Photon Counting Histograms for Diffusing Fluorophores

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The theory of photon counting histograms for fluorescent molecules diffusing through a laser spot is presented. Analytic expressions for the factorial cumulants of photon counts are obtained. For an arbitrary counting time window, it is shown how the exact histograms can be obtained by solving an appropriate reaction−diffusion equation. Our formalism reduces correctly when the molecules are immobile. The approximation used in fluorescence intensity multiple distribution analysis (FIMDA) is tested against the exact numerical solution of the problem. FIMDA works very well for a wide range of parameters except for small concentrations and long time windows.

1. Introduction

The fluorescence intensity fluctuates when a fluorescent molecule diffuses through the laser spot. Fluorescence correlation spectroscopy (FCS) provides information about the amplitude and time scale of these fluctuations (see refs 1,2). Specifically, one can determine the concentration and the average time spent by the molecule in the laser spot (i.e., the diffusion time).

Alternatively, fluorescence fluctuations can be studied using the distribution of fluorescence intensity (photon counts) integrated over a time window.[3−12] For short counting time windows, the distribution of photon counts was studied in the framework of "fluorescence intensity distribution analysis" (FIDA)[3] and the "photon counting histogram" (PCH) approach.[4] These lead to mathematically equivalent results. Both FIDA and PCH assume that molecules do not move during the time window, and therefore, the distribution of photon counts is Poissonian, averaged over the positions and the number of molecules in the observation volume. These methods allow one to determine not only the concentration but also the brightness of the species, providing information that is not accessible by FCS.

By varying the size of the photon counting window, the time dependence of fluorescence fluctuations can be studied.[5,11,12] In this case, the assumption that molecules are immobile during the time window is no longer valid. Because a diffusing molecule samples various points of the observation volume during the time window, the statistical properties of fluorescence intensity fluctuations are altered. To approximately account for the effect of diffusion on the photon counting histograms, Palo et al.[5] developed "fluorescence intensity multiple distribution analysis" (FIMDA). Recently Perroud et al.[12] introduced the photon counting multiple histogram (PCMH) method in order to approximately treat diffusion. Just like PCH and FIDA, PCMH and FIMDA are fundamentally identical. FIMDA approximates the distribution of photon counts for diffusive molecules by modifying the formula for immobile molecules so that the two factorial cumulants of the photon count distribution are exact. While FIMDA reduces correctly to the limit of static molecules, its applicability for the times comparable or greater than the diffusion time remains to be established.

In this paper, we present the theory for photon counting histograms when the fluorescent molecules diffuse through the laser spot. Specifically, we show how the theory we developed for single-molecule Förster resonance energy transfer (FRET) efficiency distributions[13] can be used to construct "few-molecule" photon counting histograms. According to our theory, the distribution of photons emitted by molecules diffusing through a spot can be found exactly by solving a reaction−diffusion equation. Other photophysical processes that influence fluorescence intensity, such as photobleaching, triplet blinking, or conformational changes, can be readily incorporated into this formalism. However, in this paper, the focus is only on the effect of diffusion. Specifically, we are interested in the dependence of photon counting histograms on the size of the time window and the concentration of the molecules. Using our formalism, we establish the range of validity of the approximation used in FIMDA to treat the effect of diffusion.

2. Theory

Consider fluorescent molecules with diffusion constant D and concentration c diffusing through a spot illuminated by the laser. When a single molecule occupies a fixed position inside the laser spot, it is usually assumed that the distribution of the number of photons detected in a time window is Poissonian. This is only true when all processes that may influence photon counting are fast compared to the time between consecutively detected photons.[13] The Poisson distribution is completely determined by the mean number of photons detected per unit time (mean count rate), which is given by the product of the detection efficiency, the quantum yield, the reciprocal lifetime, and the steady-state population of the excited state that depends on the laser intensity. The steady-state population and the detection efficiency are functions of the position $\boldsymbol{R}$ of the molecule. The mean number of photons detected per unit time that were emitted by a single molecule can be written as $\epsilon(\boldsymbol{R}) = \epsilon_0 W(\boldsymbol{R})$, where ϵ_0 is the specific brightness and $W(\boldsymbol{R})$ describes the observation volume profile normalized so that $W(0) = 1$.

Processes that are slower than the time between consecutively detected photons lead to distributions that deviate from Pois-

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sonian. These may include slow conformational changes, photobleaching, variation of the background noise, and laser intensity, etc. However, in this paper, we assume that the diffusion of the molecules through the laser spot is the only such slow process.

The generating function, $F(\lambda, T)$, of the probability to detect n photons in a time interval T, $P(n|T)$, is defined as

$$F(\lambda, T) = \sum_{n=0}^{\infty} \lambda^n P(n|T) \tag{2.1}$$

In our previous paper,[13] we obtained the generating function for a many-particle system of N independent fluorescent molecules diffusing in a volume V. By taking the thermodynamic limit ($N \to \infty$, $V \to \infty$, $N/V \to c$), we showed that the generating function can be written in a form analogous to the concentration in Smoluchowski's theory of irreversible diffusion-influenced reactions (see, for example, ref 14):

$$F(\lambda, T) = \exp(-c \int_0^T k(t|\lambda)\, \mathrm{d}t) \tag{2.2}$$

where $k(t|\lambda)$ is the analogue of the Smoluchowski time-dependent rate coefficient:

$$k(t|\lambda) = \int (1 - \lambda)\, \epsilon(\boldsymbol{R})\, g(\boldsymbol{R}, t|\lambda)\, \mathrm{d}\boldsymbol{R} \tag{2.3}$$

and $g(\boldsymbol{R}, t|\lambda)$ is the solution of the one-particle reaction−diffusion equation:

$$\frac{\partial}{\partial t} g = D\nabla^2 g - (1 - \lambda)\, \epsilon(\boldsymbol{R}) g \tag{2.4}$$

with initial condition $g(t = 0) = 1$. The first term describes diffusion, while the second term is responsible for counting photons.

Integrating eq 2.4 over t from 0 to T and over all $\boldsymbol{R}$, we find that

$$\int (1 - g(\boldsymbol{R}, T|\lambda))\, \mathrm{d}\boldsymbol{R} = \int_0^T \int (1 - \lambda)\, \epsilon(\boldsymbol{R})\, g(\boldsymbol{R}, t|\lambda)\, \mathrm{d}\boldsymbol{R}\, \mathrm{d}t \tag{2.5}$$

It then follows from eqs 2.2 and 2.3 that

$$F(\lambda, T) = \exp(c \int (g(\boldsymbol{R}, T|\lambda) - 1)\, \mathrm{d}\boldsymbol{R}) \tag{2.6}$$

When the time window is short so that molecules are effectively immobile and the diffusion term in eq 2.4 can be neglected, the generating function becomes:

$$F(\lambda, T) = \exp(c \int (e^{-(1-\lambda)\epsilon(\boldsymbol{R})T} - 1)\, \mathrm{d}\boldsymbol{R}) \tag{2.7}$$

This result for static molecules has been previously obtained in the framework of FIDA.[3] Expanding eq 2.7 in powers of λ and using eq 1, we get the distribution of photons for immobile molecules that is equivalent to the distribution in the framework of PCH analysis[4] in the thermodynamic limit.

The above formalism, eqs 2.1−2.6, rigorously solves the problem of determining the photon counting distribution for diffusing molecules. These are obtained by expanding the generating function in powers of λ. To get an explicit expression for the photon counting distribution, it is convenient to rewrite the generating function, eq 2.6, as

$$F(\lambda, T) = \exp(-\bar{N} f_0(T) + \bar{N} \sum_{n=1}^{\infty} \lambda^n f_n(T)) \tag{2.8}$$

where we have defined $f_n(T)$ as

$$-f_0(T) + \sum_{n=1}^{\infty} \lambda^n f_n(T) = \frac{1}{v} \int (g(\boldsymbol{R}, T|\lambda) - 1)\, \mathrm{d}\boldsymbol{R} \tag{2.9}$$

Here, v is the observation volume

$$v = \frac{[\int W(\boldsymbol{R})\, \mathrm{d}\boldsymbol{R}]^2}{\int [W(\boldsymbol{R})]^2\, \mathrm{d}\boldsymbol{R}} \equiv \overline{\epsilon^2} / \bar{\epsilon}^2 \tag{2.10}$$

and $\bar{N}$ is the average number of molecules in the observation volume

$$\bar{N} = cv \tag{2.11}$$

The dimensionless coefficients, $f_n(T)$, introduced above are all positive and concentration-independent. The probability of detecting no photons in the time window T, $P(0|T)$, is

$$P(0|T) = F(0, T) = \exp(-\bar{N} f_0(T)) \tag{2.12}$$

It is close to 1 when the concentration is very small. For $n \geq 1$, $f_n(T)$ are proportional to the probability of detecting n photons in time T that were emitted by a single molecule. This can be seen by expanding eq 2.8 in powers of λ in the small concentration limit so that as $\bar{N} \to 0$

$$P(n|T) = \bar{N} f_n(T), \qquad n \geq 1 \tag{2.13}$$

Thus, $f_n(T)$ for $n \geq 1$ can be considered as single-molecule (nonnormalized) distributions.

By expanding the generating function in eq 2.8 in powers of λ, we get the probability to detect n photons during time T for arbitrary concentrations. This is expressed in terms of the coefficients $f_j(T)$, with $j \leq n$ as:

$$P(n|T) = e^{-\bar{N} f_0(T)} \sum_{m=1}^{n} \frac{\bar{N}^m}{m!} \sum f_{j_1}(T) f_{j_2}(T) \ldots f_{j_m}(T) \tag{2.14}$$

where the summation is over all $j_i \geq 1$ such that

$$j_1 + j_2 + \ldots j_m = n \tag{2.15}$$

Each term in the above summation corresponds to the contribution from m molecules that emit j_1, j_2, ..., j_m photons.

2.1. Numerical Solution of the Reaction−Diffusion Equation. To get the photon counting histograms, one has to solve the reaction−diffusion equation, eq 2.4. This can be done analytically only for very special observation volume profiles. In the Appendix, we present the exact solution in Laplace space for the step function profile, i.e., $\epsilon(\boldsymbol{R}) = \epsilon_0$ if $R \leq a$, and $\epsilon(\boldsymbol{R}) = 0$ if $R > a$, where a is the radius of the observation volume. In general, eq 2.4 must be solved numerically. The coefficients $f_n(T)$ that are needed to obtain the photon counting histograms via eq 2.14 are related to the power series expansion of $g(\boldsymbol{R}, t|\lambda)$ in λ:

$$g(\boldsymbol{R}, t|\lambda) = \sum_{n=0}^{\infty} \lambda^n g_n(\boldsymbol{R}, t) \tag{2.16}$$

as (see eq 2.9)

$$f_0(T) = \frac{1}{v}\int [1 - g_0(\boldsymbol{R}, T)]\, d\boldsymbol{R} \quad (2.17a)$$

$$f_n(T) = \frac{1}{v}\int g_n(\boldsymbol{R}, T)\, d\boldsymbol{R} \quad (2.17b)$$

By substituting eq 2.16 into eq 2.4 and equating powers of λ, we find that $g_n(\boldsymbol{R}, t)$ satisfy the following set of coupled equations:

$$\frac{\partial}{\partial t}g_0 = D\nabla^2 g_0 - \epsilon(\boldsymbol{R})g_0 \quad (2.18a)$$

$$\frac{\partial}{\partial t}g_n = D\nabla^2 g_n - \epsilon(\boldsymbol{R})(g_n - g_{n-1}), \quad n \geq 1 \quad (2.18b)$$

with the initial condition $g_0(t = 0) = 1$ and $g_n(t = 0) = 0$ ($n \geq 1$). This set of equations is solved numerically. The numerical algorithm is described in the Appendix.

In the limit of a very short time window ($D \rightarrow 0$), eqs 2.18a and b are readily solved and

$$f_0(T) = \frac{1}{v}\int (1 - e^{-\epsilon(\boldsymbol{R})T})\, d\boldsymbol{R} \quad (2.19a)$$

$$f_n(T) = \frac{1}{v}\int \frac{[\epsilon(\boldsymbol{R})T]^n}{n!} e^{-\epsilon(\boldsymbol{R})T}\, d\boldsymbol{R}, \quad n \geq 1 \quad (2.19b)$$

By using this in eq 2.14, we get the photon-counting histogram for immobile molecules. This result is equivalent to that obtained previously in the framework of the PCH method[4] in the thermodynamic limit (i.e., infinite volume of the system). In contrast to the PCH method, our $f_n(T)$ are defined in such a way that they do not involve the volume of the system.

2.2. Moments of the Photon Counting Distributions. Finally, we show how our formalism can be used to obtain the factorial cumulants of the photon counts.[15,16] The factorial cumulants are defined as

$$\kappa_n = \frac{\partial^n}{\partial \lambda^n} \ln F(\lambda, T)\Big|_{\lambda=1} \quad (2.20)$$

The explicit expressions for the first cumulants in terms of the moments of photon counts ($\langle n^i \rangle = \sum_{n=0}^{\infty} n^i P(n|T)$) are:

$$\begin{aligned} \kappa_1 &= \langle n \rangle \\ \kappa_2 &= \langle n(n-1) \rangle - \kappa_1{}^2 \\ \kappa_3 &= \langle n(n-1)(n-2) \rangle - 3\kappa_1\kappa_2 - \kappa_1{}^3 \end{aligned} \quad (2.21)$$

By using the generating function, eq 2.6, in eq 2.20, we get:

$$\kappa_n = c\,\frac{\partial^n}{\partial \lambda^n}\int (g(\boldsymbol{R}, T|\lambda) - 1)\, d\boldsymbol{R}\Big|_{\lambda=1} \quad (2.22)$$

To get the explicit expression for the cumulants, we first rewrite the differential equation for g, eq 2.4, as an integral equation:

$$g(\boldsymbol{R}, t|\lambda) - 1 = (\lambda - 1)\int_0^t dt' \int G(\boldsymbol{R}, t|\boldsymbol{R}', t')\, \epsilon(\boldsymbol{R}')\, g(\boldsymbol{R}', t'|\lambda)\, d\boldsymbol{R}' \quad (2.23)$$

where $G(\boldsymbol{R}, t|\boldsymbol{R}', t') = \exp(-(\boldsymbol{R} - \boldsymbol{R}')^2/4D(t - t'))/(4\pi D(t - t'))^{3/2}$ is the free diffusion Green's function. The perturbation series expansion of g is obtained by iterating this equation. Integrating the series over $\boldsymbol{R}$, we find

$$\begin{aligned} \int (g(\boldsymbol{R}, t|\lambda) - 1)\, d\boldsymbol{R} &= (\lambda - 1)\bar{\epsilon}t \\ &+ (\lambda - 1)^2\overline{\epsilon^2}\int_0^t dt_1 \int_0^{t_1} dt_2 C_2(t_1 - t_2) \\ &+ (\lambda - 1)^3\overline{\epsilon^3}\int_0^t dt_1 \int_0^{t_1} dt_2 \int_0^{t_2} dt_3 C_3(t_1 - t_2, t_2 - t_3) \\ &+ \ldots \end{aligned} \quad (2.24)$$

where we have defined

$$\overline{\epsilon^n} = \int [\epsilon(\boldsymbol{R})]^n\, d\boldsymbol{R} \quad (2.25)$$

and $C_n(t_1 - t_2, t_2 - t_3, \ldots, t_{n-1} - t_n)$ is the n-point correlation function:

$$\begin{aligned} C_n(t_1 - t_2, \ldots, t_{n-1} - t_n)\,\overline{\epsilon^n} &= \int \epsilon(\boldsymbol{R}_1)\, G(\boldsymbol{R}_1, t_1|\boldsymbol{R}_2, t_2) \ldots \\ &\ldots \epsilon(\boldsymbol{R}_{n-1}) G(\boldsymbol{R}_{n-1}, t_{n-1}|\boldsymbol{R}_n, t_n)\, \epsilon(\boldsymbol{R}_n)\, d\boldsymbol{R}_1 \ldots d\boldsymbol{R}_n \end{aligned} \quad (2.26)$$

Note that t_n can be set to zero without loss of generality. The two-point correlation function $C_2(t)$ is the familiar fluorescence intensity autocorrelation function of diffusing molecules, normalized so that $C_2(0) = 1$:

$$C_2(t)\overline{\epsilon^2} = \int \epsilon(\boldsymbol{R}_1)\, G(\boldsymbol{R}_1, t|\boldsymbol{R}_2, 0)\, \epsilon(\boldsymbol{R}_2)\, d\boldsymbol{R}_1\, d\boldsymbol{R}_2 \quad (2.27)$$

All these autocorrelation functions can be obtained from the FCS.

By taking the derivatives of eq 2.24 with respect to λ (see eq 2.22), it follows that the factorial cumulants of the photon counts are proportional to the terms of the perturbation series:

$$\begin{aligned} \kappa_1 &= c\,\bar{\epsilon}T \\ \kappa_2 &= 2c\overline{\epsilon^2}\int_0^T dt_1\, (T - t_1) C_2(t_1) \\ \kappa_3 &= 6c\overline{\epsilon^3}\int_0^T dt_1\, (T - t_1) \int_0^{t_1} dt_2\, C_3(t_1 - t_2, t_2) \\ \kappa_n &= n!c\overline{\epsilon^n}\int_0^T dt_1 (T - t_1) \int_0^{t_1} dt_2 \ldots \\ &\qquad \ldots \int_0^{t_{n-2}} dt_{n-1}\, C_n(t_1 - t_2, \ldots, t_{n-1}) \end{aligned} \quad (2.28)$$

The first two cumulants agree with previous results.[5,16]

In summary, both the photon counting distributions and the factorial cumulants are related to the solution of the same reaction−diffusion equation. The coefficients $f_n(T)$ are found from the power series expansion of $g(\boldsymbol{R}, t|\lambda)$ around $\lambda = 0$. The cumulants κ_n are found from the power series expansion around $\lambda = 1$.

3. FIMDA

In this section, we present the analytical approximation for the distributions $f_n(T)$ used in fluorescence intensity multiple-distribution analysis (FIMDA).[5] In this approach, the generating function for the immobile molecules, eq 2.7, is modified so that the two factorial cumulants of photon counts are exact for arbitrary time windows:

$$F(\lambda, T) = \exp\left(\frac{c}{\Gamma(T)} \int (e^{-(1-\lambda)\epsilon(\boldsymbol{R})T\,\Gamma(T)} - 1)\, \mathrm{d}\boldsymbol{R}\right) \quad (3.1)$$

where

$$\Gamma(T) = \frac{2}{T^2} \int_0^T (T - t) C_2(t)\, \mathrm{d}t \quad (3.2)$$

By expanding the logarithm of this generating function in powers of $(1 - \lambda)$, one can verify that the first two terms of the expansion are the same as those in eq 2.24, and therefore, the first two factorial cumulants are indeed exact.

The coefficients $f_n(T)$ in FIMDA are obtained by expanding $\ln F(\lambda, T)$ in powers of λ:

$$f_0(T) = \frac{1}{v\Gamma(T)} \int (1 - e^{-\epsilon(\boldsymbol{R})T\Gamma(T)})\, \mathrm{d}\boldsymbol{R} \quad (3.3a)$$

$$f_n(T) = \frac{1}{v\Gamma(T)} \int \frac{[\epsilon(\boldsymbol{R})T\Gamma(T)]^n}{n!} e^{-\epsilon(\boldsymbol{R})T\Gamma(T)}\, \mathrm{d}\boldsymbol{R}, \quad n \geq 1 \quad (3.3b)$$

Note that these can be obtained from the corresponding static distributions, eq 2.19, by replacing $v \rightarrow v\Gamma(T)$ and $\epsilon(\boldsymbol{R}) \rightarrow \Gamma(T)\epsilon(\boldsymbol{R})$. In the static limit $D \rightarrow 0$, $\Gamma(T) \rightarrow \Gamma(0) = 1$.

4. Illustrative Calculations and Discussion

Spatial distribution of the emitted light in one-photon excitation experiments is often approximated by 3D Gaussian:

$$W(\boldsymbol{R}) = \epsilon(\boldsymbol{R})/\epsilon_0 = \exp\left(-\frac{2(x^2 + y^2)}{a_1^2} - \frac{2z^2}{a_2^2}\right) \quad (4.1)$$

where a_1 and a_2 are lateral and axial dimensions of the observation volume. Although it is known that 3D Gaussian is an inadequate description of the experimental profile,[9,10] for illustrative purposes, we use the isotropic version of this profile ($a_1 = a_2 = a$). In this case, the autocorrelation function, eq 2.27, is[17]

$$C_2(t) = (1 + 4Dt/a^2)^{-3/2} \quad (4.2)$$

Figure 1 compares the exact (open circles), FIMDA (solid lines), and static (dashed lines) photon-counting histograms for the molecules diffusing through the laser spot when the time window is short or comparable with the diffusion time. For the parameters we used ($a = 1\ \mu$m, $D = 10^{-3}\ \mu\mathrm{m}^2/\mu$s, $\epsilon_0 = 0.1\ \mu\mathrm{s}^{-1}$), the diffusion time, defined as the integral of the correlation function, is $\tau_{\mathrm{dif}} = \int_0^\infty C_2(t)\, \mathrm{d}t = a^2/2D = 500\ \mu$s. The diffusion time obtained from the asymptotic behavior of the autocorrelation function is $\tau'_{\mathrm{dif}} = a^2/4D = 250\ \mu$s. The three panels represent the histograms at various concentrations ($\bar{N} = 1, 0.1, 0.01$). The histograms are calculated using eq 2.14 with different $f_n(T)$. The exact $f_n(T)$ are obtained from the numerical solution of eqs 2.18−2.17, as described in the Appendix. For immobile molecules, $f_n(T)$ are given by eqs 2.19, and in the framework of FIMDA, they are given by eqs 3.3.

Figure 2 compares the exact (open circles) and the FIMDA histograms (solid lines) by using the same parameters as Figure 1 but for the time windows longer than the diffusion time. If the specific brightness, ϵ_0, is decreased or increased by a factor of 2, the agreement between the FIMDA and the exact calculations is essentially the same as in these figures.

It can be seen that the assumption of immobile molecules is applicable only at very short time windows compared to the diffusion time. The difference between the exact histogram for diffusing molecules and that for immobile molecules increases as the photon counts increase. This is because large photon counts result from molecules located in a small region close to the center of the observation volume. The residence time of a molecule in this small region is much shorter than the average diffusion time, and thus the assumption that such a molecule is static is particularly poor.

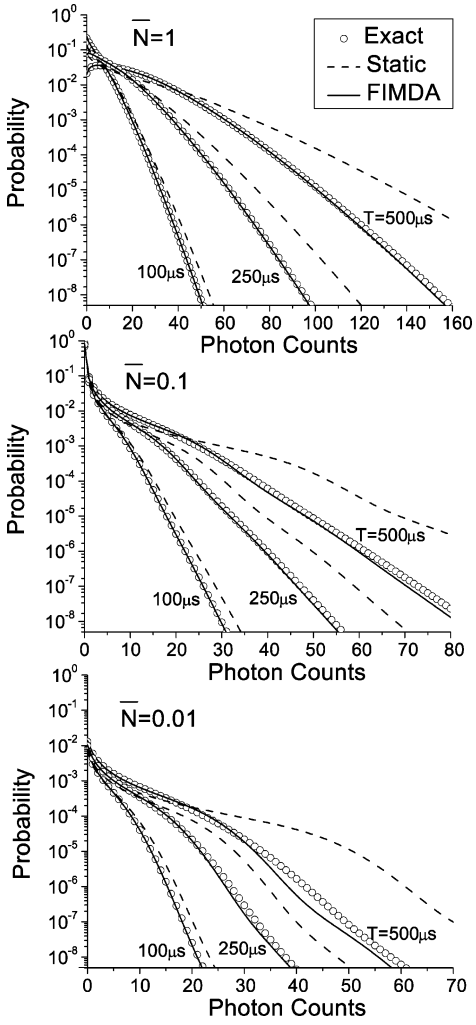

Figure 1. Photon-counting histograms, $P(n|T)$, for short time windows. Exact histograms (open circles) are obtained by solving eqs 2.18−2.17 numerically. Observation volume profile is isotropic and Gaussian with $a = 1\ \mu$m, $D = 10^{-3}\ \mu\mathrm{m}^2/\mu$s, $\epsilon_0 = 0.1\ \mu\mathrm{s}^{-1}$. Solid lines correspond to the FIMDA approximation, dashed lines are for immobile molecules.

The FIMDA approximation works very well for a wide range of parameters. For short time windows, the agreement with the exact histograms is good for both high and low concentrations. For time windows longer than the diffusion time, FIMDA is good as long as the concentration is not too small. It breaks down when there is less than 0.1 molecules in the observation volume on the average. In this regime, the undulations in the FIMDA results is an artifact due to an inadequate treatment of the higher moments.

For a fixed time window, FIMDA works better when the concentration of molecules increases. The reason is that, at high concentrations, the detected photons were more than likely emitted by several molecules. Because each of these molecules emits a small number of photons, the counting histogram is primarily determined by the single-molecule probabilities (which are proportional to $f_n(T)$ for $n \geq 1$) with small n, which are better described by FIMDA.

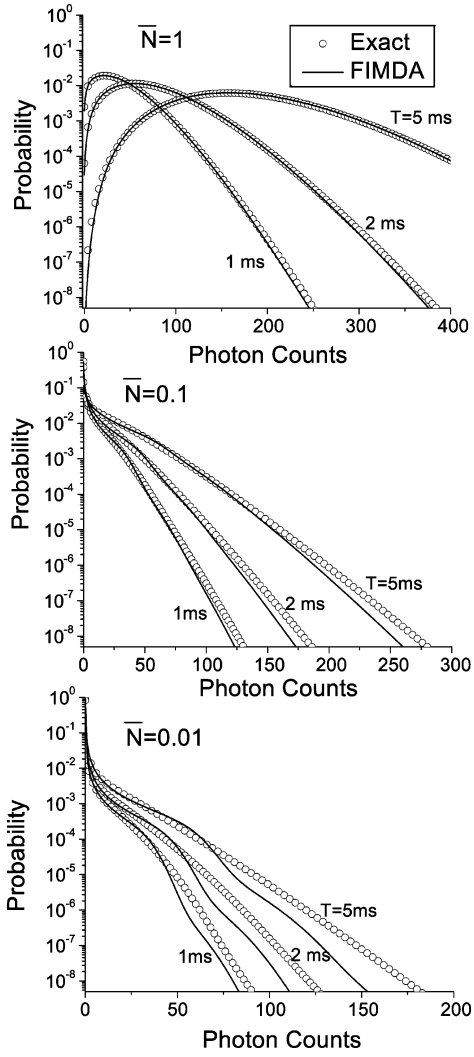

Figure 2. Same as Figure 1, for long time windows. Solid lines correspond to the FIMDA approximation.

In summary, we have developed the theory of photon counting histograms for the molecules diffusing through the laser spot, making it possible to analyze data for arbitrary time windows. For the sake of simplicity, we have explicitly considered one fluorescent species. The theory for several fluorescent species with different brightness can be readily obtained by replacing $c\,(g(\boldsymbol{R}, T|\lambda) - 1) \rightarrow \sum_j c_j(g(j, \boldsymbol{R}, T|\lambda) - 1)$ in eq 6, where $g(j, \boldsymbol{R}, T|\lambda)$ corresponds to the jth species with the concentration c_j and obeys eq 2.4 with the appropriate diffusion constant (D_j) and specific brightness (ϵ_{0j}). We have tested the approximation used in FIMDA[5] and have shown that it works remarkably well for a wide range of parameters. However, it breaks down for single-molecule measurements, where the concentrations are very small and the time windows are usually longer than the diffusion time.

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Appendix A. Numerical Solution of the Reaction−Diffusion Equation

In this appendix we describe how we solved eqs 2.18 numerically for an isotropic observation volume profile by discretizing space and time coordinates and approximating the derivatives by finite differences. In discretizing the space coordinate, we follow the variable grid algorithm described by Krissinel et al.[18] Finite differencing of the time coordinate is based on the Crank−Nicholson algorithm.

Our space grid $R(i)$, $i = 1, 2, ..., N$, is nonlinear outside the observation volume:

$$R(i) = \begin{cases} (i - 1/2)\Delta, & i \le N_{\text{in}} \\ (i - 1/2)\Delta + (R_{\text{out}} - N\Delta)\left(\dfrac{i - N_{\text{in}} - 1/2}{N_{\text{out}}}\right)^2, & \\ & N_{\text{in}} + 1 \le i \le N_{\text{in}} + N_{\text{out}} \end{cases} \quad \text{(A.3)}$$

where N_{in} and N_{out} is the number of the grid nodes inside and outside the observation volume with characteristic radius b, $N = N_{\text{in}} + N_{\text{out}}$ is the total number of the grid points, R_{out} is the outer boundary, and $\Delta \equiv b/N_{\text{in}}$ is the step size inside the volume. This grid satisfies the conditions $R(1/2) = 0$, $R(N_{\text{in}} + 1/2) = b$, $R(N + 1/2) = R_{\text{out}}$.

The Laplacian in eqs 2.18 is replaced by an $N \times N$ tridiagonal matrix $\boldsymbol{L}$ with the nonzero elements $L_{ii} = -(l_i^+ + l_i^-)$, $L_{ii\pm1} = l_i^\pm$, $L_{11} = -l_1^+$, $L_{NN} = -l_N^-$, where

$$l_i^\pm = \frac{4\pi D[R(i \pm 1/2)]^2}{v_i s(i \pm 1/2)} \quad \text{(A.4)}$$

Here, $s(i) = \mathrm{d}R(i)/\mathrm{d}i$ is the derivative of $R(i)$, and v_i is the volume of ith layer

$$v_i = \frac{4\pi}{3}([R(i + 1/2)]^3 - [R(i - 1/2)]^3) \quad \text{(A.5)}$$

This discretization corresponds to the reflecting boundary condition at $R = 0$ and $R = R_{\text{out}}$.

By applying this discretization to eqs 2.18, one gets a linear system of matrix differential equations:

$$\frac{\mathrm{d}}{\mathrm{d}t}\boldsymbol{g}_0(t) = (\boldsymbol{L} - \epsilon)\boldsymbol{g}_0 \quad \text{(A6.a)}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\boldsymbol{g}_n(t) = (\boldsymbol{L} - \epsilon)\boldsymbol{g}_n + \epsilon\boldsymbol{g}_{n-1}, \qquad n \ge 1 \quad \text{(A6.b)}$$

where $\boldsymbol{g}_n(t)$ is a vector with the components $g_n(R(i), t)$, and ϵ is the diagonal matrix, with the elements $\epsilon(R(i))$ on the diagonal.

The time derivative in the above equations is approximated by using the Crank−Nicholson algorithm, according to which a differential equation

$$\frac{\mathrm{d}}{\mathrm{d}t}f(t) = \psi(t) \quad \text{(A.7)}$$

is approximated by

$$\frac{f(t + \tau) - f(t)}{\tau} = \frac{1}{2}[\psi(t) + \psi(t + \tau)] \quad \text{(A.8)}$$

where τ is the time step. Applying the Crank−Nicholson algorithm to eq A.6, we get:

$$\boldsymbol{g}_0(t + \tau) = \boldsymbol{A}\,\boldsymbol{g}_0(t) \quad \text{(A.9a)}$$

$$\boldsymbol{g}_n(t + \tau) = \boldsymbol{A}\,\boldsymbol{g}_n(t) + \boldsymbol{B}(\boldsymbol{g}_{n-1}(t) + \boldsymbol{g}_{n-1}(t + \tau)) \quad \text{(A.9b)}$$

where

$$\boldsymbol{A} = \left(\boldsymbol{I} - \frac{\tau}{2}(\boldsymbol{L} - \boldsymbol{\epsilon})\right)^{-1}\left(\boldsymbol{I} + \frac{\tau}{2}(\boldsymbol{L} - \boldsymbol{\epsilon})\right)$$
$$\boldsymbol{B} = \frac{\tau}{2}\left(\boldsymbol{I} - \frac{\tau}{2}(\boldsymbol{L} - \boldsymbol{\epsilon})\right)^{-1}\boldsymbol{\epsilon} \tag{A.10}$$

Because our time step is constant, the matrixes $\boldsymbol{A}$ and $\boldsymbol{B}$ are calculated only once. At every time step, $\boldsymbol{g}_n(t + \tau)$ is calculated successively starting with $n = 0$.

The integration over the volume, which is necessary to calculate the coefficients $p_n(T)$ (see eq 2.17), is performed according to the rule

$$\int f(\boldsymbol{R})\, \mathrm{d}\boldsymbol{R} \rightarrow \sum_{i=1}^{N} v_i\, f\,(R(i)) \tag{A.11}$$

In Figures 1 and 2, we used $R_{\mathrm{out}} = 5\ \mu$m, $b = a = 1\ \mu$m, $D = 10^{-3}\ \mu\mathrm{m}^2/\mu$s, $N_{\mathrm{in}} = N_{\mathrm{out}} = 50$, $\Delta = b/N_{\mathrm{in}} = 0.02\ \mu$m, and $\tau = 0.1\ \mu$s.

The accuracy of the above procedure was checked for the step observation volume profile, i.e.,

$$\epsilon(R) = \begin{cases} \epsilon_0, & R \le a \\ 0, & R > a \end{cases} \tag{A.12}$$

for which eq 2.4 can be solved analytically. Specifically, in Laplace space

$$-\hat{f}_0(s) + \sum_{n=1}^{\infty} \lambda^n \hat{f}_n(s) \tag{A.13a}$$

$$= \frac{(1-\lambda)\epsilon_0}{s(s + (1-\lambda)\epsilon_0)}\left[1 + \frac{3D}{a^2}\frac{(1-\lambda)\epsilon_0(1+x)}{s(s + (1-\lambda)\epsilon_0)}\frac{y\coth(y) - 1}{y\coth(y) + x}\right] \tag{A.13b}$$

where $x = \sqrt{sa^2/D}$ and $y = \sqrt{[s+(1-\lambda)\epsilon_0]a^2/D}$. The coefficients $p_n(T)$ are obtained by first inverse Laplace transforming using the Stehfest algorithm[19] and then expanding in powers of λ using Mathematica (Wolfram Research, Champaign, IL).

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