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'True' single-molecule molecule observations by fluorescence correlation spectroscopy and two-color fluorescence cross-correlation spectroscopy

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Abstract

Fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) are a measure of fluctuations of detected light as a fluorescence molecule diffuses through a femtoliter detection volume caused by a tightly focused laser and confocal optics. Fluorescence from a single molecule can easily be distinguished from the slight background associated with a femtoliter of solvent. At a solution concentration of about 1 nM, the probability that there is an analyte molecule in the probe volume is less than one. Although fluorescence from individual molecules is collected, the data are analyzed by autocorrelation or two-color cross-correlation functions that are the average of thousands of molecules. Properties of single molecules are not obtained. I have been working on problems and opportunities associated with very dilute solutions. The molecule in the confocal probe volume is most probably the molecule that just diffused out, turned around, and diffused back in, i.e., reentered. For the first time, some theoretical results of the novel theory of the meaningful time are presented that enable study of just one single molecule over extended periods of times without immobilization or hydrodynamic focusing. Reentries that may also be called reoccurrences or encounters of a single molecule are significant because during measurement times they give rise to fluctuation phenomena such as molecule number fluctuations. Likewise, four criteria have been developed that can be used to verify that there is only one "selfsame" molecule in the laser probe volume during the experiment: (Földes–Papp, Z., 2006. What it means to measure a single molecule in a solution by fluorescence fluctuation spectroscopy. Exp. Mol. Pathol. 80 (3) 209–218).

Keywords: Single molecule; Solution; Individual molecule; Selfsame molecule; Selfsame single-molecule regime; Selfsame molecule likelihood estimator; Meaningful time theory of single-molecule analysis; Fluorescence fluctuation microscopy; Fluorescence correlation spectroscopy (FCS); Two-color fluorescence cross-correlation spectroscopy (FCCS); Single (solution)-phase single-molecule auto- and two-color cross-correlation spectroscopy (SPSM-FCS)

Introduction

Fluorescence is the luminescent emission that results from absorption of photons. Fluorescence is distinguished from its counterpart, a longer lasting afterglow called phosphorescence, by the magnitude of the decay time. Fluorescent emission ceases abruptly when the exciting energy is shut off. The decay time, or afterglow, of the emission is about 10^{-8} [s] and results in a negative frequency-shifted emission (Stokes shift).

Fluorescence microscopy and spectroscopy allow selective examination of a particular component of a complex biomolecular assembly. The growing importance in biology and medicine is due to (i) the development of confocal tools and (ii) the extraordinary development of new fluorescent probes (Kogure et al., 2006) and quantum dots (Jin et al., 2006). The structures labeled with the fluorescent molecules light up against the background. Additional experimental information can be derived from the combination of optical and biochemical responses exhibited by the fluorescent probe, such as protein–protein interaction analysis by C-terminally specific fluorescence labeling (Oyama et al., 2006), protein–protein interaction domains (Muto et al., 2006), lateral mobility of membrane

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binding protein interactions in live cells (Ohsugi and Kinjo, 2006; Ohsugi et al., 2006), rotational movement of the F1-ATPase motor (Konno et al., 2006), microenvironment and energy depletion in the cell nucleus (Pack et al., 2006), chaperonin-induced altering of the aggregation (Kitamura et al., 2006), dynamics of yeast prion aggregates in single live cells (Kawai-Noma et al., 2006), direct quantification of gene expression (Winter et al., 2004) and restriction fragment length polymorphisms (Nomura et al., 2006), just to name a few studies that very recently have demonstrated a higher-level of understanding on biochemical mechanisms by giving access to information never before seen.

Laser-induced fluorescence (LIF) is an exquisitely sensitive detection tool that, under optimal conditions, even allows single-molecule detection. Due to this inherent sensitivity, the number of applications of LIF in biology and medicine is rapidly increasing. Detecting minute quantities of biologically important molecules in solution has important applications in many fields, including molecular biology, medical diagnosis, and forensic analysis (Nie et al., 1994, 1995; Basché et al., 1997; Orrit, 2002, 2003; Andrec et al., 2003; Slaitas et al., 2003; Watkins and Yang, 2004; Kulzer and Orrit, 2004). High-sensitivity tools are needed, for example, to detect genetic disorders and the growth of tumors or to diagnose early bacterial or viral infections (Földes-Papp et al., 2004a,b, 2005b; Striebel et al., 2003, 2004). Current methods do not have the required sensitivity to adequately detect the small amounts of nucleic acids, antigens or antibodies present in clinical samples (Földes-Papp and Holm, 2002; Rigler et al., 2003; Rigler and Földes-Papp, 2003).

In the case of DNA analytics measured at the single-molecule level, specific nucleic acid sequences must be amplified by means of the polymerase chain reaction (PCR), which duplicates DNA (Földes-Papp et al., 1997a,b, 1998; Rigler et al., 1998; Gösch et al., 2000; Stephan et al., 2001; Sauer et al., 2001; Földes-Papp and Rigler, 2001; Földes-Papp and Kinjo, 2001; Földes-Papp et al., 2001a,b, 2003, 2004a). The use of many PCR cycles may introduce ambiguities arising from contamination and by mechanisms not yet fully understood (Björling et al., 1998; Földes-Papp et al., 2004a). In addition, while DNA microarray technologies are useful for analyzing the expression of a large number of genes in a small number of samples, once target genes are identified it becomes perhaps more useful to analyze a relatively small number of genes in a large number of samples. Current technologies applied in biomolecular and genomic medicine can only measure biological mechanisms as an average of a population of molecules, as only their combined effect can be detected (Földes-Papp, 2006).

In many respects, fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) are an alternative to DNA chip technology (Rigler and Földes-Papp, 2003; Földes-Papp et al., 2005a). FCS and FCCS detect the molecular Brownian movement of fluorescent particles in a very tiny volume of the laser focus. "Classical" FCS and FCCS analyze multi-particle systems in solution, single molecules with hydrodynamic focusing or immobilized single molecules (Földes-Papp and Kinjo, 2001; Földes-Papp, 2005a).

Single (solution)-phase single-molecule fluorescence auto- and two-color cross-correlation spectroscopy (SPSM-FCS) is based on the detection of the Brownian movement of fluorescent molecules *and* Poisson distribution analysis that depends upon the molar concentration of molecules of the same kind in solution or membrane (bulk phase) and the average entry frequency, as well as the presence of just a single molecule in the very tiny detection volume (about 0.2 fl and less) (Földes-Papp, 2001, 2002a,b,c, 2004a,b, 2006; Földes-Papp et al., 2005a). For the first time, SPSM-FCS was introduced for the measurement of just one single molecule in solution without amplification, replication, transcription, immobilization or hydrodynamic focusing, as described in the original articles (Földes-Papp et al., 2005a,b,c).

This original article focuses on *the novel theory of the meaningful time* that enables study of just one single molecule over extended periods of time without immobilization or hydrodynamic focusing. Some of its outcomes are first presented in the context of FCS and FCCS without any loss of generality. The molecule in the confocal probe volume is most probably the molecule that just diffused out, turned around, and diffused back in, i.e., reentered. Most people consider reentries a major problem. For the first time, it is turned into a potential opportunity. New directions in science are launched by new tools much more often than by new concepts (Dyson, 1997).

Results: a novel theory to observe just one single molecule over extended periods of times without immobilization or hydrodynamic focusing

The experimental and theoretical examples in this section shall illustrate the nature of the problem. FCS and FCCS are a measure of the fluctuations of the detected light as a fluorescence molecule diffuses through a femtoliter detection volume caused by a tightly focused laser and confocal optics. Fluorescence from a single molecule can be distinguished easily from the slight background associated with a femtoliter of solvent. At a solution concentration of about 1 nM, the probability that there is an analyte molecule in the probe volume is less than one. Although fluorescence from individual molecules is collected, the data are analyzed by autocorrelation or two-color cross-correlation functions that are the average of thousands of molecules. *Properties of single molecules are not obtained.* I have been working on problems and opportunities associated with the very dilute solutions. The molecule in the confocal probe volume is most probably the molecule that just diffused out, turned around, and diffused back in, i.e., reentered. I shall first translate the biological problem into mathematical terms.

State-of-the-art

Today, biomolecular and genomic medicine, which is still the domain of natural sciences, is bridging molecular biology

and medicine and leads to a paradigm shift in medical sciences. The future will see different areas of importance in diagnostics, analytics and also treatment, and different assumptions may be made about knowledge and responsibility (Földes-Papp, 2006). The capability to explore the behavior of each molecule in a population individually, in order to fully understand molecular mechanisms and their regulation in the cell, will open up an entirely new view of molecular and genomic medicine and diagnostic technology (Földes-Papp, 2006, 2007a). For example, biological macromolecules such as an immobilized single horseradish peroxidase molecule oscillate between different activity and conformational states (Edman et al., 1999; Bjerneld et al., 2002; Rischel et al., 2003). The quest for understanding of the biological process not only at the molecular level, but at the level of the individual molecule as well, has led to a host of new tools to satisfy that demand. Prominent among them are a variety of tools that rely on the fluorescence of dyes in response to monochromatic light.

The prerequisite of the real revolution now in progress (English et al., 2006), though not yet generally recognized not to mention practiced, is the alternative systematic development and adoption of ultrasensitive, noninvasive, multiparametric, simultaneous analytical and exploratory in vivo and ex vivo observation tools in order to gain greater descriptive insight into pathophysiological processes at the level of the individual molecule. For example, a single macromolecule such as a protein must be considered as a statistical set with fluctuations of the conformations of sequences of residues of a certain number of amino acids, and this set forms a regional as well as more extended regulatory-allosteric array of expression such as conformational maps, related probability distribution functions, and local structural fractal kinetics. They will be offset by interaction factors able to maintain the most appropriate affinities, efficiencies, and efficacies, and will require specific knowledge of the most favorable enthalpies and entropies, which concur to define the binding affinities as well as the intrinsic efficacies of individual molecules.

As single-molecule research proceeds ever more systematically with large-scale assessments of molecular interactions such as high-throughput screening of combinatorial libraries, the heterogeneity of molecules of the same kind in biological systems can no longer be ignored. It seems a central premise, validated by the long experience that suggests rules of thumb, that 3D/2D structurally similar molecules exert similar biological activities in a pool of different chemical compounds (isomers, sequentially altered oligomers, etc.). Similar chemical structures do not always have similar biological activity, even when they are designed for a biological target of known structure. The low fraction of biological activity within similar compounds occurs not only because of deficiencies in the similarity calculations. Thus, many compounds of combinatorial libraries are turned away that otherwise would be attractive to consider. It is even known from the original analysis that by not considering compounds similar to those in the screening set, one is missing some kind of active compounds: the question is how many. In contrast, this original article deals with the assessment of the single molecule while capturing (identifying) it in a specific, non-averaged state. These results require new strategies for compound acquisition at the level of individual molecules and for design of combinatorial libraries.

In biology and experimental medicine, there is a need for tools to estimate the detection of just one ("selfsame") molecule in single phases such as solutions without immobilization or hydrodynamic focusing (Yeung, 2004; Földes-Papp, 2006; Rigler and Földes-Papp, 2000, 2003).

Basic problem involved in FCS and FCCS

Thermal fluctuations of molecule and molecular assemblies can be assessed by fluorescence correlation spectroscopy (FCS). In FCS, fluctuations in the numerical density of molecules excited to fluorescence are recorded and correlated in time. FCS dates back to the early 1970s, when the first experiments have been carried out and the theoretical framework was developed (Magde et al., 1972; Elson and Magde, 1974; Ehrenberg and Rigler, 1974). In the initial phase of FCS, amplitudes $G(\tau)$ of 10^{-4} to 10^{-6} were very common and rather noisy signals were obtained. Therefore, the analysis of the signal-to-noise statistics for the correlation function $G(\tau)$ was very important (Koppel, 1974). In contrast to other photon counting applications with a Poisson statistics where the variance of the measured photon counts is given by its expectation value, the variance of the correlation function $G(\tau)$ depends upon the physical process behind the photon emission, i.e., it is model dependent. Koppel (1974) calculated the variance for a correlation function obeying an exponential decay process as can be found, for example, in triplet kinetics (Widengren et al., 1995). The signal-to-noise ratio (S/N) increases with the square root of the measurement time and with the photon counts per molecule, which are the most important results of Koppel's study, and with the square root of the number of channels involved in calculating the S/N (Koppel, 1974). The latter result is also called the Poisson noise limit since the variance is mainly determined by the photon noise. An increase in sensitivity by several orders of magnitude was achieved by the introduction of diffraction limited confocal probe volumes (Rigler et al., 1992, 1993), which by reduction of the background had made possible the detection of single molecules in solution with FCS and related tools (Rigler and Widengren, 1990; Rigler and Mets, 1992; Mets and Rigler, 1994; Nie et al., 1994, 1995; Edman et al., 1996; Wennmalm et al., 1997). At a confocal probe volume of 0.24 fl (0.24×10^{-15} l) and an amplitude $G(\tau)=1.67=1+1/\langle N\rangle$, resulting in a mean molecule number of $\langle N\rangle=1.5$ within the probe volume, that was measured with excitation of 0.25 mW at the wavelength of 514.5 nm, one rhodamine-6G molecule yields 100,000 photon counts per second signal and 100 photon counts per second background. It corresponds to a signal-to-background ratio of 1000. FCS/FCCS today represents the most sensitive type of optical spectroscopy for the study of molecular interactions.

In a typical FCS experiment in solution, fluorochromes are excited to fluorescence by a continuous wave laser and the intensity fluctuations are detected and correlated. From the amplitude of the normalized self-correlation function, the mean

number (Elson and Magde, 1974; Ehrenberg and Rigler, 1974) of fluorochromes in the probe volume and thus the concentrations of interacting molecules are obtained. From the time dependence of the correlation function, information is obtained about the dynamics of molecular behavior ranging from nanoseconds to minutes and even hours. This includes information on excited states kinetics (Ehrenberg and Rigler, 1974; Widengren et al., 1994, 1995), rotational and translational diffusion (Ehrenberg and Rigler, 1974; Elson and Magde, 1974), and chemical kinetics (Elson and Magde, 1974; Widengren and Rigler, 1998). Concentration ranges between picomolar to micromolar could be analyzed. FCS has turned out to very powerful in determining molecular interactions between nucleic acid sequences including DNA and PNA (peptide nucleic acids) (Kinjo and Rigler, 1995; Schwille et al., 1996; Oehlenschläger et al., 1996; Aich et al., 1997). In those cases, the interactions were determined from the change in diffusional motion as the DNA probe molecules bind to their much larger target DNAs.

A way to determine interactions between nucleic acids independent of differences in thermal motion was demonstrated that involved the correlation of complementary nucleic acid sequences tagged in different colors (Schwille et al., 1997; Rigler et al., 1998). The method consists of using two nucleic acid probes complementary to different sites on a target DNA sequence. The two probes are each labeled with different fluorochromes. When mixed with a sample containing the target DNA, the two probes hybridize to their respective binding sites on the same target DNA molecule (Schwille et al., 1997) or the two probes are enzymatically incorporated into the same target DNA molecule as primers in the PCR reaction (Rigler et al., 1998). The sample is then analyzed by a laser-based ultrasensitive fluorescence system capable of detecting single fluorescent molecules at two different wavelength channels simultaneously. Thus, coincident detection of both fluorochromes provides the necessary specificity to detect target DNA. Here, the two-color cross-correlation function is recorded and only the molecular species containing both colors are observed. If the target is not present, only uncorrelated events originating from free probes and/or free fluorochromes are observed at either detection channel and no two-color cross-correlation function is obtained. No matter what fluorochromes, which have, of course, to meet photophysical and photochemical requirements, and optical setups/devices are used, the primary measurement signal arises from fluctuations of the mean molecule number in a confocal femtoliter or smaller probe region. Optical separation by two-color fluorescence cross-correlation spectroscopy (FCCS) setups is usually defined in terms of cross-talk and cross-excitation/cross-emission, respectively, which can be calculated and minimized by the researcher from readily measurable quantities of the absorption/emission scenario for single labels and multiple labels bound to or incorporated into the two-color molecules (Földes-Papp et al., 2001a,b; Földes-Papp and Rigler, 2001; Földes-Papp, 2005a). In addition, for the first time relevant formulas were given and derived for the quantification of molecule numbers under different experimental conditions with substantial quenching of the two-color molecules such as single labels and multiple labels bound to or incorporated into the two-color molecules, and high-density labeling of two-color molecules with multiple green-emitting labels and one red-emitting label (Földes-Papp, 2005a, 2007b, and references therein).

At a single-molecule level, FCS and FCCS involve a basic problem. Since the principle of FCS and FCCS demands that a molecule be observed several times until an analysis can be performed, it was never clear whether the same molecule (the 'selfsame' molecule) in the probe volume always contributes to the autocorrelation and two-color cross-correlation functions or several different individual molecules cause the correlation measurements as schematically depicted in Fig. 1.

Motivation of the novel theory being developed

The strength of single-molecule measurements is the ability to discern differences between molecules in a sample (heterogeneities) that are hidden in ensemble averages. Consider a molecule in dynamic equilibrium between two conformations or a probe target complex where one of the conformations (or the complex) has a higher fluorescence yield than the other. The data stream (fluorescence intensity vs. time) will look like a square wave as the molecule oscillates between conformations. The ratio of the total on time to the total off time gives the equilibrium constant (a thermodynamic property available from bulk measurements). The distribution of on times gives the kinetic dissociation rate constant and the distribution of off

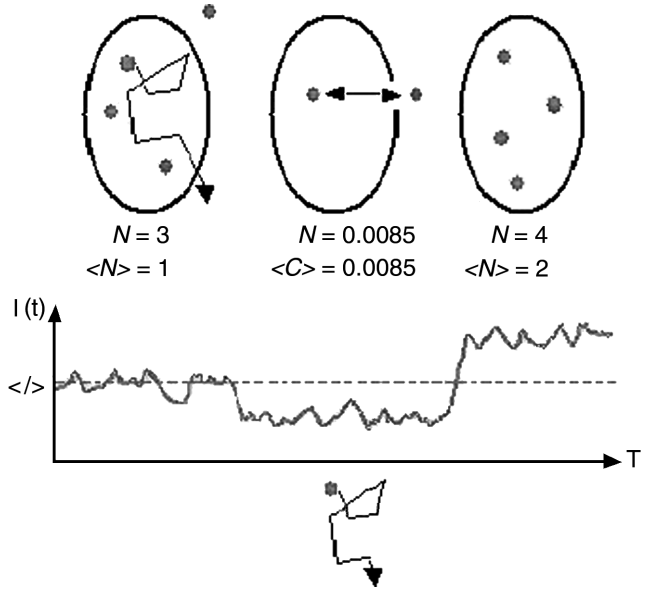

Fig. 1. Three different molecular scenarios are schematically shown for the confocal probe volume in the x–y plane (upper panel) and observed intensity fluctuations (lower panel). N denotes the molecule number in the confocal probe region and $\langle N \rangle$ is the average molecule number (observed N value that is extracted from the amplitude of the correlation curve) in the confocal probe region. If $N<1$ then N stands for the Poisson probability of finding a single molecule in the confocal probe region (arrival of a single molecule). Under this condition $N<1$, $\langle C \rangle = C$ is the average frequency with which the confocal probe region contains a single molecule. For $C \ll e^{-C}$, C equals N. $I(t)$ is the fluorescence intensity, $\langle I \rangle$ stands for a mean intensity, and T is the measurement time.

times gives the association rate constant. Deviations from an exponential distribution indicate that the reaction pathway is more complicated than a simple two-state system. FCS and FCCS are an attractive method to measure these fluctuations because their signal-to-noise (S/N) ratio for the detection of single molecules eliminates or reduces the need for polymerase chain reaction (PCR) amplification of the sample. In addition, the optimum concentration of 1 nM is good for the study of bioreactions with nanomolar equilibrium constants.

The correlation function is also a concept from mechanical statistics. Although one may calculate a correlation function of a molecule, this does not necessarily mean an ensemble average. In the dilute solution, the observation time of single molecules is restricted by the diffusion time even though reentries occur. This fluctuates because the diffusion time is defined by the statistical nature of the underlying physical process. Occasionally, one will observe the molecule in a longer time period relative to the diffusion time. But the photons usually come only in a small part of the whole observation period. Therefore, although the photon burst is observable, the statistics of the correlation function will not be good. Here is a brief calculation: assuming the transient time of a molecule is 50 μs (e.g., diffusion time), the lifetime is 5 ns, quantum yield is 100% and enough excitation power, the maximum number of photons is $50\times10^{-6}/5\times10^{-9}=10^4$ photons. Then, considering the collection efficiency function (Rigler et al., 1993) as the fraction of emitted fluorescence that goes across the pinhole, we can optimistically obtain the observable photon number on the order of 10^3 to 10^2. This value is actually overestimated because it corresponds to about 10^6 to 10^5 photon counts per second and per molecule in a usual FCS measurement using a single photon counting module with avalanche photodiode (EG&G) that results in an internal gain of about 10^3. What most investigators think about the problem is how one can extract these 10^2 to 10^3 photons associated with the specific molecule.

The mathematical expressions of the physical single-molecule reentry mechanism shall be obtained by probing its Brownian trajectories.

Mathematical formulations of the physical reentry model

The selfsame molecule diffusing through the focal periphery gives rise to some fluorescence, which is only weakly correlated with that from the sharp focal plane ('spot'). Therefore, the reentry probabilities $p_n(t)$ of just one molecule in the confocal probe region are theoretically examined in this original article. In order to explain reentries it is important to know by which motional rates of a single molecule they occur. For a better understanding of many experimental situations at the single-molecule level, I shall probe somewhat more deeply into the physical mechanism of reentries.

If we change the number of molecules in the experiments, for example, by dilution or more interestingly by the kinetics of the biochemical or immunological process under study, we will come to the situation where the measured value for the molecule number N in the probe region becomes smaller than one. This experimental situation is the starting point of the analysis. The formulations that I first present in this section are in line with the approach given in Földes-Papp (2005b), which usually seems more concrete. It should be emphasized that by putting certain restrictions on the nature of the reentry process (reentry cases) for the selfsame molecule, which is to be treated, I specify, from the mathematical point of view, the path (trajectory) by which the molecule number fluctuations may take place in the absence of external forces, but without any loss of generality. It is also logically neater and more satisfying. In other words, it is no surprise, then, that reentries that are also called reoccurrences or encounters of a single molecule in the confocal probe region are significant because during measurement times they give rise to fluctuation phenomena, such as molecule number fluctuations. These fluctuations are the fundamental physical process on which fluorescence auto- and two-color cross-correlation spectroscopy is based. For example, if the signal indicates that a molecule diffuses out of the detection volume and right back in, it is still likely the same molecule. The number of reentries that result in a useful burst size is meaningful and of interest. *But what happens if the molecule starts near a boundary?* I am particularly concerned about the situation where the selfsame molecule sits at the border of the confocal probe region, crosses in and out, and therefore has many reentries, none of which are meaningful. Such a molecular situation follows directly for the selfsame molecule from the equation of the special case of the *Selfsame Molecule Likelihood Estimator* $0\le P\left(\bigcap_{i=1}^{2} A_i\right)\le 1$ (see Földes-Papp et al., 2005a) that is characterized by the following property

$$
\begin{aligned}
P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) &= 1 - P_1 \cdot P(\xi \le q < +\infty) \\
&= 1 - P_1 \cdot \int_{\dot{z}_1}^{\dot{z}_2} \int_0^{2\pi} \int_{q=\xi}^{+\infty} n(q,\varphi,z,t)\cdot q\,\mathrm{d}q\,\mathrm{d}\varphi\,\mathrm{d}z \\
&= 1 - P_1 \cdot \int_{\dot{z}_1}^{\dot{z}_2} \int_0^{2\pi} \int_{q=\xi}^{+\infty} n(q,\varphi,z,t)\,\mathrm{d}x\,\mathrm{d}y\,\mathrm{d}z. \qquad (1)
\end{aligned}
$$

Thus, the probability along the z-axis of the confocal probe volume is equally distributed and very close to 1, i.e., it is 99.9%. n is the density function of a single fluorescent molecule, $q=\sqrt{x^2+y^2}$ is measured from its origin at $q=0$ where x and y are the Cartesian space coordinates, and t is a specified time value. Which coordinate system is more appropriate depends on the nature of the problem and its symmetries. In very good approximation, the confocal probe region (detection volume) is cylindrically symmetric rather than spherically symmetric. Therefore, I was motivated to use cylindrical polars q, ϕ, z. P_1 is the Poisson probability of finding a single molecule in the confocal probe region.

The physical model that I propose for the quantitation of single-molecule reentries is first given here. It depends upon the motional rates $k_n(t)$ and $g_n(t)$ of a single molecule. $k_n(t)$ applies for the single molecule inside the probe region and $g_n(t)$ for the

single molecule outside the probe region. Thus, all possible cases of the reentry probabilities $p_n(t)$ for the selfsame molecule during its path (trajectory) through the confocal probe region are described by the system of differential equations of the motional states $n \geq 0$

$$\frac{\mathrm{d}}{\mathrm{d}t}p_n(t) = k_{n-1}(t)p_{n-1}(t) + g_{n+1}(t)p_{n+1}(t) - [k_n(t) + g_n(t)]p_n(t), \quad n \geq 1, \tag{2a}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}p_0(t) = g_1(t)p_1(t) - k_0(t)p_0(t), \quad n = 0. \tag{2b}$$

The initial conditions are

$$p_n(0) = \begin{cases} 1 \\ 0 \end{cases}. \tag{2c}$$

An analytical solution of this physical reentry model (Eqs. (2a–2c)) is given for the meaningful time T_m in which one is able to study a single molecule in the probe region ΔV (Földes-Papp, 2006)

$$t = T_\mathrm{m} = \frac{\tau_\mathrm{diff}}{c_\mathrm{m} N_A \Delta V \cdot \exp\{-c_\mathrm{m} N_A \Delta V\}}, \tag{3}$$

where N_A is Avogadro's number of 6.023×10^{23} [mol^{-1}], c_m is the molar concentration of molecules of the same kind in the bulk phase, e.g., bulk solution, obtained by $c_\mathrm{m} = \frac{C}{N_A \cdot \Delta V}$ with C as the average frequency with which the confocal probe region contains a single molecule (see Fig. 1), and τ_diff is the diffusion time of the selfsame molecule in the probe region ΔV (confocal volume). Eq. (3) is called the 4th criterion since it specifies the time parameter t in the *Selfsame Molecule Likelihood Estimator* $0 \leq P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \leq 1$, i.e., in the 3rd criterion:

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - \frac{N}{2 \cdot \pi}\exp\left\{-\frac{\xi^2}{4 \cdot D \cdot t}\right\} = 1 - \frac{N}{2 \cdot \pi} \cdot \exp\left\{-\frac{\omega_{xy}^2}{4 \cdot D \cdot T_\mathrm{m}}\right\}. \tag{4}$$

Here, N is the 1st criterion and is measured, for example, by counting the molecule number per confocal probe volume with $N < 1$ by means of fluctuation techniques such as FCS and FCCS. D is simply the diffusivity or the diffusion coefficient. ξ is the lower limit of distances q in the axially radial direction of the confocal probe region. For example, ξ is taken as the radius ω_{xy} of the confocal volume element in the x–y plane. Evidently, it does not matter whether the three-dimensional volume element ΔV is placed on a three-dimensional object like a solution or on a two-dimensional object like a membrane. The analytical sensitivity that the probe region contains two fluorescent molecules reads

$$\ln\{P(X = 2;\ C = \Pi \cdot T) = P_2\} = 2 \cdot \ln\ C - \ln\ 2 - C, \tag{5}$$

where I record the absolute number of events X occurring in an interval of time T (e.g., measurement time); Π is the average detection probability that depends upon properties of the fluorescent molecule and the measuring device.

Another solution of the physical reentry model Eqs. (2a–c) is the time constant and the variance of the reentry probabilities $p_n(t)$

$$k = \frac{P(X \leq 1;\ C = \Pi \cdot T) = P_1 = N}{\tau_\mathrm{diff}}. \tag{6}$$

All parameters on the right hand side are measured by the correlation curves in FCS and FCCS, for example the molecule number of rhodamine green in the confocal probe region is $N = 0.0085$ and the diffusion time of a molecule like rhodamine green is $\tau_\mathrm{diff} = 26 \times 10^{-6}$ [s]. Thus, the selfsame molecule reenters the probe region 326.92 times per second.

In further view of the physical reentry model (Eqs. (2a–2c)), I also obtain its solution for the portion of meaningful reentries $p_n(t)$ that results in a useful photon burst size

$$p_{\overline{n,n}} = N. \tag{7}$$

The theoretical framework of Eqs. (2a–2c) leads to many predictions, for example, see legend to Fig. 1 in Földes-Papp et al., 2005a. Actually, some of the predictions of Eqs. (2a–2c) could be given on empirical grounds, but they are now established on a sound physical basis for the first time.

Discussion

Single-molecule tools are homogeneous. In many cases, unbound probes need not be removed from the solution before determining the concentration of the target (Braet et al., 2007-this issue). High sensitivity and selectivity can reduce the analysis time from days to hours and can eliminate or reduce the need for PCR (Földes-Papp et al., 2005a). In FCS, the auto- and two-color cross-correlation functions of the fluctuations of the fluorescence intensity as analyte molecules diffuse in and out of the femtoliter probe volume yield interesting information about the analyte molecule. This tool is a single-molecule measurement in the sense that, on the average, there is less than one molecule in the probe volume (Földes-Papp, 2001, 2002a,b,c). However, it is not a single molecule measurement in the sense that thousands of molecules contribute to the auto- and two-color cross-correlation functions (Földes-Papp, 2001, 2002a,b,c, 2004a,b; Földes-Papp et al., 2005a). The main advantage of FCS and FCCS is that the femtoliter probe volume reduces background emission to the level where the fluorescence from a single molecule is easily detected above emission from the background. Typically, the detection limit is determined by free (unbound) fluorescent probe molecules. Designing the assay so that the target and the probe carry different color fluorescent tags can reduce interference from probe molecules. Probe–target complexes are identified by the simultaneous detection of both colors. A cross-correlation analysis is used to analyze the data. I reported a more than hundredfold increase in sensitivity over ELISA (Földes-Papp, 2004b), the clinical assay currently used for these problems. Increased discrimination against background from unbound probes was first accomplished by using probes that only fluoresce when bound to the target (Földes-Papp et al.,

2005c). The small probe volume and the requirement that the average occupancy is about one restrict the useful concentration to about 1 nM for some applications (Földes-Papp and Baumann, 2004; Földes-Papp, 2001). However, Eq. (3) predicts that meaningful observation times of just one single molecule are obtained in the second range if the molar concentrations of molecules of the same kind in the bulk phase are in the femtomolar range. The new field of nanoscale medical diagnostics requires the development of advanced detection technologies that are needed to measure concentrations of biological targets at subnanomolar concentrations. Lower detection limits have driven developments of novel approaches to molecular detection.

Two-color FCS and FCCS were evaluated as an alternative to PCR to quantitate the concentration of autoantibodies and SNPs (single nucleotide polymorphisms) in the serum of subjects suffering from Goodpasture's disease and MTHFR deficiency (methylene tetrahydrofolate reductase deficiency), respectively. Quantitation of genotypes at allele concentrations of about 10^{-15} M (fM) is an impressive step supporting the movement of single-molecule tools into the field of medical diagnostics (Földes-Papp et al., 2003, 2004a, 2005c,a). The basic reason for this, as can readily be seen in Eq. (3), lies in the fact that the self-same molecule can be observed and its fluorescence emission can be correlated in time in the 60-s range of measurement times at femtomolar concentrations of molecules of the same kind in the bulk solution. Thus, true single-molecule molecule observations are possible by FCCS and FCS. This work and work by others demonstrate that single-molecule measurements are not just a passing fancy but will play an important role in bioanalytical chemistry.

A common recurring theme in my research is based on the general theme, "if you diminish the number of molecules in solution to below a certain concentration (Bark et al., 1999; Edman et al., 1999; Lagerkvist et al., 2001; Rigler et al., 2001; Bjerneld et al., 2002; Jermutus et al., 2002; Rischel et al., 2003), one can use Poisson statistics to prove that the signal is likely coming from a single molecule" (Földes-Papp, 2002a, 2004a, 2006; Földes-Papp and Baumann, 2004; Földes-Papp et al., 2005a,b). I used Poisson statistics to develop probability equations that characterize the situation where, on average, there is less than one analyte molecule in the detection volume. In particular, I presented four criteria that must be met to assure that the properties of a single molecule are being measured (Földes-Papp, 2001, 2002a,c, 2004a,b, 2006; Földes-Papp and Baumann, 2004; Földes-Papp et al., 2005a). I then applied these criteria and resulting equations to the study of single molecules without immobilization, hydrodynamic focusing, or burst-size histograms (Földes-Papp et al., 2003, 2004a, 2005a,b; Földes-Papp, 2001, 2002a,b,c, 2004a,b, Földes-Papp and Baumann, 2004).

The ca. 0.1 to 1 ms diffusion time across the femtoliter probe volume does not yield enough cycles in the dynamic equilibrium between two conformations to obtain good conversion rates — or between a probe target complex, one of which is fluorescent and the other is nonfluorescent or one of which (or the complex) has a higher fluorescence yield than the other. Often there are no cycles while the molecule is in the laser beam. I address this problem by working at very low concentrations so that the same molecule passes through the probe volume several times before another molecule is under observation including the time that the molecule is outside the confocal probe volume. Of course, the time resolution is much better when the molecule is in the confocal probe volume.

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