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Authors

Müller, Joachim D
Chen, Yan
Gratton, Enrico

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could be genetically targeted to specific sites in cells through transfection of cells with constructs that contain appropriate signaling sequences and also code for SHG-enhancing proteins.

Acknowledgments

[4] Fluorescence Correlation Spectroscopy

By Joachim D. Müller, Yan Chen, and Enrico Gratton

Introduction

Spontaneous, microscopic fluctuations are an integral part of every fluorescence measurement and add a noise component to the observed fluorescence signal. Fluorescence correlation spectroscopy (FCS) extracts information from this noise and characterizes the kinetic processes that are responsible for the signal fluctuations. For instance, the dynamic equilibrium between a fluorescent and a nonfluorescent state of a fluorophore introduces fluctuations. Another example is Brownian motion, which leads to the stochastic appearance and disappearance of fluorescent molecules in a small observation volume. FCS characterizes any kinetic process that leads to changes in the fluorescence, because the spontaneous fluctuations at thermodynamic equilibrium are governed by the same laws that describe the kinetic relaxation of a system to equilibrium. Thus, FCS offers a very convenient method for determining kinetic properties at equilibrium without requiring a physical perturbation of the sample. This is especially important for systems in which the use of perturbation techniques in extremely difficult and challenging, such as measurements in living cells.

The concept of measuring signal fluctuations has direct consequences for the experimental realization of FCS. Statistics tells us that the relative fluctuation amplitude of a signal is inversely proportional to the number of molecules simultaneously measured. Thus, the presence of a large number of molecules, as typically encountered in the macroscopic world, suppresses the effects of fluctuations and

only the ensemble average is observed. This simple statistical argument illustrates that FCS measurements require signals from a single or a very small number of molecules in order not to mask signal fluctuations. Although the concept of FCS was presented in 1972,[1] the technical challenge of single molecule detection has been overcome only in the past decade. The most important contributions are the development of stable laser light sources, the availability of photodetectors with high sensitivity, and most importantly the introduction of new microscope techniques.

The development of single molecule sensitivity has been linked to the use of optical microscopes for two reasons. First, the microscope is a very efficient optical instrument. High numerical aperture (NA) objectives capture a significant fraction of the fluorescent light that is emitted randomly in all spatial directions. Second, the microscope optics allows the generation of very small observation volumes. High numerical aperture objective focus an incoming laser beam down to a diffraction-limited spot. The radial size of the diffraction-limited spot is determined by the laws of optics, but is given in good approximation by the wavelength of the light. Although the radial dimension of the incoming light is confined by the optics of the objective, the axial direction of the light is not confined. The light leaving the focusing optics of the objective describes the shape of a double cone and fluorescence is excited everywhere within the double cone of light. Optical tricks are used to confine the light in the axial direction. In a confocal microscope a pinhole is placed in front of the detector to block fluorescence light coming from sections other than the focal plane (Fig. 1).[2] In two-photon microscopy a nonlinear optical phenomenon is used to reduce the excitation of fluorescence to the focal region.[3] A molecule that is excited with visible light at 400 nm is also excited by the simultaneous absorption of two photons of near-infrared light at 800 nm. In both cases the excited molecule returns to the ground state via emission of fluorescence (Fig. 2). Because two individual photons need to be absorbed at the location of the fluorophore, the probability of excitation is proportional to the square of the photon flux. The objective focuses the laser light and the photon flux (or intensity) is highest in the focal region. Because of the quadratic intensity dependence the two-photon excitation is restricted to the focal region and drops off drastically outside of the focal region. Consequently, no pinhole before the detector is needed for two-photon FCS, because the excitation of fluorophores is limited to the focal region by the inherent optical sectioning effect of two-photon absorption.

The optical observation volume generated by confocal and two-photon microscopy is less than 1 fl. Conventional, cuvette-based fluorescence instrumentation employs observation volumes on the order of 10 μl. The reduction of the illuminated volume by about 10 orders of magnitude drastically reduces the number

[1] D. Magde, E. Elson, and W. W. Webb, *Phys. Rev. Lett.* **29,** 705 (1972).

[2] J. B. Pawley, ed., "Handbook of Biological Confocal Microscopy." Plenum Press, New York, 1995.

[3] W. Denk, J. H. Strickler, and W. W. Webb, *Science* **248,** 73 (1990).
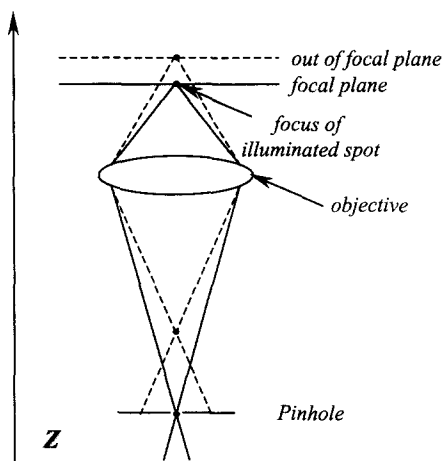
FIG. 1. Illustration of the confocal principle. Fluorescence that emerges from the focus of the illuminated spot in the sample is collected by the objective and passes through the pinhole. Fluorescence light that is excited outside of the focal region is also collected by the objective, but cannot pass through the pinhole.
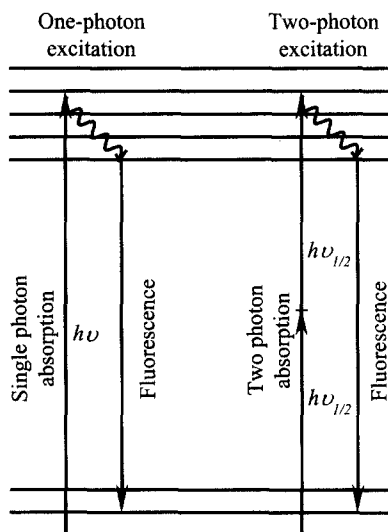
FIG. 2. Jablonski diagram of one-photon versus two-photon excitation. In one-photon excitation the absorption of a single photon of energy $h\upsilon$ promotes the molecule from the electronic ground state into an excited state. In two-photon excitation two photons of half the energy, $h\upsilon/2$, are absorbed simultaneously to reach the excited state. The molecule returns in both cases via emission of fluorescence to its ground state.

of molecules present in the observation volume. A concentration of 1 nM corresponds to an average of less than one molecule in the volume generated by the microscope. In addition, the subfemtoliter observation volumes efficiently suppress background signal from the bulk of the sample. Due to the introduction of these technological innovations, FCS measurements are now routinely performed on biological samples with single-molecule sensitivity.

Instrumentation

FCS instruments are commercially available from a number of companies, but it is relatively straightforward to build your own instrument. A typical setup of an FCS instrument is shown in Fig. 3. The light of a commercial laser source passes through a beam expander, is reflected by a dichroic mirror, passes through the objective, and is focused onto the sample. The fluorescence excited by the laser light is collected by the same objective. The dichroic mirror transmits the fluorescence signal and thereby separates the excitation light from the fluorescence of the sample. A barrier filter is added to suppress additional scattered light from the laser. The tube lens of
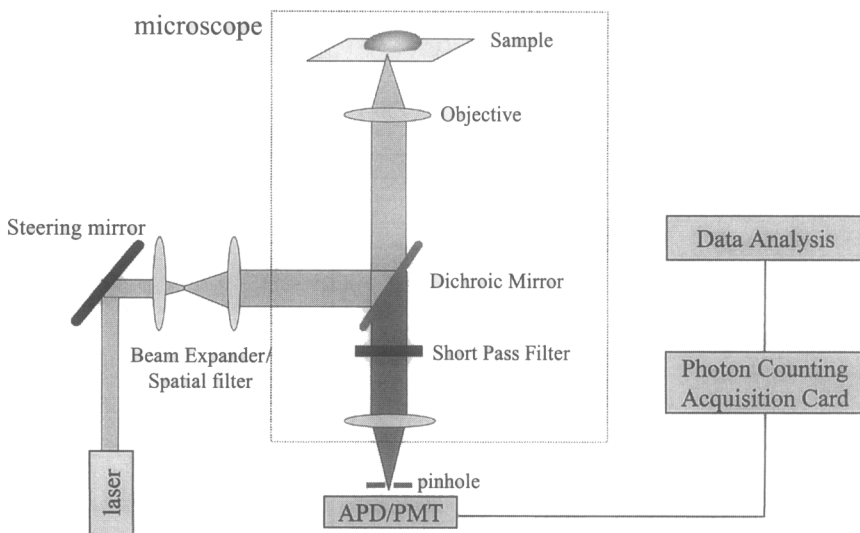
FIG. 3. Schematic diagram of an FCS instrument. The laser light passes through a beam expander, reflects on the dichroic mirror, and is focused by the objective into the sample. The fluorescence light excited in the sample is collected by the objective and passes through the dichroic mirror. The microscope tube lens focuses the fluorescence light onto a photodetector. A pinhole is placed in front of the detector for confocal FCS. Two-photon FCS does not require a pinhole. An electronic data acquisition board processes the signal from the photodetector and a computer is used to analyze the FCS data.

the microscope focuses the fluorescence light. A pinhole is placed at the location of the focus of the fluorescence light in the confocal arrangement to suppress out of focal signal contributions. The photodetector is placed directly behind the pinhole and converts the impinging fluorescent light into electronic pulses that are fed to a data acquisition board. The board works together with computer software to calculate and display the autocorrelation function. In a two-photon microscope the pinhole is not needed, because the fluorescence light originates only from the focal spot of the microscope.

The individual components of the FCS instrument will be considered next. The main requirement for the laser source is the stability of its intensity. Otherwise the laser light introduces intensity fluctuations, which cannot be separated from the fluorescence fluctuations caused by the sample. A Gaussian shape of the laser beam is recommended, because it leads to the smallest focus, which is limited only by diffraction. One-photon excitation with confocal FCS requires very little laser power; less than 1 mW of power at the sample is sufficient. Argon ion and HeNe (helium) lasers are widely used for confocal FCS. Two-photon FCS requires lasers in the near-infrared with ultrashort pulses and a high repetition frequency. The short pulses bunch photons together temporally and create at the time of the pulse an enormously high flux of photons that is required for efficient two-photon absorption. Although a number of different laser sources are available for two- and multiphoton excitation spectroscopy, almost every two-photon system uses the titanium–sapphire laser. It provides wide wavelength tunability (700–1000 nm), high average power (about 1 W), high repetition frequency (80 MHz), and short pulse width ($\approx$100 fs). Titanium–sapphire lasers have good intensity stability and are essentially turnkey systems.

Any research-grade biological microscope can serve as the body of an FCS instrument. The back aperture of the microscope objective needs to be overfilled to get the best optical performance. However, the beam diameter of most lasers is only about 1 mm wide. To overfill the back aperture, the laser beam diameter is magnified by passing through a beam expander. A spatial filter can be used as a beam expander as well and has the additional advantage that the beam is cleaned up. Dichroic mirrors and barrier filters need to be considered for each experiment separately and are available from many commercial sources.

The microscope manufacturers offer a wide range of objectives. Most FCS experiments require a high NA objective for good signal statistics. Chromatic corrections are important for FCS studies. This is particularly true for two-photon FCS, in which the wave lengths of the excitation light and the fluorescence are separated by more than 100 nm. Because FCS measurements are performed on the optical axis, field flatness is less of a concern. However, every objective behaves differently and the experimenter should characterize its performance. Pinholes are available in many different sizes and the optimal pinhole size for confocal measurements has been discussed in the literature.[2] Avalanche photodiodes (APD) and

photomultipliers (PMT) are the detectors of choice for FCS experiments. APDs have a higher quantum yield in the visible than photomultipliers and their sensitivity extends to the near infrared. However, the sensitivity of the APD drops off drastically in the blue part of the spectrum. Here, photomultipliers perform better than APDs. Two types of data acquisition cards are available for processing the detected photon counts from FCS experiments. Traditional boards take the signal from the detector and calculate the autocorrelation function electronically on board. Today these boards are all based on the multiple-tau(τ) correlator design, which calculates the autocorrelation function for evenly logarithmic spaced sampling frequencies.[4] A disadvantage of these data acquisition schemes is that they provide access only to the autocorrelation function and not to the complete time sequence of photon counts. Electronic cards that provide a time-stamp for every photon event represent a different data acquisition strategy and are commercially available.[5] The autocorrelation function is subsequently determined from the recorded photon events by software. Computers today are fast enough to calculate the autocorrelation online. The advantage of this approach is that the complete sequence of photon events is available, which allows new and different analysis techniques on the raw data, such as moment analysis, higher order correlation functions, and histogram analysis.[6–8]

Autocorrelation Function

Fluctuation measurements require statistical analysis methods to extract the information hidden in the data. FCS uses the autocorrelation function $g(\tau)$ of the fluorescence signal to analyze the intensity fluctuations,

$$g(\tau) = \frac{\langle F(t)F(t+\tau)\rangle - \langle F\rangle^2}{\langle F\rangle^2} \tag{1}$$

$F(t)$ is the fluorescence intensity at time t, the brackets $\langle\ \rangle$ indicate a time average over the fluorescence signal, $\langle F\rangle$ is the average fluorescence intensity, and τ represents the lag time. The autocorrelation function measures how long fluctuations persist and the autocorrelation curve describes the temporal decay of memory as a function of the lag time τ. The value $g(\tau)$ characterizes the residual persistence of a fluctuation after a time τ has passed. The autocorrelation function offers a very convenient way to separate processes with short and long memory.

Models are needed to extract the dynamic information encoded in the shape of the autocorrelation function. The theory of FCS and the autocorrelation function

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[5] J. S. Eid, J. D. Müller, and E. Gratton, *Rev. Sci. Instrum.* **71**, 361 (2000).

[6] H. Qian and E. L. Elson, *Biophys. J.* **57**, 375 (1990).

[7] A. G. Palmer and N. L. Thompson, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6148 (1989).

[8] Y. Chen, J. D. Müller, P. T. So, and E. Gratton, *Biophys. J.* **77**, 553 (1999).

have been reviewed in detail.[9] Here, we consider the simplest model, a single, freely diffusing species (such as a solution of fluorescein), to illustrate the use of the autocorrelation function. Before we look more closely at the autocorrelation function we want to stress that the functional form of the autocorrelation depends on the point spread function (PSF). The PSF describes the spatial intensity profile seen by the detector of the FCS instrument. The PSF depends on the microscope optics and the properties of the excitation light source. Three different PSFs have been widely used in the FCS literature. (1) The two-dimensional Gaussian PSF describes a radially symmetric Gaussian intensity profile. This PSF is a good approximation of the intensity distribution in the focal plane of the microscope. It is primarily used to describe processes on surfaces, such as on lipid membranes. (2) The three-dimensional Gaussian PSF has like the two-dimensional Gaussian PSF a Gaussian intensity profile in the radial direction. The intensity distribution in the third dimension along the optical axis is also given by a Gaussian. The three-dimensional Gaussian PSF approximately describes the spatial intensity distribution of confocal FCS experiments. The Gaussian intensity distribution along the optical axis approximates the contribution of light from out-of-focal planes in a confocal detection arrangement. (3) The Gaussian–Lorentzian PSF has been used to describe two-photon FCS. Again, a two-dimensional Gaussian describes the light intensity distribution in a plane perpendicular to the optical axis. A Lorentzian function characterizes the intensity profile along the optical axis in the absence of a confocal pinhole before the detector. Note that the intensity distribution needs to be squared, because of the quadratic intensity dependence of two-photon absorption.

The autocorrelation function for a single, freely diffusing fluorescent species is given by Eq. (2) for a two-dimensional Gaussian PSF and by Eq. (3) in the case of a three-dimensional Gaussian PSF. The autocorrelation function $g_{GL}(\tau)$ for the Gaussian–Lorentzian PSF is not shown here. It cannot be written in closed form and is given by an integral expression.[10]

$$g_{2DG}(\tau) = \frac{\gamma_{2DG}}{N}\left[\frac{1}{1 + (\tau/\tau_D)}\right] \qquad (2)$$

$$g_{3DG}(\tau) = \frac{\gamma_{3DG}}{N}\left[\frac{1}{1 + (\tau/\tau_D)}\right]\left[\frac{1}{\sqrt{1 + (\omega_0/z_0)^2(\tau/\tau_D)}}\right] \qquad (3)$$

The shape of the autocorrelation function $g_{2DG}(\tau)$ for the two-dimensional Gaussian PSF is given by a hyperbolic function (Fig. 4). Here, the diffusion time

[9] N. L. Thompson, in "Topics in Fluorescence Spectroscopy" (J. R. Lakowicz, ed.), Vol. 1, p. 337. Plenum Press, New York, 1991.
[10] K. M. Berland, P. T. C. So, and E. Gratton, *Biophys. J.* **68**, 694 (1995).
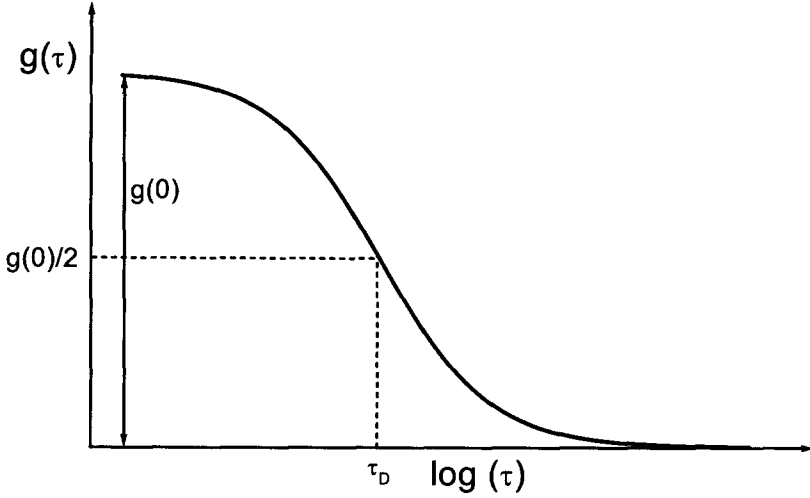
FIG. 4. Autocorrelation function for a two-dimensional Gaussian PSF. The fluctuation amplitude $g(0)$ is inversely proportional to the number of molecules N in the observation volume of the micro-scope. At the diffusion time τ_D the autocorrelation function has decayed to one-half of its value.

τ_D is the time, where the autocorrelation function has decayed to one-half of its value. The fluctuation amplitude $g(0)$ for a single species is inversely proportional to the number of molecules N in the observation volume,

$$g(0) = \frac{\gamma}{N} \qquad (4)$$

The shape of the PSF determines the value of the factor γ. Its numerical value is $\gamma_{2DG} = 0.5$ for the two-dimensional Gaussian PSF, $\gamma_{3DG} = 0.35$ for the three-dimensional Gaussian PSF, and $\gamma_{GL} = 0.076$ for the Gaussian–Lorentzian PSF. The functional shape of the autocorrelation functions characterized by Eqs. (2) and (3) is very similar. Mathematically, the only difference between the functions is an additional multiplicative factor for the three-dimensional Gaussian autocorrelation function. This factor takes the influence of the Gaussian beam profile along the axial direction into account. The beam waist ω_0 characterizes the width of the Gaussian in the radial direction and the beam waist z_0 describes the width in the axial direction.

The diffusion time τ_D characterizes the average time it takes for a molecule to diffuse through the radial part of the observation volume of the microscope. However, the diffusion time is not a constant and changes with the size of the observation volume, which depends on the laser wavelength and the optics of the instrument. The diffusion coefficient D, on the other hand, is a property of a molecule in a given solvent and thus much better suited to characterize the

experimental data than the diffusion time. The relationship between the diffusion time and the diffusion coefficient is given by

$$\tau_D = \frac{\omega_0^2}{4D} \tag{5}$$

which simply is a consequence of the Gaussian beam profile and the laws of diffusion. In the case of two-photon FCS the diffusion time is reduced by a factor of two, $\omega_0^2/8D$. It is important to remember that the diffusion coefficient depends linearly on the viscosity of the solvent. However, most experiments are performed in aqueous solution at room temperature and under these conditions the viscosity is with good approximation a constant.

We briefly compare the autocorrelation function for each PSF. The autocorrelation function $g_{GL}(\tau)$ for the Gaussian-Lorentzian PSF and its best approximation by the function $g_{3DG}(\tau)$ for the three-dimensional Gaussian PSF are shown in Fig. 5A. The differences are so minute that experimentally the two PSFs cannot be distinguished by autocorrelation analysis. For that reason two-photon FCS experiments are mostly analyzed assuming a three-dimensional Gaussian PSF instead of the mathematically more complex equations required by the Gaussian–Lorentzian PSF. Figure 5B displays the differences between the autocorrelation functions for the two-dimensional and three-dimensional Gaussian PSF. The autocorrelation functions overlap almost perfectly at early times. However, the tail of the two autocorrelation functions is clearly different. This difference is of course due to the extension of the PSF into the third dimension. Molecules have an additional path to escape from the observation volume, which leads to a faster decay of the autocorrelation function for the three-dimensional Gaussian PSF. The spatial resolution of confocal and two-photon microscopy is worse along the axial direction than in radial direction. In other words, the ratio of the beam waists ω_0/z_0 is less than one and the multiplicative factor in Eq.(3) decays much more slowly than the first term. Consequently, the three-dimensional Gaussian autocorrelation function [Eq.(3)] approaches the two-dimensional Gaussian autocorrelation function [Eq.(2)] as the axial beam waist z_0 increases. In the limiting case, when the axial beam waist is infinity, the three-dimensional autocorrelation function $g_{3DG}(\tau)$ reduces to the two-dimensional Gaussian autocorrelation function $g_{2DG}(\tau)$. Thus, if the axial beam waist z_0 is extended, then the two-dimensional Gaussian model is a fair approximation for FCS measurements in solution. However, if the radial and axial beam waists are of similar size, then the two-dimensional Gaussian model introduces a misfit of the experimental autocorrelation function and the three-dimensional PSF must be used.

Data evaluation of the autocorrelation function requires choosing models with which to fit the data. A statistical criterion is needed to judge the quality of a particular model. The reduced chi-squared (χ^2) is the most widely used tool to judge model-dependent fits of data. A χ^2 value of 1 indicates a perfect fit of the data, whereas χ^2 values larger than three typically are interpreted as a rejection of the
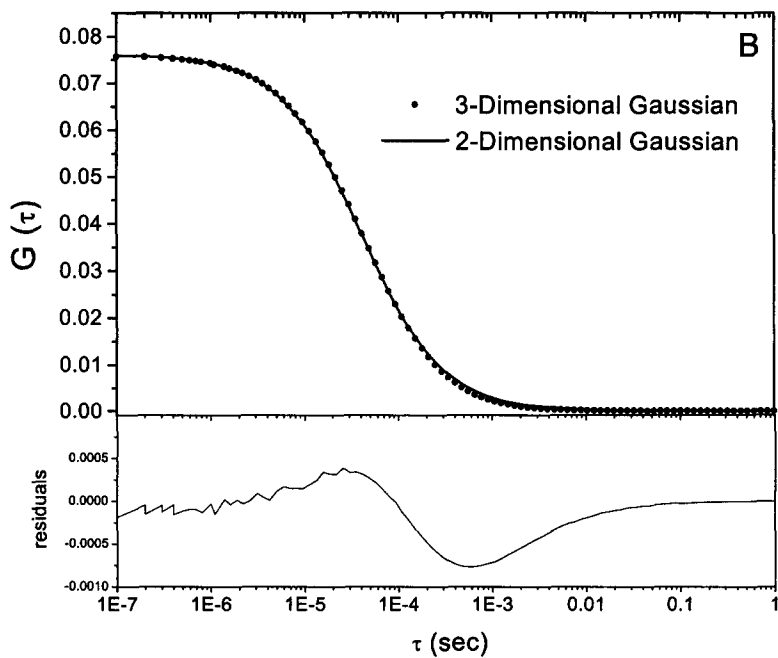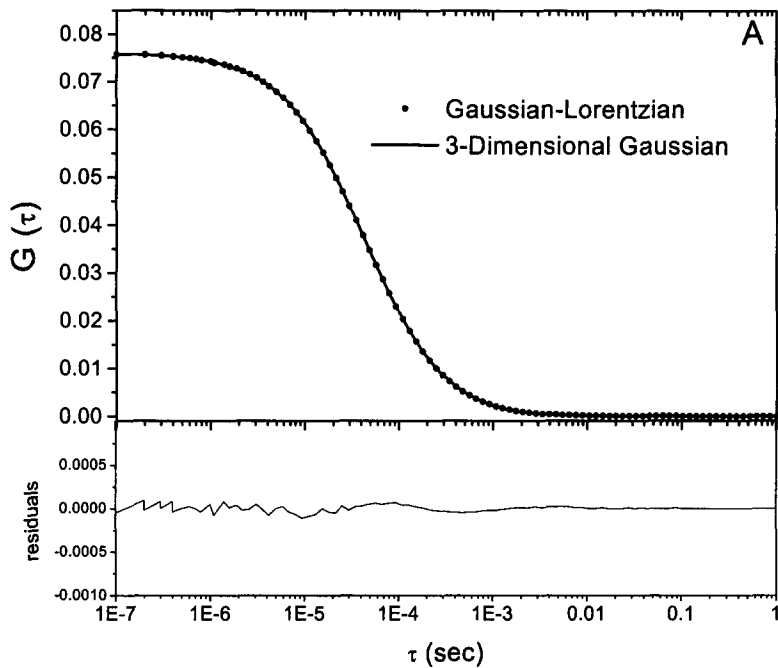
model by the data. However, χ^2-based analysis of the data requires the assignment of the correct experimental errors to each data point. The standard deviation (SD), which is a measure of the experimental error, is not trivial to determine in FCS. The correlation function alone does not provide enough information to calculate the SD and error analysis has been mostly neglected in FCS. However, especially in FCS knowledge of the experimental error is almost indispensable, because different models often lead only to minor changes in the autocorrelation function. Data evaluation based on error analysis establishes an objective procedure by which models are accepted or rejected. A detailed discussion and several methods to determine the SD from FCS data have been recently described in the literature.[11]

We have used the following strategy with good success to determine the SD of FCS data. Because it is not straightforward to calculate the SD of the autocorrelation function, we measured it experimentally. The data acquisition card used allows access to the complete sequence of photon counts and the following procedure was chosen to analyze the autocorrelation function from the raw data. The complete data set is evenly divided into records of equal length. The number of records n depends on the total length of the photon sequence, but is always larger than 10 to have enough records to determine its statistics. Each record is treated as an independent and individual experiment and the autocorrelation function of each record is determined by software. The autocorrelation functions from all individual records allow a straightforward calculation of the standard deviation of a single record $\sigma_{Record}(\tau)$. The SD $\sigma(\tau)$ of the autocorrelation function for the full data set is related to the SD $\sigma_{Record}(\tau)$ of a single record by $\sigma(\tau) = \sigma_{Record}(\tau)/\sqrt{n}$. The last equation is valid, if the n data records used to calculate the SD are statistically independent. This condition is easily verified by inspecting the autocorrelation functions calculated from each record. If these autocorrelation functions decay to zero, then all memory is lost within a single record and individual records are statistically independent from one another. Figure 6 shows the autocorrelation function of the dye rhodamine 110 and its standard deviation measured by two-photon FCS. The autocorrelation function is fit within statistical error to a model of a single species model with a three-dimensional Gaussian PSF ($\chi^2 = 1.1$).

Autocorrelation Function of Multiple Species

A major interest of FCS experiments is the detection of molecular interaction between biomolecules, such as binding between two macromolecules. These

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[11] T. Wohland, R. Rigler, and H. Vogel, *Biophys. J.* **80**, 2987 (2001).

FIG. 5. Comparison between autocorrelation functions for different PSFs. (A) The autocorrelation function for a Gaussian–Lorentzian beam profile (●) and its approximation by an autocorrelation function for a three-dimensional Gaussian PSF (solid lines). (B) Comparison between the autocorrelation function for a three-dimensional Gaussian PSF (●) and its best approximation using a two-dimensional Gaussian model (solid line).
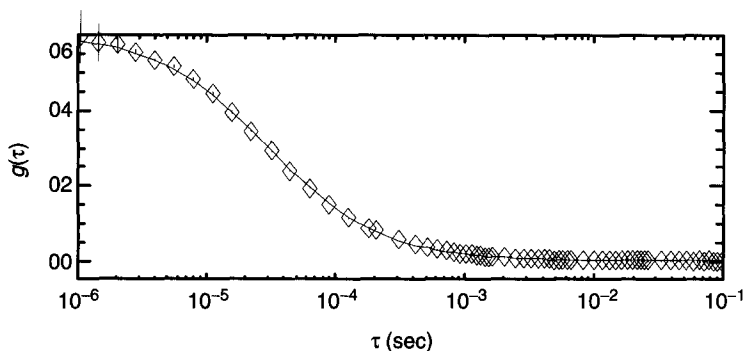
FIG. 6. The autocorrelation function of a rhodamine 110 solution was fit to a single species model for a three-dimensional Gaussian PSF.

interactions are chemical reactions between different species. In a reaction a molecule goes generally from one state into another state, for example from a free ligand to a bound complex. Chemical reaction or other kinetic processes have to occur within the diffusion time of the molecule for FCS to see them. Once a molecule has left the observation volume we have no means of measuring its state, and any other molecule entering the observation volume is statistically independent from the molecule that left the volume. Thus, for soluble proteins the direct measurement of reactions is limited to processes that are faster than approximately a few milliseconds. FCS experiments require nanomolar sample concentrations and the observation of binding at these concentrations requires dissociation coefficients K_D with values, which are nanomolar or lower. The sum of the on and off rates is under these conditions much smaller than the reaction rate limit imposed by the FCS technique. In other words, the probability of observing a biomolecular complex dissociate during its passage time through the observation volume is essentially zero. Although the binding kinetics are typically not visible in FCS measurements, the presence of two species, the free ligand and the bound complex, is reflected in the autocorrelation data. An understanding of the autocorrelation function for multiple, noninteracting species is in most cases sufficient to address binding equilibria between biomolecules with FCS.

We will briefly discuss how the presence of more than one species affects the autocorrelation function. For simplicity, we will consider only the case of two, noninteracting species. The generalization to more than two species is straightforward. Let $g_1(\tau)$ and $g_2(\tau)$ be the autocorrelation functions of species 1 and of species 2, respectively. The autocorrelation function of the mixture of two species is given by

$$g(\tau) = f_1^2 g_1(\tau) + f_2^2 g_2(\tau) \tag{6}$$

The fractional intensity f_i is defined as

$$f_i = \langle k_i \rangle / \sum_j \langle k_j \rangle \tag{7}$$

where $\langle k_i \rangle$ is the fluorescence intensity of the ith species. The fractional intensity is simply the fractional contribution of a species to the total fluorescence intensity. The autocorrelation function of a mixture is a superposition of the individual autocorrelation functions of each species. Each autocorrelation function is scaled by the square of the corresponding fractional intensity. This nonlinear scaling of the autocorrelation function has practical consequences for FCS experiments. Consider two species with the same concentration, but a difference of 2 in their molecular brightness. The fractional contribution, $f_1^2 / (f_1^2 + f_2^2)$, of the dim species to the autocorrelation function is only 20%. This simple analysis illustrates that the detection of a dim species in a sample with a bright species can be difficult experimentally.

A difference in the molecular weight of the two species gives rise to different diffusion coefficients. The autocorrelation function of such a mixture contains a fast and a slow decaying fraction (Fig. 7). The fast decay originates from the species with the larger diffusion coefficient, whereas the slow decay characterizes the species with the smaller diffusion coefficient. Fitting the autocorrelation function of the mixture to Eq. (6) separates the two components. However, a direct separation of the autocorrelation function into its two components requires

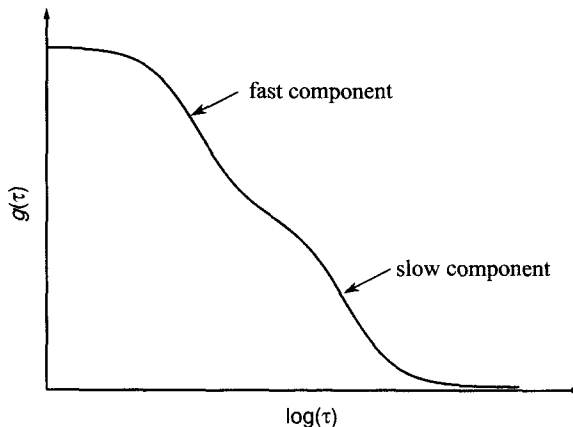FIG. 7. Theoretical autocorrelation function for a binary mixture. The autocorrelation function for two species with vastly different diffusion coefficients is shown. The fast decay component represents the species with the higher diffusion coefficient. The slow decay characterizes the slowly diffusing species. The amplitude of both components depends on the fluctuation amplitude of each species and their fractional intensities.

a minimum difference of their molecular weight ratio of 5 to 8.[12] If the molecular weight difference between the species is insufficient for a direct separation of the two components from the autocorrelation function, other approaches that are not based on the diffusion coefficient are possible. The analysis of the fluctuation amplitude $g(0)$ of a titration experiment allows the characterization of binding equilibria.[13,14] The photon count distribution offers another statistical tool to separate a mixture of species.[8,15,16] Finally, two-color FCS is an elegant technique for separating species that have been labeled with differently colored fluorophores.[17]

Calibration of Instrument

Calibration of Observation Volume

Data evaluation of the autocorrelation function requires knowledge about the dimensions of the observation volume. The radial and axial beam waists ω_0 and z_0 parameterize the PSF in the case of the three-dimensional Gaussian intensity distribution. For the Gaussian–Lorentzian PSF only the knowledge of the radial beam waist ω_0 is required. One approach to attain these parameters is the direct measurement of the PSF. A number of techniques are available for determining the PSF of the instrument. However, these methods are time consuming and often require equipment not available on a standard FCS instrument.

A relatively simple and fast, but indirect method for finding the beam waists is the calibration of the instrument with a sample of known concentration and diffusion coefficient. Experimentally, we do not directly measure diffusion coefficients, but rather measure the residence time τ_D of a molecule inside the observation volume, which is given by $\omega_0^2/4D$ (or by $\omega_0^2/8D$ in the case of two-photon FCS). Fitting the autocorrelation function of the calibration sample, while keeping the diffusion coefficient D fixed to the known value, determines the radial beam waist ω_0. If the data are fit with a three-dimensional Gaussian PSF the axial beam waist z_0 is determined as well. After the instrument has been calibrated all further experiments are analyzed with the beam waist parameters recovered from the standard sample. A sample with known diffusion coefficient is required for the calibration of the instrument. In the past, we used fluorescent spheres of known diameter for this purpose. The radius of the sphere and the viscosity of the water determine the diffusion coefficient according to the Stokes–Einstein equation. However, we found that spheres tend to aggregate as a function of time. Depending on the size

[12] U. Meseth, T. Wohland, R. Rigler, and H. Vogel, *Biophys. J.* **76,** 1619 (1999).

[13] K. M. Berland, P. T. C. So, Y. Chen, W. W. Mantulin, and E. Gratton, *Biophys. J.* **71,** 410 (1996).

[14] Y. Chen, J. D. Müller, S. Y. Tetin, J. D. Tyner, and E. Gratton, *Biophys. J.* **79,** 1074 (2000).

[15] P. Kask, K. Pälo, D. Ullmann, and K. Gall, *Proc. Natl. Acad. Sci. U.S.A.* **96,** 13756 (1999).

[16] J. D. Müller, Y. Chen, and E. Gratton, *Biophys. J.* **78,** 474 (2000).

[17] P. Schwille, F. J. Meyer-Almes, and R. Rigler, *Biophys. J.* **72,** 1878 (1997).

and the concentration of the spheres, they aggregate on a time scale of minutes to hours. Spheres also adsorb to many other materials, such as test tubes, glass slides, and biological cells. Frequently, the fluorescent intensity from the sphere samples decreases during measurements due to the adsorption of spheres to the walls of the sample holder. We found that fluorescent dyes are much better suited for the calibration procedure. Most fluorescent dyes are stable, do not tend to aggregate, and their sample preparation is straightforward.

Rhodamine 6G, which is a very bright and photostable dye, is a good choice for calibrating the observation volume. Its diffusion coefficient in aqueous solution is 280 μm^2/sec in aqueous solution. We often use fluorescein in high pH buffer (50 mM tris(hydroxymethyl)aminomethane buffer, pH 10) for calibration. Fluorescein is a pH-sensitive dye, and its spectroscopic properties vary drastically from pH 7.5 to 2. At pH > 7.5, fluorescein has a constant quantum yield and very good water solubility. The diffusion coefficient of fluorescein in aqueous solution at room temperature is 300 μm^2/sec.[18] An advantage of using fluorescein is its lack of interactions with surfaces. We have found no evidence of adsorption to container walls for a wide variety of sample holders. Another dye we frequently used is rhodamine 110; rhodamine 110 has lower water solubility than fluorescein, but on the two-photon instrument is almost a factor of two brighter than fluorescein when excited at 780 nm.

A fit of the autocorrelation function to a three-dimensional Gaussian model [Eq. (3)] also yields the axial beam waist z_0. Yet, the accurate determination of the axial beam waist z_0 is much more difficult than finding the radial beam waist ω_0. This simply reflects the fact that the axial beam profile has only a minor influence on the shape of the autocorrelation function. Autocorrelation data of excellent quality are needed to acquire the statistics necessary for an accurate determination of the axial beam waist. The larger the axial beam waist is in comparison to the radial beam waist, the harder it is to determine the parameter z_0 from FCS data, because the experimental autocorrelation function rapidly approaches the shape of the two-dimensional Gaussian correlation function $g_{2DG}(\tau)$ [Eq.(2)], as previously discussed. Analysis of the shape of the PSF for confocal microscopy predicts a beam waist ratio z_0/ω_0 of 2 to 3. However, experimentally often a ratio of 4 to 5 is found for confocal FCS, which is most likely due to aberrations. In two-photon FCS the autocorrelation function based on a Gaussian–Lorentzian beam profile is approximated by an autocorrelation function for a three-dimensional Gaussian model with a ratio of the beam waists of approximately 5.

FCS not only characterizes dynamic processes, but also measures the concentration of the sample. The fluctuation amplitude $g(0)$ of the autocorrelation function is given by the ratio of the gamma factor γ and the number of molecules N in the observation volume V [see Eq. (4)]. FCS has the remarkable property

[18] Y. Chen, University of Illinois at Urbana-Champaign (1999).

that it determines particle concentrations from statistical fluctuations, which are governed by fundamental physical principles. Traditional techniques that measure concentration require the knowledge of a molecular property, such as the extinction coefficient. In contrast, the number of fluctuations within a small observation volume that is in contact with a large surrounding bath is Poisson distributed. This law holds universally for noninteracting particles, independent of specific molecular properties. Thus, in principle, FCS offers a very attractive way for determining concentrations. However, the determination of the average number of molecules N requires knowledge of the γ factor. The value of the γ factor depends on the PSF of the instrument. As already discussed, the autocorrelation function is not a sensitive measure for distinguishing PSFs. For example, the correlation functions based on the three-dimensional Gaussian and the Gaussian–Lorentzian model are essentially identical, but their γ factor differs by a factor of 4.6. Image formation of high numerical aperture objectives is complex and the PSFs discussed so far are mathematical idealizations of the real PSF. A direct measurement of the PSF by imaging or other techniques is difficult and the proper parameterization of the measured PSF is not obvious either. Calibration of the instrument with a sample of known concentration is a practical approach to address the problem. The volume of the PSF is defined as $V = \int \mathrm{PSF}(\mathbf{r})d\mathbf{r}/\mathrm{PSF}(\mathbf{0})$, where $\mathrm{PSF}(\mathbf{r})$ is the value of the PSF at the spatial location $\mathbf{r}$. This volume definition is not a measure of the geometric extent of the PSF, but represents an effective volume of the PSF. For a Gaussian–Lorentzian PSF the volume is given by $V_{\mathrm{GL}} = \pi \omega_0^4/\lambda$ and for the three-dimensional Gaussian the volume is given by $V_{\mathrm{3DG}} = (\pi^{3/2}/8)\omega_0^2 z_0$, where λ is the wavelength of the excitation light source.

The definition of the molar concentration is the ratio of the number of molecules per volume $c = N/V N_{\mathrm{A}}$ and Avogardo's number N_{A} converts the particle number concentration into a molar concentration. The experimental γ factor is determined by $\gamma = g(0)cV/N_{\mathrm{A}}$ and allows a comparison of the measured γ factor with the theoretical γ factor of the assumed PSF. The concentration of the dye used for calibration is measured by absorption spectroscopy and its concentration determined from the known extinction coefficient according to Beer's law. Micromolar dye concentrations, which are required for absorption spectroscopy, are too high for FCS experiments and the sample solution is diluted to nanomolar concentrations. The dye used for calibration should not adsorb to surfaces, so that the sample can be diluted reliably.

Another approach taken by some researchers is a procedure where the γ factor is simply set to a fixed value of one. Such a procedure requires a different definition of the observation volume. A γ factor of one describes any PSF, which is constant within a volume of arbitrary shape and vanishes everywhere outside of the volume, such as a small cylindrical-shaped volume element. A simple geometric interpretation of the observation volume is then given by $V = 1/[g(0)cN_{\mathrm{A}}]$. Whenever relative concentrations instead of absolute concentrations are of concern, no

calibration procedure is necessary. The ratio of the number of molecules measured by FCS reflects their concentration ratio, because the γ factor stays constant, if the instrument optics and excitation light are unchanged.

Molecular Brightness

Another important quantity of FCS measurements is the molecular brightness of a fluorophore. FCS uses photon counting for detecting the fluorescence signal and the average intensity $\langle k \rangle$ is measured in counts per second (cps). The average photon count rate $\langle k \rangle$ is the product of the number of molecules N in the observation volume V and the fluorescence brightness ε of a single molecule,

$$\langle k \rangle = \varepsilon N \tag{8}$$

The molecular brightness ε is a measure of the detected fluorescence intensity of a single molecule. Molecular brightness is expressed in counts per second and per molecule (cpsm). The value of the molecular brightness is not a constant, but depends on the excitation intensity, the optical filters, the microscope optics, the quantum yield of the detector, and the molecular properties of the dye. However, the molecular brightness allows a meaningful evaluation of the performance of the instrument as long as the same experimental conditions are used.

It is good practice to measure a calibration sample under the same instrumental conditions at the beginning of each experiment. We typically start by measuring a sample of fluorescein at a particular wavelength and laser power. The fluorescein sample serves for calibrating the two-photon observation volume. In addition, we calculate the molecular brightness of the sample according to Eq. (8) and its value is fairly reproducible. A reduction in the molecular brightness from its usual value indicates a problem with the instrument, such as a misalignment of the optics, and the problem can be addressed right away. This is an important issue, because the molecular brightness is a crucial parameter in FCS measurements. The signal-to-noise ratio of FCS measurements depends on the square of the molecular brightness.[19] A reduction of the molecular brightness by a factor of 2 decreases the signal-to-noise ratio by a factor of 4. A fourfold increase of the data acquisition time is required to offset the reduced signal-to-noise ratio.

Dilution Study with FCS

The fluctuation amplitude $g(0)$ is inversely proportional to the concentration of fluorophores. The fluctuation amplitude decreases until an upper concentration limit is reached, where instrumental noise overtakes the fluorescence fluctuations of the sample. Similarly a lower concentration limit exists, where background counts overwhelm the signal counts. The exact value of the concentration limits

[19] D. E. Koppel, *Phys. Rev. A* **10**, 1938 (1974).

depends on the molecular brightness of the fluorophore and many instrumental parameters, but to get a better feel for the FCS instrument, the performance of the instrument at different sample concentrations should be experimentally determined. We probed the range of concentrations that can be measured on the two-photon FCS instrument by performing a dilution experiment. Figure 8 shows the result of measurements on fluorescein diluted from 275 to 0.27 nM. The measured fluorescence intensity $\langle k \rangle$ and the number of molecule N in the observation volume are plotted as a function of the fluorescein concentration. Both fluorescence intensity $\langle k \rangle$ and the number of molecules N exhibit a linear behavior as a function of concentration. A closer inspection of Fig. 8 indicates that both curves deviate slightly from the ideal linear curve. Plotting of the fluorescence intensity versus the average number of molecules yields a straight line and therefore suggests a slight systematic error in the successive dilution of the sample. The fluorophore concentration range measured in this experiment covers three orders of magnitude. The upper concentration limit of this experiment was about 275 nM. Signal-to-noise considerations set the lower concentration limit. Two sources of noise, the dark counts of the detector and the background counts of the buffer, determine the lower concentration limit of FCS experiments. Ideally, both the dark and background counts are uncorrelated noise sources, which add to the average photon counts measured. The APD used in this study has about 50 dark counts per second and the buffer contributed an additional 70 cps. Compared with a photon count rate of 1600 cps for fluorescein at 0.275 nM, the dark and background counts do not yet contribute significantly to the overall fluorescence signal. Yet a calculation of the fractional intensity [Eq. (7)] already indicates a suppression of the fluctuation amplitude by about 14%. We estimate based on these values that by properly taking the background and dark counts into account, the number of molecules in the sample can be measured down to about 70 pM, where the uncorrected fluctuation amplitude is half of its nominal value and uncertainties in the correction procedure are becoming a concern.

Sample Preparation

Contaminations

The extreme sensitivity of FCS allows measurements at nano- and subnanomolar concentrations. For such experiments the presence of fluorescent contaminants poses a severe problem. The water, any buffer, or cosolvent used in experiments should be checked for contaminants by FCS measurements. We once experienced contamination from our filtered, deionized water source. The molecular brightness of some contaminants rivals that of bright fluorophores and gives rise to autocorrelation curves of excellent quality. The concentration of contaminants is typically subnanomolar. Solvents, such as ethanol or dimethyl sulfoxide (DMSO), often contain fluorescent contaminants even if the solvents purchased are of spectroscopic
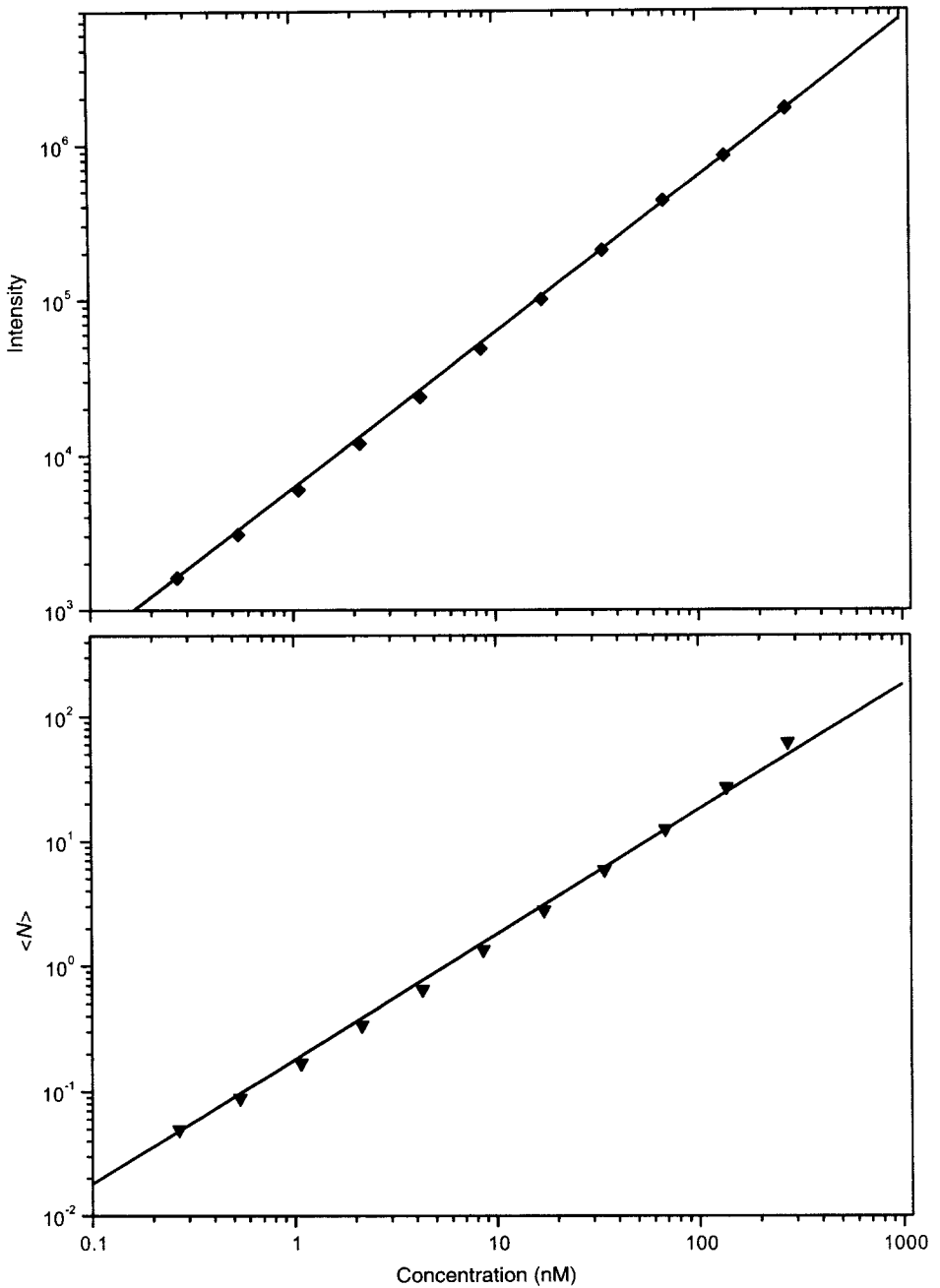
FIG. 8. Dilution study of fluorescein by FCS. Fluorescein in Tris buffer (pH 9.5) was successively diluted from 275 nM to 0.27 nM. The intensity (diamonds) and the average number of molecules N (triangles) are plotted as a function of fluorescein concentration. The solid line is a fit of the data to a straight line.

or higher grade. It is helpful to compare solvents from different sources and select the one best suited for FCS experiments. Contaminants can also be introduced by contact with glassware, pipette tips, and sample holders that are "dirty." If experiments are performed on surfaces a much greater effort is required to eliminate contaminants than for experiments in the bulk phase, because a number of contaminants are surfactants and specifically stick to surfaces. Surface substrates, such as fused silica, should be cleaned meticulously, just as done for the preparation of single molecule experiments. After solution has been added to the cleaned surface, it should be examined for the presence of fluorescent surfactants.

Sample Loss in Fluorescence Fluctuation Measurements

Some biological samples are very precious and the amount available for experiments is often very limited. In these situations it is prudent to reduce the volume and the concentration of a sample used in measurements as much as possible. In FCS experiments the observation volume is of the order of 10^{-1} μm^3 and sample concentrations range from micromoler to picomolar. It therefore is quite easy to perform fluorescence fluctuation measurements on a few microliters of a highly diluted sample. Unfortunately, there are some experimental complications that might occur and disturb the measurement.

The adsorption of a small amount of sample to the sample holder results in a loss of concentration. This is especially important for samples with large surface-to-volume ratios at low concentrations, which can lead to a significant fraction of adsorbed sample at the container walls. Hanging drop glass microscope slides with 22×22-mm square coverslips are widely used as sample holders for microscope experiments. They accommodate 90 μl of sample, and have a surface area close to 10 cm^2. We have observed sample loss due to adsorption with this assembly. We also use a commercial sample holder for cell cultures (Nalge Nunc International, Naperville, IL). This sample holder has a surface area of 3 cm^2 and holds a solution of 500 μl, which reduces the surface-to-volume ratio by at least one order of magnitude as compared to the hanging drop slide. However, the surface-to-volume ratio is only one of the variables affecting sample loss. Some biomolecules preferentially stick to particular materials. It might be necessary to try different sample cells and coat surfaces to minimize adsorption.

Determining Sample Loss

Whenever we start to work with a new fluorescent dye, we check the surface adsorption properties of the dye by performing a simple dilution experiment. Depending on the way the dilution is carried out, different results are observed if adsorption is present. For example, consider a dilution experiment of rhodamine B in water. If fresh sample holders are used for each dilution step, then the fluorescence intensity is linear with the rhodamine B concentration at submicromolar concentrations, but displays sublinear behavior at lower concentrations. However,

if the same dilution is done from high to low concentrations in the same sample holder, a superlinear dependence of the intensity as a function of concentration is observed. These results are consistent with the Langmuir isotherm of binding to surfaces. The molecules on the surface are in dynamic equilibrium with the molecules in solution. When a fresh sample holder is used for each concentration, the adsorption of fluorescent molecules to the unoccupied binding sites of the surface removes molecules from solution. When the dilution is done in the same sample holder, the molecules adsorbed to the container walls at high concentration reappear according to Le Chatelier's principle in the solution at low concentrations.

Dilution experiments are very useful for detecting sample adsorption of fluorescent molecules. However, it is very difficult to recognize adsorption when the sample is nonfluorescent. Consider the titration of a nonfluorescent receptor with a fluorescent ligand, where the receptor absorbs to surfaces. The failure to identify the adsorption of the protein leads to a misinterpretation of the experimental results. Thus, great care should be exercised when performing titration studies.

Aggregation of Macromolecules

Fluorescence fluctuation spectroscopy is an extremely sensitive technique for the detection of aggregates. Aggregates are easily identified at the molecular level through their different intensity and diffusion coefficient. An aggregated protein appears brighter, because it contains many fluorescent labels. In addition, the aggregate stays inside the excitation volume longer due to its increased mass. Consequently, the measured fluorescence intensity increases above its average value when an aggregate passes through the illumination volume. The sensitivity of FCS to detect aggregates lies in the small observation volume of the technique. If an aggregate happens to move through the observation volume, then its properties dominate the fluorescence signal detected. Even if aggregates are very rare (for example, a single event in 200 sec), a single passage through the excitation volume is sufficient to produce a clear signature. In conventional fluorometry the observation volume is about a factor of 10^{10} larger than in FCS and aggregates are very hard to detect, because their molecular characteristics are obscured once averaged over an ensemble of millions of molecules. In fact, people were surprised to learn that their sample contains aggregates, because the conventional, cuvette-based instruments they had used has shown no evidence of their presence. It is important to stress that the presence of even one single large aggregate during an FCS measurement is enough to affect the shape of the autocorrelation function. No meaningful analysis of the autocorrelation function is possible, because a single or a few events do not provide the statistics required for FCS analysis. These experiments have to be repeated under conditions in which no aggregates are present. If the data are taken with a data acquisition board that records the sequence of photon counts; the occurrence of an aggregate can then be cut out

from the original data and an autocorrelation analysis of the processed data is now possible. We have found this procedure to be very convenient and useful. Although FCS is quite sensitive in detecting large and very bright aggregates, it provides very little sensitivity in detecting the presence of small or less bright aggregates. For example, the detection of dimers or tetramers in a solution of monomers with FCS is challenging. Two-color FCS is useful for detecting small oligomers; another alternative is photon counting histogram analysis, which determines the molecular brightness heterogeneity of a sample.

FCS Measurements in Vivo

FCS is an attractive technique for intracellular applications. First, FCS determines kinetic processes from equilibrium fluctuations. Thus, no external perturbation is required to obtain kinetic information. Second, FCS provides excellent spatial resolution. The subfemtoliter observation volumes allow the investigator to probe specific organelles and other local regions within a living cell.

Experiments that extend FCS into the cellar environment involve a few challenges not encountered by measurements in vitro. (1) Cells are essentially cuvettes with a volume of a few picoliter. Photobleaching of fluorescent probes in the out-of-focal region of the laser beam is a serious problem for confocal FCS, because of the limited amount of fluorophores available in the tiny volumes of cellular compartments. We will focus here on two-photon FCS, because of its advantages over confocal FCS for in vivo applications. The nonlinear nature of the excitation process limits two-photon absorption to the focal volume of the microscope. This inherent three-dimensional sectioning effect of two-photon excitation eliminates photodamage outside of the focal volume of the microscope. This reduction in photobleaching is the principal advantage of two-photon over conventional single-photon excitation for in vivo applications.[20] (2) Cells contain molecules with intrinsic fluorescence. This autofluorescence adds a background contribution to any fluorescence measurement in the intracellular environment. In contrast to in vitro measurements, where background fluorescence can be avoided by careful sample preparation, the autofluorescence is always present and has to be considered by the investigator. (3) A living cell is a nonequilibrium system. The influence of the cellular environment, such as cellular motion, on FCS measurements is currently not sufficiently understood. For example, do we expect to see simple or anomalous diffusion inside of cells? How do other processes, such as active transport, contribute to the autocorrelation function? However, a number of FCS studies applied to cells have been reported and demonstrate that in vivo FCS experiments are feasible.[21,22]

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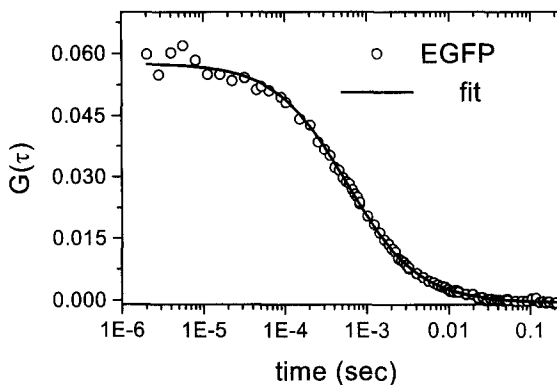
FIG. 9. Autocorrelation function of EGFP inside the nucleus. The autocorrelation function ($\bigcirc$) was fitted to a model of a single, freely diffusing species (solid line).

When working with FCS it is necessary to carefully choose the right fluorescent dyes, particularly for *in vivo* measurements. A fluorescent reporter group is needed, which should merely label the protein of interest, but not take part in any interactions with the cellular environment. Thus, the properties of a fluorophore should first be studied under *in vivo* conditions, before using it as a reporter group in an intracellular study. Green fluorescent protein (GFP) has become a vital tool for cell biology and is widely used as a reporter group for imaging and functional studies of proteins in living cells. We therefore characterized EGFP protein, which is a fairly bright fluorophore, in HeLa cells. The diffusion coefficient of EGFP *in vivo* is slowed down by a factor of 3 as compared to its value in aqueous solution. The slowing of diffusion is simply due to the increased viscosity in the cellular environment. We found that the autocorrelation function of EGFP *in vivo* is described within experimental error by a simple diffusion process (Fig. 9). We conclude from these results that EGFP does not stick to cellular components in the nucleus and cytoplasm, where the measurements were performed. Thus, EGFP is a good fluorescent reporter group for FCS measurements *in vivo*.[23]

Most of the autofluorescence in mammalian cell lines comes from NAD, flavins, and lipofuscin.[24–26] The two-photon cross section of FMN and NADH has been determined *in vitro*.[27] NADH is excited at wavelengths between 700 and

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800 nm, whereas the excitation spectrum of FMN is very broad and is excited over the whole tuning range of the titanium–sapphire laser (700–1000 nm). The two-photon excitation spectrum of lipofuscin is not known. We have measured *in vivo* at two different excitation wavelengths (780 and 895 nm). We noticed that while measuring with the same laser power (1.75 mW at the sample) in the nucleus the response of the cells was wavelength dependent. The autofluorescence intensity increases as a function of time when exciting at 780 nm, but stays constant when excited at 895 nm. Exposure to intense light sources causes oxidative stress that damages cells.[28] A telltale sign of cellular stress is the increase of its autofluorescence. We have observed dramatic and rapid increases in the autofluorescence intensity of cells after exposure to laser light. The power threshold depends on the type of cell, the wavelength of the laser light, and the laser repetition frequency. At equal power, light of longer wavelength apparently is less damaging to cells than light of shorter wavelengths. These observations illustrate the importance of ensuring that the power and wavelength of the excitation light are benign for the cells studied.

The autofluorescence intensity varies strongly between the different cellular compartments of a cell. Inside the nucleus the autofluorescence intensity is typically weak and homogeneous, whereas its intensity in the cytoplasm is stronger and spatially more heterogeneous. When performing FCS measurements inside the nucleus, we found that the fluorescence intensity was very stable. In the cytoplasm, however, the fluorescence intensity depends on the spatial location, and sometimes strong fluctuations, which persist for a few seconds, are observed, whereas at other times the intensity is as stable as inside the nucleus. These differences in the autofluorescence properties make measurement of fluorescently tagged biomolecules inside the cytoplasm much more challenging that inside the nucleus. The concentration range accessible by FCS *in vivo* is less than under *in vitro* conditions, because of the presence of autofluorescence and intensity fluctuations caused by the cell. At very low fluorophore concentrations, the autofluorescence dominates the fluorescence signal. The lowest concentration we measured was around 5 nM. The fluctuation amplitude of EGFP is small at high fluorophore concentrations and any other source of noise, such as intensity fluctuations caused by the cell or instrumental noise, starts to strongly influence the autocorrelation function. The highest concentration we were still able to measure was 300 nM.

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