Protein dynamics from single-molecule fluorescence intensity correlation functions

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Fluorescence intensity correlation functions contain information about photophysical and conformational dynamics. We propose and implement a simple procedure to analyze such functions measured in the presence of resonance energy transfer. When there is a separation of time scales and the conformational dynamics is modeled as diffusion in the potential of mean force along the interdye distance, we obtain an analytic expression for the conformational correlation time. This can be used to find the diffusion coefficient describing conformational fluctuations given the photon count rate and equilibrium distribution. © *2009 American Institute of Physics*.
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I. INTRODUCTION

Fluorescence correlation methods allow the measurement of translational, rotational, and conformational dynamics of molecules over a wide range of time scales.[1] In combination with Förster resonance energy transfer (FRET),[2] inter- and intramolecular distances between about 1 and 10 nm can be probed. FRET is based on the highly distance-dependent transfer of excitation energy between a pair of appropriate donor and acceptor fluorophores via dipole-dipole coupling.[3] If such dyes are attached to a macromolecule, changes in intramolecular distances can be monitored via the resulting changes in photon emission rates from the donor and the acceptor. If single molecule detection is used to avoid ensemble averaging, spontaneous distance fluctuations at equilibrium can be studied via intensity correlation functions.[1] Recently, this approach has been extended to probe conformational dynamics in polymers down to the nanosecond range.[4–7]

The measured intensity correlation functions are influenced by both polymer dynamics and photophysical transitions between the excited and ground states of the fluorophores. By modeling polymer dynamics as diffusion in the presence of a potential of mean force[8] and including the stochastic kinetics of the electronic transitions, the correlation functions can be calculated from combined Brownian dynamics and Monte Carlo simulations.[9] A more efficient numerical procedure for analyzing experimental data is based on discretizing the diffusion operator and expressing the correlation function as an exponential of a rate matrix that describes both electronic and conformational transitions.[5,10]

In this paper we introduce a more direct and much simpler procedure to analyze intensity correlation functions to yield information about conformational dynamics. By exploiting the separation of time scales between fast photophysical and slow conformational dynamics, we obtain an analytic expression for the conformational correlation time that can be measured by simply fitting the experimental correlation functions. Our expression depends on the distribution of interdye distances, the photon count rate, and the diffusion coefficient that describes conformational fluctuations. The first two quantities can be determined from independent measurements, so that the diffusion coefficient can be obtained directly from the experimentally determined decay rate.

II. THEORY

A. General formalism

We start with the general theory for intensity correlation functions when photon emission and conformational dynamics are described by a kinetic scheme, which includes both electronic and conformational states.[10] Let K_{ij} be the rate of $j\rightarrow i$ transition. Let $\mathbf{K}$ be the matrix of all such rates with $K_{jj}=-\Sigma_{i\neq j}K_{ij}$. Emitted photons are associated with transitions from excited to ground states. Let $\mathbf{V}$ be the matrix with off-diagonal elements equal to the rates of monitored transitions. For example, if we are monitoring only photons corresponding to the $m\rightarrow m'$ transition, $\mathbf{V}$ has only one nonzero element $V_{m'm}=\phi K^{\mathrm{rad}}_{m'm}$, where $K^{\mathrm{rad}}_{m'm}$ is the radiative transition rate and ϕ is the detection efficiency.

The intensity correlation function $g(t)$ normalized so that $g(\infty)=1$ can be expressed as[10]

$$g(t)=\frac{\langle I(t)I(0)\rangle}{\langle I\rangle^2}=\frac{\mathbf{1}^{\top}\mathbf{V}e^{\mathbf{K}t}\mathbf{V}\mathbf{p}_{ss}}{(\mathbf{1}^{\top}\mathbf{V}\mathbf{p}_{ss})^2}, \tag{1}$$

where $\mathbf{1}$ is the unit vector, $\mathbf{p}_{ss}$ is the vector of the normalized steady-state probabilities, which are obtained by solving $\mathbf{K}\mathbf{p}_{ss}=0$ with $\mathbf{1}^{\top}\mathbf{p}_{ss}=1$.

The rate matrix that combines protein dynamics and distance-dependent photon emission from the donor-acceptor pair can be formally represented as

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$$\mathbf{K} = \mathbf{K}_0(r) + \mathbf{I}\mathcal{L}_r, \tag{2}$$

where $\mathbf{K}_0(r)$ is the rate matrix that describes only electronic transitions. It depends on a conformational coordinate r through the rate of energy transfer. $\mathbf{I}$ is the identity matrix and $\mathcal{L}_r$ is the operator describing conformational dynamics. For the diffusion on a free energy profile

$$\mathcal{L}_r = D\frac{\partial}{\partial r}p_c(r)\frac{\partial}{\partial r}p_c(r)^{-1}, \tag{3}$$

where D is the diffusion coefficient and $p_c(r)$ is the normalized equilibrium distribution of the conformational coordinate r.

To directly apply Eq. (1), the operator $\mathcal{L}_r$ must be discretized.[5] Then $\mathbf{K}$ becomes a $MN \times MN$ block matrix, which is the direct product of $\mathbf{K}_0(r)$ (of size $M \times M$) and the discretized $\mathcal{L}_r$ (of size $N \times N$).

B. Separation of time scales

When conformational dynamics are slow compared to photophysical transitions, the above formalism can be simplified. For an arbitrary correlation function one can exploit time scale separation as follows. Consider the correlation function $\langle A(t)A(0)\rangle$ of quantity $A(t)$, which depends on two coupled variables. $A(t)$ changes in time due to both fast and slow fluctuations of these variables, which we refer to as fast and slow variables. At short times, the slow variable does not change; therefore, the correlation function is equal to $\langle\langle A(t)A(0)\rangle_f\rangle_s$, where $\langle A(t)A(0)\rangle_f$ is the correlation function resulting from fluctuations of the fast variable at a fixed value of the slow one and $\langle\cdots\rangle_s$ means averaging over the equilibrium distribution of the slow variable. At intermediate times that are short compared to the characteristic time of slow fluctuations and long compared to that of fast fluctuations, $A(t)$ is completely averaged over the fast variable at a fixed value of the slow variable; therefore, the correlation function is a constant, $\langle\langle A\rangle_f^2\rangle_s$, where $\langle\cdots\rangle_f$ means averaging over the fast variable at a fixed slow variable. At long times, the average $\langle A\rangle_f$ still fluctuates because of slow dynamics and the resulting correlation function is denoted by $\langle\langle A\rangle_f(t)\langle A\rangle_f(0)\rangle_s$. Combining these expressions, we can find an approximation that reduces correctly at short, long, and intermediate times:

$$\langle A(t)A(0)\rangle = \langle\langle A(t)A(0)\rangle_f\rangle_s - \langle\langle A\rangle_f^2\rangle_s + \langle\langle A\rangle_f(t)\langle A\rangle_f(0)\rangle_s. \tag{4}$$

Similarly one can construct an approximation in the "product" form:

$$\frac{\langle A(t)A(0)\rangle}{\langle A\rangle^2} = \frac{\langle\langle A(t)A(0)\rangle_f\rangle_s}{\langle\langle A\rangle_f^2\rangle_s}\frac{\langle\langle A\rangle_f(t)\langle A\rangle_f(0)\rangle_s}{\langle\langle A\rangle_f\rangle_s^2}. \tag{5}$$

When the slow and fast relaxation times are sufficiently different, these two expressions are equivalent.

C. Intensity correlation functions for slow conformational dynamics

Let us apply this approximation to the intensity correlation function when the photophysical transition rates are modulated by slow conformational dynamics. In this case the excited and ground state populations reach steady state before the conformational coordinate changes. The photon count rate (i.e., the number of photons per unit time) at a fixed conformational coordinate (the term $\langle A\rangle_f$) is

$$n(r) = \mathbf{1}^\top\mathbf{V}\mathbf{p}_{ss}^0(r), \tag{6}$$

where $\mathbf{p}_{ss}^0(r)$ is the vector of local steady-state probabilities at fixed r, $p_{ss}^0(i,r)$, which are obtained by solving $\mathbf{K}_0(r)\mathbf{p}_{ss}^0(r) = 0$ and $\mathbf{1}^\top\mathbf{p}_{ss}^0(r) = 1$, for each r.

In the present context, Eq. (5) for the intensity correlation function becomes

$$g(t) = g_{\mathrm{ph}}(t)g_c(t), \tag{7}$$

where $g_{\mathrm{ph}}(t)$ is the correlation function that describes the decay of fast photophysical fluctuations:

$$g_{\mathrm{ph}}(t) = \langle\mathbf{1}^\top\mathbf{V}e^{\mathbf{K}_0 t}\mathbf{V}\mathbf{p}_{ss}^0\rangle_c/\langle n^2\rangle_c, \tag{8}$$

where $\langle\cdots\rangle_c$ denotes an average over the equilibrium distribution of the conformational coordinate (the slow variable):

$$\langle\cdots\rangle_c \equiv \int\cdots p_c(r)dr. \tag{9}$$

The second factor in Eq. (7) is the correlation function that describes only slow fluctuations of the count rate due to conformational dynamics,

$$g_c(t) = \langle n[r(t)]n[r(0)]\rangle_c/\langle n\rangle_c^2. \tag{10}$$

Here $\langle n(t)n(0)\rangle_c$ is the correlation function of the photon count rate $n[r(t)]$, where the dynamics of $r(t)$ is described by the operator $\mathcal{L}_r$. The approximation in Eq. (7) reproduces the correlation function at short (compared to slow conformational dynamics) and long (compared to fast photophysical relaxation) times. At $t=0$, g_{ph} is zero for almost all kinetic schemes because of antibunching. This follows from Eq. (8) because $\mathbf{V}^2=0$ for all kinetic schemes, except those where the final state of one monitored transition is the initial state of another.

The correlation functions $g_{\mathrm{ph}}(t)$ and $g_c(t)$ are in general multiexponential functions of time. We further approximate these correlation functions as single exponentials that are correct at $t=0$ and at $t=\infty$:

$$g(t) \approx (1 - e^{-t/\tau_{\mathrm{ph}}})\left(1 + \frac{\langle n^2\rangle_c - \langle n\rangle_c^2}{\langle n\rangle_c^2}e^{-t/\tau_c}\right). \tag{11}$$

The decay time of the photophysical correlation function τ_{ph} can be determined by requiring the area under $1-g_{\mathrm{ph}}$ to be exact [i.e., the same as what would be obtained using Eq. (8)]. Alternately, the decay time τ_{ph} can be found by requiring the slope at $t=0$ to be exact. The decay time of the conformational correlation function τ_c is determined by requiring the area under g_c-1 to be exact:

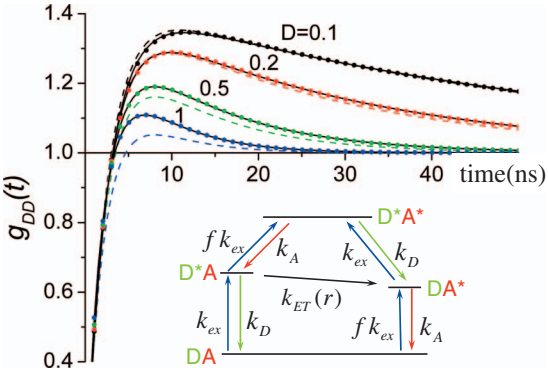

FIG. 1. Donor-donor intensity correlation functions for various diffusion coefficients. Exact results (dots) are compared with the approximate ones (dashed lines) obtained from Eqs. (11)–(16). Full lines show a two-exponential least-squares fit, Eq. (11) with three adjustable parameters. The excitation rate is $k_{ex}=0.02$ ns^{-1}, the decay rates are $k_A=k_D=0.27$ ns^{-1}, the direct excitation factor is $f=0.05$, the energy transfer rate is $k_{ET}(r)=k_D(R_0/r)^6$, Förster radius is $R_0=5.4$ nm, rms interdye distance is $\sqrt{\langle r^2\rangle}=6$ nm, $D=0.1,0.2,0.5$, and 1 nm^2 ns^{-1}. Inset: kinetic scheme for FRET with donor re-excitation and direct excitation of the acceptor.

$$\tau_c = \int_0^\infty \frac{\langle \delta n(t)\,\delta n(0)\rangle_c}{\langle \delta n^2\rangle_c} dt, \tag{12}$$

where $\delta n = n - \langle n\rangle_c$. When the conformational dynamics is described as diffusion on a free energy profile, τ_c can be found analytically using the generalization of the theory of first passage times.[11] This formalism was first applied to rotational correlation functions that determine fluorescence depolarization of probes in uniaxial liquid crystals.[12] For a system in which the conformational coordinate is restricted to the interval $r_c \le r \le l_c$, it can be shown through a simple modification of the results in Ref. 13 that

$$\tau_c = \frac{\int_{r_c}^{l_c} (p_c(r))^{-1}\left[\int_{r_c}^{r} \delta n(\rho) p_c(\rho) d\rho\right]^2 dr}{D\int_{r_c}^{l_c} \delta n(r)^2 p_c(r) dr}, \tag{13}$$

where $\int_{r_c}^{l_c} p_c(r)dr = 1$. This is one of the key results of this paper. It dramatically simplifies the analysis of experimental intensity correlation functions to yield dynamical information about conformational fluctuations.

III. RANGE OF VALIDITY AND APPLICATION TO EXPERIMENT

In order to demonstrate the utility of the above results, consider FRET between donor and acceptor fluorophores attached to a flexible polymer. Depending on the donor-acceptor distance r, the donor dye emits a photon or transfers the excitation to the acceptor resulting in emission of acceptor photons. This process can be described by the kinetic scheme shown in the inset of Fig. 1, which also includes donor re-excitation ($DA^* \to D^*A^*$) and direct excitation of the acceptor ($DA \to DA^*$ and $D^*A \to D^*A^*$).[5,6,9] We use the general formalism presented in Sec. II A to generate "exact" donor-donor intensity correlation functions.[5] These are then compared with the approximation in Eq. (11). Finally, a simple procedure to obtain information about conformational dynamics from the intensity correlation function is introduced and applied to experimental data.

The rate matrix that describes electronic transitions, $\mathbf{K}_0(r)$, for the above model is [in the basis (DA, D^*A, D^*A^*, DA^*)],

$$\mathbf{K}_0(r) = \begin{pmatrix} -k_{ex}(1+f) & k_D & 0 & k_A \\ k_{ex} & -k_D - fk_{ex} - k_{ET}(r) & k_A & 0 \\ 0 & fk_{ex} & -k_A - k_D & k_{ex} \\ fk_{ex} & k_{ET}(r) & k_D & -k_A - k_{ex} \end{pmatrix}.$$

The matrix of transitions resulting in the detected donor photons, $\mathbf{V}$, has nonzero off-diagonal elements corresponding to the transitions $D^*A \to DA$ and $D^*A^* \to DA^*$,

$$\mathbf{V} = \phi_D \begin{pmatrix} 0 & k_D^{rad} & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & k_D^{rad} & 0 \end{pmatrix}, \tag{14}$$

where ϕ_D is the detection efficiency of the donor photons. Conformational dynamics is described by diffusion in a potential of mean force that corresponds to the end-to-end distribution of a Gaussian chain:

$$p_c(r) = \frac{r^2 \exp(-3r^2/2\langle r^2\rangle)}{\int_{r_c}^{l_c} r^2 \exp(-3r^2/2\langle r^2\rangle) dr}, \tag{15}$$

where r_c and l_c are the contact radius and the contour length and $\langle r^2\rangle$ is the mean square end-to-end distance when $r_c=0$ and $l_c=\infty$.

The "exact" donor-donor intensity correlation function, Eq. (1), will now be compared with the approximation in Eq. (11). To find the required parameters, we first obtain the local steady state populations by solving $\mathbf{K}_0(r)\mathbf{p}_{ss}^0(r)=0$ and requiring that $p_{ss}^0(DA,r)+p_{ss}^0(D^*A,r)+p_{ss}^0(D^*A^*,r)+p_{ss}^0(DA^*,r)=1$. The photophysical correlation time τ_{ph} is determined by requiring that the initial slope of the correlation function be the same as that in Eq. (8). For the donor-donor autocorrelation function, this leads to

$$\tau_{ph} = k_{ex}^{-1} \frac{\langle (p_{ss}^0(D^*A,r) + p_{ss}^0(D^*A^*,r))^2\rangle_c}{\langle p_{ss}^0(D^*A,r) + p_{ss}^0(D^*A^*,r)\rangle_c}, \tag{16}$$

where k_{ex} is the excitation rate (see Fig. 1). This time does not depend on the conformational dynamics. On the other hand, the conformational correlation time τ_c in Eq. (13) is inversely proportional to the diffusion coefficient. It also depends on the normalized end-to-end equilibrium distribution and the mean donor photon count rate at fixed distance between donor and acceptor, which is found using Eqs. (6) and (14):

$$n(r) = \phi_D k_D^{rad} (p_{ss}^0(D^*A,r) + p_{ss}^0(D^*A^*,r)). \tag{17}$$

Here ϕ_D and k_D^{rad} are the detection efficiency and the radiative rate. Note that τ_c does not depend on these parameters.

Figure 1 compares the exact and approximate donor-donor intensity correlation functions for the scheme shown in the inset when the energy transfer rate is modulated by diffusive chain dynamics. The correlation functions exhibit nonmonotonic behavior. There is a dip at short times due to antibunching. After reaching a maximum, the correlation

TABLE I. Photophysical and conformational correlation times for various diffusion coefficients. The times τ_{ph}^{fit} and τ_c^{fit} are obtained by fitting the correlation functions to two exponentials. τ_c^{fit} should be compared to τ_c predicted by Eq. (13). τ_{ph}=2.52 ns for all D, as predicted by Eq. (16). The ratio $\tau_c^{fit}/\tau_{ph}^{fit}$ is a measure of the separation of time scales.

D ($nm^2\ ns^{-1}$)	τ_{ph}^{fit} (ns)	τ_c^{fit} (ns)	τ_c (ns)	$\tau_c^{fit}/\tau_{ph}^{fit}$
0.1	2.62	53.8	51.7	20
0.2	2.55	28.3	25.9	11
0.5	2.42	12.0	10.4	5
1	2.30	6.21	5.17	2.7
2	2.14	3.42	2.59	1.6

functions decay with a rate that depends on the diffusion coefficient dependent. This bunching of photons is due to conformational dynamics. When D=0.1 and 0.2 $nm^2\ ns^{-1}$, the time scales of the photophysical and conformational dynamics are well separated and the exact and approximate correlation functions agree over the whole time range. As D increases, the two processes become coupled and our approximation deteriorates.

Full lines in Fig. 1 show the two-exponential fit based on Eq. (11). The parameters τ_{ph}, τ_c, and the amplitude of the conformational correlation function were adjusted, not calculated as for the dashed lines. Although both $g_{ph}(t)$ and $g_c(t)$ are multiexponential, the two-exponential fit works very well, even when electronic transitions and conformational dynamics are coupled.

In Table I, we compare the fitted correlation times with those predicted by Eqs. (13) and (16). As expected, when fast and slow dynamics are well separated ($\tau_c^{fit}/\tau_{ph}^{fit}>10$), the fitted and calculated relaxation times agree with each other. Remarkably, even when τ_{ph}^{fit} and τ_c^{fit} differ by only a factor of 2 and Eq. (11) is a poor approximation, Eq. (13) predicts the conformational correlation time within 25%.

The above comparison suggests the following procedure for obtaining the effective diffusion coefficient of chain dynamics from the measured intensity correlation function. First, the fast and slow correlation times are determined by fitting the correlation function. Given the equilibrium distribution of the conformational coordinate and the dependence of the photon count rate on this coordinate, D can be found using Eq. (13). The accuracy of this procedure increases with increasing $\tau_c^{fit}/\tau_{ph}^{fit}$.

We now apply our procedure to experimental donor-donor intensity correlation functions measured for the unfolded subpopulation of the cold shock protein[5] [see Fig. 2(a)]. We suppose that bunching of photons and decay of the correlation function is due to polymer dynamics that results in interdye distance fluctuations. Dye orientation, which affects the photon count rate through the κ^2 factor in the energy transfer rate, is assumed to be averaged out by rapid rotational diffusion of the dyes. This assumption is supported by the observation that the decay time of the fluorescence anisotropy of the unfolded cold shock protein labeled only with a donor dye is less than 1 ns.[6] In addition, no bunching effect has been observed for rigid polyproline, which was labeled with the same dyes and with the protein labeled only

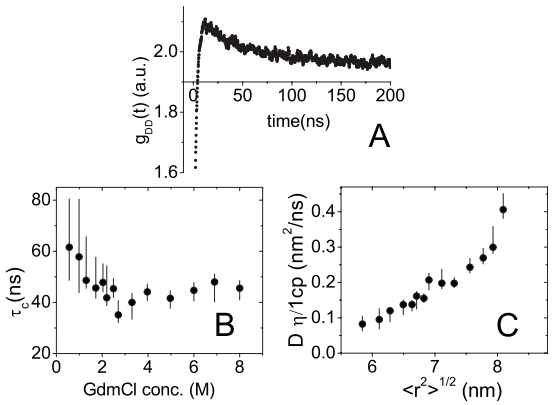

FIG. 2. (a) Intensity autocorrelation function of donor emission from unfolded cold shock protein at 4M GdmCl. (b) Denaturant dependence of the slow decay time of the correlation function. (c) Viscosity-corrected end-to-end diffusion coefficient calculated from the correlation times using Eq. (13).

with a donor dye.[5] Therefore, although dye reorientation might affect the fast antibunching part of the correlation function, it is unlikely that it will influence the slow decay time of the correlation function, τ_c.

The conformational correlation times obtained by fitting the experimental correlation functions are plotted in Fig. 2(b) as a function of the denaturant [guanidinium hydrochloride (GdmCl)] concentration. To obtain D using Eq. (13), we need to know $n(r)$ and $p_c(r)$. The end-to-end distribution was assumed to be that of a Gaussian chain with a denaturant dependent $\langle r^2\rangle$ determined by fitting the peak position of the energy transfer efficiency histogram obtained from single-molecule measurements.[14] The distance-dependent count rate was calculated for the kinetic scheme shown in the inset of Fig. 1 using the independently determined parameters given in the caption. The resulting viscosity-corrected[5] diffusion coefficients ($D\eta/1\ cp$) are plotted as a function of the rms end-to-end distance in Fig. 2(c). They are in excellent agreement with those obtained using a much more elaborate procedure involving numerical solution of a reaction-diffusion equation using finite differences.[5] The diffusion coefficient increases with the end-to-end distance. Thus the more compact the protein, the slower its internal dynamics.

It is interesting to compare the decay times determined from the donor-donor intensity correlation function with the relaxation time of the end-to-end distance correlation function $\tau_r=\int_0^\infty\langle\delta r(t)\delta r(0)\rangle_c/\langle\delta r^2\rangle_c dt$. This time can be referred to as the chain reconfiguration time. For a Gaussian chain with r_c=0 and l_c=∞, $\tau_r\approx\langle r^2\rangle/6.22D$, as can be verified by replacing $n(r)$ by r in Eq. (13). To show how the decay time of the conformational correlation function τ_c is related to the chain reconfiguration time, we plot τ_c/τ_r as a function of $\sqrt{\langle r^2\rangle}/R_0$ in Fig. 3. When the rms end-to-end distance is near the Förster radius, the correlation decay time is close to the chain reconfiguration time, as was observed experimentally.[5] If $\langle r^2\rangle$ is known, this figure can be used to determine D from the measured τ_c.

IV. CONCLUDING REMARKS

In this paper we introduced a simple procedure for analyzing fluorescence intensity correlation functions. When

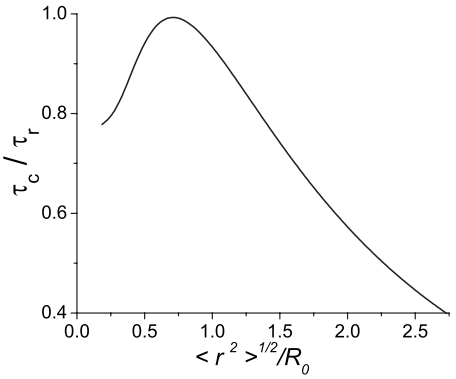

FIG. 3. The ratio of the donor-donor fluorescence intensity correlation decay time, τ_c, and the chain reconfiguration time, τ_r, as a function of the ratio of rms end-to-end distance of a Gaussian chain to the Förster radius.

there is a separation of time scales, the correlation function can be factored as a product of the correlation functions describing only fast photophysical and only slow conformational fluctuations. When the conformational dynamics are described by diffusion in the presence of a potential of mean force, the corresponding correlation time can be calculated analytically [Eq. (13)]. Given the experimentally determined τ_c, this expression can be used to obtain the effective diffusion coefficient that describes conformational dynamics.

We specifically considered only donor-donor intensity autocorrelation functions. The acceptor-acceptor and cross-correlation functions can be found analogously. The photophysical relaxation time [and the amplitude of the exponent $\exp(-t/\tau_{\mathrm{ph}})$ in the crosscorrelation function] will be different. However, using Eq. (13) and the work of Camley *et al.*,[15] it can be shown that the decay time τ_c obtained from all auto- and crosscorrelation functions is the same. This is true for any kinetic scheme describing photophysical transitions as long as it contains only one rate that depends on the conformational coordinate.

Finally, we note that there is an interesting mathematical correspondence between the procedure proposed here to analyze intensity correlation functions and that used to analyze triplet quenching.[16] In these experiments, chain dynamics are probed by measuring the quenching rate k_{obs} of the triplet state of a tryptophan located at one end of the polypeptide chain by a cysteine on the other. The observed rate depends both on how the quenching rate depends on the end-to-end distance, $q(r)$, and on how this distance r fluctuates because of conformational dynamics. The end-to-end diffusion coefficient can be extracted from k_{obs} measured as a function of viscosity using the approximate expression:[16]

$$\frac{1}{k_{\mathrm{obs}}} = \frac{1}{\langle q\rangle_c} + \frac{\int_{r_c}^{l_c}(p_c(r))^{-1}\left[\int_{r_c}^{r}\delta q(\rho)p_c(\rho)d\rho\right]^2 dr}{D\langle q\rangle_c^2}, \tag{18}$$

where $\delta q = q(r) - \langle q\rangle_c$. Note that the second term is virtually identical to Eq. (13) with the photon count rate $n(r)$ being replaced by the quenching rate $q(r)$, even though the physics of the two experiments is quite different.

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