Theory of photon statistics in single-molecule Förster resonance energy transfer

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We present the theory for the distribution of the number of donor and acceptor photons detected in a time bin and the corresponding energy-transfer efficiency distribution obtained from single-molecule Förster resonance energy-transfer measurements. Photon counts from both immobilized and freely diffusing molecules are considered. Our starting point is the joint distribution for the donor and acceptor photons for a system described by an arbitrary kinetic scheme. This is simplified by exploiting the time scale separation between fast fluorescent transitions and slow processes which include conformational dynamics, intersystem conversion to a dark state, and translational diffusion in and out of the laser spot. The fast fluorescent transitions result in a Poisson distribution of the number of photons which is then averaged over slow fluctuations of the local transfer efficiency and the total number of photons. The contribution of various processes to the distribution and the variance of the energy-transfer efficiency are analyzed. © *2005 American Institute of Physics.* [DOI: 10.1063/1.1812746]

I. INTRODUCTION

Single-molecule spectroscopy has become a powerful technique for examining individual molecules that undergo complex conformational dynamics. In several recent experiments, Förster resonance energy transfer (FRET) has been used as a distance probe to study polymer dynamics such as protein and RNA folding.[1–11] In these studies, a macromolecule labeled with donor and acceptor dyes is continuously illuminated by a laser beam. The resulting donor and acceptor fluorescent photons are simultaneously counted in equally spaced time bins. The FRET efficiency in each bin is determined as a ratio of the number of acceptor photons to the total number of photons. These FRET efficiencies are random and their distribution contains information about the molecule's conformational dynamics.

Single fluorescently tagged molecules can be detected when they are immobilized (attached to a surface, embedded in gels, or trapped in lipid vesicles)[1,9] or freely diffusing in solution.[2–6] The first kind of experiment allows one to record the fluorescent signal from a single molecule over an extended period of time, until the photobleaching of the fluorophore (on the order of a few seconds). Unfortunately, interaction with the surface may significantly alter the dynamic and photophysical properties of the molecule.

This problem is absent when the molecules are freely diffusing. In this type of experiment, a laser beam is focused into a dilute solution. When a fluorescently labeled molecule diffuses into the focal volume, a burst of donor and acceptor photons is generated. Because the residence time of a molecule in the laser spot is short, it is not possible to measure long temporal single-molecule trajectories. The fluorescence

trajectory of freely diffusing molecules consists of separate bursts of photons generated by individual molecules. Such a trajectory is divided into equal time bins and the distributions of the number of photons and the FRET efficiency are constructed.

Single-molecule transfer efficiency fluctuations can arise from a variety of sources. Usually, only the contributions from the fluctuations of the interdye distance are of interest. These must be extracted from the shot noise resulting from the discreteness of the number of photons. Other effects that may contribute to broadening of the distribution include transitions into and out of nonfluorescent states (i.e., intersystem crossing to the acceptor's triplet state) and fluctuations of the burst size due to translational diffusion through the laser spot.

In this paper we develop a comprehensive theory for the photon statistics and FRET efficiency distributions obtained from single-molecule measurements where the photons are integrated over time bins. Our approach is based on the exact generating function for the number of transitions in an arbitrary kinetic scheme. We assume that light coherence effects can be neglected for the fluorescent molecules at room temperature so that the emission of photons can be described by rate (master) equations (see Ref. 12 and references therein for quantum effects). The kinetic schemes we consider may include both discrete (e.g., fluorescent states) and continuous coordinates that describe translational diffusion and/or conformational dynamics. The formalism can handle both immobilized and freely diffusing molecules. We specifically consider the following photophysical processes:

(a)   Förster energy transfer from the donor to the acceptor dye in the simplest three state system [see Fig. 1(a)]. The donor is excited with excitation rate I, which is proportional to the laser intensity. Transfer to the ac-

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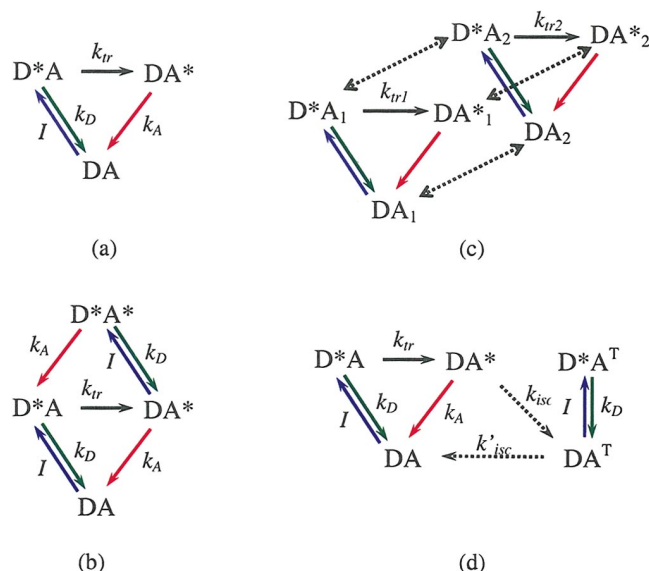
FIG. 1. (Color) Kinetic schemes for FRET models. (a) Three-state model for energy transfer from the donor to the acceptor. Generalization of this model to include (b) donor re-excitation in the excited acceptor state, (c) two-state conformational changes, and (d) intersystem crossing into the acceptor's triplet state. Blue arrows denote donor excitation, red and green arrows denote the decay of the acceptor and the donor excited states, black arrows describe FRET, and dotted arrows represent transitions due to conformational dynamics [panel (c)] and intersystem crossing [panel (d)].
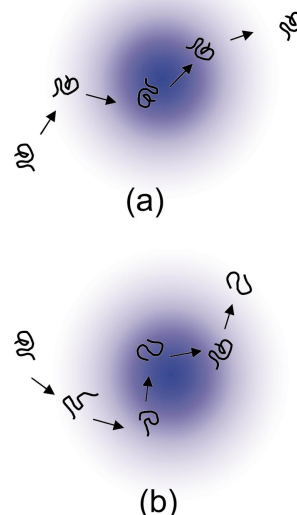

FIG. 2. (Color) Diffusion in and out of the laser spot (a) diffusion of a molecule with fixed conformation and (b) diffusion and conformational changes.

ceptor occurs with the rate k_{tr}, the excited states decay to the ground state with rates k_D and k_A. These describe both radiative and nonradiative processes.

(b)   Reexcitation of the donor when the acceptor is excited [Fig. 1(b)].

(c)   Conformational dynamics [Fig. 1(c)]. Because the energy-transfer rate k_{tr} depends on the interdye distance, the reaction scheme is a set of three-state systems for each conformation. For simplicity, only two conformations with different interdye distances are shown in Fig. 1(c) with the energy-transfer rates k_{tr1} and k_{tr2}. The two conformations can represent folded and unfolded states of a protein. Conformational changes are denoted by dotted arrows.

(d)   Intersystem crossing from the acceptor excited state to the triplet (dark) state [Fig. 1(d)]. Slow transitions, k_{isc} and k'_{isc}, are indicated by dotted arrows.

In addition, we will discuss whether fluctuations of the orientation of the donor and acceptor transition dipoles influence the FRET efficiency distribution.

These processes can occur for both immobilized and diffusing molecules. Figure 2(a) illustrates a molecule with a fixed conformation diffusing in and out of the laser spot. In this simplest case, the width of the FRET efficiency distribution is determined by shot noise and by the fluctuations of the residence time that the molecule spends inside the laser spot. Figure 2(b) shows a freely diffusing molecule with the interdye distance changing due to conformational dynamics. In this case, there are three possible sources of the FRET efficiency fluctuations, shot noise, conformational dynamics, and the fluctuations of the residence time.

In the previous paper[13] we considered the FRET efficiency distribution of immobilized single molecules with conformational dynamics in the limit that shot noise is negligible. Here we extend our study to incorporate shot noise, intersystem crossing, and translational diffusion. We will obtain the distributions for the donor and acceptor photons and for the FRET efficiency calculated in bins of duration t. We also analyze various contributions to the width of the transfer efficiency distribution and consider the influence of the bin size and the value of the photon-counting threshold.

The general formalism must consider all the different time scales in this problem. The lifetimes of the excited fluorescent states are of the order picosecond to nanosecond. The lifetime of the acceptor dark triplet state in the presence of oxygen, which is an efficient quencher, is of the order of 1 μs,[1] so that intersystem conversion to the dark state is much slower than fluorescent transitions. The same is true for translational diffusion in and out of the laser spot (the residence time of the molecule in the spot is of the order of millisecond).[2] Conformational fluctuations can occur on the picosecond to nanosecond time scale. Finally, there are two parameters related to photon counting; the average time between two consecutively detected photons and the bin size. At most 100 photons can be detected per millisecond without introducing saturation effects,[1,4] so that the average time between photons is on the microsecond to millisecond time scale and the bin size is usually not less than 100 μs.[4]

We show here that processes that are fast and slow compared to the time between two consecutively detected photons influence the photons statistics in different ways. Fast processes result in a Poisson distribution of photons and the corresponding FRET efficiency distribution. The processes that are slow or comparable with the time between consecutively detected photons broaden these distributions.

This paper is organized in a hierarchical manner, starting with the simplest description of FRET in immobilized molecules and ending by treating diffusing molecules that undergo conformational changes. In the next section we briefly review general formalism for the statistics of photons integrated over bins of duration t and consider the distributions of the number of photons for a three-state system as an example. The Poisson distribution of the number of photons is discussed in detail in Sec. II B. Separation of time scales between fast and slow transitions is exploited in Sec. II C and a general expression for the FRET distribution is obtained. In Sec. III we apply the general formalism to measurements of immobilized single molecules and specifically consider the influence of slow conformational dynamics (Sec. III A) and intersystem conversion into the acceptor's dark state (Sec. III B). Section IV presents the theory of photon statistics for freely diffusing molecules. The general formalism for a diffusing molecule in Sec. IV A is applied to a molecule with fixed conformation (Sec. IV B) and to one undergoing conformational dynamics (Sec. IV C). Some concluding remarks are made in Sec. V.

II. GENERAL FORMALISM

A. Exact distribution of the number of photons detected in a time bin

We begin by focusing on the joint distribution of the number of donor and acceptor photons detected in a time bin. In this paper we will not explicitly consider the effect of background noise. Suffice it to say that our analysis applies to probability distributions that are obtained from experiment by deconvoluting the noise. To calculate the joint distribution, we generalize our previous work[14] which involved a "counting parameter." This method is, in turn, a generalization of a technique used to analyze a simple model of microtubule growth.[15] A similar method was used to treat single-molecule photon emission events in the framework of the optical Bloch equation.[16] We start with the exact treatment of the problem and then derive approximate equations for the generating function of the joint probability taking advantage of the time scale separation of the various processes.

Consider a general kinetic scheme where the probability of finding the system in state n, $p_n(t)$, obeys the rate equation

$$\frac{d}{dt}p_n(t) = \sum_m K_{nm}p_m(t),\tag{2.1}$$

where K_{nm} is the rate constant for the transition $m \to n$. Suppose that transitions $i_D \to j_D$ and $i_A \to j_A$ result in the emission of donor and acceptor photons, respectively. We are interested in the joint probability $P(N_A, N_D|t)$ of the number N_A of $i_A \to j_A$ transitions and of the number N_D of $i_D \to j_D$ transitions during the bin time t when the system is initially in the steady state and in an arbitrary state at the end of the bin. These probabilities can be obtained from the generating function $F(\lambda_A, \lambda_D, t)$ for the joint probability

$$F(\lambda_A, \lambda_D, t) \equiv \sum_{N_A, N_D=0}^{\infty} \lambda_A^{N_A} \lambda_D^{N_D} P(N_A, N_D|t).\tag{2.2}$$

It was shown[14] that this generating function can be obtained by introducing counting parameters into the rate matrix. Specifically, we multiply the off-diagonal elements $K_{j_A i_A}$ and $K_{j_D i_D}$ of the rate matrix by the counting parameters λ_A and λ_D and solve the rate equations with the modified rate matrix $K_{nm}(\lambda_A, \lambda_D)$

$$\frac{d}{dt}f_n(t) = \sum_m K_{nm}(\lambda_A, \lambda_D)f_m(t),\tag{2.3}$$

with the steady-state initial conditions. Here $f_n(t)$ is the generating function for the system in state n at the end of the bin. Then

$$F(\lambda_A, \lambda_D, t) = \sum_n f_n(t).\tag{2.4}$$

This result follows from our previous work.[14] An alternative proof is given in Appendix A. Note that $F(\lambda_A = \lambda_D = 1, t) = 1$.

This procedure is readily generalized. For example, if both $i_1 \to j_1$ or $i_2 \to j_2$ transitions result in emission of donor photons, then the same counting parameter λ_D must be put in front of the off-diagonal elements $K_{j_1 i_1}$ and $K_{j_2 i_2}$. When the transition $i_\alpha \to j_\alpha$ describes both radiative and nonradiative decays, $K_{j_\alpha i_\alpha} = K_{j_\alpha i_\alpha}^{rad} + K_{j_\alpha i_\alpha}^{nr}$, $\alpha = A, D$, only the radiative rate constant $K_{j_\alpha i_\alpha}^{rad}$ is multiplied by the counting parameter. If in addition not every photon is detected due to low detection efficiency $\phi_{A,D}$, then the decay rate is split into two parts, one that corresponds to the detected photons ($K_{j_\alpha i_\alpha}^0 = \phi_\alpha K_{j_\alpha i_\alpha}^{rad}$) and the other to nonradiative or nondetected transitions ($K_{j_\alpha i_\alpha} - K_{j_\alpha i_\alpha}^0$, $\alpha = A, D$). The counting parameter must be put in front of the rate constant that corresponds to detected transitions.

To illustrate this formalism, we consider a three state system with ground (DA), excited donor (D^*A), and excited acceptor (DA^*) states [Fig. 1(a)]. Suppose that the acceptor and donor radiative decay rates are k_A^{rad} and k_D^{rad} and the detection efficiencies for the acceptor and donor photons are ϕ_A and ϕ_D. Then the generating function is found by solving the following rate equations:

$$\frac{d}{dt}f_{DA}(t) = -If_{DA} + (k_D - k_D^0 + \lambda_D k_D^0)f_{D^*A}$$
$$+ (k_A - k_A^0 + \lambda_A k_A^0)f_{DA^*},$$

$$\frac{d}{dt}f_{D^*A}(t) = If_{DA} - (k_D + k_{tr})f_{D^*A},\tag{2.5}$$

$$\frac{d}{dt}f_{DA^*}(t) = k_{tr}f_{D^*A} - k_A f_{DA^*},$$

where $k_A^0 = \phi_A k_A^{rad}$, and $k_D^0 = \phi_D k_D^{rad}$ are the effective rate constants for the transitions that result in detected photons. These equations are solved with the steady state initial conditions that are found from the unmodified rate equations [i.e., Eq. (2.5) with $\lambda_A = \lambda_D = 1$]

$$p_{ss}(DA) = \frac{k_A(k_D + k_{tr})}{k_A(k_D + k_{tr}) + I(k_A + k_{tr})}, \tag{2.6a}$$

$$p_{ss}(DA^*) = \frac{I k_{tr}}{k_A(k_D + k_{tr})} p_{ss}(DA). \tag{2.6c}$$

$$p_{ss}(D^*A) = \frac{I}{k_D + k_{tr}} p_{ss}(DA), \tag{2.6b}$$

Solving Eq. (2.5) in Laplace space $[\hat{f}(s) = \int_0^\infty f(t) \times \exp(-st) dt]$ we get

$$\hat{F}(\lambda_A, \lambda_D, s) = \hat{f}_{DA}(s) + \hat{f}_{D*A}(s) + \hat{f}_{DA*}(s)$$

$$= \frac{1 - n_A(1 - \lambda_A)\hat{\mathcal{R}}_A(s) - n_D(1 - \lambda_D)\hat{\mathcal{R}}_D(s)}{s + n_A(1 - \lambda_A)[1 - s\hat{\mathcal{R}}_A(s)] + n_D(1 - \lambda_D)[1 - s\hat{\mathcal{R}}_D(s)]}. \tag{2.7}$$

Here n_A and n_D are the mean numbers of detected acceptor and donor photons per unit time that are proportional to the steady-state probabilities of the excited states

$$n_A = k_A^0 p_{ss}(DA^*), \quad n_D = k_D^0 p_{ss}(D^*A) \tag{2.8}$$

and $\hat{\mathcal{R}}_{A,D}(s)$ are the Laplace transforms of the relaxation function[17] of the excited acceptor and donor state starting from the ground state,

$$\hat{\mathcal{R}}_A(s) = \frac{s + I + k_D + k_{tr} + k_A}{(s + k_A)(s + I + k_D + k_{tr}) + I k_{tr}}, \tag{2.9a}$$

$$\hat{\mathcal{R}}_D(s) = \frac{s + k_A - I k_{tr}/k_A}{(s + k_A)(s + I + k_D + k_{tr}) + I k_{tr}}. \tag{2.9b}$$

These relaxation functions describe the approach of the excited donor and acceptor populations to steady state.

Having the generating function in hand, it is easy to get the mean and the variance of the number of photons by evaluating derivatives of this function with respect to $\lambda_{A,D}$ at $\lambda_{A,D} = 1$. The mean number of acceptor (donor) photons detected during time t is equal to $n_{A(D)}t$. The variance of the number of photons at long times becomes[14]

$$\lim_{t \to \infty} (\langle N_\alpha^2 \rangle - \langle N_\alpha \rangle^2) = n_\alpha t(1 - 2n_\alpha \tau_\alpha), \quad \alpha = A, D, \tag{2.10}$$

where τ_A and τ_D are the effective relaxation times required for the excited states to relax to steady state,

$$\tau_\alpha = \int_0^\infty \mathcal{R}_\alpha(t') dt' \equiv \hat{\mathcal{R}}_\alpha(0), \quad \alpha = A, D. \tag{2.11}$$

The first term on the right-hand side of Eq. (2.10), $n_\alpha t$, is the variance corresponding to the Poisson distribution of photons. The second term represents the deviation from Poisson statistics. This is always negative for the acceptor photons and can be both positive and negative for the donor photons, depending on the parameters. The deviation becomes small in the fast relaxation limit, i.e., when the relaxation time is short compared with the time between two consecutively detected photons, $n_\alpha|\tau_\alpha| \ll 1$.

Finally, expanding Eq. (2.7) in powers of the λ's, we get the Laplace transform of the joint probability. The exact distribution is complicated. However, in certain circumstances, the distribution of the number of photons can be approxi-

mated by the Poisson distribution which can be obtained from Eq. (2.7) by formally setting $\hat{\mathcal{R}}_\alpha(s)$ to zero. The physical meaning of this will be considered in detail in the next section.

The results for the three-state model can be used to treat triplet blinking[12,18,19] when fluorescence intensity jumps between "on" (high) and "off" (background) intensity levels due to intersystem crossing into a long-lived triplet state. The distribution of the number of photons emitted by a blinking molecule has a peak at zero number of photons because of the time that the molecule spends in a dark (off) state, a broad peak corresponding to a bright (on) state, and a flat region in between.[18] Reinterpreting (DA, D^*A, and DA^*) states in Fig. 1(a) by (D, D^*, and D^T) and considering k_{tr} and k_A as intersystem crossing rates, the probability of the number of detected photons N_D is obtained by taking derivatives of the generating function, Eq. (2.7) over λ_D at $\lambda_A = 1$ ($N_A, N_D > 0$),

$$\hat{P}(N_D|s) = \frac{n_D^{N_D}[1 - s\hat{R}_D(s)]^{N_D - 1}}{(s + n_D[1 - s\hat{R}_D(s)])^{N_D + 1}}. \tag{2.12}$$

This is a special case of a general expression we obtained previously.[14] The corresponding variance for a three-state blinking molecule is given in Eq. (2.10). This was recently obtained[12] using the probability distribution of the times between consecutive photons. To calculate this function, Molski et al.[20,21] introduced an absorbing boundary that "captures" the photon once it is emitted. Their equations are equivalent to Eq. (2.5) with $\lambda_A = 1$ and $\lambda_D = 0$. The distribution of photons from a three-state blinking molecule has also been considered[19] by an alternative approach using Mandel's formula. This approximation is analogous to the one used in Sec. III B. Our exact result reduces to that in Ref. 19 in the limit of slow intersystem crossing.

B. Poisson distribution of photons

Even in the two-state system, the distribution of the number of photons emitted in time t is not Poissonian because after emitting a photon, the system must be re-excited before the next photon can be emitted.[22] Since this takes time, a photon is correlated with the previously emitted photon. However, when the relaxation to steady state is fast

compared to the time between two consecutively detected photons, the distribution of photons can be approximated by the Poisson distribution. For the three-state system, Fig. 1(a), when the relaxation is fast, $f_\alpha(t)$ in Eq. (2.5) can be approximated by their steady state, $p_{ss}(\alpha)$, multiplied by a weight factor $f_0(t)$ depending solely on time,

$$f_\alpha(t) \approx p_{ss}(\alpha)f_0(t), \quad \alpha = DA, \ D^*A, \ DA^*. \quad (2.13)$$

This standard procedure is called the local equilibrium (here steady-state) approximation by Zwanzig.[23] Using Eq. (2.13) in Eq. (2.5) and summing up these equations, we get

$$\frac{d}{dt}f_0(t) = -[(1-\lambda_A)n_A + (1-\lambda_D)n_D]f_0, \quad (2.14)$$

with $f_0(0) = 1$. Here $n_{A,D}$ are the mean number of photons per unit time defined in Eq. (2.8). Solving this equation, we get the generating function

$$F(\lambda_A, \lambda_D, t) = \sum_\alpha p_{ss}(\alpha)f_0(t)$$
$$= f_0(t) = e^{-[(1-\lambda_A)n_A + (1-\lambda_D)n_D]t}. \quad (2.15)$$

Expanding this generating function in powers of the λ's, we obtain the uncorrelated Poisson distribution for the number of donor and acceptor photons

$$P(N_A, N_D|t) = \frac{(n_A t)^{N_A}}{N_A!} \frac{(n_D t)^{N_D}}{N_D!} e^{-(n_A+n_D)t}. \quad (2.16)$$

This is valid when the relaxation times of the excited states, Eq. (2.11), are short compared to the time between two consecutively detected photons, $n_\alpha|\tau_\alpha| \ll 1$, $\alpha = A, D$, so that the system comes to a steady state before the next photon is detected. Also the bin size must be longer than the relaxation time, $t \gg |\tau_{A,D}|$, which is fulfilled if there is at least one photon in a bin on average and the previous condition is true. These conditions can also be deduced from the variance, Eq. (2.10), and from the generating function, Eq. (2.7). Using the explicit expressions for the three-state system for $n_{A,D}$, Eq. (2.8), and $\tau_{A,D}$, Eqs. (2.9) and (2.11), the distribution of emitted photons becomes Poissonian for low detection efficiency, $\phi_{A(D)} \ll 1$, or low excitation rate, $I \ll Min(k_A, k_D + k_{tr})$, which frequently occurs in experiments.

The above derivation is actually valid in general case if one uses the approximation (2.13) in Eq. (2.3) (see Appendix B). Thus, we have shown that the distribution of the number of photons is Poissonian for all processes that are fast compared to the time between two consecutively detected photons. Indeed, in this case, the photon detection events become independent, which results in the Poisson distribution. In FRET experiments, the relaxation times of the excited states [$\tau_D = (k_D + k_{tr})^{-1}$ and $\tau_A = \tau_D + k_A^{-1}$ for low intensities for the system on Fig. 1(a)] are on the picosecond to nanosecond time scale. These times are short compared to microsecond to millisecond time between consecutively detected photons and, therefore, this system is Poissonian. The same is true for the energy transfer with donor reexcitation, Fig. 1(b), and for conformational dynamics, Fig. 1(c), when the rate constants are in picosecond to nanosecond time scale.

The Poisson distribution is characterized by the mean number of photons per unit time, $n_{A,D}$, which is obtained from the steady-state probabilities. For the three-state system in Fig. 1(a), they are given in Eq. (2.8). For the four-state system with donor re-excitation, Fig. 1(b), the mean numbers of donor and acceptor photons per unit time are equal to $k_D^0[p_{ss}(D^*A) + p_{ss}(D^*A^*)]$ and $k_A^0[p_{ss}(DA^*) + p_{ss}(D^*A^*)]$, respectively. As another example, consider the influence of fast angular fluctuations of the dyes which modulate the energy-transfer rate via the orientational factor κ^2. If the orientational fluctuations are fast compared to the time between consecutively detected photons, then the distribution of photons is Poissonian with the mean number per unit time

$$n_A = k_A^0 \int p_{ss}(DA^*, \Omega)p_{eq}(\Omega)d\Omega,$$
$$n_D = k_D^0 \int p_{ss}(D^*A, \Omega)p_{eq}(\Omega)d\Omega, \quad (2.17)$$

where $p_{eq}(\Omega)$ is the equilibrium distribution of the angle between the transition dipoles. The steady-state probabilities, $p_{ss}(DA^*, \Omega)$ and $p_{ss}(D^*A, \Omega)$, are obtained from the equation which describes both fluorescent transitions and orientational dynamics,

$$(L_\Omega + K_{3st})p_{ss} = 0, \quad (2.18)$$

where L_Ω is the orientational operator, K_{3st} is the matrix of rate constants for the three-state system [Eq. (2.5) with $k_{tr} \to k_{tr}(\Omega)$ at $\lambda_A = \lambda_D = 1$], and p_{ss} is the vector of the steady-state probabilities. This equation can be easily solved in two limiting cases. If the orientational fluctuations are fast compared to the donor's lifetime, then $k_{tr}(\Omega)$ is averaged out to $\int k_{tr}(\Omega)p_{eq}(\Omega)d\Omega$ (i.e., the orientational factor $\kappa^2 = 2/3$) which is then substituted into Eq. (2.6) instead of k_{tr}. If the orientational fluctuations are slower than the acceptor's lifetime (but still faster than the time between consecutively detected photons), then the steady-state probabilities are calculated for fixed Ω [Eq. (2.6) with $k_{tr} \to k_{tr}(\Omega)$] and then averaged according to Eq. (2.17). If the fluorescent transitions and orientational dynamics are on the same time scale, then one must solve the above equation explicitly.

The situation is completely analogous when interdye distance r also fluctuates on the picosecond to nanosecond time scale. Thus, fast fluctuations of the relative dipole orientation or interdye distance affect the mean number of photons per unit time but do not lead to deviations from the Poisson distribution of the number of photons.

1. FRET efficiency distribution

We now turn to the consideration of the distribution for the FRET efficiency $E = N_A/(N_A + N_D)$ when the distribution of the number of photons is uncorrelated Poissonian. The transfer efficiency is calculated for each bin excluding those with $N_A = N_D = 0$. In experiments,[2,4] an additional threshold is usually applied at $N_A + N_D \geq N_T$, which allows one to reduce the influence of the noise from the background. The transfer efficiency distribution $P(E|t)$, which depends on the bin size t, is obtained from the *normalized* joint distribution for the number of photons by

$$P(E|t) = \sum_{\substack{N_A,N_D \\ N_A+N_D \geqslant N_T}}^{\infty} P(N_A,N_D|t)\,\delta\!\left(E - \frac{N_A}{N_A+N_D}\right).$$

$$(2.19)$$

It should be noted that because the transfer efficiency is a rational number, the efficiency distribution is a discontinuous function. Using the Poisson distribution of photons, Eq. (2.16), in this definition we get

$$P(E|t) = \rho_p\!\left(E \left| \frac{n_A}{n_A+n_D}, (n_A+n_D)t\right.\right),$$

$$(2.20)$$

where we have introduced the following notation for the FRET efficiency distribution for Poissonian photons (and hence the subscript p) for future convenience

$$\rho_p(E|\epsilon,N) = \mathcal{N}_p^{-1}(N) \sum_{\substack{N_A,N_D \\ N_A+N_D \geqslant N_T}}^{\infty} \frac{[\epsilon N]^{N_A}}{N_A!}\frac{[(1-\epsilon)N]^{N_D}}{N_D!}$$

$$\times e^{-N}\delta\!\left(E - \frac{N_A}{N_A+N_D}\right),$$

$$(2.21)$$

$\mathcal{N}_p^{-1}(N)$ is the normalization factor

$$\mathcal{N}_p(N) \equiv \sum_{\substack{N_A,N_D \\ N_A+N_D \geqslant N_T}}^{\infty} \frac{[\epsilon N]^{N_A}}{N_A!}\frac{[(1-\epsilon)N]^{N_D}}{N_D!} e^{-N}$$

$$= e^{-N}\sum_{j=N_T}^{\infty}\frac{N^j}{j!}.$$

$$(2.22)$$

This factor behaves approximately as a step function at $N = N_T$ [i.e., $\mathcal{N}_p(N)\approx 0$ for $N<N_T$ and $\mathcal{N}_p(N)\approx 1$ for $N>N_T$]. It can be seen from Eq. (2.20) that N is the mean number of donor and acceptor photons detected in time t.

The distribution in Eq. (2.21) has the following properties. The mean efficiency, $\langle E\rangle = \int_0^1 dE\,E\rho_p(E|\epsilon,N)$, does not depend on the value of the threshold, N_T, or the total mean number of photons, N, and is given by

$$\langle E\rangle = \epsilon.$$

$$(2.23)$$

The variance of this distribution, $\sigma_p^2(\epsilon,N)\equiv\langle E^2\rangle - \langle E\rangle^2$, is given by

$$\sigma_p^2(\epsilon,N) = \epsilon(1-\epsilon)\psi_p(N),$$

$$(2.24a)$$

$$\psi_p(N) = \sum_{j=N_T}^{\infty}\frac{N^j}{j!j}\,\bigg/\,\sum_{j=N_T}^{\infty}\frac{N^j}{j!}.$$

$$(2.24b)$$

Replacing the numerator in Eq. (2.24b) by the larger value $\Sigma_{j=N_T}^{\infty} N^j/(j!N_T)$, we get the following rigorous upper bound for the variance:

$$\sigma_p^2(\epsilon,N) < \frac{\epsilon(1-\epsilon)}{N_T}.$$

$$(2.25)$$

Figure 3 plots the variance $\sigma_p^2(\epsilon,N)$ for $\epsilon=1/2$ as a function of the mean total number of photons, N. For $N\gg 1$, it approaches $\epsilon(1-\epsilon)/N$ if $N\gg N_T$ and to $\epsilon(1-\epsilon)/N_T$ if $N\ll N_T$.

With the mean and variance in hand, one can approximate $\rho_p(E|\epsilon,N)$ by the Gaussian
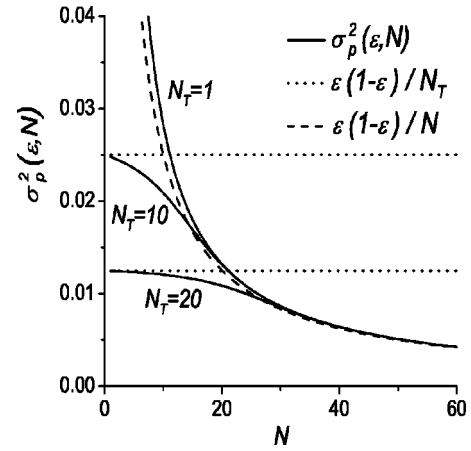

FIG. 3. Variance of the FRET efficiency distribution for photons with Poissonian statistics as a function of the mean total number of donor and acceptor photons, N, for different values of the threshold, $N_T=1,10,20$. The mean FRET efficiency is $\langle E\rangle=\epsilon=0.5$. Dotted and dashed lines are the approximations for small, $N\ll N_T$, and large, $N\gg N_T$, numbers of photons, respectively. The dotted lines are the upper bounds for the variance, Eq. (2.25).

$$\rho_p(E|\epsilon,N) \approx [2\pi\sigma_p^2(\epsilon,N)]^{-1/2}\exp\!\left(-\frac{(E-\epsilon)^2}{2\sigma_p^2(\epsilon,N)}\right).$$

$$(2.26)$$

This approximation has the exact mean and variance but has a nonzero probability when $E>1$ and $E<0$.

Another approximation for the efficiency distribution is the beta distribution,[2] which for $N_T=1$ is

$$\rho_p(E|\epsilon,N) \approx E^{\epsilon N-1}(1-E)^{(1-\epsilon)N-1}\frac{\Gamma(N)}{\Gamma(\epsilon N)\Gamma((1-\epsilon)N)}.$$

$$(2.27)$$

This distribution has the correct mean and the variance

$$\sigma_B^2(\epsilon,N) = \frac{\epsilon(1-\epsilon)}{N+1},$$

$$(2.28)$$

which becomes exact for large N. The beta distribution with the correct variance can be obtained from Eq. (2.27) by setting $N\to 1/\psi_p(N)-1$, where $\psi_p(N)$ is given in Eq. (2.24b) for arbitrary cutoff.

In Fig. 4 we plot the exact histograms for the transfer efficiency together with the Gaussian (solid line) and the beta (dashed line) distributions. The histograms look "noisy" because the numbers of photons are integers, and the transfer efficiency can be only certain rational numbers. The "noise" is reduced when the mean number of photons is increased. For a sufficiently large total number of photons, the distribution approaches the delta function centered on the mean efficiency.

C. Slow dynamics

The distribution of photons is not Poissonian when slow processes occur with the characteristic times comparable or
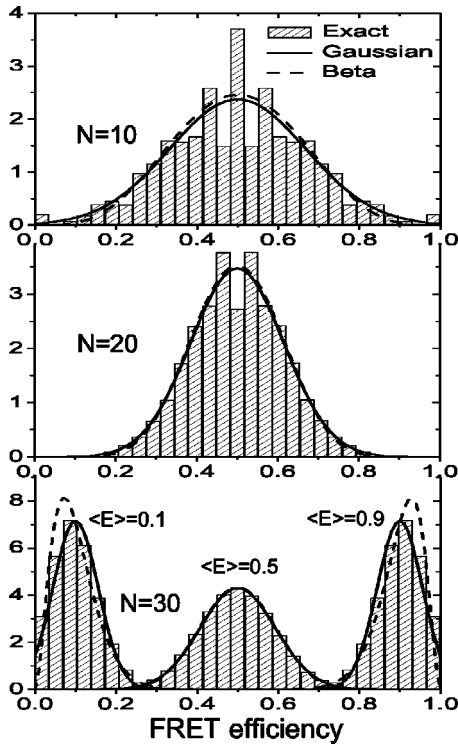
FIG. 4. Exact histograms of the FRET efficiency for the Poisson distribution of photons at different mean total numbers of photons detected in time t, $N=10,20,30$. Mean transfer efficiency is $\langle E \rangle = 0.5$, in the lower panel the histograms for $\langle E \rangle = 0.1, 0.9$ are also given. Threshold value $N_T = 1$ and the histogram step is 0.034. The solid line corresponds to the Gaussian distribution, Eq. (2.26) and the dashed line is the beta distribution, Eq. (2.27).

longer than the average time between two consecutively detected photons. These may include conformational changes, intersystem conversion to the dark triplet acceptor state (the lifetime of the triplet state is of the order of 1 μs),[1] and translational diffusion in and out of the laser spot for freely diffusing molecules (the residence time in the spot is of the order of millisecond).[2] How is the Poisson distribution modified by these slow processes? To answer this question, one must solve Eq. (2.3), taking all states into consideration. This equation can be significantly simplified if we exploit the time scale separation between fast (fluorescent transitions) and slow (conformational changes, intersystem crossing) dynamics.

To do so, we generalize the derivation of the generating function for the Poisson distribution in Sec. II B to include slow transitions using the local steady-state approximation analogous to Eq. (2.13) (see Appendix B). Let us denote all slow variables collectively by r. When the slow coordinate is fixed, the distribution of photons is Poissonian with the mean numbers of acceptor (donor) photons detected per unit time, $n_A(r)$ and $n_D(r)$, depending on r. Slow dynamics of r modulates $n_{A,D}(r)$. As shown in Appendix B, the generalization of Eq. (2.14) is

$$\frac{d}{dt}f_0(r,t) = \mathcal{L}_r f_0(r,t) - [(1-\lambda_A)n_A(r)$$
$$+ (1-\lambda_D)n_D(r)]f_0(r,t), \qquad (2.29)$$

with the steady-state initial condition. Here $\mathcal{L}_r$ is the operator that describes the slow dynamics. For two state conformational changes [see Fig. 1(c)], $\mathcal{L}_r$ is a 2×2 matrix, for diffusion in a potential of mean force $U(r)$, $\mathcal{L}_r$ is the Smoluchowski operator $\nabla D \exp(-\beta U)\nabla \exp(\beta U)$. The generating function is obtained using the generalization of Eq. (2.15)

$$F(\lambda_A, \lambda_D, t) = \sum_r f_0(r,t). \qquad (2.30)$$

Thus, by exploiting the separation of time scales, we have reduced the solution of the whole kinetic scheme to solution of Eq. (2.29), which depends only on the slow variables. All the information about fast fluctuations is contained in the mean numbers of photons per unit time, $n_{A,D}(r)$, which are obtained from the steady-state probabilities calculated at fixed r. For example, for the three-state system in Fig. 1(c), when the energy transfer rate $k_{tr}(r)$ depends only on the distance between the donor and acceptor which fluctuates due to slow conformational changes, $\mathcal{L}_r$ is the operator that describes the conformational dynamics and $n_{A,D}(r)$ are given by Eq. (2.8) with the steady-state probabilities, Eqs. (2.6), with $k_{tr} \to k_{tr}(r)$. The only influence of additional fast fluctuations is to change $n_{A,D}(r)$.

Solving Eq. (2.29), one could get the joint probability by expanding the generating function in powers of the λ's. However, it is more convenient to carry out the expansion formally before solving this equation. In this way, it is possible to represent the joint probability distribution in the form of Mandel's formula[24] which was originally used to relate photon statistics with the fluctuations of the incident light intensity. We begin by formally solving Eq. (2.29) and substituting it into Eq. (2.30),

$$F(\lambda_A, \lambda_D, t) = \left\langle e^{-(1-\lambda_A)\int_0^t dt' n_A(r(t')) - (1-\lambda_D)\int_0^t dt' n_D(r(t'))} \right\rangle, \qquad (2.31)$$

where $\langle \cdots \rangle$ denotes averaging over trajectories of slow coordinate, $r(t)$, which starts from the equilibrium distribution. This equation can be rewritten as follows:

$$F(\lambda_A, \lambda_D, t) = \int_0^\infty dW_A dW_D P_w(W_A, W_D | t)$$
$$\times e^{-(1-\lambda_A)W_A - (1-\lambda_D)W_D}, \qquad (2.32)$$

where $P_w(W_A, W_D | t)$ is the joint probability distribution of the number of acceptor and donor photons, W_A and W_D, when the fluctuations are due only to slow dynamics

$$P_w(W_A, W_D | t) = \left\langle \delta\left(W_A - \int_0^t n_A(r(t'))dt' \right) \right.$$
$$\left. \times \delta\left(W_D - \int_0^t n_D(r(t'))dt' \right) \right\rangle. \qquad (2.33)$$

Expanding Eq. (2.32) in powers of the λ's, we get Mandel's formula[24] for the joint distribution of the number of acceptor and donor photons

$$P(N_A, N_D|t) = \int_0^\infty dW_A dW_D P_w(W_A, W_D|t)$$

$$\times \frac{W_A^{N_A}}{N_A!} \frac{W_D^{N_D}}{N_D!} e^{-W_A - W_D}. \quad (2.34)$$

Thus, due to the separation of time scales, the probability distribution of the number of photons can be expressed as a double integral of the product of the Poisson distribution of the number of photons with average numbers W_A and W_D and the probability distribution of W_A and W_D due to slow fluctuations. The latter can be obtained from the generating function, Eqs. (2.29) and (2.30), by taking the inverse Laplace transforms [see Eq. (2.32)].

Turning to the FRET efficiency distribution, it is convenient to replace the integration variables W_A and W_D by

$$\epsilon = \frac{W_A}{W_A + W_D}, \quad N = W_A + W_D. \quad (2.35)$$

Then the distribution for the numbers for the donor and acceptor photons becomes

$$P(N_A, N_D|t) = \int_0^\infty dN \int_0^1 d\epsilon P_{en}(\epsilon, N|t)$$

$$\times \frac{[\epsilon N]^{N_A}}{N_A!} \frac{[(1-\epsilon)N]^{N_D}}{N_D!} e^{-N}, \quad (2.36)$$

where

$$P_{en}(\epsilon, N|t) = \left\langle \delta\left(\epsilon - \int_0^t \frac{n_A(r(t'))}{N} dt' \right) \right.$$

$$\left. \times \delta\left(N - \int_0^t [n_A(r(t')) + n_D(r(t'))] dt' \right) \right\rangle. \quad (2.37)$$

Subscript en indicates that this is the distribution of the efficiency and the total number of photons measured in a time window of duration t due solely to slow dynamics.

Normalizing this distribution and substituting it into the definition of the FRET efficiency distribution, Eq. (2.19), and using the definition of the FRET efficiency distribution for Poisson statistics $\rho_p(E|\epsilon, N)$, Eq. (2.21), we get

$$P(E|t) = \int_0^\infty dN \int_0^1 d\epsilon \rho_p(E|\epsilon, N) P_{en}(\epsilon, N|t) \mathcal{N}_p(N)/\mathcal{N}_{en}(t), \quad (2.38)$$

where $\mathcal{N}_p(N)$ is given by Eq. (2.22) and $\mathcal{N}_{en}(t) = \int_0^\infty dN \int_0^1 d\epsilon P_{en}(\epsilon, N|t) \mathcal{N}_p(N)$ is the normalization factor. This is one of the key formal results in this paper. It shows how the energy-transfer efficiency distribution is affected by shot noise and by slow fluctuations of both the transfer efficiency and the total number of photons. Transfer efficiency distribution resulting from Poisson statistics (shot noise) is given by $\rho_p(E|\epsilon, N)$ which is a function of the mean transfer efficiency, ϵ, and mean total number of photons, N. Probability distribution $P_{en}(\epsilon, N|t)$ accounts for the slow fluctuations of N and ϵ. It is obtained from the solution of Eq. (2.29).

Because of the time scale separation, these contributions are independent and, consequently, Eq. (2.38) has a simple structure.

III. CONFORMATIONAL DYNAMICS AND INTERSYSTEM CROSSING FOR IMMOBILIZED SINGLE MOLECULES

A. Conformational dynamics

We begin by considering how conformational dynamics that occur on a time scale slower or comparable to the time between two consecutively detected photons influence the FRET efficiency distribution. Conformational fluctuations of the macromolecule influence the rate of the Förster energy transfer that depends on the interdye distance r, $k_{tr}(r) \propto r^{-6}$. The kinetic scheme for such system is a set of three-state systems for each r [see Fig. 1(c)]. Single-molecule experiments are focused on getting information about conformational dynamics through the fluctuations of interdye distance r.

Consider the case when the total number of detected photons does not fluctuate (see Appendix C for the general case) because of conformational changes,

$$n_A(r) + n_D(r) = n_0. \quad (3.1)$$

This condition is fulfilled for equal decay rates, $k_A = k_D$, and equal quantum yields and detection efficiencies, $k_A^0 = k_D^0$, of the excited donor and acceptor states. Indeed, it follows from Eqs. (2.6) and (2.8) that $n_0 = (Ik_A^0)/(I + k_A)$ is a constant. The transfer efficiency which is defined as

$$\mathcal{E}(r) = \frac{n_A(r)}{n_A(r) + n_D(r)} = \frac{n_A(r)}{n_0}, \quad (3.2)$$

fluctuates due to conformational dynamics. For the three-state system with equal decay rates, quantum yields and detection efficiencies,

$$\mathcal{E}(r) = \frac{k_{tr}(r)}{k_{tr}(r) + k_D}. \quad (3.3)$$

In this case we get from Eqs. (2.36)–(2.38) that the distribution of the number of donor and acceptor photons is

$$P(N_A, N_D|t) = \int_0^1 d\epsilon P_c(\epsilon|t) \frac{[\epsilon n_0 t]^{N_A}}{N_A!} \frac{[(1-\epsilon)n_0 t]^{N_D}}{N_D!} e^{-n_0 t}, \quad (3.4)$$

and the FRET efficiency distribution is

$$P(E|t) = \int_0^1 d\epsilon \rho_p(E|\epsilon, n_0 t) P_c(\epsilon|t), \quad (3.5)$$

where $\rho_p(E|\epsilon, N)$ is defined in Eq. (2.21) and $P_c(\epsilon|t)$ is the distribution of the transfer efficiency, $\mathcal{E}(r)$, averaged over time t

$$P_c(\epsilon|t) = \left\langle \delta\left(\epsilon - \frac{1}{t} \int_0^t \mathcal{E}(r(t')) dt' \right) \right\rangle. \quad (3.6)$$

The subscript c indicates that this distribution results only from conformational fluctuations. This distribution describes the fluctuations of the FRET efficiency $\mathcal{E}[r(t)]$ averaged in a bin provided shot noise is negligible. We have previously

considered[13] the properties of this distribution and showed how it can be evaluated for various models of conformational dynamics. Equation (3.5) generalizes our previous result to include shot noise.

It readily follows from the above equations that the mean of the FRET efficiency is equal to the equilibrium average of $\mathcal{E}(r)$

$$\langle E \rangle = \langle \mathcal{E} \rangle \equiv \sum_r \mathcal{E}(r) p_{eq}(r), \qquad (3.7)$$

and that the FRET efficiency variance is

$$\sigma^2(t) = \langle E \rangle (1 - \langle E \rangle) \psi_p(n_0 t) + \sigma_c^2(t)[1 - \psi_p(n_0 t)]. \qquad (3.8)$$

The first term here is the variance due to shot noise, Eq. (2.24), and $\sigma_c^2(t)$ is the variance of the distribution $P_c(\epsilon | t)$ which comes solely from the conformational fluctuations,[13]

$$\sigma_c^2(t) = \frac{2}{t^2} \int_0^t (t - t') \langle \delta \mathcal{E}(t') \delta \mathcal{E}(0) \rangle dt', \qquad (3.9)$$

where $\delta \mathcal{E} = \mathcal{E} - \langle \mathcal{E} \rangle$. At long times this becomes

$$\sigma_c^2(t) \approx \frac{2}{t} \int_0^\infty \langle \delta \mathcal{E}(t') \delta \mathcal{E}(0) \rangle dt' = \frac{2 \tau_c}{t} \langle \delta \mathcal{E}^2 \rangle, \qquad (3.10)$$

where $\tau_c = \int_0^\infty \langle \delta \mathcal{E}(t') \delta \mathcal{E}(0) \rangle / \langle \delta \mathcal{E}^2 \rangle dt'$ is the relaxation time of the autocorrelation function of $\mathcal{E}$.

When the total number of photons is large, $n_0 t \gg 1$, it follows from Eq. (2.24b) that $\psi_p(n_0 t) \ll 1$. Thus the FRET efficiency variance, Eq. (3.8), is just the sum of the conformational and shot noise variances. If the bin size is large compared to the conformational relaxation time, $t > \tau_c$, then for $n_0 t > N_T$,

$$\sigma^2(t) = \frac{\langle \mathcal{E} \rangle (1 - \langle \mathcal{E} \rangle)}{n_0 t} + \langle \delta \mathcal{E}^2 \rangle \frac{2 \tau_c}{t}. \qquad (3.11)$$

It follows from this that shot noise can be neglected if the bin size is large enough and if the conformational relaxation time is long compared to the time between two consecutively detected photons, $n_0 \tau_c \gg 1$. These results for the FRET efficiency distribution and for the variance are completely general and do not depend on a model of conformational dynamics.

In general, distribution $P_c(\epsilon | t)$ can be found numerically (see Ref. 13 for the details). For transitions between two conformations [e.g., Fig. 1(c)], the distribution can be found analytically. For example, the two conformations may represent folded and unfolded states of a protein. If the local efficiency fluctuates between two values E_1 and E_2 with the transition rates k_1 and k_2,

$$E_1 \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E_2,$$
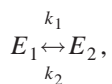
the distribution $P_c(\epsilon | t)$ for $E_2 < \epsilon < E_1$, denoted by $P_{2st}(\epsilon | t)$, is[13,25]
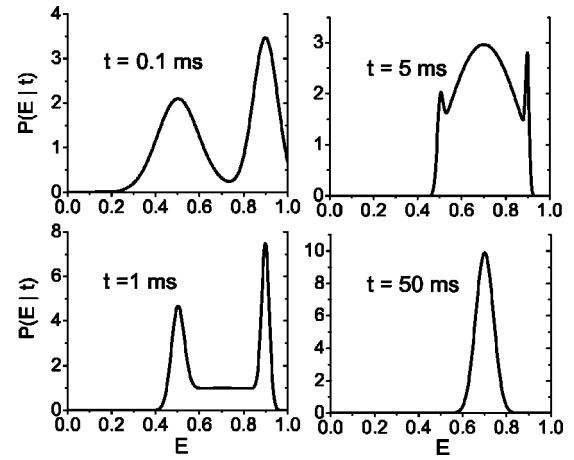

FIG. 5. FRET efficiency distribution for immobilized molecules with two conformational states, Fig. 1(c), with different bin sizes $t = 0.1, 1, 5, 50$ ms and $N_T = 1$, $E_1 = 0.9$, $E_2 = 0.5$, $\tau_c = 1$ ms, and $n_0 = 300$ ms^{-1}.

$$P_{2st}(\epsilon | t) = \exp(-kzt) \left\{ p_{eq}^{(1)} \delta(\epsilon - E_1) + p_{eq}^{(2)} \delta(\epsilon - E_2) \right.$$
$$\left. + \frac{2kt p_{eq}^{(1)} p_{eq}^{(2)}}{E_1 - E_2} [I_0(y) + kt(1 - z) I_1(y)/y] \right\}, \qquad (3.12)$$

where $x = (\epsilon - E_2)/(E_1 - E_2)$, $k = k_1 + k_2$, $p_{eq}^{(1)} = 1 - p_{eq}^{(2)} = k_2 / k$, $y = 2kt \sqrt{p_{eq}^{(1)} p_{eq}^{(2)} x(1 - x)}$, $z = p_{eq}^{(1)}(1 - x) + p_{eq}^{(2)} x$, and $I_n(y)$ are modified Bessel functions of the first kind. $P_{2st}(\epsilon | t)$ is equal to zero beyond the interval $E_2 < \epsilon < E_1$.

Using this distribution in Eq. (3.5), we obtain the FRET efficiency distribution for the kinetic scheme with two conformations shown in Fig. 1(c). Illustrative results are shown in Fig. 5, where $E_1 = 0.9$, $E_2 = 0.5$, and $k_1 = k_2 = 1/2$ ms^{-1}, so that conformational relaxation time is $\tau_c = 1$ ms. $\rho_p(E | \epsilon, n_0 t)$ was approximated by the Gaussian distribution in Eq. (2.26) with $n_0 = 300$ counts/ms. For bin times short compared to the conformational relaxation time, the FRET efficiency distribution is a superposition of two peaks with widths due to shot noise. These peaks first narrow as the observation time increases because the shot noise decreases. Then, when the bin time becomes comparable to the conformational relaxation time, a third peak centered at the average efficiency of the two states appears. For even larger bins, the distribution becomes Gaussian eventually approaching $\delta(E - \langle E \rangle)$.

B. Intersystem crossing

How does conversion into the dark triplet state change the statistics of the donor and acceptor photons in a three-state system? Consider the kinetic scheme for intersystem crossing between two subsystems, (DA, D^*A, DA^*) and (DA^T, D^*A^T) shown in Fig. 1(d). Because the intersystem crossing is much slower than the lifetime of the donor and acceptor states (triplet lifetimes are of the order of microsecond),[1] this five-state system can be considered as a
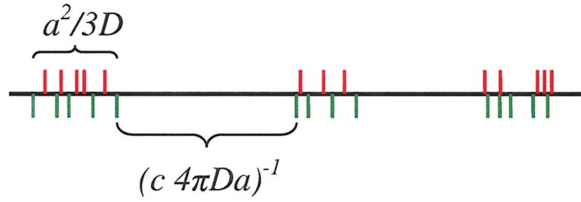
FIG. 6. (Color) Schematic representation of donor photon (green) and acceptor (red) photons emitted by diffusing molecules.

three-state system with two conformations, bright and dark. In the triplet state, only the donor dye is excited, so that k_{tr} in this state is set to zero. When the sum of donor and acceptor photons, Eq. (3.1), does not fluctuate, one can use the results of the previous section. At times longer than the three-state system's relaxation time and shorter than the conversion time, local equilibrium is achieved with the local transfer efficiency equal to $E_1 = k_{tr}/(k_D + k_{tr})$ in the bright (for the acceptor photons) state and $E_2 = 0$ in the triplet state. The rates of transitions between bright and dark states can be found from the appropriate special case of Eq. (B7) in Appendix B to be $k_1 = k_{isc} p_{ss}(DA^*)$ and $k_2 = k'_{isc} p_{ss}(DA^T)$ with $p_{ss}(DA^*)$ defined in Eq. (2.6b) and $p_{ss}(DA^T) = k_D/(I + k_D)$. Since the total number of photons is $N = n_0 t$, the efficiency distribution is

$$P(E|t) = \int_0^1 d\epsilon \, \rho_p(E|\epsilon, n_0 t) P_{2st}(\epsilon|t), \qquad (3.13)$$

where the Poissonian transfer efficiency distribution, $\rho_p(E|\epsilon, n_0 t)$, is defined in Eq. (2.21) and the distribution for the two-state system, $P_{2st}(\epsilon|t)$, is given in Eq. (3.12).

The mean transfer efficiency, Eq. (3.8), in this case is

$$\langle E \rangle = E_1 \frac{k_2}{k_1 + k_2}, \qquad (3.14)$$

and the variance, Eq. (3.8), is

$$\sigma^2(t) = \sigma_p^2(\langle E \rangle, n_0 t) + \langle E \rangle (E_1 - \langle E \rangle)$$
$$\times \psi_{2st}(t)[1 - \psi_p(n_0 t)], \qquad (3.15)$$

where $\psi_{2st}(t) = 2[\tau_c/t - (1 - e^{-t/\tau_c})\tau_c^2/t^2]$ with $\tau_c = (k_1 + k_2)^{-1}$. At large bin sizes, $n_0 t \gg 1$ and $n_0 t \gg N_T$,

$$\sigma^2(t) \approx \frac{1}{n_0 t} \langle E \rangle (1 - \langle E \rangle) + \frac{2\tau_c}{t} \langle E \rangle (E_1 - \langle E \rangle). \qquad (3.16)$$

It follows from this result that intersystem conversion can only contribute to FRET efficiency broadening if τ_c is comparable or longer than n_0^{-1}. For example, for 1-μs triplet lifetimes, the count rate would have to be as high as 10^6 s^{-1} for this effect to be observed.

IV. DIFFUSION THROUGH THE LASER SPOT

In this section we generalize our previous results to treat freely diffusing molecules. A photon trajectory obtained for diffusing molecules at low concentrations consists of separate bursts of donor and acceptor photons with long gaps between them (see Fig. 6). One burst corresponds to one molecule that diffuses through the laser spot. The molecule may enter the spot several times before it leaves the spot forever. The next burst of photons corresponds to a different molecule. The burst size is of the order of $a^2/3D$, where a is the effective radius of the spot and D is the diffusion constant of the molecule, and the time between the bursts is the order of $(c 4\pi D a)^{-1}$,[26] where c is the bulk concentration of the molecules. For small c these times are well separated. Bin size should be smaller than the time between the bursts in order to contain the photons from a single molecule.

Diffusion of the molecule in and out of the laser spot gives rise to extra fluctuations of the number of acceptor and donor photons per unit time, $n_A(\mathbf{R}, r)$ and $n_D(\mathbf{R}, r)$. In addition to the conformational coordinate r, these now depend on three-dimensional translational coordinate $\mathbf{R}$ through the excitation rate $I(\mathbf{R})$ and the detection efficiencies $\phi_A(\mathbf{R})$ and $\phi_D(\mathbf{R})$ which are anisotropic in general case. Specifically, for the three-state system

$$n_A(\mathbf{R}, r) = \frac{k_A^{\text{rad}} \phi_A(\mathbf{R}) I(\mathbf{R}) k_{tr}(r)}{k_A[k_D + k_{tr}(r)] + I(\mathbf{R})[k_A + k_{tr}(r)]},$$

$$n_D(\mathbf{R}, r) = \frac{k_D^{\text{rad}} \phi_D(\mathbf{R}) I(\mathbf{R}) k_A}{k_A[k_D + k_{tr}(r)] + I(\mathbf{R})[k_A + k_{tr}(r)]}. \qquad (4.1)$$

The fluctuations due to translational diffusion are on the time scale of the burst size which is larger than the time between consecutive photons. Therefore they will be treated using the appropriate generalization of Mandel's formula, Eq. (2.34).

A. General formalism for diffusing molecules

We begin by treating the general case when the concentration of the noninteracting diffusing molecules is arbitrary. Then we will take the small concentration limit. Suppose that there are M noninteracting molecules with coordinates $\{\mathbf{R}_1, r_1, \ldots \mathbf{R}_M, r_M\}$ diffusing in a volume V. Then the probability of the number of donor and acceptor photons detected during time t is obtained from Eq. (2.34) using the following generalization of Eq. (2.33):

$$P_w(W_A, W_D|t) = \left\langle \delta \left(W_A - \sum_j \int_0^t n_{Aj}(t') dt' \right) \right.$$
$$\left. \times \delta \left(W_D - \sum_j \int_0^t n_{Dj}(t') dt' \right) \right\rangle, \qquad (4.2)$$

where $n_{Aj}(t) \equiv n_A(\mathbf{R}_j(t), r_j(t))$ and $n_{Dj}(t) \equiv n_D(\mathbf{R}_j(t), r_j(t))$ are the numbers of acceptor and donor photons per unit time emitted by the jth molecule. The symbol $\langle \cdots \rangle$ denotes averaging over all trajectories of all the particles starting from the equilibrium distribution.

Using the Fourier representation of the δ function, $\delta(x) = \int_{-\infty}^{\infty} d\nu \exp(i\nu x)/(2\pi)$, we get

$$P_w(W_A, W_D|t)$$

$$= \frac{1}{(2\pi)^2} \int_{-\infty}^{\infty} d\nu_A d\nu_D e^{i\nu_A W_A + i\nu_D W_D} S(\nu_A, \nu_D, t), \qquad (4.3)$$

where $S(\nu_A, \nu_D, t)$ is

$$S(\nu_A, \nu_D, t) = \left\langle e^{-i\sum_j \int_0^t (\nu_A n_{Aj}(t') + \nu_D n_{Dj}(t')) dt'} \right\rangle. \qquad (4.4)$$

To find $S(\nu_A,\nu_D,t)$, we use the Kubo-Anderson formalism[27–30] according to which

$$S(\nu_A,\nu_D,t)=\int d\mathbf{R}_1 dr_1\cdots d\mathbf{R}_M dr_M f_M \qquad (4.5)$$

where $f_M(\mathbf{R}_1,r_1,\ldots,\mathbf{R}_M,r_M,t|\nu_A,\nu_D)$, is the solution of the equation that describes the diffusive and conformational dynamics of all the molecules in the presence of imaginary "sink" terms

$$\frac{\partial}{\partial t}f_M=\sum_{j=1}^{M}[D\nabla^2_{\mathbf{R}j}+\mathcal{L}_{rj}-i\nu_A n_A(\mathbf{R}_j,r_j)$$
$$-i\nu_D n_D(\mathbf{R}_j,r_j)]f_M, \qquad (4.6)$$

with the equilibrium initial condition $f_M(t=0)=\Pi_{j=1}^{M}p_{eq}(r_j)/V^M$, where $p_{eq}(r)$ is the equilibrium conformational distribution. The first two terms on the right-hand side of the above equation describe the translational diffusion and conformational dynamics.

Alternatively, this equation can be obtained by generalizing Eq. (2.29) to incorporate translational diffusion of the molecules. Comparing Eqs. (4.3) and (2.32), one can see that $S(\nu_A,\nu_D,t)$ is the generating function $F(\lambda_A,\lambda_D,t)$ at $\lambda_A=1-i\nu_A$ and $\lambda_D=1-i\nu_D$.

Equations of this kind appear in the theory of irreversible diffusion-influenced stochastically gated bimolecular reactions.[31,32] If $i\nu_A$ and $i\nu_D$ were real, Eq. (4.6) would describe the kinetics of the reaction between a protein and M ligands. The ligands undergo conformational fluctuations which influence the intrinsic reaction rate via the sink function $i\nu_A n_A(\mathbf{R}_j,r_j)+i\nu_D n_D(\mathbf{R}_j,r_j)$. $S(\nu_A,\nu_D,t)$ would then be the survival probability or relative concentration of the protein in the presence of ligands with concentration $c=M/V$. To find $S(\nu_A,\nu_D,t)$, we exploit the independence and equivalence of the molecules to factorize f_M

$$S(\nu_A,\nu_D,t)$$
$$=\int d\mathbf{R}_1 dr_1\cdots d\mathbf{R}_M dr_M\prod_{i=1}^{M}f_1(\mathbf{R}_i,r_i,t|\nu_A,\nu_D)$$
$$=\left[\int d\mathbf{R}dr f_1(\mathbf{R},r,t|\nu_A,\nu_D)\right]^M$$
$$=\left(1-\frac{1}{V}\int d\mathbf{R}dr[p_{eq}(r)-g(\mathbf{R},r,t|\nu_A,\nu_D)]\right)^M, \quad (4.7)$$

where $f_1(\mathbf{R},r,t|\nu_A,\nu_D)$ is a one-particle distribution function and $g(\mathbf{R},r,t|\nu_A,\nu_D)=f_1(\mathbf{R},r,t|\nu_A,\nu_D)V$. In the thermodynamic limit, $M\to\infty$, $V\to\infty$, $M/V\to c$, we get

$$S(\nu_A,\nu_D,t)$$
$$=\exp\left(-c\int d\mathbf{R}dr[p_{eq}(r)-g(\mathbf{R},r,t|\nu_A,\nu_D)]\right), \quad (4.8)$$

where the one-particle function $g(\mathbf{R},r,t|\nu_A,\nu_D)$ obeys

$$\frac{\partial}{\partial t}g=D\nabla^2_{\mathbf{R}}g+\mathcal{L}_rg-[i\nu_A n_A(\mathbf{R},r)+i\nu_D n_D(\mathbf{R},r)]g.$$
$$(4.9)$$

Initially, $g(t=0)=p_{eq}(r)$. Equation (4.8) can be rewritten in the form of the survival probability in Smoluchowski's theory of irreversible diffusion-influenced reactions[33]

$$S(\nu_A,\nu_D,t)=\exp\left[-c\int_0^t k(t'|\nu_A,\nu_D)dt'\right], \qquad (4.10)$$

where $k(t'|\nu_A,\nu_D)$ is analogous to the Smoluchowski time-dependent rate coefficient

$$k(t|\nu_A,\nu_D)=\int d\mathbf{R}dr[i\nu_A n_A(\mathbf{R},r)+i\nu_D n_D(\mathbf{R},r)]g$$
$$(4.11)$$

This can be proved by integrating Eq. (4.9) over t, $\mathbf{R}$, and r, and using it in Eq. (4.8).

Fourier transforming $S(\nu_A,\nu_D,t)$ with respect to ν_A and ν_D according to Eq. (4.3) and using the result in Eq. (2.34), we get the probability distribution of the number of photons for arbitrary concentration of the macromolecules. The first term in the expansion of Eq. (4.10) in powers of c contributes to the probability of events with zero photons. At small concentrations, these events are the most probable. The second (linear in c) term leads to the joint distribution for the donor and acceptor photons for single-molecule measurements.

Replacing the variables W_A and W_D in the Mandel's formula, Eq. (2.34), by ϵ and N [see Eq. (2.35)] we find that at low concentrations, the distribution of the number of photons is given by Eq. (2.36) and the FRET efficiency distribution by Eq. (2.38) with

$$P_{en}(\epsilon,N|t)\sim\frac{cN}{(2\pi)^2}\int_{-\infty}^{\infty}d\nu_A d\nu_D e^{i\nu_A\epsilon N+i\nu_D(1-\epsilon)N}$$

$$\times\int_0^t k(t'|\nu_A,\nu_D)dt'. \qquad (4.12)$$

The symbol $\sim$ indicates that the distribution is not normalized. Note that Eq. (4.12) can be used only for the bin sizes smaller than the time between the photon bursts.

Thus we have reduced the problem of calculating the photon and FRET efficiency distribution to the solution of one-particle equation, Eq. (4.9), which incorporates translational diffusion and conformational dynamics.

B. Diffusing molecules with the same conformation

We begin by considering a molecule with fixed conformation ($\mathcal{L}_r=0$) [see Fig. 2(a)]. This is the simplest case where diffusion influences the photon counting statistics. To find the distribution $P_{en}(\epsilon,N|t)$, one must solve Eq. (4.9) with $\mathcal{L}_r=0$. This diffusion equation with a sink term can be solved analytically only for few specific laser spot geometries. To solve it approximately, we use the Wilemski-Fixman approximation[34] which has been found to work very well[33] as long as the translational diffusion is not too slow. This approximation will be tested below in the present context for a simple case that can be solved exactly. We further assume that the number of acceptor and donor photons, which now depend on the translational coordinate only, $n_A(\mathbf{R})$ and $n_D(\mathbf{R})$, have the same dependence on $\mathbf{R}$, i.e.,

$n_A(\mathbf{R})/n_D(\mathbf{R}) = $ const. For the three-state system, Eq. (4.1), this is true when detection efficiencies of the donor and acceptor photons are equal. Then the Laplace transform of $k(t|\nu_A, \nu_D)$ in the Wilemski-Fixman approximation is given by:[33]

$$\frac{v}{s\hat{k}(s|\nu_A,\nu_D)} = \frac{1}{i\nu_A\bar{n}_A + i\nu_D\bar{n}_D} + \hat{C}(s), \qquad (4.13)$$

where $v = [\int n_\alpha(\mathbf{R})d\mathbf{R}]^2/\int n_\alpha^2(\mathbf{R})d\mathbf{R}$, $\alpha = A,D$ is the effective volume of the laser spot, $\bar{n}_D = \int n_D(\mathbf{R})d\mathbf{R}/v$ and $\bar{n}_A = \int n_A(\mathbf{R})d\mathbf{R}/v$ are the mean numbers of the donor and acceptor photons per unit time averaged over the laser spot, and $C(t)$ is the correlation function,

$$C(t) = \frac{\int n_\alpha(\mathbf{R})G(\mathbf{R},t|\mathbf{R}',0)n_\alpha(\mathbf{R}')d\mathbf{R}d\mathbf{R}'}{\int n_\alpha^2(\mathbf{R})d\mathbf{R}}$$

$$\equiv \frac{\langle n_\alpha(\mathbf{R}(t))n_\alpha(\mathbf{R}(0))\rangle}{\langle n_\alpha^2\rangle}, \qquad \alpha = A,D, \qquad (4.14)$$

where $G(\mathbf{R},t|\mathbf{R}',0) = \exp[-|\mathbf{R}-\mathbf{R}'|^2/(4Dt)]/\sqrt{(4\pi Dt)^3}$ is the free-diffusion Green's function. This correlation function, which is just the intensity-intensity correlation function, can be obtained from the photon trajectory and independently from fluorescence correlation spectroscopy experiments.[35]

Substituting $\hat{k}(s|\nu_A,\nu_D)$ in Eq. (4.13) into the Laplace transform of Eq. (4.12) and inverting the Fourier transform, we find that the joint probability of the efficiency and the total number of photons is

$$P_{en}(\epsilon,N|t) \sim \delta(\epsilon - \bar{n}_A/\bar{n}_0)cv P_d(N|t), \qquad (4.15)$$

where $\bar{n}_0 = \bar{n}_A + \bar{n}_D$ and the Laplace transform of $P_d(N|t)$ is given by

$$\hat{P}_d(N|s) = \frac{1}{s^2\hat{C}^2(s)\bar{n}_0}\exp\left(-\frac{N}{\bar{n}_0\hat{C}(s)}\right). \qquad (4.16)$$

The delta-function dependence in Eq. (4.15) arises from the assumption $n_A(\mathbf{R})/n_D(\mathbf{R}) = $ const and so FRET efficiency does not depend on the position of the molecule in the laser spot. Fluctuations of the total number of photons due to diffusion through the laser spot give rise to the distribution $P_d(N|t)$ (the subscript d refers to the diffusion), which leads to deviations from the Poisson statistics of photons.

It is interesting to note that for the step-function spot (i.e., a spot with the constant light intensity $I(\mathbf{R}) = I_0$ for $|\mathbf{R}| < a$ and $I(\mathbf{R}) = 0$ for $|\mathbf{R}| > a$), the distribution of the number of photons $P_d(N|t)$ is related to the residence time distribution $P_r(\tau|t)$. This is the probability density of the time τ that a molecule (with a uniform initial distribution) spends inside the spot during observation time t provided that the trajectories where the molecule never visited the spot are neglected. Since for the step-function spot the number of photons and the residence time are related by $N = \bar{n}_0\tau$, $P_r(\tau|t) = P_d(\bar{n}_0\tau|t)$ up to a normalization factor. For infinite observation time, the residence time distribution in the Wilemski-Fixman approximation is obtained from Eq. (4.16) by taking limit $s \to 0$

$$P_r(\tau|\infty) = \tau_{dif}^{-1}\exp(-\tau/\tau_{dif}), \qquad (4.17)$$

where $\tau_{dif} = \hat{C}(0) = 2a^2/(5D)$ for the step-function spot. The corresponding exact distribution can be found from the work of Berezhkovskii et al.[36] by noting that $P_r(\tau|\infty)$ is the same as the residence time distribution of a molecule starting out on the surface of the spot, since the contribution of configurations starting inside the spot is negligible,

$$P_r(\tau|\infty) = \frac{2D}{a^2}\sum_{j=0}^{\infty}\exp[-\pi^2(j+1/2)^2\tau D/a^2]. \qquad (4.18)$$

For long residence times $\tau > \tau_{dif}$, this distribution decays as $\exp[-\pi^2\tau/(10\tau_{dif})]$. The exponent $\pi^2/10 \approx 0.987$ differs from the approximate one in Eq. (4.17) only by 1.3%, showing that the Wilemsky-Fixman approximation is reasonable.

1. Distributions for the numbers of photons

Joint distribution of the number of photons is obtained by substituting $P_{en}(\epsilon,N|t)$, Eqs. (4.15), and (4.16) into Eq. (2.36) and performing integration over ϵ and N. For $N_A + N_D > 0$, we obtain the following Laplace transform of the joint distribution:

$$\hat{P}(N_A,N_D|s) \sim cv\int_0^{\infty}d\tau\hat{P}_d((\bar{n}_A+\bar{n}_D)\tau|s)$$

$$\times \frac{(\bar{n}_A\tau)^{N_A}}{N_A!}\frac{(\bar{n}_D\tau)^{N_D}}{N_D!}e^{-(\bar{n}_A+\bar{n}_D)\tau}, \qquad (4.19a)$$

$$\sim \frac{cv}{s^2\hat{C}_c(s)}\frac{[\bar{n}_A\hat{C}_c(s)]^{N_A}[\bar{n}_D\hat{C}_c(s)]^{N_D}}{[1+(\bar{n}_A+\bar{n}_D)\hat{C}_c(s)]^{N_A+N_D+1}}$$

$$\times \frac{(N_A+N_D)!}{N_A!N_D!}. \qquad (4.19b)$$

By summing this over N_A, we get that the distribution of the number of donor photons is

$$\hat{P}(N_D|s) \sim \frac{cv}{s^2\hat{C}(s)}\frac{[\bar{n}_D\hat{C}(s)]^{N_D}}{[1+\bar{n}_D\hat{C}(s)]^{N_D+1}}. \qquad (4.20)$$

The distribution of acceptor photons is obtained by replacing $D \to A$.

The behavior of these distributions depends on the bin size which should be compared with the diffusion correlation time,

$$\tau_{dif} = \int_0^{\infty}C(t)dt \equiv \hat{C}(0). \qquad (4.21)$$

For large bin sizes, $t \gg \tau_{dif}$ [but $t \ll (c4\pi Da)^{-1}$], the distributions of the number of photons become independent on the bin size because the number of photons cannot be greater than that in an entire burst. The residence time distribution in this limit is approximately exponential, $\sim \exp(-\tau/\tau_{dif})$. Therefore, the distribution of the total number of photons that fluctuates due to translational diffusion is also exponen-
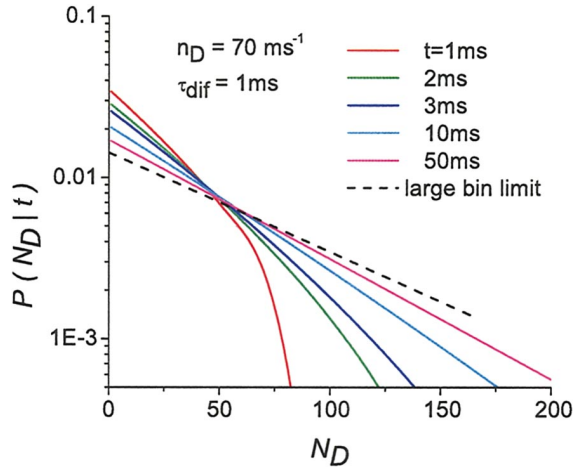
FIG. 7. (Color) Distribution of the number of donor photons emitted by a diffusing molecule for different bin sizes. The distribution is obtained by numerical inversion of the Laplace transforms in Eq. (4.20) for the isotropic Gaussian laser spot with the correlation function $C(t)=(t+2t/\tau_{diff})^{-3/2}$. $\tau_{dif}=1$ ms, $\bar{n}_D=70$ ms^{-1}.

tial, $P_d(N|t)\sim\exp(-N/[(\bar{n}_A+\bar{n}_D)\tau_{dif}])$. In this limiting case, the joint distribution of the number of acceptor and donor photons can be obtained by averaging the Poisson distribution of the number of photons for an immobilized molecule given in Eq. (2.16) over the exponential distribution of the residence time. Thus for $t\gg\tau_{dif}$,

$$P(N_A,N_D|t)\sim\frac{[\bar{n}_A\tau_{dif}]^{N_A}[\bar{n}_D\tau_{dif}]^{N_D}}{[1+(\bar{n}_A+\bar{n}_D)\tau_{dif}]^{N_A+N_D+1}}\frac{(N_A+N_D)!}{N_A!N_D!}.$$
(4.22)

The corresponding distribution for the number of donor photons is

$$P(N_D|t)\sim\frac{[\bar{n}_D\tau_{dif}]^{N_D}}{[1+\bar{n}_D\tau_{dif}]^{N_D+1}}.$$
(4.23)

For bins small compared with the diffusion time, $t\ll\tau_{dif}$, we get from Eq. (4.16) that the distribution for the total number of photons is $P_d(N|t)\sim\delta(N-(\bar{n}_A+\bar{n}_D)t)$, i.e., the total number of photons does not fluctuate. This result is valid only for the step-function spot because it was based on the Wilemski-Fixman approximation. In general, the total number of photons, N, changes with the location of the molecule in the spot and $P_d(N|t)\sim\int d\mathbf{R}\delta(N-[n_A(\mathbf{R})+n_D(\mathbf{R})]t)$ in this limit.

As an illustration of how diffusion through the laser spot dramatically changes the distribution of the number of photons, consider the special case of an isotropic Gaussian laser spot,

$$n_\alpha(\mathbf{R})=n_\alpha(0)\exp\left(-\frac{R^2}{2a^2}\right),\quad \alpha=A,D.$$
(4.24)

The correlation function decays as power law[35]

$$C(t)=(1+Dt/a^2)^{-3/2}.$$
(4.25)

The corresponding diffusion correlation time defined in Eq. (4.21) is $\tau_{dif}=2a^2/D$. Figure 7 plots the distribution of the number of donor photons obtained by numerical inversion of

the Laplace transform in Eq. (4.20) for an isotropic Gaussian spot using the Stehfest algorithm.[37] Events with zero number of photons (the most probable events) are excluded from this distribution. The dashed line in Fig. 7 shows the limiting bin-independent distribution, Eq. (4.23). Because of diffusion, this limit is approached as a slow power law.

2. Transfer efficiency distribution

Using Eq. (4.15) in Eq. (2.38), we get the FRET efficiency distribution resulting from shot noise and diffusion,

$$P(E|t)=\int_0^\infty dN\rho_p(E|\bar{n}_A/\bar{n}_0,N)P_d(N|t)\mathcal{N}_p(N)/\mathcal{N}_d(t),$$
(4.26)

where the Laplace transform of $P_d(N|t)$ in the Wilemski-Fixman approximation is given in Eq. (4.16) and $\mathcal{N}_d(t)$ is the normalization factor,

$$\mathcal{N}_d(t)=\int_0^\infty dNP_d(N|t)\mathcal{N}_p(N).$$
(4.27)

The above distribution is obtained by averaging the FRET efficiency distribution due to shot noise over the total number of photons N fluctuating due to diffusion.

For small bin sizes, $t\ll\tau_{dif}$, this distribution reduces to that for Poisson statistics, Eq. (2.20). For large bins, $t\gg\tau_{dif}$, but smaller than the time between the photon bursts, the FRET efficiency distribution becomes independent on the bin size once the bin is large enough to contain entire bursts.

Using this distribution we find that the average efficiency is not influenced by diffusion,

$$\langle E\rangle=\frac{\int n_A(\mathbf{R})d\mathbf{R}}{\int[n_A(\mathbf{R})+n_D(\mathbf{R})]d\mathbf{R}}=\frac{\bar{n}_A}{\bar{n}_0}.$$
(4.28)

The variance of the FRET efficiency distribution is

$$\sigma_d^2(t)=\langle E\rangle(1-\langle E\rangle)\psi_d(t),$$
(4.29a)

$$\psi_d(t)=\int_0^\infty dN\psi_p(N)P_d(N|t)\mathcal{N}_p(N)/\mathcal{N}_d(t),$$
(4.29b)

where $\psi_d(t)$ is obtained from $\psi_p(N)$, Eq. (2.24b), by averaging over the distribution of N. It is interesting that the variance of the FRET efficiency resulting from the shot noise and diffusion through the laser spot is not given by the sum of the variances as it was for conformational fluctuations, Eq. (3.11). Instead, the variance due to shot noise is averaged over the fluctuations of the total number of photons.

Using $\psi_p(N)<N_T^{-1}$ [see Eq. (2.24b)] in the formula for the variance, one finds the following rigorous upper bound to the FRET efficiency variance, the same as for the Poisson statistics, Eq. (2.25)

$$\sigma_d^2(t)<\frac{\langle E\rangle(1-\langle E\rangle)}{N_T}.$$
(4.30)

Note that this result was obtained without involving the Wilemski-Fixman approximation.

Finally, within the framework of the Wilemski-Fixman approximation for $P_d(N|t)$, Eq. (4.16), we obtain an explicit result for the Laplace transform of the normalization factor,
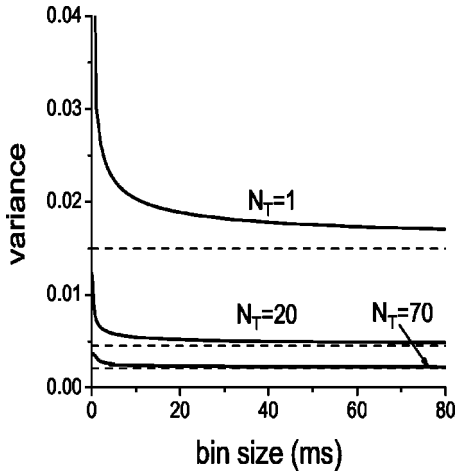
FIG. 8. Variance of the FRET efficiency for a diffusing molecule as a function of the bin size t for different values of the threshold N_T (solid lines). Dashed lines show asymptotic values for large bins, Eq. (4.33). τ_{dif} $=1$ ms, $\bar{n}_0 = \bar{n}_A + \bar{n}_D = 70$ ms^{-1}, $\langle E \rangle = 1/2$.

FRET efficiency

FIG. 9. FRET efficiency distribution for different bin sizes $t = 0.5$, 1, and 10 ms and different values of the threshold $N_T = 1$, 10, and 20. The distance between the donor and acceptor is fixed. Solid and dashed lines correspond to diffusing and immobilized molecules, respectively. $\tau_{dif} = 1$ ms, $\bar{n}_0 = \bar{n}_A + \bar{n}_D = 70$ ms^{-1}, and $\langle E \rangle = 1/2$. The distributions were calculated using the Gaussian approximation, Eq. (2.26), with the variance in Eq. (4.29a) for a diffusing molecule and in Eq. (2.24) for an immobilized molecule.

$$\hat{\mathcal{N}}_d(s) = \frac{1}{s^2 \hat{C}(s)} \left[\frac{\bar{n}_0 \hat{C}(s)}{1 + \bar{n}_0 \hat{C}(s)} \right]^{N_T}, \tag{4.31}$$

and for the Laplace transform of the product $\psi_d(t)\mathcal{N}_d(t)$,

$$[\widehat{\psi_d \mathcal{N}_d}](s) = \frac{1}{s^2 \hat{C}(s)} \sum_{j=N_T}^{\infty} \frac{1}{j} \frac{[\bar{n}_0 \hat{C}(s)]^j}{[1 + \bar{n}_0 \hat{C}(s)]^{j+1}}. \tag{4.32}$$

Figure 8 plots the variance of the transfer efficiency, Eq. (4.29a), obtained by numerical inversion of Eqs. (4.32) and (4.31) for isotropic Gaussian spot using Stehfest algorithm,[37] as a function of the bin size. It can be seen that the variance approaches a constant limiting value for large bin sizes. For large thresholds ($N_T \gg 1$) when the bin size is larger than the diffusion time, $t > \tau_{dif}$, the variance hardly depends on the bin size (however, it strongly depends on the value of the threshold) and can be approximated by its asymptotic value,

$$\sigma_d^2(t) \to \langle E \rangle (1 - \langle E \rangle) \sum_{j=0}^{\infty} \frac{1}{j + N_T} \frac{[\bar{n}_0 \tau_{dif}]^j}{[1 + \bar{n}_0 \tau_{dif}]^{j+1}}. \tag{4.33}$$

If the bin size is large enough so that several bursts are included in the bin, the variance will decrease as the bin size is increased further.

The influence of translational diffusion is illustrated in Fig. 9 which plots the FRET efficiency distributions for diffusing molecules (solid line) and for immobilized molecules (dashed line) with a fixed distance between the donor and acceptor. For large bins, the effective bin size for the diffusing molecules becomes the burst duration and the FRET efficiency distribution does not narrow with increasing bin size as what happens for shot noise.

C. Diffusion and conformational fluctuations

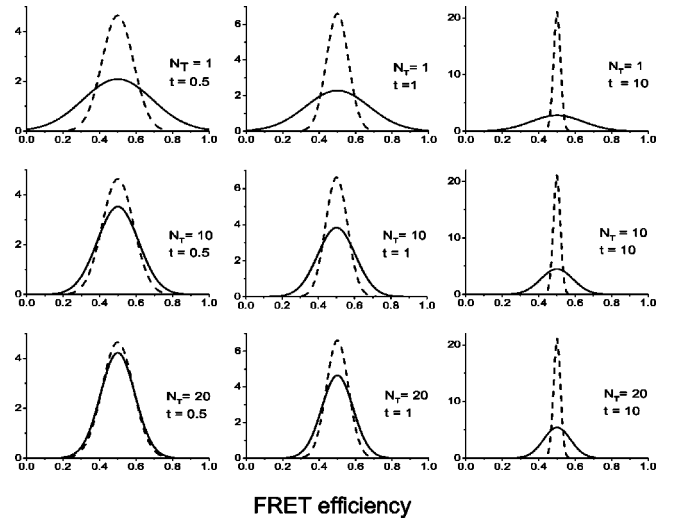We now consider the influence of both diffusion and conformational fluctuations on the distribution of the number of photons and of the FRET efficiency. In addition, it is assumed here that the total number of photons fluctuates solely due to translational diffusion,

$$n_A(\boldsymbol{R},r) + n_D(\boldsymbol{R},r) = n_0(\boldsymbol{R}), \tag{4.34}$$

while the efficiency depends only on the conformational coordinate,

$$\frac{n_A(\boldsymbol{R},r)}{n_A(\boldsymbol{R},r) + n_D(\boldsymbol{R},r)} = \frac{n_A(\boldsymbol{R},r)}{n_0(\boldsymbol{R})} = \mathcal{E}(r). \tag{4.35}$$

Let us first consider the limiting cases where one of the two fluctuating variables is static. If the bin size is much smaller than the diffusion time, $t \ll \tau_{dif}$, then translational coordinate can be considered as static and we have

$$P_{en}(\epsilon, N|t) \sim P_c(\epsilon|t) \int d\boldsymbol{R} \, \delta[N - n_0(\boldsymbol{R})t], \tag{4.36}$$

where $P_c(\epsilon|t)$ is defined in Eq. (3.6). Using this in Eq. (2.38), we get the generalization of Eq. (3.5)

$$P(E|t) = \int d\boldsymbol{R} \int_0^1 d\epsilon \, \rho_p(E|\epsilon, n_0(\boldsymbol{R})t)$$
$$\times P_c(\epsilon|t) \mathcal{N}_p[n_0(\boldsymbol{R})t] / \mathcal{N}_0(t), \tag{4.37}$$

where $\mathcal{N}_0(t) = \int d\boldsymbol{R} \mathcal{N}_p[n_0(\boldsymbol{R})t]$ is the normalization factor. The mean of this distribution is the equilibrium average of $\mathcal{E}(r)$, Eq. (3.7), and the variance is obtained from Eq. (3.8) by averaging $\psi_p[n_0(\boldsymbol{R})t]$ over $\boldsymbol{R}$

$$\sigma^2(t) = \langle E \rangle (1 - \langle E \rangle) \psi_0(t) + \sigma_c^2(t)[1 - \psi_0(t)], \tag{4.38a}$$

$$\psi_0(t) = \int d\boldsymbol{R} \, \psi_p[n_0(\boldsymbol{R})t] \mathcal{N}_p[n_0(\boldsymbol{R})t] / \mathcal{N}_0(t). \tag{4.38b}$$

The other limit is obtained when the bin size is much smaller than the conformational relaxation time, $t \ll \tau_c$. Then conformational coordinate is static and we have

$$P_{en}(\epsilon, N | t) \sim c v P_d(N | t) \int dr \, p_{eq}(r) \delta[\epsilon - \mathcal{E}(r)]. \quad (4.39)$$

The corresponding FRET efficiency distribution is

$$P(E | t) = \int dr \, p_{eq}(r) \int_0^\infty dN \rho_p(E | \mathcal{E}(r), N) P_d(N | t)$$

$$\times \mathcal{N}_p(N) / \mathcal{N}_d(t). \quad (4.40)$$

This distribution has the structure analogous to Eq. (4.37), i.e., it averages the FRET efficiency distribution for diffusing molecules with one fixed conformation, $P_d(N | t)$, over many conformations. Again, the mean is the same, $\langle E \rangle = \langle \mathcal{E}(r) \rangle$, and the variance is

$$\sigma^2(t) = \langle E \rangle (1 - \langle E \rangle) \psi_d(t) + \langle \delta \mathcal{E}^2 \rangle [1 - \psi_d(t)], \quad (4.41)$$

where $\psi_d(t)$ is given in Eq. (4.29b). Here the first term is the variance due to shot noise and diffusion at fixed conformation [see Eq. (4.29a)] and the second term comes from the static conformational distribution.

In both these limits, $t \ll \tau_{dif}$ or $t \ll \tau_c$, the rigorous upper bound to the variance can be obtained by using $\psi_p(N) < N_T^{-1}$ and Eq. (4.30)

$$\sigma^2(t) < \frac{\langle E \rangle (1 - \langle E \rangle)}{N_T} + \sigma_c^2(t). \quad (4.42)$$

Note that the above result does not depend on specific forms of the distribution due to conformational dynamics, $P_c(\epsilon | t)$, or due to diffusion, $P_d(N | t)$.

To find the distribution $P_{en}(\epsilon, N | t)$ in the general case, one should solve Eq. (4.9). Instead of killing ourselves trying to solve this equation, we use the following physically transparent approximation for $P_{en}(\epsilon, N | t)$. Because of the diffusion in and out of the spot, the effective time during which conformational dynamics occur can be approximated by the residence time τ which is related to the fluctuating number of photons by $\tau = N / \bar{n}_0$, where $\bar{n}_0 = \bar{n}_A + \bar{n}_D = \int n_0(R) dR / v$ is the total number of donor and acceptor photons per unit time averaged over the spot. Consequently, the distribution of the transfer efficiency ϵ and of the number of photons N is a product of the distribution for the number of photons due to diffusion, $P_d(N | t)$, Eq. (4.16), and the transfer efficiency distribution due to conformational fluctuations, $P_c(\epsilon | t)$, Eq. (3.6), evaluated for $t = N / \bar{n}_0$ (i.e., the residence time)

$$P_{en}(\epsilon, N | t) \approx c v P_c(\epsilon | N / \bar{n}_0) P_d(N | t). \quad (4.43)$$

Substituting this into Eq. (2.38), we get the FRET efficiency distribution which takes into account shot noise, conformational fluctuations, and the fluctuations of the total number of photons due to diffusion through the laser spot,

$$P(E | t) = \int_0^\infty dN \int_0^1 d\epsilon \rho_p(E | \epsilon, N) P_c(\epsilon | N / \bar{n}_0) P_d(N | t)$$

$$\times \mathcal{N}_p(N) / \mathcal{N}_d(t). \quad (4.44)$$

This distribution correctly reduces to Eq. (4.40) for an ensemble of rigid molecules. In the limit of slow diffusion, it gives Eq. (4.37) for only the step-function spot.

For the two-state conformational dynamics, FRET efficiency distribution, Eq. (4.44), is given explicitly by using $P_{2st}(\epsilon | N / \bar{n}_0)$, Eq. (3.12), for $P_c(\epsilon | N / \bar{n}_0)$. This distribution describes diffusing molecules, Fig. 2(b), with conformational fluctuations due, for example, to two-state folding, Fig. 1(c).

The mean of this distribution is the equilibrium average, $\langle E \rangle = \langle \mathcal{E}(r) \rangle$, and the variance is

$$\sigma^2(t) = \sigma_d^2(t) + \sigma_{dc}^2(t), \quad (4.45a)$$

$$\sigma_{dc}^2(t) = \int_0^\infty dN \sigma_c^2(N / \bar{n}_0)$$

$$\times [1 - \psi_p(N)] P_d(N | t) \mathcal{N}_p(N) / \mathcal{N}_d(t). \quad (4.45b)$$

Here $\sigma_d^2(t)$, Eq. (4.29a), accounts for the shot noise and the fluctuation of the number of photons due to diffusion for fixed conformations. The second term, $\sigma_{dc}^2(t)$, results from the conformational fluctuations but is also influenced by the diffusion into the spot. Note that the variance in Eq. (4.45a) can be obtained from the variance due to shot noise and conformational fluctuations, Eq. (3.8), by setting $\bar{n}_0 t = N$ and averaging over N with the distribution $P_d(N | t)$ and the factor $\mathcal{N}_p(N)$ which accounts for the threshold.

For bins large compared to the conformational correlation time, $t \gg \tau_c$, and to the diffusion time, $t \gg \tau_{dif}$, the integral in Eq. (4.45b) can be evaluated by neglecting $\psi_p(N)$ and using $\sigma_c^2(t)$ in Eq. (3.10), $\mathcal{N}_p(N)$ in Eq. (2.22), and $\hat{P}_d(N | s)$ and $\hat{\mathcal{N}}_d(s)$ in Eqs. (4.16) and (4.31) taken in the limit $s \to 0$. Using $\sigma_d^2(t)$ in Eq. (4.33) we find

$$\sigma^2(t) \to \left(\frac{\langle E \rangle (1 - \langle E \rangle)}{1 + \bar{n}_0 \tau_{dif}} + \frac{2 \langle \delta \mathcal{E}^2 \rangle \tau_c}{\tau_{dif}} \right)$$

$$\times \sum_{j=0}^\infty \frac{1}{j + N_T} \left(\frac{\bar{n}_0 \tau_{dif}}{1 + \bar{n}_0 \tau_{dif}} \right)^j, \quad (4.46)$$

$$\sigma^2(t) < [\langle E \rangle (1 - \langle E \rangle) + 2 \langle \delta \mathcal{E}^2 \rangle (\bar{n}_0 + 1 / \tau_{dif}) \tau_c] / N_T,$$

where the second relation represents the upper bound for the variance. This relation can be used to estimate the broadening of the FRET efficiency distribution due to slow conformational changes.

The above result can also be used to estimate the broadening of the FRET efficiency distribution resulting from transitions into the acceptor's triplet state. In this case $\langle \delta \mathcal{E}^2 \rangle = \langle E \rangle (E_1 - \langle E \rangle)$, where $E_1 = k_{tr} / (k_D + k_{tr})$ is the transfer efficiency in the bright state, τ_c is the correlation time for the triplet transitions (see Sec. III B). As in the case of an immobilized molecule, the intersystem conversion can contribute to the FRET efficiency width only if τ_c is comparable (or slower) than the time between consecutively detected photons emitted by the molecule inside the laser spot.

V. CONCLUDING REMARKS

Transitions that are fast and slow compared with the time between two consecutively detected photons influence photon statistics differently. Fast transitions include the photophysical processes shown in Figs. 1(a) and 1(b). When the system comes to the steady state before the next photon is detected, consecutively detected photons are not correlated with each other and, therefore, the distribution of photons is Poissonian. The corresponding FRET efficiency distribution depends only on the mean FRET efficiency and the mean total number of photons. For large total number of photons, this can be approximated by the Gaussian distribution. When the total number of photons is smaller than 20, the FRET efficiency histogram becomes "noisy." This "noise," which cannot be eliminated, reflects the fact that the distribution is a discontinuous function.

The transitions that are slow or comparable with the time between two consecutively detected photons, such as conformational changes, Fig. 1(c), lead to deviations from Poisson statistics and to broadening of the FRET distribution. By exploiting the separation of time scales, we were able to express the total distribution in terms of the distributions due to the fast and slow fluctuations. Specifically, the resulting distribution is the Poisson distribution of photons or corresponding FRET efficiency distribution averaged over slow fluctuations of both FRET efficiency and the total number of photons, Eqs. (2.36) and (2.38).

Slow fluctuations of the total number of photons due to translational diffusion in and out of the laser spot [Fig. 2(a)] lead to the effective truncation of the bin size at the diffusion time, i.e., the time that a molecule spends inside the spot. Consequently, the FRET efficiency distribution of diffusing molecules becomes bin independent for bins larger than the diffusion time and the corresponding variance approaches a constant as the bin size is increased as long as it is smaller than the time between photon bursts.

The variance of the FRET efficiency distribution that results from shot noise and/or slow fluctuations of the total number of photons (e.g., due to diffusion in and out of the laser spot) is rigorously bounded, i.e., $\sigma^2(t) < \langle E \rangle (1 - \langle E \rangle)/N_T$, when the detection efficiencies of the donor and acceptor photons are the same. If the variance exceeds this value, slow fluctuations of the FRET efficiency must be present and/or the detection efficiencies must be significantly different.

Shot noise for immobilized molecules can be decreased by increasing the bin size or the value of the threshold. However, in order to get information about conformational distribution and dynamics, the bin size should not exceed the conformational relaxation time. Therefore, ideally the bin size for immobilized molecules should be within the limits $n_0^{-1} \ll t \lesssim \tau_c$. For the diffusing molecules, this is true for the bin sizes smaller than the diffusing time. For the bins larger than the diffusion time, the influence of shot noise can be reduced only by applying a threshold.

In this paper we have presented a rather complete analysis of the various processes that can contribute to the broadening of the FRET efficiency distribution. One of our surprising results is that fluctuations in the orientation of the fluorophores do not lead to broadening of the FRET efficiency distribution contrary to what has been suggested.[3,5] Since the fluorescence anisotropy of the probes decays on the nanosecond time scale, the angular motion of the dyes is fast compared with the the time between two consecutively detected photons. Therefore, angular fluctuations on the nanosecond time scale cannot directly contribute to the broadening of the energy-transfer efficiency, although the mean FRET efficiency could be affected.

APPENDIX A: EXACT EQUATION FOR THE GENERATING FUNCTION

To prove Eqs. (2.2) and (2.3), consider only transitions $i_A \rightarrow j_A$. The generalization to different types of transitions is straightforward. The probability of N_A $i_A \rightarrow j_A$ transitions during time t when the system is initially in state m and ends up in state n, $P_{nm}(N_A|t)$, is obtained by repeated convolution of the probability with no transitions,

$$P_{nm}(N_A|t) = G'_{nj_A}(t) * K_{j_A i_A} G'_{i_A j_A}(t) * K_{j_A i_A}$$
$$\times G'_{i_A j_A}(t) \cdots * K_{j_A i_A} G'_{i_A m}(t), \quad (A1)$$

where $G'_{kl}(t)$ is the probability of being in state k at time t initially starting in state l, provided no $i_A \rightarrow j_A$ transitions were made during time t. There are N_A factors $K_{j_A i_A}$ in the above expression, $*$ denotes time convolution, $f_1(t) * f_2(t) \equiv \int_0^t f_1(t-t') f_2(t') dt'$. The generating function $f_{nm}(\lambda_A, t) = \sum_{N_A=0}^{\infty} \lambda_A^{N_A} P_{nm}(N_A|t)$ for these probabilities satisfies the integral equation

$$f_{nm}(t) = G'_{nm}(t) + \int_0^t G'_{nj_A}(t-t') \lambda_A K_{j_A i_A} f_{i_A m}(t') dt', \quad (A2)$$

as can be verified by the recursion of this equation. The probability of no transitions, $G'_{nm}(t)$, is obtained from the kinetic equations when the $i_A \rightarrow j_A$ transition is irreversible

$$\frac{d}{dt} G'_{nm}(t) = \sum_i K_{ni} G'_{im} - \delta_{nj_A} K_{j_A i_A} G'_{i_A m}, \quad (A3)$$

with $G'_{nm}(0) = \delta_{nm}$. Differentiating Eq. (A2) and using Eq. (A3) we get

$$\frac{d}{dt} f_{nm}(\lambda_A, t) = \sum_i K_{ni} f_{im} - \delta_{nj_A}(1-\lambda_A) K_{j_A i_A} f_{i_A m}, \quad (A4)$$

with initial condition $f_{nm}(\lambda_A, 0) = \delta_{nm}$. Multiplying this equation by $p_{ss}(m)$ and summing over m, we find that $f_n(t) = \sum_m f_{nm}(t) p_{ss}(m)$ satisfies Eq. (2.3) when $\lambda_D = 1$.

APPENDIX B: STEADY-STATE APPROXIMATION. DERIVATION OF EQS. (2.14) AND (2.29)

Here we apply the local steady-state approximation to the modified rate equation for the generating function, Eq. (2.3), with

$$K(\lambda_A,\lambda_D)=K-V_A-V_D+\lambda_A V_A+\lambda_D V_D, \tag{B1}$$

where V_A and V_D are the matrices whose only nonzero element corresponds to the transitions resulting in the detected acceptor (donor) photons, $[V_\alpha]_{nm}=\delta_{nj_\alpha}\delta_{mi_\alpha}\phi_\alpha K^{\text{rad}}_{j_\alpha i_\alpha}$, $\alpha=A,D$.

To get Eq. (2.14), we use the approximation $f_n(t)\approx p_{ss}(n)f_0(t)$ in Eq. (2.3). After summing the equation over n and using $\Sigma_n K_{nm}=0$, we find that only V_A and V_D, which are multiplied to (λ_A-1) and (λ_D-1), appear in the equation for $f_0(t)$. As a result we get Eq. (2.14) for the generating function and the Poisson distribution for the number of photons, Eq. (2.15), where $n_{A,D}$ are the mean number of photons per unit time.

To get Eq. (2.29) for the generating function, we generalize the above derivation to include slow transitions. Let us divide the entire set of states into groups and label the states by the double index ir where i corresponds to the fast coordinate in a group labeled by r. Transitions $ir\rightarrow i'r$ are fast while $ir\rightarrow i'r'$ are slow. Then the matrix of rate constants, K, can be partitioned so that $M_{i,i'}(r)$ corresponds to the fast transitions $i'\rightarrow i$ at fixed slow variable r and $L_{i,i'}(r,r')$ to the slow transitions between the groups $i'r'\rightarrow ir$. Putting the counting parameter in front of the appropriate terms of $M_{i,i'}(r)$, we can rewrite Eqs. (2.2) and (2.3) as

$$F(\lambda_A,\lambda_D,t)=\sum_{i,r}f_i(r,t|\lambda_A,\lambda_D), \tag{B2}$$

where $f_i(r,t|\lambda_A,\lambda_D)\equiv f_i(r,t)$ satisfies

$$\frac{d}{dt}f_i(r,t)=\sum_{i',r'}L_{ii'}(r,r')f_{i'}(r',t)$$

$$+\sum_{i'}M_{ii'}(r|\lambda_A,\lambda_D)f_{i'}(r,t) \tag{B3}$$

with the steady-state initial conditions. At this point we exploit the separation of time scales and assume that at times longer than the relaxation time of the fast subsystem

$$f_i(r,t)\approx p_{ss}(i,r)f_0(r,t). \tag{B4}$$

This is analogous to Eq. (2.13), but now the weight factor $f_0(r,t)$ depends on the slow index r. Here $p_{ss}(i,r)$ is the steady-state probability of the state i of the fast subsystem with a fixed value of slow variable r that can be obtained by solving

$$\sum_{i'}M_{ii'}(r)p_{ss}(i',r)=0. \tag{B5}$$

Substituting $f_i(r_0,t)$ in Eq. (B4) into Eqs. (B2) and (B3), summing the latter over i, using the normalization condition, $\Sigma_i p_{ss}(i,r)=1$, and $\Sigma_i M_{ii'}(r)=0$, we obtain

$$F(\lambda_A,\lambda_D,t)=\sum_{i,r}f_i(r,t)=\sum_r f_0(r,t), \tag{B6}$$

where $f_0(r,t)$ satisfies Eq. (2.29) with $\mathcal{L}_r$ which is the operator that describes the slow dynamics

$$\mathcal{L}_r f(r)\equiv\sum_{i'r'}L_{ii'}(r,r')p_{ss}(i',r')f(r'). \tag{B7}$$

APPENDIX C: FLUCTUATING TOTAL NUMBER OF PHOTONS

In this appendix we consider what happens if the total number of the photons, $n_A(r)+n_D(r)$, fluctuates. It follows from the explicit expressions for $n_A(r)$ and $n_D(r)$ for the three-state system, Eqs. (2.8) and (2.6), that the following linear combination is independent of r:

$$n_A(r)+\gamma n_D(r)=n_0, \tag{C1}$$

where $\gamma=k_A^0(I+k_D)/[k_D^0(I+k_A)]$ and $n_0=Ik_A^0/(I+k_A)$ is the same as in Eq. (3.1). At low laser intensity, γ is the ratio of the products of the detection efficiencies and quantum yields for donor and acceptor photons. To try to account for differences in the fluorescence quantum yields and the detection efficiencies of the donor and acceptor channels, the FRET efficiency can be computed in each bin as $E=N_A/(N_A+\gamma N_D)$.[3,4] Proceeding in the same way as before but using the substitution $N=W_A+\gamma W_D$ and $\epsilon=W_A/(W_A+\gamma W_D)$, we find that the generalization of the FRET efficiency distribution to the case $\gamma\neq 1$ is

$$\rho_\gamma(E|t)=\int_0^1 d\epsilon\,\rho_{p\gamma}(E|\epsilon,n_0 t)P_{c\gamma}(\epsilon|t), \tag{C2}$$

where $P_{c\gamma}(\epsilon|t)=\langle\delta(\epsilon-1/t\int_0^t\mathcal{E}_\gamma[r(t')]dt')\rangle$ is the distribution of the modified FRET efficiency $\mathcal{E}_\gamma(r)$,

$$\mathcal{E}_\gamma(r)=\frac{n_A(r)}{n_A(r)+\gamma n_D(r)}. \tag{C3}$$

For the three-state system this becomes

$$\mathcal{E}_\gamma(r)=\frac{k_{tr}(r)}{k_{tr}(r)+k_A(I+k_D)/(I+k_A)}, \tag{C4}$$

$\rho_{p\gamma}(E|\epsilon,n_0 t)$ is the FRET efficiency distribution for Poissonian donor and acceptor photons,

$$\rho_{p\gamma}(E|\epsilon,N)=\mathcal{N}_p^{-1}(Nv(\epsilon))$$

$$\times\sum_{\substack{N_A,N_D\\N_A+N_D\geqslant N_T}}^{\infty}\frac{[\epsilon N]^{N_A}}{N_A!}\frac{[(1-\epsilon)N/\gamma]^{N_D}}{N_D!}$$

$$\times e^{-Nv(\epsilon)}\delta\!\left(E-\frac{N_A}{N_A+\gamma N_D}\right), \tag{C5}$$

where $v(x)=x+(1-x)/\gamma$. This reduces to Eq. (2.21) when $\gamma=1$. It can be shown that the modified FRET efficiency distribution is related to that with $\gamma=1$ by the following transformation:

$$\rho_{p\gamma}(E|\epsilon,N)=\rho_p[q(E)|q(\epsilon),Nv(\epsilon)]\frac{dq(E)}{dE}, \tag{C6}$$

where $q(x) = \gamma x/(1 - x + \gamma x)$. The mean and the variance of this distribution can only be expressed as double sums over N_A and N_D. These can be used to construct a Gaussian approximation analogous to Eq. (2.26).

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$$P(N_D|t) = \frac{(1 - \gamma^2)^{N_D} \tau^{N_D} e^{-\tau}}{(2\gamma)^{N_D} N_D! \sqrt{8\,\gamma\tau/\pi}} \left[2\gamma(N_D + \tau) I_{N_D - 1/2}(\gamma\tau) \right.$$
$$\left. + (1 + \gamma^2)\tau I_{N_D + 1/2}(\gamma\tau) \right],$$

where $\tau = (I + k_D)t/2$, $\gamma = \sqrt{1 - 4n_D/(I + k_D)}$, and $I_n(z)$ are modified Bessel functions of the first kind.

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