Fluorescence Fluctuation Spectroscopic Approaches to the Study of a Single Molecule Diffusing in Solution and a Live Cell without Systemic Drift or Convection: A Theoretical Study

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Abstract: Reentries of a single molecule in the confocal, femtoliter-sized probe region (about 10^{-16} L and less) are significant because during measurement times they give rise to fluctuation phenomena such as molecule number fluctuations at the single-molecule level in solution without immobilization or hydrodynamic focusing. These fluctuations are the fundamental physical process on which, for example, fluorescence correlation spectroscopy and two-color fluorescence cross-correlation spectroscopy are based. The reentries of just one molecule in the confocal probe region are theoretically examined in this original article using a hidden, continuous-time Markov model. The system is not set up to have systemic drift or convection. It is found that the reentries obey certain conditions and analytical expressions for the reentry probabilities are obtained first. In particular, the time constant of the mean value and the variance of the reentry probabilities are obtained. The fractions of non-meaningful reentries and meaningful reentries are found for these experimental situations. Therewith, the concentration dependence of the meaningful time that one can study bimolecular reactions of the selfsame molecule in the confocal probe region is derived for the first time. The meaningful time in the probe volume is proportional to the diffusion time of the selfsame molecule and related inversely to the size of the given confocal probe volume. For small molecules, i.e. small diffusion times at a given size of the confocal probe region, one needs lower concentrations of molecules of the same kind in the bulk phase, whereas large molecules can be studied at higher concentrations. The selfsame molecule scenario is compared with the molecular scenario that a second molecule enters the probe volume at random as a function of the meaningful time. The analytical solutions of the physical reentry model (mechanism) hold for the one-, two- (membrane), or three- (solution, live cell) dimensional Brownian motion.

Key Words: Single molecule, re-entries, meaningful time for measuring a single molecule diffusing in solution or a live cell without systemic drift or convection, quantitative relationships, theory, fluorescence fluctuation spectroscopic approaches, fluorescence correlation spectroscopy, two-color fluorescence cross-correlation spectroscopy, molecule number fluctuations.

INTRODUCTION

Biomedical science has been steadily zooming in on the biochemical and molecular biological phenomena that underlie higher levels of organization. And these phenomena – once by necessity taken as averages of enormous numbers of individual processes – are themselves beginning to be seen as yet another layer to be understood in terms of their constituent parts of single molecules and their interactions. Yet these are the events that cause biological changes. Data from the measurement of a single molecule can reveal information about kinetic processes not normally accessible by ensemble measurements, such as variations in kinetic rates, memory effects, and the lifetimes of transient intermediates.

Many biological processes, such as ligand binding, are masked by averaging under ensemble conditions. The challenge for life sciences is to enable discovery of the actual single-molecule biochemistry that underlies biological functions. The constituents of these systems are very often at low concentrations in the incipient stage of their biological formation, and they must be measured on a wide range of time scales from the microsecond to the second range and even longer.

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The quest for understanding of the biological process not only on the molecular level, but at the level of the *individual* molecules, has led to a host of new tools for achieving those goals. Prominent among these are a variety of technologies that rely on fluorescence in response to monochromatic light [1-17].

Among the several approaches to the study of single molecules, fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) are the most utilized techniques for direct observation of sparse molecules by immobilization or hydrodynamic focusing [18].

Solvent viscosity, analyte molecular weight, size of the probe volume and concentration dependence of the probability of detecting a single target molecule, double-occupancies and transient events in the probe region, as well as significant signal overlapping were experimentally examined by means of laser-induced fluorescence [19], fluorescence monitoring of single influenza viruses [20], fluorescence microscopy [21], fluorescence photomicroscopy and digital video microscopy [22-24], fluorescence flow cytometry [25, 26], and confocal fluorescence microscopy [27, 28]. Keller and co-workers [26] first used laser-induced fluorescence for single-molecule detection in flowing samples. In 1990, the first paper on the detection of individual molecules passing

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in a hydrodynamic flow through the probe region one by one with one dye molecule per second was published from the laboratory of Keller [29]. Since this paper, the area of single-molecule detection has grown tremendously with emphasis not just on observing single molecule signatures, but applying single-molecule detection to basic chemical and biological problems in applied and fundamental studies [30]. Several spectroscopies have proven to be platforms for single-molecule detection, such as wide-field epi-illumination, near-field optical scanning, laser scanning and multiphoton microscopy [31]. The platforms are combined with analytical methods of fluorescence polarizing anisotropy, fluorescence resonance energy transfer or quenching and fluorescence lifetime measurements. Rigler and co-workers [32, 33] first reported the use of fluorescence correlation spectroscopy (FCS) for single-molecule detection in solution. Eigen and Rigler [34] first suggested its application in molecular diagnostics and evolutionary biology. Most of the experimental single-molecule studies were combined with simulation results. In addition, theoretical and simulation methods were applied which directly operate on the photon arrival trajectories of a single molecule by evaluating a likelihood function without the need of averaging over many molecules [35-39].

Typically, FCS is performed on a single molecule, for example a single enzyme molecule [18, 40-42], by immobilization or adsorption of the molecule on a surface so that one can observe its behavior over a period of time. Miniaturization is also having a big impact on sensitivity of FCS by applying zero-mode waveguides consisting of subwavelength holes in a metal film for parallel analysis of single-molecule dynamics at high ligand concentration (e.g., micromolar concentrations) [18]. Such guides can provide zeptoliter observation volumes (1 zeptoliter = 10^{-21} L) [18]. For direct observation of single enzyme activity, enzymes are absorbed (immobilized) onto the bottom of the waveguide in the presence of a solution containing the fluorescent tagged ligand molecules. There are technical hurdles associated with doing these experiments resulting from immobilization. Unbound enzyme molecules had to be removed by a washing step and inactivation (denaturation) of enzyme molecules occurred because the surface of the metal film is a very unnatural environment for biological molecules. Another way that was suggested using FCS in single-molecule studies is just to take a very diluted solution so that all the occasional low molecules flow through FCS illumination and certainly single molecules will be detected one by one by means of hydrodynamic focusing [15]. In this way, one can average the behavior of many single molecules but one cannot follow the behavior of a single molecule over a period of time. But in fact, FCS/FCCS is more fundamentally and more intrinsically suited to single-molecule measurement. Single-molecule live-cell imaging is still in its early days, and opportunities are plentiful to keep many scientists busy for a long time. Originally, single-molecule work was restricted to a handful of researchers, but over time, it will become easier, because one will eventually have better probes and push-button devices that will make things simple enough for "everybody" to use it [1].

Quantitative understanding of molecular interactions at the level of single molecules within single cells is the next step in basic and applied biomedical research for the analysis of the dynamics and localization of molecules in a variety of physiological and pathophysiological processes [1-7]. However, what is the measurement time in which one is able to study just one single molecule in solution without immobilization or hydrodynamic focusing? Existing theories are inadequate since they do not predict the meaningful time as a function of the concentration of other molecules of the same kind in the bulk solution. This situation produces considerable concern, and experimental hypotheses differ according to which single-molecule analysis methods are thought to have greater validity. This subject is clearly at the forefront of research and should be of great interest to experimental medical scientists. Reentries of a single molecule into the confocal probe region are significant, because during measurement times they give rise to fluctuation phenomena, such as molecule number fluctuations at the single-molecule level. These fluctuations are the fundamental physical process on which fluorescence auto- and two-color cross-correla-tion spectroscopy is based. The single-molecule literature offers no reasoning and no information relevant to the objectives of the presented original research work:

- the time for a molecule to find the probe volume at an initial concentration in the bulk solution,
- the number of meaningful reentries,
- the meaningful time in the probe volume,
- the probability that the entering molecule is the original molecule.

For the first time, exact analytical relationships are found for the above-given experimental situations in solution without immobilization and hydrodynamic focusing and within a live cell. The selfsame molecule diffuses across the microscopic laser focus and is experimentally identified when it diffuses in and out the tiny illuminated probe region many times. The passing through the confocal probe region ΔV causes temporal fluctuations in the fluorescence intensity traces, i.e. signal bursts. 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 but none of them are meaningful. 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 of just one molecule in the confocal probe region are theoretically examined in this original article using a hidden, continuous-time Markov model. 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

probe more deeply into the physical mechanism of reentries. The system is not set up to have systemic drift or convection.

THEORY AND METHODS

Motivation of the Novel Theory on the Meaningful Time to Measure Just one Single Molecule in Solution and within Live Cells

Utilization of the unique advantage of FCS and FCCS for biomolecule quantification of kinetic and dynamic interactions has not been realized in order to achieve measurements of a single molecule diffusing in solution without immobilization or hydrodynamic focusing. Fig. (**1**) shows spontaneous fluctuations in a number of molecules in the small illuminated confocal probe volume of about one femtoliter (10^{-15} L). The characteristic diffusion fluctuations into and out of the confocal probe volume take a characteristic period of time, let us say a millisecond. The fluctuations are stochastic. It does not matter how precisely we measure the individual fluctuations, we still cannot get the kinds of parameters such as rate constants and binding constants that we want. We have to measure many fluctuations and then to calculate the correlation function. If we obtain a thousand fluctuations or ten thousand fluctuations, it might take a second or ten seconds or even more. That is why it is so difficult to be able to make these kinds of measurements on just a single molecule in solution without immobilization or hydrodynamic focusing. Even measurements of two, three, or a few molecules at a time are not acceptable. If one simultaneously measures two, three, or a few single molecules (events), it is the same situation as if one would average over 10^{17} molecules. Single-molecule properties are not obtained.

We actually know very little about the extent to which a single molecule (molecular species) freely diffusing in solution is represented by a given intensity fluctuation, even if we have a scale and we may even know whether it is a linear or a nonlinear scale. When measuring low-concentration targets (< 1 nM), the detected fluorescence signals become digital since the average number of molecules in the confocal probe volume is smaller than unity (< 1.0). Fluorescence bursts are only detected when single fluorescent molecules pass through the confocal probe volume.

Just because there is an average molecule number $<N>= 1.2$ or $<N>= 1$ in the confocal probe volume, one cannot say that this is a single molecule (Fig. **1**). There are many questions that we might want to ask. One of them is this: how many fluctuations do we have to get from a single molecule in solution? If we obtain some kind of correlation function over some period of data accumulation (collection time) then how can we judge that the correlation function represents just a single molecule? If we want to perform a single-molecule measurement in solution or within a live cell then we do not want to collect (integrate) data longer than we have to. So, what is the minimum time that we need to measure the correlation function to get a measurement with one single molecule only? This time would be the "meaningful time" of the single-molecule measurement. These are the kinds of questions that have to be asked and answered for the first time if we want to extend FCS/FCCS to studies of just a single molecule in solution (or membrane) without immobilization or hydrodynamic focusing. And the same holds true for application examples that are supposed if we want to measure whether or not a ligand binds to a single partner molecule.

The Poisson probability P_1 that the confocal probe volume (observation volume) ΔV contains a single fluorescent molecule, $\chi = 1$, is $\ln\{N = P(X \le 1, \Pi \cdot T = C)\} = \ln\{N = P_1\} = \ln C - C$, where C relates to the molar concentration c_m of other molecules of the same kind in the bulk solution (or membrane) by $C \equiv c_m N_A \Delta V$ with $N_A = 6.023 \cdot 10^{23}$ $[mol^{-1}]$ as Avogadro's number. This relationship was newly identified (for detail see ref [43, 44]); the main difference to other Poisson analyses in the literature is that the final expressions no longer contain the detection probability $\prod$; it cancelled out.

All the Poisson events of $\chi = 1$, $\chi = 2$, $\chi = 3$, and so forth contribute to the molecule number fluctuations of just one molecule in the confocal probe volume (the confocal probe region is the laser focus, i.e. the detection volume). One has a discrete, infinitely stretched populations of molecules, whose statistics must obey this condition exactly. Poisson statistics was used to develop probability equations that characterize the situation where, on average, there is less than one analyte molecule in the detection volume (Fig. **1**). In particular, I presented three criteria that must be met to be assured that the properties of a single molecule are being

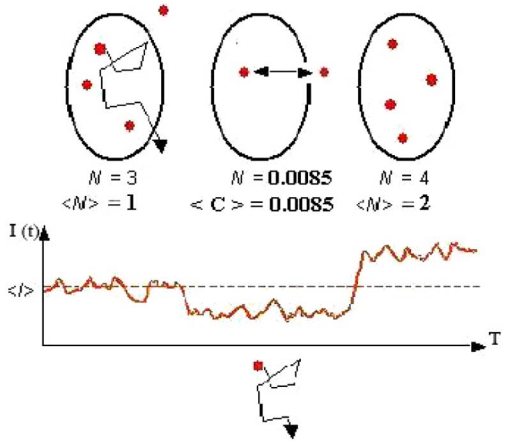

Fig.1. Three different molecular scenarios in solution are schematically shown for the confocal probe volume of 0.2 femtoliter ($2 \cdot 10^{-16}$ L) in the x-y plane (upper panel) and observed intensity fluctuations (lower panel). *N* denotes the molecule number in the confocal probe region and <*N*> is the average molecule number that is the observed *N* value extracted from the amplitude of the correlation curve. **If the observed *N* value becomes $N < 1$ 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, $N < 1$, $<C> = C$ is the average frequency that the confocal probe region contains a single molecule. For $C << e^{-C}$, *C* equals *N*. I(*t*) is the fluorescence intensity, <*I*> stands for a mean intensity, and T is the measurement time for data collection. There is no systemic drift or convection. Modified from Z. Földes-Papp 2007, ref. [50].

measured and applied these criteria and resulting equations to the study of molecules with FCS and FCCS at the single-molecule level without immobilization or hydrodynamic focusing [17, 31, 43-48] (see Wakatsuki, Fee, and Elson [51], and references therein). The question that the *criteria 1*, *2*, and *3* address pertains to the probabilities for a molecule to be within a finite detection volume. These probabilities are called the SELFSAME MOLECULE LIKELIHOOD ESTIMATOR $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ [17, 45, 49]

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - P_1 \cdot P\left(\xi \le q < +\infty\right) = 1 - \frac{N}{2 \cdot \pi} \cdot \exp\left\{-\frac{\xi^2}{4 \cdot D \cdot t}\right\}, \quad (\textbf{\textit{criterion 3a}}) \ (1)$$

where $P_1 = N < 1$ is the *criterion 1*. *t* is a specified time. *D* is the diffusivity or the diffusion coefficient of the single molecule. First *criterion 1* (and criterion *2* that is the analytical sensitivity to detect two molecules at a time, $P_2 = \ln\{P(X = 2, \Pi \cdot T = C)\} = \ln\{P_2\} = 2 \cdot \ln C - \ln 2 - C$) must be fulfilled, and then *criterion 3a* (equation (1)) can be applied. *Criterion 3a* gives the probability for the independent events of finding a single fluorescent molecule (A_1) in the confocal detection/observation volume (arrival) and moving of this single fluorescent molecule (A_2) over a fixed distance (departure). The fixed distance is the lower limit ξ that is, for example, the radius of the confocal probe region in the x-y focal plane with $q = \xi = \omega_{xy}$. $P\left(\xi \le q < +\infty\right)$ is the frequency distribution of the diffusive spreading for the same molecule. q is the axially radial distance with, e.g. $q = \sqrt{x^2 + y^2}$, where (q,ϕ, z) are the cylindrical polars and (x, *y*, *z*) the Cartesian coordinates as shown in Fig. (**2**) [50, 43, 17]. The system is not set up to have systemic drift or convection in the bulk solution or in the live cell.

Special Cases of the the SELFSAME MOLECULE LIKELIHOOD ESTIMATOR for the axially-symmetric, cylindrical volume element in terms of cylindrical polars (q,ϕ, z) with radial diffusion in space (three-dimensional)

For the single molecule, *criterion 3a*, i.e. equation (1), is the probability of entering the confocal detection volume ΔV and being inside the lower limit of distance $q = \xi = \omega_{xy}$. *Criterion 3a* is the so-called '*q* contribution' in the x-*y* focal plane of the probability $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ (see Fig. **2**). For further interpretation, let us consider now the effect that the SELFSAME MOLECULE LIKELIHOOD ESTIMATOR $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ (the complement of the probability of *P* in Fig. (**2**) applied to the arrival and departure of a single molecule) has within the angular range $0 \le \phi \le 2\pi$ of the axially-symmetric volume element (q,ϕ, z) with three-dimensional, radial diffusion in space. An additional angular integration of the density function for the diffusive spreading of the single molecule, $n(q,\phi,t)$, which depends on the radius and the time *t* only

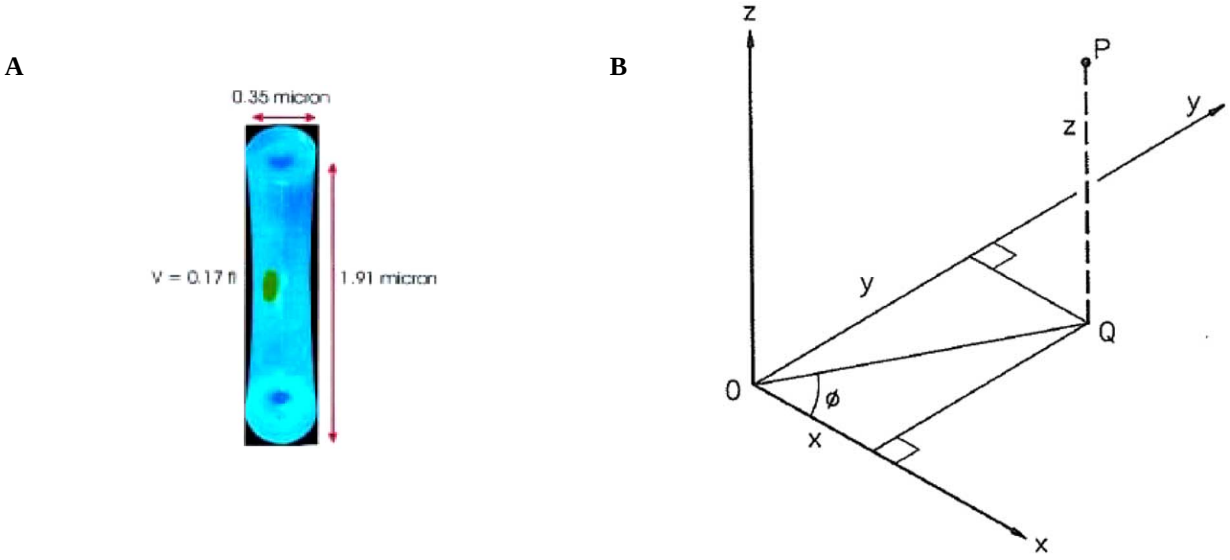

Fig. (2). Fluorescence fluctuation spectroscopic approaches detect the random Brownian motion of fluorescent molecules in a probe region of about 0.2 fL ($2 \cdot 10^{-16}$ [L]) and less within the sample.

$\xi = \omega_{xy} = 0.175 \cdot 10^{-6}[m]$ and $2 \cdot \omega_z / unit = 2 \cdot \dot{\omega}_z = 1.91 \cdot 10^{-6}$.

A: The green fluorescent molecule, e.g. rhodamine green, diffuses out and in the focal plane of the confocal probe volume (probe region) and thereby causes fluctuations in the fluorescence intensity.

B: The single molecule *P*, e.g. rhodamine green, in three dimensions has cylindrically polar coordinates (q, ϕ, z). *Q* is the foot of the perpendicular from the single molecule *P* into the focal x-y plane of the detection/observation volume. ϕ is the angle between 0*Q* and the x-axis. q is the length 0*Q*. Modified from ref. [17].

$$n(q,\varphi,t) = \frac{1}{4\cdot\pi\cdot D\cdot t}\cdot\exp\left\{-\frac{q^2}{4\cdot D\cdot t}\right\} \qquad (2)$$

yields

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - P_1\cdot P\left(\xi \le q < +\infty\right)$$

$$= 1 - P_1\cdot\int_0^{2\cdot\pi}\int_{q=\xi}^{+\infty}\left(n(q,\varphi,t)\cdot q\right)dqd\varphi$$

$$= 1 - N\cdot 2\cdot\pi\cdot\int_{q=\xi}^{+\infty}\left(\frac{1}{4\cdot\pi\cdot D\cdot t}\cdot\exp\left\{-\frac{q^2}{4\cdot D\cdot t}\right\}\cdot q\right)dq$$

$$= 1 - N\cdot\exp\left\{-\frac{\xi^2}{4\cdot D\cdot t}\right\}. \qquad (\textbf{\textit{criterion 3b}}) \ (3)$$

The *criterion 3b* means that the probability within the angular range $0 \le \phi \le 2\pi$ is equally distributed, and therefore it is not subject to changes with a confidence given by equation (3) [17, 49]. However, the measurements do not permit any angle resolution. In other words, *criterion 3b* is the so-called '*q*-ϕ contribution' of the probability $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ within $0 \le \phi \le 2\pi$. The same reasoning holds true along the z-axis and results in the so-called '*q*-*z* contribution' of the probability $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ within $2\cdot\omega_z$

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - P_1\cdot P\left(\xi \le q < +\infty\right)$$

$$= 1 - \frac{N\cdot\dot{\omega}_z}{\pi}\cdot\exp\left\{-\frac{\xi^2}{4\cdot D\cdot t}\right\}, \qquad (\textbf{\textit{criterion 3c}}) \ (4)$$

where $2\cdot\omega_z/unit = 2\cdot\dot{\omega}_z$ is a ratio and equals the double numerical value of the extracted ω_z at a given size of the confocal probe region [17, 49]. Therefore, the so-called '*q*-ϕ-*z* contribution' of the probability $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ within $0 \le \phi \le 2\pi$ and $2\cdot\omega_z$ is

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) = 1 - P_1\cdot P\left(\xi \le q < +\infty\right)$$

$$= 1 - 2\cdot N\cdot\dot{\omega}_z\cdot\exp\left\{-\frac{\xi^2}{4\cdot D\cdot t}\right\}. \qquad (\textbf{\textit{criterion 3d}}) \ (5)$$

Taken together, as claimed in refs [17, 43-45], the numerical values of the SELFSAME MOLECULE LIKELIHOOD ESTIMATOR $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ are different within the lower limit of distance $q = \xi = \omega_{xy}$, the angular range $0 \le \phi \le 2\pi$ and within the lower limit of distance $2\cdot\omega_z$, but the probabilities $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ are always equally distributed and are not subject to changes with confidences given by the *criteria 3a-3d*. For example, I take the measured values N = 0.14, D = $4.5\cdot10^{-12}$ [m^2/s], $q = \xi = \omega_{xy;blue} = 0.159\cdot10^{-6}$ [m], $2\cdot\omega_z/unit = 2\cdot\dot{\omega}_z$ = $1.91\cdot10^{-6}$, and t = 60 [s] (measurement time) [47] and obtain the following set of confidences according to *criteria 3a-d*: 0.978, 0.860, 0.999, 0.999. Actually, there is an entire family of the SELFSAME MOLECULE LIKELIHOOD ESTIMATOR $0 \le P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \le 1$ that depends on whether the diffusive spreading of a single molecule $P\left(\xi \le q < +\infty\right)$ is taken, e.g., three-dimensional in solution or two-dimensional in a membrane (see also ref. [45]).

Physical Model Proposed for the First Time to Quantify Single Molecule Reentries

I have been working on problems and opportunities associated with very dilute solutions. The molecule in the 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 here.

The system is not set up to have systemic drift or convection (no external forces) in the bulk phase (solution, live cell). The molecules of the same kind in the bulk phase have approached, for example, a local, macroscopic steady state. Let us further assume that there are motional states (transitions), which result in molecule number fluctuations within the probe region and are caused by reentries of the selfsame molecule. Therefore, I shall distinguish between several meaningful and non-meaningful reentry cases. A valuable aid to the physical mechanism of single-molecule reentries is the stochastic fluctuation analysis of Brownian motion. This is based on the fact that the random vibrations of the liquid's molecules batter the larger particles. The resultant motion reveals the size of the molecules and the molecule number in the confocal probe region. If we change the number of molecules in the experiments, for example, by dilution or more interestingly by the kinetics of the biochemical or immu-

nological process under study, we come to the situation where the measured value for the molecule number in the probe region becomes smaller than one. This experimental situation is the starting point of the analysis. The mathematical expressions of the physical single-molecule reentry mechanism shall be first obtained by probing its Brownian trajectories. A biochemical or chemical system fluctuates at the single-molecule level, but if we decrease the number of molecules in the bulk phase, we measure the number fluctuations from just one ("selfsame") molecule only.

I consider three different time moments s, t and $t + \Delta t$ of the above characterized stochastic trajectory of a single molecule $\{X(q,t) = X(t), t \geq 0\}$, for which the random variable $X(t)$ has the values $X(s) = i$ and $X(t + \Delta t) = n$. The selfsame molecule has four natural possibilities to move at random from the thermodynamic state i to the state n (Fig. **3**):

(i) Meaningful reentries. The selfsame molecule is inside the confocal probe region ΔV and diffuses out from motional state i to the motional state $n - 1$ outside the probe region. Then it diffuses in from the motional state $n - 1$ to n. Hence, the random variable $X(t)$ makes the transition from $X(s) = i$ to $X(t) = n - 1$ during the time interval $[s, t)$ and afterwards the transition from $X(t) = n - 1$ to $X(t + \Delta t) = n$ during the time interval $[t, t + \Delta t)$. These reentries (transitions) contribute to the fluorescence intensity fluctuations in the SPSM-FCS (single-phase single-molecule fluorescence auto- or two-color cross-correlation spectroscopy) experiments. They are the number of reentries that results in a useful burst size.

(ii) Meaningful reentries. The selfsame molecule is just outside the confocal probe region ΔV and diffuses in from motional state i to the motional state $n + 1$ inside the probe region. Then it diffuses out from the motional state $n + 1$ to n. Hence, the random variable $X(t)$ makes the transition from $X(s) = i$ to $X(t) = n + 1$ during the time interval $[s, t)$ and afterwards the transition from $X(t) = n + 1$ to $X(t + \Delta t) = n$ during the time interval $[t, t + \Delta t)$. These reentries (transitions) contribute to the fluorescence intensity fluctuations in the SPSM-FCS (single-phase single-molecule fluorescence auto- or two-color cross-correlation spectroscopy) experiments. They are the number of reentries that results in a useful burst size.

(iii) Non-meaningful reentries that also include no reentries. The selfsame molecule is inside the confocal probe region ΔV and diffuses from motional state i to the motional state n inside the probe region. For example, the molecule sits at the inner border of the probe volume and crosses in and out by many reentries but none of them are meaningful because the selfsame molecule remains in the motional state n. Of course, there is the other possibility that the selfsame molecule is just outside the confocal probe region ΔV and diffuses from motional state i to the motional state n outside the probe volume. Hence, the random variable $X(t)$ makes the transition from $X(s) = i$ to $X(t) = n$ during the time interval $[s, t)$, but afterwards it remains in $X(t) = n$ during the time interval $[t, t + \Delta t)$.

(iv) Meaningful reentries. The selfsame molecule is either inside or outside the confocal probe region ΔV and diffuses from motional state i to the motional state $n \pm a$ with $|a| > 1$ either outside or inside the probe region. Then, it diffuses back from motional state $n \pm a$ to n. Hence, the random variable $X(t)$ makes the transition from $X(s) = i$ to $X(t) = n \pm a$ during the time interval $[s, t)$ and afterwards the transition from $X(t) = n \pm a$ to $X(t + \Delta t) = n$ during the time interval $[t, t + \Delta t)$.

The physical model described above is analytically solved in the section **Appendix** and holds for the one-, two- (membrane), or three- (solution, live cell) dimensional Brownian motion.

RESULTS AND DISCUSSION

The Reentry Probabilities $p_n(t)$

For clarity of presentation of the stochastic fluctuation analysis, the models considered in this original article are limited to special solutions of the physical process of single-molecule reentries in the probe region, i.e. of the system of differential equations (A9). Let us take the motional rates of the selfsame molecule as follows

$$\left.\begin{aligned} & k_n(t) = k = const \\ & and \\ & g_n(t) = 0 \end{aligned}\right\} \tag{6}$$

Hence,

$$\frac{d}{dt} p_n(t) = k p_{n-1}(t) - k p_n(t), \; n \geq 1, \tag{7a}$$

$$\frac{d}{dt} p_0(t) = -k p_0(t), \tag{7b}$$

and the initial conditions

$$\left.\begin{aligned} & p_0(0) = 1 \\ & p_n(0) = 0 \end{aligned}\right\} \tag{7c}$$

With the ansatz

$$p_n(t) = e^{-kt} u_n(t), \tag{8a}$$

I obtain

$$\frac{d}{dt} u_n(t) = k u_{n-1}(t), \; n > 1, \tag{8b}$$

$$\frac{d}{dt} u_1(t) = k \;, \; u_0(t) = 1, \tag{8c}$$

and the initial conditions

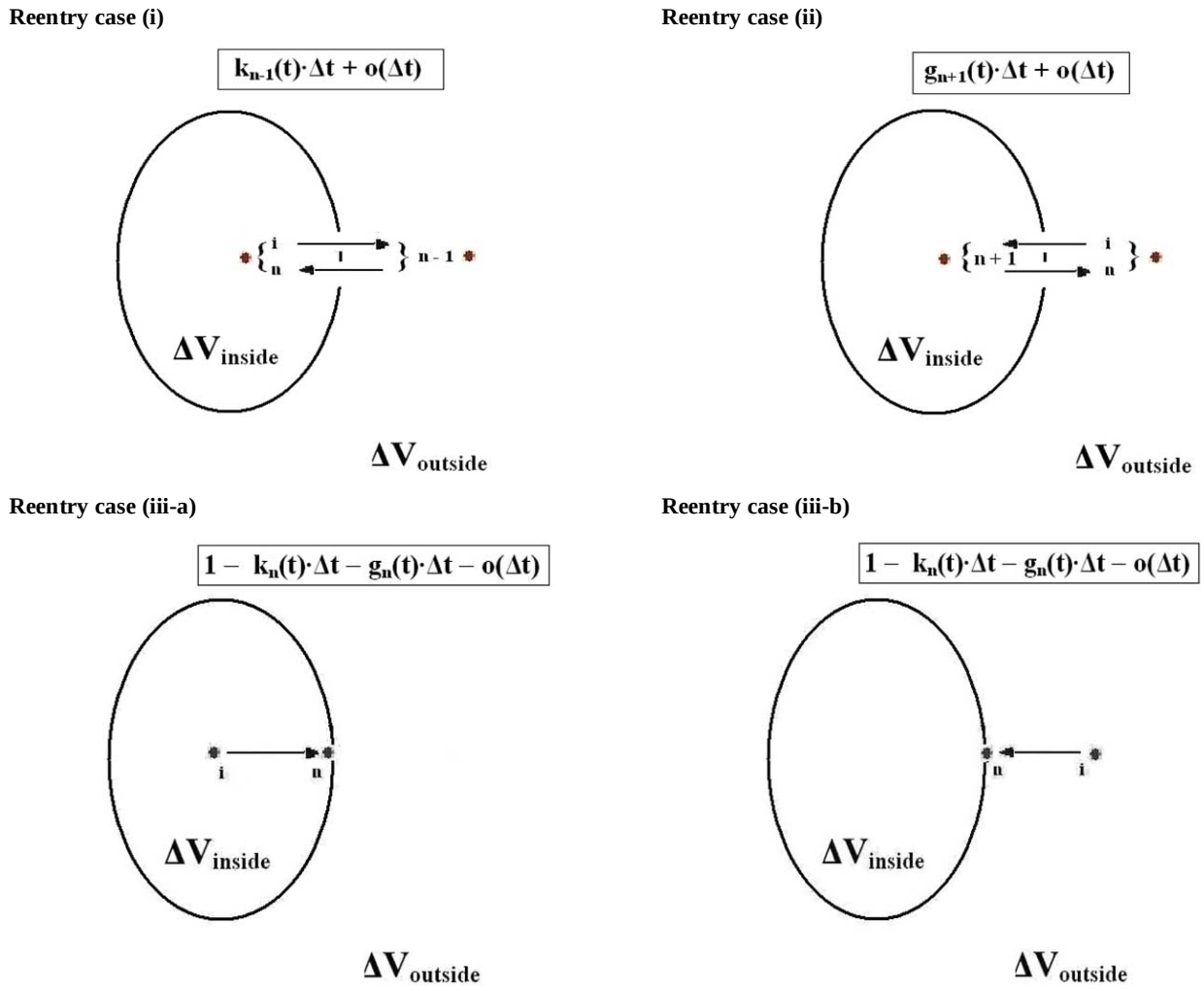

Fig. (3). Schematic view of the reentry cases (i) to (iii) for just a single molecule.

$$\left.\begin{aligned} u_0(0) &= 1 \\ u_n(0) &= 0 \end{aligned}\right\} \tag{8d}$$

From this the function $u_n(t)$ is developed as follows

$$u_1(t) = kt, u_2(t) = \frac{(kt)^2}{2!}, \ldots, u_n(t) = \frac{(kt)^n}{n!} \quad , \tag{9}$$

and hence

$$p_n(t) = \frac{(kt)^n}{n!} e^{-kt} = \frac{(u_1)^n}{n!} e^{-u_1} \quad , n = 0, 1, 2, \ldots \tag{10}$$

The way to satisfy the condition (10) is for u_1 to be the probability of finding the single fluorescent molecule in the confocal probe region ΔV

$$u_1 = P(X \leq 1; C = \Pi \cdot T) = N \text{ , with } 0 < N < 1 \text{ ;} \tag{11}$$

(see ref. [44] for the derivation of the probability of finding a molecule in the light 'cavity'). Π is the constant or average detection probability per unit time T of the experiment. It depends on the molecular properties of the fluorescent molecule and instrumental parameters of the measuring device. N is the time-averaged number of specific fluorescent molecules in the confocal probe region. N becomes $N < 1$ below a specified molar concentration of the bulk phase, which is the 'critical' molar concentration, and then equals the Poisson probability of finding a single molecule in the observation volume. C is the average frequency number (mean value) of specific fluorescent molecules in the probe region. With the specified characteristic time parameter $t = \tau_{diff} = const$ for a given size of the confocal probe region ΔV, which is the measurable diffusion time of the single fluorescent molecule, I first obtain the straightforward analytical solution to the time constant k of the mean value and the variance (u_1) of the reentry probabilities $p_n(t)$

$$k = \frac{N}{\tau_{diff}} \quad , \tag{12}$$

where $k_n(t) = k$ and $g_n(t) = 0$. The quantities on the right hand side of the found equation (12) are directly measured by the SPSM-FCS (single-phase single-molecule fluorescence auto- or two-color cross-correlation spectroscopy) experiments. For example (Fig. (**1**), middle part: N = 0.0085),

$k = \frac{0.0085}{26 \cdot 10^{-6}\ [s]} = 326.92\ [s^{-1}]$. Thus, the selfsame molecule reenters the probe region 326.92 times per second [17, 46, 48].

Non-Meaningful Reentries $p_{n,n}$

Now, I evaluate the portion of non-meaningful reentries, which also includes no reentries. If the selfsame molecule does not diffuse out or in the confocal probe region ΔV then there is a non-meaningful molecular situation and therefore no temporal fluctuations in the fluorescence intensity traces of that molecule. For example, the selfsame molecule sits at the border of the probe region and crosses in and out. To solve the problem, I start with the equation (A3). With the motional rates $k_n(t) = k$ and $g_n(t) = 0$ (equation (6)) for the selfsame molecule, I get the portion of molecular transitions by which the selfsame molecule remains in its motional state n

$$p_{n,n}(t, t + \Delta t) = 1 - k\Delta t - o(\Delta t). \tag{13}$$

With the equations (A8), the molecular transitions $i = l =$ n can be written as

$$p_n = p_n(0) p_{n,n}(t) \tag{14a}$$

with the initial conditions

$$p_n(0) = \begin{cases} 1 \\ 0 \end{cases} \tag{14b}$$

and thus,

$$p_n(0) = 1 \cdot p_{n,n}(t), \text{ or} \tag{14c}$$

$$p_n(0) = 0 \cdot p_{n,n}(t) = 0\ . \tag{14d}$$

Hence, the reentry probability for non-meaningful reentries including no reentries is

$$p_n = p_{n,n} = p_{n,n}(t, t + \Delta t) = 1 - k\Delta t - o(\Delta t). \tag{15}$$

For $\lim_{\Delta t \to 0} \frac{o(\Delta t)}{\Delta t} = 0$ and $\lim_{\Delta t \to 0} \left[\frac{o(\Delta t)}{\Delta t} \cdot \Delta t \right] = 0$, it follows

$$p_{n,n} = 1 - kt\ . \tag{16}$$

With the specified characteristic time parameter $t = \tau_{diff} = const$ for a given size of the confocal probe region ΔV, $p_{n,n}$ is written as

$$p_{n,n} = 1 - k\tau_{diff} = 1 - N\ . \tag{17}$$

For example, $p_{n,n} = 1 - 0.0085 = 0.9915$. The fraction of non-meaningful reentries is 99.15%.

Meaningful Reentries $p_{\overline{n,n}}$ and Meaningful Time T_m

The equation (17) implies that the fraction of meaningful reentries is thus

$$p_{\overline{n,n}} = 1 - p_{n,n} = k\tau_{diff} = N\ . \tag{18}$$

Hence, the meaningful time T_m in the confocal probe volume ΔV is

$$T_m = \frac{1}{k} = \frac{\tau_{diff}}{N} = \frac{\tau_{diff}}{P(X \le 1; C = \Pi \cdot T)}\ . \tag{19}$$

Since

$$P(X \le 1; C = \Pi \cdot T) = N = P_1 = \frac{C^1 \cdot e^{-C}}{1!} \tag{20}$$

(see refs.[43] and [44-46, 50]), I obtain for the meaningful time

$$T_m = \frac{\tau_{diff}}{C \cdot e^{-C}}\ . \tag{21}$$

With the definition of the molar concentration c_m of other molecules of the same kind in the bulk phase (e.g. in the bulk solution), C is given by

$$C \equiv c_m N_A \Delta V\ , \tag{22}$$

where N_A is the Avogadro's number of $6.023 \cdot 10^{23}$ [mol^{-1}]. Substituting equation (22) into the equation (21) yields the concentration dependence of the meaningful time T_m that one can study the selfsame single molecule in the confocal probe region ΔV of a given size

$$T_m = \frac{\tau_{diff}}{c_m N_A \Delta V \cdot \exp\{-c_m N_A \Delta V\}}\ . \tag{23a}$$

As can be inferred from Table **1**, the less the molar concentration of molecules of the same kind in the bulk phase, e.g. the bulk solution, the larger the meaningful time in the probe volume is for the selfsame molecule. I first derived the relation

$$T_m \propto \frac{1}{c_m}\ . \tag{23b}$$

One needs a lower molar concentration to study bimolecular reactions of small molecules. On the other hand, if the diffusion time is large, i.e. a bigger molecule is studied, the meaningful time in the probe volume increases proportional to the size of the molecule as the relation

$$T_m \propto \tau_{diff} \tag{23c}$$

follows directly from the equation (23a) at a given size of the confocal probe region. This relationship equation (23c) is very useful for applications with biomolecules such as DNA or proteins. The theoretical model (23a) also yields the relation

$$T_m \propto \frac{1}{\Delta V}\ . \tag{23d}$$

The meaningful time is related inversely to the size of the confocal probe volume. The smaller the probe region, the longer the selfsame molecule can be observed.

Equation (23a) specifies the time parameter *t* in the *criteria 3a-d*.

Goodwin and co-workers first measured single-stranded mRNA and DNA at about three femtomolar bulk concentration ($3 \cdot 10^{-15}$M) by means of two-color fluorescence cross-correlation spectroscopy (FCCS) [53]. Földes-Papp and co-workers first measured double-stranded, genomic DNA at about thirty femtomolar ($30 \cdot 10^{-15}$M) bulk allele concentration by means of FCCS [54].

Chance that the Reentering Molecule is not the Original Molecule

As Function of the Meaningful Time T_m and the Molar Concentration c_m

According to the derived equations (1), (3)-(5) and (21), there is the random chance that the *reentering* molecule is *not* the original molecule [17, 31, 43-48, 50, 38, 39]. Now, I focus on the probability of such a molecular situation as function of the meaningful time T_m and the molar concentration c_m of the bulk phase. For this reason, I take the two-dimensional Poisson probability distribution of finding fluorescent molecules in the detection volume ΔV of the bulk phase. I consider the time moments $t \neq 0$ and $t = t + \Delta t$. The random variables $X(t,q) = X(t)$ and $X(t + \Delta t, q) = X(t + \Delta t)$, i.e. the trajectories of the molecules, specify the two-dimensional Poisson probability distribution. I look at the Brownian movement at two different time moments and evaluate the probability function for the molecules $\chi_t, \chi_{t+\Delta t} \geq 0$

$$p_\chi(t) = P\left(X(t) = \chi_t, X(t+\Delta t) = \chi_{t+\Delta t}\right) \quad , \tag{24}$$

$$t < t + \Delta t, \chi_t \leq \chi_{t+\Delta t} \ .$$

Hence

$$p_\chi(t) = P\left(X(t) - X(0) = \chi_t, X(t+\Delta t) - X(t) = \chi_{t+\Delta t} - \chi_t\right)$$

$$= P\left(X(t) = \chi_t\right) \cdot P\left(X(t+\Delta t) - X(t) = \chi_{t+\Delta t} - \chi_t\right). \tag{25}$$

I get

$$p_\chi(t) = \frac{(\Pi \cdot t)^{\chi_t} \cdot \exp\{-\Pi \cdot t\}}{\chi_t!} \cdot \frac{(\Pi \cdot \Delta t)^{\chi_{t+\Delta t} - \chi_t} \cdot \exp\{-\Pi \cdot \Delta t\}}{(\chi_{t+\Delta t} - \chi_t)!} \ . \tag{26}$$

The two-molecule scenario $\chi_t = 1, \chi_{t+\Delta t} = 2$ results in

$$p_{1,2}(t) = (\Pi \cdot t) \cdot \exp\{-\Pi \cdot t\} \cdot (\Pi \cdot \Delta t) \cdot \exp\{-\Pi \cdot \Delta t\}. \tag{27}$$

With the specified time values $t = T$, $\Delta t = T_m = f\left(c_m, \tau_{diff}, \Delta V\right)$ as the meaningful time in the probe volume ΔV (equation (23a)) and with the equation (20), equation (27) yields

$$p_{1,2}(T_m) = N \cdot \Pi \cdot T_m \cdot e^{-\Pi \cdot T_m} \ . \tag{28}$$

Because we do not know the detection probability Π per time unit T of the experiment, I rearrange the equation (28). In the time $t = T + T_m$, the second molecule $\chi_{t+\Delta t} = 2$ enters the probe volume that now contains two molecules. With the Poisson probability P_2 of finding two molecules in the probe volume, I get from equation (28)

$$p_{1,2}(T_m) = 2 \cdot N \cdot P_2 \cdot \frac{1}{\Pi \cdot T_m} \ . \tag{29}$$

Table 1. Concentration dependence of the meaningful time T_m, which is derived for the first time (equation (23a)) for a solution or a membrane. Exemplified for rhodamine green in solution at a diffusion time $\tau_{diff} = 26 \cdot 10^{-6}$[s] of a confocal probe volume $\Delta V = 0.2 \cdot 10^{-15}$[L]. The finite contributions of the Poisson events (see Fig. 1) result in very short meaningful times in the nanomolar and picomolar concentration ranges of the bulk solution, whereas these contributions become negligibly small in femtomolar bulk solutions yielding meaningful times in the 60-second range and even longer. The results aim not to just measure highly diluted target solutions of femtomolar (10^{-15} M) bulk concentration or less [52]; the high dilution is merely a prerequisite to measure just a single molecule and its kinetics as well as dynamic interactions within an acceptable time frame underlying the new 'meaningful time concept'.

Molar concentration of the bulk phase c_m [mol / L]	Experimental $N < 1$ value that can be measured	Corresponding C value	Meaningful time T_m in the confocal probe volume [s]
10^{-9} (nM)	0.107	0.121	$2.4347 \cdot 10^{-4} \approx 0.2$ [ms]
10^{-10}	$1.19 \cdot 10^{-2}$	$1.21 \cdot 10^{-2}$	$2.1846 \cdot 10^{-3} \approx 2$ [ms]
10^{-12} (pM)	$1.21 \cdot 10^{-4}$	$1.21 \cdot 10^{-4}$	$2.1587 \cdot 10^{-1} \approx 215$ [ms]
$21.6 \cdot 10^{-15}$ (fM)	$2.6 \cdot 10^{-6}$	$2.6 \cdot 10^{-6}$	$9.99 \approx$ 10 [s]
$3.6 \cdot 10^{-15}$ (fM)	$4.3 \cdot 10^{-7}$	$4.3 \cdot 10^{-7}$	$0.5991 \cdot 10^{2} \approx$ 1 [min]

Because the system is not set up to have systemic drift or convection (no external forces), the average molecule number does not change during the time interval $t = T + T_m$. Thus, I arrive at the following expressions

$$p_{1,2} = 2 \cdot N \cdot P_2 \cdot \frac{1}{C} , \quad (30a)$$

and

$$p_{1,2} = N \cdot C \cdot e^{-C} . \quad (30b)$$

Under the experimental conditions $C << e^{-C}$, C becomes $C \cong N$ (ref. [43]) and I finally get

$$p_{1,2} \cong N^2 \cdot e^{-N} . \quad (30c)$$

The equation (24) also holds true for multiple time values $\Delta t = \lambda \cdot T_m$ with the parameter λ. By using equation (28), I obtain the analytical expressions for the time and concentration dependences of $p_{1,2}$ at a given size of the confocal probe volume

$$p_{1,2}(T_m, \lambda) = N \cdot \lambda \cdot (\Pi \cdot T_m) \cdot e^{-\lambda \cdot (\Pi \cdot T_m)} , \ \lambda = 1,2,3,\ldots , \quad (31a)$$

$$p_{1,2}(T_m, \lambda) = \lambda \cdot N \cdot C \cdot e^{-\lambda \cdot C} , \quad (31b)$$

and

$$p_{1,2}(T_m, \lambda) \cong \lambda \cdot N^2 \cdot e^{-\lambda \cdot N} . \quad (31c)$$

Since the novel ideas presented in this subsection are important, let us summarize them. $p_{1,2}(T_m, \lambda)$ is the probability that the reentering molecule is a different molecule than the original molecule, where Δt is taken as the meaningful time. A visualization of the probability $p_{1,2}(T_m, \lambda)$, which depends on the meaningful time $T_m = f(c_m, \tau_{diff}, \Delta V)$, is shown in Fig. (**4**). Fig. **4** gives a graphical meaning to the idea of $p_{1,2}(T_m, \lambda)$. To study just one molecule in the probe region, i.e. the selfsame molecule, we want $p_{1,2}(T_m, \lambda)$ to be very close to zero. As I let $\Delta t = \lambda \cdot T_m$ on the curve become larger, $p_{1,2}(T_m, \lambda)$ approaches zero, but in between, I find a local maximum at $\Delta t = 9 \cdot T_m = 2.19$ [ms]. Here is where the idea of $p_{1,2}(T_m, \lambda)$ becomes important. This should not be paradoxical, since I find the maximum value of $p_{1,2}(T_m, \lambda)$ in some 'small' time region at $\lambda = \frac{1}{N}$.

As Function of the Time from Last Entry

Next, I want to know the probability that the reentering molecule is a different molecule than the original molecule, where the increment Δt is the time from last entry. To start with my molecular analysis I look at an example. For $n = 0$ in the equation (10), I get

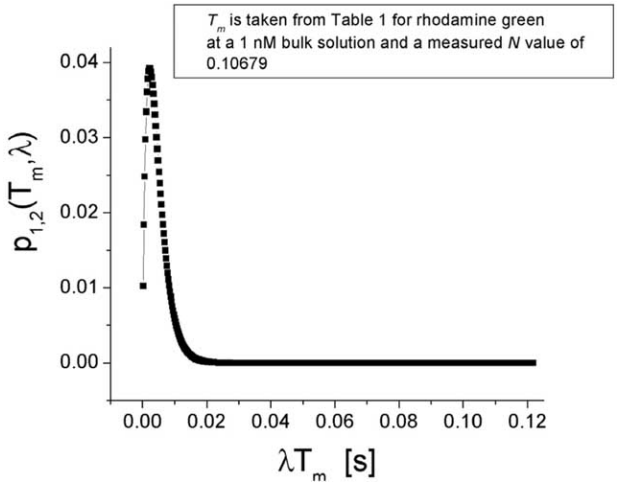

Fig. (4). Probability that the reentering molecule is not the original molecule as a function of the meaningful time T_m and multiple of it $\lambda \cdot T_m$.

$$p_0(t) = e^{-N} . \quad (32)$$

This is the probability of no reentering event, e.g. there is just no molecule close to the border of the probe region diffusing out and in. It is important not to confuse t with Δt. The equation (32) shows that t and Δt have now different meanings. I sum up all the reentering events being a positive integer, which happen with the original molecule from last entry

$$p_{>0}(\Delta t) = \sum_{i=1}^{+\infty} p_i(\Delta t) = \sum_{i=1}^{+\infty} \left[e^{-k \cdot \Delta t} \cdot u_i(\Delta t) \right]$$

$$= \sum_{i=1}^{+\infty} \left[e^{-k \cdot \Delta t} \cdot \frac{(k \cdot \Delta t)^i}{i\,!} \right] = e^{-k \cdot \Delta t} \cdot \left(e^{k \cdot \Delta t} - 1 \right)$$

$$= 1 - e^{-k \cdot \Delta t} . \quad (33)$$

The subscript >0 below $p = f(\Delta t)$ is to remind us that more than no event occurs in the waiting time Δt. The equation (33) is developed as

$$p_{>0}(\Delta t) = 1 - \left[1 - \frac{k \cdot \Delta t}{1} + \frac{(k \cdot \Delta t)^2}{2!} - \ldots \right] = k \cdot \Delta t + o(\Delta t) \quad (34)$$

For $\lim_{\Delta t \to 0} \frac{o(\Delta t)}{\Delta t} = 0$ and $\lim_{\Delta t \to 0} \left[\frac{o(\Delta t)}{\Delta t} \cdot \Delta t \right] = 0$, it follows

$$P(\Delta t \le t) = p_{>0}(\Delta t) = k \cdot \Delta t . \quad (35a)$$

In this form, we see that equation (33) is the probability distribution of the stochastic variable 'waiting time for the

next entry' or, generally speaking, 'time from last entry' of the original molecule. *P* designates the relative frequency of the stochastic variable time from last entry. Hence

$$k \cdot \Delta t = \frac{N}{\tau_{diff}} \cdot \Delta t \ , \tag{35b}$$

and

$$\Delta t = \frac{\tau_{diff}}{N} = \frac{1}{k} = T_m \ . \tag{35c}$$

Thus, the 'waiting time for the next entry' or the 'time from last entry' of the original molecule depends upon the meaningful time T_m. In addition, the equation (35c) suggests that the time from last entry is regarded as the meaningful time $T_m = f\left(c_m, \tau_{diff}, \Delta V\right)$. This turns out to be

$$\Delta t = \frac{\tau_{diff}}{c_m N_A \Delta V \cdot \exp\left\{- c_m N_A \Delta V\right\}} \ . \tag{35d}$$

The relationship (35d), which is now first introduced, is characterized by the differential

$$\lim_{dt=\Delta t \to 0} \frac{dp_{>0}}{\Delta p_{>0}} = 1 \tag{36}$$

for $p_{>0} = f\left(\Delta t\right)$, as studied with the equations (35). Although $\Delta p_{>0}$ and $dp_{>0}$ are different, $dp_{>0}$ is very close to $\Delta p_{>0}$ for sufficiently small Δt. I, therefore, use $\Delta p_{>0}$ and $dp_{>0}$ interchangeably when it is understood that the limit will be taken or that the result (equation (35d)) approximates the differential equations (A9). Furthermore, even if I do not take the limit $\Delta t \to 0$, $dp_{>0}$ is almost the same as $\Delta p_{>0}$. Thus,

$$\frac{dP\left(\Delta t \le t\right)}{dt} = \frac{d}{dt} p_{>0}\left(\Delta t\right) = k = \frac{N}{\tau_{diff}} \ . \tag{37}$$

The equation (37) is interpreted as the density $k = \frac{N}{\tau_{diff}}$ of the stochastic variable time from last entry of the selfsame (original) molecule. The probability that Δt is between two time moments $t_1 < t_2$ is determined by the motional rate *k* found for the first time.

CONCLUSIONS

Molecular analysis in terms of single molecules is becoming a necessity in new tools for experimental and molecular medicine, medical genomics and proteomics, biochemistry, molecular biology and immunology [1]. The achieved detection sensitivity in analytical chemistry allows real-time measurements of a single molecule in solution or membrane or within a live cell [1-7]. Modern diagnostic assays are generally capable of detecting specific molecules below ten.

Fluorescence Correlation Spectroscopy (FCS) and two-color Fluorescence Cross-Correlation Spectroscopy (FCCS) provide 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 be distinguished easily from the small 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 an autocorrelation or two-color crosscorrelation function that is the average of thousands of molecules. Properties of single molecules are not obtained.

A valuable aid to the physical mechanism of single-molecule reentries is the stochastic fluctuation analysis of Brownian motion. For the first time, I derived/found exact analytical relationships for the physical process of entry and reentry of just one molecule in the confocal probe region. The system is not set up to have systemic drift or convection (no external forces) in the bulk phase (solution, live cell, membrane). This is important for studying single-molecule interactions in single phases, e.g., solution and membrane, without external forces. The single-molecule events in the confocal probe volume (detection volume) are Poisson distributed as a function of the average frequency number of molecules, *C*, the presence of a single fluorescent molecule in the minute detection volume, the molar concentration, c_m, of the bulk phase, and a specified molar bulk concentration, c_m^*, as the upper limit for a meaningful probabilistic assignment ('critical' concentration). For a confocal probe volume $\Delta V = 0.2 \cdot 10^{-15}[L]$, c_m^* is roughly one nanomolar. Because of the introduction of this distribution, one has a discrete, infinitely stretched population of molecules, whose statistic samples must obey exactly this condition. There are one-molecule events, two-molecule events, three-molecule events, and so forth with certain Poisson probabilities. All these Poisson events contribute to the molecule number fluctuations of just one molecule in the probe regions. These finite contributions result in very short meaningful times in the nanomolar and picomolar concentration ranges of the bulk phase as exemplified for a solution (see Table **1**), whereas these contributions become negligibly small in femto- and attomolar bulk concentrations yielding meaningful times in the 60-second range and even longer (Table **1**). The experiments performed so far [17, 31, 43-48, 50, 54, 53] and the theory are consistent within the assumptions made.

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APPENDIX

The transition probabilities for all reentry cases (i) to (iv) given in the section Theory and Methods are determined by the motional rates *k* and *g* of the single molecule:

(i) $p_{i,n-1}(s,t)$, $p_{n-1,n}(t,t+\Delta t) = k_{n-1}(t)\Delta t + o(\Delta t)$; (A1)

(ii) $p_{i,n+1}(s,t)$, $p_{n+1,n}(t,t+\Delta t) = g_{n+1}(t)\Delta t + o(\Delta t)$; (A2)

(iii) $p_{i,n}(s,t)$, $p_{n,n}(t,t+\Delta t) = 1 - k_n(t)\Delta t - g_n(t)\Delta t - o(\Delta t)$; (A3)

$o(\Delta t)$ is given by

(iv) $p_{i,n\pm a}(s,t)$, $p_{n\pm a,n}(t,t+\Delta t) = o(\Delta t)$. (A4)

The reentries do not depend on each other. With the equations (A1), (A2), (A3) and (A4), I obtain for the four possible reentry cases

$$p_{i,n}(s,t+\Delta t) = k_{n-1}(t)p_{i,n-1}(s,t)\Delta t + g_{n+1}(t)p_{i.,n+1}(s,t)\Delta t + \left[1 - k_n(t)\Delta t - g_n(t)\Delta t\right]p_{i,n}(s,t) + o(\Delta t). \quad \text{(A5)}$$

From the equation (A5) it follows immediately

$$\frac{p_{i,n}(s,t+\Delta t) - p_{i,n}(s,t)}{\Delta t} = k_{n-1}(t)p_{i,n-1}(s,t) + g_{n+1}(t)p_{i,n+1}(s,t) - \left[k_n(t) + g_n(t)\right]p_{i,n}(s,t) + \frac{o(\Delta t)}{\Delta t} \quad \text{(A6)}$$

Under the conditions $\Delta t \to 0$ and $\lim_{\Delta t \to 0} \frac{o(\Delta t)}{\Delta t} = 0$, the equation (A6) is written as

$$\frac{\partial p_{i,n}(s,t)}{\partial t} = k_{n-1}(t)p_{i,n-1}(s,t) + g_{n+1}(t)p_{i,n+1}(s,t) - \left[k_n(t) + g_n(t)\right]p_{i,n}(s,t), \text{ for } n \geq 1; \quad \text{(A7a)}$$

I take $n = 0$ and get

$$\frac{\partial p_{i,0}(s,t)}{\partial t} = g_1(t)p_{i,1}(s,t) - k_0(t)p_{i,0}(s,t), \quad \text{(A7b)}$$

since the term $k_{-1}(t)$ vanishes and $g_0(t) = 0$, otherwise the value −1 appears. Because the total probabilities

$$p_l(t) = P(X(t) = l) \quad \text{(A8a)}$$

are defined by

$$p_l = \sum_i p_i(0)p_{i,l}(t) \quad \text{(A8b)}$$

for $0 \leq p_{i,l}(t)$, I obtain the system of differential equations for $p_n(t)$ with $n \geq 0$

$$\frac{d}{dt}p_n(t) = k_{n-1}(t)p_{n-1}(t) + g_{n+1}(t)p_{n+1}(t) - \left[k_n(t) + g_n(t)\right]p_n(t), \; n \geq 1, \quad \text{(A9a)}$$

$$\frac{d}{dt}p_0(t) = g_1(t)p_1(t) - k_0(t)p_0(t), \; n = 0. \quad \text{(A9b)}$$

The initial conditions are

$$p_n(0) = \begin{cases} 1 \\ 0 \end{cases}. \quad \text{(A9c)}$$

REFERENCES

[1] Arnaud, C.H. (2007) *Chem. Eng. News*, **85** (23), 13-17.
[2] Cohen, A.E.; Moerner, W.E. (2007) *Proc. Natl. Acad. Sci. USA*, published online, May 11, 2007.
[3] Moerner, W.E. (2007) *Proc. Natl. Acad. Sci. USA*, in press.
[4] Moerner, W.E. (2006) *Nat. Methods*, **3**, 781-782.
[5] Elf, J.; Li, G.-W. and Xie, X.S. (2007) *Science*, **316**, 1191-1194.
[6] Kim, S.; Blainey, P.C.; Schroeder, C.M. and Xie, X.S. (2007) *Nat. Methods*, **4**, 397-399.
[7] Rasnik I.; McKinney, S.A. and Ha, T. (2006) *Nat. Methods*, ***3***, 891-893.
[8] Nir, E.; Michalet, X.; Hamadani, K.; Laurence, T.A.; Neuhauser, D.; Kovchegov, Y. and Weiss, S. (2006) *J. Phys. Chem. B*, **110**, 22103-22124.
[9] Kapanidis, A.N.; Margeat, E.; Ho, S.O.; Kortkhonjia, E.; Weiss. S. and Ebright, R.H. (2006) *Science*, **314**, 1144-1147.
[9] Shav-Tal, Y.; Darzacq, X.; Shenoy, S.M.; Fusco, D.; Janicki, S.M.; Spector, D.L. and Singer, R.H. (2004) *Science*, **304**, 1797-1800.
[10] Zhang, J.; Campbell, R.E.; Ting, A.Y. and Tsien, R.Y. (2002) *Nat. Rev. Mol. Cell Biol.*, **3**, 906-918.
[11] Földes-Papp, Z.; Kinjo, M. (2001) *Fluorescence Correlation Spectroscopy - Theory and Applications*, (Rigler, R.; Elson, E.L. Eds.) Springer Series in Physical Chemistry, Volume 65, Springer, Boston, pp. 25-64.
[12] Földes-Papp, Z.; Angerer, B.; Ankenbauer, W.; Baumann, G.; Birch-Hirschfeld, E.; Björling, S.; Conrad, S.; Hinz, M.; Rigler, R.; Seliger, H.; Thyberg, P. and Kleinschmidt, A.K. (1998) *Fractals in Biology and Medicine - Vol. 2*, (Losa,G.A.; Merlini, D.; Nonnenmacher, T.F.; Weibel, E.R., Eds.), Birkhauser, Boston, pp. 238-254.
[13] Földes-Papp, Z.; Angerer, B.; Thyberg, P.; Hinz, M.; Wennmalm, S.; Ankenbauer, W.; Seliger, H.; Holmgren, A. and Rigler, R. (2001) *J. Biotechnol.*, **86**, 203-224.
[14] Földes-Papp, Z.; Angerer, B.; Ankenbauer, W. and Rigler, R. (2001) *J. Biotechnol.*, **86**, 237-253.
[15] Stephan, J.; Dörre, K., Brakmann, S.; Winkler, T.; Wetzel, T.; Lapczyna, M.; Stuke, M.; Angerer, B.; Ankenbauer, W.; Földes-Papp, Z.; Rigler, R. and Eigen, M. (2001) *J. Biotechnol*., **86**, 255-267.
[16] Bjerneld, E.J.; Földes-Papp, Z.; Käll, M. and Rigler, R. (2002) *J. Phys. Chem. B*, **106**, 1213-1218.
[17] Földes-Papp, Z.; Baumann, G.; Kinjo, M. and Tamura, M. (2005) Encyclopedia of Medical Genomics & Proteomics, (Fuchs, J.; Podda, M, Eds.), Marcel Dekker, New York, USA, (distinguished article entry).
[18] Levene, M.J.; Korlach, J.; Turner, S.W.; Foquet, M.; Craighead, H.G. and Webb, W.W. (2003) *Science*, **299**, 682-686.
[19] Rotman, B. (1961) *Proc. Natl. Acad. Sci. USA*, ***47***, 1981-1991.
[20] Georgiou, G.N.; Morrison, I.E. and Cherry, R.J. (1989) *FEBS Lett.*, **250**, 487-492.
[21] Hirschfeld, T. (1976) *Appl. Opt.*, **15**, 2965-2966.
[22] Barak, L.S. and Webb, W.W. (1981) *J. Cell. Biol.*, **90**, 595-604.
[23] Barak, L.S. and Webb, W.W. (1982) *J. Cell. Biol.*, **95**, 846-852.
[24] Ghosh, R.N. and Webb, W.W. (1994) *Biophys. J.*, **66**, 1301-1318.
[25] Watson, J.V. and Walport, M.J. (1986) *J. Immunol. Methods*, **93** (2), 171-175.
[26] Dovichi, N.J.; Martin, J.C.; Jett, J.H. and Keller, R.A. (1983) *Science*, **219**, 845-847.
[27] Li, L.-Q. and Davis, L.M. (1993) *Rev. Sci. Instrum.*, **64**, 1524-1529.
[28] Nie, S.; Chiu, D.T. and Zare, R.N. (1994) *Science*, **266**, 1018-1021.
[29] Shera, E.B.; Seitzinger, N.K.; Davis, L.M.; Keller, R.A. and Soper, S.A. (1990) *Chem. Phys. Lett.*, **174**, 553-557.
[30] Soper, S.A. and Goodwin, P.M. (2005) *PITTCON 2005*, Orlando, FL.
[31] Földes-Papp, Z. and Baumann, G. (2004) *Curr. Pharm. Biotechnol.*, **5** (2), 163-172.
[32] Rigler, R. and Mets, U. (1992) *SPIE Proc. Soc. Int. Opt. Eng.*, **1921**, 239-248.
[33] Rigler, R.; Mets, U.; Widengren, J. and Kask, P. (1993) *Eur. Biophys. J.*, **22**, 169-175.
[34] Eigen, M. and Rigler, R. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 5740-5747.
[35] Basché, T.; Moerner, W.E.; Orit, M. and Wild, U.P., Eds. (1997) *Wiley-VCH, Weinheim.*
[36] Orrit, M. (2003) *Science*, **302**, 239-240.
[37] Kulzer, F. and Orrit, M. (2004) *Annu. Rev. Phys. Chem.*, **55**, 585-611.
[38] Andrec, M.; Levy, R.M. and Talaga, D.S. (2003) *J. Phys. Chem. A*, **107**, 7454-7464.
[39] Watkins, L.P. and Yang, H. (2004) *Biophys. J.*, **86**, 4015-4029.
[40] Lu, H.; Xun, L. and Xie, X.S. (1998) *Science*, **282**, 1877-1882.
[41] Edman, L.; Földes-Papp, Z.; Wennmalm, S. and Rigler, R. (1999) *Chem. Phys.*, **247**, 11-22.
[42] English, B.P.; Min, W.; van Oijen, A.M.; Lee, K.T.; Luo, G.; Sun, H., Cherayil, B.J.; Kour, S.C. and Xie, X.S. (2006) *Nat. Chem. Biol.*, **2** (2), 87-94.
[43] Földes-Papp, Z. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 11509-11514.
[44] Földes-Papp, Z. (2002) *Exp. Biol. Med.*, **227** (5), 291-300.
[45] Földes-Papp, Z. (2002) *Pteridines*, **13**, 73-82.
[46] Földes-Papp, Z. (2004) *J. Immunol. Meth.*, **286** (1-2), 1-11.
[47] Földes-Papp, Z. (2004) *J. Immunol. Meth.*, **286** (1-2), 13-20.
[48] Földes-Papp, Z. (2002) *J. Immunol. Meth.*, **260**, 117-124.
[49] Földes-Papp, Z. (2006) *Exp. Mol. Pathol.*, **80** (3), 209-218.
[50] Földes-Papp, Z. (2007) *Exp. Mol. Pathol.*, **82** (2), 147-155.
[51] Wakatsuki, T.; Fee, J.A. and Elson, E.L. (2004) *Curr. Pharm. Biotechnol.*, **5** (2), 181-189.
[52] Whitemore, C.D.; Olsson, U.; Larsson, R.A.; Hindsgaul, O.; Palcic, M.M. and Dovichi, N.J. (2007) *Electrophoresis*, in press (July 31).
[53] Nolan, R.L.; Cai, H.; Nolan, J.P. and Goodwin, P.M. (2003) *Anal. Chem.*, **75**, 6236-6243.
[54] Földes-Papp, Z.; Kinjo, M.; Tamura, M. and Birch-Hirschfeld, E. (2005) *Exp. Mol. Pathol.*, **78** (3), 177-189.