

Supporting Information

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SI Text

Delay Time Distribution and Photon Count Rates. In this section, we obtain explicit expressions for the delay time distribution, count rates, and FRET efficiency. In *Fixed energy transfer rate*, we consider the simplest case when the energy transfer rate is fixed, derive Eq. 2 in the main text, and discuss the γ -factor. In the next section (*Influence of dynamics on lifetimes and count rates*), we present various general relations for the case of fluctuating energy transfer rate. When these fluctuations are slow compared to the lifetime of the excited state, the general relations simplify and we derive Eq. 3 in the main text (*Average fluorescence lifetime, count rates and FRET efficiency when dynamics are slower than the lifetime*).

Fixed energy transfer rate. Let us assume that the donor and acceptor photophysics are described by the three-state kinetic scheme shown in Fig. 1A of the main text. The excited donor decays to the donor ground state with rate constant k_D (which includes both radiative and nonradiative processes), emitting a photon with radiative rate constant k_D^{rad} . The lifetime of the donor in the absence of acceptor is $\tau_D = k_D^{-1}$. Because the donor excited state can also decay by transferring its energy to the acceptor with rate constant k_{ET} , the lifetime of the donor excited state in the presence of acceptor is $\tau^* = (k_D + k_{\text{ET}})^{-1}$.

First consider the distribution $\mathcal{P}(\delta t)$ of delay times (i.e., the time between the laser pulse and the photon arrival). The delay times correspond to the decay of the excited state through the radiative channel. In general, the distribution of the delay times differs from the distribution of the excited-state lifetimes, which reflect the decay of the excited state through any channel. However, for the simple three-state kinetic scheme, the distributions are the same (single-exponential) independent of the decay route:

$$\mathcal{P}(\delta t) = (k_D + k_{\text{ET}})e^{-(k_D + k_{\text{ET}})\delta t} = e^{-\delta t/\tau^*}/\tau^*. \quad [\text{S1}]$$

The mean of this distribution is

$$\langle \delta t \rangle \equiv \int_0^\infty t \mathcal{P}(t) dt = (k_D + k_{\text{ET}})^{-1} = \tau^*. \quad [\text{S2}]$$

The efficiency of energy transfer, ϵ , can be defined as the probability that the excited donor state transfers its energy to the acceptor rather than decaying to its ground state. For the kinetic scheme in Fig. 1A, this is

$$\epsilon = \frac{k_{\text{ET}}}{k_D + k_{\text{ET}}}. \quad [\text{S3}]$$

Because $k_D/(k_D + k_{\text{ET}}) = 1 - k_{\text{ET}}/(k_D + k_{\text{ET}})$, it follows that the ratio of the lifetimes of the donor excited state in the presence and absence of the acceptor is related to the FRET efficiency by

$$\tau^*/\tau_D = 1 - \epsilon. \quad [\text{S4}]$$

Now consider the donor and acceptor count rates n_A and n_D that determine the Poisson distribution of photons in a bin. They can be expressed in microscopic terms as follows (see again Fig. 1A in the main text). The number of donor photons detected per unit time is the product of the probabilities that (i) the donor is excited by the laser pulse (p_{ex}), (ii) the donor decays to its ground state [$k_D/(k_D + k_{\text{ET}})$], (iii) it does so by emitting a photon

($\phi_D = k_D^{\text{rad}}/k_D$, the fluorescence quantum yield of donor in the absence of acceptor), and (iv) the photon is detected (ζ_D , called the detection efficiency), divided by the time between pulses, Δ . This argument and a similar one for n_A results in

$$n_D = p_{\text{ex}} \phi_D \zeta_D \Delta^{-1} \frac{k_D}{k_D + k_{\text{ET}}} \quad n_A = p_{\text{ex}} \phi_A \zeta_A \Delta^{-1} \frac{k_{\text{ET}}}{k_D + k_{\text{ET}}}, \quad [\text{S5}]$$

where ϕ_A and ζ_A are the acceptor quantum yield and detection efficiency, respectively.

The ratio of the donor and acceptor photon count rates is

$$n_D/n_A = \phi_D \zeta_D k_D / \phi_A \zeta_A k_{\text{ET}}. \quad [\text{S6}]$$

Interestingly, it turns out that this ratio does not depend on the donor quantum yield (ϕ_D) or the donor lifetime (k_D). Although the energy transfer rate constant is usually written as $k_{\text{ET}} = k_D(R_0/r)^6$, in fact R_0^6 is proportional to the donor quantum yield (1). Thus the factor $\phi_D k_D$ in Eq. S6 is canceled by the same factor in k_{ET} . An important consequence is that donor quenching (which increases the nonradiative decay rate and lowers the quantum yield) does not influence the ratio of the acceptor and donor count rates.

It follows from Eqs. S3 and S6 that the efficiency ϵ is related to the count rates by

$$\epsilon = \frac{n_A}{n_A + \gamma n_D} \quad [\text{S7}]$$

where we have defined the γ -factor as the ratio of the products of the detection efficiencies and quantum yields:

$$\gamma = \frac{\zeta_A \phi_A}{\zeta_D \phi_D}. \quad [\text{S8}]$$

Let us now consider how the above parameters can be obtained from the binned experimental photon trajectory (i.e., from the random N_A , N_D , τ in bins of length T). The fluorescence lifetime in a bin averaged over all bins is

$$\langle \tau \rangle \equiv \left\langle \sum_{i=1}^{N_D} \delta t_i / N_D \right\rangle = \langle \delta t \rangle = \tau^*. \quad [\text{S9}]$$

Because the distributions of both N_A and N_D are Poissonian with count rates n_A and n_D , it follows that

$$\langle N_{A,D} \rangle = n_{A,D} T. \quad [\text{S10}]$$

Thus the relation in Eq. S4 can be rewritten (using Eqs. S7 and S10) in terms of the averages over bins as

$$\langle \tau \rangle / \tau_D = 1 - \frac{\langle N_A \rangle}{\langle N_A \rangle + \gamma \langle N_D \rangle}. \quad [\text{S11}]$$

If one wants to convert photon counts N_A and N_D into FRET efficiency before further analysis, one needs to define the random energy transfer efficiency E in terms of N_A and N_D . In the past (2) we advocated the definition $E = N_A/(N_A + N_D)$ because the average of E over all bins is exactly given by

$$\langle E \rangle = \left\langle \frac{N_A}{N_A + N_D} \right\rangle = \frac{n_A}{n_A + n_D}. \quad [\text{S12}]$$

This is true for a Poisson distribution relation of photon counts even in the presence of a cutoff imposed on the number of photons. The quantity $n_A/(n_A + n_D)$ is commonly called an apparent FRET efficiency in contrast to the “true” FRET efficiency ε . Using Eqs. S7 and S12, we can rewrite Eq. S4 in terms of the measured $\langle E \rangle$ and $\langle \tau \rangle$ (3)

$$\langle \tau \rangle / \tau_D = 1 - \frac{\langle E \rangle}{\gamma + (1 - \gamma)\langle E \rangle}. \quad [\text{S13}]$$

For $\gamma = 1$, this reduces to Eq. 2 of the main text.

Now, one can make Eq. 2 of the main text valid (to an excellent approximation) even when $\gamma \neq 1$ by defining the random FRET efficiency in a bin as $E' = N_A/(N_A + \gamma N_D)$. By using the approximation

$$\left\langle \frac{A}{B} \right\rangle \approx \frac{\langle A \rangle}{\langle B \rangle} \left(1 - \frac{\langle \delta A \delta B \rangle}{\langle A \rangle \langle B \rangle} + \frac{\langle \delta B^2 \rangle}{\langle B \rangle^2} \right), \quad [\text{S14}]$$

where $\delta X = X - \langle X \rangle$, one can show (using $\langle \delta N_A \delta N_D \rangle = 0$ and $\langle \delta N_{A,D}^2 \rangle = \langle N_{A,D} \rangle$ for uncorrelated photons with Poisson statistics) that

$$\langle E' \rangle = \left\langle \frac{N_A}{N_A + \gamma N_D} \right\rangle \approx \frac{n_A}{n_A + \gamma n_D} \left(1 + \frac{\gamma(\gamma - 1)n_D}{(n_A + \gamma n_D)^2 T} \right). \quad [\text{S15}]$$

The correction term is small when the average total number of photons in a bin is large (say, greater than 10). With this definition of the random FRET efficiency in a bin, the relation between the bin averaged fluorescence lifetimes and FRET efficiencies is

$$\langle \tau \rangle / \tau_D = 1 - \langle E' \rangle + \frac{(\gamma - 1)\langle E' \rangle(1 - \langle E' \rangle)}{\langle N_A \rangle + \gamma \langle N_D \rangle}. \quad [\text{S16}]$$

The last term in the above relation is usually very small. If this term is neglected and the primes on E' are dropped, we again recover Eq. 2 in the main text. Thus, when $\gamma \neq 1$, even for the simplest case, there are a few subtleties that fortunately can be easily handled in practice.

Influence of dynamics on lifetimes and count rates. When the energy transfer rate k_{ET} fluctuates, the relationship between the FRET efficiency and fluorescence lifetime becomes more complicated. We begin by expressing the quantities of interest (i.e., excited-state lifetime, fluorescence lifetime, FRET efficiency, and count rates) in terms of the population of the donor excited state.

Consider a donor that is prepared in its excited state D^* at $t = 0$. In the simplest case considered above (see Fig. 1A in the main text), its population decays as $p(D^*, t) = \exp(-(k_D + k_{\text{ET}})t)$, which is actually the probability to be in the excited state at time t (i.e., the survival probability of D^*). When the energy transfer rate k_{ET} fluctuates, the decay of the excited state is multiexponential and is formally given by the path average

$$p(D^*, t) = \left\langle \exp(-k_D t - \int_0^t k_{\text{ET}}(t') dt') \right\rangle_{x(t)}, \quad [\text{S17}]$$

where x denotes all coordinates (distance, orientation) that can influence the energy transfer rate. For a model of the dynamics described by an operator \mathcal{L} , the evaluation of the path average can be reduced to the solution of

$$\frac{\partial p(D^*, x, t)}{\partial t} = [\mathcal{L} - k_D - k_{\text{ET}}(x)]p \quad [\text{S18}]$$

subject to the equilibrium initial condition $p(D^*, x, 0) = p_{\text{eq}}(x)$. The equilibrium population $p_{\text{eq}}(x)$ satisfies $\mathcal{L}p_{\text{eq}}(x) = 0$ and $\int p_{\text{eq}}(x) dx = 1$. The population of the donor excited state is

$$p(D^*, t) = \int p(D^*, x, t) dx. \quad [\text{S19}]$$

Now we present expressions for various quantities of interest in terms of the donor excited-state population.

1. The light intensity in ensemble measurements, $I(t)$, is related to $p(D^*, t)$ by

$$I(t)/I(0) = p(D^*, t). \quad [\text{S20}]$$

2. The distribution of the donor excited-state lifetime is the probability density that the excited state disappears (to any state, radiatively or nonradiatively) between time t and $t + dt$. Because $[p(D^*, t) - p(D^*, t + dt)]$ is $[-dp(D^*, t)/dt]dt$, the probability density is given by $-dp(D^*, t)/dt$. This distribution is normalized:

$$\int_0^\infty (-dp(D^*, t)/dt) dt = p(D^*, 0) - p(D^*, \infty) = 1.$$

The mean lifetime of the excited state, $\langle \tau^* \rangle$, is

$$\begin{aligned} \langle \tau^* \rangle &= \int_0^\infty t(-dp(D^*, t)/dt) dt = \int_0^\infty p(D^*, t) dt \\ &= \int_0^\infty I(t)/I(0) dt. \end{aligned} \quad [\text{S21}]$$

The second equality is obtained by integrating by parts and using $p(D^*, \infty) = 0$.

3. The FRET efficiency, ε , is the probability that the excited state decays by transferring its energy to the acceptor and is given by

$$\varepsilon = \int_0^\infty \int k_{\text{ET}}(x) p(D^*, x, t) dx dt. \quad [\text{S22}]$$

4. The probability that the excited state decays to the ground state is $1 - \varepsilon$ and is equal to

$$\begin{aligned} 1 - \varepsilon &= \int_0^\infty \int k_D p(D^*, x, t) dx dt = k_D \int_0^\infty p(D^*, t) dt \\ &= \langle \tau^* \rangle / \tau_D, \end{aligned} \quad [\text{S23}]$$

where we have used Eqs. S19 and S21. This equation is the general relation between the mean lifetime of the excited state and the FRET efficiency. It is simply a statement that the excited state can decay either by transferring its energy to the acceptor or by returning to its ground state.

5. The normalized distribution of excited-state lifetimes on condition that the excited state decays to its ground state through the radiative channel is the same as the distribution of delay times, $\mathcal{P}(\delta t)$, and is given by

$$\mathcal{P}(\delta t) = \frac{p(D^*, \delta t)}{\int_0^\infty p(D^*, t) dt} = \frac{I(\delta t)}{\int_0^\infty I(t) dt} \quad [\text{S24}]$$

so that the mean delay time is

$$\langle \delta t \rangle = \int_0^\infty t \mathcal{P}(t) dt = \frac{\int_0^\infty t p(D^*, t) dt}{\int_0^\infty p(D^*, t) dt} = \frac{\int_0^\infty t I(t) dt}{\int_0^\infty I(t) dt}. \quad [\text{S25}]$$

The mean delay time is equal to the average fluorescence lifetime in a bin, $\langle \tau \rangle = \langle \sum_{i=1}^N \delta t_i / N_D \rangle = \langle \delta t \rangle$. However, only when $p(D^*, t)$ is a single exponential are the lifetime of the excited state, $\langle \tau^* \rangle$, and the fluorescence lifetime, $\langle \tau \rangle$, equal.

6. Photon count rates (fluorescence intensities) can be shown to be (4)

$$n_D = p_{\text{ex}} \phi_D \zeta_D \Delta^{-1} (1 - \varepsilon) \quad n_A = p_{\text{ex}} \phi_A \zeta_A \Delta^{-1} \varepsilon, \quad [\text{S26}]$$

where ε is defined in Eq. S22. From these expressions, it follows that relationship [S7] between FRET efficiency and photon count rates is valid even in the presence of submicrosecond fluctuations of the energy transfer rate.

Thus, in the presence of dynamics on a timescale comparable to the lifetime of the excited state, the problem of calculating the count rates and the delay time distribution is rather involved. However, there is a simple relation between the excited state (not fluorescence!) lifetime and FRET efficiency (photon count rates):

$$\langle \tau^* \rangle / \tau_D = 1 - \varepsilon = 1 - \frac{n_A}{n_A + \gamma n_D} = 1 - \langle E \rangle, \quad [\text{S27}]$$

where n_A and n_D are the donor and acceptor count rates and γ is the ratio of the products of the detector efficiencies and quantum yields, Eq. S8. The last equality holds when the random FRET efficiency in a bin is defined as $E = N_A / (N_A + \gamma N_D)$ and the number of photons in a bin is sufficiently large.

Average fluorescence lifetime, count rates and FRET efficiency when dynamics are slower than the lifetime. When the fluctuations of the energy transfer rate are slower than the donor lifetime, it follows from Eqs. S18 and S19 that the population of the donor excited state is

$$p(D^*, t) = \int \exp(-[k_D + k_{\text{ET}}(x)]t) p_{\text{eq}}(x) dx \\ \equiv \langle \exp(-(k_D + k_{\text{ET}})t) \rangle, \quad [\text{S28}]$$

where we have replaced the averaging over various paths by the averaging over the distribution of interdy distance and/or dye orientation, $\langle (\dots) \rangle = \int (\dots) p_{\text{eq}}(x) dx$.

The average lifetime of the excited state, from Eq. S21, is

$$\langle \tau^* \rangle = \int \frac{p_{\text{eq}}(x)}{k_D + k_{\text{ET}}(x)} dx = \langle (k_D + k_{\text{ET}})^{-1} \rangle. \quad [\text{S29}]$$

The distribution of delay times, from Eq. S24, is (5)

$$\mathcal{P}(\delta t) = \frac{\langle \exp[-(k_D + k_{\text{ET}})\delta t] \rangle}{\langle (k_D + k_{\text{ET}})^{-1} \rangle} \quad [\text{S30}]$$

and so the average delay time, which is equal to the average fluorescence lifetime (see Eq. S25), is

$$\langle \tau \rangle = \langle \delta t \rangle = \int \frac{\int [k_D + k_{\text{ET}}(x)]^{-2} p_{\text{eq}}(x) dx}{\int [k_D + k_{\text{ET}}(x)]^{-1} p_{\text{eq}}(x) dx} = \frac{\langle (k_D + k_{\text{ET}})^{-2} \rangle}{\langle (k_D + k_{\text{ET}})^{-1} \rangle}. \quad [\text{S31}]$$

Thus the average lifetime of the donor excited state is not the same as the average fluorescence lifetime. Physically, the reason is that states with smaller energy transfer rates are more likely to

emit donor photons. As a simple example, consider a molecule with two equally populated states with large and small energy transfer rates, which correspond to short and long lifetimes. The average lifetime of the excited state (i.e., the lifetime when every decay of the excited state is counted) is just a simple arithmetic average of the short and long lifetimes. To find the average fluorescence lifetime, only those events that result in a donor photon should be taken into account. There are more donor photons emitted from the state with the longer lifetime (small energy transfer rate), therefore, this state has a larger weight.

Under the above conditions (slow fluctuations of the energy transfer rate), the count rates are (see Eqs. S22 and S26)

$$n_A = p_{\text{ex}} \phi_A \zeta_A \Delta^{-1} \left\langle \frac{k_{\text{ET}}}{k_D + k_{\text{ET}}} \right\rangle \\ n_D = p_{\text{ex}} \phi_D \zeta_D \Delta^{-1} \left\langle \frac{k_D}{k_D + k_{\text{ET}}} \right\rangle. \quad [\text{S32}]$$

With these count rates, the mean FRET efficiency is

$$\langle E \rangle = \frac{n_A}{n_A + \gamma n_D} = \int \frac{k_{\text{ET}}(x)}{k_D + k_{\text{ET}}(x)} p_{\text{eq}}(x) dx = \left\langle \frac{k_{\text{ET}}}{k_D + k_{\text{ET}}} \right\rangle. \quad [\text{S33}]$$

To get Eq. 3 in the main text, we write $(k_D + k_{\text{ET}})^{-1} = \tau_D [1 - k_{\text{ET}} / (k_D + k_{\text{ET}})]$ and so

$$\langle (k_D + k_{\text{ET}})^{-1} \rangle = \tau_D (1 - \langle E \rangle) \\ \langle (k_D + k_{\text{ET}})^{-2} \rangle = \tau_D ((1 - \langle E \rangle)^2 + \sigma_c^2)$$

where σ_c^2 is the FRET efficiency variance:

$$\sigma_c^2 \equiv \langle k_{\text{ET}}^2 / (k_D + k_{\text{ET}})^2 \rangle - \langle k_{\text{ET}} / (k_D + k_{\text{ET}}) \rangle^2 \\ = \int \frac{k_{\text{ET}}^2}{(k_D + k_{\text{ET}})^2} p_{\text{eq}}(x) dx - \left(\int \frac{k_{\text{ET}}}{k_D + k_{\text{ET}}} p_{\text{eq}}(x) dx \right)^2. \quad [\text{S34}]$$

Using these expressions in Eq. S31, we get Eq. 3 in the main text.

Finally, the second moment of the delay time distribution in Eq. S30 is

$$\langle \delta t^2 \rangle = \int_0^\infty t^2 \mathcal{P}(t) dt = 2 \frac{\langle (k_D + k_{\text{ET}})^{-3} \rangle}{\langle (k_D + k_{\text{ET}})^{-1} \rangle}. \quad [\text{S35}]$$

This expression can be used to simulate fluorescence lifetimes in a bin (see *Methods*) when the fluctuations of the energy transfer rate are on a timescale between the donor lifetime (nanoseconds) and the interphoton time (microseconds). In the absence of the fluctuations, the above equation reduces to $\langle \delta t^2 \rangle = 2(k_D + k_{\text{ET}})^{-2} = 2\langle \delta t \rangle^2$, as expected for exponentially distributed delay times.

Joint Distribution of Photon Counts and Fluorescence Lifetimes. In this section, we derive Eq. 5 in the main text for the joint distribution of the numbers of photons and lifetime in the presence of conformational dynamics on a timescale longer than the interphoton time. For the sake of simplicity, consider photons of one color emitted by a molecule with interconverting conformational states. We are interested in finding the joint distribution $P(N, \tau)$ of detecting N photons during bin time T and lifetime in a bin defined as $\tau = \sum_{i=1}^N \delta t_i / N$, where δt_i is the time interval between the laser pulse and the detection of i th photon.

For a single conformational state, the photons are uncorrelated and the joint distribution is

$$P(N, \tau) = \frac{(nT)^N}{N!} e^{-nT} P(\tau|N), \quad [\text{S36}]$$

where n is the mean number of photons per unit time (the count rate) and $P(\tau|N)$ is the distribution of the lifetimes in bins containing N photons:

$$P(\tau|N) = \int \delta(\tau - \sum_{i=1}^N \delta t_i / N) \prod_{i=1}^N \mathcal{P}(\delta t_i) d\delta t_i. \quad [\text{S37}]$$

Here $\mathcal{P}(\delta t)$ is the distribution of the delay times. The lifetime distribution $P(\tau|N)$ is known analytically (the gamma-distribution) only when the delay times are exponentially distributed. When there are dynamics on nanosecond to microsecond timescale, the delay time distribution $\mathcal{P}(\delta t)$ is not exponential and depends on the details of the submicrosecond dynamics.

When conformational states, $s(t)$, change on a timescale comparable to or slower than the interphoton time, the photons become correlated. Conformational changes modulate the count rate, $n(t) \equiv n(s(t))$, and the delay time distribution, $\mathcal{P}(\delta t|t) \equiv \mathcal{P}(\delta t|s(t))$. Our first step is to find the joint distribution $\bar{P}(N, \tau)$ of N photons and lifetime τ for a specific conformational trajectory $s(t)$. To this end, consider a sequence of photons detected at t_1, \dots, t_N with the delay times $\delta t_1, \dots, \delta t_N$ in the interval $[0, T]$. The probability distribution to detect such sequence of photons is

$$e^{-\int_{t_N}^T n(t) dt} \mathcal{P}(\delta t_N | t_N) n(t_N) \dots \mathcal{P}(\delta t_2 | t_2) n(t_2) \\ \times e^{-\int_{t_1}^{t_2} n(t) dt} \mathcal{P}(\delta t_1 | t_1) n(t_1) e^{-\int_0^{t_1} n(t) dt}.$$

Here, reading right to left, $\exp(-\int_0^{t_1} n(t) dt)$ is the probability that no photons are detected between initial time and t_1 , $n(t_1)$ is the probability density to detect a photon at t_1 , $\mathcal{P}(\delta t_1 | t_1)$ is the probability density that this photon has the delay time δt_1 , etc. This distribution is then multiplied by $\delta(\tau - \sum_{i=1}^N \delta t_i / N)$ and integrated with respect to all δt_i as in Eq. S37. In addition, because the photons can be detected at any time during bin time, one must also integrate with respect to all t_i . Thus the joint distribution for a specific conformational trajectory is

$$\bar{P}(N, \tau) = e^{-\int_0^T n(t) dt} \int \delta(\tau - \sum_{i=1}^N \delta t_i / N) \prod_{i=1}^N n(t_i) \mathcal{P}(\delta t_i | t_i) d\delta t_i dt_i. \quad [\text{S38}]$$

Here the integrals with respect to δt_i are from zero to infinity, the integrals with respect to t_i are time-ordered so that $0 \leq t_1 \leq t_2 \dots \leq t_N \leq T$.

We now use the Fourier representation of the δ -function

$$\delta(\tau - \sum_{i=1}^N \delta t_i / N) = \int_{-\infty}^{\infty} e^{i\omega\tau - i\omega \sum_{i=1}^N \delta t_i / N} d\omega / 2\pi \quad [\text{S39}]$$

in Eq. S38 and integrate first with respect to δt_i and then t_i using the identity

$$\int_0^T dt_1 \int_{t_1}^T dt_2 \dots \int_{t_{N-1}}^T dt_N \prod_{i=1}^N f(t_i, \omega) = \left(\int_0^T f(t, \omega) dt \right)^N / N!$$

where we have defined

$$f(t, \omega) \equiv n(t) \int_0^{\infty} \mathcal{P}(\delta t_i | t) \exp(-i\omega \delta t_i / N) d\delta t_i. \quad [\text{S40}]$$

In this way we get

$$\bar{P}(N, \tau) = e^{-\int_0^T n(t) dt} \int \frac{d\omega}{2\pi} e^{i\omega\tau} \left(\int_0^T f(t, \omega) dt \right)^N / N! \quad [\text{S41}]$$

This equation can be rewritten as

$$\bar{P}(N, \tau) = \frac{[\bar{n}T]^N}{N!} e^{-\bar{n}T} \bar{P}(\tau|N) \quad [\text{S42}]$$

where $\bar{n}T = \int_0^T n(t) dt$ and

$$\bar{P}(\tau|N) = \int \frac{d\omega}{2\pi} e^{i\omega\tau} \left(\frac{\int_0^T f(t, \omega) dt}{\bar{n}T} \right)^N$$

Using Eq. S40 for $f(t, \omega)$ and applying the Fourier representation of the δ -function in Eq. S39 backward, $\bar{P}(\tau|N)$ can be written as

$$\bar{P}(\tau|N) = \int \delta(\tau - \sum_{i=1}^N \delta t_i / N) \prod_{i=1}^N \bar{\mathcal{P}}(\delta t_i) d\delta t_i, \quad [\text{S43}]$$

where we have defined $\bar{\mathcal{P}}(\delta t)$ as

$$\bar{\mathcal{P}}(\delta t) = \frac{\int_0^T \mathcal{P}(\delta t | t) n(t) dt}{\int_0^T n(t) dt} \quad [\text{S44}]$$

Thus $\bar{P}(\tau|N)$ is the distribution of $\tau = \sum_{i=1}^N \delta t_i / N$, where delay times δt are distributed according to $\bar{\mathcal{P}}(\delta t)$.

Comparing Eqs. S42 and S43 and Eqs. S36 and S37, we see that, for a specific conformational state trajectory, the joint distribution has the same form as that for uncorrelated photons, but with the parameters \bar{n} and $\bar{\mathcal{P}}(\delta t)$ that depend on the state trajectory $s(t)$. This distribution is then averaged over all possible state trajectories:

$$P(N, \tau) = \left\langle \frac{[\bar{n}T]^N}{N!} e^{-\bar{n}T} \bar{P}(\tau|N) \right\rangle_{s(t)}. \quad [\text{S45}]$$

The above reasoning can be readily extended to photons of two colors, resulting in the joint distribution $P(N_A, N_D, \tau)$ of the number of the acceptor and donor photons and donor lifetime given in Eq. 5 in the main text.

Joint Distribution for a Two-State Molecule. Consider a molecule with two states that interconvert with the transition rate constants k_1 ($1 \rightarrow 2$) and k_2 ($2 \rightarrow 1$). The photon count rates in state s ($s = 1, 2$) are n_{A_s} and n_{D_s} . The distribution of the donor delay times is $\mathcal{P}_s(\delta t)$. The first two moments of the delay time distribution are $\langle \delta t \rangle_s$ and $\langle \delta t^2 \rangle_s$. For the exponential delay time distribution, $\mathcal{P}_s(\delta t) = \exp(-\delta t / \tau_s) / \tau_s$, the moments are $\langle \delta t \rangle_s = \tau_s$ and $\langle \delta t^2 \rangle_s = 2\tau_s^2$. The joint distribution of the numbers of acceptor and donor photons and donor fluorescence lifetimes is (see Eq. 7 in the main text)

$$P(N_A, N_D, \tau) = \int_0^1 \frac{[\bar{n}_A T]^{N_A} [\bar{n}_D T]^{N_D}}{N_A! N_D!} e^{-(\bar{n}_A + \bar{n}_D)T} \bar{P}(\tau|N_D) P(\theta|T) d\theta, \quad [\text{S46}]$$

where $\bar{n}_A = n_{A1}\theta + n_{A2}(1 - \theta)$, $\bar{n}_D = n_{D1}\theta + n_{D2}(1 - \theta)$, and θ is the fraction of time spent in the first state during bin time T . $P(\theta|T)$ is the distribution of θ , which is known analytically (6):

$$P(\theta|T) = p_1 e^{-k_1 T} \delta(\theta) + p_2 e^{-k_2 T} \delta(1 - \theta) + 2k_0 T p_1 p_2 (I_0(y) + k_0 T(1 - z) I_1(y)/y) e^{-z k_0 T}, \quad [\text{S47}]$$

where $k_0 = k_1 + k_2$, $p_1 = k_2/k_0 = 1 - p_2$, $y = 2k_0 T \sqrt{p_1 p_2 \theta(1 - \theta)}$, $z = p_2 \theta + p_1(1 - \theta)$, and $I_n(y)$ is the modified Bessel function of the first kind of order n . $\bar{P}(\tau|N_D)$ is the distribution of $\tau = \sum_{i=1}^{N_D} \delta t_i / N_D$, where δt_i have distribution $\bar{\mathcal{P}}(\delta t)$:

$$\bar{\mathcal{P}}(\delta t) = \frac{\mathcal{P}_1(\delta t) n_{D1} \theta + \mathcal{P}_2(\delta t) n_{D2} (1 - \theta)}{n_{D1} \theta + n_{D2} (1 - \theta)}. \quad [\text{S48}]$$

Note that this distribution varies from bin to bin.

The mean ($\bar{\tau}$) and variance ($\bar{\sigma}^2$) of $\bar{P}(\tau|N_D)$ can be expressed in terms of the moments of the delay time distribution $\bar{\mathcal{P}}(\delta t)$ as

$$\bar{\tau} = \int_0^\infty t \bar{\mathcal{P}}(t) dt \quad \bar{\sigma}^2 = \left(\int_0^\infty t^2 \bar{\mathcal{P}}(t) dt - \bar{\tau}^2 \right) / N_D. \quad [\text{S49}]$$

Using Eq. S48 for $\bar{\mathcal{P}}(\delta t)$, we have

$$\bar{\tau} = f(\theta) \langle \delta t \rangle_1 + [1 - f(\theta)] \langle \delta t \rangle_2 \quad [\text{S50}]$$

$$\bar{\sigma}^2 = (f(\theta) \langle \delta t^2 \rangle_1 + [1 - f(\theta)] \langle \delta t^2 \rangle_2 - \bar{\tau}^2) / N_D,$$

where $f(\theta) = n_{D1} \theta / [n_{D1} \theta + n_{D2} (1 - \theta)]$. For large photon counts, $N_D \gg 1$, $\bar{P}(\tau|N_D)$ can be approximated by a gamma-distribution with parameters that ensure that mean and variance are correct:

$$\bar{P}(\tau|N_D) \approx \beta^\alpha \tau^{\alpha-1} e^{-\beta \tau} / \Gamma(\alpha) \quad \alpha = \bar{\tau}^2 / \bar{\sigma}^2, \quad \beta = \bar{\tau} / \bar{\sigma}^2, \quad [\text{S51}]$$

where $\Gamma(\alpha)$ is the gamma-function. The resulting lifetimes are always positive. Moreover, the distribution in Eq. S51 becomes exact when the delay times are exponentially distributed and $\theta = 0$ or $\theta = 1$.

Two-State Dynamic Lines. In this section, we consider the joint distribution of FRET efficiencies and fluorescence lifetimes, $P(E, \tau)$, for a two-state molecule in the limit of large photon counts (no shot noise) and derive equations for the two-state dynamic lines (Eqs. 8–10 in the main text). Random FRET efficiency is defined as $E = N_A / (N_A + \gamma N_D)$, where γ is the correction factor which was discussed earlier in *Fixed energy transfer rate*. The distribution $P(E, \tau)$ is related to the distribution of photon counts and lifetimes, $P(N_A, N_D, \tau)$ as

$$P(E, \tau) = \sum_{N_A, N_D} \delta \left(E - \frac{N_A}{N_A + \gamma N_D} \right) P(N_A, N_D, \tau). \quad [\text{S52}]$$

In the limit of no shot noise, the Poisson distributions and $\bar{P}(\tau|N_D)$ in Eq. 5 in the main text for $P(N_A, N_D, \tau)$ become δ -functions and the summation over photon counts can be replaced by integration, so that we have

$$P(E, \tau) = \int \left\langle \delta \left(E - \frac{N_A}{N_A + \gamma N_D} \right) \delta(N_A - \bar{n}_A T) \times \delta(N_D - \bar{n}_D T) \delta(\tau - \bar{\tau}) \right\rangle_{s(t)} dN_A dN_D$$

$$= \left\langle \delta \left(E - \frac{\bar{n}_A}{\bar{n}_A + \gamma \bar{n}_D} \right) \delta(\tau - \bar{\tau}) \right\rangle_{s(t)}, \quad [\text{S53}]$$

where we have evaluated the integrals over N_A and N_D . For a two-state system, this becomes

$$P(E, \tau) = \int \delta \left(E - \frac{\bar{n}_A}{\bar{n}_A + \gamma \bar{n}_D} \right) \delta(\tau - \bar{\tau}) P(\theta|T) d\theta, \quad [\text{S54}]$$

where $P(\theta|T)$ is given by Eq. S47 and

$$\bar{n}_A = n_{A1} \theta + n_{A2} (1 - \theta) \quad \bar{n}_D = n_{D1} \theta + n_{D2} (1 - \theta)$$

$$\bar{\tau} = \frac{\tau_1 n_{D1} \theta + \tau_2 n_{D2} (1 - \theta)}{n_{D1} \theta + n_{D2} (1 - \theta)}. \quad [\text{S55}]$$

Here n_{As} and n_{Ds} are the photon count rates of states $s = 1, 2$, and τ_s is the first moment of the delay time distribution of state s [$\tau_s = \int_0^\infty t \mathcal{P}_s(t) dt$]. Integration with respect to θ now allows us to express θ in terms of E , n_{As} , and n_{Ds} , and then to express $\bar{\tau}$ in terms of E , $\varepsilon_s = n_{As} / (n_{As} + \gamma n_{Ds})$, and τ_s , $s = 1, 2$. In this way, we find

$$P(E, \tau) = \delta(\tau - f(E)) \int \delta \left(E - \frac{\bar{n}_A}{\bar{n}_A + \gamma \bar{n}_D} \right) P(\theta|T) d\theta, \quad [\text{S56}]$$

where

$$f(E) = \frac{\tau_1 - \tau_2}{\varepsilon_1 - \varepsilon_2} \left(\frac{\tau_1 \varepsilon_2 - \tau_2 \varepsilon_1}{\tau_1 - \tau_2} - E + \frac{(\varepsilon_2 - E)(E - \varepsilon_1)}{1 - E} \right). \quad [\text{S57}]$$

The first term in the above equation for $P(E, \tau)$ (the δ -function) leads to the equation for the two-state dynamic line (Eq. 8 in the main text). The second term (the integral) is the FRET efficiency distribution in the absence of shot noise which we considered previously in the special case that $\gamma = 1$ and $n_{A1} + n_{D1} = n_{A2} + n_{D2}$ (7).

Note that the above derivation does not use specific expressions for the count rates and lifetimes of the states and, therefore, is valid for any value of the γ -factor. In the special case that the energy transfer rate only fluctuates on timescale faster than the fluorescence lifetime, the FRET efficiencies and lifetimes of the states are related as $\tau_s / \tau_D = 1 - \varepsilon_s$ and the equation for the two-state line simplifies (see Eqs. 9 and 10 in the main text). The γ -factor in this case is the ratio of the acceptor and donor quantum yields and detection efficiencies.

The above derivation for the two-state dynamic lines can be extended to a multistate molecule, in which case the two-state dynamic lines correspond to only those bins during which the molecule visits two states.

Two-State Dynamic Lines for Diffusing Molecules. In this section, we prove that Eqs. 8–10 in the main text for the two-state dynamic lines are also valid for diffusing molecules. The count rates depend on the location of the molecule in the laser spot through the dependence of the excitation probability (p_{ex}) and the detection efficiencies ($\zeta_{s,D}$) in Eqs. S5 or S26. Thus, when a molecule diffuses through the confocal volume, the donor and acceptor count rates, $n_{A,D}(R, s)$ (which depend on state s and location in the spot R) fluctuate due to transitions between states and due to diffusion. The distribution of the delay times, $\mathcal{P}(\delta t|s)$, is modulated only by the interconversion between the states.

Our starting point is the generalization of Eq. S53 for the FRET efficiency and lifetime distribution in the absence of shot noise

$$P(E, \tau) = \left\langle \delta \left(E - \frac{\bar{n}_A}{\bar{n}_A + \gamma \bar{n}_D} \right) \delta(\tau - \bar{\tau}) \right\rangle_{s(t), R(t)}. \quad [\text{S58}]$$

The time average count rates and lifetime are now given by $\bar{n}_{A,D} = \int_0^T n_{A,D}(R(t), s(t)) dt / T$ and $\bar{\tau} = \int_0^T n_D(R(t), s(t)) \tau(s(t)) dt / \bar{n}_D$. Note that, in contrast to the count rates, the fluctuating delay time distribution and its first moment $\tau(s(t)) = \int_0^\infty t' \mathcal{P}(t'|s(t)) dt'$ do not depend on the location of the molecule in the laser spot.

In the special case of a molecule with two conformational states, labeled 1 and 2, the random state variable $s(t)$ can either be 1 or 2. For the sake of convenience, we introduce the Kronecker δ -function $\delta_{1,s(t)}$, which indicates when the molecule is in state 1 [i.e., it is 1 when $s(t) = 1$ and 0 otherwise]; $\delta_{2,s(t)}$ indicates when the molecule is in state 2. Because the molecule must be in one of the two states, $\delta_{1,s(t)} + \delta_{2,s(t)} = 1$. Consequently, we can split the time-averaged count rates into two contributions as

$$\bar{n}_A = \bar{n}_{A1} + \bar{n}_{A2} \quad \bar{n}_D = \bar{n}_{D1} + \bar{n}_{D2}, \quad [\text{S59}]$$

where we have defined $\bar{n}_{Ai} = \int_0^T n_A(R(t), i) \delta_{i,s(t)} dt / T$ and $\bar{n}_{Di} = \int_0^T n_D(R(t), i) \delta_{i,s(t)} dt / T$ with $i = 1, 2$. Here, for example, $n_A(R(t), 1)$ is the fluctuating acceptor count rate as the molecule in state 1 diffuses through the laser spot.

Similarly, we can express $\bar{\tau}$ as

$$\bar{\tau} = \frac{\bar{n}_{D1} \tau_1 + \bar{n}_{D2} \tau_2}{\bar{n}_{D1} + \bar{n}_{D2}} \quad [\text{S60}]$$

or, equivalently,

$$(\bar{\tau} - \tau_1) \bar{n}_{D1} + (\bar{\tau} - \tau_2) \bar{n}_{D2} = 0, \quad [\text{S61}]$$

where τ_1 (τ_2) is the first moment of the delay time distribution of state 1(2).

To obtain the desired result, we only need to exploit the fact that if the donor and acceptor detection efficiencies have the same dependence on R , then the γ -factor, Eq. S8, does not change as the molecule diffuses through the spot. Then it follows from Eq. S26 that

$$\begin{aligned} n_A(R(t), 1) &= \frac{\gamma \varepsilon_1}{1 - \varepsilon_1} n_D(R(t), 1) \\ n_A(R(t), 2) &= \frac{\gamma \varepsilon_2}{1 - \varepsilon_2} n_D(R(t), 2), \end{aligned} \quad [\text{S62}]$$

where ε_i is the FRET efficiency in state $i = 1, 2$, which does not depend on the location in the laser spot. Then multiplying both sides of the first (second) equation by $\delta_{1,s(t)}$ ($\delta_{2,s(t)}$) and integrating with respect to t from 0 to T , we find

$$\bar{n}_{A1} = \frac{\gamma \varepsilon_1}{1 - \varepsilon_1} \bar{n}_{D1} \quad \bar{n}_{A2} = \frac{\gamma \varepsilon_2}{1 - \varepsilon_2} \bar{n}_{D2}. \quad [\text{S63}]$$

These identities allow us to write \bar{n}_A in Eq. S59 as

$$\bar{n}_A = \frac{\gamma \varepsilon_1}{1 - \varepsilon_1} \bar{n}_{D1} + \frac{\gamma \varepsilon_2}{1 - \varepsilon_2} \bar{n}_{D2}. \quad [\text{S64}]$$

Therefore, we can rewrite the equality $E = \bar{n}_A / (\bar{n}_A + \gamma \bar{n}_D)$, which is the consequence of the first δ -function in Eq. S58, in terms of \bar{n}_{D1} and \bar{n}_{D2} as

$$\left(\frac{E}{1-E} - \frac{\varepsilon_1}{1-\varepsilon_1} \right) \bar{n}_{D1} + \left(\frac{E}{1-E} - \frac{\varepsilon_2}{1-\varepsilon_2} \right) \bar{n}_{D2} = 0. \quad [\text{S65}]$$

Now this equality and Eq. S61 can both be valid for nonzero \bar{n}_{Di} only if the determinant of the coefficients vanishes:

$$\begin{vmatrix} \bar{\tau} - \tau_1 & \bar{\tau} - \tau_2 \\ \frac{E}{1-E} - \frac{\varepsilon_1}{1-\varepsilon_1} & \frac{E}{1-E} - \frac{\varepsilon_2}{1-\varepsilon_2} \end{vmatrix} = 0. \quad [\text{S66}]$$

The solution of this secular equation is $\bar{\tau} = f(E)$, where $f(E)$ is given in Eq. S57 and Eq. 8 of the main text. Thus in the limit of large photon counts (no shot noise),

$$P(E, \tau) = \delta(\tau - f(E)) \left\langle \delta \left(E - \frac{\bar{n}_A}{\bar{n}_A + \gamma \bar{n}_D} \right) \right\rangle_{s(t), R(t)} \quad [\text{S67}]$$

so that Eqs. 8–10 for the dynamic two-state lines hold for diffusing molecules. The only assumption required is that the acceptor and donor detection efficiencies have the same dependence on the position of the molecule in the laser spot.

Generating Function in the Presence of Diffusion and Conformational Dynamics. Our previous derivation of a formula for the generating function of the probability of finding N_A acceptor and N_D donor photons in a bin (2, 8) can be readily generalized to include donor fluorescence lifetimes. The result is

$$\begin{aligned} \sum_{N_A, N_D} \int_0^\infty \lambda_A^{N_A} (\lambda_D e^{-z\tau})^{N_D} P(N_A, N_D, \tau) d\tau \\ = \exp \left(c \mathbf{1}^\top \int (\mathbf{g} - \mathbf{p}) dR \right), \end{aligned} \quad [\text{S68}]$$

where c is the concentration of diffusing molecules, \mathbf{p} is the column vector of equilibrium probabilities of M discrete states, $\mathbf{1}^\top$ is the row vector with all elements equal to 1, and the vector $\mathbf{g}(R, t)$ satisfies

$$\frac{d\mathbf{g}}{dt} = [\mathbf{D}_r \nabla^2 + \mathbf{K} - (1 - \lambda_A) \mathcal{N}_A - \mathcal{N}_D + \lambda_D \hat{\mathcal{P}} \mathcal{N}_D] \mathbf{g} \quad [\text{S69}]$$

subject to the initial condition $\mathbf{g}(t=0) = \mathbf{p}$. Here \mathbf{D}_r is a diagonal matrix with the translational diffusion coefficients of the various states on the diagonal, \mathcal{N}_A (\mathcal{N}_D) is the diagonal matrix with elements $n_{As}(R)$ [$n_{Ds}(R)$], $s = 1, \dots, M$, where R specifies the location of the molecule in the laser spot and $\hat{\mathcal{P}}$ is a diagonal matrix with elements $\int_0^\infty \exp(-zt) \mathcal{P}_s(t) dt$, where $\mathcal{P}_s(\delta t)$ is the delay time distribution in state s .

Joint Distribution for Diffusing Molecules. In this section, we consider the joint distribution for diffusing molecules with multiple interconverting conformational states. As mentioned in *Two-State Dynamic Lines for Diffusing Molecules*, the donor and acceptor count rates, $n_{A,D}(R, s)$, depend on the conformational state s and the location in the spot R . The count rates fluctuate due to transitions between states and due to diffusion. The distribution of the delay times, $\mathcal{P}(\delta t|s)$, is modulated only by the interconversion between the states.

The averaging in Eq. 5 (main text) should be performed over both the state trajectories, $s(t)$, and the trajectories of the diffusing molecule, $R(t)$. The joint distribution can be written as

$$P(N_A, N_D, \tau) = \left\langle \frac{[\bar{n}_A T]^{N_A} [\bar{n}_D T]^{N_D}}{N_A! N_D!} e^{-(\bar{n}_A + \bar{n}_D) T} \bar{P}(\tau | N_D) \right\rangle_{s(t), R(t)} \quad [\text{S70a}]$$

$$= \left\langle \frac{[\bar{n} T]^{N_A + N_D}}{(N_A + N_D)!} e^{-\bar{n} T} \frac{(N_A + N_D)!}{N_A! N_D!} \bar{e}^{-N_A} (1 - \bar{e})^{N_D} \bar{P}(\tau | N_D) \right\rangle_{s(t), R(t)} \quad [\text{S70b}]$$

where we have defined $\bar{n} = \bar{n}_A + \bar{n}_D$, $\bar{n}_{A,D} = \int_0^T n_{A,D}(t) dt / T$, $n_{A,D}(t) \equiv n_{A,D}(R(t), s(t))$, and $\bar{e} = \bar{n}_A / (\bar{n}_A + \bar{n}_D)$, $\bar{P}(\tau | N_D)$ is

the distribution of $\tau = \sum_{i=1}^{N_D} \delta t_i / N_D$, where δt_i are distributed according to

$$\bar{\mathcal{P}}(\delta t) = \frac{\int_0^T \mathcal{P}(\delta t | s(t)) n_D(t) dt}{\int_0^T n_D(t) dt}. \quad [\text{S71}]$$

We now assume that the total count rate does not depend on the conformational state, $n_A(R, s) + n_D(R, s) = n(R)$, so that the \bar{n} depends only on the diffusive trajectories. This assumption implies that the γ -factor is equal to 1. If the donor and acceptor detection efficiencies have the same dependence on R , it follows from Eq. S26 that the apparent FRET efficiency, defined as $n_A(R, s) / (n_A(R, s) + n_D(R, s)) = \varepsilon(s)$, does not depend on the location in the laser spot. Then $\bar{\varepsilon}$ can be written as $\bar{\varepsilon} = \int_0^T n(R(t)) \varepsilon(s(t)) dt / \int_0^T n(R(t)) dt$. Note that $\bar{\varepsilon}$, because it involves the fluorescence intensity, still depends on the diffusive trajectory. Only if we assume that fluctuations of $n(R(t))$ due to diffusion are small (i.e., it is essentially constant during the bin time), can we write $\bar{\varepsilon} \approx \int_0^T \varepsilon(s(t)) dt / T$, which is why the distribution does not exactly factor. If we make the same quasi-static approximation to the distribution of lifetimes—i.e.,

$$\begin{aligned} \bar{\mathcal{P}}(\delta t) &= \frac{\int_0^T n(R(t)) [1 - \varepsilon(s(t))] \mathcal{P}(\delta t | s(t)) dt}{\int_0^T n(R(t)) [1 - \varepsilon(s(t))] dt} \\ &\approx \frac{\int_0^T [1 - \varepsilon(s(t))] \mathcal{P}(\delta t | s(t)) dt}{\int_0^T [1 - \varepsilon(s(t))] dt} \end{aligned} \quad [\text{S72}]$$

—then the averaging over diffusion and state trajectories in Eq. S70b can be decoupled, so that

$$\begin{aligned} P(N_A, N_D, \tau) \\ \approx P(N_A + N_D) \frac{(N_A + N_D)!}{N_A! N_D!} \langle e^{\bar{N}_A} (1 - \bar{\varepsilon})^{N_D} \bar{P}(\tau | N_D) \rangle_{s(t)}, \end{aligned} \quad [\text{S73}]$$

where $P(N_A + N_D) = \langle [\bar{n}T]^{N_A + N_D} \exp(-\bar{n}T) / (N_A + N_D)! \rangle_{R(t)}$ is the distribution of the sum of donor and acceptor photons, which depends on diffusing trajectories and is taken from the data.

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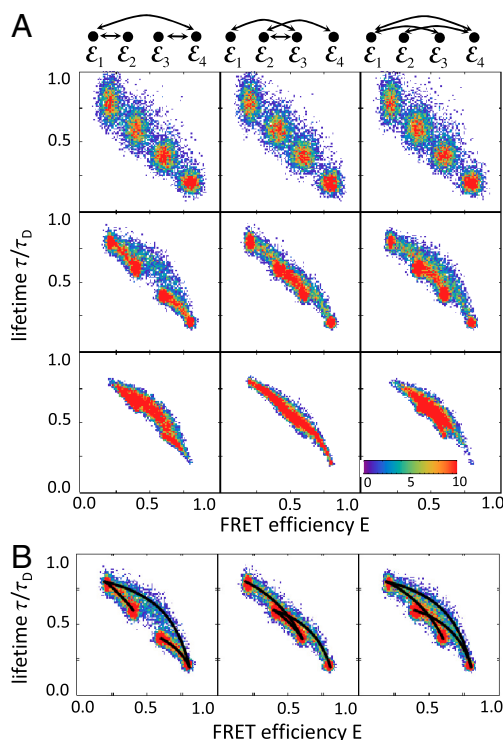


Fig. S1. FRET efficiency and lifetime density histograms for a molecule with four interconverting states with three different connectivities as a function of increasing bin time (rows, top to bottom $T = 1, 6,$ and 30 ms). FRET efficiencies of the states are $\varepsilon_1 = 0.2, \varepsilon_2 = 0.4, \varepsilon_3 = 0.6,$ and $\varepsilon_4 = 0.8$. The relative lifetimes are $0.8, 0.6, 0.4,$ and 0.2 . The histograms were simulated using the algorithm discussed in *Methods* with $n_{As} + n_{Ds} = 100 \text{ ms}^{-1}, s = 1, 2, 3, 4$. (B) The superposition of the histogram for $T = 6$ ms with the two-state dynamic lines (black) calculated using Eq. 9 in the main text for all pairs of directly connected states.

