

Fluorophore–Quencher Distance Correlation Functions from Single-Molecule Photon Arrival Trajectories[†]

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We generalize and simplify the method of Yang and Xie (*J. Chem. Phys.* **2002**, *117*, 10965) to obtain distance correlation functions from photon arrival trajectories of single fluorophores whose lifetime, $[k(r)]^{-1}$, depends on the distance to a quencher. It is assumed that this distance does not change during the fluorescence lifetime. The experimental trajectory is first transformed by replacing the delay time (i.e., the interval between the photon arrival and the nearest laser pulse) by a certain function of this delay time. This function is the inverse Laplace transform of $r(k)$, which is the solution of $k(r) = 1$. The correlation function of the transformed data then directly gives the distance correlation function. Illustrative examples include Förster energy transfer and quenching due to electron transfer.

1. Introduction

Single-molecule spectroscopy is a powerful tool to study the dynamics of biological molecules and has already provided novel insights into the nature of protein folding, RNA and DNA dynamics and other conformational changes.^{1–14} Such experiments exploit the fact that the emission properties of a fluorophore depend on the distance to another fluorophore label (e.g., Förster energy transfer) or a naturally occurring aromatic amino acid (e.g., quenching by electron transfer).

Recent advances in single-molecule photon counting allow one to monitor the lifetime of the fluorophore in the presence of a quencher. The lifetime depends on the distance between the fluorophore and quencher. To monitor the lifetimes, a single molecule is excited by a train of laser pulses and both chronological time t_i and the delay time τ_i (the time between the photon arrival and the nearest pulse) are registered for each photon.

When there is no motion during the time interval between excitation and emission, each delay time arises from an exponential distribution with a mean lifetime that depends on the fluorophore–quencher distance during the interval. The simplest way to analyze photon arrival trajectories is to divide them into bins containing many photons. Then the mean lifetime of the photons in a bin is determined and converted into a distance. This is meaningful only if the conformation of the molecule did not change during the bin time. This method allows one to measure distance fluctuations that are slower than the bin size (and thus much slower than the time between photons).

Recently, Yang and Xie^{15–17} proposed a clever method of analyzing such photon arrival trajectories to yield information about the fluctuations of the fluorophore–quencher distance on a time scale ranging from the time between consecutive photons to the bleaching time. This photon-by-photon analysis avoids binning and thus its resolution is limited by the time between consecutive photons and not by the bin size. In this paper we simplify and generalize their method.^{16,17} Specifically, we show that in order to get the fluorophore–quencher distance correla-

tion function and equilibrium averages of powers of the distance, the delay time trajectory should be transformed by replacing each delay time by a certain function of that time. The correlation function or the average of the transformed data then directly give the distance correlation function and the moments. We also generalize the method to handle diffusion through the laser spot.

2. Delay Time Moments and Correlation Function

In this paper we assume that the fluorophore–quencher distance varies only on a time scale longer than the intrinsic fluorophore lifetime. In this case, the light intensity $F(t)$ obtained after excitation of a bulk sample by the δ -function laser pulse is given by

$$\frac{F(t)}{F(0)} = \int e^{-k(r)t} p_{eq}(r) dr \equiv \langle e^{-k(r)t} \rangle \quad (1)$$

where the lifetime $[k(r)]^{-1} = [k_F + k_Q(r)]^{-1}$ is determined by the fluorophore's intrinsic decay rate to the ground state (both radiative and nonradiative), $k_F = k_F^{rad} + k_F^{nrad}$, and the quenching rate, $k_Q(r)$, that depends on the fluorophore–quencher distance r . $p_{eq}(r)$ is the equilibrium distribution of fluorophore–quencher distances and the angular brackets denote averaging over this distribution, namely, for any function $g(r)$

$$\langle g \rangle \equiv \int g(r) p_{eq}(r) dr$$

In this section we obtain microscopic expressions for averages and correlation functions that can be readily calculated from single-molecule photon arrival trajectories. On the basis of these results, in the next section we will show how to get averages and correlation functions of the fluorophore–quencher distance.

Consider a single-molecule photon arrival trajectory where both the delay time τ_i (relative to the nearest laser pulse) and the chronological time t_i for each photon i are recorded. The intervals between the laser pulses contain no more than one photon. From such a trajectory, one can get the delay time distribution, the mean, and the time dependence of the correla-

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tion function of any function of the delay time, $f(\tau)$. For a sufficiently large number of photons, n , the mean is related to the delay time distribution of photons, $p(\tau)$, by

$$\frac{1}{n} \sum_{i=1}^n f(\tau_i) = \int_0^\infty f(\tau) p(\tau) d\tau \quad (2)$$

The sum in the left-hand side of the above equality is over all photons. Note that in this definition the mean is normalized using the total number of photons, n , not the number of laser pulses, N .

Now we obtain an analytical expression for the delay time distribution, $p(\tau)$. For fixed fluorophore–quencher distance r , the delay times, τ_i , are distributed exponentially, $k(r) \exp(-k(r)\tau)$. To find $p(\tau)$ for fluctuating fluorophore–quencher distances, we begin by considering the probability that a photon was emitted when the fluorophore–quencher distance was r . The probability of emitting a photon is proportional to the quantum yield, $k_F^{rad}/k(r)$. Thus, the normalized probability density that a detected photon was emitted at r is $[k(r)]^{-1} p_{eq}(r)/\langle k^{-1} \rangle$. Multiplying this by the delay time distribution for fixed r , we find that the joint probability density $p(\tau, r)$ that a photon with delay time τ was emitted when the fluorophore–quencher distance was r is

$$p(\tau, r) = \frac{1}{\langle k^{-1} \rangle} e^{-k(r)\tau} p_{eq}(r) \quad (3)$$

It can be readily checked that $\int dr \int_0^\infty d\tau p(\tau, r) = 1$. The normalized distribution of the delay times is obtained from this by averaging over r :

$$p(\tau) = \int p(\tau, r) dr = \frac{\langle e^{-k(r)\tau} \rangle}{\langle k^{-1} \rangle} \quad (4)$$

To obtain the average over the single-molecule delay time trajectory, we substitute eq 4 into eq 2 and perform integration over the delay time. In this way, we find that

$$\frac{1}{n} \sum_{i=1}^n f(\tau_i) = \frac{\langle \hat{f}(k(r)) \rangle}{\langle k^{-1} \rangle} \quad (5)$$

where we have used the notation $\hat{f}(k)$ for the Laplace transform of $f(\tau)$

$$\hat{f}(k) = \int_0^\infty f(\tau) e^{-k\tau} d\tau \quad (6)$$

As a simple application of eq 5 we set $f(\tau) = \tau$ and find that the mean of the delay times over a photon arrival trajectory is

$$\frac{1}{n} \sum_{i=1}^n \tau_i = \frac{\langle k^{-2} \rangle}{\langle k^{-1} \rangle} \quad (7)$$

Note that this does not coincide with the mean lifetime $\langle k^{-1} \rangle$, which is simply $1/p(0)$ (see eq 4).

By comparison of eqs 1 and 4 it can be seen that the distribution of the delay times obtained from single-molecule trajectory decays the same way as the bulk fluorescence intensity, specifically,

$$\frac{F(t)}{F(0)} = \frac{p(t)}{p(0)} \quad (8)$$

Since the mean lifetime $\langle k^{-1} \rangle = 1/p(0)$ is equal to $\int_0^\infty F(t)/F(0) dt$, it follows from eqs 2 and 8 that the averages over photon arrival trajectories are related to the averages over bulk fluorescence intensity via

$$\frac{1}{n} \sum_{i=1}^n f(\tau_i) = \int_0^\infty f(t) F(t) dt / \int_0^\infty F(t) dt \quad (9)$$

The same reasoning can be applied to the correlation function of $f(\tau)$ defined as $\sum_{i=1}^N f(\tau_{i+l\Delta}) f(\tau_i) / n$ for discrete times $t = l\Delta$, where Δ is the time between consecutive laser pulses and l is an integer greater or equal to 1. In this definition, the sum is over laser pulses, N is the total number of pulses, $f(\tau_i) = 0$ if there is no a photon between the i th and the $(i+1)$ th pulses and n is the total number of photons. This correlation function is given by

$$\frac{1}{n} \sum_{i=1}^N f(\tau_{i+l\Delta}) f(\tau_i) = \int_0^\infty d\tau \int_0^\infty dr dr_0 f(\tau) p(\tau, r, t | r_0) f(\tau_0) p(\tau_0, r_0) \quad (10)$$

where $p(\tau_0, r_0)$ is the joint probability density that the photon at $t = 0$ has the delay time τ_0 and was emitted when the fluorophore–quencher distance was r_0 , eq 3, and $p(\tau, r, t | r_0)$ is the conditional probability density to detect a photon at a time t later with delay time τ that is emitted when the fluorophore–quencher distance is r given it was r_0 initially. This conditional probability density is given by the product of the following: (1) the conditional probability density $G(r, t | r_0)$ that the fluorophore–quencher distance is r at time t provided it was r_0 initially, (2) the probability to detect a photon at time t later than the initial one, which is equal to the ratio of the total number of photons to the total number of laser pulses, n/N , and (3) the conditional probability density that the detected photon has the delay time τ given it is emitted when the fluorophore–quencher distance is r , $\exp(-k(r)\tau)/\langle k^{-1} \rangle$. This can be obtained by dividing the corresponding joint probability $p(\tau, r)$, eq 3, by the distance equilibrium distribution. In this way we get

$$p(\tau, r, t | r_0) = \frac{n}{N} \frac{1}{\langle k^{-1} \rangle} e^{-k(r)\tau} G(r, t | r_0) \quad (11)$$

This differs from $p(\tau, r)$, eq 3, by the replacement of $p_{eq}(r)$ by the conditional probability density $G(r, t | r_0)$. In addition, the factor n/N is the probability that a photon was actually detected some time after the initial one. Integrating this over τ , r , and r_0 with the weight factor $p_{eq}(r_0)$ gives the probability to detect a photon in the interval between two laser pulses

$$\int dr dr_0 \int_0^\infty d\tau p(\tau, r, t | r_0) p_{eq}(r_0) = \frac{n}{N} \quad (12)$$

Using eq 11 in eq 10 and integrating over τ , we find

$$\frac{N}{n^2} \sum_{i=1}^N f(\tau_i) f(\tau_{i+l\Delta}) = \frac{\langle \hat{f}(t) \hat{f}(0) \rangle}{\langle k^{-1} \rangle^2} \quad (13)$$

where we defined the correlation function $\langle g(t)g(0) \rangle$ as

$$\langle g(t)g(0) \rangle \equiv \langle g(r(t))g(r(0)) \rangle = \int g(r) G(r, t | r_0) g(r_0) p_{eq}(r_0) dr dr_0$$

Can one choose $f(\tau)$ so that the corresponding autocorrelation

function calculated from the photon arrival trajectory directly gives the autocorrelation function of the fluorophore–quencher distance?

3. Distance Moments and Correlation Function

The key idea of this paper is the following. Suppose we can find a function $f(\tau)$ such that $\hat{f}(k(r)) = r$, i.e.,

$$\int_0^\infty f(\tau) \exp(-k(r)\tau) d\tau = r \quad (14)$$

Then we can get the mean distance and the distance correlation function from the photon trajectory by using eqs 5 and 13.

Thus, to get the fluorophore–quencher distance correlation function we first find $f(\tau)$ by taking the inverse Laplace transform (in k space) of the reciprocal function $r(k)$ (which is the solution of the equation $k(r) = 1$) and then transform the original delay time trajectory by replacing $\tau_i \rightarrow f(\tau_i)$. The mean and the correlation function of the transformed data result in the mean distance and the distance correlation function:

$$f(\tau) = \frac{1}{2\pi i} \int_{c-i\infty}^{c+i\infty} r(k) e^{k\tau} dk \quad (15a)$$

$$\langle r \rangle = \frac{\langle k^{-1} \rangle}{n} \sum_{i=1}^n f(\tau_i) \quad (15b)$$

$$\langle r(t)r(0) \rangle = \frac{N\langle k^{-1} \rangle^2}{n^2} \sum_{i=1}^N f(\tau_{i+t/\Delta}) f(\tau_i) \quad (15c)$$

In the above integral, the integration contour should be to the right of all the singularities of $r(k)$ in the complex plane. In practice, one can usually find $f(\tau)$ using Laplace transform tables.

Similarly, to get averages and correlation functions of r^m one should take the inverse Laplace transform of $r^m(k)$ and use this function to transform the original delay time trajectory:

$$f_m(\tau) = \frac{1}{2\pi i} \int_{c-i\infty}^{c+i\infty} r^m(k) e^{k\tau} dk \quad (16a)$$

$$\langle r^m \rangle = \frac{\langle k^{-1} \rangle}{n} \sum_{i=1}^n f_m(\tau_i) \quad (16b)$$

$$\langle r^m(t)r^m(0) \rangle = \frac{N\langle k^{-1} \rangle^2}{n^2} \sum_{i=1}^N f_m(\tau_{i+t/\Delta}) f_m(\tau_i) \quad (16c)$$

The above results involve the mean lifetime $\langle k^{-1} \rangle$. As shown in the previous section, this can be easily obtained from either single-molecule or bulk experiments.

We illustrate the above theory for Förster energy transfer. In this case the decay rate and its reciprocal function are

$$k(r) = k_F + k_0 \left(\frac{R_0}{r} \right)^6 \quad (17a)$$

$$r(k) = \frac{R_0 k_0^{1/6}}{(k - k_F)^{1/6}} \quad (17b)$$

The inverse Laplace transform¹⁸ of $r^m(k)$ gives the transformation rule for the delay time trajectory required to get the distance correlation functions and the moments:

$$f_m(\tau) = R_0^m \frac{(k_0 \tau)^{m/6}}{\Gamma(m/6) \tau} \exp(k_F \tau) \quad (18)$$

Using this transformation in eq 16, we get the moments and the distance correlation function which are the same as those in eqs A2 and A3 of ref 16.

As another example, consider electron transfer with

$$k(r) = k_F + k_0 \exp(-r/R_0) \quad (19a)$$

$$r(k) = -R_0 \ln \frac{k - k_F}{k_0} \quad (19b)$$

Since the inverse Laplace transform of the logarithm function does not exist, one cannot directly apply eqs 15 and 16 to get the mean and the correlation function of the fluorophore–quencher distance. The same problem arises in the method presented by Yang and Xie.¹⁶ However, this can be circumvented in the following way. Consider the transformation

$$f(\tau) = \frac{\exp(k_F \tau)}{\tau + \epsilon/k_0} \quad (20)$$

where ϵ is a regularization constant with the only requirement being that $0 < \epsilon \ll 1$. The Laplace transform of this function

$$\begin{aligned} \hat{f}(k) &= \int_0^\infty \frac{\exp(-(k - k_F)\tau)}{\tau + \epsilon/k_0} d\tau \\ &= \ln \left(\frac{k - k_F}{k_0} \right) - \ln \epsilon e^\gamma + \epsilon(\dots) \end{aligned} \quad (21)$$

where $\gamma = 0.577$ is Euler's constant, results in $\hat{f}(k(r)) \sim r/R_0 - \ln \epsilon e^\gamma$ for small ϵ . Therefore, using $f(\tau)$ in eq 20 in eqs 5 and 13, we find that

$$\frac{\langle k^{-1} \rangle}{n} \sum_{i=1}^n f(\tau_i) \rightarrow \langle r/R_0 \rangle - \ln \epsilon e^\gamma \quad (22a)$$

$$\begin{aligned} \frac{N\langle k^{-1} \rangle^2}{n^2} \sum_{i=1}^N f(\tau_{i+t/\Delta}) f(\tau_i) \rightarrow \\ \langle (r(t)/R_0 - \ln \epsilon e^\gamma)(r(0)/R_0 - \ln \epsilon e^\gamma) \rangle \end{aligned} \quad (22b)$$

as $\epsilon \rightarrow 0$. For a given ϵ one can obtain the distance correlation function using the above relation. Alternately, the constant $\ln \epsilon e^\gamma$ can be removed by taking the following difference:

$$\begin{aligned} \langle \delta r(t) \delta r(0) \rangle = \\ \frac{R_0^2 \langle k^{-1} \rangle^2}{n^2} \left(N \sum_{i=1}^N f(\tau_{i+t/\Delta}) f(\tau_i) - \left(\sum_{i=1}^n f(\tau_i) \right)^2 \right) \end{aligned} \quad (23)$$

where $\delta r = r - \langle r \rangle$. Equations 22 and 23 are identities to order ϵ , so in practice, ϵ should be systematically decreased until the correlation function converges.

Recently Xie and co-workers¹⁵ studied a system where the quenching of the fluorophore is well described by an exponential $k_Q(r)$. They obtained $\langle \delta r(t) \delta r(0) \rangle$ from the delay time correlation function by assuming that the underlying dynamics can be described by a non-Markovian Gaussian process.^{19,20} It would be interesting to compare this result with that obtained from our model-independent expression given in eq 23.

Finally, we point out that the above method can be easily generalized to single-molecule measurements of diffusing molecules. When a molecule diffuses in and out of the laser focal spot, the probability of excitation by the pulse, $I(\mathbf{R})$, fluctuates because the laser intensity is a function of the translational coordinate \mathbf{R} . This does not influence the results for the moments. However, the correlation function is modified. Applying the same reasoning as before we get:

$$\frac{N}{n^2} \sum_{i=1}^N f(\tau_i) f(\tau_{i+t/\Delta}) = \frac{\langle \hat{f}(t) \hat{f}(0) \rangle}{\langle k^{-1} \rangle^2} C_d(t) \quad (24)$$

where

$$C_d(t) = \frac{\int I(\mathbf{R}) G_d(\mathbf{R}, t | \mathbf{R}_0) I(\mathbf{R}_0) d\mathbf{R} d\mathbf{R}_0}{(\int I(\mathbf{R}) d\mathbf{R})^2} \quad (25)$$

and $G_d(\mathbf{R}, t | \mathbf{R}_0)$ is the Green's function for translational diffusion. This correlation function can be obtained from fluctuations of the fluorescence intensity measured for the fluorophore with the absence of the quencher.²¹ For example, in the case of a Gaussian laser spot

$$I(\mathbf{R}) = I(0) \exp\left(-\frac{x^2 + y^2}{2a_1^2} - \frac{z^2}{2a_2^2}\right)$$

the correlation function decays as the power law²¹

$$C_d(t) = (1 + Dt/a_1^2)^{-1} (1 + Dt/a_2^2)^{-1/2} \quad (26)$$

Since $C_d(t) \rightarrow 0$ for times longer than the diffusion time (i.e., the average time that the molecule spends in the laser spot), the experiment now contains information about conformational dynamics only on a time scale that is less than a few diffusion times.

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