Triple Fluorescence Energy Transfer in Covalently Trichromophore-Labeled DNA

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DNA is a unique molecule that can be used to separate donor and acceptor for fluorescence energy transfer (ET)[1] and long-range photoinduced electron-transfer studies.[2] ET has been used extensively as a spectroscopic ruler for biological structures,[3a−c] and ET primers and terminators are markedly superior to single dye-labeled reagents in DNA sequencing and analysis.[4a−b] Molecular beacons using both organic dyes[5] and metal complexes[6] for genetic analysis were also developed. Most of the reported ET systems using DNA as a backbone are based on one donor−acceptor pair.[7a−b] An ET system involving three chromophores that are located on separate strands of oligonucleotides has also been reported.[8] Recently, we developed a novel approach for constructing a large number of combinatorial fluorescence energy transfer tags from a small number of chromophores for multiplex biological assays.[9] We report here the systematic study of the photophysical properties of an ET system consisting of three different fluorophores that are covalently linked to a single-stranded (ss) DNA molecule.

The trichromophore-labeled DNA that has a scaffold of 26 nucleotides (Chart 1) was constructed by solid-phase phosphoramidite synthesis and selective solution-coupling chemistry. The structure of the compound was confirmed by MALDI-TOF mass spectrometry.[10] The 5′-end of the DNA was linked to 6-carboxyfluorescein (**F**). *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (**R**) was positioned four nucleotides away from **F**. A cyanine-5 monofunctional dye (**Cy**) was attached to the DNA six nucleotides from **R**. Twelve thymidine nucleotides followed the triple ET moiety with a cytidine at the 3′-end for DNA sequencing evaluations. The trichromophore-labeled DNA is designated as **F-4-R-6-Cy-13** (number refers to the nucleotide residues) where **F** acts as the donor for **R** and **Cy**, **R** acts as an acceptor for **F** and a donor for **Cy**, while **Cy** acts as a final acceptor for both **F** and **R**.

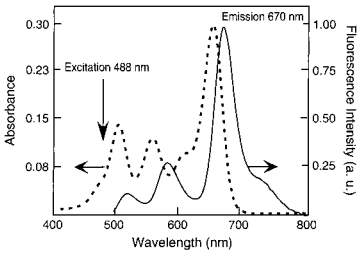

Figure 1. Absorption (···) and emission (−) spectra of **F-4-R-6-Cy-13**.

Chart 1. Molecular Structure of **F-4-R-6-Cy-13**

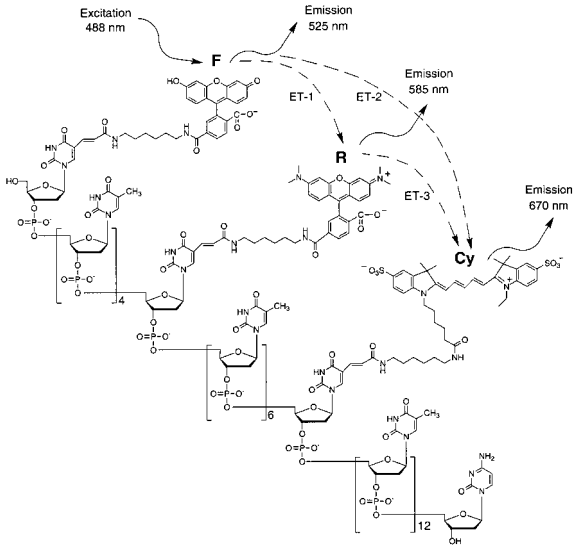

Figure 1 shows the spectra of **F-4-R-6-Cy-13** that exhibits the characteristic absorption and emission of **F** (λ_{abs}^{max}: 496 nm, λ_{em}^{max}: 525 nm), **R** (λ_{abs}^{max}: 555 nm, λ_{em}^{max}: 585 nm), and **Cy** (λ_{abs}^{max}: 643 nm, λ_{em}^{max}: 670 nm). The unique interaction of the three chromophores that are separated by defined number of nucleotides allows efficient ET to take place with dominant fluorescence emission from **Cy** at 670 nm with 488 nm excitation, leading to a "Stokes shift" of 182 nm. The quenching efficiency for **F** (Q_F) is 99%, and the overall fluorescence quantum yield (ϕ) for **F-4-R-6-Cy-13** is 0.13,[10] while ϕ for the **Cy** monomer is 0.27.[11]

To evaluate the ET property of **F-4-R-6-Cy-13** in ssDNA with different lengths, we used it as a primer in Sanger sequencing method[12] to produce DNA extension fragments terminated by biotinylated dideoxycytidine triphosphate (ddCTP-biotin) on a template generated from human genomic DNA. The solid-phase sequencing chemistry using ddCTP-biotin and streptavidin-coated magnetic beads allows the isolation of pure DNA extension fragments which are free from false terminations.[13] These ssDNA fragments, analyzed by a three-color capillary array electrophoresis (CAE) system and resolved at single base pair (bp) resolution, produced an electropherogram as shown in Figure 2. All the peaks for each of the DNA fragments (92 to 110 bp are shown) extended by **F-4-R-6-Cy-13** display a constant fluorescence emission signature of 2:3:7 (blue:green:red) that is defined by the ratio of the fluorescence intensity from each of the three detection channels (**F**, blue, 520 ± 20 nm; **R**, green, 585 ± 20 nm; **Cy**,

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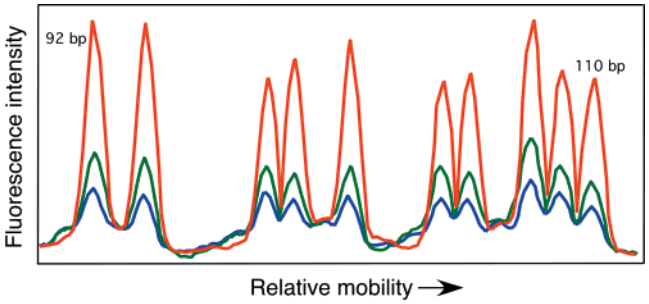

Figure 2. Electropherogram of the DNA sequencing fragments generated with **F-4-R-6-Cy-13** as a primer and separated on a CAE sequencer. **F** channel, blue; **R** channel, green; **Cy** channel, red.

Table 1. Polarization Data (*P*) for the Dye-Labeled DNAs (5′-T_{25}C-3′)

single-stranded DNA[a]	P_{FF}	P_{RR}	P_{CyCy}	P_{FR}	P_{RCy}	P_{FCy}
F-4-R-6-Cy-13	0.13	0.22	0.28	0.045	0.10	0.009
F-4-R-20	0.11	0.20		0.039		
F-11-Cy-13	0.065		0.27			0.010
5-R-6-Cy-13		0.27	0.28		0.050	
F-25	0.043					
5-R-20		0.16				
12-Cy-13			0.29			

[a] The number in each ssDNA refers to the number of nucleotides.

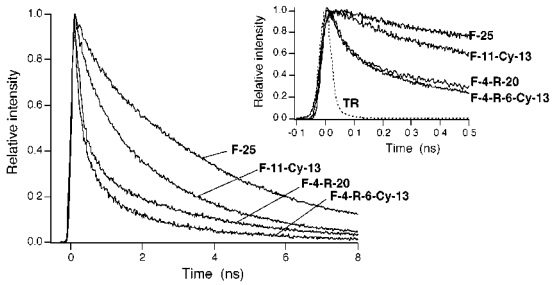

Figure 3. Fluorescence decay of **F** recorded from 510 to 540 nm after laser excitation (2 ps) of **F** at 488 nm for **F-25**, **F-11-Cy-13**, **F-4-R-20**, and **F-4-R-6-Cy-13 (**1X TBE buffer solution, pH = 8.5). TR: Temporal response at 488 nm using the scattered laser light.

red, 670 ± 20 nm). This indicates that the ssDNA fragments with different lengths have the same ET efficiency, displaying a fluorescence signature dominated by **Cy** emission in the entangled linear polyacrylamide polymer during electrophoresis. Thus, the trichromophore-labeled ssDNA with enhanced fluorescence emission intensity and a large "Stokes shift" can be used as a primer for DNA sequencing and PCR-based genetic analyses.

We have also synthesized a series of fluorescently labeled DNA with one and two chromophores (structures shown in Table 1), consisting of the same sequence as **F-4-R-6-Cy-13**, to systematically analyze the photophysics of the trichromophore-labeled DNA. Figure 3 shows the fluorescence ET decay kinetics of **F** after its excitation with laser pulses (2 ps pulse width) at 488 nm. **F-25** shows a fluorescence lifetime of approximately 3.5 ns. Introduction of the ET acceptor dyes, **R** and **Cy** to form **F-4-R-20** (Q_F = 95%; ϕ = 0.22) and **F-11-Cy-13** (Q_F = 72%; ϕ = 0.43), decreases the fluorescence lifetime of **F** because of ET quenching. Due to the complexity of kinetics, the fluorescence decay rate constants will be determined in the future.

Qualitatively, Figure 3 shows that the fluorescence lifetime of **F** in **F-4-R-20** is shorter than that in **F-11-Cy-13** due to an increase in donor−acceptor distance and a reduced spectral overlap between **F** and **Cy**.[14] For **F-4-R-6-Cy-13**, ET from singlet excited states of **F** can occur to produce singlet excited states of **R** (*ET-1*; Chart 1) and **Cy** (*ET-2*), respectively. Therefore, **F** shows the shortest lifetime in **F-4-R-6-Cy-13**. In addition, ET from singlet excited states of **R**, generated by *ET-1*, can produce singlet excited states of **Cy** (*ET-3*). By time-resolved fluorescence spectroscopy (λ_{ex} = 488 nm) of **F-4-R-6-Cy-13** and kinetic analysis of the individual dye components, the energy-transfer processes *ET-1*, *ET-2*, and *ET-3* were directly observed by delayed growth of the fluorescence from **R** and **Cy**.[10]

Fluorescence depolarization studies provided further information for the photophysics of **F-4-R-6-Cy-13**. Table 1 shows the polarization values (*P*) for the individual fluorophore and ET components in the fluorescently labeled DNAs, respectively. The *P* values were determined by exciting one fluorophore with linear polarized light and analyzing the depolarization of the fluorescence at different wavelengths corresponding to the individual fluorophores. The P_{FF} value of 0.043 for **F-25** is the lowest because the linear polarization can be lost due to the high level of rotation of the molecule during its fluorescence lifetime. For the two-dye (**F-11-Cy-13**, **F-4-R-20**)- and three-dye (**F-4-R-6-Cy-13**)-labeled DNAs, the polarization values P_{FF} are higher than that of **F-25** (Table 1) due to reduced fluorescence lifetime of **F**, which is caused by ET quenching. The photophysics derived from the order of P_{FF} values (**F-25** < **F-11-Cy-13** < **F-4-R-20** < **F-4-R-6-Cy-13**) agrees with that of the fluorescence lifetimes (**F-25** > **F-11-Cy-13** > **F-4-R-20** > **F-4-R-6-Cy-13**) (Figure 3). Fluorescence polarization can be partly transferred from one dye to another dye during energy transfer. For example, some fluorescence polarization of **R** can be observed after excitation of **F** (P_{FR}) in **F-4-R-20** and **F-4-R-6-Cy-13**. Because of the shorter lifetime of **R** in **F-4-R-6-Cy-13**, which is caused by *ET-3*, P_{FR} for **F-4-R-6-Cy-13** is higher than that of **F-4-R-20**. Similar interpretations can be applied to other *P* values in Table 1.

These results illustrate that a covalent assembly of a triple fluorescence ET system can be constructed to have enhanced acceptor emission and a large "Stokes shift". We used the trichromophore-labeled DNA as a primer to produce nucleic acid sequencing fragments with constant fluorescence signature which is independent of the length of DNA fragments. The covalent trichromophoric labeling approach can further be extended to more than three chromophores and can be applied to broad applications in biological labeling and imaging.

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Supporting Information Available: Details of fluorescently labeled DNA synthesis and characterization by mass spectrometry, DNA sequencing fragments generation, absorbance, steady state and time-resolved fluorescence, quenching efficiency, and quantum yield calculation and polarization studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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