Single-Molecule FRET with Diffusion and Conformational Dynamics

Irina V. Gopich* and Attila Szabo

Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

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Under relatively mild conditions, we show how one can extract information about conformational dynamics from Förster resonance energy transfer (FRET) experiments on diffusing molecules without modeling diffusion. Starting from a rigorous theory that does treat diffusion, we first examine when the single-molecule FRET efficiency distribution can be decomposed into the measured distribution of the total number of photons and the efficiency distribution of an immobilized molecule in the absence of shot noise. If the conformation does not change during the time the molecule spends in the laser spot, this is possible when (I) the efficiency is independent of the location in the laser spot and (II) the total number of photons does not depend on conformation. This decomposition is approximate when the conformation changes during the diffusion time. However, it does provide a simple framework for analyzing data. This is illustrated for a two-state system where the FRET efficiency distribution can be found analytically for all values of the interconversion rates. If the arrival time of each donor and acceptor photon can be monitored, we introduce an alternative procedure that allows one to rigorously extract the rates of conformational changes when the above two conditions hold. In this case, the pattern of colors in the photon trajectory depends solely on conformational dynamics. This can be exploited in the framework of statistical inference because the likelihood function, which must be optimized with respect to the model rate parameters, depends only on how the conformation changes during the interval between photons with specified colors.

I. Introduction

Single-molecule Förster resonance energy transfer (FRET) measurements on freely diffusing molecules contain information about conformational dynamics because the rate of transfer depends on the donor−acceptor distance.[1−17] In these experiments, a molecule diffuses through a spot illuminated by a laser, and the donor is excited (see Figure 1). The probability that a photon is emitted either by the donor or by the acceptor depends on the distance between them. These photons are recorded generating a photon trajectory consisting of bursts of photons separated by long gaps. The times between successive photons and their "color" is determined in general by both conformational and diffusive dynamics.

The standard way of analyzing such data is based on the construction of FRET efficiency histograms. These can be obtained from the photon trajectory in two different ways which we shall refer to as bin and burst analysis. In bin analysis, the photon trajectory is divided into equally spaced time bins. The vast majority of these contain only noise (e.g., detector dark counts) and are discarded by imposing a threshold on the total number of photons. The FRET efficiency is defined as the ratio of the number of acceptor photons in a bin to the total number of photons above some threshold. This ratio fluctuates because of various stochastic processes of which only conformational dynamics is usually of interest. In burst analysis, a burst of photons of variable duration is identified using a search algorithm.[1,14,18] The definition of what constitutes a burst is necessarily subjective because of the stochastic nature of diffusion and the inhomogeneous intensity profile of the laser spot (see Figure 1a). The FRET efficiency is defined as the ratio of the number of acceptor photons to the total number of photons in a burst. The FRET efficiency histogram can be constructed by averaging over all bursts or only those with the same duration. An advantage of this procedure is that the observation time can be extended to the duration of exceptionally long bursts. A disadvantage is that the statistics of photons depend on the details of the burst search algorithm used.

The FRET efficiency distribution is influenced by a variety of stochastic processes occurring over a wide range of time scales. These include photophysical ones such as excitation, radiative and nonradiative decay, energy transfer, and dye blinking. Diffusion through the laser spot with an inhomogeneous illumination profile influences the fluorescence intensity. Fluctuations in the relative orientation of the dyes as well as the distance between them modulate the rate of energy transfer and thus influence photon statistics. Even the detection of emitted photons is a random process.

We have previously developed a general formalism that rigorously treats all of these processes when the photon trajectory is analyzed using bins of equal duration.[19,20] We found that fluctuations faster than the interphoton time (which is usually on the microsecond time scale) influence only the mean FRET efficiency, $\langle E\rangle$. The shape of the FRET efficiency distribution depends on dynamical processes that are slower than the interphoton time. For diffusing molecules, we proved that if the width of the distribution is larger than $\sqrt{\langle E\rangle(1-\langle E\rangle)/N_T}$, where N_T is the threshold on the total number of photons, such slow processes must be in play. We showed that the calculation of the joint distribution of donor and acceptor photons in a bin could be reduced to the solution of a "simple" equation that describes the influence of diffusion and conformational changes and involves only the photon count rates. Although this

* Corresponding author.

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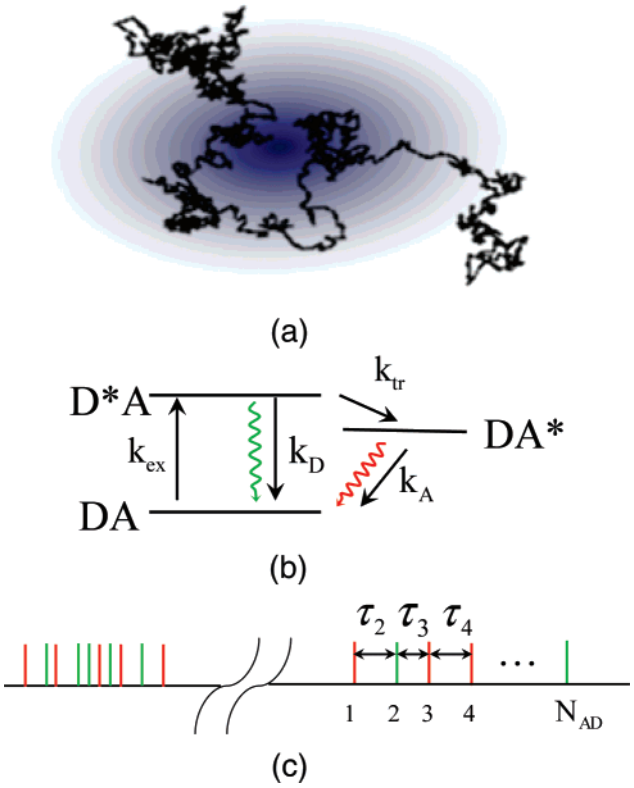

Figure 1. (a) Diffusion of a single molecule through a laser spot with an inhomogeneous intensity profile. (b) The simplest kinetic scheme that describes FRET: k_{ex} is the excitation rate, k_{tr} is the energy transfer rate, $1/k_A$ and $1/k_D$ are the acceptor and donor lifetimes. (c) The resulting photon trajectory consists of bursts of photons separated by long gaps.

constitutes an elegant exact solution of the problem, this formalism was not easy to use in practice to analyze experiments. The fundamental difficulty is that the photon statistics turn out to be sensitive to the precise shape and profile of the observation volume. Small deviations from a Gaussian shape of the laser spot result in large changes in the distribution of the total number of photons.[21]

In the framework of burst analysis, this difficulty has recently been circumvented independently by Antonik et al.[13] and Nir et al.[14] when conformational changes do not occur during the time the molecule spends in the laser spot. Their basic idea is to use the experimentally determined distribution of the total number of photons to bypass the problem of modeling the role of diffusion. For a rigid molecule, using this distribution and the mean efficiency, the shape of the FRET efficiency histogram can be predicted. By comparing with the experimentally determined efficiency distribution, one can determine whether processes other than shot noise are in play.

After providing the necessary background, we begin this paper by showing that this procedure[13,14] is valid for rigid molecules if (I) the FRET efficiency is independent of the location of the molecule in the laser spot and (II) the sum of donor and acceptor count rates is independent of conformation, when only one molecule is in the laser spot at a given time.

When conformational dynamics and diffusion through the laser spot are on the same time scale, it is no longer possible to rigorously express the FRET efficiency histogram in terms of the experimentally determined distribution of the total number of photons and some function that depends only on conformational dynamics. To treat this case, we introduce two procedures, one approximate and the other that is completely rigorous when the above two conditions hold. The approximate procedure involves the distribution of the total number of photons obtained either by using bins or bursts of equal duration. For two-state conformational changes, this approach can be implemented analytically and used to describe the shape of the FRET efficiency distribution for arbitrary values of the interconversion rates.

Our rigorous procedure is based on the fact that, when the above two conditions hold, all conformational information contained in any given trajectory segment is coded into the pattern of photon colors. Given the interphoton times, one can decode this pattern by constructing the likelihood of observing all the trajectory segments for a proposed model of the conformational dynamics. The model parameters are then determined by optimizing this likelihood. The performance of different models can be compared using standard inference methods. This procedure has the promise of becoming routinely used to obtain kinetic information from single-molecule FRET experiments.

Finally, it should be emphasized that we shall assume here that the experimental photon trajectory has been pre-processed and the influence of noise minimized. In addition, complications due to processes such as photobleaching have been circumvented, for example, by using alternating-laser excitation (ALEX).[22] Finally, if the ratio of the acceptor and donor detection efficiencies and quantum yields (γ) is not equal to unity, it should artificially forced to be one by randomly disregarding the appropriate number of donor or acceptor photons.[14]

II. Fluctuations Faster Than Interphoton Times

We shall refer to dynamical processes that are faster than the average time between detected photons as "fast". The interphoton time is usually on the order of 10 μs (due primarily to low detection efficiency). Thus, fast processes include photophysical ones such as excitation, decay, energy transfer, and so forth, as well as changes in the dye orientations and interdye distances that occur on the submicrosecond time scale. They exclude translational diffusion through the laser spot.

When all fluctuations are fast, the photon statistics are essentially Poissonian, and the donor and acceptor photons are uncorrelated. Specifically, the joint probability of detecting N_A acceptor and N_D donor photons in a time bin T is

$$P(N_A, N_D|T) = \frac{(n_A T)^{N_A}}{N_A!}\frac{(n_D T)^{N_D}}{N_D!}e^{-(n_A+n_D)T} \qquad (1)$$

where n_A and n_D are the mean numbers of acceptor and donor photons per unit time (i.e., the count rates). The FRET efficiency distribution is a projection of this joint distribution to a one-dimensional distribution such that $N_A/(N_A + N_D) = \text{constant} = E$. For Poisson statistics, the mean FRET efficiency is $\langle E\rangle = n_A/(n_A + n_D)$, independent of any threshold on the total number of photons that may have been imposed.

Thus, when all fluctuations are fast, the photon statistics are completely characterized by the mean count rates n_A and n_D. These are determined by the steady-state populations of the corresponding excited states, the detection efficiencies and possible leakage of the donor photons into the acceptor channel (crosstalk). The steady-state populations depend on all fast processes including excitation, decay, energy transfer, direct excitation of the acceptor, reexcitation of the donor, and conformational dynamics on the submicrosecond time scale. They can be obtained by solving the appropriate steady-state rate equations that describe all fast processes.[20] The solution of these equations simplifies when the dynamics are either faster

or slower than the fluorophore lifetimes. If the dynamics are faster (as is commonly assumed for dye reorientation), the count rates can be obtained using a conformationally averaged transfer rate (i.e., using $\kappa^2 = 2/3$). On the other hand, if the dynamics are slower than the fluorophore lifetimes (as is commonly assumed for the end-to-end distance fluctuations of a polymer), one must average the count rates obtained for each conformation over an equilibrium distribution of conformations.[9,17,20]

As the simplest possible example, consider a donor−acceptor pair (DA) with a fixed interdye distance, r (see Figure 1b). The donor is excited with rate k_{ex} to form D*A. The donor can either emit a photon or decay nonradiatively, or the excitation can be transferred to the acceptor to form DA*. If reorientational dynamics of the dyes is fast compared with the donor fluorescence lifetime ($1/k_D$), then the transfer rate is $k_{tr} = k_D(R_0/r)^6$, where R_0 is the Förster radius. The mean numbers of detected photons per unit time are $n_A = \phi_A\eta_A k_A p_{ss}(DA^*)$ and $n_D = \phi_D\eta_D k_D p_{ss}(D^*A)$, where $\phi_{A,D}$ and $\eta_{A,D}$ are the quantum yields and detection efficiencies of acceptor and donor photons and $p_{ss}(DA^*)$ and $p_{ss}(D^*A)$ are the steady-state populations of the acceptor and donor excited states. These populations can be found by solving the steady-state rate equations corresponding to the kinetic scheme in Figure 1b. For low laser intensity when $k_{ex} \ll k_A$, $p_{ss}(D^*A) = k_{ex}/(k_D + k_{tr})$ and $p_{ss}(DA^*) = k_{tr}p_{ss}(D^*A)/k_A$. Even when $\gamma = \phi_A\eta_A/\phi_D\eta_D$ is not equal to unity, one should define the FRET efficiency in a bin as $E = N_A/(N_A + N_D)$ (rather than $N_A/(N_A + \gamma N_D)$) because only then is the average FRET efficiency given by $\langle E\rangle = \langle N_A\rangle/(\langle N_A\rangle + \langle N_D\rangle) = n_A/(n_A + n_D)$ for Poissonian statistics. By using the above expressions for the steady-state populations, it follows that the "theoretical" and "experimental" FRET efficiencies are related by[17] $(1 + r^6/R_0^6)^{-1} = \langle E\rangle/(\langle E\rangle + \gamma(1 - \langle E\rangle))$.

The joint distribution in eq 1 can be rewritten as the product of a Poisson distribution of the total number of photons $N_{AD} = N_A + N_D$ and a binomial distribution involving the mean FRET efficiency

$$P(N_A, N_D \mid T) = \frac{(nT)^{N_{AD}}}{N_{AD}!}e^{-nT}\frac{N_{AD}!}{N_A!N_D!}\mathcal{E}^{N_A}(1-\mathcal{E})^{N_D} \quad (2)$$

where we have defined $n \equiv n_A + n_D$ as the count rate of the sum of acceptor and donor photons and $\mathcal{E} \equiv \langle E\rangle = n_A/(n_A + n_D)$ as the mean FRET efficiency. The above binomial distribution is the joint probability of detecting N_A acceptor and N_D donor photons on condition that the total number of photons is N_{AD}. The corresponding mean FRET efficiency (i.e., N_A/N_{AD} averaged over the binomial distribution) is equal to $\mathcal{E}$, and the variance of the FRET efficiency is equal to $\mathcal{E}(1 - \mathcal{E})/N_{AD}$.

Before considering how to generalize these results when the dynamics are slower than the interphoton time, it should be pointed out that even for fast processes the distribution of photons is not exactly Poissonian. When rare events are monitored, deviations from Poisson statistics can be detected. For example, photons separated by nanoseconds are correlated because of antibunching[23] and/or conformational dynamics on the nanosecond time scale.[24] To interpret such experiments, a more general theory[25] that does not exploit the separation of time scales should be used.[24]

III. Translational Diffusion and Slow Conformational Dynamics

Diffusion through the laser spot is slow compared with the interphoton times. In addition, there may be conformational changes that are slow in this sense. The photon count rates $n_A(R,r)$ and $n_D(R,r)$ depend on the position of the molecule in the laser spot, R, through the excitation rate and detection efficiencies and on a conformational coordinate r through the energy transfer rate. Since both these coordinates fluctuate, the photon counts also fluctuate in time. In this case, eq 1 for the joint probability of N_A acceptor and N_D donor photons in a time bin T formally generalizes to

$$P(N_A, N_D \mid T) = \left\langle \frac{[\int_0^T \sum_i n_{Ai}(t)\,dt]^{N_A}}{N_A!}\frac{[\int_0^T \sum_i n_{Di}(t)\,dt]^{N_D}}{N_D!} e^{-\int_0^T \sum_i [n_{Ai}(t)+n_{Di}(t)]\,dt}\right\rangle_{R,r} \quad (3)$$

where we have defined $n_{Ai,Di}(t)$ as the acceptor and donor fluctuating count rates of the ith molecule. The average $\langle ...\rangle_{R,r}$ is over the trajectories of all diffusing molecules at concentration c starting from the uniform distribution and over all conformations starting from the equilibrium distribution.

We have shown previously[20] how the problem of evaluating the above "path integral" can be reduced to the solution of a reaction−diffusion equation. Specifically, the generating function of the joint probability distribution is

$$\sum_{N_A,N_D=0}^{\infty}\lambda_A^{N_A}\lambda_D^{N_D}P(N_A, N_D|T) = \left\langle \exp\left(-\int_0^T \sum_i [(1-\lambda_A)n_{Ai}(t) + (1-\lambda_D)n_{Di}(t)]\,dt\right)\right\rangle = \exp\left(c\int (g(R,r,T) - p_{eq}(r))\,dR\,dr\right) \quad (4)$$

where c is the concentration of diffusing molecules and $g(R, r, t)$ is the solution of the one-particle reaction-diffusion equation

$$\frac{\partial}{\partial t}g(R,r,t) = (D\nabla_R^2 + \mathcal{L}_r)g - [(1-\lambda_A)n_A(R,r) + (1-\lambda_D)n_D(R,r)]g \quad (5)$$

with the equilibrium initial condition that $g(R, r, 0) = p_{eq}(r)$. Here, the three-dimensional Laplacian describes translational diffusion with conformation-independent diffusion constant D. The operator $\mathcal{L}_r$ describes conformational dynamics. For two-state conformational changes, $\mathcal{L}_r$ is just the 2×2 rate matrix. If conformational dynamics is described as diffusion in a potential of mean force $U(r)$, then $\mathcal{L}_r$ is the Smoluchowski operator $\partial/\partial r D_c(r)\exp(-\beta U)\partial/\partial r \exp(\beta U)$ where $D_c(r)$ is a conformation-dependent diffusion coefficient and $\beta = (k_B T)^{-1}$. In the single-molecule limit when there is only one molecule in the laser spot at a given time, the exponential in eq 4 is expanded to linear order in the concentration.

In principle, the above formalism completely solves the problem of how the photon statistics are influenced by diffusion and slow conformational dynamics. In practice, however, the formalism is not easy to use to interpret the experimental data. Even if one is willing to use numerical methods (see ref 26 where we have used this formalism to calculate photon counting histograms in the absence of conformational changes), the results are very sensitive to the precise dependence of the count rate on the translational coordinate (i.e., the shape of the observation volume).[21]

The analysis of FRET experiments with diffusing molecules can, however, be simplified when only one molecule is in the laser spot at a given time and the following two conditions hold to a good approximation:

(I) The FRET efficiency is independent of the location of the molecule in the laser spot:

$$\frac{n_A(R,r)}{n_A(R,r)+n_D(R,r)} = \mathcal{E}(r) \tag{6}$$

This condition is satisfied when the observation volumes at the wavelength of the donor and acceptor photons are the same.

(II) The count rate of the sum of donor and acceptor photons is independent of the conformational coordinate

$$n_A(R,r) + n_D(R,r) = n(R) \tag{7}$$

where $n(R)$ is the count rate of the total number of photons. At low laser intensity, this requires that the product of the detection efficiency and quantum yield of donor and acceptor photons be the same for all conformations ($\gamma = 1$). If this is not the case, γ should be made equal to unity by randomly disregarding the appropriate number of either donor or acceptor photons.[14]

When the above conditions are satisfied, the count rates can be factored:

$$n_A(R,r) = n(R)\mathcal{E}(r) \qquad n_D(R,r) = n(R)(1-\mathcal{E}(r)) \tag{8}$$

Consequently, the intensity correlation functions also factor and can be written as a product of a correlation function that depend only on diffusion and one that depends only on conformational dynamics. By taking the ratio of the acceptor intensity and the total intensity correlation functions, one can determine the efficiency correlation function, which is a direct measure of conformational dynamics:

$$\frac{\langle n_A(t)n_A(0)\rangle}{\langle n(t)n(0)\rangle} = \langle \mathcal{E}(r(t))\mathcal{E}(r(0))\rangle \tag{9}$$

Similar expression holds for the donor intensity correlation function. These are valid for times greater than the relaxation time of the fast processes.

We shall now consider how the above conditions simplify the analysis of the photon statistics when either slow conformational dynamics or translational diffusion are absent. Then, we will propose an approximate procedure to handle the case when conformational changes occur during the time the diffusing molecule is in the laser spot.

A. Diffusion without Conformational Dynamics. Let us begin with a molecule that has only one conformation. Then $\mathcal{E}(r) \equiv \mathcal{E}$ does not fluctuate. Using eq 8 in eq 3 in the single-molecule limit (i.e., ignoring the sum over all molecules), we can factor the joint distribution as

$$P(N_A, N_D|T) = P(N_{AD}|T)\frac{N_{AD}!}{N_A!N_D!}\mathcal{E}^{N_A}(1-\mathcal{E})^{N_D} \tag{10}$$

where $P(N_{AD}|T)$ is the distribution of the sum of donor and acceptor photons ($N_{AD} = N_A + N_D$) in the low concentration limit

$$P(N_{AD}|T) = \left\langle \frac{[\int_0^T n(t)\,dt]^{N_{AD}}}{N_{AD}!} e^{-\int_0^T n(t)\,dt} \right\rangle_R \tag{11}$$

that depends only on translational diffusion. For a rigid molecule, this factorization requires only condition I to hold.

An analogous relation holds for an ensemble of conformations that do not interconvert during the diffusion time:

$$P(N_A, N_D|T) = P(N_{AD}|T)\frac{N_{AD}!}{N_A!N_D!}\int \mathcal{E}(r)^{N_A}(1-\mathcal{E}(r))^{N_D}p_{eq}(r)\,dr \tag{12}$$

where $p_{eq}(r)$ is the normalized equilibrium conformational distribution. When the conformational space is discrete, the integral over r is replaced by a sum over conformations. This equation is valid only in the single-molecule limit and when both conditions I and II are met.

Antonik et al.[13] and Nir et al.[14] used eqs 10 and 12 in conjunction with burst analysis. They dramatically simplify the analysis of FRET efficiency histograms of diffusing molecules in the absence of conformational changes because the distribution of the total number of photons does not need to be modeled but can be obtained directly from the experimental data. One can check whether the experimentally determined FRET efficiency distribution is consistent with a single or multiple noninterconverting conformations by comparing it with the one calculated using eqs 10 or 12 with the experimentally determined distribution of the total number of photons. If there is only one conformer, the only adjustable parameter is the mean FRET efficiency ($\mathcal{E}$ in eq 10). If there are two conformers, then there are three adjustable parameters (the mean efficiencies of the two states and their relative population) and so on. If the conformation distribution is continuous, then one can use eq 12 with some assumed functional forms of $\mathcal{E}(r)$ and $p_{eq}(r)$ containing a few adjustable parameters.

Another way of establishing the existence of multiple conformers is to simply examine the variance of the FRET efficiency distribution.[20] Suppose one obtains the FRET efficiency distribution using eq 12 with a threshold N_T. Then one can show that the mean of the resulting distribution is the equilibrium conformational average of $\mathcal{E}(r)$; $\langle E\rangle = \int \mathcal{E}(r)p_{eq}(r)\,dr \equiv \langle\mathcal{E}\rangle_c$. The variance is

$$\langle E^2\rangle - \langle E\rangle^2 = \sigma_c^2(1-\langle N_{AD}^{-1}\rangle) + \langle E\rangle(1-\langle E\rangle)\langle N_{AD}^{-1}\rangle \tag{13a}$$

$$\langle E^2\rangle - \langle E\rangle^2 < \sigma_c^2 + \langle E\rangle(1-\langle E\rangle)/N_T \tag{13b}$$

where $\sigma_c^2 \equiv \langle\delta\mathcal{E}^2\rangle_c \equiv \int(\mathcal{E}(r) - \langle\mathcal{E}\rangle_c)^2p_{eq}(r)\,dr$ is the variance due to conformational heterogeneity and $\langle N_{AD}^{-1}\rangle \equiv \langle(N_A + N_D)^{-1}\rangle$ is the average of the reciprocal of the total number of photons. This average can be calculated from the experimentally determined distribution of the total number of photons as $\langle N_{AD}^{-1}\rangle = \sum_{N_{AD}=N_T}^{\infty} N_{AD}^{-1}P(N_{AD}|T)/\sum_{N_{AD}=N_T}^{\infty}P(N_{AD}|T)$. If one uses bursts rather than bins, then the variance is the same but $\langle N_{AD}^{-1}\rangle$ must be calculated by averaging over all bursts that contribute to the FRET efficiency peak.

For a single diffusing conformer, the FRET efficiency variance is $\langle E\rangle(1-\langle E\rangle)\langle N_{AD}^{-1}\rangle$. The influence of diffusion is reflected only in the value of $\langle N_{AD}^{-1}\rangle$. This variance is always less than $\langle E\rangle(1-\langle E\rangle)/N_T$, in agreement with our previous work.[20] Thus, if the observed FRET efficiency distribution is wider than this, multiple conformations with different efficiencies must be present. However, whether these interconvert on a time scale that is the same as or slower than the diffusion time, requires further analysis (e.g., by examining the bin size dependence of the variance or the intensity correlation functions in eq 9).

B. Conformational Dynamics without Diffusion. Next, consider an immobilized molecule undergoing conformational dynamics. When condition II (see eq 7) holds, the sum of the donor and acceptor count rates, $n(R) \equiv n$, does not depend on conformation and hence does not fluctuate. It then follows from eqs 3 and 8 that the joint distribution can be written as

$$P(N_A, N_D|T) = \frac{(nT)^{N_{AD}}}{N_{AD}!} e^{-nT} B_c(N_A, N_D|T) \quad (14)$$

where $B_c(N_A, N_D|T)$ is the generalization of the binomial distribution to account for conformational dynamics and is given by

$$B_c(N_A, N_D|T) = \frac{N_{AD}!}{N_A! N_D!} \int_0^1 \epsilon^{N_A} (1-\epsilon)^{N_D} P_c(\epsilon|T)\, d\epsilon \quad (15)$$

where $P_c(\epsilon|T)$ is the FRET efficiency distribution due solely to conformational dynamics.[19,20] It is defined as $P_c(\epsilon|T) = \langle \delta(\epsilon - (1/T)\int_0^T \mathcal{E}(r(t))\, dt)\rangle_r$ where $\delta(x)$ is the Dirac δ function and the average is over conformational trajectories starting from the equilibrium distribution.

The distribution $P_c(\epsilon|T)$ depends on the model used to describe conformational dynamics and can be found by solving an equation with the same structure as eq 5 but without the translational diffusion term ($D = 0$). We have previously developed a numerical procedure for calculating this distribution for an arbitrary number of discrete conformational states and used a Gaussian polymer chain as an illustration.[19] The mean of this distribution is $\langle \mathcal{E} \rangle_c = \int \mathcal{E}(r) p_{eq}(r)\, dr$ for all T and its variance is

$$\sigma_c^2(T) = \frac{2}{T^2} \int_0^T (T-t) \langle \delta\mathcal{E}(t) \delta\mathcal{E}(0) \rangle\, dt \quad (16)$$

where $\delta\mathcal{E} = \mathcal{E} - \langle \mathcal{E} \rangle_c$. Thus, the variance is determined by the fluctuations of the efficiency due to conformational dynamics through its autocorrelation function. For large bins, $\sigma_c^2(T) \approx 2\tau_c \langle \delta\mathcal{E}^2 \rangle / T$, where τ_c is the relaxation time of this autocorrelation function ($\tau_c = \int_0^\infty \langle \delta\mathcal{E}(t)\delta\mathcal{E}(0)\rangle_c / \langle \delta\mathcal{E}^2 \rangle_c\, dt$).

The distribution $P_c(\epsilon|T)$ has a simple structure when the observation time T is either shorter or longer than the conformational relaxation time τ_c. When $T \ll \tau_c$, $P_c(\epsilon|T) = \int \delta(\epsilon - \mathcal{E}(r)) p_{eq}(r)\, dr$. When $T \gg \tau_c$, then $P_c(\epsilon|T)$ is a Gaussian with mean $\langle \mathcal{E} \rangle_c$ and variance $\sigma_c^2(T)$.

When the conformational dynamics can be described by transitions between two discrete states, $P_c(\epsilon|T)$ can be found analytically.[19,27] By using this result in eq 15, the generalization of the binomial distribution becomes ($\mathcal{E}_2 \geq \mathcal{E}_1$)

$$B_c(N_A, N_D|T) = \frac{N_{AD}!}{N_A! N_D!} \Bigg(\mathcal{E}_1^{N_A} (1-\mathcal{E}_1)^{N_D} p_1\, e^{-k_1 T} + \mathcal{E}_2^{N_A} (1-\mathcal{E}_2)^{N_D} p_2\, e^{-k_2 T} + \frac{2kTp_1p_2}{\mathcal{E}_2 - \mathcal{E}_1} \int_{\mathcal{E}_1}^{\mathcal{E}_2} \epsilon^{N_A} (1-\epsilon)^{N_D} [I_0(y) + kT(1-z) I_1(y)/y]\, e^{-kzT}\, d\epsilon \Bigg) \quad (17)$$

where k_1 and k_2 are the transition rates, $\mathcal{E}_1$ and $\mathcal{E}_2$ are the transfer efficiencies, $k = k_1 + k_2$, $p_1 = 1 - p_2 = k_2/k$, $y = 2kT(p_1p_2(\mathcal{E}_2 - \epsilon)(\epsilon - \mathcal{E}_1)/(\mathcal{E}_2 - \mathcal{E}_1)^2)^{1/2}$, $z = (p_1(\epsilon - \mathcal{E}_1) + p_2(\mathcal{E}_2 - \epsilon))/(\mathcal{E}_2 - \mathcal{E}_1)$, and $I_n(y)$ are modified Bessel functions of the first kind. This expression can be used to calculate the shape of the FRET efficiency distribution for all values of the interconversion rates.

Finally, we note that if the FRET efficiency distribution is calculated using eq 14, then the mean FRET efficiency is equal to the equilibrium average of $\mathcal{E}(r)$, $\langle E \rangle = \langle \mathcal{E} \rangle_c$. The variance is given by eq 13a with σ_c^2 replaced by the bin-time dependent $\sigma_c^2(T)$ given in eq 16 and $\langle N_{AD}^{-1} \rangle$ calculated by averaging over a Poisson distribution of the total number of photons.

C. Conformational Dynamics and Diffusion. When conformational dynamics occur during the time the molecule spends in the laser spot, it is not possible to factor the joint distribution into a product of the distribution of the sum of the acceptor and donor photons and a term that depends solely on conformational dynamics as in eqs 10 and 14. This is the case for both bins and bursts even when the two conditions, discussed in the beginning of the section, hold. The simplest approximation can be found by combining the first factor in eq 10 with the second factor in eq 14 so that

$$P(N_A, N_D|T) \approx P(N_{AD}|T) B_c(N_A, N_D|T) \quad (18)$$

The first factor is the distribution of the sum of acceptor and donor photons that can be determined experimentally. The second one is the dynamical generalization of the binomial distribution to describe conformational changes given in eq 15.

This approximation is only useful when the molecule is quasi-immobilized during the observation time. In other words, during time T, the molecule should only explore a region of the observation volume where the intensity and hence the total count rate does not fluctuate significantly. In the context of bin analysis, it should be used only for bin sizes that are shorter than the diffusion time. In addition, one should impose a sufficiently high threshold to eliminate events where the molecule transiently leaves and then immediately re-enters the laser spot.

In the framework of burst analysis, eq 18 should be used for bursts with the same duration. These can be obtained by chopping all bursts into segments of duration T (and then $2T$, $3T$, etc. until there is insufficient data). Since the bursts have been preselected, one can use longer time windows than is possible when the entire photon trajectory is binned. However, T should not be much longer than the mean diffusion time as determined by the area under the FCS intensity correlation function normalized to unity at $t = 0$. The longer the time window, the more likely it is that the total count rate fluctuated significantly because of the nonuniform nature of the observation volume profile. In addition, it becomes increasingly probable that the photons were emitted by two molecules that were simultaneously within the laser spot.

As an illustration, we consider how the FRET efficiency distribution of a two-state system behaves when the conformational relaxation time is slower than, equal to, or faster than the observation time. The FRET efficiency distribution is a slice of the joint distribution $P(N_A, N_D|T)$ for which $N_A/(N_A + N_D) = E$. It can be visualized by constructing the FRET efficiency histogram, FEH(E), which is the probability that $N_A/(N_A + N_D)$ falls in the interval between $E - h/2$ and $E + h/2$, where h is the histogram step size. The FRET efficiency histogram is easily constructed using

$$\mathrm{FEH}(E) = \mathcal{N}^{-1} \sum_{N_{AD}=N_T}^{\infty} \sum_{N_A=[(E-h/2)N_{AD}]+1}^{[(E+h/2)N_{AD}]} P(N_A, N_{AD} - N_A|T) \quad (19)$$

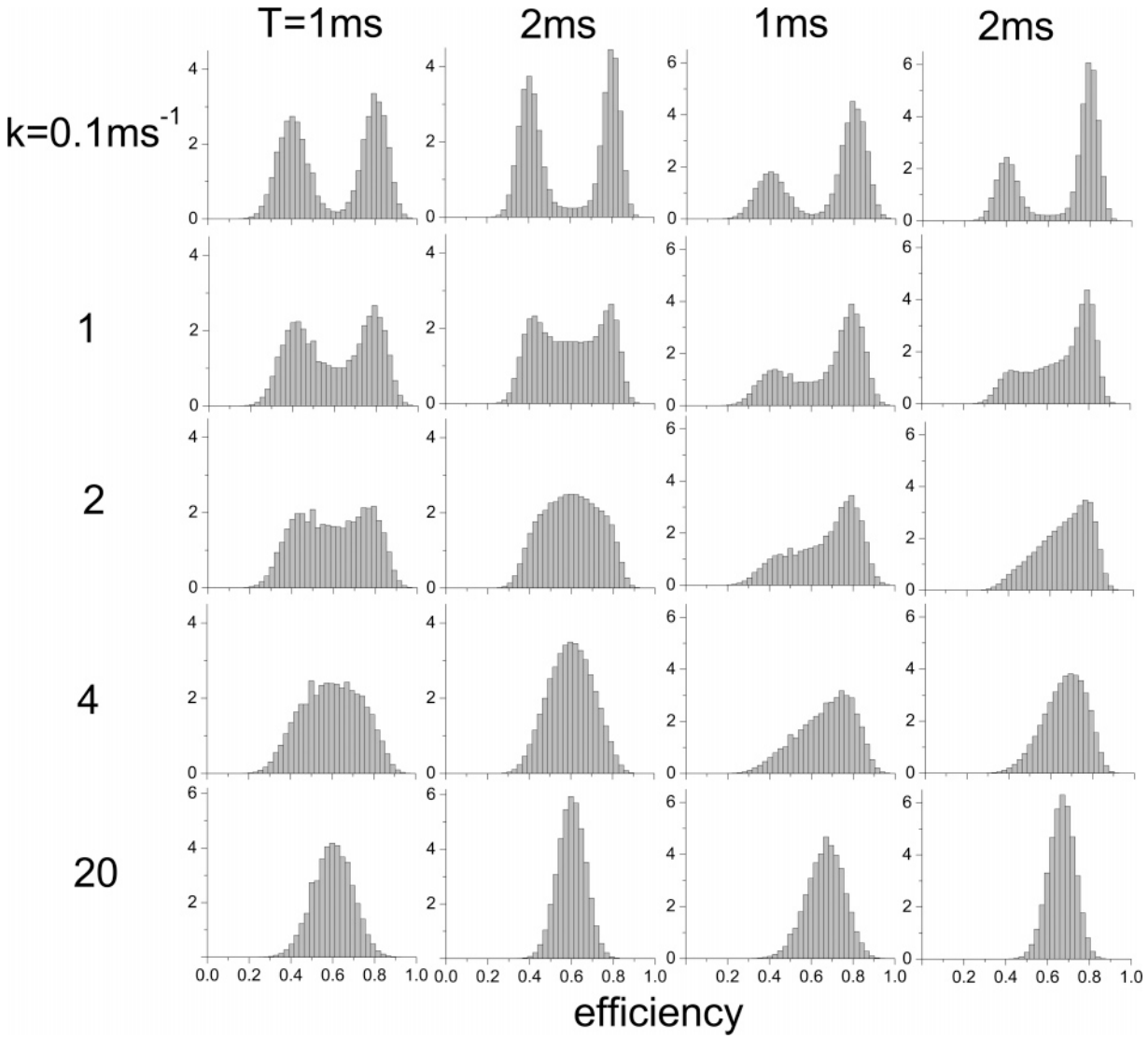

Figure 2. FRET efficiency histograms of a diffusing molecule obtained from bursts of size T. The molecule undergoes transitions between two conformational states with low ($E_1 = 0.4$) and high ($E_2 = 0.8$) transfer efficiency. The histograms are shown for different values of the relaxation rate $k = k_1 + k_2$ (the rows) and the burst duration (the columns). In the first two columns, the equilibrium populations of two states are the same ($k_1 = k_2$). In the third and forth columns, the populations differ by a factor of 2 ($k_1 = 2k_2$).

where we have imposed a threshold N_T on the total number of photons. Here, $[x]$ is the whole part of x, that is, the largest integer not exceeding x, and $\mathcal{N} = \sum_{N_{AD}=N_T}^{\infty}\sum_{N_A=0}^{N_{AD}} P(N_A, N_{AD} - N_A|T)$ is the normalization factor. This recipe works for bins as well as bursts and can be used to predict the FRET distribution from the joint distribution of acceptor and donor photons that is constructed from the experimentally determined distribution of the total number of photons via eqs 10, 12, 14, or 18.

Consider two conformations with FRET efficiencies $\mathcal{E}_1 = 0.4$ and $\mathcal{E}_2 = 0.8$ that interconvert with rates k_1 and k_2. For illustrative purposes, we assume that the distribution of the total number of photons is Poissonian with count rate $n = 50$ ms^{-1}. The threshold value $N_T = 30$. The FRET distribution is calculated using eq 19 ($h = 1/41$) with the joint distribution obtained from eqs 14 and 17. Figure 2 shows the FRET efficiency distributions for different values of the burst size T (the columns) and the conformational relaxation rate $k = k_1 + k_2$ (the rows). In the first two columns, the populations of the two states are equal ($k_1 = k_2$). In the third and forth columns, $k_1 = 2k_2$; hence, the peak at $\mathcal{E}_2 = 0.8$ is higher than the peak at $\mathcal{E}_1 = 0.4$.

When conformational dynamics are slow ($kT \ll 1$), the FRET efficiency distribution is a superposition of two peaks with widths determined by shot noise. Increasing T in this regime decreases the shot noise because more photons are collected; therefore, the peaks become narrower. When the conformational relaxation time is comparable to the observation time, transitions between the two states give rise to a plateau between the peaks. When $kT \sim 1$, the shape of the distribution is sensitive to the value of the observation time. As kT increases, the distribution eventually becomes a Gaussian centered on the average efficiency (the last row). The width of the Gaussian is determined by both shot noise and conformational dynamics. The measured variance is the sum of the variance due to shot noise, $\langle E\rangle(1 - \langle E\rangle)\langle(N_A + N_D)^{-1}\rangle$, and the variance due to conformational dynamics, $2\langle\delta\mathcal{E}^2\rangle_c/kT = 2p_1p_2(\mathcal{E}_2 - \mathcal{E}_1)^2/kT$, which decreases as T increases.

IV. Decoding the Pattern of Photon Colors

We have seen that when conformational changes occur during the time a diffusing molecule spends in the laser spot, the joint distribution of donor and acceptor photons can only be approximately factored (see eq 18) even when conditions I and II hold. We will now show how to extract information about conformational dynamics from single-molecule FRET photon trajectories in a rigorous way without having to model diffusion

when conditions I and II are met and the photon arrival times can be determined.

The basic idea is simple. If the sum of acceptor and donor count rates is conformation independent (condition II, eq 7), then the arrival times of the photons (irrespective of their color) in any trajectory segment cannot depend on conformational dynamics. If the efficiency is independent of where the molecule is in the laser spot (condition I, eq 6), the color of these photons (i.e., whether they were emitted by the donor or the acceptor) cannot depend on diffusion. Thus, all information about conformational dynamics must be coded in the color pattern as long as all photons in the trajectory segment were generated by the same molecule. These segments could but need not be bursts as they are usually defined. They may be "superbursts" containing short gaps resulting from the same molecule exiting and entering the laser spot several times before another molecule enters.

Arguably, the best way (i.e., one that uses all available information) to decode the observed pattern of photon colors is to use Bayesian inference.[28] Such procedures have been recently applied to a variety of different problems in single-molecule spectroscopy.[11,18,29−32] In the present context, one first constructs the probability of occurrence or likelihood of a set of bursts within the framework of a model of conformational dynamics and then varies the model parameters so as to optimize the likelihood (or more commonly its logarithm). Suppose we are given a burst with N_{AD} photons where the time between the (i − 1)th and ith photon is τ_i, $i = 2, ..., N_{AD}$ (see Figure 1c). Suppose that the conformational state of the molecule is specified by the interdye distance r (which may be effectively discrete) that slowly fluctuates. The conditional probability that the interdye distance is r at time t, given that it was r_0 at $t = 0$, is the conformational propagator or Green's function which we denote here as $G_c(r, t|r_0)$. Then the likelihood (L) of the observed color pattern of this burst can be written as

$$L = \int \left(\prod_{i=2}^{N_{AD}} f_i(r_i)\, G_c(r_i,\tau_i|r_{i-1}) \right) f_1(r_1)\, p_{eq}(r_1)\, dr_1 ... dr_{N_{AD}} \quad (20)$$

where $f_i(r_i)$ is the probability that the ith photon, which was emitted when the interdye distance was r_i, has a specific color. If the ith photon was emitted by the acceptor, then $f_i(r) = \mathcal{E}(r)$, otherwise, $f_i(r) = 1 - \mathcal{E}(r)$, where $\mathcal{E}(r)$ is the conformation-dependent FRET efficiency. When the conformation does not change during the burst, then the above likelihood simplifies to

$$L = \int \mathcal{E}(r)^{N_A} (1 - \mathcal{E}(r))^{N_D} p_{eq}(r)\, dr \quad (21)$$

where N_A (N_D) is the number of acceptor (donor) photons. The likelihood of a set of bursts is the product of the likelihood of each burst.

When the conformational space is discrete or when it is approximated as such, the integrals in the above expressions are replaced by sums. Specifically, for a system with M interconverting states, the analog of eq 20 is

$$L = \mathbf{1}^T \left(\prod_{i=2}^{N_{AD}} \mathbf{F}_i\, e^{\mathbf{K}\tau_i} \right) \mathbf{F}_1 \mathbf{p}_{eq} \quad (22)$$

where $\mathbf{K}$ is the $M \times M$ rate matrix that describes the transitions among the states, $\mathbf{1}^T$ is a row vector with unit elements, $\mathbf{F}_i$ is a diagonal matrix with the efficiencies of the various conformers on the diagonal if the ith photon is an acceptor photon and one minus the efficiencies otherwise, and $\mathbf{p}_{eq}$ is a column vector of the equilibrium populations. The matrix exponential, $\exp(\mathbf{K}\tau_i)$, for various i's is most efficiently calculated by first diagonalizing the rate matrix $\boldsymbol{K}$ and then using the spectral representation. If the interphoton times are much shorter than the conformational relaxation time, the approximation $\exp(\boldsymbol{K}\tau_i) \approx \mathbf{I} + \boldsymbol{K}\tau_i$ may be useful, where $\mathbf{I}$ is the unity matrix.

As the simplest application of the above formalism, consider a system with two interconverting states with efficiencies $\mathcal{E}_1$ and $\mathcal{E}_2$. These states can correspond to two conformers with long and short interdye distances. Alternately, this model can describe slow "blinking" of acceptor photons in a molecule with a single conformer, if state 1 is "dark" and state 2 is "bright" so that $\mathcal{E}_1 = 0$.[20] For a two-state system, employing the same notation as used in eq 17, $(\exp \boldsymbol{K}\tau_i)_{\alpha\beta} = p_\alpha + (\delta_{\alpha\beta} - p_\alpha) \exp(-k\tau_i)$, $\alpha, \beta = 1, 2$ ($\delta_{\alpha,\beta} = 1$ if $\alpha = \beta$ and equals 0 otherwise). The likelihood function in eq 22 for a given set of trajectory segments can be calculated by repeated multiplication of 2×2 matrices. For example, for the burst in Figure 1c, the likelihood function is $\mathbf{1}^T(\mathbf{I} - \mathbf{E})\, ...\, \mathbf{E}\, e^{\mathbf{K}\tau_4}\mathbf{E}\, e^{\mathbf{K}\tau_3}(\mathbf{I} - \mathbf{E})\, e^{\mathbf{K}\tau_2}\mathbf{E}\mathbf{p}_{eq}$, where $\mathbf{E}$ is the 2×2 diagonal matrix with $\mathcal{E}_1$ and $\mathcal{E}_2$ on the diagonal, $\mathbf{p}_{eq}$ is the two-state vector of equilibrium populations, and τ_i are the interphoton times. The two adjustable parameters (k and p_1) could be determined by maximizing the likelihood. This suffices in simple cases when the likelihood surface has a single dominant maximum. In general, one should explore the entire surface using Monte Carlo methods.

Once the model rate parameters have been determined, one can see how good they are by comparing the experimental FRET efficiency histogram calculated from the set of bursts with the predicted one. This can be obtained by first erasing all the colors from the bursts and then recoloring them randomly in accordance with the proposed model. The FRET efficiency histogram can then be calculated from the recolored photon trajectory and compared with the experimental one. It will be interesting to compare the performance of this method with that of the simpler but approximate procedure based on eqs 17 and 18 when applied to experimental data obtained for a macromolecule that undergoes conformational changes on the time scale of the diffusion time.

V. Concluding Remarks

In this paper, we considered a variety of practical procedures for extracting information about conformational dynamics from FRET experiments on freely diffusing molecules. We showed that the FRET efficiency histogram approach developed independently by the Seidel[13] and Weiss[14] groups for rigid molecules, which involves the experimentally determined distribution of the total number of photons, is valid when (I) the FRET efficiency does not depend on the position in the laser spot and (II) the sum of the count rates is the same for all conformations. When a molecule has several conformational states interconverting on a time scale much slower than the diffusion time, then one can extract the populations of the various conformers either using the approach of Antonik et al.[13] based on eq 12 or by using the maximum likelihood method based on eq 21.

When conformational dynamics occurs on a time scale comparable to the diffusion time and if the above two conditions hold, one can estimate this time scale from the decay of the efficiency correlation function obtained from the ratio of the acceptor and the total intensity correlation functions (see eq 9). To extract more information about the nature of conformational changes, we proposed and illustrated an approximate method that involves fitting the FRET efficiency histogram using eq

18 as well as a rigorous procedure based on decoding the pattern of photon colors in a set of bursts, each generated by the same molecule. These procedures should prove useful in the analysis of the ever-increasing amount of data that is being collected as single-molecule spectroscopy matures.

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