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Theory of Fluorescence Correlation Spectroscopy on Single Molecules

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A theoretical framework is presented that connects “classic” fluorescence correlation spectroscopy (FCS) treating averages of many freely diffusing molecules and FCS single-molecule analysis. By assuming a general two-state emission dynamics of the single molecule, expressions describing the autocorrelation of the total fluorescence fluctuations are derived. By studying an idealized experimental situation, the relation between signal-to-noise and signal-to-background ratios are discussed. Under appropriate conditions it is possible to make statistically feasible measurements of single molecule dynamics despite low signal-to-background. The quantum yield ratio of the background molecules to the single molecule as well as the position of the single molecule inside the detection volume are crucial in obtaining good signal-to-noise ratios in single-molecule experiments.

1. Introduction

Fluorescence Correlation Spectroscopy (FCS) is used in a wide range of experiments involving temporal dynamics of the fluorescence from molecules in solution.[1] The fluorescence fluctuations can have different origins such as Brownian motion of the molecules,[2] rotational Brownian motion of the molecule,[3] chemical reactions changing the fluorescence properties of the molecule,[2] or singlet−triplet interactions in a molecule.[4] Recently, FCS has been applied to experiments involving the study of single immobilized molecules.[5,6] The autocorrelation of the detected fluorescence is a very informative addition to the fluorescence intensity trace of which both are recorded in real time during the experiment.

So far, the experimental introduction of FCS on single immobilized molecules[5,6] has not been accompanied by a more in depth theoretical treatment that penetrate the connection between “classic” FCS with many particles undergoing Brownian motion and the case of a single immobilized molecule. In this paper, we derive autocorrelation functions describing the situation with a single immobilized molecule in a background of many freely diffusing molecules. Furthermore, we study the statistical properties of the autocorrelation function with the aim to investigate the feasibility to do single molecule analysis under different experimental conditions.

In the typical single-molecule experiment, effort is put in to optimize the conditions in the direction of highest possible ratio between the fluorescence intensity signal of the single molecule and the fluorescence signal of the background (e.g., S/B ratio = single molecule fluorescence intensity/background fluorescence intensity). However, many experiments on single molecules studied under biologically relevant conditions have an inherent fluorescence background, which often is difficult to reduce without paying a price of less biological significance.[6] It is therefore motivated to make a more detailed investigation with aim to penetrate how the experimental conditions in a typical single molecule experiment affect the visibility of the spectroscopic dynamic features of the single molecule. The visibility is here defined as the signal-to-noise ratio (S/N = signal/standard deviation of the signal) of the autocorrelation of the emission dynamics of the single molecule in a background. The analysis is carried out assuming an idealized situation ignoring limited measuring time, and the discrete representation of time in the measurement equipment.[7] Also, so-called shot-noise effects[8] will not be included in the present analysis, which means that we assume that the absolute values of the quantum yields are not very small. This should not induce a restriction to the present analysis since it makes use of the differences (ratios) of the quantum yields of the single molecule and the background.

The variance of the autocorrelation function enables for the S/N ratios of a given model to be defined yielding an analytical expression between S/B and S/N ratios. One of the goals in the present paper, to assess various experimental conditions versus the visibility of single molecule dynamics in a background, can then be achieved. An analysis is made on the interplay of the single molecule and the background fluorescence with regard to physical parameters. Examples of such parameters are the optical properties of the molecules, the position of the single molecule in relation to the excitation light profile, the excitation light intensity, autocorrelation time scales implied by diffusion times, and the dynamics of the single molecule.

The expressions of the variance should also be useful as weight functions in evaluation (fitting) of experimental data to a given model. However, we point out that the best possible weight function is obtained by the variance of the experimental autocorrelation data when available.[9]

2. Derivation of the Correlation Function

2.1. No Background Fluorescence. Consider a general expression of the autocorrelation of the fluorescence intensity fluctuations as given in[3]

$$G(\tau) = \langle I \rangle^2 + \langle \delta I(0)\delta I(\tau) \rangle \qquad (1)$$

In eq 1, $I(t)$ denotes the detected photon flux at time t. $\delta I(t)$ denotes the photon flux deviation from its mean value at time

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t. A stationary state is assumed in which photons are detected with equal probability in the sample volume V. Also[3]

$$\langle \delta I(0)\delta I(\tau)\rangle = \int_V \int_V \left[f(\mathbf{r},\tau|\mathbf{r}_0) - \frac{\langle C(\mathbf{r})\rangle}{M}\right]\langle C(\mathbf{r}_0)\rangle \, \mathrm{d}\mathbf{r}\, \mathrm{d}\mathbf{r}_0 \quad (2)$$

where $f(\mathbf{r},\tau|\mathbf{r}_0)\mathrm{d}\mathbf{r}\,\mathrm{d}t$ denotes the probability that a molecule that emitted a photon within the interval [0,dt] from a small volume centered around $\mathbf{r}_0$ emits another photon within the interval [τ,τ+dt] from a small volume centered around $\mathbf{r}$. M is the number of molecules in the total sample volume V. No particle transport can take place through the surface of V. In this paper we will assume that V is infinitely large as compared to all other characteristic volumes. Below, we hence set V equal to full three-dimensional space ($V = R^3$). $\langle C(\mathbf{r})\rangle \mathrm{d}\mathbf{r}\,\mathrm{d}t$ is the unconditional probability to detect a photon from a very small volume centered around $\mathbf{r}$ in a time period dt originating from any of the total M sample molecules.

In a single-molecule experiment we have $M = 1$. For simplicity we will assume that the spectroscopic fluctuations of the single molecule consist of two states, one fluorescent state and one nonfluorescent state. The binary description of the molecule's spectroscopic fluctuation is supported by the experimental conditions of a large set of single molecule experiments.[5,6,10−12]

$\langle C(\mathbf{r})\rangle$ is related to the molecular concentration $c_\mathrm{m}(\mathbf{r})$ per unit volume, the laser light intensity $i(\mathbf{r})$, the probability that a laser light photon incident at the molecule will give rise to an by the molecule emitted photon (q_m), and the probability that the molecule is in its fluorescent state p_f. We have

$$p_\mathrm{f} = \lim_{\tau\to\infty} f(\tau) \quad (3)$$

where $f(\tau)$ is the probability that the immobilized single molecule situated at $\mathbf{r}_\mathrm{m}$ is in its fluorescent state time τ given that the molecule was in its fluorescent state time 0. The difference between the conditional probability $f(\tau)$ and the unconditional probability p_f gives the correlation between two detected photons. Observe that $f(\tau)$ is a conditional probability that provide information about local variations of the stationary fluorescence intensity $I(t)$.

Hence,

$$\langle C(\mathbf{r})\rangle \mathrm{d}\mathbf{r}\,\mathrm{d}t = c_\mathrm{m}(\mathbf{r})i(\mathbf{r})q_\mathrm{m}p_\mathrm{f}\mathrm{d}\mathbf{r}\,\mathrm{d}t \quad (4)$$

In the case of a single immobilized molecule, the concentration $c(\mathbf{r})$ is concentrated to one single spot $\mathbf{r}_\mathrm{m}$:

$$c_\mathrm{m}(\mathbf{r}) = \delta(\mathbf{r} - \mathbf{r}_\mathrm{m}) \quad (5)$$

Equations 3−5 substituted into eq 2 yields

$$\langle \delta I(0)\delta I(\tau)\rangle = \int_{R^3} f(\mathbf{r},t|\mathbf{r}_\mathrm{m})p_\mathrm{f}q_\mathrm{m}i(\mathbf{r}_\mathrm{m})\mathrm{d}\mathbf{r} - [\mathrm{p}_\mathrm{f}q_\mathrm{m}i(\mathbf{r}_\mathrm{m})]^2 \quad (6)$$

The molecule always emits fluorescence at the same single spot $\mathbf{r}_\mathrm{m}$, hence

$$f(\mathbf{r},\tau|\mathbf{r}_\mathrm{m})d\mathbf{r}\,\mathrm{d}t = f(\tau)i(\mathbf{r}_\mathrm{m})q_\mathrm{m}\delta(\mathbf{r} - \mathbf{r}_m)\mathrm{d}\mathbf{r}\,\mathrm{d}t \quad (7)$$

The analysis in this paper is based on an immobilized single molecule that shifts its fluorescent properties between one nonfluorescent state and one fluorescent state. The fluorescent state is characterized by that a photon incident at the molecule with probability q_m will give rise to a by the molecule emitted photon.

Combining eqs 6 and 7 yields

$$\langle \delta I(0)\delta I(\tau)\rangle = (f(\tau) - p_\mathrm{f})p_\mathrm{f}[i(\mathbf{r}_\mathrm{m})q_\mathrm{m}]^2 \quad (8)$$

The mean intensity from the single molecule is equal to

$$\langle I\rangle = \int_{R^3} c(\mathbf{r})i(\mathbf{r})q_\mathrm{m}p_\mathrm{f}\, d\mathbf{r} = i(\mathbf{r}_\mathrm{m})q_\mathrm{m}p_\mathrm{f} \quad (9)$$

Equationss 8 and 9 in eq 1 gives the expression of the normalized autocorrelation functions for the case of a single immobilized molecule without background fluorescence,

$$\frac{G(\tau)}{\langle I\rangle^2} = G_\mathrm{N}(\tau) = 1 + \left[\frac{f(\tau)}{p_\mathrm{f}} - 1\right] = \frac{f(\tau)}{p_\mathrm{f}} \quad (10)$$

2.2. Background Fluorescence. In the case of fluorescence from freely diffusing molecules, in addition to the fluorescence of the single molecule, the photon flux $I(t)$ will have two origins. First, the fluorescence from the single molecule ($I_\mathrm{m}(t)$) and second the fluorescence from the freely diffusing molecules ($I_\mathrm{f}(t)$),

$$I(t) = I_\mathrm{m}(t) + I_\mathrm{f}(t) \quad (11)$$

Hence,

$$\begin{aligned}\langle \delta I(0)\delta I(\tau)\rangle &= \langle \delta(I_\mathrm{m}(0) + I_\mathrm{f}(0))\delta(I_\mathrm{m}(\tau) + I_\mathrm{f}(\tau))\rangle = \\ &\langle \delta I_\mathrm{m}(0)\delta I_\mathrm{m}(\tau) + \delta I_\mathrm{m}(0)\delta I_\mathrm{f}(\tau) + \delta I_\mathrm{f}(0)\delta I_\mathrm{m}(\tau) + \\ &\qquad \delta I_\mathrm{f}(0)\delta I_\mathrm{f}(\tau)\rangle = \\ &\langle \delta I_\mathrm{m}(0)\delta I_\mathrm{m}(\tau)\rangle + \langle \delta I_\mathrm{m}(0)\delta I_\mathrm{f}(\tau)\rangle + \langle \delta I_\mathrm{f}(0)\delta I_\mathrm{m}(\tau)\rangle + \\ &\qquad \langle \delta I_\mathrm{f}(0)\delta I_\mathrm{f}(\tau)\rangle \quad (12)\end{aligned}$$

We still have one single immobilized molecule, eq 2 with $M = 1$ provides the expression of $\langle \delta I_\mathrm{m}(0)\delta I_\mathrm{m}(\tau)\rangle$. On the other hand, the total number of freely diffusing fluorescent molecules within the volume V is very large in the typical FCS experiment wherefore eq 2 derived for an ensemble of background molecules yield

$$\langle \delta I_\mathrm{f}(0)\delta I_\mathrm{f}(\tau)\rangle = \int_{R^3}\int_{R^3} f_\mathrm{f}(\mathbf{r},\tau|\mathbf{r}_0)\langle C_\mathrm{f}(\mathbf{r}_0)\rangle \mathrm{d}\mathbf{r}\,\mathrm{d}\mathbf{r}_\mathbf{0} \quad (13)$$

where the indices "f" indicate that the entities are defined for the case of freely diffusing molecules.

In this paper we will assume that

$$\langle \delta I_\mathrm{m}(0)\delta I_\mathrm{f}(\tau)\rangle = \langle \delta I_\mathrm{f}(0)\delta I_\mathrm{m}(\tau)\rangle = 0 \quad (14)$$

meaning that the fluorescence from the single molecule is not correlated with the fluorescence of the free molecules, and vice versa. This assumption is based on experimental facts from the study of single immobilized enzyme molecules[6] where the time scales of the dynamics of the single molecule and the characteristic passage time of the background molecules through detection volume are very different.

Using eqs 8 and 12−14 in eq 12, we obtain

$$\begin{aligned}\langle \delta I(0)\delta I(\tau)\rangle = q_\mathrm{f}^2 c \int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},\tau|\mathbf{r}_\mathbf{0})i(\mathbf{r}_\mathbf{0})\mathrm{d}\mathbf{r}\,\mathrm{d}\mathbf{r}_\mathbf{0} + \\ (f(\tau) - p_\mathrm{f})p_\mathrm{f}[i(\mathbf{r}_\mathrm{m})q_\mathrm{m}]^2 \quad (15)\end{aligned}$$

where c is the homogeneous concentration per unit volume of free molecules, q_f is the probability that a laser light photon incident at the free molecule will give rise to an emitted photon. $p(\mathbf{r},\tau|\mathbf{r_0})\mathrm{d}\mathbf{r}\mathrm{d}t$ is the probability that a freely diffusing molecule is situated in a small volume around $\mathbf{r}$ within the time $[\tau,\tau+\mathrm{d}t]$, provided that it was situated in a very small volume around $\mathbf{r}_0$ at time $[0,\mathrm{d}t]$. Assuming that the background molecules are undergoing Brownian motion with diffusion constant D, the solution is obtained by the Fokker−Planck equation as

$$p(\mathbf{r},\tau|\mathbf{r_0}) = \frac{1}{(4\pi D\tau)^{3/2}} \exp\left(-\frac{(\mathbf{r}-\mathbf{r_0})^2}{4D\tau}\right) \tag{16}$$

The laser light intensity profile is given by[13]

$$i(\mathbf{r}) = i_0 \exp\left(-2\frac{x^2+y^2}{w^2}\right)\exp\left(-2\frac{z^2}{z_0^2}\right) \tag{17}$$

where w and z_0 are constants >0 and the z direction is parallel with the laser beam. The maximum laser light intensity is given by i_0. Inserting eqs 16 and 17 in eq 15 and executing the integrals then yield

$$\langle \delta I(0)\delta I(\tau)\rangle = q_\mathrm{f}^2\, i_0^2 c\, \frac{\pi^{3/2}}{8} \frac{w^2 z_0}{\left(1+\frac{4D}{w^2}\,\tau\right)\sqrt{1+\frac{4D}{z_0^2}\tau}} + (f(\tau)-p_\mathrm{f})p_\mathrm{f}[i(\mathbf{r}_\mathrm{m})q_\mathrm{m}]^2 \tag{18}$$

Also,

$$\langle I\rangle = q_\mathrm{f} i_0 c\, \frac{\sqrt{2}}{4}\, w^2 z_0 \pi^{3/2} + q_\mathrm{m} i(\mathbf{r}_\mathrm{m})p_\mathrm{f} \tag{19}$$

If eq 18 and eq 19 are inserted into eq 1, the general expression of the normalized autocorrelation function follows

$$G_\mathrm{N}(\tau) = \frac{G(\tau)}{\langle I\rangle^2} = 1 + \frac{q_\mathrm{f}^2\, i_0^2 c\, \frac{\pi^{3/2}}{8}\frac{w^2 z_0}{\left(1+\frac{4D}{w^2}\tau\right)\sqrt{1+\frac{4D}{z_0^2}\tau}} + (f(\tau)-p_\mathrm{f})p_\mathrm{f}[i(\mathbf{r}_\mathrm{m})q_\mathrm{m}]^2}{\left(q_\mathrm{f} i_0 c\frac{\sqrt{2}}{4}\, w^2 z_0 \pi^{3/2} + q_\mathrm{m} i(\mathbf{r}_\mathrm{m})p_\mathrm{f}\right)^2} \tag{20}$$

If the fluorescence intensity from the immobilized single molecule is much larger than the fluorescence intensity from the freely diffusing molecules, and also the photophysical properties of the immobilized molecule and the free molecules are assumed equal so that $q = q_\mathrm{m} = q_\mathrm{f}$,

$$G_\mathrm{N}(\tau) = \frac{f(\tau)}{p_\mathrm{f}} + c\frac{\pi^{3/2}}{8[i(\mathbf{r}_\mathrm{m})p_\mathrm{f}]^2}\frac{w^2 z_0}{\left(1+\frac{4D}{w^2}\right)\tau\sqrt{1+\frac{4D}{z_0^2}\tau}} = \frac{f(\tau)}{p_f} + \frac{1}{[i(\mathbf{r}_\mathrm{m})p_\mathrm{f}]^2}\frac{\frac{3}{32}\sqrt{\pi}N}{\left(1+\frac{4D}{w^2}\tau\right)\sqrt{1+\frac{4D}{z_0^2}\tau}} \tag{21}$$

In eq 21, N denotes the average number of freely diffusing molecules that are inside the open volume element (VE) from where the fluorescence is detected from, defined by all points having $i_0\exp(-2)$ or higher laser light intensity. The volume V_VE of the VE is that of an ellipsoid,

$$V_\mathrm{VE} = \frac{4}{3}\pi w^2 z_0 \tag{22}$$

and

$$N = V_\mathrm{VE}\, c \tag{23}$$

If the fluorescence from the freely diffusing molecules is very much larger than the fluorescence of the immobilized single molecule,

$$G_\mathrm{N}(\tau) = 1 + \frac{1}{\epsilon_\mathrm{E} N}\frac{1}{\left(1+\frac{4D}{w^2}\tau\right)\sqrt{1+\frac{4D}{z_\mathrm{o}^2}\tau}} \tag{24}$$

The constant

$$\epsilon_\mathrm{E} = \frac{3}{4}\sqrt{\pi} \approx 1.33 \tag{25}$$

Observe that the constant ϵ_E is not an implication of the special case of an immobilized single molecule and background fluorescence considered here. It is a general correction term for autocorrelation models that describe fluorescence fluctuations from molecules undergoing Brownian motion in three dimensions excited to fluorescence by the laser light profile as given by eq 17. If a Gaussian profile is used in the perpendicular plane to the laser beam ($x-y$ directions), but a uniform profile in the direction of the laser beam (z direction) $\epsilon_\mathrm{E} = 1$.[2]

3. Statistical Aspects of the Autocorrelation Function

3.1. Variance of the Autocorrelation Function. In the following section, all entities having the subscript “exp” are considered experimental observables. Consider an experimental measurement of the fluorescence autocorrelation function,

$$G_\mathrm{exp}(\tau) = \langle I\rangle_\mathrm{exp}^2 + \langle \delta I(0)\delta I(\tau)\rangle_\mathrm{exp} \tag{26}$$

If a measurement is performed N times, the best estimate of the autocorrelation functions is

$$G_\mathrm{exp}(\tau) = \frac{1}{N}\sum_{i=1}^{N} G_{\mathrm{exp}_i}(\tau) = \frac{1}{N}\sum_{i=1}^{N} I_{\mathrm{exp}_i}^2 + \frac{1}{N}\sum_{i=1}^{N}\delta I(0)\delta I(\tau)_{\mathrm{exp}_i} \tag{27}$$

We will assume that the measurements are independent. The variance of the correlation function is defined by

$$\mathrm{Var}(G_{\mathrm{exp}}(\tau)) = \frac{1}{N}\sum_{i=1}^{N}\left(G_{\mathrm{exp}_i}(\tau) - \frac{1}{N}\sum_{j=1}^{N} G_{\mathrm{exp}_j}(\tau)\right)^2 =$$

$$\frac{1}{N}\sum_{i=1}^{N}(G_{\mathrm{exp}_i}(\tau))^2 - \left(\frac{1}{N}\sum_{i=1}^{N}G_{\mathrm{exp}_j}(\tau)\right)^2 =$$

$$\frac{1}{N}\sum_{i=1}^{N}(I^2_{\mathrm{exp}_i} + \delta I(0)\delta I(\tau)_{\mathrm{exp}_i})^2 -$$

$$\left(\frac{1}{N}\sum_{i=1}^{N}(I^2_{\mathrm{exp}_i} + \delta I(0)\delta I(\tau)_{\mathrm{exp}_i})\right)^2 =$$

$$\frac{1}{N}\sum_{i=1}^{N}(I^2_{\mathrm{exp}_i})^2 + \frac{2}{N}\sum_{i=1}^{N}(\delta I(0)\delta I(\tau)_{\mathrm{exp}_i})^2 +$$

$$\frac{2}{N}\sum_{i=1}^{N} I^2_{\mathrm{exp}_i}\,\delta I(0)\delta I(\tau)_{\mathrm{exp}_i} - \left(\frac{1}{N}\sum_{i=1}^{N}I^2_{\mathrm{exp}_i}\right)^2 -$$

$$\left(\frac{1}{N}\sum_{\mathrm{i}=1}^{N}\delta I(0)\delta I(\tau)_{\mathrm{exp}_i}\right)^2 - 2\,\frac{1}{N}\sum_{i=1}^{N}I^2_{\mathrm{exp}_i}\frac{1}{N}\sum_{i=1}^{N}\delta I(0)\delta I(\tau)_{\mathrm{exp}_i} =$$

$$= [\text{independent measurements}] =$$

$$\frac{1}{N}\sum_{i=1}^{N}(I^2_{\mathrm{exp}_i})^2 - \left(\frac{1}{N}\sum_{i=1}^{N}I^2_{\mathrm{exp}_i}\right)^2 + \frac{1}{N}\sum_{i=1}^{N}(\delta I(0)\delta I(\tau)_{\mathrm{exp}_i})^2 -$$

$$\left(\frac{1}{N}\sum_{i=1}^{N}\delta I(0)\delta I(\tau)_{\mathrm{exp}_i}\right)^2 =$$

$$\mathrm{Var}(I^2_{\mathrm{exp}}) + Var(\delta I(0)\delta I(\tau)_{\mathrm{exp}}) \qquad (28)$$

In the limit when $N \rightarrow \infty$,

$$\lim_{N\rightarrow\infty}\mathrm{Var}(I^2_{\mathrm{exp}}) = \mathrm{Var}(I^2) \qquad (29)$$

$$\lim_{N\rightarrow\infty}\mathrm{Var}(\delta I(0)\delta I(\tau)_{\mathrm{exp}}) = \mathrm{Var}(\delta I(0)\delta I(\tau)) \qquad (30)$$

The variance is given by the mean-square deviation fluctuation:

$$\mathrm{Var}(I^2) = \langle(I^2(\tau) - \langle I\rangle^2)^2\rangle = \langle(\delta(I^2))^2\rangle \qquad (31)$$

$$\mathrm{Var}(\delta I(0)\delta I(\tau)) = \langle(\delta I(0)\delta I(\tau)|_t - \langle\delta I(0)\delta I(\tau)\rangle)^2\rangle = \langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle \qquad (32)$$

In eq 32, t denotes time (brackets denotes time averages). See Figure 1 for a complementary description of the involved entities. For the mean-square squared-intensity fluctuation, eq 33, the result is given by

$$\langle(\delta(I^2))^2\rangle = c^2q_{\mathrm{f}}^4\int_{R^3} i^4(\mathbf{r})\mathrm{d}\mathbf{r} + p_{\mathrm{f}}^2(q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4 \qquad (33)$$

or explicitly

$$\langle(\delta(I^2))^2\rangle = c^2q_{\mathrm{f}}^4\,i_0^4\,\frac{\sqrt{2}}{32}\pi^{3/2}w^2z_0 + p_{\mathrm{f}}^2(q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4 \qquad (34)$$

For the case of eq 32, the interpretation is somewhat abstract since the physical meaning is that of "the fluctuation of the fluctuations" of the fluorescence intensity (Figure 2). An expression is derived in the appendix yielding the following expression,

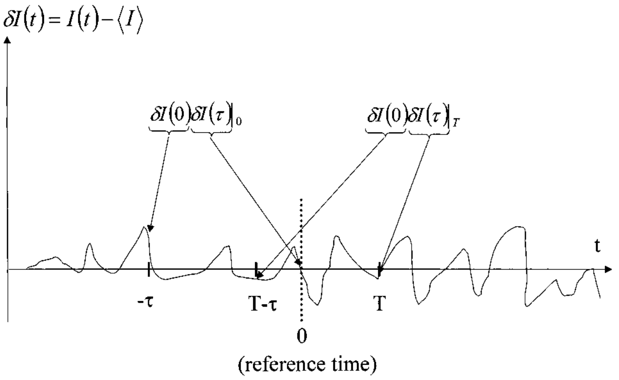

Figure 1. Definitions of the components used in the analysis and derivation of $\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle$.

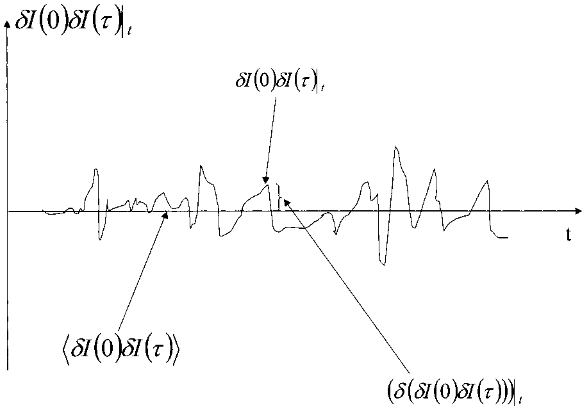

Figure 2. Description of the quantity $\delta I(0)\delta I(\tau)|_t$ that fluctuates around its mean value $\langle\delta I(0)\delta I(\tau)\rangle$. At a given time t the deviation from the mean value, referred to as the fluctuations of the fluctuations, is denoted $(\delta(\delta I(0)\delta I(\tau)))|_t$. The time-average of the squared value of $(\delta(\delta I(0)\delta I(\tau)))|_t$ is equal to $\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle$, the variance of $\langle\delta I(0)\delta I(\tau)\rangle$.

$$\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle =$$

$$cq_{\mathrm{f}}^4\int_{R^3}\int_{R^3} i^2(\mathbf{r}')p(\mathbf{r}',0|\mathbf{r}'',-\tau)i^2(\mathbf{r}')\mathrm{d}\mathbf{r}'\,\mathrm{d}\mathbf{r}'' +$$

$$c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},0|\mathbf{r}',-\tau)i(\mathbf{r}')\mathrm{d}\mathbf{r}\,\mathrm{d}\mathbf{r}'\right)^2 +$$

$$c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i^2(\mathbf{r})d\mathbf{r}\right)^2 + (q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4(p_{\mathrm{f}}(f(\tau) - p_{\mathrm{f}}) -$$

$$((f(\tau) - p_{\mathrm{f}})p_{\mathrm{f}})^2) \qquad (35)$$

An explicit form of eq 35 is obtained upon execution of the integrals:

$$\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle = cq_{\mathrm{f}}^4\,i_0^4\frac{\sqrt{2}\pi^{3/2}}{32}\frac{w^2z_0}{\left(1 + \frac{8D}{w^2}\tau\right)\sqrt{1 + \frac{8D}{w^2}\tau}} +$$

$$c^2q_{\mathrm{f}}^4\,i_0^4\left(\frac{\pi^{3/2}}{8}\frac{w^2z_0}{\left(1 + \frac{4D}{w^2}\tau\right)\sqrt{1 + \frac{4D}{z_0^{\,2}}\tau}}\right)^2 +$$

$$c^2q_{\mathrm{f}}^4\,i_0^4\,\frac{\sqrt{2}}{32}\pi^{3/2}w^2z_0 + (q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4(p_{\mathrm{f}}(f\,(\tau) - p_{\mathrm{f}}) -$$

$$((f(\tau) - p_{\mathrm{f}})p_{\mathrm{f}})^2) \qquad (36)$$

3.2. *S*/*N* Ratios. Here, the signal-to-noise (*S*/*N*) ratio is defined by

$$S/N = \frac{\langle \delta I(0)\delta I(\tau)\rangle}{[\mathrm{Var}(\delta I(0)\delta I(\tau))]^{1/2}} \tag{37}$$

or explicitly using eq 20 and eq 36

$$S/N = \frac{q_{\mathrm{f}}^{2}\, i_{0}^{2} c\, \dfrac{\pi^{3/2}}{8} \dfrac{w^{2}z_{0}}{\left(1+\dfrac{4D}{w^{2}}\tau\right)\sqrt{1+\dfrac{4D}{z_{0}^{2}}\tau}} + (f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}[i(\mathbf{r}_{\mathrm{m}})q_{\mathrm{m}}]^{2}}{\left[\begin{array}{l} c q_{\mathrm{f}}^{4}\, i_{0}^{4} \dfrac{\sqrt{2}\pi^{3/2}}{32} \dfrac{w^{2}z_{0}}{\left(1+\dfrac{8D}{w^{2}}\tau\right)\sqrt{1+\dfrac{8D}{z_{0}^{2}}\tau}} + \\ c^{2} q_{\mathrm{f}}^{4}\, i_{0}^{4} \left(\dfrac{\pi^{3/2}}{8} \dfrac{w^{2}z_{0}}{\left(1+\dfrac{4D}{w^{2}}\tau\right)\sqrt{1+\dfrac{4D}{z_{0}^{2}}\tau}}\right)^{2} + c^{2} q_{\mathrm{f}}^{4}\, i_{0}^{4} \dfrac{\sqrt{2}}{32}\pi^{3/2} w^{2} z_{0} + \\ (q_{\mathrm{m}} i(\mathbf{r}_{\mathrm{m}}))^{4}(p_{\mathrm{f}}(f(\tau)-p_{\mathrm{f}}) - ((f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}})^{2}) \end{array}\right]^{1/2}} \tag{38}$$

If the background is assumed very small ($N \rightarrow 0$), we obtain

$$S/N = \sqrt{\frac{(f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}}{(1-((f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}))}} \tag{39}$$

If we study separately the signal from the single immobilized molecule to the total noise, we finally obtain

$$S_{m}/N = \frac{(f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}}{\left[\begin{array}{l} \dfrac{q_{\mathrm{f}}^{4}\, i_{0}^{4}}{q_{\mathrm{m}}^{4}\, i_{\mathrm{m}}^{4}} \dfrac{3\sqrt{2}\pi^{3/2}}{128} \dfrac{N}{\left(1+\dfrac{8D}{w^{2}}\tau\right)\sqrt{1+\dfrac{8D}{z_{0}^{2}}\tau}} + \\ c^{2} \dfrac{q_{\mathrm{f}}^{4}\, i_{0}^{4}}{q_{\mathrm{m}}^{4}\, i_{\mathrm{m}}^{4}} \left(\dfrac{\pi^{3/2}}{8} \dfrac{w^{2}z_{0}}{\left(1+\dfrac{4D}{w^{2}}\tau\right)\sqrt{1+\dfrac{4D}{z_{0}^{2}}\tau}}\right)^{2} + \\ \qquad \dfrac{\sqrt{2}}{32} c^{2} \dfrac{q_{\mathrm{f}}^{4}\, i_{0}^{4}}{q_{\mathrm{m}}^{4}\, i_{\mathrm{m}}^{4}} \pi^{3/2} w^{2} z_{0} + \\ + (p_{\mathrm{f}}(f(\tau)-p_{\mathrm{f}}) - ((f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}})^{2}) \end{array}\right]^{1/2}} \tag{40}$$

4. Discussion

The expression eq 20 gives the most general expression of the autocorrelation function in the case of a single immobilized molecule in a background of freely diffusing molecules. It shows how physical parameters and the dynamics of the background molecules and the single molecule together contribute to the autocorrelation function. The amplitude of the background term is proportional to N when S_{m}/B is large, but proportional to N^{-1} when S_{m}/B is small. However, for large S_{m}/B the optical parameters q_{m}, and $i(\mathbf{r}_{\mathrm{m}})$ play a more dominant role as they directly influence the amplitude by their squared value (eq 21).

One main objective of the present paper is to discuss the relation between S_{m}/B ratios and the S_{m}/N ratios. The eq 40 provides the help necessary to accomplish this. Clearly, if the single molecule dynamics and the diffusion of background molecules are in the same time range, the S_{m}/N ratio is highly dependent on the S_{m}/B ratio. More specifically, the quantum yield ratio ($q_{\mathrm{m}}/q_{\mathrm{f}}$) and the excitation intensity dependent on the position of the single molecule ($\mathbf{r}_{\mathrm{m}}$) inside the VE influence the S_{m}/N to its second order. The concentration of the free molecules (c) influences the S_{m}/N ratio to its first-order value or its value to the power of $^{1}/_{2}$ (eq 40).

A single molecule experiencing emission dynamics on a slower time range than the diffusion of the background molecules[6] will be less sensitive to bad quantum yield of the single molecule as well as its position in the VE according to eq 40.

Can a single molecule experiment be statistically feasible even though the S_{m}/B ratio is low? Assume that the time scale of the emission dynamics of the single molecule is larger than the time scale of the background dynamics. Then, on the relevant time scale of the single molecule dynamics, the S_{m}/N is given by

$$S_{\mathrm{m}}/N = \frac{(f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}}{\sqrt{(ac^{2}+bc)\dfrac{q_{\mathrm{f}}^{4}\, i_{0}^{4}}{q_{\mathrm{m}}^{4}\, i_{\mathrm{m}}^{4}} + (f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}\,(1-((f(\tau)-p_{\mathrm{f}})p_{\mathrm{f}}))}} \tag{41}$$

where

$$a = \frac{4+\sqrt{2}}{32}\pi^{3/2} w^{2} z_{0} \tag{42}$$

and

$$b = \frac{\sqrt{2}\pi^{3/2}}{32} w^{2} z_{0} \tag{43}$$

Hence, if the S_{m}/B is small due to a high concentration of background molecules but the quantum yield of the free molecules is low so that $q_{\mathrm{f}}^{4}\, i_{0}^{4}/q_{\mathrm{m}}^{4}\, i_{\mathrm{m}}^{4} \ll 1$, the S_{m}/N is almost as good as in the absence of any background.

Clearly, the above case is a constructed situation that might not be the most common case in practice. However, we have proved that there are situations in which the S_{m}/B does not give full insight into the actual statistical accuracy in a measurement of single molecule spectroscopic dynamics. The statistical quality of a single molecule FCS experiment is assessed via the eq 41, or in the more general case via eq 40, and not via the S_{m}/B alone.

The general conclusion is however that based on eqs 40 and 41, the quantum yield ratio of the background molecule to the single molecule as well as the position of the single molecule inside the VE are the most crucial physical parameters in obtaining good S_{m}/N ratio and hence good quality in a single molecule experiment using FCS.

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Appendix: Calculation of $\langle(\delta(\delta I(0)\delta I(\tau)))^{2}\rangle$

The fluctuations of the fluctuations of the fluorescence intensity can be depicted according to Figure 2. The signal $\delta I(0)\delta I(\tau)|_{t}$ can be studied as a not averaged signal that fluctuates around a mean value (Figure 2). We define

$$\delta(\delta I(0)\delta I(\tau))|_{\mathrm{t}} = \delta I(0)\delta I(\tau)|_{\mathrm{t}} - \langle \delta I(0)\delta I(\tau)\rangle \tag{A1}$$

Multiplying both sides of eq A1 by $\delta(\delta I(0)\delta I(\tau))|_t$ and averaging then yields

$$\langle \delta(\delta I(0)\delta I(\tau))|_t\, \delta(\delta I(0)\delta I(\tau))|_{t'}\rangle = \langle(\delta I(0)\delta I(\tau)|_t - \langle \delta I(0)\delta I(\tau)\rangle)(\delta I(0)\delta I(\tau)|_{t'} - \langle \delta I(0)\delta I(\tau)\rangle)\rangle \tag{A2}$$

Calculation of the right-hand side of Eq. A2 using the fact that $I(t)$ is a stationary process so that we can set $T = |t - t'|$ yields

$$\langle[\delta(\delta I(0)\delta I(\tau))\delta(\delta I(0)\delta I(\tau))]|_T\rangle = \langle \delta I(0)\delta I(\tau)|_0\, \delta I(0)\delta I(\tau)|_T\rangle - \langle \delta I(0)\delta I(\tau)\rangle^2 \tag{A3}$$

If we let T approach zero, we then obtain the expression of the variance,

$$\langle[\delta(\delta I(0)\delta I(\tau))]^2\rangle = \langle[\delta I(0)\delta I(\tau)]^2\rangle - \langle \delta I(0)\delta I(\tau)\rangle^2 \tag{A4}$$

If we study separately the variance for the freely diffusing molecules, we note that the first argument of the right-hand side of eq A3 as derived for the background molecules has two main components. The *first* component is equal to the probability that one of the background molecules emits a photon from a small volume around $\mathbf{r}$ in the time interval $[-\tau,-\tau+\mathrm{d}t]$ (this probability is equal to $cq_f i(\mathbf{r})\mathrm{d}\mathbf{r}\ \mathrm{d}t$) and then emits photons within the time intervals $[-\tau+T,-\tau+T+\mathrm{d}t]$, $[0,\mathrm{d}t]$ and $[T, T+\mathrm{d}t]$ (Figure 2), from small volumes around the respective positions $\mathbf{r}'$,$\mathbf{r}''$, and $\mathbf{r}'''$. The *second* component is the group combinations of two molecules each emitting two photons in the above time intervals. There exist three such combinations for two molecules emitting two photons per molecule.

It is worth mentioning that eq A3 does not describe the general joint probability to observe any four photons, for which the case would be much more complicated with many more combinatorial possibilities. Rather, eq A3 describes the second-order moment of the fluctuations from the mean $\langle \delta I(0)\delta I(\tau)\rangle$. Therefore, since $\langle \delta I(t)\rangle = 0$ for any t (ref 2), if more than two molecules are used to create the four photons there will be no contribution to the average fluctuations of the fluctuation of the fluorescence intensity.

Since the background molecules are undergoing Brownian motion according to eq 16, the stochastic changes of the spatial position of each background molecule in time is a Markov process. The probability of a molecular trajectory is therefore equal to the product of the probabilities of all pairwise positional movements that define the entire trajectory. Using this and taking the above discussion regarding components of eq A3 into account, for the first term of the right-hand-side of eq A3, we have

$$\begin{aligned}\langle \delta I(0)\delta I(\tau)|_0\, \delta I(0)\delta I(\tau)|_T\rangle_{\mathrm{f}} = {} & cq_{\mathrm{f}}^4 \int_{R^3}\int_{R^3}\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},-\tau + T|\mathbf{r}',-\tau)i(\mathbf{r}')p(\mathbf{r}',0|\mathbf{r}'',-\tau + T)i(\mathbf{r}'')p(\mathbf{r}'',T|\mathbf{r}''',0) \times \\ & i(\mathbf{r}''')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}''\ \mathrm{d}\mathbf{r}''' + c^2q_{\mathrm{f}}^4 \int_{R^3}\int_{R^3}\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},T - \tau|\mathbf{r}',-\tau)i(\mathbf{r}')i(\mathbf{r}'')p(\mathbf{r}'',0|\mathbf{r}''',T)i(\mathbf{r}''')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}''\ \mathrm{d}\mathbf{r}''' + \\ & c^2q_{\mathrm{f}}^4 \int_{R^3}\int_{R^3}\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},0|\mathbf{r}',-\tau)i(\mathbf{r}')i(\mathbf{r}'') \times p(\mathbf{r}'',T - \tau|\mathbf{r}''',T)i(\mathbf{r}''')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}''\ \mathrm{d}\mathbf{r}''' + \\ & c^2q_f^4\int_{R^3}\int_{R^3}\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},T|\mathbf{r}',-\tau)i(\mathbf{r}')i(\mathbf{r}'') \times p(\mathbf{r}'',0|\mathbf{r}''',T - \tau)i(\mathbf{r}''')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}''\ \mathrm{d}\mathbf{r}'''\end{aligned} \tag{A5}$$

if $T < \tau$ (Figure 2).

When T approaches zero we obtain

$$\begin{aligned}\lim_{T\to 0}\langle[\delta(\delta I(0)\delta I(\tau))\delta(\delta I(0)\delta I(\tau))]_{\mathrm{T}}\rangle_{\mathrm{f}} = {} & cq_{\mathrm{f}}^4 \int_{R^3}\int_{R^3}\int_{R^3}\int_{R^3} i(\mathbf{r})\delta(\mathbf{r} - \mathbf{r}')i(\mathbf{r}')p(\mathbf{r}',-\tau|\mathbf{r}'',0)i(\mathbf{r}'') \times \\ & \delta(\mathbf{r}'' - \mathbf{r}''')i(\mathbf{r}''')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}''\ \mathrm{d}\mathbf{r}''' + \\ & 2c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},0|\mathbf{r}',-\tau)i(\mathbf{r}')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\right)^2 + \\ & c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i(\mathbf{r})\delta(\mathbf{r} - \mathbf{r}')i(\mathbf{r}')d\mathbf{r}d\mathbf{r}'\right)^2 = \\ & cq_{\mathrm{f}}^4\int_{R^3}\int_{R^3} i^2(\mathbf{r}')p(\mathbf{r}',0|\mathbf{r}'',-\tau)i^2(\mathbf{r}')d\mathbf{r}'d\mathbf{r}'' + \\ & 2c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},0|\mathbf{r}',-\tau)i(\mathbf{r}')\mathrm{d}\mathbf{r}\ \mathrm{d}\mathbf{r}'\right)^2 + \\ & c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i^2(\mathbf{r})d\mathbf{r}\right)^2 = \langle[\delta(\delta I(0)\delta I(\tau))]^2\rangle_{\mathrm{f}}\end{aligned} \tag{A6}$$

For the case of the single immobilized molecule using the definition of an expectation value of a random variable we obtain by direct application of the right-hand side of eq A4

$$\begin{aligned}\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle_{\mathrm{m}} = {} & (q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4(p_{\mathrm{f}}(f(\tau) - p_f) - ((f(\tau) - \\ & p_{\mathrm{f}})p_{\mathrm{f}})^2) = (q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4(p_{\mathrm{f}}(f(\tau) - p_{\mathrm{f}}))(1 - p_{\mathrm{f}}(f(\tau) - p_{\mathrm{f}}))\end{aligned} \tag{A7}$$

Because we have assumed (recall eq 14) that the emission fluctuations from the single molecule and the background are uncorrelated, we obtain

$$\langle[\delta(\delta I(0)\delta I(\tau))]^2\rangle = \langle[\delta(\delta I(0)\delta I(\tau))]^2\rangle_{\mathrm{f}} + \langle[\delta(\delta I(0)\delta I(\tau))]^2\rangle_{\mathrm{m}} \tag{A8}$$

so that

$$\begin{aligned}\langle(\delta(\delta I(0)\delta I(\tau)))^2\rangle = {} & cq_{\mathrm{f}}^4\int_{R^3}\int_{R^3} i^2(\mathbf{r}')p(\mathbf{r}',0|\mathbf{r}'',-\tau)i^2(\mathbf{r}')\mathrm{d}\mathbf{r}'\ \mathrm{d}\mathbf{r}'' + \\ & c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i(\mathbf{r})p(\mathbf{r},0|\mathbf{r}',-\tau)i(\mathbf{r}')d\mathbf{r}d\mathbf{r}'\right)^2 + \\ & c^2q_{\mathrm{f}}^4\left(\int_{R^3}\int_{R^3} i^2(\mathbf{r})d\mathbf{r}\right)^2 + (q_{\mathrm{m}}i(\mathbf{r}_{\mathrm{m}}))^4(p_{\mathrm{f}}(f(\tau) - p_{\mathrm{f}}) - \\ & ((f(\tau) - p_{\mathrm{f}})p_{\mathrm{f}})^2)\end{aligned} \tag{A9}$$

References and Notes

(1) For a review, see: Thompson, N. L. Fluorescence Correlation Spectroscopy. In *Topics in Fluorescence Spectroscopy*; Lakowicz, J. R., II, Ed.; Plenum Press: New York, 1995; pp 337−410.

(2) Elson, E.; Magde, D. *Biopolymers* **1974**, *13*, 1.

(3) Ehrenberg, M.; Rigler, R. *Chem. Phys.* **1974**, *4*, 390.

(4) Widengren, J.; Mets, Ü.; Rigler, R. *J. Phys. Chem.* **1995**, *99*, 13368.

(5) Wennmalm, S.; Edman, L.; Rigler, R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10641.

(6) Edman, L.; Földes-Papp, Z.; Wennmalm, S.; Rigler, R. *Chem. Phys.* **1999**, *247*, 11.

(7) Koppel, D. E. *Phys. Rev. A* **1974**, *10*, 1938.

(8) Qian, H. *Biophys. Chem.* **1990** *38*, 49.

(9) Wohland, T.; Rigler, R.; Vogel, H. Manuscript in preparation.

(10) Lu, H. P.; Xun, L.; Xie, X. S. *Science* **1998**, *282*, 1877.

(11) Jia, Y.; Talaga, D. S.; Lau, W. L.; Lu, H. S. M.; DeGrado, W. F.; Hochstrasser, R. M. *Chem. Phys.* **1999**, *247*, 69.

(12) Ha, T.; Ting, A. Y.; Liang, J.; Deniz, A. A.; Chemla, D. S.; Schultz, P. G.; Weiss, S. *Chem. Phys.* **1999**, *247*, 107.

(13) Rigler, R.; Mets, Ü.; Widengren, J.; Kask, P. *Euro. Biophys. J.* **1993**, *22*, 169.