

Using fluorescence resonance energy transfer to measure distances along individual DNA molecules: Corrections due to nonideal transfer

Chandran R. Sabanayagam, John S. Eid, and Amit Meller^{a)}

Rowland Institute at Harvard, Harvard University, Cambridge, Massachusetts 02142

(Received 24 September 2004; accepted 8 December 2004; published online 3 February 2005)

Single molecule fluorescence resonance energy transfer has been extensively used to measure distance changes and kinetics in various biomolecular systems. However, due to complications involving multiple de-excitation pathways of the dyes, the absolute inter-dye distance information has seldom been recovered. To circumvent this we directly probe the relative variations in the quantum yield of individual fluorophores. B-DNA was used as a scaffold to position the donor (Cy3 or TMR) at precise distances from the acceptor (Cy5) within the Förster radius. We found that the variation in the Cy3 quantum yield is ~ 5 times larger than that of TMR. By taking into account the molecule-to-molecule variability in the acceptor/donor quantum yield ratio, the apparent fluorescence resonance energy transfer efficiencies were scaled to yield the theoretical values. We obtained very good agreement with a physical model that predicts distances along B-DNA. © 2005 American Institute of Physics. [DOI: 10.1063/1.1854120]

Elucidating the various structures and dynamics of nucleic acids is essential to understanding their specific functions at the molecular level. A sensitive tool used to measure distances at the nanometer scale is fluorescence resonance energy transfer (FRET), which exploits a strong donor-acceptor distance dependence (R^{-6}).¹ FRET has been extensively used to study different nucleic acid forms, for example: A-, B- and Z-DNA,^{2,3} quadruplexes,⁴ four-way junctions,⁵ hairpins,⁶ single-stranded DNAs,⁷ and catalytic RNAs.⁸

With single molecule FRET (SM-FRET) the dynamics of individual DNA or RNA molecules can be probed.^{9,10} SM-FRET can be detected from freely diffusing molecules in solution,¹¹ or confined molecules using a variety of immobilization methods.⁹ In either case, SM-FRET has been typically used to probe relative variations in FRET efficiency. Thus, the absolute inter-dye distance information has not been directly recovered, complicating the interpretation of the SM data and the comparison with bulk experiments and models. Here we describe a method to quantify the SM-FRET efficiency of immobilized DNA molecules based on the measurement of all possible photon contributions to the donor and acceptor channels. Unlike previous studies in which the fluorophores were linked to the 3' and/or to the 5' terminus of DNA,^{3,12,13} we present results obtained from internally labeled DNA molecules, which provides a rigid scaffold to attach the fluorophores (i.e., no distance fluctuations due to end fraying).

The DNA constructs were hairpins having a 30 basepair complementary region connected by a (dT)₅ linker. The middle dT residue of the linker contained a C₆-biotin moiety to allow the hairpin to be immobilized on a streptavidin surface. The donor was chemically conjugated to a single

amine-modified dT residue on a partial DNA hairpin. Then, the partial hairpin was completed by DNA polymerase with dTTP-Cy5, dATP, dCTP, and dGTP nucleotides. The partial hairpin was designed to allow only one site for dT incorporation. Five FRET labeled DNA constructs with different donor-acceptor distances, R_{DA} , were separately prepared using two donor-acceptor pairs, TMR-Cy5 and Cy3-Cy5. A schematic representation of the final constructs is shown in Fig. 1.

Single molecule FRET measurements were performed using an automated confocal microscope (514.5 nm, 10 μ W excitation) with a flow cell permitting the continuous exchange of an enzymatic oxygen scavenging system, as previously described.¹⁴ Direct excitation of Cy5 and the leakage of the acceptor emission into the donor channel were both $<1\%$. Fluorescence lifetime and anisotropy measurements were performed using time-correlated single-photon counting. Anisotropy was calculated using: $r(t) = (I_{VV}(t) - GI_{VH}(t)) / (I_{VV}(t) + 2GI_{VH}(t))$, with G for our instrument equal to 1.

For each molecule, the donor and acceptor intensities were collected until two seconds after both fluorophores photobleached. Figure 2 shows typical time traces for the two FRET pairs on the DNA construct, *ss9*. In order to extract the true acceptor and donor intensities used to calculate the FRET efficiency, each trace must be corrected for background, channel cross-talk, variations in the fluorophores' quantum yields, and the corresponding detection efficiencies of the two channels. Thus, the FRET efficiency determined from the sensitized acceptor emission (assuming negligible acceptor leakage into the donor channel) is given by

$$E = \frac{I_A - \beta I_D}{I_A + \gamma I_D}, \quad (1)$$

where I_A and I_D are the background corrected intensities from the acceptor and donor channels, respectively, β is the

^{a)}Authors to whom correspondence should be addressed. Electronic mail: meller@rowland.harvard.edu

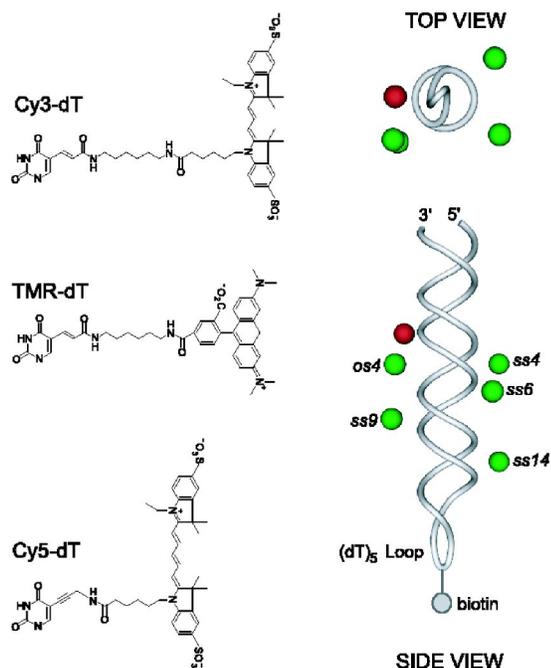


FIG. 1. Structure of the donors (TMR, Cy3) and acceptor (Cy5) linked to the C-5 methyl group of thymine (left). Composite representation of the five different FRET DNA constructs (right). The donor positions are shown in green and the acceptor position is shown in red. The oligonucleotide names describe the spatial relation between the donor and acceptor dyes of the final products, for example, *os4* denotes a four basepair separation, with the dyes located on opposite strands, whereas *ss4* denotes a 4 base separation of the dyes on the same strand.

leakage of the donor emission into the acceptor channel, and γ is a factor that includes the quantum yields of the fluorophores and the detection efficiencies of the two channels. The γ factor is defined as, $\gamma = \phi\eta / \alpha_D - \beta$, where ϕ is the ratio of acceptor to donor quantum yields, $\eta = 0.97$ is the ratio of the detection efficiencies in the acceptor and donor channels,¹⁴ and α_D is the fraction of spectral overlap of the donor emission with the donor channel. Thus, $\gamma + \beta$ is directly proportional to ϕ . The γ factor is determined experimentally from the ratio $(\langle I_A \rangle - \langle I'_A \rangle) / (\langle I_D \rangle - \langle I'_D \rangle)$, where $\langle I \rangle$ and $\langle I' \rangle$ are calculated from the average photon counts 100 ms before and after acceptor photobleaching, respectively. Donor leakage, β , is calculated from the ratio $\langle I'_A \rangle / \langle I'_D \rangle$. Equation (1) is similar to the derivation given by others,¹⁵ however unlike previous studies that use a common mean value for β and γ , we instead correct each trace individually.

FRET efficiency determination using the donor-quenching method¹⁶ was adopted for our single-molecule analysis, by comparing the donor intensities before and after photobleaching of the acceptor dye:

$$E = 1 - \frac{I_D}{I'_D}, \quad (2)$$

where I_D is the donor intensity during FRET, and I'_D is the intensity after the acceptor has photobleached. While Eq. (2) does not require any correction factors it suffers from signal-to-noise issues (I_D is very low for high FRET efficiency) and does not discern between quenching due to energy transfer

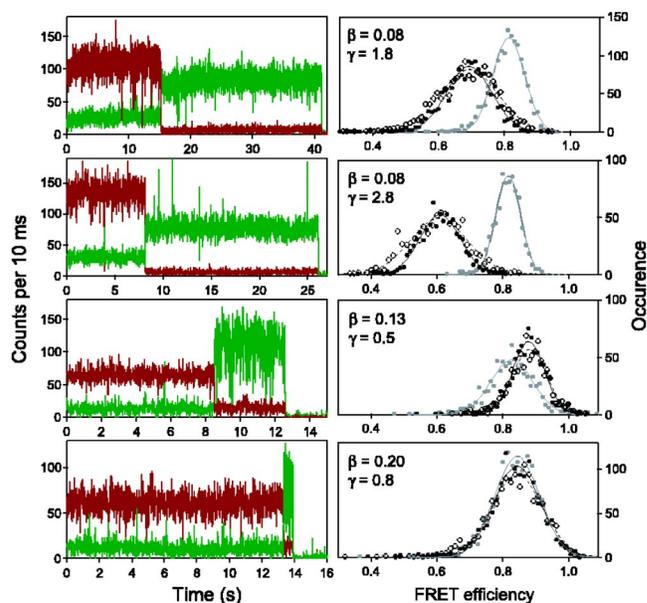


FIG. 2. Left panels show single-molecule fluorescence time traces (I_A red or black, I_D green or gray) and corresponding FRET efficiencies (right panels) from the *ss9* construct. The top two traces are from the Cy3-Cy5 pair, and the bottom two are from the TMR-Cy5 pair. The right panels each show three FRET efficiency histograms calculated by (1) Eq. (1) with $\gamma = 1$ and $\beta = 0$ (gray circles); (2) Eq. (1) with γ and β values calculated from each molecule as given in the trace (black circles); and (3) the donor-quenching energy transfer efficiencies calculated from Eq. (2) (empty circles).

and that due to other processes. Agreement between the two efficiencies calculated using Eqs. (1) and (2) requires that β and γ are properly determined. For both donor-acceptor pairs we display two traces with different γ values and compare the energy transfer efficiency distributions determined by Eq. (1) and Eq. (2) (Fig. 2). For reference we also show the FRET efficiency derived for the ideal case where $\gamma = 1$ and $\beta = 0$. The transfer efficiency distributions obtained by the two methods are in excellent agreement for both FRET pairs, and are either shifted to the right (TMR-Cy5) or left (Cy3-Cy5) with respect to the idealized case.

The β and $\gamma + \beta$ distributions of the two FRET pairs were compiled from over 2500 molecules. The peak value for β is 0.13 for TMR-Cy5 and 0.09 for Cy3-Cy5 (data not shown). The β distributions are well defined for the two donor fluorophores, with the expected higher value of the TMR leakage into the acceptor channel due to its red-shifted emission profile relative to that of Cy3. The distributions of $\gamma + \beta$ are given in Fig. 3 (inset). Note that these distributions are not symmetric and are clearly dissimilar. For the TMR-Cy5 pair, the peak value for $\gamma + \beta$ is 0.60 with a FWHM of 0.15; for the Cy3-Cy5 pair the peak value is 2.13 with a FWHM of 0.78. The average quantum yield ratio of Cy3 to TMR can be estimated by $(\gamma + \beta)_{\text{TMR}} / (\gamma + \beta)_{\text{Cy3}} = \alpha \phi_{\text{Cy3}} / \phi_{\text{TMR}}$, where $\alpha = \alpha_{\text{Cy3}} / \alpha_{\text{TMR}} = 1.7$ for our setup, yielding $\phi_{\text{Cy3}} / \phi_{\text{TMR}} = 0.47$, which is in close agreement to the 0.5 value calculated from Dietrich *et al.*¹³ The Cy3-Cy5 pair has a wider $\gamma + \beta$ distribution compared to the TMR-Cy5 pair that we attribute to a greater intermolecular variability in the quantum yield of Cy3 as compared with TMR.

We obtained the energy transfer statistics for each construct by calculating the most probable transfer efficiency,

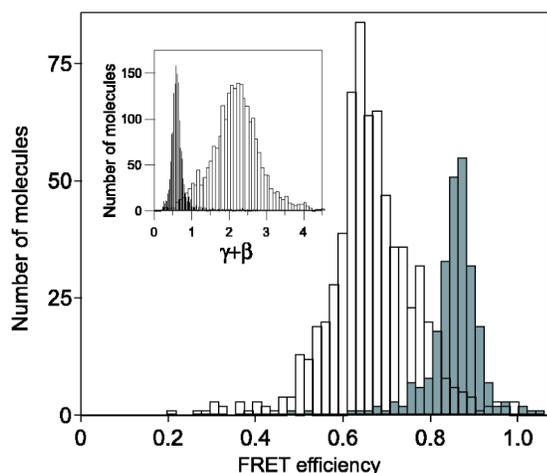


FIG. 3. Single molecule histograms of FRET efficiency calculated using Eq. (1) for the *ss9* construct with the Cy3-Cy5 pair (empty bars) and the TMR-Cy5 pair (gray bars). The inset shows the distributions of $\gamma+\beta$ (proportional to the acceptor-donor quantum yield ratio) for Cy3-Cy5 (empty bars) and TMR-Cy5 (gray bars) used to determine the SM-FRET efficiencies. Data in this figure was compiled from approximately 2500 individual traces.

E_{SM} , for each individual trace (Fig. 2). Then the histogram of the E_{SM} values for a set of over 500 molecules was used to determine the peak value E_{SM}^* . The histogram of E_{SM} values represents a transfer efficiency distribution in which each molecule is equally weighted (Fig. 3). An alternative method for calculating the FRET efficiency is to histogram the entire concatenated data set, and obtain the peak value, E_{all}^* ; this is the common analysis approach for single molecule data.¹⁷ Note however, that E_{all}^* weighs each molecule's efficiency by its duration. Regardless of which method is employed, the calculated FRET efficiency at the single molecule level will be incorrect if the γ and β factors are not individually determined. Moreover, the average FRET efficiency will shift due to the asymmetric nature of the γ distribution if an average value of γ is used to calculate E_{all}^* . For example, in the case of Cy3-Cy5 the γ distribution is skewed toward lower values, thus using the average γ will result in a shift towards higher FRET efficiencies. The different analyses used to calculate the FRET efficiency agree to within 4%.²²

The FRET efficiency is also given by: $E = 1 - \tau_{DA} / \tau_D$, where τ_{DA} and τ_D are the lifetimes of the donor in the presence and absence of energy transfer, respectively. We measured the fluorescence lifetime and anisotropy in bulk for the free donor, the free acceptor, the donor conjugated to DNA, and the FRET-labeled DNA constructs for the two FRET pairs (Fig. 4). The lifetime of the Cy3 is extremely short (<0.1 ns) in agreement with its known low quantum yield (<0.04). The Cy3 coupled to DNA displayed lifetimes (amplitudes) of 0.22 ns (0.8) and 0.86 ns (0.2), as shown in Fig. 4(a). The free TMR yielded a single lifetime of 2.4 ns, and the TMR coupled to DNA displayed a second lifetime component at 4.4 ns with a relative amplitude of 0.14 [Fig. 4(b)]. The double exponential lifetime distributions are likely due to a fast exchange between two conformational states of the dye coupled to DNA: a state in which the dye is primarily surrounded by polar water molecules (shorter lifetime), and a state in which the dye stays in close proximity to the less-

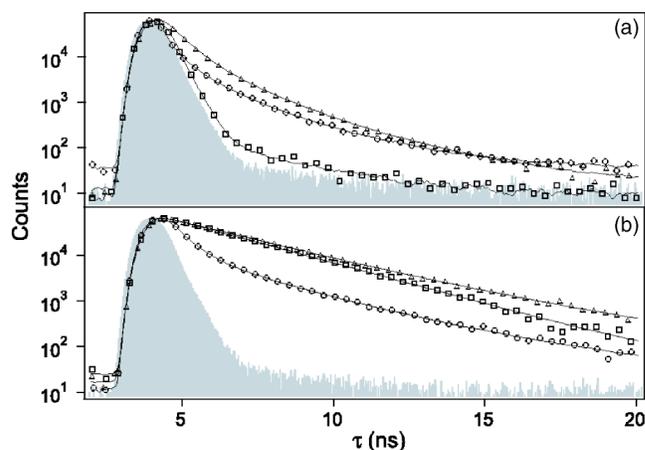


FIG. 4. Time correlated single photon counting lifetime measurements of free donor (squares), donor conjugated to DNA (triangles), and donor-Cy5, conjugated to *ss9* (circles) for (a) Cy3 and (b) TMR. Lines are deconvolution fits and the shaded region in both panels is the instrument response function. The lifetimes (amplitudes) for free TMR and TMR-DNA are 2.4 ns (1.0); 2.4 ns (0.86), 4.4 ns (0.14). The lifetime (amplitudes) of free Cy3 and Cy3-DNA are 0.15 ns (1.0); 0.22 ns (0.8), 0.86 ns (0.2). The rotational correlation times for TMR-DNA, Cy3-DNA, and Cy5-DNA are 1.11 ns, 0.63 ns, and 1.03 ns, respectively, indicating that the dyes are relatively free.

polar DNA (longer lifetime).¹⁸ Upon acceptor conjugation, both donors show at least four lifetimes (Fig. 4).

Given the complexity of the donor lifetimes, it is reasonable to expect that many donor relaxation pathways are present. In addition, other complications exist. Cy5 is known to isomerize between a fluorescent *trans* and a dark *cis* state,¹⁹ and to have a significant triplet state, especially in an oxygen depleted environment.²⁰ Donor triplet to acceptor triplet energy transfer and donor singlet to acceptor triplet transfer are both possible, and although some studies have indicated that those efficiencies are similar to the traditional singlet-singlet transfer process,²⁰ it is unclear that this would always be the case, especially if different donor fluorophores are used. Closer inspection of the traces given in Fig. 2 indeed shows a significant donor intensity during FRET, for a donor-acceptor separation that should theoretically yield nearly 100% efficiency, and thus should result in negligible donor intensity. Therefore, for a certain fraction of time some processes other than energy transfer take place, and this fraction differs in magnitude depending on the donor fluorophore.

A quantitative delineation of the fraction and magnitude of all possible photophysical processes requires an involved multiparameter approach capable of discriminating the multiple lifetime components for each molecule.²¹ This is beyond the scope of the current communication. Instead, we fit the data using two variables, R_0 , the Förster radius, and a scaling factor, c , that accounts for all other (non-FRET) processes, yielding an apparent FRET efficiency:

$$E_{app}(R_{DA}) = c[1 + (R_{DA}/R_0)^6]^{-1}. \quad (3)$$

We estimated R_{DA} based on a model structure of B-DNA in solution:³ $R_{DA} = \sqrt{(3.4\Delta n + L)^2 + d^2 + a^2 - 2da \cos \theta}$, where Δn is the number of basepairs separating the donor and acceptor having radial lengths, $d=20$ Å and $a=15$ Å, respec-

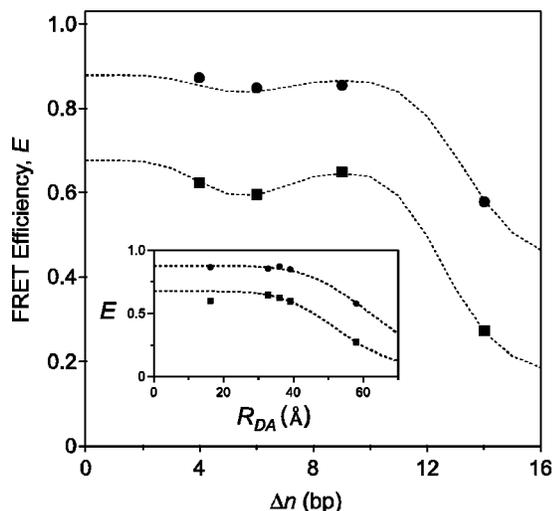


FIG. 5. Inset: FRET efficiency vs R_{DA} calculated using a model for B-DNA (see text). Two FRET pairs, TMR-Cy5 (circles) and Cy3-Cy5 (squares) were separately conjugated to five different DNA constructs. The lines are fits using Eq. (3), yielding scaling factors (0.88 TMR, 0.69 Cy3) and the Förster radii (65 Å TMR, 54 Å Cy3). Main figure: The FRET efficiency expressed as a function of Δn (same strand labeling only) measured using Eq. (1) and (2). The lines are fits using Eq. (3) (expressed as a function of Δn), with the same parameters as in the inset.

tively (which includes the physical linkers and the radius of B-DNA), $\theta = \Delta n \times 36^\circ + \theta_0$ is the angle between the dyes where θ_0 is the angular separation of the dyes for $\Delta n = 0$, and L represents the axial separation between the dyes for the case of $\Delta n = 0$. For the constructs used in this study, the donor and acceptor fluorophores are linked to the C5-methyl group of internal thymines. Considering the physical geometry and the structural rigidity of internal bases we set $L = 0$ and use $\theta_0 = 0^\circ$ and $\theta_0 = 240^\circ$ when the dyes are conjugated to the same strand and opposite strands, respectively.

Figure 5 shows the measured energy transfer efficiencies plotted as a function of Δn and R_{DA} . We use Eq. (3) to fit the FRET efficiency values as a function of R_{DA} with $c = 0.88$ and $R_0 = 65$ Å for TMR-Cy5, and $c = 0.68$ and $R_0 = 54$ Å for Cy3-Cy5 (inset). The data shows excellent agreement with Eq. (3) yielding Förster radii in accord with the published values.¹³ The main figure shows the energy transfer efficiency as a function of Δn with the same fit lines as in the inset expressed as a function of Δn . The fact that an excellent agreement was obtained using a single scaling factor for all inter-dye separations indicates that photophysical effects and FRET can be decoupled in this distance range.

We have presented a method to use FRET at the single molecule level in order to determine the inter-dye distance on internally labeled DNA molecules. Our method involves the determination of the quantum yield ratio ϕ for each FRET trace, which is used to correct the efficiency according to Eq. (1). Using this correction we obtain excellent agreement between FRET efficiencies calculated based on sensitized acceptor emission [Eq. (1)] and donor quenching [Eq. (2)]. We have found that the intermolecular variations in ϕ are ~ 5 times larger for the Cy3-Cy5 pair as compared with

TMR-Cy5, regardless of the donor-acceptor separation, indicating that the variability in ϕ is intrinsic to the FRET pair. Moreover, the asymmetric ϕ distributions indicate that the FRET efficiency of a single molecule requires individual correction.

The FRET efficiency calculated from our data agrees very well with a theoretical estimation based on the B-DNA structure, when a scaling factor c is introduced [Eq. (3)]. The scaling constant reflects the fact that competing de-excitation pathways exist, and we found that for the DNA constructs studied, its value is intrinsic to the donor-acceptor pair. Based on the fit we found $c = 0.88$ for TMR-Cy5 and 0.68 for Cy3-Cy5. These numbers can be used to obtain the inter-dye distances of similar internally labeled DNAs, and in particular be applied to cases in which R_{DA} fluctuates in time. The recovery of the time-dependent physical distance between the dyes in the SM-FRET experiment is especially important for cases where a complicated relationship between the DNA kinetics and its structure is expected.

This work was supported by NSF Grant No. PHY-0417067. We acknowledge Rodd J. Pribik for his help in the lifetime data collection.

- ¹T. Förster, *Ann. Phys. (Leipzig)* **2**, 55 (1948); L. Stryer, *Annu. Rev. Biochem.* **47**, 819 (1978).
- ²E. A. Jares-Erijman and T. M. Jovin, *J. Mol. Biol.* **257**, 597 (1996); P. R. Selvin, *Methods Enzymol.* **246**, 300 (1995); C. Mao, W. Sun, Z. Shen *et al.*, *Nature (London)* **397**, 144 (1999).
- ³R. M. Clegg, A. I. H. Murchie, A. Zechel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2994 (1993).
- ⁴L. Ying, J. J. Green, H. Li *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14629 (2003).
- ⁵D. M. Lilley and R. M. Clegg, *Q. Rev. Biophys.* **26**, 131 (1993); C. Gohlke, A. I. H. Murchie, D. M. Lilley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11660 (1994).
- ⁶J. R. Grunwell, J. L. Glass, T. D. Lacoste *et al.*, *J. Am. Chem. Soc.* **123**, 4295 (2001).
- ⁷M. C. Murphy, I. Rasnik, W. Cheng *et al.*, *Biophys. J.* **86**, 2530 (2004).
- ⁸T. Tuschl, C. Gohlke, T. M. Jovin *et al.*, *Science* **266**, 785 (1994).
- ⁹T. Ha, Th. Enderle, D. F. Ogletree *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6264 (1996).
- ¹⁰S. Weiss, *Science* **283**, 1676 (1999); S. Weiss, *Nat. Struct. Biol.* **7**, 724 (2000); T. Ha, *Methods* **25**, 78 (2001).
- ¹¹P. J. Rothwell, S. Berger, O. Kensch *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1655 (2003); M. Margittai, J. Widengren, E. Schweinberger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15516 (2003).
- ¹²D. G. Norman, R. J. Grainger, D. Uhrin *et al.*, *Biochem. J.* **39**, 6317 (2000); J. Widengren, E. Schweinberger, S. Berger *et al.*, *J. Phys. Chem. A* **105**, 6851 (2001).
- ¹³A. Dietrich, V. Buschmann, C. Müller *et al.*, *Rev. Mol. Biotechnol.* **82**, 211 (2002).
- ¹⁴C. R. Sabanayagam, J. S. Eid, and A. Meller, *Appl. Phys. Lett.* **84**, 1216 (2004).
- ¹⁵Y. Jia, D. S. Talaga, W. L. Lau *et al.*, *Chem. Phys.* **247**, 69 (1999); T. Ha, A. Y. Ting, J. Liang *et al.*, *Chem. Phys.* **247**, 107 (1999).
- ¹⁶R. M. Clegg, *Methods Enzymol.* **211**, 353 (1992); C. Berney and G. Danuser, *Biophys. J.* **84**, 3992 (2003).
- ¹⁷G. Haran, *J. Phys.: Condens. Matter* **15**, R1291 (2003).
- ¹⁸C. Eggeling, J. R. Fries, L. Brand *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1556 (1998).
- ¹⁹J. Widengren and P. Schwille, *J. Phys. Chem. A* **104**, 6416 (2000).
- ²⁰P. Tinnefeld, V. Buschmann, K. D. Weston *et al.*, *J. Phys. Chem. A* **107**, 323 (2003); J. Hofkens, M. Cotlet *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13146 (2003).

²¹R. Kuhnemuth and C. A. M. Seidel, *Single Mol.* **2**, 251 (2001).

²²See EPAPS Document No. E-JCPSA6-122-711505 for FRET efficiencies measured at the single molecule level for five different R_{DA} distances, and two different donor-acceptor pairs. A direct link to this document may be

found in the online article's HTML reference section. The document may also be reached via the EPAPS homepage (<http://www.aip.org/pubservs/epaps.html>) or from <ftp.aip.org> in the directory /epaps/. See the EPAPS homepage for more information

EPAPS Document No. E-JCPSA6-122-711505

construct	Δn	D-A pair	$\frac{R_{DA}}{R_0}$	$E = \frac{I_A - \beta I_D}{I_A + \gamma I_D}$		$E = 1 - \frac{I_{DA}}{I_D}$	
				E_{SM}^*	E_{all}^*	E_{SM}^*	E_{all}^*
<i>ss4</i>	4	TMR-Cy5	0.54	0.87	0.89	0.87	0.89
<i>ss6</i>	6	TMR-Cy5	0.59	0.85	0.87	0.85	0.86
<i>ss9</i>	9	TMR-Cy5	0.50	0.85	0.87	0.85	0.87
<i>ss14</i>	14	TMR-Cy5	0.89	0.58	0.59	0.59	0.60
<i>os4</i>	4	TMR-Cy5	0.27	0.87	0.89	0.86	0.88
<i>ss4</i>	4	Cy3-Cy5	0.69	0.62	0.65	0.63	0.67
<i>ss6</i>	6	Cy3-Cy5	0.74	0.56	0.62	0.61	0.64
<i>ss9</i>	9	Cy3-Cy5	0.59	0.65	0.68	0.66	0.69
<i>ss14</i>	14	Cy3-Cy5	1.07	0.27	0.29	0.27	0.31
<i>os4</i>	4	Cy3-Cy5	0.32	0.60	0.62	0.61	0.64