Measurements of Single Molecules in Solution and Live Cells Over Longer Observation Times Than Those Currently Possible: The Meaningful Time

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Abstract: Monitoring translational diffusion of single molecules in solution or in a living cell, particularly DNA and proteins brings valuable information unperturbed by interaction with an artificial surface. The article derives theoretical relationships for time intervals during which just one molecule in the effective probe region can be studied, the time we call meaningful time. This time is greater than the transit time of the molecule through the detection volume, as a single molecule will likely reenter the detection volume several times during measurement. From the infinitely stretched molecular Poisson distribution of single molecules or particles, we select the contribution of the selfsame molecule or particle by applying rules for choosing appropriate statistics for the single-molecule trajectories. The results point to a useful and sensitive predictive power of the derived relationships. The meaningful time relationships are the criteria to check the experimental single molecule data measured under conditions of normal and anomalous Brownian diffusion of the molecules of interest. At femtomolar bulk concentration, it would be possible to observe an individual molecule over a second time interval or longer during which biological processes — and not conformational biophysical changes — are just starting.

Keywords: Single molecule, Brownian motion, translational normal diffusion, translational anomalous diffusion, solution, live cell, theoretical relationships for time intervals during which just one molecule in the effective probe region can be studied, meaningful time, single molecule spectroscopy, single molecule imaging.

1. INTRODUCTION

One of the reasons for exploring single molecules in solution or in a live cell is to ask whether behavior differs from one copy of a molecule to the next copy. Each molecule experiences its own environment that in turn influences its behavior. In addition, some molecules, such as enzymes, have different behaviors at different times, which are smeared out if only ensemble averages are measured. For some biological questions, single-molecule methods are not just desirable, but a necessity. For instance, at the cellular level, gene expression is inherently a single-molecule problem. Any cell contains at most two copies of the genetic DNA, depending on the stage of the cell cycle. The number of copies of a particular messenger RNA is small. And many of the final products — the proteins — are produced in small quantities, sometimes only a single copy. However, it is difficult to detect interactions of a single molecule that diffuses freely in solution or even in a live cell. The intention of this original research article is to focus on physical relationships of the time intervals during which just one molecule in the effective probed region can be studied without immobilization (attachment, firm binding) or hydrodynamic flow. These characteristic time intervals we call meaningful time since they bring significant information on the molecular processes of just one molecule (one and the same molecule).

What is a theoretical model from which we may start? In 1990, the laboratory of Richard A. Keller in Los Alamos produced the first paper on the detection of individual molecules passing through the probe region one by one in a hydrodynamic flow with one dye molecule per second [1]. Since the publication of this paper, the area of single-molecule detection has grown tremendously with emphasis not just on observing single molecule signatures, but also on applying single-molecule detection to basic chemical and biological problems in applied and fundamental studies. Typically, single-molecule measurements, for example with a single enzyme molecule, are performed by absorption (immobilization, firm binding, attachment) of the molecule on a surface [2] or on intracellular structures [3] so that its behavior can be observed over a period of time. Miniaturization is also having a major impact on the sensitivity of detection 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). Such guides can provide zepto-liter observation volumes (1 zeptoliter = 10^{-21} L) [4]. For direct observation of single enzyme activity, enzymes are absorbed (immobilized, firmly bound, attached) 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 absorption (immobilization, firm binding, attachment). Most of the experimental single-molecule studies are combined with simulation results. In addition, theoretical and simulation methods were applied that operate directly on the photon arrival trajectories of a single mole-

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cule by evaluating a likelihood function without having to average over many molecules.

In the case of single molecules freely diffusing through a solution, Brownian motion causes these molecules to be randomly transported through small (e.g., femtoliter-sized) sampling volumes in three dimensions or in two dimensions (cell membrane). Translational normal diffusion in solution or translational anomalous diffusion in a live cell allows us to monitor molecules that are not immobilized or attached (firmly bound) to subcellular structures. As modeling of reentries is the primary purpose of this paper, the physical distinction of cases of meaningful and non-meaningful reentries was first made in ref. [5]. For example, if the measuring signal indicates that a molecule diffuses out of the observation volume ΔV and right back in, it is still likely the same molecule. The number of reentries of one and the same molecule that results in a useful burst size is meaningful and of interest. But if the molecule sits at the border of the probe region ΔV, crosses in and out, and therefore has many reentries, none of those reentries results in a useful burst size. Those reentries of one and the same molecule are non-meaningful. The entries of new molecules of the same kind into ΔV are simply called 'entries'. Ways to precisely quantify just one and the same single molecule are in high demand.

2. THEORETICAL RESULTS AND DISCUSSION

In the very dilute solutions associated with fluctuation spectroscopy and imaging, 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. The challenging goal is the theoretical and experimental demonstration of reentries of a single molecule or particle into the probe volume [5]. For the molecular number $N < 1$ measured in the probe volume ΔV, the physical meaning of $N < 1$ is probabilistic. Therefore, probabilistic approaches are used in order to derive the physical relationships; the derived formulas do not depend upon the geometry of ΔV or of the underlying translational diffusive process (three-dimensional diffusion in solution and in the cytoplasm/nucleoplasm of live cells, two-dimensional diffusion in membranes of live cells). The correctness of the theoretical approaches and the formulas was shown by simulation experiments based on classical mean-field approximation using the diffusion equation for ergodic normal motion of single 24-nm and 100-nm nanospheres [6] and for anomalous motion in live cells [7, 8].

2.1. Meaningful Time for Studying Just One Molecule in Solution and in Live Cells

On the second-time scale where biology takes place, data on fluorescence fluctuation traces are rare or they are not proven to be the signature from an individual molecule or particle. When single fluorescent molecules or single fluorescent bead particles in a liquid traverse the laser excitation volume, fluorescent photons are generated. The photon bursts can be analyzed for their brightness and duration, providing information on molecular size and identity, and the trajectory of the molecule and particle, respectively.

When measuring low-concentration targets (e.g. < 1nM), the detected fluorescence signals become digital since the average number of molecules

$$\langle C \rangle = C \tag{1}$$

is smaller than unity (<1.0) in the probe (observation) volume ΔV [6]. With the portion of meaningful reentries $p_{\overline{n,n}}$, the meaningful time T_m during which just one molecule in the effective probe region can be studied, is equated to

$$T_m = \frac{1}{k} = \frac{\tau_{diff}}{P(X \leq 1; C = \Pi \cdot T)} = \frac{\tau_{diff}}{N}, \tag{2}$$

where τ_{diff} is the translational diffusion time of the single molecule, N stands for the molecule number in the probe volume under the condition $N < 1$ that corresponds to the Poisson probability of finding a single molecule in the probe region (arrival of a single molecule), Π is the detection probability of a single molecule depending upon many parameters like molecular properties of the fluorescent molecule and instrumental parameters of the measuring device [5]. $\Pi \cdot T = C$ is the average number of molecules in the probe volume ΔV, i.e. the mean value of X. T is simply the measurement time. k is the mean value of the reentry probability $p_n(t)$. The main difference from other single-molecule Poisson analyses in the literature is that the final expressions no longer contain the detection probability Π; it cancels out. The same relationship (2) holds true for $\tau_{diff}(t)$ with T_m becoming simply $Tm = T_m(t)$ [7]. The above relationship (2) for time intervals during which just one molecule in the effective probe region can be studied — the time we call meaningful time — has particular relevance for single-molecule analysis in experimental and theoretical photon streams.

The meaningful-time relationship (2) considers how low the concentration of the bulk phase should be in order to obtain single-molecule events. Observables that have been successfully applied to single-molecule studies are the photon counting histogram, Förster resonance energy transfer (FRET), the lifetime of the excited state, or the emission spectrum of a fluorescent molecule. An alternative method based on real-time single-molecule imaging in live cells was proposed by Seisenberger *et al.* 2001 [9]. The visualization of virus trajectories projected onto the transmitted light images that were taken with an epifluorescent microscope setup for fluorescence detection generated fluorescence spots ΔV. Typically, a cell was infected with 10 to 1000 virus particles/molecules. However, only if the concentration of a molecule is small enough, each burst of emitted 'fluorescent' photons in the probe volume ΔV can be approximated by a single molecule or a virus single particle.

2.2. Stretched Distribution of Other Molecules of the Same Kind in Solution and in Live Cells

In solution or in a live cell, there might be a stretched distribution of other molecules of the same kind for the Poisson events $X \leq \chi = 1$ molecule, $X \leq \chi = 2$ molecules,

$X \leq \chi = 3$ molecules, and so forth, yielding for the molecule number fluctuation of just one molecule in the observation volume ΔV

$$T_m(t) = \frac{\tau_{diff}(t)}{c_m \cdot N_A \cdot \Delta V \cdot \exp\{-c_m \cdot N_A \cdot \Delta V\}} , \qquad (3)$$

where c_m is the molar concentration of other molecules of the same kind in the bulk phase and N_A is Avogadro's number of $[mol^{-1}]$.

Why should we care about these findings? The physical relationship (3) opens a clear window on the rapid advancements that are being made in cell and molecular biology. The equation (3) takes into account the concentration effects c_m of other molecules of the same kind and the size of the probe region ΔV in single molecule spectroscopy and imaging. For example, a concentration as low as 0.1 molecule per observation volume ΔV (N=0.1) may not be small enough for single-molecule Förster resonance energy transfer (FRET) efficiency measurements of molecules diffusing through a laser spot ΔV [10]. Exemplifying a translational diffusion time τ_{diff} of 26 μs and a probe volume ΔV of 0.2 femtoliter, the single-molecule observation time T_m is 0.2435 ms given by the meaningful-time relationship (2). Thus, $N = 0.1$ is small enough for single-molecule Förster resonance energy transfer efficiency measurements of molecules with $\tau_{diff} = 26\mu s$ diffusing through a laser spot $\Delta V = 0.2 \cdot 10^{-15}$ L for the time interval of 0.2435 ms under the experimental conditions.

The chance that the reentering molecule is not the original molecule depends upon the waiting time for the next entry of another molecule of the same kind. The probability density function is the way to describe this behavior [5].

$$\frac{dP(\Delta t \leq t)}{dt} = \frac{d}{dt} p_{>0}(\Delta t) = k = \frac{1}{T_m} . \qquad (4)$$

T_m corresponds to the Markov waiting time for the next entry of a single molecule and a single particle, respectively, that is not the original one diffusing through a laser spot ΔV: Proceeding along the 3D-trajectory of a single molecule or a single particle, we show that the occurrence of non-meaningful reentries is much more likely than that of meaningful reentries underlying the random character of Brownian motion without systemic drift or convection [6-8]. Entries, meaningful reentries and non-meaningful reentries had different frequency, amplitude and time distributions under the different experimental conditions [6-8].

2.3. Estimated Errors in Measuring the Meaningful Time for Studying Just One Molecule in Solution and in Live Cells

Repeated measurements of a quantity always show slight variations due to the impossibility of reproducing highly accurate measurements exactly. The single-molecule observation time T_m is a function of directly measurable quantities but cannot be easily measured directly. The standard deviation of these measurements is a measure of the accuracy of the repeatability. Provided the variances var of $\tau_{diff}(t)$, c_m and ΔV are small, then

$$\text{var } T_m = \left(\frac{\partial T_m}{\partial \tau_{diff}}\right)^2 \cdot \text{var } \tau_{diff} + \left(\frac{\partial T_m}{\partial c_m}\right)^2 \cdot \text{var } c_m + \left(\frac{\partial T_m}{\partial \Delta V}\right)^2 \cdot \text{var } \Delta V . \quad (5)$$

For $\tau_{diff} = const$ and hence $T_m = const$,

$$\text{var } T_m = \frac{1}{c_m^2 \cdot N_A^2 \cdot \Delta V^2} \cdot \text{var } \tau_{diff} + \frac{\tau_{diff}^2}{c_m^4 \cdot N_A^2 \cdot \Delta V^2} \cdot \text{var } c_m + \frac{\tau_{diff}^2}{c_m^2 \cdot N_A^2 \cdot \Delta V^4} \cdot \text{var } \Delta V .$$

$$(6)$$

The standard deviation SD_{Tm} of T_m is

$$SD_{T_m} = \sqrt{\text{var } T_m} . \qquad (7)$$

SD_{Tm} is in the same unit as T_m, i.e. in seconds.

Almost all the papers about single molecules diffusing freely in solution or in a living cell at longer observation times than in the lower millisecond range are not true single molecule data. Those experimental data are averages over many molecules and they do not tell us anything about one molecule. This appears to be the situation in single molecule biophysics without immobilization (attachment, firm binding to subcellular structures) of molecules.

Present fluorescence fluctuation imaging and spectroscopy are not sensitive enough to decrease the bulk concentration of molecules down to the femtomolar concentration range. At femtomolar bulk concentration, it would be possible to observe an individual molecule over a second time interval or longer during which biological processes — and not conformational biophysical changes — are just starting. We were only able to go down to the lower picomolar concentration range of the dye being used [11, 12]. Of course, that also is an issue of the photophysical properties of the fluorescent probes used, but that is beyond the scope of this original research article. Right now, just one thing is really clear: there is a lot more interesting work waiting for us out there, and it is time to "get high on single molecule biophysics" [13].

3. CONCLUSIONS

There are two different perspectives with opposite outcomes. The first outcome is that there is the concentration effect per probe ΔV of other molecules of the same kind in the bulk phase for single-molecule spectroscopy and single-molecule imaging [14]. The meaningful time T_m gives the measurement time for studying just one molecule in solution and in live cells as the molecule diffuses through the detection volume and subsequently reenters to transit the detection volume again. The second outcome is that it is virtually impossible to study a specific "single molecule" under such conditions because of the random movement of molecules in and out of the sampling volume ΔV in solution, as concluded by Li *et al.* [15]. The different perspectives and outcomes have to do with the burden of evidence in single-molecule measurements and the way we are looking at the theory. All of this is explained by the theory, which is the starting point.

CONFLICT OF INTEREST

The author confirms that this article content has no conflicts of interest.

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