

## Luminescence Resonance Energy Transfer

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Fluorescence resonance energy transfer (FRET), in which a fluorescent donor molecule transfers energy via a nonradiative dipole–dipole interaction to an acceptor molecule (which is usually a fluorescent molecule), is a standard spectroscopic technique for measuring distances in the 10–70-Å range. Upon energy transfer, which depends on the  $R^{-6}$  distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced, and the acceptor fluorescence is increased, or sensitized.<sup>1</sup>

We have used a luminescent europium chelate as donor and an organic dye, CY-5, as acceptor. This luminescence resonance energy transfer (LRET) has several advantages over the more conventional FRET.<sup>2</sup> The distance at which 50% of the energy is transferred ( $R_0$ ) is large, 70 Å; the donor lifetime is single exponential and long (0.63 ms in H<sub>2</sub>O; 2.5 ms in D<sub>2</sub>O), making lifetime measurements facile and highly accurate; the orientation dependence ( $\kappa^2$ ) of energy transfer is minimized by the donor's multiple electronic transitions and long lifetime, limiting uncertainty in the measured distance due to orientation effects to  $\pm 12\%$  in the worst case;<sup>3</sup> the sensitized emission of the acceptor can be measured with little or no interfering background, yielding a >50-fold improvement in signal to background over standard donor–acceptor pairs and enabling distances several times  $R_0$  to be measured.<sup>4</sup> We also measure the sensitized emission lifetime which, in our case, is independent of total concentration and incomplete labeling.

We have used both terbium<sup>5</sup> and europium as donors, and the results for europium are presented here. The donor–acceptor model system is known in Figure 1. The double-stranded DNA oligomer serves as a rigid tether to establish a defined distance between the europium donor and the CY-5 acceptor. The points of attachment of the donor and acceptor are separated by 42 Å, although there is some uncertainty about the exact position of the dyes due to the flexible six-carbon linkers used for attachment.<sup>6,7</sup>

Figure 2 shows the spectral characteristics which lead to the unusually large  $R_0$  of 70 Å in D<sub>2</sub>O (56 Å in H<sub>2</sub>O).  $R_0$  is determined

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(1) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*; W. H. Freeman and Co.: San Francisco, 1980; Vol. 2.

(2) Lanthanides in energy transfer have primarily been used in diffusion-enhanced FRET (Stryer, L.; Thomas, D. D.; Meares, C. F. In *Annual Review of Biophysics and Bioengineering*; L. J. Mullins, Ed.; Annual Reviews, Inc.: Palo Alto, CA, 1982; Vol. 11; pp 203–222). They have also been used with multichromophoric allophycocyanin (Mathis, G. *Clin. Chem.* 1993, 39, 1953) and as isomorphous replacements in calcium-binding proteins (Horrocks, W. D., Jr.; Holmquist, B.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* 1975, 72, 4764. Cronce, D. T.; Horrocks, W. D., Jr. *Biochemistry* 1992, 31, 7963).

(3) Stryer, L. *Annu. Rev. Biochem.* 1978, 47, 819–846.

(4) The ability to measure energy transfer well beyond  $R_0$  using sensitized emission has been shown by Bruno and Horrocks. They used terbium as the acceptor and tyrosine as the donor. With an  $R_0$  of 3 Å they measured out to 12 Å. (Bruno, J.; Horrocks, W. D., Jr.; Zauhar, R. J. *Biochemistry* 1992, 31, 7016.)

(5) Selvin, P. R.; Hearst, J. E. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

(6) Cardullo, R.; Agrawal, S.; Flores, C.; Zamecnik, P. C.; Wolf, D. E. *Proc. Natl. Acad. Sci. U.S.A.* 1988, 85, 8780.

(7) Clegg, R. M.; Murchie, A. I.; Zechel, A.; Lilley, D. M. *Proc. Natl. Acad. Sci. U.S.A.* 1993, 90, 2994.

from standard equations<sup>1</sup> based on a calculated spectral overlap ( $J$ ) of  $6.55 \times 10^{15} \text{ nm}^4 \text{ M}^{-1}$ , an orientation factor ( $\kappa^2$ ) of  $2/3$ , an index of refraction of 1.33, and a quantum yield for europium luminescence in D<sub>2</sub>O of 1 (0.25 in H<sub>2</sub>O).<sup>8</sup> In calculating  $R_0$  it is important to use the quantum yield of the lanthanide emission, and not the quantum yield of the entire chelate, and to include in the spectral overlap ( $J$ ) calculation only those transitions which are electric dipole. The europium emission at 617 nm, which is used here for energy transfer, has been shown to be "forced" electric dipole,<sup>9</sup> and hence Förster's theory of energy transfer is applicable. The europium emission at 596 nm cannot couple to an acceptor because it is a magnetic dipole transition and so is not included in the spectral overlap calculation.<sup>10</sup>

We can measure the sensitized emission of the acceptor without significant interference from either donor emission or direct acceptor fluorescence. At 668 nm, europium is nearly silent (europium emission at 668 nm is 125 times less than at its 617-nm maximum) and by using pulsed excitation and gating off the detector for 90  $\mu\text{s}$ , the direct fluorescence of the carbostyryl sensitizer in the donor complex and the direct fluorescence of the CY-5 are completely eliminated, while the europium stays excited and capable of energy transfer.<sup>11</sup>

Figure 3 shows such a dark-background sensitized emission experiment. Here the ratio of donor to acceptor strands is approximately 1:0.6; we intentionally add less acceptor than donor to show the ability of our system to analyze heterogeneous signals (see Figure 4). The average fraction of energy transfer in Figure 3 is 57%. The signal at 668 nm arises from sensitized emission of CY-5, i.e., fluorescence due only to energy transfer. We calculate the signal/background at 668 nm to be 94:1 (where background is due to a small amount of europium luminescence), a factor of 50–100 improvement in signal/background over the sensitized emission signal from fluorescein–rhodamine, one of the best donor–acceptor pairs, attached to the same 10-mer (data not shown).

Figure 4 shows lifetime data corresponding to Figure 3. The donor-only signal is single exponential with a lifetime of 2.52 ms. The donor quenching signal fits a biexponential extremely well ( $\chi^2 = 0.998$ ):  $y = 63\% \exp(-t/(0.22 \text{ ms})) + 37\% \exp(-t/(2.40 \text{ ms}))$ . The long-time component corresponds to the donor-only species. That the long-time component nearly equals the donor-only lifetime is an internal control which shows that intermolecular energy transfer is at most 5%. The short-time component arises from intramolecular energy transfer in the hybridized donor–acceptor complex and corresponds to 91% quenching (1–0.22 ms/2.52 ms) and a donor–acceptor distance of 46 Å. (Fluorescein–rhodamine on the same DNA with C-6 linkers yields 22% energy transfer.) A titration with increasing acceptor concentration increases the fraction of the short-time component but does not change its lifetime, as expected. At a 2-fold excess of acceptor strand, a 10% component corresponding to donor-only signal remains, presumably due to unhybridized donor strands.

Figure 4 also shows the lifetime of the sensitized emission.

(8) The exact quantum yield is difficult to determine although the long lifetime and lack of radiationless deactivation mechanisms make it likely that the quantum yield is close to 1 in D<sub>2</sub>O. This assumption has given distances in agreement with X-ray crystallography studies (see ref 4). In H<sub>2</sub>O, the quantum yield is decreased because there are 1.3 water molecules in the primary coordination sphere of the lanthanide in our chelate (data not shown) (Horrocks, W. D., Jr.; Sudnick, D. R. *J. Am. Chem. Soc.* 1979, 101, 334).

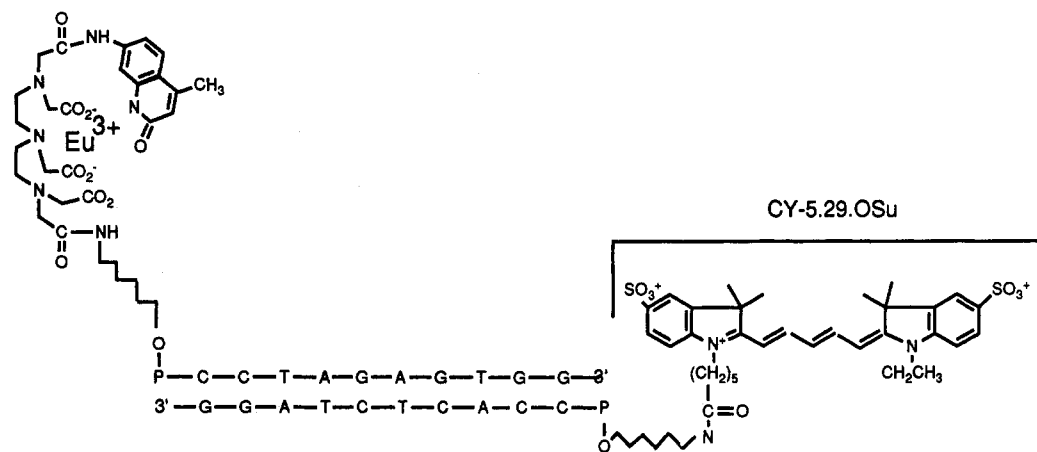
(9) Bunzli, J.-C. G. In *Lanthanide Probes in Life, Chemical and Earth Sciences, Theory and Practice*; Bunzli, J.-C. G., Choppin, G. R., Eds.; Elsevier: New York, 1989; pp 219–293.

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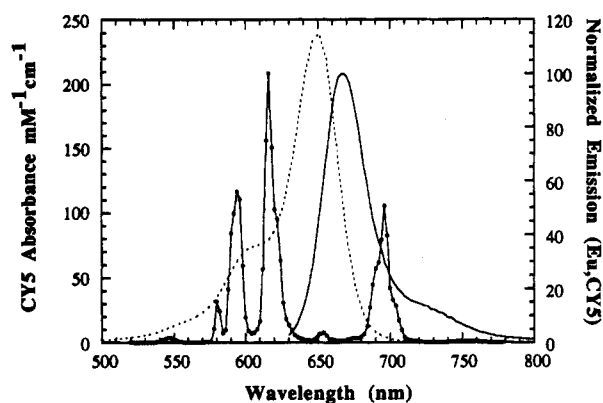
(11) Morrison has used gated integration to increase the signal to background of the sensitized emission with organic dyes. Morrison, L. E. *Anal. Biochem.* 1988, 174, 101.

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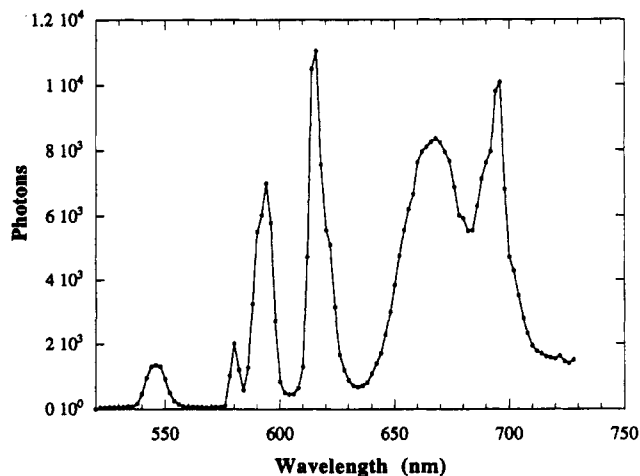
(13) Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* 1993, 4, 105.



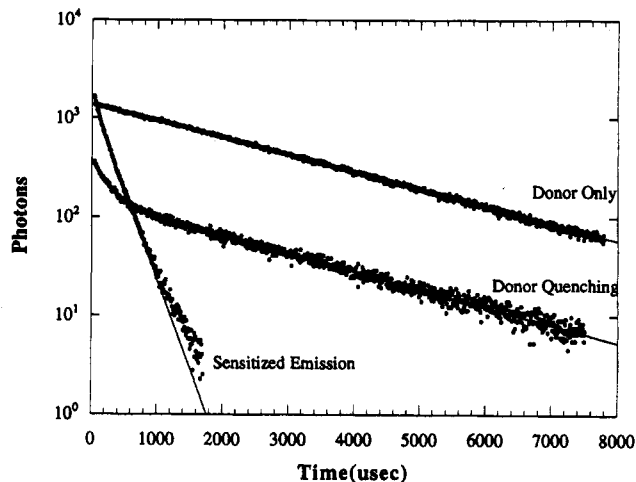
**Figure 1.** Schematic diagram of double-stranded DNA with europium chelate (donor) at one 5' end and CY-5 at the other 5' end. The europium chelate, (diethylenetriaminepentaacetic acid-carboxystyryl 124-Eu: trivial name, DTPA-cs124-Eu) was made by a modification of the procedure of Bailey,<sup>12</sup> starting with the dianhydride of DTPA (Sigma), carboxystyryl 124 (Aldrich), and the synthetic DNA base-protected and on the column to ensure that labeling occurs only at the 5' amino group. The cs124 effectively increases the absorption cross section of the europium to approximately  $8000 \text{ M}^{-1} \text{ cm}^{-1}$  at 337 nm, where we excited the donor with a pulsed nitrogen laser. A 100-fold excess of EDTA did not remove any noticeable amount of europium from the DTPA-cs124 chelator. The acceptor was 5' labeled with CY-5<sup>13</sup> (Biological Detection Systems) via standard methods. Unlabeled complementary DNA oligomers were made as controls. All DNA was reversed-phase HPLC purified.



**Figure 2.** Spectra of DNA labeled with either CY-5 or DTPA-cs124-Eu. Dashed and solid lines are the absorption and emission spectra of CY-5, respectively. Solid line with circles is the emission spectrum of DTPA-cs124-Eu on DNA. The small signal at 548 nm is due to contaminating terbium. All data shown are at  $0.5 \mu\text{M}$  donor strand concentration in 10 mM Tris-HCl pH 8.0, 10 mM  $\text{MgCl}_2$ , 150 mM NaCl,  $\text{D}_2\text{O}$  at  $5^\circ\text{C}$ . Decreasing concentration by 2- and 4-fold yielded the same results. Emission spectroscopy was done on a laboratory-built spectrometer utilizing a pulsed nitrogen laser, a photon-counting detector, and a multichannel scalar with  $2\text{-}\mu\text{s}$  time resolution. The CY-5 emission spectrum shown above was obtained on a steady-state SPEX fluorimeter.



**Figure 3.** Emission spectrum of a mixture of donor-strand DNA and acceptor strand in approximately a 1:0.6 ratio (see Figure 1). The signal is collected with a 7.5-ms gate after a  $90\text{-}\mu\text{s}$  delay.



**Figure 4.** Lifetime data corresponding to Figure 3, showing a donor-only lifetime of 2.5 ms, a biexponential donor quenching corresponding to a mixture of donor-only and donor-acceptor complexes, and a largely single exponential sensitized emission signal. The latter signal is insensitive to donor-only or acceptor-only species. The donor-only lifetimes on single-stranded and double-stranded DNA differ by less than 5%.

The sensitized emission lifetime signal is fit to a biexponential ( $r^2 = 0.999$ :  $y = 40\% \exp(-t/(59 \mu\text{s})) + 60\% \exp(-t/(0.25 \text{ ms}))$ ). The short-time component is due to direct fluorescence of the acceptor and can be eliminated by gating the detector. The 0.25-ms component is due to an energy transfer of 90% ( $1-0.25 \text{ ms}/2.52 \text{ ms}$ ), in excellent agreement with the short-time component of the donor quenching. A very small long-time component ( $\approx 1\%$ ) can be seen due to direct donor fluorescence.

In summary, luminescence energy transfer yields results consistent with a Förster theory assuming that the appropriate parameters are used. On the basis of the large  $R_0$ , the ease and reproducibility of our lifetime measurements, and the excellent signal to background, we expect distances significantly greater than  $100 \text{ \AA}$  to be measurable.

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