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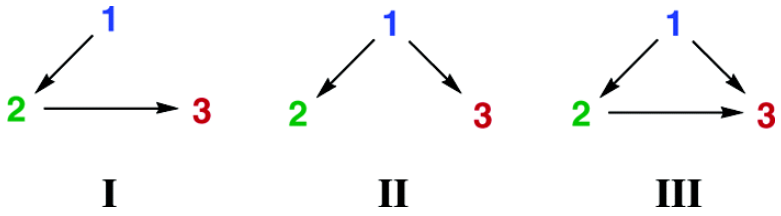

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Two-Step FRET as a Structural Tool

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Abstract: The power of FRET to study molecular complexes is expanded by the use of two or more donor/acceptor pairs. A general theoretical framework for distance measurements in three-chromophore systems is presented. Three energy transfer schemes applicable to many diverse situations are considered: (I) two-step FRET relay with FRET between the first and second chromophores and between the second and third, (II) FRET from a single donor to two different acceptors, and (III) two-step FRET relay with FRET also between the first and third chromophores. Equations for the efficiencies involving multiple energy transfer steps are derived for both donor quenching and sensitized emission measurements. The theory is supported by experimental data on model systems of known structure using steady-state donor quenching, lifetime quenching, and sensitized emission. The distances measured in the three-chromophore systems agree with those in two-chromophore systems and molecular models. Finally, labeling requirements for diagnosis of the energy transfer scheme and subsequent distance measurements are discussed.

Introduction

Förster resonance energy transfer (FRET) provides information about the structure and dynamics of macromolecules and molecular assemblies.[1−6] Being a fluorescence technique, it is sensitive, selective, and adaptable to a wide variety of systems ranging from single molecules to living cells.[7,8] The FRET experiment requires two chromophores, a fluorescent donor and an acceptor. The efficiency of energy transfer from excited donor to acceptor depends on the inverse sixth power of the distance between the two chromophores.[9] FRET has widespread applications both as a qualitative indicator of proximity and as a quantitative measure of distance in the 10−90 Å range through Förster theory.

Recently, the conventional FRET system with one donor/acceptor pair has been expanded to include three or more different chromophores. The three-chromophore systems comprise two donor/acceptor pairs that share a common chromophore. One system was designed with two parallel one-step (**1**/**2** and **1**/**3**) energy transfers from a single donor to two different acceptors for monitoring multicomponent binding interactions.[10] Other systems were designed with parallel one-step (**1**/**3**) and two-step (**1**/**2** and **2**/**3**) energy transfers for applications such as biomimetic photosynthetic reaction centers,[11−13] multiplex biological assays,[14] multivalent interactions on cell surfaces,[15] and DNA sequencing and tertiary structure.[16,17] Two-step FRET offers several advantages over one-step FRET: higher efficiency of long-range transfer,[18] larger Stokes shift, and better detection sensitivity for acceptor fluorescence,[15] with less effort than combinatorial one-step FRET. The efficiencies of three-chromophore systems have not been used for distance measurements except in the following cases. Experimental efficiencies in lamellar thin films were simulated by Monte Carlo calculations using Förster theory.[11] Distances were measured in a DNAzyme using equations developed for parallel one- and two-step FRET.[17]

This paper presents a general approach to FRET in three-chromophore systems based on Förster theory. Equations for time-resolved and steady-state intensities of the chromophores are given for three cases: (I) two-step FRET, (II) two parallel one-step FRETs, and (III) parallel one- and two-step FRET. Strategies for diagnosing each case in the absence of prior structural knowledge as well as methods for distance determination are described and tested with steady-state and time-resolved measurements on well-defined model systems. We also

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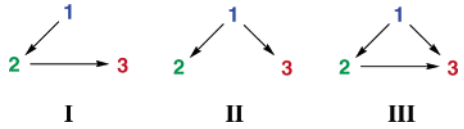
Scheme 1. Three-Chromophore Systems for Energy Transfer



discuss the requirements and relative merits of various FRET methods for three-chromophore systems.

Theory

The energy transfer efficiency E_{ij} measures the rate of energy transfer k_{Tij} from donor i to acceptor j relative to the sum of all the rates for deactivation of the excited state

$$E_{ij} = k_{Tij}/(k_{ri} + k_{nri} + k_{Tij}) \quad (1)$$

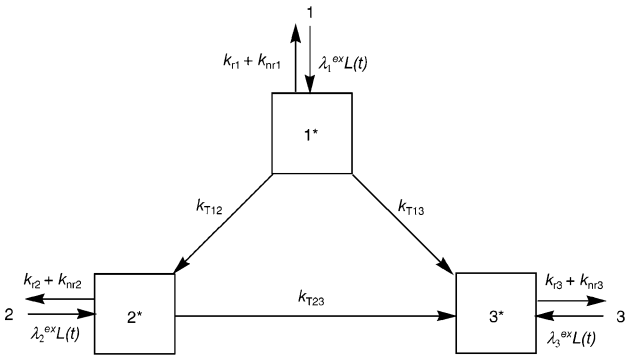
where k_{ri} is the radiative rate, and k_{nri} is the total nonradiative rate of the donor in the absence of acceptor. E_{ij} is related to the through-space distance r_{ij} between the two chromophores by

$$E_{ij} = R_{0ij}^{\ 6}/(R_{0ij}^{\ 6} + r_{ij}^{\ 6}) \quad (2)$$

where R_{0ij} is the Förster distance for the donor/acceptor pair i/j. Measurement of the one-step FRET efficiency, whether by quenching of donor emission or by sensitized emission of acceptor, allows quantitative distance determination. Extracting distances from efficiencies of three-chromophore systems is more complicated.

Three scenarios for a three-chromphore system are depicted in Scheme 1: (I) two-step FRET (FRET1 → 2 → 3); (II) two parallel one-step FRETs from one donor to two different acceptors with *no* two-step FRET (FRET1 → 2 and FRET1 → 3); and (III) parallel one- and two-step FRET (FRET1 → 3 and FRET1 → 2 → 3). The three-chromophore FRET systems can be described using the general irreversible excited-state reaction shown in Scheme 2, where the asterisk denotes an excited chromophore and $\lambda_i^{ex}L(t)$ is direct excitation of chromophore i.

Scheme 2. Excited-State Reaction



The decay of excited chromophores is described by the following system of differential equations

$$d[1^*]/dt = -(k_{r1} + k_{nr1} + k_{T12} + k_{T13})[1^*] \quad (3a)$$

$$d[2^*]/dt = -(k_{r2} + k_{nr2} + k_{T23})[2^*] + k_{T12}[1^*] \quad (3b)$$

$$d[3^*]/dt = -(k_{r3} + k_{nr3})[3^*] + k_{T23}[2^*] + k_{T13}[1^*] \quad (3c)$$

Assuming only direct excitation of **1**, $[1^*]_0 = N_0$, and δ-pulse excitation, the solution to the system of differential equations yields the fluorescence decay $[i^*](t)$ of chromophore i[19]

$$[1^*](t) = N_0 e^{-(k_{r1}+k_{nr1}+k_{T12}+k_{T13})t} \quad (4a)$$

$$[2^*](t) = \frac{N_0 k_{T12}[e^{-(k_{r1}+k_{nr1}+k_{T12}+k_{T13})t} - e^{-(k_{r2}+k_{nr2}+k_{T23})t}]}{(k_{r2} + k_{nr2} + k_{T23}) - (k_{r1} + k_{nr1} + k_{T12} + k_{T13})} \quad (4b)$$

$$[3^*](t) = N_0 \times \frac{\begin{bmatrix}\{(k_{r2} + k_{nr2} + k_{T23}) - (k_{r3} + k_{nr3})\} \times \\ \{k_{T13}^2 + 2k_{T13}(k_{r1} + k_{nr1} - k_{T23}) + k_{T12}k_{T23}\} \times \\ e^{-(k_{r1}+k_{nr1}+k_{T12}+k_{T13})t} - k_{T12}k_{T23}\{(k_{r3} + k_{nr3}) - \\ (k_{r1} + k_{nr1} + k_{T12} + k_{T13})\}e^{-(k_{r2}+k_{nr2}+k_{T23})t} + \\ \{[(k_{r2} + k_{nr2} + k_{T23}) - (k_{r1} + k_{nr1} + k_{T12} + k_{T13})] \\ \times [(k_{r2} + k_{nr2})k_{T13} + k_{T12}k_{T23} + k_{T13}k_{T23}] + \\ k_{T13}(k_{r3} + k_{nr3})[(k_{r1} + k_{nr1} + k_{T12} + k_{T13}) + \\ (k_{r2} + k_{nr2} - k_{T23})]\}e^{-(k_{r3}+k_{nr3})t}\end{bmatrix}}{\begin{bmatrix}[(k_{r3} + k_{nr3}) - \\ (k_{r2} + k_{nr2} + k_{T23})] \times [(k_{r3} + k_{nr3}) - \\ (k_{r1} + k_{nr1} + k_{T12} + k_{T13})] \times [(k_{r2} + k_{nr2} + k_{T23}) \\ - (k_{r1} + k_{nr1} + k_{T12} + k_{T13})]\end{bmatrix}} \quad (4c)$$

Integrating $[i^*](t)$ over time provides the fluorescence intensity I_i, which we express relative to N_0.

Case I. When the three chromophores are arranged so that no energy transfer occurs from **1** to **3**, $k_{T13} \rightarrow 0$. $[1^*](t)$ reports one-step energy transfer from **1** to **2** (FRET1 → 2)

$$[1^*](t) = N_0 e^{-(k_{r1}+k_{nr1}+k_{T12})t} \quad (5)$$

and $I_1 = 1/(k_{r1} + k_{nr1} + k_{T12})$. The energy transfer efficiency E_{12} from **1** to **2** can be measured by donor quenching of **1**

$$E_{12} = 1 - I_1/\tau_1 = k_{T12}/(k_{r1} + k_{nr1} + k_{T12}) \quad (6)$$

where the lifetime $\tau_1 = 1/(k_{r1} + k_{nr1})$ is proportional to the intensity of **1** in the absence of energy transfer. $[2^*](t)$ reports both energy accepted from **1** (FRET1 → 2) and energy transfer from **2** to **3** (FRET2 → 3)

$$[2^*](t) = \frac{N_0 k_{T12}[e^{-(k_{r1}+k_{nr1}+k_{T12})t} - e^{-(k_{r2}+k_{nr2}+k_{T23})t}]}{(k_{r2} + k_{nr2} + k_{T23}) - (k_{r1} + k_{nr1} + k_{T12})} \quad (7)$$

and $I_2 = [k_{T12}/(k_{r1} + k_{nr1} + k_{T12})][1/(k_{r2} + k_{nr2} + k_{T23})]$. The Förster equation for energy transfer efficiency measured by sensitized emission ratios the enhanced acceptor intensity to the acceptor intensity in the absence of energy transfer from the donor.[20] Thus, E_{12} can also be measured by sensitized emission of **2**

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$$E_{12} = I_2/\tau_2' \quad (8)$$

where $\tau_2' = 1/(k_{r2} + k_{nr2} + k_{T23})$ is the lifetime of **2** in the presence of energy transfer to **3**. Direct excitation of **2** at λ_2^{ex} gives only one-step energy transfer from **2** to **3** (FRET2 → 3). E_{23} is determined by equations analogous to eqs 5 and 6 for FRET1 → 2.

The decay of the third chromophore [3*](t) results solely from the two-step FRET relay (FRET1 → 2 → 3)

$$[3^*](t) = \left[\frac{N_0 k_{T12} k_{T23}}{(k_{r1} + k_{nr1} + k_{T12}) - (k_{r2} + k_{nr2} + k_{T23})}\right] \times \left[\frac{e^{-(k_{r1}+k_{nr1}+k_{T12})t} - e^{-(k_{r3}+k_{nr3})t}}{(k_{r1} + k_{nr1} + k_{T12}) - (k_{r3} + k_{nr3})} + \frac{e^{-(k_{r2}+k_{nr2}+k_{T23})t} - e^{-(k_{r3}+k_{nr3})t}}{(k_{r3} + k_{nr3}) - (k_{r2} + k_{nr2} + k_{T23})}\right] \quad (9)$$

The intensity I_3 is as follows

$$I_3 = \left(\frac{k_{T12}}{k_{r1} + k_{nr1} + k_{T12}}\right)\left(\frac{k_{T23}}{(k_{r2} + k_{nr2} + k_{T23})}\right)\left(\frac{1}{k_{r3} + k_{nr3}}\right) \quad (10)$$

The two-step FRET relay efficiency E_{relay} is measured by sensitized emission of **3**

$$E_{relay} = I_3/\tau_3 = E_{12} \times E_{23} \quad (11)$$

where $\tau_3 = 1/(k_{r3} + k_{nr3})$ is the lifetime of **3**. As expected, the relay efficiency is simply the product of efficiencies for each FRET step. E_{relay} depends on the R_0 values and distances of the two FRET steps in the relay, **1**/**2** and **2**/**3**, but does *not* provide a through-space distance r_{13} between **1** and **3**. However, measurement of the relay efficiency and either one-step FRET efficiency gives the two distances r_{12} and r_{23}. In the special case of a sequential linear arrangement of chromophores, the through-space distance $r_{13} = r_{12} + r_{23}$.

Case II. When the three chromophores are arranged so that no two-step FRET occurs, $k_{T23} \rightarrow 0$. Energy transfer from a single donor to multiple identical acceptors has been treated previously.[21−23] In Case II, energy transfer occurs from donor **1** to two different acceptors, **2** and **3**. The donor fluorescence decay [1*](t) is given by eq 4a and $I_1 = 1/(k_{r1} + k_{nr1} + k_{T12} + k_{T13})$. The energy transfer efficiency E_{2A} from **1** to both **2** and **3** is measured by donor quenching of **1** in 123 relative to **1** in the absence of both acceptors

$$E_{2A} = 1 - I_1/\tau_1 = \frac{k_{T12}}{k_{r1} + k_{nr1} + k_{T12} + k_{T13}} + \frac{k_{T13}}{k_{r1} + k_{nr1} + k_{T12} + k_{T13}} = E'_{12} + E'_{13} \quad (12)$$

where now E'_{1j} is an apparent energy transfer efficiency from **1** to acceptor j in the presence of energy transfer to acceptor k. Individual apparent efficiencies E'_{1j} of energy transfer to acceptor j can be measured by donor quenching of **1** in 123 relative to **1** in the absence of acceptor j

$$E'_{1j} = 1 - I_1/\tau_1' = k_{T1j}/(k_{r1} + k_{nr1} + k_{T1j} + k_{T1k}) \quad (13)$$

where $\tau_1' = 1/(k_{r1} + k_{nr1} + k_{T1k})$ is the lifetime of **1** in the presence of energy transfer to k.

The fluorescence decay [j*](t) of acceptor j reports energy accepted from **1** in the presence of acceptor k

$$[j^*](t) = \frac{N_0 k_{T1j}[e^{-(k_{r1}+k_{nr1}+k_{T12}+k_{T13})t} - e^{-(k_{rj}+k_{nrj})t}]}{(k_{rj} + k_{nrj}) - (k_{r1} + k_{nr1} + k_{T12} + k_{T13})}, j = 2, 3 \quad (14)$$

and $I_j = [k_{T1j}/(k_{r1} + k_{nr1} + k_{T12} + k_{T13})][1/(k_{rj} + k_{nrj})]$. The apparent efficiency E'_{1j} can also be measured by sensitized emission of j

$$E'_{1j} = I_j/\tau_j = k_{T1j}/(k_{r1} + k_{nr1} + k_{T12} + k_{T13}) \quad (15)$$

As an apparent efficiency, E'_{1j} *cannot* be used to calculate distances directly from eq 1, because it depends on the R_0 values and distances of *both* donor/acceptor pairs, **1**/**2** and **1**/**3**. However, if both apparent efficencies E'_{12} and E'_{13} are known, the distances r_{12} and r_{13} can be calculated[17]

$$r_{1j} = R_{01j}[(1 - E'_{1j} - E'_{1k})/E'_{1j}]^{1/6} \quad (16)$$

Case III. When the three chromophores are arranged so that the two-step FRET relay is accompanied by one-step FRET from **1** to **3**, two energy transfers complicate the decays of both **1** and **2**. [1*](t) reports energy transfer to acceptors **2** and **3** as in Case II above. The fluorescence decay [2*](t) of **2** is similar to Case I, except that energy transfer from **1** to **3** competes with the energy transfer from **1** to **2** as described by eq 4b, so that $I_2 = [k_{T12}/(k_{r1} + k_{nr1} + k_{T12} + k_{T13})][1/(k_{r2} + k_{nr2} + k_{T23})] = E'_{12}\tau_2'$. Likewise E'_{12} can be measured by sensitized emission of **2** as in eq 8. The fluorescence decay [3*](t) reports energy accepted from both one- and two-step FRET as given by eq 4c. The intensity I_3 of the third chromophore is as follows

$$I_3 = \left[\left(\frac{k_{T12}}{k_{r1} + k_{nr1} + k_{T12} + k_{T13}}\right)\left(\frac{k_{T23}}{k_{r2} + k_{nr2} + k_{T23}}\right) + \frac{k_{T13}}{k_{r1} + k_{nr1} + k_{T12} + k_{T13}}\right]\left(\frac{1}{k_{r3} + k_{nr3}}\right) \quad (17)$$

Ratioing eq 17 to the intensity of **3** in the absence of energy transfer τ_3 yields the expected expression for the total efficiency E_{tot} of the parallel one- and two-step FRET

$$E_{tot} = I_3/\tau_3 = E'_{12} \times E_{23} + E'_{13} \quad (18)$$

which collapses to eq 11 for E_{relay} when $k_{T13} \rightarrow 0$.

Experimental Section

Materials. Alexa Fluors 350 (**1**), 488 (**2**), and 594 (**3**) in the carboxylic acid, succinimidyl ester form and ChromaTide Alexa Fluor 488- and 594−5-dUTPs were purchased from Molecular Probes (Eugene, OR). Oligodeoxynucleotides were purchased unpurified from Operon Technologies (Alameda, CA). HPLC grade methanol and acetonitrile, ACS grade dimethyl sulfoxide (DMSO), and ethanol were purchased from Fisher Scientific.

Oligodeoxynucleotides. 43-mer, 5′-AGGCAGGCAGGTGAGTTC-CGCAAC**T**CCGACAGCAGTACCATCG-3′; 23-mer, 5′-CGATGG-TAC**T**GCTGTCGGAGTGG-3′; 22-mer, 5′-TGCGGAAC**T**CACCT-

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GCCTGCCT-3′; and 21-mer, 5′-CGATGGTAC**T**GCTGTCGGAGT-3′. **T** represents C6dT with an internal amino linker for conjugation of fluorescent probes. **T** is replaced by T in unlabeled sequences. Cases I and III use the same 43- and 22-mers. For the third strand, Case I uses the 23-mer, which has a 2-nucleotide tail, and Case III uses the 21-mer (see Figure 2). Prior to labeling, oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis, eluted from gel slices by an Elutrap (Schleicher & Schuell), and further purified by C18 Sep-pak (Waters) followed by size exclusion using a 1 mL spin column of Bio-Gel P-2 (BioRad). Oligonucleotide concentrations were determined from absorbance at 260 nm $A(260)$ using extinction coefficients ϵ_O calculated by the nearest neighbor approach.[24,25] The $\epsilon_O(260)$ values are 417 000, 219 700, 190 000, and 199 300 cm^{-1} M^{-1} for 43-, 23-, 22-, and 21-mer, respectively.

Oligonucleotide was reacted with ~20-fold molar excess of fluorescent probe (~1 mg dissolved in 5 μL of anhydrous DMSO) in 0.2 M Na_2CO_3, pH 9.5 at 37 °C for 2 h, ethanol precipitated on dry ice, and purified as described above. The ratio of bound probe D to oligonucleotide O was determined from absorbance measurements of the oligoconjugate

$$[D] = A(\lambda^{max})/0.3\epsilon_D(\lambda^{max}) \tag{19a}$$

$$[O] = \{A(260) - 0.3\epsilon_D(260)[D]\}/0.3\epsilon_O(260) \tag{19b}$$

where λ^{max} is the probe absorption peak. Typical $[D/O]$ values were 0.96−1.2. Spectroscopic ratiometric measurements are not sufficient to confirm the presence of a single probe on the oligonucleotide, because the extinction coefficient of the probe may change upon conjugation.[26,27] Moreover, nonspecific labeling at site(s) other than the reactive **T** was observed using different reaction conditions and using the above reaction conditions with one lot of a different fluorescent probe. The presence of a single probe was established by Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry in the Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign. All oligoconjugates used in this study contained one probe.

DNA duplexes were formed by mixing equimolar amounts of two or three complementary oligonucleotides in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and heating to 95 °C for 3 min followed by slow cooling to room temperature. Duplexes were purified by native gel electrophoresis at 5 °C to maximize duplex yield, eluted by Elutrap, and further purified by Sep-pak and P-2 spin column. Duplexes are named by the chromophore(s) present.

Molecular Modeling. A linear DNA duplex was generated using InsightII98.0 (Accelrys, San Diego, CA). C6dT amino linkers were generated using the Builder module of InsightII and attached to thymidine C5 without minimization. Through-space distances were measured between nitrogens of C6dT amino linkers in the duplex.

UV−Vis and Steady-State Fluorescence. Samples were prepared in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 and placed in 45-μL quartz cells with 3-mm path length. Absorption spectra were measured on a Cary 3E UV−vis Spectrophotometer. Fluorescence excitation and emission spectra were acquired in ratio mode on a SLM Aminco 8100 spectrofluorometer (SLM Instruments, Urbana, IL) under magic angle conditions. Temperature was maintained by a circulating water bath at 5 °C to stabilize duplex DNA in the low-salt buffer. Sample compartment was purged with nitrogen to prevent condensation. Emission spectra were blank subtracted and corrected.

Fluorescence quantum yields ϕ were measured relative to a reference fluorophore using

$$\phi = \frac{\phi_{ref} F A_{ref}(\lambda^{ex}) n^2}{F_{ref} A(\lambda^{ex}) n_{ref}^{\ 2}} \tag{20}$$

where F is the integrated corrected emission spectrum, $A(\lambda^{ex})$ is absorbance at the excitation wavelength, and n is the refractive index of the medium. Quantum yields of fluorescent probes on duplex DNA were determined using 23-mer−**1**/43-mer, 23-mer/43-mer−**2**, and 43-mer/22-mer−**3**.

Time-Resolved Fluorescence. Fluorescence decays were measured by time-correlated single photon counting using picosecond laser excitation. A DCM dye laser system was used for 330 nm excitation.[28,29] A Ti-sapphire laser system was used for 465 nm excitation with the following modifications.[30] The doubled output was passed through a beam splitter, focused by a concave lens, and used to excite the sample. A portion of the doubled output was detected, amplified, and used as the stop timing signal.

Fluorescence intensity decays $I(t)$ were deconvolved assuming a sum of exponentials

$$I(t) = \Sigma_i \alpha_i e^{-t/\tau_i} \tag{21}$$

with amplitudes α_i and lifetimes τ_i of component i. Number-average lifetimes $\bar{\tau}$ proportional to the steady-state intensity were calculated from $\bar{\tau} = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i$.

Energy Transfer. Efficiency E was measured by quenching of donor fluorescence using

$$E = 1 - (F_{ij}/F_i) = 1 - (\bar{\tau}_{ij}/\bar{\tau}_i) \tag{22}$$

where F_{ij} and F_i are intensities and $\bar{\tau}_{ij}$ and $\bar{\tau}_i$ are average lifetimes of donor i in the presence and absence of acceptor j. Efficiency E was also measured by sensitized emission using

$$E = [\epsilon_j(\lambda_i^{ex})/\epsilon_i(\lambda_i^{ex})]\{[F_{ij}(\lambda_j^{em})/F_j(\lambda_j^{em})] - 1\} \tag{23}$$

where $\epsilon_{i(j)}(\lambda_i^{ex})$ is the donor (acceptor) extinction coefficient at the donor excitation wavelength λ_i^{ex} and $F_{ij}(\lambda_j^{em})$ and $F_j(\lambda_j^{em})$ are acceptor intensities at the acceptor emission wavelength λ_j^{em} in the presence and absence of donor.[31]

The Förster distance R_0 on duplex DNA was determined for each donor/acceptor pair from

$$R_0 = 0.211[\kappa^2 n^{-4} \phi_i J]^{1/6}\ \text{Å} \tag{24}$$

where κ^2 is an orientation factor, n is the refractive index of the medium, ϕ_i is donor i quantum yield, and J is the spectral overlap integral. For random orientation of donor emission and acceptor absorption transition dipoles, $\kappa^2 = 2/3$. The anisotropy decay of **1** on duplex DNA (23-mer−**1**/43-mer) consisted primarily of a 120-ps rotational correlation time (>90%) with a small amount of 15-ns motion (data not shown). Similar results were reported for **2** on an 18-mer duplex.[32] Therefore, we assumed $\kappa^2 = 2/3$. The probes are pendent on DNA immersed in buffer, so we used the refractive index of water, $n = 1.333$.

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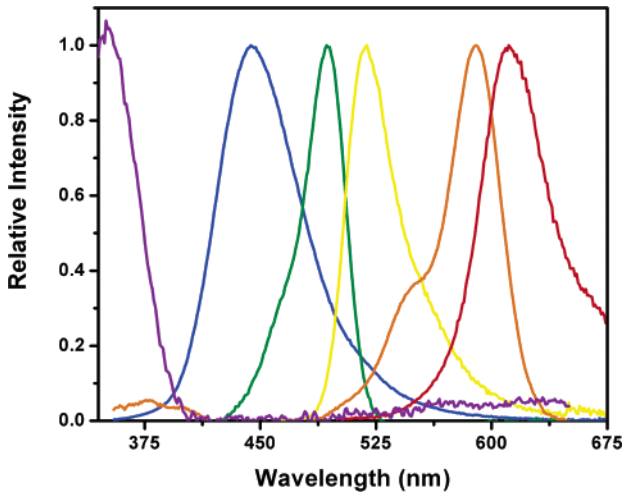

Figure 1. Absorption and emission spectra of fluorescent probes on duplex DNA. **1**: $A_1(\lambda^{max})$ = 345 nm (purple); $F_1(\lambda^{max})$ = 445 nm (blue). **2**: $A_2(\lambda^{max})$ = 492 nm (green); $F_2(\lambda^{max})$ = 518 nm (yellow). **3**: $A_3(\lambda^{max})$ = 588 nm (orange); $F_3(\lambda^{max})$ = 616 nm (red).

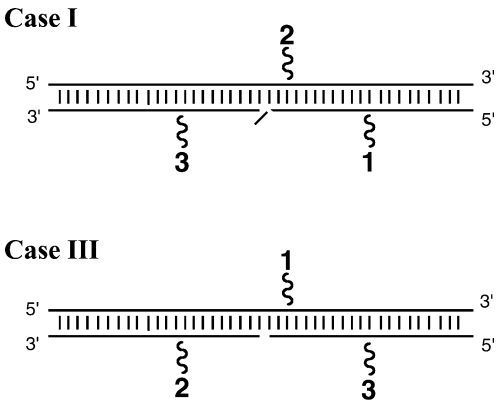

Figure 2. DNA models with fluorescent probes. Through-space distances between labeling sites were measured by molecular modeling: r_{calc} = 40 Å between sites on top strand and 5′ bottom strand; r_{calc} = 53 Å between sites on top strand and 3′ bottom strand; r_{calc} = 68 Å between sites on 3′ and 5′ bottom strands.

The spectral overlap integral J of donor emission spectrum and acceptor absorption spectrum is calculated from

$$J = \int F_i(\lambda)\epsilon_j(\lambda)\lambda^4\, d\lambda \,/ \int F_i(\lambda) d\lambda \ \mathrm{M^{-1}\,cm^{-1}\,nm^4} \qquad (25)$$

where $F_i(\lambda)$ is the corrected donor emission spectrum and $\epsilon_j(\lambda)$ is the acceptor extinction coefficient at wavelength λ in units of M^{-1} cm^{-1}.

Results

Model System. In addition to the requirement for overlap of donor emission and acceptor absorption spectra, desirable characteristics of FRET probes include photostability, high extinction coefficient and quantum yield, and environmental insensitivity. We chose Alexa Fluors 350 (**1**), 488 (**2**), and 594 (**3**) to create a two-step FRET relay with donor/acceptor pairs **1**/**2** and **2**/**3**. The spectral overlap of the chromophores is shown in Figure 1. Excitation of **1** (or **2**) leads to some emission that results from direct excitation of **2** and **3** (or **3**), not just FRET. This requires appropriate controls in sensitized emission measurements to correct for direct excitation of the other chromophore(s). Quantum yields, spectral overlap integrals, and R_0 values are given in Table 1. Quantum yields were also measured for dUTP- and oligoconjugates to monitor environmental effects.[26,27,33] Probes **2** and **3** were insensitive to environment. Probe **1** showed ~30% drop in quantum yield for duplex DNA compared to oligoconjugate.

Table 1. Quantum Yields, Spectral Overlap Integrals, and Förster Distances[a]

chromophore	conjugate	λ_i^{ex}	ϕ	J, $M^{-1}\,cm^{-1}\,nm^4$	R_0, Å
1	oligonucleotide	330	0.55 ± 0.01[b]		
1	duplex		0.38 ± 0.01[b]		
2	dUTP	450	1.0 ± 0.01[c]		
2	oligonucleotide		0.90 ± 0.06[c]		
2	duplex		1.00 ± 0.09[c]		
3	dUTP	540	0.94 ± 0.03[d]		
3	oligonucleotide		1.0 ± 0.2[d]		
3	duplex		0.90 ± 0.02[d]		
1/**2**	duplex			9.18×10^{14}	43.5 ± 0.2
2/**3**	duplex			1.68×10^{15}	56.2 ± 0.1
1/**3**	duplex			1.84×10^{14}	32.6 ± 0.1

[a] 5 °C. Errors are standard deviations of 3−12 experiments. [b] Quinine sulfate in 1 N H_2SO_4, ϕ_{ref} = 0.55.[34] [c] Fluorescein in 1 M NaOH, ϕ_{ref} = 0.95.[35] [d] Cresyl violet in methanol, ϕ_{ref} = 0.54.[36]

The three chromophores are attached to duplex DNA to model Cases I and III (Figure 2). We chose DNA as a model system for three reasons: (1) DNA sequences and labeling positions are conveniently designed and commercially available at reasonable cost. (2) Labeling reactions involve simple chemistry and straightforward purification strategies. (3) Most importantly, duplex DNA provides a rigid scaffold with facile distance estimation by molecular modeling. Our DNA model comprises three single strands: a 43-mer Watson strand with 22- and 21-nucleotide sequences that are complementary to two shorter Crick strands. The distances between labeling sites on DNA were designed based on the measured R_0 values for each FRET pair. Reasonable FRET detection limits are 0.5 $R_0 < r < 1.5$ R_0. If the distance between donor and acceptor is >1.5 R_0, then the efficiency is too low to measure accurately. For Case I, sequential through-space distances between **1** and **2** and between **2** and **3** were engineered close to the R_0 value for each FRET pair to create FRET1 → 2 → 3. The through-space distance between **1** and **3** is ~2.5 R_{013}, eliminating FRET1 → 3. For Case III, the relative position of the three chromophores was permuted to allow FRET1 → 3.

Conventional FRET. Distances in the models for Cases I and III were determined by conventional one-step FRET with single donor/acceptor pairs to compare with the results in three-chromophore systems. Efficiencies were measured by both donor quenching and sensitized emission using eqs 22 and 23. This combinatorial approach requires five duplexes for Case I and six duplexes for Case III: a duplex with each donor/acceptor pair (12, 23, and 13) and a duplex with each chromophore alone (1, 2, and 3). Minor differences in concentration of DNA duplexes were adjusted using $A(\lambda^{max})$ for each chromophore. The experimental and calculated distances r_{ij} are given in Table 2 and in the legend of Figure 2.

Similar values were obtained for efficiencies and distances by donor quenching and sensitized emission. Values for donor quenching using steady-state and lifetime measurements agree within error, though the errors are generally smaller using lifetime quenching. The efficiencies using sensitized emission appear consistently lower, and the distances correspondingly larger. Efficiencies were also measured by sensitized emission

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Table 2. One-Step FRET Efficiencies and Distances between Donor/Acceptor Pairs in DNA Models[a]

	FRET pair	donor quenching			sensitized emission		
case	*i*/*j*	duplexes	E_{ij}	r_{ij}, Å	duplexes	E_{ij}	r_{ij}, Å
I	**1**/**2**	12, 1	0.56 ± 0.04[b]	41.8 ± 0.7	12, 2	0.48 ± 0.06[b]	44 ± 1
			0.67 ± 0.09[c]	39 ± 1			
	2/**3**	23, 2	0.56 ± 0.07[d]	54 ± 1	23, 3	0.40 ± 0.09[d]	60 ± 2
			0.54 ± 0.01[e]	54.7 ± 0.3			
	1/**3**	13, 1	0		13, 3	0	
III	**1**/**2**	12, 1	0.53 ± 0.05[b]	43 ± 1	12, 2	0.30 ± 0.09[b]	50 ± 2
			0.48 ± 0.02[c]	42.4 ± 0.1			
	2/**3**	23, 2	0.30 ± 0.09[d]	65 ± 3	23, 3	0.3 ± 0.1[d]	62 ± 4
			0.29 ± 0.01[e]	65.2 ± 0.4			
	1/**3**	13, 1	0.5 ± 0.1[b]	33 ± 1	13, 3	0.2 ± 0.1[b]	40 ± 3
			0.45 ± 0.02[c]	33.4 ± 0.3			

[a] Duplexes of three complementary oligonucleotides are named by the chromophore(s) present, 5 °C. Errors are standard deviations of 3 experiments. [b] Steady-state, $\lambda_1^{ex} = 345$ nm. [c] Lifetime, $\lambda_1^{ex} = 330$ nm. [d] Steady-state, $\lambda_2^{ex} = 492$ nm. [e] Lifetime, $\lambda_2^{ex} = 465$ nm.

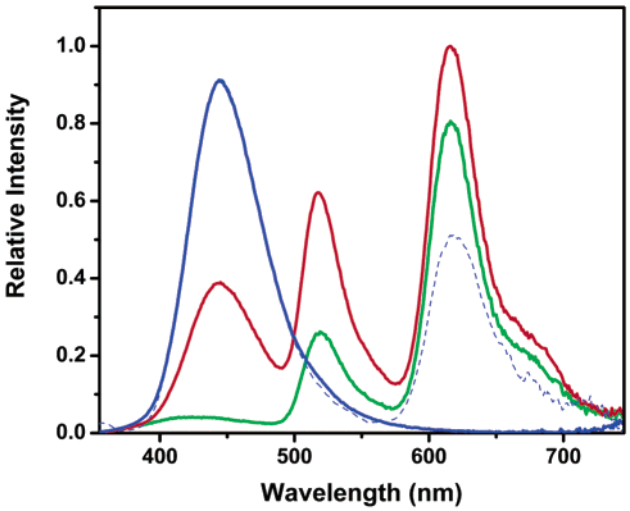

Figure 3. Emission spectra of Case I duplexes excited at $\lambda_1^{ex} = 345$ nm. 1 (blue−), 13 (blue- - -), 123 (red), 23 (green).

using the $(\text{ratio})_A$ method.[2,26] The value of $E_{23} = 0.40 \pm 0.06$ obtained for **2**/**3** in Case I agrees with the results in Table 2. However, a low value of $E_{12} = 0.10 \pm 0.01$ was obtained for **1**/**2** in Case I and negative values were obtained for both **1**/**2** and **1**/**3** in Case III. The $(\text{ratio})_A$ method uses emission intensity data collected at both donor and acceptor excitation wavelengths. The failure of this method in our hands for **1**/**2** and **1**/**3** but not for **2**/**3** was attributed to wavelength-dependence of the fluorometer excitation train at shorter wavelength.

In our DNA models, two distances between labeling sites are measured with two different donor/acceptor pairs: the shortest distance by **1**/**2** in Case I and **1**/**3** in Case III, and the intermediate distance by **2**/**3** in Case I and **1**/**2** in Case III. Distances measured with different donor/acceptor pairs are 7−11 Å larger in Case I than Case III, perhaps due to the tail. The experimental distances are quite close to the through-space distances calculated from molecular models in Case I, whereas the experimental distances are smaller in Case III.

Case I. Diagnosis of the energy transfer steps with three chromophores requires three duplexes: 123, 1, and 13. If the intensity of **1** is the same in 1 and 13, no FRET1 → 3 occurs and both Cases II and III can be excluded. To confirm a two-step FRET relay, the intensity of **1** must be greater in 1 than in 123 upon excitation at λ_1^{ex} and the sensitized emission of **3** in 123 must be greater than direct excitation of **3** in 13 upon excitation at λ_2^{ex}. The three emission spectra at λ_1^{ex} are shown in Figure 3, along with the spectrum of 23 demonstrating greater intensity of **3** in 123. After Case I has been confirmed, the efficiency E_{relay} can be measured by sensitized emission of **3** in 123 at λ_1^{ex} using a modified form of eq 23

$$E = [\{\epsilon_2(\lambda_1^{ex}) + \epsilon_3(\lambda_1^{ex})\}/\epsilon_1(\lambda_1^{ex})][(F_{123}/F_{23}) - 1] \quad (26)$$

and duplex 23, which corrects for direct excitation of both **2** and **3**. The measured value of $E_{relay} = 0.2 \pm 0.1$ is in good agreement with the value of 0.19 ± 0.05 calculated using eq 11 and sensitized emission data for E_{12} and E_{23} from Table 2. However, it is lower than the values of 0.31 ± 0.05 or 0.36 ± 0.07 calculated using steady-state donor quenching or lifetime quenching data from Table 2.

In Case I, distance determination requires two efficiency measurements, E_{12} and E_{23}. Both one-step FRET efficiencies can be measured in 123 by donor quenching of **1** at λ_1^{ex} and of **2** at λ_2^{ex}. FRET1 → 2 uses duplexes 123 and 1; FRET2 → 3 uses 123 and an additional duplex, either 12 or 2. Alternatively, the two one-step efficiencies can be measured by sensitized emission of **2** at λ_1^{ex} and of **3** at λ_2^{ex} using eq 23. FRET1 → 2 uses duplexes 123 and 23; FRET2 → 3 uses 123 and either 13 or 3. The measured efficiencies and distances for Case I are summarized in Table 3. The values obtained from one-step FRET measurements on the three-chromophore system agree well with the corresponding values in Table 2 for individual donor/acceptor pairs. Calculation of one of the one-step efficiencies E_{ij} from E_{relay} and the other one-step efficiency E_{jk} using eq 11 gives values consistent with the results in Table 2 within the large propagated errors. The donor quenching or sensitized emission values for E_{12} from Table 3 give $E_{23} = 0.4 \pm 0.2$ and $r_{23} = 62 \pm 5$ Å or $E_{23} = 0.4 \pm 0.2$ and $r_{23} = 59 \pm 5$ Å. Conversely, the donor quenching or sensitized emission values for E_{23} give $E_{12} = 0.4 \pm 0.2$ and $r_{12} = 48 \pm 4$ Å or $E_{12} = 0.5 \pm 0.3$ and $r_{12} = 44 \pm 4$ Å.

Case II. Two parallel one-step FRETs is distinguished from Cases I and III by the absence of FRET2 → 3. If the intensity of **3** is the same in 123 and 13 upon excitation at λ_2^{ex}, no FRET2 → 3 occurs. To confirm one-step FRET from **1** to both **2** and **3**, the intensity of **1** in 123, 13, and 1 must be greatest in 1 and lowest in 123 upon excitation at λ_1^{ex}. After Case II has been confirmed, the efficiency of one-step FRET to two acceptors E_{2A} can be measured by donor quenching of **1** at λ_1^{ex} using duplexes 123 and 1. In Case II, distance determination requires two apparent efficiencies. These can be obtained from E_{2A} and

Table 3. FRET Efficiencies and Distances in Three-Chromophore Model Systems[a]

	FRET pairs	donor quenching			sensitized emission		
case	*ijlk*	duplexes	*E*	r_{ij}, Å	duplexes	*E*	r_{ij}, Å
I	**1/2**	123, 1	$0.62 \pm 0.04^{b,f}$	40.1 ± 0.7	123, 23	$0.5 \pm 0.2^{b,f}$	42 ± 3
			$0.66 \pm 0.02^{c,f}$	39.0 ± 0.7			
	2/3	123, 2	$0.54 \pm 0.07^{d,f}$	55 ± 1	123, 3	$0.45 \pm 0.04^{d,f}$	58.1 ± 0.9
			$0.57 \pm 0.01^{e,f}$	53.6 ± 0.3			
	1/2/3				123, 23	$0.2 \pm 0.1^{b,g}$	n/a
III	**1/2** & **1/3**	123, 1	$0.65 \pm 0.08^{b,h}$	n/a			
			$0.65 \pm 0.3^{c,h}$				
	2/3	123, 2	$0.3 \pm 0.1^{d,f}$	65 ± 4	123, 3	$0.4 \pm 0.2^{d,f}$	62 ± 6
			$0.29 \pm 0.01^{e,f}$	65.2 ± 0.4			
	1/2/3 & **1/3**				123, 23	$0.31 \pm 0.08^{d,i}$	n/a
	1/2	123, 13	$0.37 \pm 0.03^{b,j}$	43 ± 1^{k}	123, 23	$0.22 \pm 0.04^{b,j}$	51 ± 2^{k}
			$0.36 \pm 0.01^{c,j}$	42.4 ± 0.4^{k}			
	1/3	123, 12	$0.3 \pm 0.1^{b,j}$	33.5 ± 0.3^{k}	eq 18	0.24 ± 0.08^{j}	38 ± 3^{k}
			$0.31 \pm 0.01^{c,j}$	33.4 ± 0.3^{k}			

[a] Duplexes of three complementary oligonucleotides are named by the chromophore(s) present, 5 °C. Errors are standard deviations of 3 experiments. [b] Steady-state, $\lambda_1^{ex} = 345$ nm. [c] Lifetime, $\lambda_1^{ex} = 330$ nm. [d] Steady-state, $\lambda_2^{ex} = 492$ nm. [e] Lifetime, $\lambda_2^{ex} = 465$ nm. [f] E_{ij}. [g] E_{relay}. [h] E_{2A}. [i] E_{tot}. [j] E'_{1j}. [k] Calculated using eq 16.

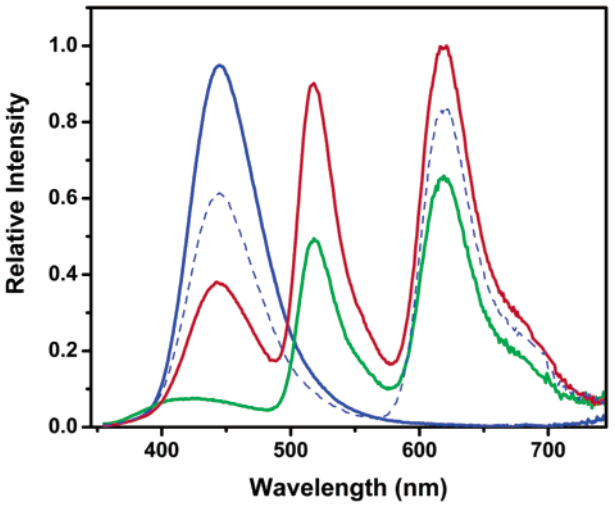

Figure 4. Emission spectra of Case III duplexes excited at $\lambda_1^{ex} = 345$ nm. 1 (blue−), 13 (blue- - -), 123 (red), 23 (green).

an additional efficiency measurement, either E'_{12} or E'_{13}. Both apparent efficiencies E'_{1j} can be measured in 123 by donor quenching using duplexes 123 and 1k. Alternatively, E'_{1j} can be measured by sensitized emission of *j* upon excitation at λ_1^{ex} using duplexes 123 and jk or j.

Our system of three fluorescent probes **1**, **2**, and **3** cannot simultaneously demonstrate the two extremes represented by Cases I and II. However, Case III below encompasses an example of energy transfer from a single donor to two different acceptors. The donor/acceptor pairs for Case I were chosen with R_{013} < both R_{012} and R_{023} to minimize FRET1 → 3. A linear arrangement of three chromophores with **1** in the center and **2** and **3** on opposite sides allows maximal distance between **2** and **3**. Given the R_0 values for our system, it is not possible to place **2** and **3** far enough apart to eliminate FRET2 → 3 while maintaining both FRET1 → 2 and FRET1 → 3. Donor acceptor pairs for Case II should have both R_{012} and $R_{013} > R_{023}$.

Case III. Parallel one- and two-step FRET shows one-step FRET from **1** to both **2** and **3** upon excitation at λ_1^{ex} as in Case II as well as sensitized emission of **3** upon excitation at λ_2^{ex} as in Case I. Emission spectra are shown in Figure 4. After Case III has been confirmed, the efficiency E_{tot} can be measured by sensitized emission of **3** using duplexes 123 and 23 as in Case I. In Case III, distance determination requires three efficiency measurements, E'_{12}, E'_{13}, and E_{23}. E_{23} can be measured by donor quenching of **2** or by sensitized emission of **3** as described above for Case I, and E'_{12} can be measured by sensitized emission of **2** analogously to Case I. The remaining apparent efficiency E'_{13} calculated from eq 18 as well as the distances for Case III are given in Table 3. The values of E_{23} and the three distances agree well with the values in Table 2.

Alternatively, both apparent efficiencies E'_{1j} can be measured directly by donor quenching of **1** as described for Case II. Here again, the values of E_{23} and the three distances in Table 3 agree with Table 2. Finally, the efficiency of one-step FRET to two acceptors E_{2A} can be measured as described for Case II. The apparent efficiencies can then be calculated from eqs 12 and 18 using the measured values for E_{tot}, E_{2A}, and E_{23}. The results are $E'_{12} = 0.5 \pm 0.2$ and $E'_{13} = 0.2 \pm 0.2$ using E_{23} measured by donor quenching of **2**, or $E'_{12} = 0.6 \pm 0.3$ and $E'_{13} = 0.1 \pm 0.3$ using E_{23} measured by sensitized emission of **3**. Obviously, the large propagated errors are a disadvantage of this approach.

Discussion

Three-chromophore FRET systems offer several advantages. First, three-chromophore systems report the simultaneous proximity of three species and provide the ability to measure two or three distances in a complex. Structural information about the assembly can then be inferred from the relative positions of individual components of the complex. For example, in Case I where no FRET1 → 3 occurs, r_{13} must be >1.5 R_{013}. This restricts the position of **3** relative to **1** to a minimal distance of $r_{13} \approx 1.75\ R_{013}$ and a maximal distance of $r_{13} = r_{12} + r_{23}$ for a linear arrangement of **1**, **2**, and **3**. Second, in the case of linear or near linear arrangement of the three chromophores, two-step FRET extends the distance range for detection of simultaneous proximity. For example, assuming $R_0 = 55$ Å for the two FRET pairs and a detection limit of 1.5 R_0, one-step FRET at a distance $r = 83$ Å has an efficiency $E_{ij} = 0.08$. A two-step FRET relay with $E_{relay} = 0.08$ corresponds to a total distance $r = 127$ Å. Thus, the detectable distance range increases by as much as 50%. Third, three-chromophore systems require fewer labeled samples to measure two or three distances than conventional one-step FRET. Without prior structural knowledge, three-

Table 4. Labeling Requirements for Three-Chromophore Systems

		distance measurement method		
case	diagnosis	donor quenching	sensitized emission	$(\text{ratio})_A$
I	123, 1, 13	123, 1, 12 or 2	123, 23, 13 or 3	123, 1, 2
II	123, 1, 13	123, 12, 13	123, 23	123, 1, 2
III	123, 1, 13	123, 12, 13	123, 23, 13 or 3	123, 1, 2

chromophore systems require the same three labeled samples to diagnose the energy transfer steps: 123, 1, and 13. Once diagnosed, Cases I−III require at most three labeled samples for distance measurements by any method, one of which is an additional sample: 12, 2, or 23 (Table 4). Preparation of four labeled samples, while cumbersome, is still preferable to the five or six necessary for combinatorial one-step FRET.

Steady-state FRET provides a mean distance averaged over all species present and may overestimate the actual interchromophore distance. Time-resolved FRET (tr-FRET) can identify relative populations of a heterogeneous mixture of conformations, the mean distance associated with each population, and the width of the distance distribution.[37,38] Through the use of donor decays, tr-FRET can obtain structural, energetic, and dynamic information. Application of tr-FRET to three-chromophore systems is only straightfoward for Case I, where the efficiency of each one-step FRET depends on distance of a single donor/acceptor pair. Just like conventional one-step FRET, donor decays in 123 can report multiple distances of donor/acceptors pairs for **1**/**2** at λ_1^{ex} and for **2**/**3** at λ_2^{ex}. The apparent efficiencies in Cases II and III depend on distances of two donor/acceptor pairs, vastly complicating the analysis of conformational heterogeneity by tr-FRET.

While this work was in progress, Liu and Lu reported a three-chromophore system to monitor conformational changes in a DNAzyme in the presence of varying concentrations of Zn^{2+}.[17] Energy transfer efficiencies and distances were derived for each FRET pair in Case III and measured using the $(\text{ratio})_A$ method of sensitized emission. Although these authors took a different mathematical approach, their treatment is congruent with the results presented here and converges to Case I when $k_{T13} \rightarrow 0$ and Case II when $k_{T23} \rightarrow 0$. Our approach allows direct measurement of all individual efficiencies by donor quenching. This has the advantage of reducing error propagation to calculated distances. Lifetime quenching as well as the $(\text{ratio})_A$ method are inherently more accurate than steady-state donor quenching and sensitized emission using eqs 23 or 26, both of which are susceptible to errors in sample concentration. In the DNAzyme, fluorescein, tetramethylrhodamine, and Cy5 were attached to oligonucleotides at the two 5′ ends of the duplex and one internal site. In our models, the three Alexa Fluors were attached at internal sites to eliminate the possibility of end-stacking effects on the orientation factor κ^2.[39] Moreover, the Alexa Fluors have tailored FRET characteristics, including pH insensitivity within the range pH 4−10,[40] and little or no change in spectral properties upon conjugation to DNA. Finally, our approach is completely general and can be used with any energy transfer method. This makes it applicable to blue-absorbing chromophores, thereby extending the spectral range available for multi-chromophore FRET and allowing greater flexibility in instrumentation.

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