

Single molecules in Fluorescence Fluctuation Spectroscopy: effective volume and photon counting histogram

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ABSTRACT

This paper presents a stochastic theory for the interpretation of photon counting histograms in fluorescence fluctuation spectroscopy (FFS). New concepts of an effective volume and a single molecule probability distribution are introduced to characterize a molecular species. Whereas the effective volume corresponds to the visibility of a molecular species in a given confocal setup, the single molecule probability distribution gives the signal measured for a single visible molecule. Specific properties of the effective volume and the single molecule probability distribution are discussed. Advantages arise for the high precision measurements of concentrations, mixtures, and binding constants especially for complex molecular environment, e.g. in flow systems and cell compartments.

Keywords: fluorescence fluctuation spectroscopy, photon counting distribution, effective volume, single molecule, stochastic theory

1. INTRODUCTION

Fluorescence fluctuation spectroscopy (FFS)¹ has been established as a standard method in a broad field of applications especially in biophysics and biochemistry. The photon counting histogram (PCH) approach^{2,3} is an alternative method to the well established fluorescence correlation spectroscopy (FCS)^{4,5} and also known in similar methods as fluorescence intensity fluctuation analysis (FIDA)⁶ and burst size distribution analysis (BSDA).⁷ FCS as well as the PCH approach are based on the recording of photon counts emitted from molecules in a small detection volume V .

In FCS the size of the detection volume V_{FCS} determines the mean transit time of a particle as well as, via the mean number of molecules $\langle m \rangle$, the concentration $c = \langle m \rangle / V_{\text{FCS}}$. The mean number of molecules $\langle m \rangle$ is defined by the well known relation $\langle m \rangle := g(t \rightarrow \infty) / (g(t \rightarrow 0) - g(t \rightarrow \infty))$ where $g(t)$ represents the autocorrelation function. For given model systems, e.g. Gaussian observation volume profiles, analytical expressions can be derived for the volume V_{FCS} . The volume turns out to depend on the characteristic dimensions of the laser excitation intensity but not on other experimental parameters such as the overall laser power, the molecular concentration, the molecular brightness, etc. By this property the FCS becomes an universal, easy to use, and stable method. Applying FCS different species can be resolved based on their typical transition time but the brightness of the species is not utilized, see however.⁸

Despite the great success of the PCH approach to overcome this limitation of the FCS a similarly rigorous and sustainable theory is lacking for the analysis of photon counting distributions. This work suggest a theoretical platform for the *a priori* prediction of photon counting histograms. Our approach aims to provide a new physical insight into FFS and to make the PCH approach extendable to complex experimental tasks, e.g. flow systems and cell compartments.

The primary objective of this work is the determination of the contribution of a single molecule to a measured photon counting histogram. The number of molecules contributing to the signal vary due to local particle fluctuation. In a thermodynamical steady state the average number of contributing molecules, however, is constant. This average number of molecules remained undetermined in the context of previous theoretical models. The previous models are based, implicitly or explicitly, on the choice of an arbitrary detection volume.

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The choice of an arbitrary detection volume has little or no effect on the model considering a measurable photon counting distribution and, hence, may be seen as a pure technical aspect without any physical meaning. A drawback of this conception is that neither the average number of contributing molecules nor the contribution of a single molecule is well defined and extractable from experimental data. The term "photon counting histogram of a single molecule" is applied in FIDA and the PCH approach as an abstract construction implemented to compute the photon counting distribution for a given concentration, i.e. for a superposition of various multi-molecular contributions. It depends on an arbitrarily chosen volume parameter and, consequently, eludes physical interpretation.

The single molecule signal, as one would like to see this term intuitively, is the most pure information on a molecular species to be extracted from a fluorescence fluctuation experiment. It is the fingerprint of a molecular species and it contains all information about a molecular species relevant in the given experimental set-up independent of the concentration. For a given theoretical model the single molecule contribution can be simulated easily, but precise analytical formulas are lacking even for simple models. In the following we derive a definition of the single molecule probability distribution which makes its rigorous computation feasible for given models and makes it extractable from experiments. The effective volume introduced in this work is coupled to the average number of molecules contributing to a measured photon counting distribution. The average number of contributing molecules, i.e. concentrations, becomes extractable from experimental data by applying simple analytical expressions. This applies as well to mixtures of different species, where the conception of the effective volume leads to important consequences. Since the analysis of moments is of high practical interest, analytical expression and interrelation are derived for the moments of the total photon counting histogram, the moments of the single molecule distribution, and the moments of the molecular brightness function. A brightness function of simple Gaussian profile serves as example to demonstrate and discuss the consequences of our considerations. Of special interest are the properties of the single molecule probability distribution shown for this illustrative model.

2. THEORY

In single molecule experiments the situation is as follows: Photons emitted from molecules in a small observation volume V hit a detector for photons. Discrete time points $t_i, i = 0, 1, 2, \dots$ divide the observation time into equidistant intervals ($t_i - t_{i-1} = \Delta T$ for all $i = 1, 2, 3, \dots$). The number of photons counted in the time interval $t \in [t_{i-1}, t_i)$ present a sequence of numbers $\{n_1, n_2, n_3, \dots\}$. A completed measurement gives a finite number of counting rates n_1, n_2, \dots, n_N . The number of time intervals ($\#k$) in which n photons are counted gives the photon counting distribution ($\#k(n)$).

Dividing each element of the sequence $\#k(n)$ by the total number of intervals $N = \sum_{n=0}^{\infty} \#k(n)$ gives a real number

$$p_N(n) = \frac{\#k(n)}{N} \quad (1)$$

which can be interpreted — at least in the limit of a "great many" time intervals measured — as the probability to measure n photons in a time interval ΔT . The corresponding physical probability distribution is defined by

$$p(n) = \lim_{N \rightarrow \infty} p_N(n) \quad (2)$$

For simplicity we assume all photons to be produced by molecules of interest and ignore any background noise produced e.g. by the detector hardware or scattered light. Incorporating background noise is straightforward and its influence on $p(n)$ will be discussed later.

Molecules near the laser focus produce a high contribution to the photon count rate, whereas molecules far away from the laser focus give rise to a low or no contribution. The contribution of a single molecule can be measured by immobilizing it at a given position \vec{r} , on a surface (e.g. a glass surface) or in a matrix (e.g. a gel). The series of photon counts n_i recorded for this set up gives the probability distribution $p(n)$. The so measured average value of photon counts $\langle n \rangle = \sum_n n p(n)$ is called the molecular brightness μ at position \vec{r} .

Table 1. Random variables and probability distributions

random variable	assigned distribution	source	equation random variable	equation distribution
N_{exp}	$P_{\text{exp}}(n)$	measurement	$N_{\text{exp}} = N_{\text{sig}} + N_{\text{noise}}$	$P_{\text{exp}}(n) = (P_{\text{sig}} \otimes P_{\text{noise}})(n)$
N_{noise}	$P_{\text{noise}}(n)$	background	$N_{\text{noise}} \xrightarrow{k} N_{\text{noise}} + 1$	$P_{\text{noise}}(n) = \text{Poi}(n, \langle \text{noise} \rangle)$
N_{sig}	$P_{\text{sig}}(n)$	M molecules ^(*1)	$N_{\text{sig}} = N_1^{(1)} + N_1^{(2)} + \dots + N_1^{(M)}$	$P_{\text{sig}}(n) = \sum_{m=0}^n Q(m) P_m(n)$
N_m	$P_m(n)$	m molecules ^(*2)	$N_m = N_1^{(1)} + N_1^{(2)} + \dots + N_1^{(m)}$	$P_m(n) = (P_1^{(1)} \otimes P_1^{(2)} \otimes \dots \otimes P_1^{(m)})(n)$
N_1	$P_1(n)$	single molecule ^(*3)	$N_1 \xrightarrow{\mu(\vec{r})} N_1 + 1$	$P_1(n) = \int_V \text{Poi}(n, \mu(\vec{r})) p(\vec{r}) dV$

*1 fluctuating number of molecule, M random variable with probability distribution $Q(m)$
 *2 fixed number of molecule, m fixed integer
 *3 single molecule at position \vec{r} , where \vec{r} is a random variable

2.1. Random variables and probability distributions

In terms of probability theory the number of photon measured during a time interval can be formalized by a random variable N_{exp} to which a probability distribution $P_{\text{exp}}(n)$ is assigned, see Table 1. Not all photons measured may have been produced by fluorescent molecules. A more or less significant part of the measured events may originate from Raman scattering of laser light, by thermal background noise of electronic devices or other sources. Such contributions to the photon counting rate are summarized by a random variable N_{noise} and we get the number of measured photons as sum

$$N_{\text{exp}} = N_{\text{sig}} + N_{\text{noise}} \quad , \quad (3)$$

where N_{sig} is the random variable for the photons produced by fluorescent molecules. The production of background events are — in general — well approximated by a Markov process

$$N_{\text{noise}} \xrightarrow{k} N_{\text{noise}} + 1 \quad , \quad (4)$$

with constant production rate k . The solution of this master equation with initial condition $N_{\text{noise}} = 0$ at $t = 0$ is given by the well-known Poisson distribution

$$P_{\text{noise}}(n) = \text{Pois}(n, \lambda) \equiv e^{-\lambda} \frac{\lambda^n}{n!} \quad , \quad (5)$$

with $\lambda \equiv k \Delta T$.

A number of identical molecules may produce photons and, hence, the random variable N_{sig} is the sum of identical random variables N_1 each of which is associated to the photon production of a single molecule:

$$N_{\text{sig}} = N_1^{(1)} + N_1^{(2)} + \dots + N_1^{(M)} \quad . \quad (6)$$

M is the number of molecules contributing to the signal N_{sig} . Typically in confocal optical set ups the number of contributing molecules fluctuate and the number M is an random variable itself (named identically). The probability distribution assigned to M is denoted $Q(m)$. Assuming the random variables M and N_1 to be independent from each other a separation

$$P_{\text{sig}}(n) = \sum_{m=0}^n Q(m) P_m(n) \quad (7)$$

sums up the simultaneous contributions of $m = 1, 2, 3, \dots$ molecules. The effective contribution of a fixed number of m molecules is denoted by P_m . Note, that each contributing molecule give rise to at least one photon event and, hence, the number of contributing molecules m can not exceed the number of photons n in the signal. The probability distribution $P_m(n)$ is assigned to the random variable

$$N_m = N_1^{(1)} + N_1^{(2)} + \dots + N_1^{(m)} \quad , \quad (8)$$

where $m \in \mathbb{N}$ is a fixed integer (and not a random variable). Each single molecule produce photons by the characteristic probability distribution $P_1(n)$. The photon production of a single molecule is a stochastic process where, however, the rate depends on the location of the molecule or, more precisely, on the molecular brightness $\mu(\vec{r})$ at its position \vec{r} . Since the motion of a molecule is completely independent from the process of photon production, Mandel's classical formula¹:

$$P_1(n) = \int_V Poi(n, \mu(\vec{r})) p(\vec{r}) dV \quad (9)$$

is widely applied in the approximation of very short bin width ($\Delta T \rightarrow 0$). $p(\vec{r})$ denote the the probability density to find the molecule at position \vec{r} . The validity of several steps is the description above deserve a closer look, but we abstain from discussing these aspects from a more rigorous point of view. This is out of the scope of this work and we refer to the discussion relevant for our work in the text below.

2.2. How to extract the single molecule distribution?

A task of fluorescence fluctuation spectroscopy is to extract the probability distribution $P_1(n)$ from a measured photon counting histogram $P_{\text{exp}}(n)$. $P_1(n)$ is the fingerprint of a type of molecule to identify it in a probe, to determine fractions of different molecules in mixtures, or to compute fraction bounds in binding assays. In the proceeding paper we will formulate recurrence formulas to compute the expected experimental distribution $P_{\text{exp}}(n)$ from a given single molecule distribution $P_1(n)$. Vice versa these recurrence formulas can be inverted to compute $P_1(n)$ from an experimental distribution $P_{\text{exp}}(n)$. Therefore we follow the line described above in reversionary direction: $P_1 \rightarrow P_m \rightarrow P_{\text{sig}} \rightarrow P_{\text{exp}}$. The distribution P_m is easily given by m-times convolution of the single molecule distribution:

$$P_m(n) = (P_1 \otimes P_1 \otimes \dots \otimes P_1)(n) = \sum_{i=1}^n P_{m-1}(n-i) P_1(i) \quad \text{for} \quad m = 2, 3, \dots \quad (10)$$

The knowledge of the signal of $m = 1, 2, 3, \dots$ molecules enables the computation of the signal form a small volume in which the number of molecules fluctuate. To proceed with step $P_m \rightarrow P_{\text{sig}}$ and equation (7) we have to know the probability $Q(m)$ to receive photons from $m = 1, 2, 3, \dots$ molecules. The diffusion process of molecules in and out of a small volume is stochastic birth and death process. It is shown quit generally that in thermal equilibrium the detailed balance principle holds so that we always get the Poisson distribution⁹:

$$Q(m) = e^{\langle m \rangle} \frac{\langle m \rangle^m}{m!} \quad , \quad (11)$$

where $\langle m \rangle$ is the average number of molecules in the volume. Adding background signal in the step $P_{\text{sig}} \rightarrow P_{\text{exp}}$ is simple and applying the method of probability-generating functions we finally get the recurrence formula¹⁰:

$$P_{\text{exp}}(n) = \frac{\langle n \rangle_{\text{noise}}}{n} P_{\text{exp}}(n-1) + \frac{1}{n} \sum_{i=1}^n i P_{\text{exp}}(n-i) \left[\sum_{s=1}^N \langle m \rangle^{(s)} P_1^{(s)}(i) \right] \quad (12)$$

with

$$P_{\text{exp}}(n = 0) = \exp \left[-\langle n \rangle_{\text{noise}} - \sum_{s=1}^N \langle m \rangle^{(s)} \right] . \quad (13)$$

The index (s) accounts for N different species in a sample. Equation (12) is now the requested relation either to compute the expected experimental distribution P_{exp} from a known single molecule distribution P_1 or *vice versa* to extract the single molecule signal from a measured trace.

Parameters of relation (12) are the average number of background photons $\langle n \rangle_{\text{noise}}$ and the average number of contributing molecules $\langle m \rangle$. Whereas the background signal $\langle n \rangle_{\text{noise}}$ can be measured easily, the determination of the average number of contributing molecules $\langle m \rangle$ is a tricky problem. In the PCH approach,³ the FIDA,⁶ and their successor^{11–14} the number of molecule are computed by the simple relation $\langle m \rangle = c V$, where c is the concentration of the fluorescent species and V is a rather arbitrarily chosen volume. On one hand V have to be large enough not to cut away too much contribution from the tail of the molecular brightness $\mu(\vec{r})$.^{15,16} A typical choice for V in applications is the size of V_{FCS} multiplied with an appropriate factor of three to ten. Consequently, P_1 obtained from a measurement depends strongly on the choice of V and hence, analytical methods based on the single molecule distribution P_1 have not been exploited in the past.

A corresponding relation between the moments of a measured PCH and the moments of the single molecule distribution can be derived based on Eq. 12. For a description of the method of moments we refer to the works of Qian and Elson,² and Müller.¹⁷ The moment m_k of a distribution $P(n)$ is given by

$$m_k(P) := \sum_{n=0}^{\infty} P(n) n^k . \quad (14)$$

Eq. 12 gives the relation

$$m_k(P_{\text{exp}}) = \sum_{l=0}^{k-1} \binom{k-1}{l} m_l(P_{\text{exp}}) \left(\langle n \rangle_{\text{noise}} + \sum_{s=1}^N \langle m \rangle^{(s)} m_{k-l}(P_1^{(s)}) \right) \quad (15)$$

between the moments of the measured probability distribution $P_{\text{exp}}(n)$ and the moments of single molecule distributions $P_1^{(s)}(n)$.

2.3. Effective volume and number of contributing molecule

Mandel's classical formula (9) results in the simple solution $P_1(n) = \delta_{0n}$ in the limes of infinite large integration volumes $V \rightarrow \mathbb{R}^3$. This reflects the physical fact that an individual molecule in an infinite volume corresponds to a zero concentration and can not give rise to any photon collection event at all. Physical situation in real life measurement corresponds to a non-zero concentration c . Here the average number $\langle m \rangle$ of molecules contributing to each counting rate is given by

$$\langle m \rangle := \int_{\mathbb{R}^3} [1 - Poi(n = 0, \mu(\vec{r}))] c dV , \quad (16)$$

where cdV denote the number of molecules in volume element dV and the factor $[1 - Poi(n = 0, \mu(\vec{r}))]$ describes the probability of each molecule in dV to give rise to at least one photon count event. Introducing an effective volume

$$V_{\text{eff}} := \int_{\mathbb{R}^3} [1 - Poi(n = 0, \mu(\vec{r}))] dV , \quad (17)$$

gives the simple relation $\langle m \rangle = c V_{\text{eff}}$. The effective volume V_{eff} is not readily described by the optical set up but also depends on the properties of the molecular species considered. A molecule is defined to be "inside" this volume during a particular time interval if and only if it contributes to the count rate. The probability to

contribute to the count rate is non-zero for a molecule at any location. Consequently the effective volume V_{eff} can not be determined by spatial boundaries. Nevertheless the process of "entering" and "leaving" this volume is a stochastic process similar to the diffusion process into and out of a small physical volume.

By definition a "single molecule" inside the effective volume cannot produce any count rate of zero. Consequently the term to have a "single molecule" inside the effective volume is conceptionally very different from having a "single molecule" in any spatial volume. Physically the single molecule probability distribution $P_1(n)$ can be measured in a Gedankenexperiment by considering only non-zero count rates in which all photon events during the time interval originate solely from a single molecule. Superposition of signals originating from several molecule have to be ignored. Applying this definition of a single molecule in a effective volume V_{eff} Mandel's formula¹ reads:

$$P_1(n) = 1/V_{\text{eff}} \int_{\mathbb{R}^3} \text{Poi}(n, \mu(\vec{r})) \, dV \quad , \quad (18)$$

with $P_1(n=0) := 0$. According to the considerations above a particle species is characterized by two specific quantities: the effective volume V_{eff} and single particle distribution $P_1(n)$. The single particle distribution $P_1(n)$ can be distinguished from the photon counting histogram of a single molecule in the PCH approach by the specific property $P_1(n=0) = 0$. In the PCH approach the condition $P_1(n=0) = 1 - \sum_{n=1}^{\infty} P_1(n)$ is chosen to obtain correct normalization and $P_1(n)$ depends on an rather arbitrary integration volume. Our quantities, V_{eff} and $P_1(n)$, have a defined physical meaning. They are rich in the sense that they contain all information specific for the molecule in the experimental set up; they are pure in the sense that they characterize the properties of a molecular species without any averaging processes due to the simultaneous contribution of several particles.

Applying equation Eq. 18 the moments of the single molecule distribution $P_1^{(s)}(n)$ are related via

$$m_k(P_1^{(s)}) = \sum_{l=1}^k \left\{ \begin{matrix} k \\ l \end{matrix} \right\} \langle \mu^l \rangle^{(s)} \quad (19)$$

to the moments of the molecular brightness:

$$\langle \mu^l \rangle^{(s)} := \frac{1}{V_{\text{eff}}^{(s)}} \int_{\mathbb{R}^3} \left(\mu^{(s)}(\vec{r}) \right)^l \, dV \quad . \quad (20)$$

The Stirling numbers of the second kind are given in the Karamata notation (using braces). Note, that in products of the type $\langle m \rangle^{(s)} m_{k-l}(P_1^{(s)})$ the effective volume cancels away.

In the case of mixtures a specific molecular species s has to be distinguishable from other species based on its characteristic moments $m_k(P_1^{(s)})$. To obtain these characteristic moments $m_k(P_1^{(s)})$, a probability distribution $P_{\text{exp}}(n)$ has to be measured for samples containing the species s at a known concentration c_s . We note, however, that the accuracy of measured moments of high orders suffers from large statistical errors. This fact restricts the applicability of the method of momentum to small numbers of molecular species. Note, also, that the classical Stieltjes problem¹⁸ of mathematical statistics applies. If only a finite number of moments are known the associated probability distribution may vary in an extreme broad range. Two distributions may have identical moments $m_k, k = 1, 2, \dots, M$ even if they diverge. Since this is true for any large but finite number of moments the method of moments may, in certain cases, become insufficient to characterize a diversity of molecular species.

2.4. Illustrative example: Gaussian brightness function

In theoretical considerations, the most popular model for the brightness function is the spatial Gaussian profile

$$\mu(\vec{r}) := \mu_{\text{max}} \exp(-2r^2/a^2) \quad , \quad (21)$$

where μ_{max} is the brightness of a molecule in the center of the laser focus and a denotes the waist parameter of the laser beam. The limitations of such a bold approximation to account for the complex three-dimensional spatial brightness function has been demonstrated by several groups, see¹⁹⁻²¹ and literature therein. We remark that our approach is applicable to any spatial brightness function. Approximations by Gauss functions, however,

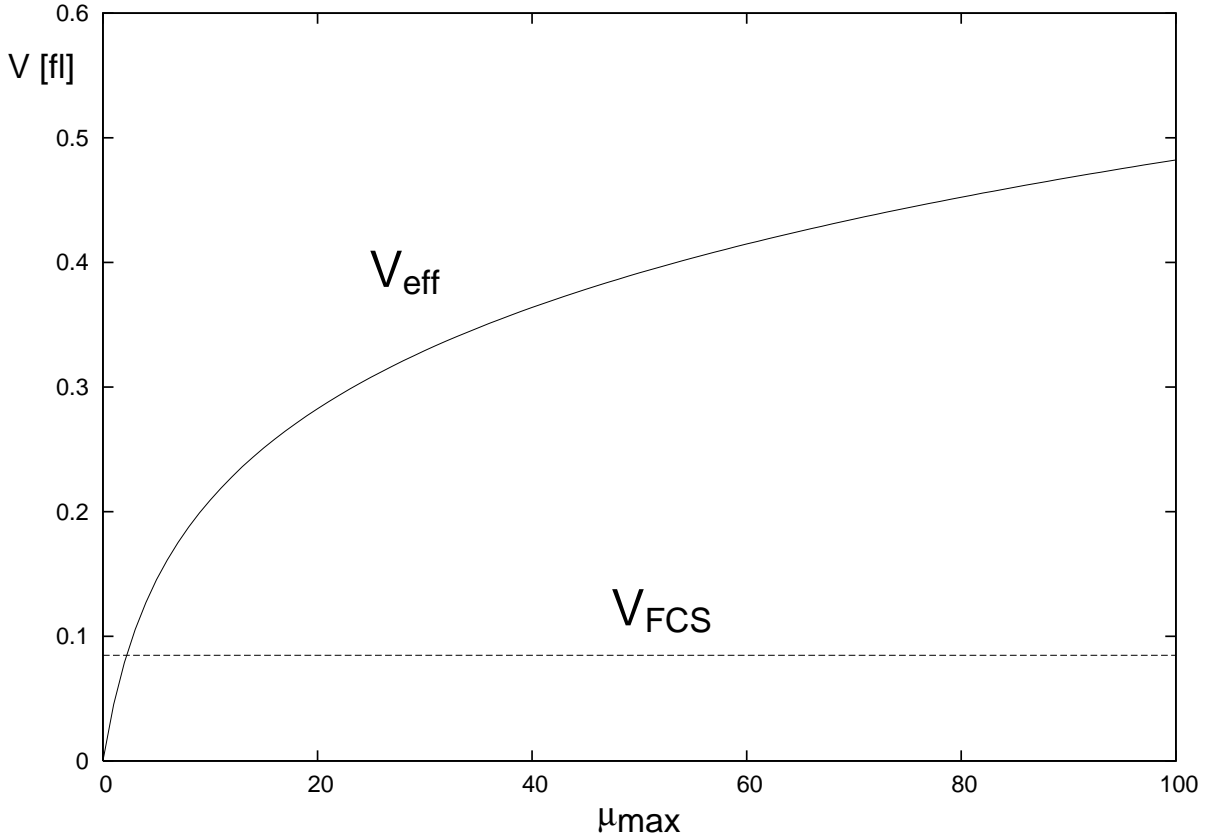


Figure 1. The effective volume V_{eff} versus the molecular brightness parameter μ_{max} , see Gaussian shape model Eq. 21. The waist parameter of the laser beam is $a = 0.3\mu\text{m}$. V_{eff} becomes identical to $V_{\text{FCS}} = \pi^{\frac{3}{2}} a^3 \approx 0.15 \text{ fl}$ at $\mu_{\text{max}} \approx 5.3$ counts/bin. The effective volume V_{eff} varies nonlinearly with the molecular brightness μ_{max} .

are usually easy to handle and contraction of Gauss functions have been proven to converge to any integrable spatial function.²² This is one of the reasons why they have been applied extensively in modern quantum chemistry for decades. In the following the Gaussian profile serves as an illustrative example.

Inserting the Gaussian profile Eq. 21 into the definition of the effective volume Eq. 17 and the single molecule function Eq. 18, respectively, a transformation of coordinates $r \rightarrow \mu$, and the integration over the rotational symmetry yield

$$V_{\text{eff}} = \frac{\pi a^3}{\sqrt{2}} F(\mu_{\text{max}}) \quad , \quad (22)$$

and

$$P_1(n) = \frac{1}{n! F(\mu_{\text{max}})} Q(n, \mu_{\text{max}}) \quad (23)$$

with

$$F(\mu_{\text{max}}) := \int_{\mu_{\text{max}}}^0 \frac{1 - e^{-\mu}}{\mu} \sqrt{-\ln(\mu/\mu_{\text{max}})} d\mu \quad , \quad (24)$$

and

$$Q(n, \mu_{\text{max}}) := \int_{\mu_{\text{max}}}^0 e^{-\mu} \mu^{n-1} \sqrt{-\ln(\mu/\mu_{\text{max}})} d\mu \quad . \quad (25)$$

Numerical Romberg integration may be applied to compute the functions F and Q . Note, that Q is finite only for $n \geq 1$ and not defined for $n = 0$. The effective volume V_{eff} becomes identical to the corresponding detection

volume in FCS $V_{\text{FCS}} = \pi^{3/2} a^3$ for $F(\mu_{\text{max}}) = \sqrt{2\pi}$, i.e. for certain value of μ_{max} . In the case of a non-isotropic Gaussian shape function $\mu(\vec{r})$ the factor a^3 above has to be replaced by the product of waist parameters $a_x a_y a_z$ describing the different sizes of the brightness function in x , y , and z direction, respectively. Note, that in the Gaussian type model the effective volume V_{eff} , i.e. the number of contributing molecules $\langle m \rangle = V_{\text{eff}} c$, increase non-linearly in the molecular brightness characterized in this model by μ_{max} , see Fig. 1. This fact is important especially for the resolution of mixtures containing species of different brightness.⁸

The moments $\langle \mu^k \rangle$ can be computed explicitly:

$$\langle \mu^k \rangle^{(s)} = \frac{\left(\mu_{\text{max}}^{(s)}\right)^k}{k^{3/2} F(\mu_{\text{max}}^{(s)})} \quad (26)$$

and we get the moments of the single molecule function in the form of

$$m_k(P_1^{(s)}) = \frac{1}{F(\mu_{\text{max}}^{(s)})} \sum_{l=1}^k \left\{ \begin{matrix} k \\ l \end{matrix} \right\} \frac{\left(\mu_{\text{max}}^{(s)}\right)^l}{\sqrt{l^3}} \quad (27)$$

Inserting these relations into Eq. 15 gives a relation between the moments $m_k(p_{\text{tot}})$ and μ_{max} . Moments for various shape functions $\mu(\vec{r})$ are discussed in the work of Kask *etal.*²³

Furthermore, for a Gaussian type model Eq. 21 the maximal molecular brightness is determined via Mandel's Q -parameter

$$Q := \frac{m_2(p_{\text{tot}}) - m_1^2(p_{\text{tot}}) - m_1(p_{\text{tot}})}{m_1(p_{\text{tot}}) - \langle n \rangle_{\text{noise}}} = \frac{\mu_{\text{max}}}{\sqrt{8}} \quad (28)$$

by the first and second moment of a measured distribution. Applying Eq. 26 the integrals Eq. 25 and Eq. 24 can be represented as power series in μ_{max} , as e.g.

$$V_{\text{eff}} = \left(\sqrt{\frac{\pi}{2}} a\right)^3 \mu_{\text{max}} \sum_{k=0}^{\infty} \frac{1}{(k+1)^{5/2}} \frac{(-\mu_{\text{max}})^k}{k!} \quad (29)$$

2.5. Simulation

Extensive molecular dynamic simulations have been performed to test the concept of a single molecule distribution $P_1(n)$ and the effective volume V_{eff} introduced in this work. Simulation and model parameters have been chosen carefully not to influence the results. A random walk simulation on a $512 \times 512 \times 512$ grid with grid parameter of $g = 5 \times 10^{-9} \text{m}$ is applied to describe the diffusion of particle with typical diffusion constant of $D = 2.8 \times 10^{-10} \text{m}^2 \text{s}^{-1}$. The bin width of $1 \mu\text{s}$, the experimental parameters and properties of the fluorescent particle are chosen to result in a brightness parameter of $\mu_{\text{max}} = 16.9499$ and waist parameter $a = 0.3 \times 10^{-6} \text{m}$ for the simple Gaussian brightness model Eq. 21, see¹⁰ for details. This parameter choice lead to a rather small effective volume of $V_{\text{eff}} = 0.264 \text{fl}$ compared to the simulation volume of 16.78fl . During simulation the brightness was averaged over the diffusive motion of the particles and the number of photon counts measured for a bin is computed by a single call of a Poisson deviates.²⁴ For a numerical treatment of the Master equation including triplet state excitation we refer to the work of Palo *etal.*¹²

Since the fluctuations of the number of molecules turn out to influence the simulated photon count distribution appropriate birth and death processes have been chosen to describe the diffusive motion of particles in and out of the simulation volume. In this way the fluctuation of the number of molecules in the simulation volume is Poissonian, whereas simple reflecting or periodic boundary conditions produce a constant number of particles on the grid.

Table 2. The first three moments and Mandel's Q-parameter of simulated single molecule distributions.

$m_1(P_1)$	3.394 ± 0.003	3.408^*
$m_2(P_1)$	23.61 ± 0.03	23.83^*
$m_3(P_1)$	249.5 ± 0.6	253.1^*
Q	2.56 ± 0.01	2.58^*
* analytical value		

2.6. Results

Fig. 2 shows the photon counting distribution of a single molecule. Ten independent numerical solutions of the Master equation are compared to the analytical solution Eq. 23 plotted as line. Each simulation is performed for a diffusion time of 10s, i.e. 10^7 bins of $1\mu\text{s}$ each. In agreement with the statistical fluctuations observed in measurements⁶ the residuals shown in the lower part of the figure are greater than the expected ones by a factor of about three. The first three moments and Mandel's Q-parameter of the simulated single molecule distributions are compared to the analytical value in Table 2. The moments and the Q-parameter are slightly lower than the analytical values. Simulation with longer bin-width indicate that this deviation is not an artificial effect of the numerics but a result of the reduction of μ_{max} due to the diffusive motion of the molecule, see section Discussion.

Simulations for concentrations in the range $c = 10^{-10}\text{M}$ to $c = 10^{-7}\text{M}$ have been performed. The mean number of molecules contributing to the signal vary between 0.0159 and 15.9 for $c = 10^{-10}\text{M}$ and $c = 10^{-7}\text{M}$, respectively. The mean total numbers of molecules are between 1.01 and 1010 in these simulations. The result of simulation shows no systematic deviation from the analytical formula. For concentrations $c \geq 10^{-9}\text{M}$ the first two moments and Mandel's Q-parameter in Table 3 are identical to the analytical values. A statistical significant reduction of the moments and the Q-parameter is observed for the lowest simulated concentration of $c = 10^{-10}\text{M}$ only.

Table 3. The first first two moments and Mandel's Q-parameter for various concentrations.

$c[\text{M}]$	m_1	m_2	Q
10^{-7}	54.28 ± 0.02	3323 ± 2	5.97 ± 0.05
	54.26^*	3323^*	5.99^*
10^{-8}	5.422 ± 0.008	67.2 ± 0.2	5.96 ± 0.04
	5.426^*	67.4^*	5.99^*
10^{-9}	0.542 ± 0.002	4.08 ± 0.02	5.99 ± 0.04
	0.543^*	4.09^*	5.99^*
10^{-10}	0.0529 ± 0.0007	0.371 ± 0.007	5.96 ± 0.2
	0.0543^*	0.382^*	5.99^*
* analytical value			

Based on the recursion relation Eq. 12 the single molecule distribution can be extracted from a measured distribution. Applying these method to the simulated distribution for the concentrations in the range $c = 10^{-10}\text{M}$ to $c = 10^{-8}\text{M}$ reproduces the single molecule distribution. For the higher concentration $c = 10^{-7}\text{M}$ the method

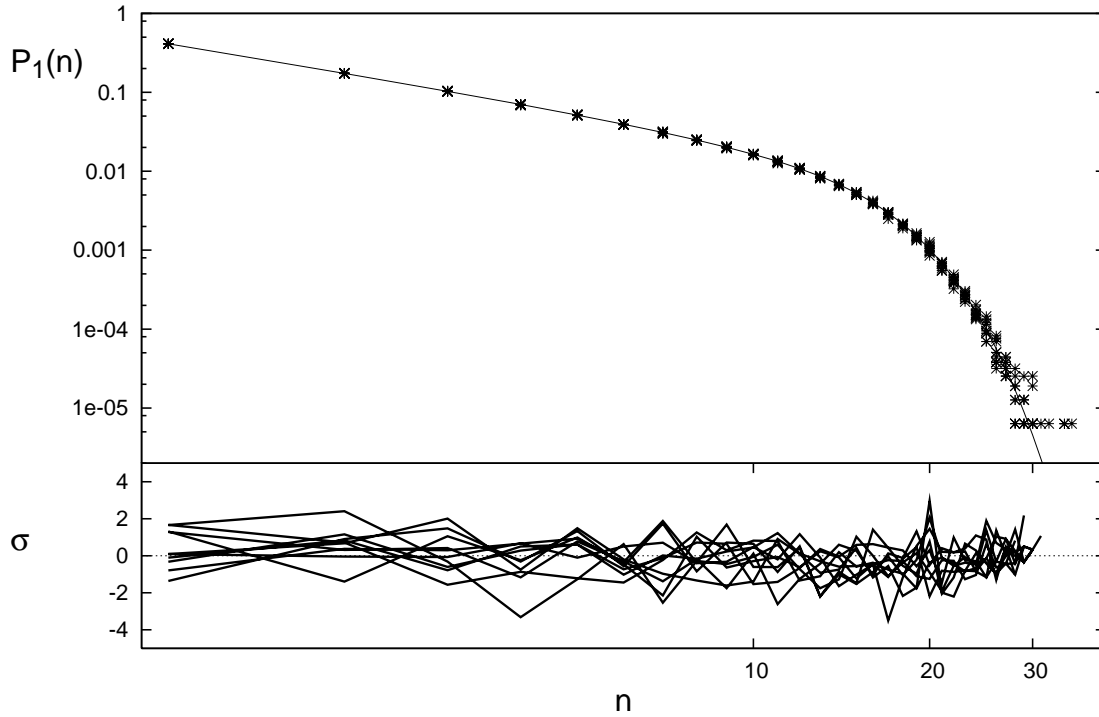


Figure 2. Photon counting distribution of a single diffusing molecule. Shown are ten numerical solutions of the Master equation (dots) and the analytical solution (line). Assumed are a Gaussian brightness function with $\mu_{\max} = 16.9499$ counts per bin, and a diffusion constant of $D = 2.8 \times 10^{-10} \text{ m}^2\text{s}^{-1}$. The simulations are performed for a diffusion time of 10s, i.e. 10^7 bins of $1\mu\text{s}$ each. The lower part shows the residuals for each of the ten numerical solutions. The residuals are weighted by the estimated statistical variance assuming independent measurement for each bin. In agreement with statistical variances in measurements⁶ the variances in the results of the simulations are greater than the expected ones by a factor of about three.

fails, because of the low statistical significance of the zeroth element $p(n = 0, c = 10^{-7}M)$. The first five moments of the extracted single molecule distribution are identical to the analytical values; the variances increase from about 0.1% for $c = 10^{-8}M$ to 1% for $c = 10^{-10}M$. Similar statistical significance has been obtained for the moments $\langle \mu^l \rangle$ of molecular brightness function $\mu(\vec{r})$, see Eq. 20. Therefore the inversion of relation Eq. 19 is utilized. Fig. 3 shows the single molecule functions $P_1(n)$ which have been extracted from the ten simulated experimental data for the concentration $c = 10^{-10}M$.

3. DISCUSSION

In this work the concept of molecular brightness applied in the PCH approach, FIDA and their successors is replaced by an effective volume and single molecule distribution which aims to characterize a molecular species for a given experimental setup. The single molecule distribution of a molecular species can be measured or simulated using molecular dynamic models, but a rigorous theoretical description has been lacking so far. We define the single molecule distribution on firm mathematical grounds and demonstrate its computation for a specific case. Our approach is mainly motivated by theoretical considerations but has several advantages for practical tasks, e.g. for the high sensitive determination of concentrations and for the analysis of mixtures.²⁵ Since the effective volume and the single molecule distribution are quantities which in practical applications should be measured they can serve as molecular fingerprint especially in complex environments.

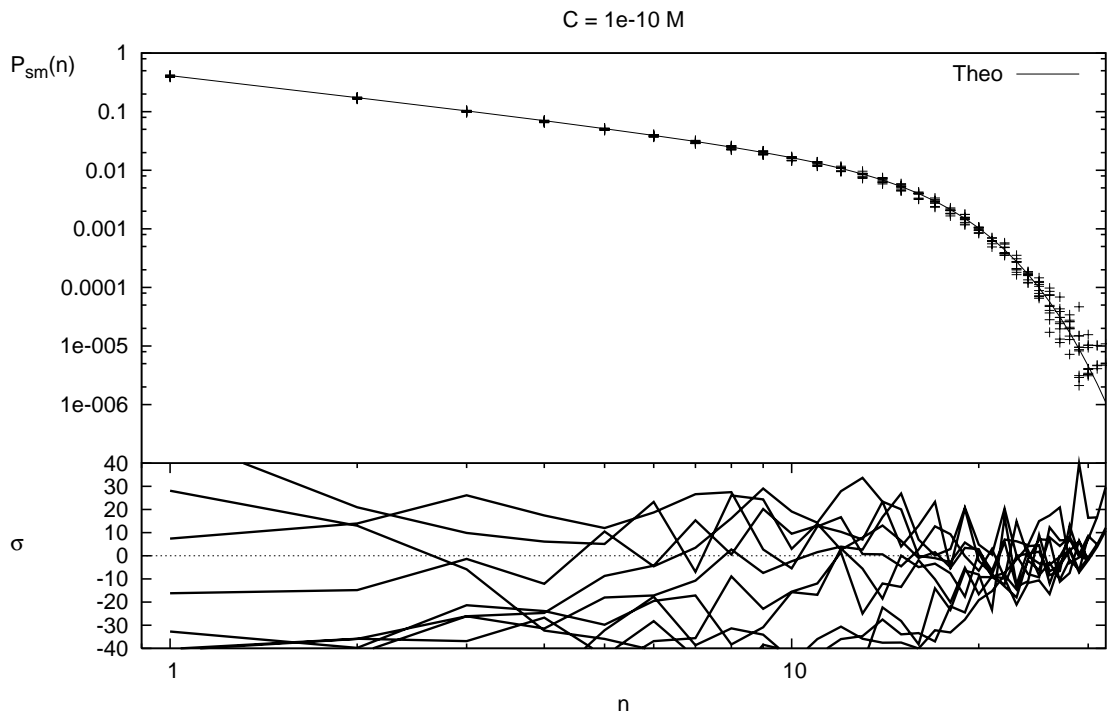


Figure 3. The single molecule functions $P_1(n)$ has been extracted from ten simulated experimental data for the concentration $c = 10^{-10}\text{M}$, see Eq. 12. Each simulation is represented by a cross. A line is drawn through the analytical values. The lower part shows the residuals for each of the ten extracted single molecule distributions.

This applies as well to measurements in micro-structured flow systems or inside cell compartments. Here the effects shaping the single molecule distribution may be completely unknown without inflicting the precision of the measurement, e.g. of concentrations in mixtures or binding constants. Modelling the effects responsible for the shape of the single molecule distribution is a theoretical task for itself. Exploring the analytical relations described above will improve measurement by FFS with high precision and under complex conditions.

In our illustrative example we discuss the case of molecular brightness function of Gaussian shape. This shape function is also a basic model for analysis in FCS. Compared to FCS we neglect the effect of diffusion completely, but it is obvious that the diffusive motion of a single molecule influences the single molecule distribution at least in the case of long bin width. Deriving a theoretical model for the dependence of the single molecule distribution on the molecular diffusion constant will open opportunities for alternatives to FCS. The discussion of an illustrative example including diffusion effects, e.g. based of molecular brightness function of Gaussian shape, is straight forward and intriguing, but out of the scope of this work. A worthwhile extension of our approach would be the discussion to subfractional focal volumes in stimulated emission depletion²⁶ and the discussion of the probability distribution of on and off periods.²⁷

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