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Review

What it means to measure a single molecule in a solution by fluorescence fluctuation spectroscopy

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Abstract

Traditional methodologies in micro- and nanofluidics measure biological mechanisms as an average of a population of molecules as only their combined effect can be detected. Fluorescence fluctuation spectroscopy methods such as fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) are used as alternative experimental approaches in ultrasensitive analytics at the single-molecule level. However, what is the measurement time in which one is able to study just one single molecule in solution without immobilizing it? 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 bulk solution. This situation produces considerable concern, and experimental hypotheses differ according to which single-molecule detection 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. As will be seen in this article, it is worthwhile to obtain a correct form of the meaningful-time relationship through theoretical means. The new ideas are comprehensively presented, and this relationship is a new concept at this time. The meaningful time for studying just one molecule without immobilization specifies the time parameter in the selfsame molecule likelihood estimator. Possible users for this concept are those working in biotechnological applications dealing with gene technology. Furthermore, the concept is of interest for a great number of medical, pharmaceutical and chemical laboratories. It may serve as a foundation for further work in single-cell biology. It is suspected that heterogeneities play a much larger role inside the cell than in free solution — a perfect opportunity for single-molecule studies and, thus, a novel hypothesis regarding structure and dynamics of cellular networks is first presented for the minimal neurotrophin network model.

Keywords: Cutting-edge technologies; Microfluidics; Nanofluidics; Single-molecule analysis; Fluorescence fluctuation spectroscopy; Fluorescence correlation spectroscopy (FCS); Two-color fluorescence cross-correlation spectroscopy (FCCS); Single (solution)-phase single-molecule fluorescence auto- and two-color cross-correlation spectroscopy (SPSM-FCS); Individual molecule; Selfsame molecule likelihood estimators; Selfsame single-molecule regime; Meaningful time; Cellular network; Neurotrophin network; Neuron; Neurodegenerative disease

Contents

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Introduction

After studying the cell and its constituents and working all the way down to the genome and the proteome, scientists have generated huge amount of data during the last decade that can now be deciphered to shed light on the basic principles of life. Among the new technologies that are making this progress possible, the DNA chip is only one example that has helped address many molecular biology problems, such as rapid identification of genetic disorders (Földes-Papp et al., 2005a). This article is mainly based on a recently published collection of important review articles on the areas: "Hot topic: the way down from single genes and proteins to single molecules (Guest Editors: Z. Földes-Papp, J. Enderlein, J. Widengren, M. Kinjo)" in Current Pharmaceutical Biotechnology, Volume 4, number 6 (2003); Volume 5, numbers 1–3 (2004). For detailed discussion of the original papers, I refer to these contributions, which were written by leading specialists in fields that cover a wide range of current topics on biomedical and biotechnological aspects of target screening and characterization. Several articles survey the latest developments and future prospects of experimental cell and molecular biology and molecular medicine. They are devoted to discussing multidisciplinary topics at a level that will be useful to experimental scientists. I will try to convey the main trends and update some of these papers. For clarity, I will omit such controversies as have in the meanwhile been resolved. Should I have started this article from the top downward, beginning with medicine and clinical practice based on clinical ("empirical") thinking, where there is a serious error by failing to account accurately for gene or protein interactions? Alternatively, should I have proceeded from the bottom upward, beginning with single molecules and their behavior, which often seems to be different from that of the ensemble in biological systems? My answer is a compromise, working in both directions from the middle.

When all molecules of the same kind in a sample have the same property in the time domain, the sample is commonly described as being homogeneous with respect to this property. Heterogeneity refers to a sample containing molecules of the same kind with diverse property value. Now, what is the measurement time in which one is able to study a single molecule in solution without immobilizing it? The existing theories are inadequate since there is no prediction of the meaningful time in which one can study just one single molecule as a function of the concentration of other molecules of the same kind in the bulk solution. This situation produces considerable concern, and various experimental hypotheses have been proposed to explain the discrepancy, hypotheses that differ according to which single-molecule detection methods are thought to have greater validity. As will be seen in this article, it is worthwhile to obtain such a relationship through theoretical means. The new ideas are presented comprehensively and are not created by forcing sets of data into the wrong kinds of equations. The problem of establishing a valid meaningful-time relationship in confocal microscopy was ignored for several years because the existing equations seemed adequate and, perhaps, various groups had been distracted by more exciting developments in single-molecule detection. As obvious as this solution may now seem, it is nonetheless a new concept. Possible users for this concept are those working in biotechnological applications dealing with gene technology (Földes-Papp et al., 2005b). And unlike relative gene expression information that can be obtained by microarrays, it may offer a way to make absolute measurements, which are what clinical analysis requires. Furthermore, the concept will be of interest to a great number of medical, pharmaceutical and chemical laboratories. It may also serve as a foundation for work in single-cell biology, i.e., the chemistry and dynamics of intracellular chemical reactions. It is suspected that

heterogeneities play a much larger role inside the cell than in free solution—a perfect opportunity for single-molecule studies.

Micro- and nanofluidics

The technical solutions for high mutation and recombination rate studies are power-low fluidic chips. However, the technologies in the laboratories are not available in a cheap-easy form. For example, all the devices for PCR-plus separation are constructed on the same principle; it does not matter what they are used for. A phase transition change valve makes a T or a T is made without a valve. Thermocycling is liquid (biochemical reactions). Detection and typing of a DNA sample are easily performed, for example, by UV detection with a restriction enzyme. Thus, the state-of-the-art in microfluidics is that different systems at different scales are available; almost any macrosystem can be micro, and there are many applications (see Vilkner et al., 2005; Yang et al., 2005). Microfluidics-based chemical separation and reaction schemes as constructed and reported are mostly performed in linear one-dimensional separations. Today's liquid chromatography separations are all at the upper pressure limit. How do we overcome the limit? For example, with flow counterbalance electrophoresis on chip. This novel direction in chemical/biochemical analytics offers the possibility of using nanofluidic functional elements such as electrodynamic separation in nanochannels (Zheng et al., 2004a,b) without any matrix (gel). The ionic transport in nano–micro devices is currently at the top-up experimental stage.

Another major trend is seen as manipulation versus observation. Most previous and current work is manipulative. It still does not tell us very much about the biology. Observation means a minimum of manipulation. A laminar flow is a very unnatural environment. Using diffusion (Brody and Yager, 1997) instead of hydrodynamic flow makes possible, for example, microchannel culturing and as a result the cloning of embryos on a large scale.

The foundations of microarray and biochip technologies are firmly rooted in the rapid advances in nucleic acid chemistry and biochemistry. The overwhelming majority of the early-phase trials using these developments were not primarily aimed at clinical efficacy but instead at assessing the biological problems to be solved before genomics, epigenomics and proteomics could deliver useful information for clinical applications. In an attempt to better understand these problems, investigators returned to basic studies of gene expression, immunology and virology. Finally, the cloning and sequencing of the human genome have significantly expanded the range of actual and potential molecular targets. The discovery and optimization of molecular targets, e.g., disease-specific proteins, receptors, enzymes or genes, is a time-consuming and extremely expensive process. Therefore, in today's preclinical research, there is only one key to success: scientific teamwork and the use of the most advanced biotechnological approaches. The advances in biotechnology directly affect improvements in human health. That especially holds true in our time of rapid changes.

Single-molecule analysis

A further trend is single-molecule detection in microfluidics. It has the advantages of small volume platform, digital analysis, elimination of processing steps, approach for real-time measurements and automation of sample preparation. This is definitely good work and is likely the trigger for further investigations on the behavior of single molecules. Cell solution analysis includes indicator as well as marker diagnostics and predictor/risk factor studies. However, as there are many variations and dispersions from microdevice to microdevice because of different dimensions (scales), one has to use self-calibrating chips.

Current technologies can only measure biological mechanisms as an average of a population of molecules as only their combined effect can be detected. This simplification ignores the fact that biological macromolecules oscillate between different activity states and are exposed to numerous levels of regulation. There might be a completely different pathological picture if in a heterogeneous status 50% of a gene product is not active, compared to a homogeneous state, where all products are active at 50% of their maximal level. Current technology can hardly distinguish these possibilities, yet they are fundamentally different and might be at the root of pathological processes. To be able to explore the behavior of each molecule in a population individually to fully understand molecular mechanisms and their regulation in the cell will open up an entirely new view of molecular medicine and diagnostic technology. Research into single-molecule detection will make molecular mechanisms and their pathology in diseases like molecular motor diseases and protein folding diseases, just to name a few, available to the more established biomedical and biotechnological approaches. For example, instead of speculating on how a protein structure changes during a disease state, the changing process can be monitored. Nucleic acid analysis is no longer limited to analysis of various constants under ensemble conditions. The fundamental characteristics of DNA and RNA changes within live cells can be analyzed with the help of single-molecule detection.

We feel that the time has come to present and discuss recent achievements in single-molecule spectroscopy, nanoscale imaging technology and bioanalysis that are creating a new array of opportunities for the biomedical sectors (Földes-Papp et al., 2005c). The rapid development of single-molecule spectroscopy and nanoscale imaging technology is accompanied by technical development of confocal fluorescence fluctuation spectroscopy and related theoretical modeling (Földes-Papp et al., 2005d). In recent years, their applications have been demonstrated in biology, life sciences and chemistry. Biochemical analysis at the nanoscale is approaching the solution phase. The impact of nanoscale

analysis is mainly in genetics. In 2000, the draft of the human genome was completed as were its sequencing contents in 2005. It all goes back to 1990, when the human genome project started. Today, nanoscale analysis has reached the test stage, where we have to do the hard staff (Földes-Papp et al., 2003, 2004a,b,c). The critical experimental condition will have to be that there are only a few fluorescent molecules in the detection volume. This article does not focus the discussion on the nanoscale confinement of optical excitation and fluorescence fluctuation detections in fluorescence fluctuation spectroscopy. Blom et al. (2006) very recently reviewed that issue.

Since the first report of the use of quantum dots (QD) as fluorescent probes in 1998 (Bruchez et al., 1998; Chan and Nie, 1998), QDs have been recognized as a promising and fascinating field in life sciences when rationally aimed. The main domain for application of quantum dots (QD) as fluorescent probes is still cellular and biomedical imaging, whereas less attention is paid to QD-based chemical and biochemical sensing. Jin et al. (2005) reported the first application of water-soluble semiconductor QDs for the optical detection of the neurotransmitter acetylcholine. Possible prospects for applications are very high, in the areas of pharmacology, enzyme applications, biosensors, etc. Applications of QD-based fluorescent probes to the detection of biologically important molecules such as DNA or RNA are still lacking. That also demonstrates that our methods and tools in that area are rather crude and require improvement (Nomura and Kinjo, 2004).

Single molecules in solution

Here, I deal with particular problems from a different perspective. That means I use different models to look at them properly. I believe that the different models stand for the complexity of ultrasensitive analytics down to the single molecule without immobilizing it and for the angle at which one can look at the problems.

What is the meaning of the molecule number N in the confocal probe region, if N becomes smaller than one?

Over the past two decades, a variety of optical single-molecule detection (SMD) techniques in solutions or on a solid surface have been developed, greatly advancing fundamental biochemical and biophysical studies. In solution, SMD is mainly based on the use of confocal fluorescence fluctuation spectroscopy, which detects the emission from a single fluorescent molecule when it diffuses through a femtoliter-sized detection volume (for FCS and FCCS, see Földes-Papp, 2005b). Such a small detection volume is defined by a high-numerical-aperture objective and the collection efficiency function. This small detection volume significantly reduces the background noise originating from spurious fluorescence from impurities and Raman scattering of solvent molecules, so that a high signal-to-noise ratio (SNR) and thereby high sensitivity are achieved. The smaller the detection volume, the higher the sensitivity can be pushed. When measuring low-concentration targets (<1 nM), the detected fluorescence signals become digital since the average number of molecules in the detection volume is smaller than unity (<1.0). In laser-induced fluorescence detection of single molecules, fluorescence bursts are detected only when single fluorescent molecules pass through the detection volume, i.e., the focused laser beam. Since the SNR of the single-molecule fluorescence bursts does not decrease with decreasing concentrations of the molecules, SMD provides an ideal platform for analysis of low-concentration targets. For example, if $N = 0.1$, it is the Poisson probability that one has to screen 10 volume elements in the sample to find a single molecule. This view of $0 < N < 1$ was commonly accepted in the literature (Rigler et al., 2001; Zander et al., 2002).

How can we distinguish between the two possibilities of measuring different fluorescent molecules one by one and measuring the same fluorescent molecule without immobilization of the molecule and hydrodynamic flow or photon burst analyses (photon count statistics)?

Just because there is an average of one molecule in the observation volume or to rephrase it, to screen ($1/N$) volume elements, where $0 < N < 1$, to find one single molecule, it is not possible to say that this is an individual molecule. Performing fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) in solution 'at the single-molecule level' can stand for events involving one, two, three and a few molecules, but not lots of molecules. If one simultaneously measures two, three or a few single molecules (events), it is the same situation as if one averages over 10^{17} molecules; it is meaningless for investigations of an individual molecule.

I show that the fluorescence fingerprint comes from a single-molecule event and, in addition, for the first time, that it comes from one and the same single, individual molecule ('selfsame' event). There has never before been a theoretical basis for making such claims without a proper photon burst size analysis (burst count rate statistics) of fluorescence intensity traces or immobilization. Measuring what is truly happening in a single molecule requires confirmation that only one molecule (one and the same molecule) is present.

The 'selfsame' (individual) fluorescent molecule is defined by its probability density in the observation volume within the sample, that is, the selfsame molecule likelihood estimator (criterion (3), see below). The meaningful time T_m describes the time in which one can study the individual, selfsame molecule. The theoretical basis of an increase in the observation time of a single molecule is explained here in words. This meaning of 'identical' was widened to a new, more complex idea, and this concept of bioanalysis represents a key for the entry of spectroscopically based single-molecule measurements into clinical laboratories in order to address immunological and clinical diagnostics. It was the author's original idea to undertake the theoretical and practical analysis of single molecules without immobilization, hydrodynamic flow or photon burst size histograms.

What is the probability of finding a single molecule in the confocal probe region as a function of the molar concentration of other molecules of the same kind in the bulk solution?

The probability P_1 that the detection volume (observation volume) ΔV contains a single (specific) fluorescent molecule, $x = 1$, is

$$\ln\{N = P(X = 1, \Pi \cdot T = C)\} = \ln\{N = P_1\} = \ln C - C, \quad (1)$$

where C is the average frequency of molecules in ΔV and relates to the molar concentration of other molecules of the same kind c_m in the bulk solution by

$$C \equiv c_m N_A \Delta V.$$

$N_A = 6.023 \cdot 10^{23}$ mol^{-1} is Avogadro's number. This relationship (criterion (1)) was newly identified (see Földes-Papp et al., 2005d).

What is the probability of finding two single molecules in the confocal probe region as a function of the molar concentration of other molecules of the same kind in the bulk solution?

The probability P_2 that the observation volume contains two (specific) fluorescent molecules, $x = 2$, reads

$$\ln\{P(X = 2, \Pi \cdot T = C)\} = \ln\{P_2\} = 2 \cdot \ln C - \ln 2 - C. \quad (2)$$

The second criterion now describes the 'analytical sensitivity' that the observation volume (detection volume) contains 'a single fluorescent molecule'.

Can criteria (1) and (2) be experimentally verified?

As previously shown by Z. Földes-Papp (Földes-Papp et al., 2001; see Table 1 therein), it was experimentally confirmed that the Poisson probability P_1 for the measured occurrence of a single-molecule event is $P_1 = N \cong C$ under the experimental conditions $C \ll e^{-C}$. With this relationship, single-molecule events are established in homogeneous phases (e.g., solution or a membrane) without photon burst analysis. However, the single-molecule events can involve measuring different single fluorescent molecules one by one or measuring the same single, individual fluorescent molecule only.

Table 1
Concentration dependence of the meaningful time T_m found for the first time (criterion (4))

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 probe volume [s]
10^{-9} (nM)	0.10679	0.12046	$2.4347 \cdot 10^{-4}$
10^{-10}	$1.1902 \cdot 10^{-2}$	$1.2046 \cdot 10^{-2}$	$2.1846 \cdot 10^{-3}$
10^{-12} (pM)	$1.2045 \cdot 10^{-4}$	$1.2046 \cdot 10^{-4}$	$2.1587 \cdot 10^{-1}$
10^{-15} (fM)	$1.2046 \cdot 10^{-7}$	$1.2046 \cdot 10^{-7}$	$2.1584 \cdot 10^{2}$
10^{-18} (aM)	$1.2046 \cdot 10^{-10}$	$1.2046 \cdot 10^{-10}$	$2.1584 \cdot 10^{5}$

There are one-molecule events, two-molecule events, three-molecule events, and so on, with certain Poisson probabilities. All these Poisson events contribute to the molecule number fluctuations of just one molecule in the probe region. These finite contributions result in very short meaningful times in the nanomolar and picomolar concentration ranges of the bulk solution, whereas these contributions become negligibly small in femto- and attomolar bulk solutions, yielding meaningful times in the 60-s range and even longer.
Exemplified for rhodamine green at a diffusion time $\tau_{diff} = 26 \cdot 10^{-6}$ [s] of a confocal probe volume $\Delta V = 0.2 \cdot 10^{-15}$ [L]. Reproduced from Földes-Papp, 2005a.

Is there a statistical estimation for the observation of the same single, individual molecule in, for example, FCS and two-color FCCS that depends on the measurable Poisson probability $P_1 = N$?

The selfsame molecule likelihood estimators—the general case. I have previously proposed the general case of the selfsame molecule likelihood estimator, i.e., Eq. (11) in Földes-Papp, 2002

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

This selfsame molecule likelihood estimator $P\left(\overline{\bigcap_{i=1}^{2} A_i}\right)$ gives the probability for the independent events of finding (arrival of) a single fluorescent molecule (A_1) in the confocal detection/observation volume (confocal probe region) and of moving (departure) of this single fluorescent molecule (A_2) over a fixed distance (lower limit ξ). P_1 is the measured probability of finding a single fluorescent molecule in the detection volume, and $P(\xi \le q < +\infty)$ is the frequency distribution of the diffusive spreading for the same single molecule. The measured dimensions of the detection volume, e.g., the focus of the excitation laser beams, give the lower limit of distance ξ. There is in fact an entire family of valid likelihood estimators of the same structure as the above equation, although they may differ with respect to the properties of $P(\xi \le q < +\infty)$, for example, by diffusion with hydrodynamic flow or with a macroscopic concentration gradient which I do not consider here. Finding the most suitable estimator to use is still an interesting field of investigation.

Is there a straightforward analytical solution of the selfsame molecule likelihood estimator?

There is the special case which can easily be used by an experimenter who is not familiar with the physics and mathematics behind the formulas. The special case of the novel selfsame molecule likelihood estimators $P\left(\overline{\bigcap_{i=1}^{2} A_i}\right)$ is

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

Here, N is criterion (1) and is measured, for example, by counting the molecule number per confocal probe region with fluorescence fluctuation techniques such as fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS), D is the diffusivity of a single molecule and t is a time parameter which has to be

specified (see criterion (4)). First, criteria (1) and (2) must be fulfilled and then criterion (3) can be used. All quantities on the right hand side of criterion (3) are obtained by the experiment, e.g., by single (solution)-phase single-molecule fluorescence auto- and two-color cross-correlation spectroscopy (SPSM-FCS).

With criterion (3), we are led to the answer to the question of whether we observe different single fluorescent molecules one by one or an individual single fluorescent molecule (one and the same single molecule) during the measurement time T. The latter case is the interesting one because we can probe the members of the usual ensemble, averaging one at a whole time from milliseconds up to hours. Most straightforwardly, all we need to do is measure the Poisson probability of N in order to find a single fluorescent molecule in the volume of detection. The measurements of one single fluorescent molecule (i.e., one and the same single fluorescent molecule) within the bulk phase (e.g., solution) are called the 'selfsame'-single-fluorescent-molecule regime. It is a new single-molecule regime because it is based on the analysis of the absolute molecule number N that is obtained directly from the functional FCS auto- or two-color cross-correlation curves. Criterion (3) exactly defines the 'selfsame' molecule, i.e., one individual fluorescent molecule (the same single fluorescent molecule), for example, in the SPSM-FCS experiments 'at the single-molecule level'.

What are the properties of the special case of the selfsame molecule likelihood estimator?

A change in the symmetry of the confocal detection volume, e.g., by a 'cellular' distortion, would change the calculation. For an additional angular integration of the density function of the diffusive spread of the single molecule, $n(q, \phi, t)$, in the above example, the calculation result 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 of 86.0% (Földes-Papp et al., 2005d). However, the measurements do not permit any angle resolution. The same reasoning holds true along the z axis of the given confocal detection volume with a probability of about 99.9%, where $2 \cdot \omega_z/\text{unit} = 2 \cdot \dot{\omega}_z = 1.91 \cdot 10^{-6}$ is a ratio and equals the double numerical value of the extracted ω_z at a given size of the confocal femtoliter and less probe region (Földes-Papp et al., 2005d). But, in fact, the cylindrical polars (q, ϕ, z) are more appropriate to consider the diffusive rate for a molecule that does not begin at the origin Cartesian coordinates $(x, y, z) = 0$. If a molecule is near the upper or lower edge of the confocal probe region when its diffusive spread begins, i.e., $z \neq 0$, it is also taken into account.

The probabilistic definition of just one single molecule in the confocal probe region (the selfsame molecule likelihood estimators $P\left(\overline{\cap_{i=1}^{2} A_i}\right)$, for example, criterion (3)) does not explicitly contain the meaningful time T_m in which one can study the selfsame molecule

During measurement times T, reentries of a single molecule in the probe region give rise to fluctuation phenomena, such as molecule number fluctuations at the single-molecule level. These fluctuations are the physical process on which fluorescence auto- and two-color cross-correlation spectroscopy is based. Motional states, which result in molecule number fluctuations within the probe region, are caused by the occurrence or non-occurrence of meaningful reentries of the selfsame molecule (Földes-Papp, 2005a; Földes-Papp et al., 2005d). What happens if the molecule starts near a boundary? A valuable aid to the physical mechanism of single-molecule reentries is the stochastic fluctuation analysis (Földes-Papp, 2005a). There are one-molecule events, two-molecule events, three-molecule events, and so on, with certain Poisson probabilities. All these Poisson events contribute to the molecule number fluctuations of just one molecule in the probe regions in ultrasensitive assays. These finite contributions result in very short meaningful times in the nanomolar and picomolar concentration ranges of the bulk solution, whereas these contributions become negligibly small in femto- and attomolar bulk solutions, yielding meaningful times in the 60-s range and even longer (Földes-Papp et al., 2005d). The concentration dependence of the meaningful time T_{m} in which one can study molecular reactions of the selfsame molecule in the probe region was first given in Földes-Papp (2005a)

$$T_{\mathrm{m}} = \frac{\tau_{\mathrm{diff}}}{c_{\mathrm{m}} N_A \Delta V \cdot \exp\{-c_{\mathrm{m}} N_A \Delta V\}}. \tag{4}$$

τ_{diff} is the diffusion time of the selfsame molecule at a given size of the confocal probe region ΔV (detection volume). c_{m} is the molar concentration of other molecules of the same kind in the bulk phase. Criterion (4) specifies the time parameter t in criterion (3). The experiments performed so far and the theory are consistent within the assumptions made.

What is the link between the selfsame molecule likelihood estimators $P\left(\overline{\cap_{i=1}^{2} A_i}\right)$, for example, criterion (3) and the meaningful time T_m in which one can study the selfsame molecule in the confocal probe region?

It is the concentration dependence of the meaningful time T_{m} as given in Table 1. For a meaningful time T_{m}=2.1584 · 10^2 [s] = 3.59 [min] in the probe volume, exemplified for rhodamine green at a diffusion time $\tau_{\mathrm{diff}} = 26 \cdot 10^{-6}$ [s] of a confocal probe volume $\Delta V = 0.2 \cdot 10^{-15}$ [L] (see Table 1), we need the upper limit of a measured value of $N = 1.2046 \cdot 10^{-7}$, which corresponds to a 10^{-15} M (fM) bulk solution of other molecules of the same kind. Therefore,

$$P\left(\overline{\bigcap_{i=1}^{2} A_i}\right) \cong 1 - \frac{N}{2\pi} = 1 - \frac{1.2046 \cdot 10^{-7}}{2\pi} = 0.99999998.$$

This value of the special case of the selfsame molecule likelihood estimator defines the lower limit of measuring just one rhodamine green molecule in the confocal probe region in the above example.

For the first time, the additional condition is found, which is the meaningful time T_{m} in the probe volume and multiples λT_{m} of it with $\lambda \gg (1/N)$, which determines the lower limit of the selfsame molecule likelihood estimator to exactly represent

only one single molecule (the selfsame molecule) in the probe region for a continuous observation (measurement) of this molecule during the measurement time λT_m.

On the other hand, if the diffusion time is large, that is, a larger molecule is studied, the meaningful time in the probe volume increases proportional to the size of the molecule as the relation

$$T_m \sim \tau_{diff}$$

follows directly from criterion (4) at a given size of the confocal probe region. This relationship is very useful for applications with biomolecules such as DNA or proteins. The theoretical model also yields the relation

$$T_m \sim \frac{1}{\Delta V}.$$

The meaningful time is reciprocally proportional to the probe volume. The smaller the probe region, the longer the selfsame molecule can be observed.

Is it possible to measure an individual, single immune molecule in a complex biological environment such as serum by the new algorithms, that is, the 'selfsame single-molecule regime'?

The Poisson probability $N < 1$ with $P_1 = N \cong 0.14$ of finding a single two-color molecule complex was observed in solution (Földes-Papp et al., 2004d). The Poisson probability of finding two two-color molecule complexes is about 1% and can be neglected. The average diffusion coefficient of the molecule complex was experimentally determined with $D = \frac{(\omega^2_{x,y;\text{blue}} + \omega^2_{x,y;\text{red}})}{8 \cdot \tau_{gr}} = 4.5 \cdot 10^{-12} m^2/s$. The measurement times were kept at 60 s. The novel quantity $P(A_1 \cap A_2)$ was determined to be

$$\frac{N}{2 \cdot \pi} \cdot \exp\left\{-\frac{\xi^2}{4 \cdot D \cdot t}\right\} = \frac{0.14}{2 \cdot \pi} \cdot \exp\left\{-\frac{(0.159 \cdot 10^{-6})^2 [\text{m}^2]}{4 \cdot 4.5 \cdot 10^{-12} [\text{m}^2/s] \cdot 60[s]}\right\} = 22.28 \cdot 10^{-3},$$

where $\xi = \omega_{x,y;\text{blue}} = 0.159$ μm (micron). Hence, the probability that the same large immune complex is measured under the given experimental reaction conditions is 1 − 0.02228 = 0.97772. Just one individual complexed molecule of an autoantibody target in serum was assayed with a confidence of about 97.8%, but the value does not describe the meaningful time T_m in which one can study the selfsame molecule which was first described in Földes-Papp, 2005a. For the individual complexed molecule of an autoantibody target in serum, the meaningful time T_m is 19.36 ms under the experimental conditions given (Földes-Papp et al., 2005d).

Thus, the detection is improved by two orders of magnitude over conventional state-of-the-art ELISAs. This opens new ground for immunological applications. For example, it allows analysis of smallest amounts of biomolecules that are difficult to handle experimentally. The results obtained in this study point towards constructing 'calibration curves', i.e., towards functional dose–response curves that fall within the scope of further studies on the expected heterogeneity of the reaction behavior of individual biomolecules.

Is it possible to overcome PCR-based amplification of DNA, e.g., genomic DNA, by ultrasensitive analytics down to the single-molecule level without clone-by-clone mutational scanning, post-PCR cloning or single-molecule PCR?

The requirement for PCR prior to detection is likewise the major bottleneck for individual and multiplex SNP methods. One of the greatest advantages of PCR, its sensitivity, can also be one of its greatest problems in medical diagnostics. The chance that a primer will not be fully extended, i.e., will not reach the other primer-binding site, depends on a number of experimental and empirical conditions. Jumping artifact is unacceptable in any experiments studying DNA recombination because products of jumping PCR, once they are cloned, are indistinguishable from genuine recombinant DNA molecules. At present, the "drop-out" of amplification artifacts is mainly achieved by optimized protocols and specific modes of detection such as dual-labeled hybridization LightCycler® real-time PCR probes, dual-labeled fluorogenic TaqMan® real-time PCR probes and two-color fluorescence cross-correlation spectroscopy (Földes-Papp et al., 2005b).

In Földes-Papp et al., 2005b, a novel two-color hybridization approach for unamplified double-stranded genomic DNA employing molecular methods is first shown. It was a logical step to take. Measurements of individual (single) molecules in solution are not so much a question of the sensitive detection of fluorescence photons, but rather of the efficient suppression of fluorescence background signals. The approach uses the inherently lower fluorescence background of light-up nonlinear fluorescence hybridization probes (superquencher molecular beacons) that contain built-in 5′-fluorescent dyes and 3′-nonfluorescent quenchers with perfectly matched spectral overlaps. The obtained allele sensitivity of femtomolar and less and the specificity of the described molecular interactions allow PCR-based allele distinction to be circumvented. The results present an alternative to existing hybridization approaches that are currently used with and without amplification at the 'many-molecule' level and the 'single-molecule' level. This novel approach quantifies the methylene tetrahydrofolate reductase (MTHFR) genotypes at site C677T (the heterozygous mutated, the homozygous mutated and the normal) directly onto the genomic DNA. This original research work was a basic study and is not intended for diagnosis and follow-up of patients so far.

Are there implications for experimental approaches and data handling?

In many biological systems, the number of molecules is very small, as in a cell, and many molecules are macromolecules, which have many internal degrees of freedom. Single-molecule detection and the spatial selection amplify the sensitivity to heterogeneities. These heterogeneities can be intrinsic to biological systems in which kinetic behaviors involve complex pathways. Yet, these are the events that cause biological changes. Biological processes, such are receptor occupation, are masked by averaging. In the single-molecule literature applying no external forces, there are many intuitive arguments with

respect to the time for a molecule to find the probe volume at an initial concentration in the bulk phase, the number of meaningful reentries, the meaningful time in the probe volume and the probability that the entering molecule is the original molecule. For the first time, exact analytical relationships were derived/found for these arguments (1–4). The selfsame molecule likelihood estimators are an enhanced tool for the rapid finding of appropriate experimental conditions to perform measurements with just one molecule. The meaningful time T_m describes the time in which one can study the individual, selfsame molecule. To get the next selfsame fluorescent molecule, the position of the confocal probe region within the sample is moved, for example, by the joystick. The developed algorithms make it possible to distinguish between the observation of single molecules one by one and the observation of just one, the selfsame, individual molecule at a time, without immobilization or hydrodynamic flow.

The cellular network

The study of the role of proteins in metabolic pathways and the analysis of protein complexes are examples that demonstrate that proteins only fulfil their function in interaction with other proteins. Proteome research shows that even enzyme activities and protein complex formation are elements of a cellular network of protein reactions and regulation. Fundamental questions in cell biology are how this network in the cell is organized, how complex it actually is and whether it is rather to be understood structurally, in a regulatory manner, or genetically. A protein depends on all the other proteins of its cellular compartment (e.g., cell organelles) through the dependence of all cell proteins on the same however limited resources provided by the cell: energy like ATP, building blocks such as amino acids, space and water (Brown, 1991). If a gene is mutated, it not only impairs the encoded protein itself. For example, in the case of a disease, the specific effect of overexpression causes macromolecular crowding resulting in unspecific hydrophobic and electrostatic protein–protein interactions, membrane adsorption, loss of solubility and diffusivity of proteins, change in conformation, self and hetero-assembly, precipitation and finally distortion of the physiological protein degradation (Minton, 2000). Indications for such a process are plaques and inclusion bodies like those that arise in the brain in neurodegenerative diseases. An opposite scenario occurs if the gene expression of a protein is suppressed or switched off for some reason. Since each of these processes impairs cellular energy conditions and thus the flow rate of the metabolic pathways, the overexpression of proteins can result in underexpression of other proteins and vice versa. In such a way, the primary specific and local effect can lead to a network effect. Theoretical evolutionary considerations come to the conclusion that the concentration of each protein in the cell is kept at a minimum level, which is just compatible with the function of the protein. The flow rate in the metabolic pathways is upregulated to the maximum (Brown, 1991). The dynamic interaction and cellular function of a cellular protein are modulated by up to 100 different proteins at different sites in the cell. In other words, the number of interactions within a cell is enormous. To follow all details seems to be impossible. This exacerbates the importance of new model strategies to improve on the situation.

The stochastic nature of molecular concentrations cannot be neglected if the number of participating molecules is small, as in live cells, or if the reactions occur far from thermodynamic equilibrium. In the cell biology and biology systems literature, this point of view is not studied, either experimentally or theoretically. The relatively small number of genes cannot explain the complexity of biological systems; there are other contributing factors. In this context, the organization of a neuron, in both time and space, is clearly of fundamental importance. Networks of interacting chemical species rather than individual chemical species control the biological function of neurons. The networks include gene regulation, cell physiology, signal transduction, cell adhesion and cell mobility as shown for a neurotrophin network in Table 2. Such a ‘minimal’ network consists of the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and their receptors like the tropomycine-related kinase (Trk) A, B, C and p75 as well as microtubule-associated protein 2 (MAP-2) and the cytoskeleton (Table 2).

Now, the hypothesis is that such a ‘minimal’ cellular network controls biological function in the following way. Regulator genes have a high degree of dynamics, adaptation as well as flexibility, and they regulate both physiological and pathological processes. I also assume that there are physiological genes which induce the respective physiological function of the neuron together with the regulator genes. Pathogenic genes induce the disease state or a disease-like state if regulator genes are suppressed or even eliminated or if regulator genes cannot properly adapt. The important novel element in this cellular network hypothesis is that patterns of fluctuations of the chemical species rather than a mean cellular concentration (flow rate) lead to a loss of physiological gene activity and the reappearance of pathogenic activity. This hypothesis is different to all the other concepts existing in genetics, cell biology, biochemistry and molecular biology (see, for example, Hoehe et al., 2003) and single-cell and single-molecule analysis. The genetic dysregulation of the “brain” is taken as the most important pathogenic principle of neurodegenerative diseases. A current goal of cell biology is to understand the structure and mechanisms of a cellular network of proteins based on the protein–protein interactions and protein modifications. If we knew the rules that determine the cellular up- and down-regulation of proteins, we would be able to develop new strategies for understanding and perhaps treating complex genetic disorders.

Table 2
Activation of target signals of a ‘minimal’ neurotrophin network

Gene regulation	Transcription factors, unknown factors
Cell physiology	NGF, BDNF, NT-3
Signal transduction	Receptors, TrkA, TrkB, TrkC, p75
Cell adhesion	Integrins
Cell mobility	Cytoskeleton, MAP-2, β-actin

Conclusions

Biochemical and molecular biology analysis at the nanoscale is approaching solution-phase genetics, and it is going for smaller (nanofluidics and nanochannels). Cell solution analysis includes indicator as well as marker diagnostics and predictor/risk factor studies. However, there are many variations and dispersions from microdevice to microdevice because of different dimensions (scales). Thus, one has to use self-calibrating chips.

In biology and experimental medicine, there is a need for algorithms to estimate the detection of just one ("selfsame") molecule in single phases such as solutions or membranes. The requirements for such algorithms are very stringent (Földes-Papp et al., 2005d):

(i) The estimation of the probability that just one, the selfsame, molecule is measured during the collection time (measurement time) must be based on the data collected from one particular measurement only. It cannot be based on a set of related measurements because there are variations in the probability from one measurement to another.
(ii) The estimation must be capable of detecting the selfsame molecule without immobilization or hydrodynamic flow.
(iii) The estimation must be computed from the measurement of the molecule numbers N, and not from the emitted photon bursts themselves.

Requirement (iii) may appear simple, but it is important and of outstanding practical value since the most common measurements do not yield emitted photon bursts or lifetime statistics. A convenient way to meet requirement (iii) is to use fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS). Single (solution)-phase single-molecule fluorescence auto- or two-color cross-correlation spectroscopy (SPSM-FCS) is based on the detection of the Brownian motion of fluorescent molecules *and* Poisson distribution analysis 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 ('critical' concentration), c_m^*, for a meaningful probabilistic assignment (Földes-Papp et al., 2005d). With a confocal probe volume $\Delta V = 0.2 \cdot 10^{-15}$ [L], c_m^* is roughly 1 nM. Because of the introduction of this distribution, one has a discrete, infinitely stretched population of molecules, whose statistic samples must obey this condition exactly (1–3). The observation of an individual, complex biological molecule in its natural environment is also possible over extended periods of time (criterion (4)).

Many theoretical models of molecular interactions, i.e., biochemical and chemical reactions, are described at the single-molecule level, although our knowledge of the biochemical/chemical structure and dynamics primarily originates from the investigation of many-molecule systems. At present, there are four experimental platforms for observing the movement and the behavior of single fluorescent molecules: wide-field epi-illumination, near-field optical scanning, and laser scanning confocal and multiphoton microscopy. The platforms are combined with analytical methods such as fluorescence resonance energy transfer (FRET), fluorescence auto- or two-color cross-correlation spectroscopy (FCS), fluorescence polarizing anisotropy, fluorescence quenching and fluorescence lifetime measurements. This original contribution focuses on counting and characterization of diffusing single molecules in a single phase like a solution or a membrane without hydrodynamic flow or immobilization on a surface (e.g., glass substrate). This can be achieved, for example, by fluorescence auto- or two-color cross-correlation spectroscopy, as demonstrated in the article.

The impact of single-molecule detection in the fields of biological and medical sciences is significant. It revolutionizes how researchers and physicians view problems such as molecular motor diseases, protein folding diseases and enzymatic kinetics just to name a few. For example, instead of using computer simulation, a researcher can now visualize how a molecular motor moves, as in studies of myosin and the F1-ATPase motor. Instead of speculating on how a protein structure changes during a disease state, the changing process can be monitored. Enzymatic analysis is no longer limited to analysis of various constants under ensemble conditions. The fundamental characteristics of the enzyme can be analyzed with the help of single-molecule detection. Single-molecule detection has the potential of becoming the future of how assays are performed. It is accurate because it measures a population distribution rather than an ensemble average, which can be more indicative of pathological changes. It is also economical because single-molecule detection requires a very small sample. A main problem that presently prevents single-molecule detection from becoming a useful, practical medical application is the time constraint. Hence, there is a need for an assay that can rapidly measure activity from hundreds of individual molecules simultaneously to shorten the time required to perform an assay (Lee and Brody, 2005).

A major challenge of current biological and medical research is to discover how genetic information is used to generate hugely complex cells. Theoretical activities in this field range from modeling of biological macromolecules in all detail to computer simulation of long dynamics of simplified cellular network models. A novel hypothesis on the dynamics of a cellular network is presented. The important element of this cellular network hypothesis is that patterns of fluctuations of chemical species rather than a mean cellular concentration (flow rate) lead to a loss of physiological gene activity and the re-appearance of pathogenic activity. The structure and dynamics can be characterized by using single-molecule detection and analysis.

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