecules on average. For the first time, the correlation curve is interpreted in Poissonian probability terms (5, 37). The measured probability of finding a single molecule in the detection volume (unit volume) is here $N = 0.018$ (Poisson-distributed below a critical bulk concentration), where N is obtained using Eq. [2]. See section Solution-Phase Single-Molecule FCS (SPSM-FCS) for further explanations.

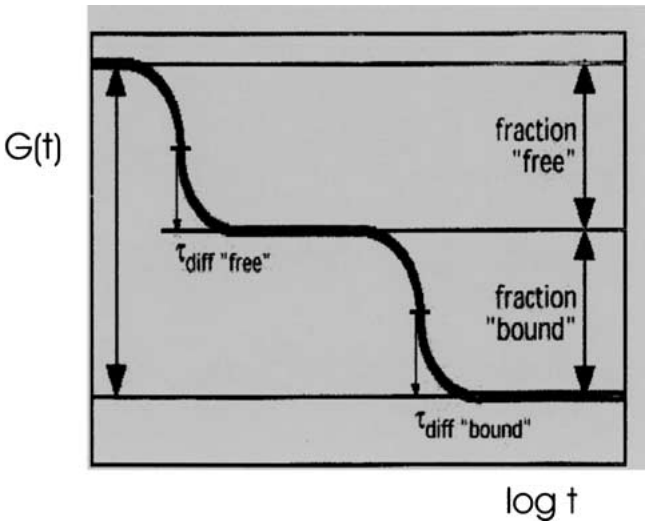

Figure 5. Schematic representation of conventional FCS data evaluation by means of the multicomponent auto-correlation function for two molecular species with the same fluorescence efficiency (quantum yield). The fluorescence intensity fluctuations arise from molecules of different size diffusing through the focus of the excitation laser. The 'individual' auto-correlation curves for the free species and the bound species are superimposed as shown in an idealized way. The different molecules (free and bound species) can be separated by their different diffusion times $\tau_{diff\ "free"}$ and $\tau_{diff\ "bound"}$. The total molecule number of species i is $N_{tot} = \Sigma_{i=1}^{i=n} N_i$ by which the diffusion term in Eq. [2] has to be multiplied. In our example of $i = 1$ and $i = 2$, we obtain from Eq. [2] an auto-correlation function with two diffusion times, one for species 1 (free) and one for species 2 (bound). Up to three species with different diffusion times can be resolved and their amounts quantitated using one fluorophore. Thus, the amount of ligand being the free species (fast diffusion) and the receptor-bound species (slow diffusion of the complex) can be quantitated and measured as a function of concentration and thereby, affinity and interaction kinetics can be determined. The striking feature is the short experimental time needed to quantitate the amount of free dye and functional complexes. Therefore, FCS is useful in high-throughput applications such as for quality control in automated selections or to quantitate the influence of various experimental conditions in optimization strategies.

In Figure 6, we show an example for interactions of a phage-displaced antibody fragment directed against the Hepatitis C virus E2 protein (21). We use two separate colors for the labeling of the phage-antigen complex. The green auto-correlation of the rhodamine green-labeled anti-phage antibody by itself allows the detection of all phages (Fig. 6A). By using the red auto-correlation of the Cy5-labeled recombinant E1/E2 protein, we observe the specific binding of antigen to phages (Fig. 6B). The FCS auto-correlation analysis of the absolute molecule number N in two separate colors (see Equation [2]) shows that about 70% of the phage population is inactive with respect to the binding of the cognate antigen. The FCS auto-correlation analysis in two colors offers a great advantage in a safer detection system differentiating target organisms from inactive "background" populations.

Interactions of a single-chain antibody fragment (scFv) with its cognate antigen while still attached to the ribosome were studied by conventional FCS (22). In experiments with purified scFv, conventional FCS was capable of resolving

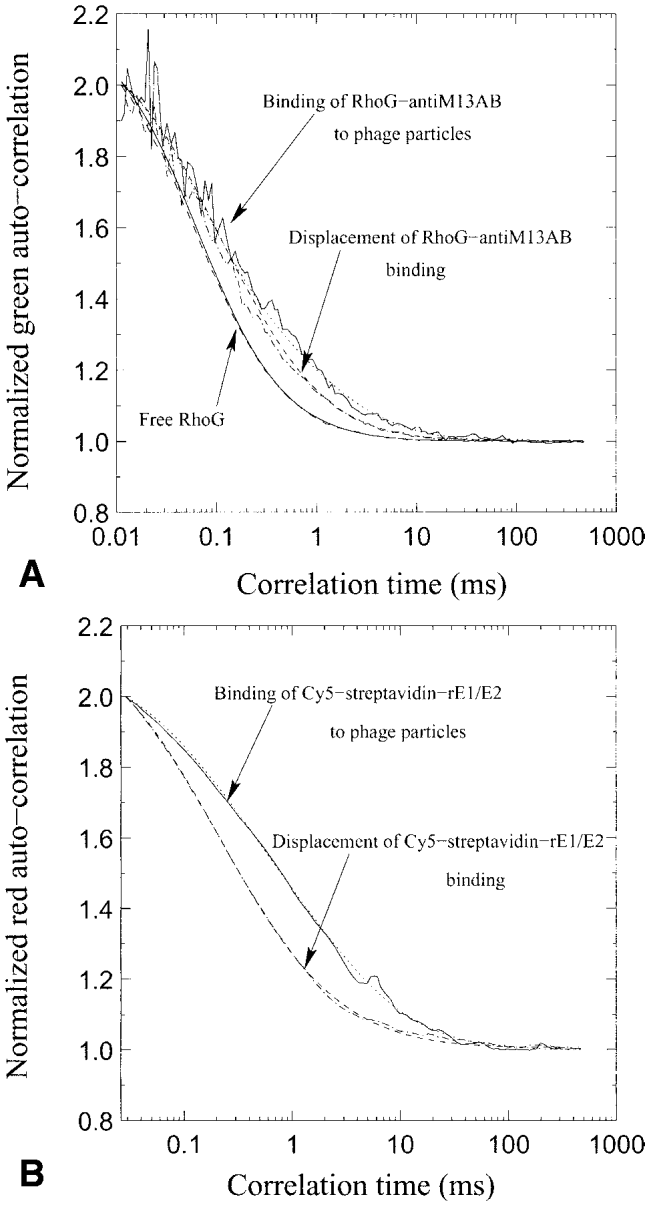

Figure 6. In FCS a mixture of soluble fluorescent molecules with different molecular weights can be analyzed by a single measurement. FCS auto-correlation analysis is shown for free rhodamine green, binding of rhodamine green anti-M13 Ab's to phages, displacement using free unlabeled ant-M13 Ab's **(A),** and for interactions between phages and recombinant E1/E2 protein **(B).** The biotinylated recombinant E1/E2 was tagged with Cy5-streptavidin. The displacement was performed using free, untagged antigen. For experimental detail, see ref (21). The fluorescence fluctuations in the measurement volume are due to the variations in concentration of the fluorescent dye and dye-tagged complexes by Brownian diffusion. From the autocorrelation function, the absolute, average number of molecules and their respective diffusion coefficients were calculated. The preparations of the labeled compounds contained approximately 25–40% free rhodamine green dye and Cy5 dye, respectively. The diffusion times of the free dyes were therefore included in all data fittings. However, higher purity of labeled compounds can significantly improve the statistics of FCS analysis. Further purification steps are necessary to reduce the background noise and to increase the sensitivity of a FCS-based assay in solution-phase.

the difference in diffusion time between free and bound labeled antigen. Ribosome-displayed antibody fragments generated by *in vitro* translation, in which neither the protein nor the mRNA leaves the ribosome due to the absence of a stop codon and stabilizing buffer conditions, could be shown to specifically bind the antigen. The antibody-antigen interaction was specific as shown by inhibition or displacement with unlabelled antigen and by control experiments with a non-cognate antibody fragment. The diffusion time of the ternary ribosomal complex (mRNA-ribosome-displayed protein) was determined to 1.2 msec with a hydrodynamic radius of 17.3 nm.

Additionally, it was found that triplet state kinetics could be conveniently measured by FCS (23, 24). The high environmental sensitivity of the triplet state parameters indicates that FCS can be used to probe molecular microenvironments. The total amount and rate of fluorescence emitted per molecule are important parameters determining the photophysics.

Two-Color Cross-Correlation FCS

Conventional two-color cross-correlation FCS is a way to coincidently measure fluorescence intensity fluctuations in two colors. We consider here two fluorescent labels with, for example, green (g) and red (r) emission maximum, which are excited within two focused, stationary laser beams. In this way, we were able to distinguish between the specific interactions caused by molecular species carrying both colors and those interactions obtained from molecular species containing only one color, for example in the Goodpasture experiment (5). The optimal approach to data analysis is the two-color cross-correlation function. The all-around form of the two-color cross-correlation function is the same as that one of Equation [2], but τ_D has the meaning of the crosscorrelated (weighted) diffusion time with $\tau_D = \tau_{gr} = \omega_g^2 + \omega_r^2 / 8D = \tau_g + \tau_r / 2$ and ω as well as z are the half axes of the cross-correlated (green and red overlapping) volume of detection (25). Further, in the general case that n green and m red labels are incorporated or bound to the same target, the two-color cross-correlation function (for its all-around form, see Equation [2]) has to be expanded to

$$\frac{1}{N} = \frac{N_g \cdot \left(\frac{Q_B^R}{Q_R^R}\right) + N_{gr} \cdot R \cdot \left(n \cdot \left(n \cdot \frac{Q_B^R}{Q_R^R} + m\right)\right)}{[N_g + N_{gr} \cdot R_g \cdot n] \cdot \left[N_r + N_g \cdot \left(\frac{Q_B^R}{Q_R^R}\right) + N_{gr} \cdot R_r \cdot \left(n \cdot \frac{Q_B^R}{Q_R^R} + m\right)\right]} \quad [3]$$

where N_{gr} is the absolute mean number of two-color molecules in the detection volume (26). The parameter $Q_{\lambda_{ex}}^{\lambda_{em}}$ stands for the relative fluorescence detection efficiencies of the free dyes at different excitation and emission wavelengths. We express $Q_{\lambda_{ex}}^{\lambda_{em}}$ as the relative photon counts per molecule and per second (27). Since photons of the green emitted signals (green fluorophores) can be detected in the red channel (crosstalk), Equation [3] also gives the relative contribution of the crosstalk (spilling over) Q_B^R in one direction (from the green channel to the red channel) on $G(\tau)$ at the limit $\tau = 0$; the other case Q_R^G (red to green) can be neglected (27). This occurs no matter how excellent the properties of the optical components are. In our optical setup, we measure here for rhodamine green (1 nM) $Q_B^G = 1.0$ and $Q_B^R = 0.045$ (crosstalk), and for Cy5-dCTP (0.1 nM) $Q_R^R = 2.19$ and $Q_R^G = 0$ (crosstalk). The absolute emission rate in photon counts per molecule and per second is 67×10^3 for rhodamine green. N_g is the number of free unbound green molecules, and N_r is the number of free unbound red molecules. The important parameter R describes the relative quantum yield difference between bound and free labels; it is defined by $R_g = Q_{B,bound}^G / Q_{B,free}^G$, $R_r = Q_{R,bound}^R / Q_{R,free}^R$, and $R = R_g \cdot R_r$ (26). Taken together, if we change the number of two-color molecules in the sample, for example, by the kinetics of the biochemical or immunological process under study, we come to a situation where we cannot neglect the molecules carrying only one color (see Equation [3]). We realize that N is the number of two-color molecules ($N = N_{gr}$) only if there is a large excess of two-color molecules over molecules containing one color; this, of course, is a very special and restricted case. Detailed considerations of these problems have been presented (25–29).

Two-color cross-correlation FCS was advantageously used to characterize a new type of DNA for single-molecule DNA sequencing that is revolutionizing molecular biology and medicine (29, 30). We developed a labeling and analysis strategy for high-density labeling of DNA (29, 31). High-density labeling of DNA by amplification reactions or by filling-up reaction requires suitable dye-tagged dNTPs and an appropriate DNA polymerase, which we selected for this type of labeling (31). The polymerase found is a thermostable $3' \rightarrow 5'$ exonuclease-deficient Tgo DNA polymerase. We gave the proof of principle that the naturally occurring nucleoside monophosphate dTMP was totally replaced in the DNA by its respective fluorescent analog rhodamine green-X-dUMP. In our approach, the two-color fluorescence cross-correlation resulted from fluorescence intensity fluctuations for which n green labels and one red label were incorporated into the same target molecule.

Furthermore, the green and red cross-correlated fluorescence signal can emerge from the background of nonincorporated or non-specifically incorporated 5′-tagged primers (26, 27). 5′-tagged amplification primers are an example

for interactions of two ligands with different colors at the same target.

A main goal of studying protein function is to understand the structural basis of enzyme specificity. Ribonuclease T1 is an ideal model for doing this. Phage display technology offers the possibility of combining phenotype and genotype of a protein. The RNAse T1 was displayed on the surface of the phage M13 (32). A gapped heteroduplex of a ribonucleotide and two smaller desoxyribonucleotides was used as substrate for the phage-displayed RNAse T1 (32). The heteroduplex was labeled with rhodamine green and Cy5. The time course of the substrate hydrolysis was followed in solution by the drop in the amplitude of the cross-correlated signal. The analysis strategy to prove (33) and to screen (34) for enzymatic reactions was first developed by others (33–35).

SPSM-FCS

This section extending the published studies is intended as an addition to a novel approach. The rational foundation of SPSM-FCS is first presented. It is mandatory for gaining confidence in the interpretation of experimental FCS results on a complex single molecule in solution. Formulating a problem mathematically is illuminating for two reasons. First, it is the way of discovering the various kinds of effects. Second, it suggests what needs to be measured.

Our novel methodology of SPSM-FCS is based on conventional FCS or conventional two-color cross-correlation FCS *and* Poisson distribution analysis depending upon a critical bulk concentration of a fluorescent compound in solution-phase at room temperature. A single-molecule event is proven without burst analysis of the intensity traces and without immobilization. We presented a procedure for dealing with systems where the number of molecules is small enough that (i) random number fluctuations are observable, and (ii) the random number fluctuations make it possible to assign, in a submicroscopic region (unit volume of detection) of a bulk solution, a probabilistic value to such a property (5, 6, 38). Our analysis is a simple but profound one (5). We say that if, in the absence of a macroscopic concentration gradient, the specific fluorescent molecules tend to a stable uniform steady state in the bulk solution, then the numbers of the molecules (events) are Poisson-distributed in the unit volume of detection as follows. We record the absolute number of events X occurring in an interval of time T (infinite number of periods of T units of time) by means of FCS. The events (x) happen with a constant detection probability P per unit time. Now the probability that an event does not take place in the interval T is

$$P(X > x) = 1 - \int_0^T \underbrace{P \cdot e^{P \cdot t}}_{\substack{\text{probability that} \\ \text{an event occurs} \\ \text{between } t+dt}} dt = 1 - (1 - e^{-P \cdot T}) = e^{-P \cdot T}. \quad [4]$$

The probability that one event takes place in the interval T (measurement time) is

$$P(X = 1) = \int_0^T \underbrace{P \cdot e^{-P \cdot t_0}}_{\substack{\text{probability that} \\ \text{one event occurs} \\ \text{between } t_0 + dt_0}} \cdot \underbrace{e^{-P \cdot (T - t_0)}}_{\substack{\text{probability that an} \\ \text{event does not occur} \\ \text{in the remaining part} \\ \text{of the interval } T - t_0}} dt_0 = P \cdot T \cdot e^{-P \cdot T}. \quad [5]$$

The probability that two events take place in the interval T is

$$P(X = 2) = \int_0^T P \cdot e^{-P \cdot t_0} \cdot \underbrace{P \cdot (T - t_0) \cdot e^{-P \cdot (T - t_0)} dt_0}_{\substack{\text{probability that an event} \\ \text{occurs in the remaining} \\ \text{part of the interval } T - t_0}} = \frac{(P \cdot T)^2}{2} \cdot e^{-P \cdot T}. \quad [6]$$

Finally, we represent the SPSM-FCS experiment by a probability ("observable") that x events take place in the interval T

$$P(X \le x; P \cdot T) = \int_0^T P \cdot e^{-P \cdot t_0} \cdot \frac{1}{(x-1)!} \cdot P^{x-1} \cdot (T - t_0)^{x-1} \cdot e^{-P \cdot (T - t_0)} dt_0 = \frac{(P \cdot T)^x}{x!} \cdot e^{-P \cdot T}. \quad [7]$$

We describe the SPSM-FCS experiment (5) by a system of integral equations. $P \cdot T$ is the average number of specific fluorescent molecules in the unit volume of detection, meaning the mean value of x. x represents the "actual" number of specific fluorescent molecules in the unit volume. The value x is always a small integer, for example, $x = 1$ in Figure 3. Now, we use C to denote the (true) mean value of the population of specific fluorescent molecules (the average number) and summarize our approach of SPSM-FCS by

$$P(X \le x; C) = \frac{C^x \cdot e^{-C}}{x!}. \quad [8]$$

For the first time, we have established this condensed, time-averaged Poisson-distributed relationship (Equation [8]). The analytical relationship of Equations [7] and [8] is between the bulk concentration of fluorescent molecules in solution $c = C/(N_A \cdot V)$ and the measurable probability $P(X = 1; C) = N$, where N is the measured absolute number of specific fluorescent molecules in the detection volume below a critical bulk concentration c^* of specific fluorescent molecules (5). N_A is Avogadro's number with 6.022×10^{23} M^{-1}, and V is the confocal volume of detection (unit volume). The proof of our approach was presented by means of experiments with the fluorophore rhodamine green

(see Table 1 in Ref. (5)). In other words, the correlation functions do not only state the probability that a molecule emits a photon at a time τ given an emission at time zero from the same molecule as known hitherto (36). Below a critical bulk concentration, they are analytical expressions for the probability to detect a single molecule in the detection volume as proved. Thus, our remaining efforts are devoted to measuring $N = P(X = 1; C)$, meaning the probability to find a single molecule in solution (see Fig. 4B).

In Figure 7A, circulating Goodpasture autoantibodies were quantified in cross-correlation mode by binding to the rhodamine green-labeled antigen and a secondary antibody tagged with the Cy5. We measured an averaged, absolute number of the complexed autoantibodies of $N = 0.125$. The absolute number was related to the average frequency C that the volume of detection contained a single complexed two-color immune molecule with $C = 0.146$ (see Equation [8] and Ref. (5)). For this case, the probability that the confocal volume element contained one single complexed molecule was 99.1% (see Equation [8]: $P(X = 2; C) = P_2 = 9.09 \cdot 10^{-3}$). It was possible to distinguish between the specific binding interactions of the sandwiched immune complex carrying both colors in the same molecule (Fig. 7A) and the crosstalk caused by rhodamine green-labeled antigen and free rhodamine green of the preparation in the same compartment (Fig. 7B).

Measuring the Selfsame Single Fluorescent Molecule by SPSM-FCS

In SPSM-FCS, the single-molecule event can be caused by measuring different fluorescent molecules one by one or by measuring the selfsame single fluorescent molecule only. For the first time, we developed the theoretical and experimental framework for quantifying a *selfsame* fluorescence-tagged biological molecule under various experimental conditions in solution without immobilization (see Equation [9] and Ref. (37))

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - \frac{N}{2 \cdot \pi} \cdot \exp\left\{-\frac{\xi^2}{4 \cdot D \cdot T}\right\}. \qquad [9]$$

All quantities on the right hand side of Equation [9] can be measured by the SPSM-FCS experiment because they are directly related to the time-dependent fluorescence intensity fluctuation of the molecular species in the observation vol-

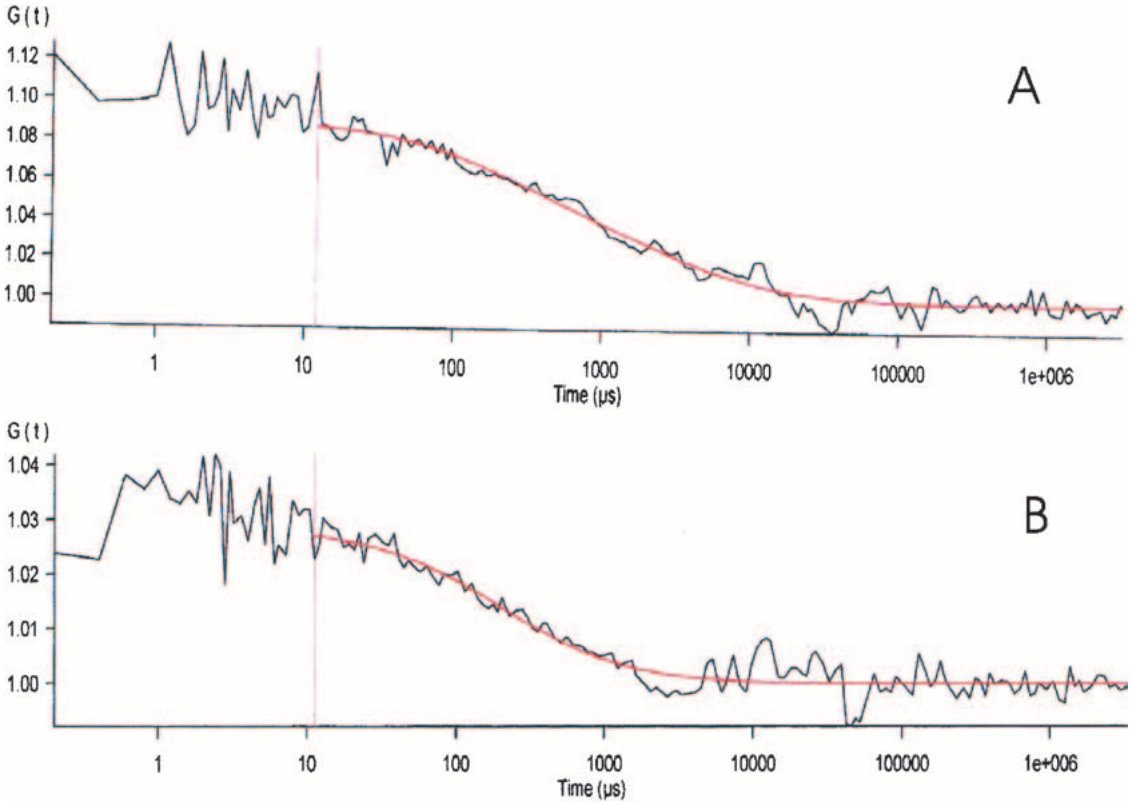

Figure 7. Two-color cross-correlation FCS of the Goodpasture autoantibody in patient serum (5). The measured curves are depicted in black. The red curves are the two-color crosscorrelated fluorescence signals of the form of Eq. [2]. **A:** The cross-correlated fluorescence signals of the huge two-color complex are superimposed by the unspecific cross talk of the free dye rhodamine green and the free rhodamine green-tagged antigen in the preparation which can be corrected for (see **B**). In the cross-correlation mode we determined $N_{gr} = 0.138$ for the huge two-color immune complex. This is about the same absolute number of the autoantibody complex determined in the red auto-correlations of $N_{r,bound} = 0.111$ with a relative fraction of 82.7% compared to 17.3% of free Cy5. **B:** The control serum of a healthy person who has no Goodpasture syndrome. The crosscorrelated fluorescence signals reflect the unspecific cross talk of the free dye rhodamine green and the free rhodamine green-tagged antigen. The results of SPSM-FCS analysis exemplified by the two-color immune complex demonstrate that this new type of analysis is a powerful and elegant way for identifying, quantifying and studying individual single target molecules in solution-phase without any immobilization (5, 6, 37, 38).

ume (37). Here, the novel quantity $P\,(\overline{\cap_{i=1}^{2} A_i})$ stands for the probability function of the complement of the independent events A of (i) finding a single fluorescent molecule (A_1) in the confocal volume of observation and of (ii) moving away (A_2) a lower limit of distance ξ out of the focal plane by this single fluorescent molecule (37). ξ is experimentally given by the measured dimensions of the femtoliterized observation volume. T is the measurement time of the SPSM-experiment, D is the measured diffusion coefficient of the molecule, and N is the observable (averaged) absolute number of fluorescent molecules in the observation volume below a critical bulk concentration of the fluorescent molecules in the solution. Our novel quantity (left hand side of Equation [9]) represents the probability of measuring the individual selfsame single fluorescent molecule in solution within a lower limit of distance ξ, which is given by the measured dimensions of the detection volume of the excitation laser beams (37). We formulated and solved the problem of distinguishing between different fluorescent molecules one by one and the individual selfsame single fluorescent molecule in the stochastic SPSM-FCS experiment (37). This represents a major breakthrough in quantifying stochastic SPSM-FCS experiments, and it opens up a new field of promising analyses, for example, in clinical immunology.

Increasing needs for more sensitivity and the detection of single-molecule activity are intriguing fields in the development of modern immunology. The qualities of sensitivity and single-molecule activity are goals for functional and structural characterization of immune reactions. Immunological reactions are not fundamentally different from other chemical processes. They obey physical and chemical laws, and they enable us to study live processes at the molecular level now (6). Single-molecule conformational dynamics, single-molecule catalysis, single-molecule protein folding, and cell surface single-molecule studies are fields of single-molecule detection in life sciences. The studies are performed on simple molecules or simple biomolecules serving as models. However, single-molecule analysis can occur in two distinct ways, one in which the molecules pass through the detection volume one by one, and another one in which the molecule does not leave the detection volume. We first developed the novel theoretical approach that allows one to visualize and quantify the selfsame immune complex in solution at room temperature without immobilization (37). This is the first time that a selfsame individual molecule of an immune complex is assayed in solution (6). The novel stochastic concept was first derived and its main characteristics were demonstrated using (i) the free fluorophore rhodamine green for simpler experimental realization and (ii) the free large two-color compound of an immune complex. The immunoassay is carried out in serum of a patient with Goodpasture syndrome. All we need is the experimental determination of the Poisson probability for the absolute number of fluorescent molecules in the detection volume by Equation [8] depending upon a critical bulk concentration. Individual events can be studied by their molecular interactions with the selfsame single, large, and complex molecule from healthy and sick people. The advantages of the presented novel theory over current procedures used in single-molecule analysis are discussed in Reference (6). Furthermore, in a very real way, we mathematically prove that the apparent velocity of molecular interactions for the reaction coordinate of a *selfsame* single fluorescent molecule in the observation volume of a liquid phase is given by the expression for the transition probability of a generalized continuous Markovian process (37).

Concluding Remarks

Life scientists should be exposed to FCS for two reasons. First, FCS-based assays are capable of detecting single fluorescent molecules. These types of assays are based on recording the Brownian motion of molecules. Second, life scientists get much more out of the information in FCS studies if they are familiar with the theoretical (mathematical) and experimental basis for the facts. In addition, the history of FCS from physical principles to biological applications makes a good story that leads one's thinking in new directions. The analysis of the behavior of single complex molecules is a growing field devoted to detailed understanding of the molecular mechanisms of biological and immune processes. FCS can contribute to these new research lines as shown in the article.

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