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On the Possibility of Long-Wavelength Long-Lifetime High-Quantum-Yield Luminophores

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Abstract

We describe an approach to creating a new class of luminophores which display both long wavelength emissions exceeding 600 nm and long lifetimes. These luminophores are based on resonance energy transfer (RET) from a long lifetime donor to a short lifetime but long wavelength acceptor. We demonstrated the possibility of obtaining these desirable spectral properties using donors and acceptors noncovalently bound to DNA. The donor was a ruthenium (Ru) metal–ligand complex in which one of the diimine ligands intercalated into double-helix DNA. The acceptors were either nile blue, TOTO-3, or TO-PRO-3. Upon binding of the acceptor to donor-labeled DNA, we found that the acceptor quantum yield was remarkably enhanced so that the wavelength-integrated intensities of the donor and acceptor bound to DNA were many-fold greater than the intensity of the donor and acceptor alone when separately bound to DNA. The origin of this effect is efficient energy transfer from the donor. Under these conditions the effective overall quantum yield approaches that of the acceptor. Importantly, the increased quantum yield can be obtained while maintaining usefully long apparent acceptor lifetimes of 30 to 80 ns. The effect of an increased quantum yield from a low quantum yield donor may find use in assays to detect macromolecular binding interactions. These results suggest the synthesis of covalently linked donor–acceptor pairs with the desirable spectral properties of long wavelength emission, high quantum yield, and moderately long lifetimes for gated detection.

There is considerable interest in the development and use of fluorophores with red or near infrared (NIR)³ emission (1, 2). Long wavelength excitation and emission are valuable because the background autofluorescence from biological samples and optical components typically decreases at longer incident wavelengths. Red and NIR probes are now routinely used in protein labeling (2, 3), chromatography (4, 5), measurements in blood (6, 7), noninvasive medical testing (8–10), and DNA analysis (11–13). An advantage of the presently available red-NIR fluorophores is their high extinction coefficient, which in turn allows high sensitivity detection. However, the high extinction coefficients are also a disadvantage because a high probability for absorption also results in a high rate of

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³Abbreviations used: A, acceptor; AO, acridine orange; bp, base pair; bpy, 2,2′-bipyridine; CT-DNA, calf thymus DNA; D, donor; DA, donor–acceptor; dppz, dipyrro [3,2-a:2′,3′-c]phenazine; EB, ethidium bromide; LED, light-emitting diode; MLC, metal–ligand complex; NB, nile blue A perchlorate; RET, resonance energy transfer; Ru–BD, [Ru(bpy)₂(dppz)](PF₆)₂; NIR, near infrared; PMT, photomultiplier tube; DMSO, dimethylsulfoxide; FISH, fluorescence *in situ* hybridization.

emission, which results in short decay times (14). Consequently, most known red-NIR fluorophores display lifetimes below 4 ns, and often below 1 ns. These fluorophores typically display small Stokes' shifts, and scattered light is most difficult to eliminate at wavelengths close to the excitation wavelength. Also, the autofluorescence from samples is typically on the ns timescale, and the signal-to-background ratio cannot be significantly improved by gated detection after the excitation pulse. Hence, the signals detected with red or NIR probes can be affected by scattered light and/or sample autofluorescence.

In the present paper we report a new approach to obtaining luminophores with long emission maxima, large Stokes' shifts and high effective quantum yields. These luminophores are typified in Scheme 1, which shows a long lifetime donor (D) which is covalently linked to an acceptor (A), with spectral properties such that resonance energy transfer occurs with moderate to high efficiency. In this case the D-to-A distance is assumed to be $0.7 R_0$, where R_0 is the Förster distance. This separation results in approximately 90% transfer. The donor is assumed to be a luminescent transition metal–ligand complex (MLC). Many such MLCs are known, and they can display a wide range of absorption and emission wavelengths and long decay times ranging from 100 ns to 10 μ s (15, 16). In recent years these complexes have been developed for use as luminescent probes (17, 18) for studies of protein dynamics, immunoassays, and chemical sensing (19–24).

While the MLCs are useful luminophores, they possess several disadvantages. The extinction coefficients are low, typically near $10,000 \text{ M}^{-1} \text{ cm}^{-1}$, and the emission spectra are broad. Broad emission spectra result in significant spectral overlap of the emission spectra of various MLCs, and an inability to use measurements at multiple emission wavelengths to resolve multiple species in macroscopic or microscopic samples. Broad emission spectra also reduce sensitivity because the autofluorescence over the wide range of wavelengths contributes to the measured intensity.

We have now realized that most of the disadvantages of the MLCs could be overcome by the use of a tandem probes in which a long lifetime MLC is the donor and a high quantum yield but short lifetime fluorophore is the acceptor (Scheme 1). In such luminophores RET will result in excitation of the acceptor throughout the donor decay. Assume the donor lifetime in the absence of acceptor is $\tau_D = 1000 \text{ ns}$. The presence of a single acceptor at a distance of $0.7 R_0$ will reduce the lifetime to about 100 ns. For the moment assume that the acceptor does not absorb at the donor excitation wavelength (λ_D^{ex}). In this case the acceptor is excited solely by RET from the donor. Since the acceptor lifetime is short ($\tau_D = 1 \text{ ns}$), the acceptor intensity will closely follow the donor intensity. Hence the acceptor will display the same decay time as the donor and the acceptor decay time (τ_{AD}) will be near 100 ns. Most acceptors will display some absorption at the donor excitation wavelength. In this case the acceptor emission will typically display two decay times, a nanosecond component due to directly excited acceptor and long decay time near 100 ns due to RET from the donor. The long lifetime emission acceptor can be readily isolated with gated detection, which is readily accomplished with photomultiplier tubes (PMTs) (25–27). Gated detection is frequently used in immunoassay based on the lanthanides (28, 29).

An important advantage of such a RET probe (Scheme 1) is an increase in the effective quantum yield of the long lifetime luminophore. This effect is illustrated in Scheme 2. Suppose the donor and acceptor both display quantum yields of unity ($Q_D = Q_A = 1.0$). In this case (top) RET quenches the donor and results in an equivalent increase in the emission intensity of the acceptor. The integrated or total intensity of the donor and acceptor remains the same in the presence or absence of RET.

A surprisingly different result is obtained if the donor displays a low quantum yield. For example, the commonly used ruthenium MLCs have quantum yields of 0.05 or less. In this case the donor emission without RET is much weaker (Scheme 2, bottom). However, RET to a nearby acceptor still results in the same increased intensity of the acceptor. More specifically, the transfer efficiency can approach unity even though the donor quantum yield is low. A favorable result of efficient RET from the donor is that the wavelength integrated intensity of the D–A pair can be almost 20-fold larger than that of the donor or acceptor alone. More specifically, for 100% transfer efficiency, the overall quantum yield becomes the quantum yield of the acceptor. These considerations suggest that tandem RET probes based on MLC donors can be used to create long lifetime probes, with red-NIR emission, with the added advantage of an increased effective quantum yield. Additionally, the modular design of these probes allows practical and rational adjustment of the spectral properties including the excitation and emission wavelengths and the decay times.

Given the simplicity of this idea we examined the literature for suggestions of designing luminophores using low quantum yield donors. There are numerous primary reports and review articles on RET, and the concept of using the acceptor emission to measure the transfer efficiency is not new (30–33). Additionally, Selvin and coworkers have already noted the usefulness of measuring the long lifetime acceptor emission with lanthanide donors to selectively detect D–A pairs (34) and to provide a long decay time for the acceptor (35, 36). Donors and acceptors with short decay times have been covalently linked for use in DNA sequencing (37, 38) and as high-affinity dyes which bind noncovalently to DNA (39, 40). The novelty of the present report is recognition that the effective quantum yield of the luminophore could be increased by rapid energy transfer. Such an increase in effective quantum yield was not important for the biochemical uses of RET (30–33) because most organic donors have good quantum yields. An increased effective quantum yield of the donor has not been important for RET with the lanthanides because the chelated lanthanide donors often display quantum yields near unity. The value of the RET-enhanced quantum yield only becomes apparent because of the development of the long lifetime MLC probes and their low quantum yields. In retrospect, the possibility of increasing the effective quantum yield of the donor was observed by the enhancement of lanthanide emission when bound to essentially nonluminescent DNA or nucleotides (41–43). However, to the best of our knowledge, the practical utility of the RET-enhanced effective quantum yield with low quantum yield donors has not been previously reported.

THEORY

The theory and application of RET have been described in numerous reviews (44–46). Here we discuss those aspects of RET needed to demonstrate the occurrence of the RET enhanced quantum yield. The rate of energy transfer from a donor to an acceptor is given by

$$k_T = \frac{1}{\tau_D} \left(\frac{R_0}{r} \right)^6,$$

[1]

where τ_D is the donor lifetime in the absence of acceptor, r is the donor-to-acceptor distance, and R_0 is the Förster distance at which RET is 50% efficient. The value of R_0 is dependent on the spectral properties of the donor and acceptor and can be accurately calculated from the spectral properties of the donor and acceptor.

Consider the donor–acceptor pair (Scheme 1) with the hypothetical spectral properties shown in Scheme 2. The donor is assumed to have a lifetime $\tau_D = 1000$ ns in the absence of RET, and the acceptor a lifetime of $\tau_A = 1$ ns when directly excited. The efficiency of energy transfer is given by the ratio of the transfer rate to the total rate of donor deactivation, which is the reciprocal of the lifetime in the presence of RET (τ_{DA}). Hence the transfer efficiency (E) from the donor is given by

$$E_D = \frac{k_T}{k_T + \tau_D^{-1}}.$$

[2]

The transfer efficiency as measured using this donor can be determined experimentally from the relative intensities of the donor (E_D) in the absence (F_D) and presence of acceptor (F_{DA}):

$$E_D = 1 - \frac{F_{DA}}{F_D}.$$

[3]

The transfer efficiencies can also be calculated from the donor decay times in the absence (τ_D) or presence (τ_{DA}) of acceptors:

$$E_D = 1 - \frac{\tau_{DA}}{\tau_D}.$$

[4]

Equation [4] is valid only when the donor decay is a single exponential. For the D–A pair shown in Scheme 2 the donor will display a lifetime (τ_{DA}) of 100 ns in the presence of acceptor. This decay time is the reciprocal of the sum of the deactivation rates of the donor,

$$\tau_{DA} = 1/(k_T + \tau_D^{-1}).$$

[5]

The acceptor is expected to show two decay times, one due to directly excited acceptors ($\tau_A = 1$ ns) and one due to RET from the donor (τ_{AD}). This long component will have a decay time essentially equivalent to that of the donor ($\tau_{AD} = 100$ ns) because the decay time of the acceptor is much shorter than that of the donor. Hence one can obtain a long lifetime component from the acceptor, which if directly excited would display a short lifetime.

It is known that the transfer efficiency can be measured using the intensity of the acceptor emission (30). The transfer efficiency as seen from the acceptor (E_A) is given by

$$E_A = \frac{\epsilon_A}{\epsilon_D} \left(\frac{F_{AD}}{F_A} - 1 \right),$$

[6]

where ϵ_A and ϵ_D are the molar extinction coefficients of the donor (ϵ_D) and acceptor (ϵ_A) at the excitation wavelength, and F_A and F_{AD} are the acceptor intensities in the absence and presence of the donor, respectively. The use of ϵ_A and ϵ_D is only appropriate for a one-to-one ratio of donor to acceptor. In the present report the donors and acceptors are not covalently linked and are not present in a one-to-one molar ratio. In this case Eq. [6] becomes

$$E_A = \frac{OD_A}{OD_D} \left(\frac{F_{AD}}{F_D} - 1 \right),$$

[7]

where OD_D and OD_A refers to the optical density of the donor or acceptor at the donor excitation wavelength.

In the present report we used donors and acceptors noncovalently bound to DNA, so that Eq. [7] is appropriate. However, it is easier to consider a single D–A pair. For a covalently linked D–A pair assume that the acceptor absorbs 10-fold less at the excitation wavelength than the donor, that is, $\epsilon_A/\epsilon_D = 0.1$. This is possible because long-wavelength probes frequently show weak absorption between their long wavelength and short wavelength absorption maxima. Assume additionally that the transfer efficiency is $E_A = 90\%$. Substitution of these assumed values into Eq. [6] yields $F_{AD}/F_D = 10$. Hence RET results in a 10-fold enhancement of the acceptor emission (Scheme 2, bottom).

What is the origin of the larger overall quantum yield for the tandem luminophore when the donor quantum yield is low? This can be understood by considering the factors which determine the intensity of the donor and acceptor. One can readily show that the total emission from a tandem luminophore $F_T = F_D + F_A$ is given by

$$F_T = k\epsilon_D Q_D (1 - E) + k(\epsilon_A + \epsilon_D E) Q_A,$$

[8]

where Q_D and Q_A are the quantum yields of the donors and acceptors, respectively, and E is the actual transfer efficiency, and k is a constant containing the excitation intensity, concentration and instrumental factors. This expression assumes the sample is diluted so that the amount of light absorbed is directly proportional to the concentration. In the absence of energy transfer the total intensity is given by

$$F_T = k(\epsilon_D Q_D + \epsilon_A Q_A),$$

[9]

as expected for a mixture of two noninteracting fluorophores. Suppose the transfer efficiency is 100%. In this case the overall quantum yield of the tandem luminophore becomes

$$F_T = k(\epsilon_A + \epsilon_D)Q_A.$$

[10]

This result shows that all the photons absorbed by the donor are transferred to the acceptor, and the effective quantum yield becomes that of the acceptor. Examination of Eq. [8] shows that as the transfer efficiency increases the absorbed energy is shifted from the low quantum yield donor to the high quantum acceptor, irrespective of the quantum yield of the donor in the absence of energy transfer.

Given the magnitude of this effect one may wonder why such large enhancements of the acceptor emission have not been reported. One explanation is that acceptors frequently absorb as strongly as the donors at the donor excitation wavelengths. For instance, suppose $\epsilon_A/\epsilon_D = 1.0$. In this case a transfer efficiency of 90% results in a 1.9-fold or 90% enhancement of the acceptor emission. A common occurrence is when the acceptor absorbs more strongly than the donor. Suppose $\epsilon_A/\epsilon_D = 10$ (Scheme 3). In this case a transfer efficiency of 90% results in $F_{AD}/F_A = 1.09$, so the acceptor enhancement is only 9% (Eq. [6]). Also, when working with typical organic fluorophores the quantum yields of the donors and acceptors are usually comparable so there is a shift in emission wavelength without an overall increase in the total intensity (Scheme 2, top).

It is informative to consider the time-dependent decays of the donor and acceptor in the presence of energy transfer. These expressions can be obtained using the known solution for a reversible excited state reaction (47–51) and setting the reverse transfer rate to zero. Assuming that the donor emission and the acceptor emission can be observed separately, the time-dependent changes of the donor and acceptor are then given by

$$I_{DA}(t) = C_D \alpha_D \exp(-t/\tau_{DA})$$

[11]

$$I_{AD}(t) = C_A [\beta_A \exp(-t/\tau_A) - \beta_A \exp(-t/\tau_{DA})].$$

[12]

In these expressions α_D and β_A are the time zero amplitudes of the intensity decays, respectively, and C_D and C_A are constants which depend on the sample concentration and the experimental apparatus. Also, we have assumed that the donor and directly excited

acceptors display a single exponential decay. The pre-exponential factors for the acceptor decay are equal and opposite and are given by

$$\beta_A = \frac{k_T \lambda_A}{\Gamma_D - \Gamma_A + k_T}.$$

[13]

In this expression λ_A is the radiative decay rate of the acceptor and Γ_D and Γ_A are the decay rates of the donor in the absence of acceptors, and the directly excited acceptor, respectively. These values are the reciprocals of the decay times in the absence of energy transfer, $\Gamma_D = \tau_D^{-1}$ and $\Gamma_A = \tau_A^{-1}$.

Suppose the rate of transfer is very rapid ($\tau_{DA} \rightarrow 0$ or $k_T \rightarrow \infty$). Then the acceptor decay becomes that would be observed if the acceptor were directly excited:

$$I_{AD}(t) = C_A \lambda_A \exp(-t/\tau_A).$$

[14]

The more interesting result is when the decay time of the donor is much longer than that of the acceptor; that is, $\tau_D \gg \tau_A$. Assuming the acceptor displays a reasonable quantum yield, the acceptor decay becomes

$$I_{AD}(t) = \frac{C_A k_T \lambda_A}{\Gamma_A - k_T} [\exp(-t/\tau_{DA}) - \exp(t/\tau_A)].$$

[15]

Following decay of the short component with a decay time τ_A the acceptor decay displays a lifetime equivalent to the donor decay time in the presence of energy transfer,

$$I_{AD}(t) = C \exp(-t/\tau_{DA}),$$

[16]

where C is a constant.

MATERIALS AND METHODS

Materials.

CT-DNA, Tris-HCl, and EDTA were obtained from Sigma (St. Louis, MO). Ru-BD was synthesized by the method described previously (52, 53). AO, EB, TOTO-3, and TO-PRO-3 were purchased from Molecular Probes (Eugene, OR) and NB was from Aldrich (Milwaukee, WI). All reagents were used without further purification and water was deionized with a Milli-Q system. To convert CT-DNA into linear fragments comparable in length to one persistent length, about 5 mg/ml solution of CT-DNA was sonicated approximately 10 min while submerged in an ice bath. The sonicated DNA solution was centrifuged for 1 h at 75,000g to remove titanium particles and undissolved DNA. All experiments were undertaken at room temperature in 2 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA.

Absorption and steady-state fluorescence measurement.

AO, EB, and Ru-BD served as donors and NB, TOTO-3, and TO-PRO-3 were used as acceptors. About 5–10 mM stock solutions of AO, Ru-BD, and NB were prepared in dimethylformamide (DMF) and about a 10 mM stock solution of EB were made in DMSO. The final DMF concentration in all solutions was less than 1% (v/v). The concentration of DNA was quantified using a molar extinction coefficient of $13,300 \text{ M}^{-1} \text{ cm}^{-1}$ (expressed as bp) at 260 nm. The DNA concentration was 1 mM bp while the concentrations of AO, EB, and Ru-BD were 5, 10, and 20 μM , respectively. Concentrations of the probes were determined using the extinction coefficients in Table 1. The highest acceptor concentrations of Ru-BD/NB, Ru-BD/TOTO-3 and Ru-BD/TO-PRO-3 D-A pairs were 120, 60, and 120 μM , respectively. Because TOTO-3 and TO-PRO-3 were supplied as 1 mM stock solutions in DMSO, the maximum percentages of DMSO in the Ru-BD/TOTO-3 and Ru-BD/TO-PRO-3 D-A pairs were 6 and 12% (v/v), respectively. In preliminary experiments, we found that DMSO increased the steady-state fluorescence intensity of Ru-BD (data not shown). Hence, we added aliquots of DMSO to obtain 6 and 12% (v/v) DMSO in all Ru-BD/TOTO-3 and Ru-BD/TO-PRO-3 D-A pairs, respectively, to equalize the effect of DMSO. UV-visible absorption spectra were measured with a Hewlett-Packard 8453 diode array spectrophotometer with ± 1 nm resolution. Steady-state fluorescence measurements were carried out using an Aminco SLM AB2 spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY) under magic angle conditions. The excitation wavelengths of AO, EB, and RuBD were 470, 518, and 440 nm, respectively.

Frequency-domain fluorescence measurements.

Measurements were performed using the instruments described previously (54) and modified with a data acquisition card from ISS, Inc. (Urbana, IL) (55). The excitation source was a blue LED LNG992CFBW (Panasonic, Japan) with luminous intensity of 1500 mcd, and an LED driver LDX-3412 (ILX Lightwave, Boseman, MO) provided 30 mA of current at frequencies from 1 to 9.3 MHz. A 450RD55 interference filter (Omega Optical, Inc., Brattleboro, VT) and a 4–96 color glass filter (Corning Glass Work, Corning, NY) were used to isolate the excitation wavelength. Rhodamine B in water was utilized as a lifetime

standard. The transmission curves of the filters for isolating the emission from the donor, D–A pairs, and acceptors are shown below (Fig. 6).

RESULTS

Steady-State Spectra

We use DNA with noncovalently bound donors and acceptors to test the possibility of creating long lifetime luminophores with high quantum yields. We chose three donors, AO, EB, and Ru-BD. These structures are shown in Fig. 1. When bound to DNA the quantum yields decrease in this respective order (Table 1). As acceptors, we selected Nile blue (NB), TOTO-3, and TO-PRO-3 (Fig. 1), which display increasing quantum yields in the listed order. We chose to examine dyes noncovalently bound to DNA because this approach allowed us to select donors and acceptors with various quantum yields, without the need for chemical synthesis. Also, this approach allowed us to adjust the concentrations of donors and acceptors to observe trends in the spectra. Based on the theory described above, we expect the largest overall increase in the total emission of the tandem luminophore to occur with RET between the lowest quantum yield donor (Ru-BD) and the highest quantum yield acceptor (TO-PRO-3).

Figure 2 shows the emission spectra of AO bound to DNA with increasing amounts of acceptor. With the high quantum yield AO donor the NB acceptor emission is almost undetectable (Fig. 2, top inset). The quantum yield of the TOTO-3 acceptor is higher than that of NB, and the quantum yield of TO-PRO-3 is higher still. As may be expected, the acceptor emission becomes more easily detectable as the acceptor quantum yields increase. In each case the observed acceptor emission is due to RET from the donor. No significant acceptor emission was found for the acceptors bound to DNA in the absence of donor (dashed lines). An interesting aspect of Fig. 2 is that it demonstrates an effect opposite to that emphasized in this paper. RET from a high quantum yield donor (AD) to a low quantum yield acceptor (NB) decreases the total emission from the donor and acceptor.

Figures 3 and 4 show emission spectra with the same acceptors, but with EB and Ru-BD as the donors. Examination of these spectra shows that the enhancement of the acceptor emission is larger for Ru-BD than for EB. Also, the largest enhancements are seen for TO-PRO-3, the acceptor with the highest quantum yield (Fig. 4, bottom). In this case the acceptor emission is increased many-fold by energy transfer from the Ru-BD donor. Also, the emission from the D–A system is considerably larger than that of the donor alone bound to DNA, or the acceptor alone bound to DNA (dashed line). This effect is the opposite of that found for the AO/NB D–A pair. In this case the weakly fluorescent NB received most of the energy by RET, but still emits with its own low quantum yield. For the Ru-BD/TO-PRO-3 D–A pair the strongly fluorescent TO-PRO-3 receives most of the energy absorbed by the donor, in spite of the low intrinsic quantum yield of the donor.

While we expected to see an increased overall quantum yield due to RET from Ru-BD, we were surprised by the magnitude of the effect. In the absence of energy transfer the intensity of the acceptor is proportional to $\epsilon_A Q_A$, where ϵ_A refers to the extinction coefficient of the acceptor at the donor excitation wavelength. If transfer is 100% effective the intensity of the

acceptor is proportional to $(\epsilon_A + \epsilon_D)/\epsilon_A$. According to Table 1 this ratio is near 4. Examination of Fig. 4 (bottom) indicates that the acceptor enhancement is greater than 4. To further this effect we examined the excitation spectra of the D–A pair, and the acceptor alone, when bound to DNA. On the same relative scale the acceptor alone displays essentially no emission upon excitation at 450 nm (Fig. 5). The lower panel shows the ratio of these excitation spectra, which become close to 40 at 450 nm. This ratio is larger than expected from the extinction coefficients listed in Table 1. It appears that excitation of TO-PRO-3 near 450 nm results in less emission than predicted by its absorption spectrum. This effect could be due to the presence of nonfluorescent absorbing impurities or absorption of nonfluorescent conformers of TO-PRO-3 at 450 nm. It is known that this class of dyes display weak fluorescence in water or when there is torsional motions about the central methine bridge (56). Irrespective of the origin of this low intensity, the acceptor enhancement seen in Fig. 4 is consistent with the excitation spectrum for this D–A pair.

Time-Resolved Decays

Following demonstration of the enhanced acceptor emission we questioned whether the acceptors would display adequately long lifetimes. Frequency-domain intensity decays were measured through filters selected to isolate the desired emission wavelengths (Fig. 6). Observation at 610 nm results in selective observation of the donor emission, and observation at 670 or 700 nm selects the acceptor emission.

Figures 7–9 show the frequency-domain data for three D–A pairs. In these data Ru–BD is always the donor. The acceptor is NB, TOTO-3, or TO-PRO-3, respectively. In the absence of acceptors, the mean Ru–BD lifetime is near 100 ns (Table 2). The Ru–BD lifetime is only moderately decreased by the acceptor. For instance, for any of the acceptors, a ratio of 0.03 acceptors per base pair results in a mean donor lifetime near 70 ns. This was initially surprising given the 2-fold or larger quenching of the Ru–BD intensity by these acceptor concentrations. However, we can explain this difference in intensity and lifetime quenching as due to a range of D-to-A distances in the labeled DNA. More specifically, most of the acceptor emission results from the more closely spaced D–A pairs. In contrast, the observed donor emission in the presence of acceptors is increased by the higher intensities of those donors most distant from acceptors, which are also the donors with the longer lifetimes.

The bottom panels of Figs. 7–9 show the frequency response observed for the longer wavelength regions dominated by the acceptor emission. In each case the mean decay times are near 30 ns for observation at the acceptor emission wavelengths. While the frequency responses are multiexponential, we did not find visually obvious contributions from the directly excited acceptors with their 0.3- to 2.3-ns lifetimes. The apparent acceptor lifetimes are shorter than the apparent donor lifetimes because the acceptor emission is enriched for the shorter distances D–A pairs which have a shorter donor lifetime.

It is informative to examine the intensity decays in the time-domain reconstructed from the frequency-domain data (Fig. 10). The decays of the directly excited acceptors are short, and emission from the directly excited acceptors will not be observed if the detection is off-gated for the first 10–20 ns following the excitation pulse. The donor decays, even in the presence of acceptors, are long lived. Also, following a brief transition period out to 10–40 ns, the

acceptor decay rates are comparable to that of the quenched donors. This long lived emission from the donors can be used for biophysical or analytical purposes.

An important conclusion from these experiments is that the apparent acceptor decays are adequately long for off-gating of the autofluorescence from biological samples. Hence the use of MLC-acceptor pairs provides an opportunity to obtain luminophores which display long lifetimes, high quantum yields, and long emission wavelengths.

DISCUSSION

In the preceding sections we presented data suggesting an approach to obtaining long wavelength emission while retaining desirably long lifetimes. By consideration of the well known characteristics of Förster transfer, one can predict that suitable designed D-A pairs will display even more favorable properties. For instance, the acceptor decay times for the DNA bound probes were shorter than the donor decay times. We explained this effect as due to a range of donor-to-acceptor distances for the probes randomly bound to DNA. It is well known that unique D-to-A distances can be obtained with polyproline spacers (57) or with double-stranded DNA as the spacer (58, 59). In such cases the donor decay times will decrease in proportion to the transfer efficiency, and the acceptor decay times will be similar to the donor decay times. The results for a donor and acceptor separated by a single distance are expected to be comparable to that shown in Scheme 1, where a 1- μ s decay time donor, with 90% transfer efficiency, results in a luminophore with a 100-ns lifetime. Since metal-ligand complexes are known with decay times as long as 42 μ s (60, 61), one can predict 4- μ s decay time luminophores with 90% transfer.

Another advantage of these RET probes is that the emission spectra of red and NIR fluorophores are typically narrow on the wavelength scale, whereas the emission spectra of the MLCs are broad. Since autofluorescence from biological samples is typically distributed broadly on the wavelength scale, the concentration of the emission into a narrow spectral range by the acceptor should improve detectability of these luminophores.

Another possible application of this phenomena is for the study of macromolecular association reactions, such as protein-protein interactions, DNA hybridization (62–64), fluorescence *in situ* hybridization (FISH) (65), or the use of molecular beacons (66, 67). As an example, suppose it was necessary to test for binding of donor-labeled oligonucleotides to a mixture of acceptor-labeled oligonucleotides. When using a RuMLC donor and one of the acceptors used in this report, most of the species labeled with donor or acceptor alone will display little emission. In contrast the D–A pairs due to macromolecular association will be brightly fluorescent. Additionally, the acceptor emission will be long lived. Using time-gated detection brightly fluorescent spots may become apparent against a background of weakly stained chromatin and/or short decay time. These spectral properties could be useful for detection of oligonucleotide hybridization on DNA arrays (68, 69). Such arrays are becoming widely used for analysis of gene expression (70–72).

In summary, we have described a generic approach to obtaining an unusual combination of spectral properties by using appropriate D–A pairs. This approach can be used to create D–A

pairs which act as a single luminophore, or this effect can be used to detect interactions in samples containing species labeled with the donor or acceptor. This approach may also be useful in studies of macromolecular folding, as illustrated by the use of RET to study ribozyme structures (73, 74). One can also imagine long lifetime donors linked to pH, Ca^{2+} , or other analyte-sensitive fluorophores (75, 76). If the analyte sensitive fluorophore displays distinct emission spectra with and without bound analyte, then there will be a long lived component in the emission with the spectral characteristics of each form. Finally, one can imagine the use of the enhanced emission and inhibition of the enhancement for use in macromolecular binding assays in high throughput screening (77, 78). There appear to be numerous applications of our approach in biochemical and biomedical research.

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FIG. 1.
Chemical structures of the donors and acceptors used in this report.

FIG. 2.

Emission spectra of the acridine orange donor [Donor] = 5 μ M bound to DNA in the presence of the acceptors nile blue (top), TOTO-3 (middle), or TO-PRO-3 (bottom). The insets show the acceptor region with the amplitude increased by a factor of 40. The dashed lines show the emission of acceptor alone with DNA, but without donor, at the highest acceptor concentration used in the figure. The donor is present at 1 donor per 200 base pairs. The number for the donor-acceptor (DA) pair is the number of base pairs for each acceptor.

FIG. 3.

Emission spectra of ethidium bromide donor [Donor] = 10 μ M bound to DNA in the presence of the acceptors nile blue (top), TOTO-3 (middle), or TO-PRO-3 (bottom). The dashed lines show the emission of the acceptor alone with DNA, but without donor, at the highest used acceptor concentration. The donor is present at 1 donor per 100 base pairs. The number for the donor-acceptor (DA) pair is the number of base pairs for each acceptor.

FIG. 4.

Emission spectra of Ru-BD($[\text{Ru}(\text{bpy})_2 \text{dppz}]^{2+}$) donor [Donor] = 20 μM bound to DNA in the presence of the acceptors nile blue (top), TOTO-3 (middle), and TO-PRO-3 (bottom). The dashed lines show the emission spectra of the acceptor alone with DNA, but without donor, at the highest used acceptor concentration. One donor is present per 50 base pairs. The number for the donor-acceptor (DA) pair is the number of base pairs for each acceptor.

FIG. 5. Uncorrected excitation spectra of Ru-BD and TO-PRO-3 bound to DNA (---) and of TO-PRO-3 alone bound to DNA (—). (Bottom) Ratio of the two excitation spectra.

FIG. 6. Transmission spectra of the emission filters used for measuring the frequency-domain intensity decays (—). The dashed lines show representative emission spectra of the donor alone (D) and donor plus acceptor (DA) samples.

FIG. 7.

Frequency-domain intensity decays of Ru-BD bound to DNA in the absence and presence of the Nile Blue acceptor. The solid dots represent the phase or modulation values and the solid lines the best multiexponential fits to the data. In the middle and bottom panels the dotted lines represent the donor-alone and acceptor-alone frequency responses, respectively.

FIG. 8.

Frequency-domain intensity decay of Ru-BD bound to DNA in the absence and presence of the TOTO-3 acceptor. See legend to Fig. 7.

FIG. 9.

Frequency-domain intensity decay of Ru-BD bound to DNA in the absence and presence of the TO-PRO-3 acceptor. See legend to Fig. 7.

FIG. 10.
Time-domain intensity decay of Ru-BD and acceptor complexes with DNA.

SCHEME 1.

A potential long wavelength, long lifetime luminophore based on a long lifetime donor (D) and a short lifetime acceptor (A).

SCHEME 2.

Intuitive description of resonance energy transfer from a high quantum yield donor ($Q_D = 1.0$, top) and a low quantum yield donor ($Q_D = 0.1$, bottom). For both panels $\epsilon_A/\epsilon_D = 0.1$. For the low quantum yield donor RET results in an increase in the overall quantum efficiency of the tandem luminophore.

SCHEME 3.

Effect of RET on the acceptor emission when the acceptor absorbs more strongly than the donor.

TABLE 1
Quantum Yields (Q), Decay Times (τ), and Molar Extinction Coefficients ($\epsilon/\lambda_{\text{max}}$) of Fluorophores in DNA

Probe	Donor/acceptor	Q^a	$\langle \tau \rangle$ (ns) ^b	$\epsilon/\lambda_{\text{ex}}$ (M ⁻¹ cm ⁻¹ /nm)	$\epsilon/\lambda_{\text{max}}$ (M ⁻¹ cm ⁻¹ /nm)
AO	Donor	0.392	5.0	23,300/470	53,000/500
EB	Donor	0.219	21.9	5,200/518	5,200/518
Ru-BD	Donor	0.008	84.0	13,000/440	13,000/440
NB	Acceptor	0.004	0.32	1,180/440	42,900/656
TOTO-3	Acceptor	0.06	2.3	2,240/440	154,000/642 ^c
TO-PRO-3	Acceptor	0.11	1.8	200/440	102,000/642 ^c

^aThe following compounds were used as quantum yield references: in the case of AO, 3-aminofluoranthene in DMSO ($Q = 0.32$); EB in methanol ($Q = 0.06$) for EB; 4-dicyanomethylene-2-methyl-6-(*p*-dimethylaminostyryl)-*H*-pyran in methanol ($Q = 0.38$) in the case of RuBD and NB; and fluorescein in 0.1 M NaOH ($Q = 0.92$) for TOTO-3 and TO-PRO-3.

^bMean lifetime calculated using $\langle \tau \rangle = \sum f_i \tau_i$, where f_i is the fractional steady state contribution of each component to the total emission.

^cFrom Molecular Probes, Inc.

TABLE 2

Multiexponential Intensity Decay Analyses of the Ru-BD Donor and Acceptors Bound to Calf Thymus DNA

	n^a	$\langle \tau \rangle^b$	a_i	f_i	τ_i	χ^2_R
Donor/acceptor Ru-BD/NB ^c						
Ru-BD	2	84	0.36	0.13	24	0.91
			0.64	0.87	93	
NB	1	0.32	1.00	1.00	0.32	0.85
DA Obs. 610 nm	2	75	0.50	0.15	16	1.40
			0.50	0.85	86	
DA Obs. 700 nm.	3	30	0.95	0.51	1.9	0.90
			0.04	0.21	22	
			0.01	0.28	87	
Donor/acceptor Ru-BD/TOTO-3 ^c						
Ru-BD	2	98	0.42	0.18	33	0.99
			0.58	0.82	111	
TOTO-3	1	2.3	1.00	1.00	2.3	1.30
DA Obs. 610 nm	2	73	0.79	0.19	5.8	1.07
			0.21	0.81	90	
DA Obs. 670 nm.	3	39	0.83	0.41	5.6	1.02
			0.12	0.24	23	
			0.05	0.35	88	
Donor/acceptor Ru-BD/TO-PRO-3 ^c						
Ru-BD	2	114	0.42	0.17	38	1.02
			0.58	0.83	130	
TO-PRO-3	1	1.8	1.00	1.00	1.8	1.02
DA Obs. 610 nm	2	83	0.62	0.19	14	0.81
			0.38	0.81	99	
DA Obs. 670 nm.	3	24	0.83	0.48	5.1	1.05
			0.15	0.33	19	
			0.02	0.19	78	

^aNumber of decay times in the multiexponential fit.^b $\langle \tau \rangle = \sum \tau_i f_i$ where f_i is the steady state contribution of each component.^cAll acceptor concentrations are 0.03 bp⁻¹.