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Spectroscopic Features of Dual Fluorescence/ Luminescence Resonance Energy-Transfer Molecular Beacons

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Molecular beacons have the potential to become a powerful tool in gene detection and quantification in living cells. Here we report a novel dual molecular beacons approach to reduce false-positive signals in detecting target nucleic acids in homogeneous assays. A pair of molecular beacons, each containing a fluorescence quencher and a reporter fluorophore, one with a donor and a second with an acceptor fluorophore, hybridize to adjacent regions on the same target resulting in fluorescence resonance energy transfer (FRET). The detection of a FRET signal leads to a substantially increased signal-to-background ratio compared with that seen in single molecular beacon assays and enables discrimination between fluorescence due to specific probe/target hybridization and a variety of possible false-positive events. Further, when a lanthanide chelate is used as a donor in a dual-probe assay, extremely high signal-to-background ratios can be achieved owing to the long lifetime and sharp emission peaks of the donor and the time-gated detection of acceptor fluorescence emission. These new approaches allow for the ultrasensitive detection of target molecules in a way that could be readily applied to real-time imaging of gene expression in living cells.

The ability to monitor and quantify the level of gene expression in living cells in real time can provide important information concerning the production, processing, localization, and transport of specific mRNA in different conditions. Technologies currently available for in vitro analysis and quantification of gene expression such as real-time PCR, Northern blotting, expressed sequence tag, serial analysis of gene expression, and DNA microarrays are powerful tools for many applications; however, they are not capable of quantifying gene expression in living cells. The ability to accurately detect endogenous genes in living cells is a formidable task. Traditional hybridization-based assays such as in situ hybridization achieve high signal-to-background ratios by washing away unbound probes.[1] However, the use of washing or other dilution-based approaches to reduce background is not possible in vivo, so different strategies are required to reduce background in visualizing probe−target hybridization events in living cells and tissues. Thus, probes for in vivo applications must be able to convert target recognition *directly* into a measurable signal with high signal-to-background ratio. In addition to differentiating between bound and unbound probes, the approach must be able to distinguish signals arising from true target recognition from a variety of false-positive events.

Molecular beacons have the potential to detect probe−target hybridization in vivo. Molecular beacons are dual-labeled oligonucleotide probes with a reporter fluorophore at one end and a quencher at the opposite end;[2] they are designed to have a target-specific probe sequence positioned centrally between two short self-complementary segments that, in the absence of target, anneal to form a stem−loop (i.e., hairpin) structure. In this configuration, the fluorophore is in close proximity with the quencher and the molecular beacon is in the "dark" state.[3] Upon hybridization with a complementary target the hairpin opens, separating the fluorophore and quencher, restoring fluorescence, and resulting in the "bright" state. Transition between the dark and bright states allows for the differentiation between bound and unbound probes[4,5] with a high signal-to-background ratio and eliminates the need to wash away unbound probes. As an added benefit, the hairpin structure offers a competing reaction with probe−target hybridization and results in improved specificity compared with linear oligonucleotide probes.[6,7] However, when conventional molecular beacons are used for gene detection in living cells, a variety of factors in the intracellular environment can lead to false-positive signals. For example, probes can be degraded by nucleases or opened by nucleic acid binding proteins; either of these events will lead to a fluorescence signal in the absence of probe−target hybridization.[8−12]

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Fluorescence resonance energy-transfer (FRET) probes can also be used to distinguish specific probe−target hybridization from background. FRET is a process where energy from one fluorophore is transferred to a second fluorophore if they are in close proximity (usually <6 nm). This assay method uses two linear oligonucleotide probes, one labeled with a donor fluorophore and a second labeled with an acceptor fluorophore. Probes are designed to hybridize to adjacent regions on a nucleic acid target such that the two fluorophores will lie within FRET range only when both probes are hybridized to the same target molecule.[13−15] When hybridized to a target nucleic acid, excitation of the donor fluorophore will be transferred to the acceptor fluorophore and will result in fluorescence emission at a wavelength characteristic of the acceptor, not the donor. Therefore, the dual FRET linear probes possess the ability to distinguish between target recognition and false-positive signals. A disadvantage of using these linear probes is the high background fluorescence resulting from the spectral overlap necessary for FRET. Further, linear probes have a reduced ability to discriminate targets with single-nucleotide polymorphisms (SNPs) compared with molecular beacons.[7]

To overcome the shortcomings of conventional molecular beacons and two-probe assays in performing cellular imaging, we have developed a new approach that uses a pair of molecular beacons labeled with a donor and an acceptor fluorophore, respectively (dual FRET molecular beacons). The probe sequences are chosen such that the molecular beacons hybridize to adjacent regions on a single nucleic acid target (Figure 1A). Emission of the acceptor fluorophore upon donor excitation serves as a positive signal in the FRET-based detection assay, which is readily differentiable from none-FRET false-positive signals due to probe degradation and nonspecific probe opening. This new approach combines the low background signal and high specificity of molecular beacons with the ability of two-probe assays to differentiate between true target recognition and false-positive signals. We demonstrated that the dual FRET molecular beacons have the potential to become a powerful tool for real-time gene detection and quantification in living cells with high specificity, signal-to-background ratio, and sensitivity.

As an alternative approach, a variant of the two-probe assay was tested that employs a lanthanide chelate as the donor and a molecular beacon with an organic fluorophore as an acceptor molecule. In contrast to organic fluorophores that have a fluorescence lifetime of ∼10 ns, lanthanide chelates can have emission lifetimes greater than 1 ms.[16,17] Further, lanthanide chelates such as those with europium and terbium have narrow emission peaks, making them an ideal donor for luminescence resonance energy transfer (LRET).[18] By using pulse excitation and time-gated

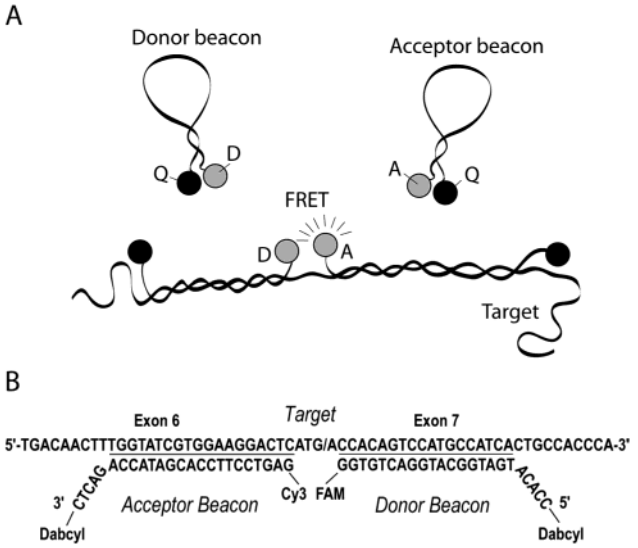

Figure 1. (A) Hybridization of the donor and acceptor molecular beacons to adjacent regions on the same nucleic acid target resulting in FRET. By detecting FRET, fluorescence signals due to probe/target binding can be distinguished from that due to molecular beacon degradation and nonspecific interactions. In the figure, letters Q, D, and A represent quencher, donor fluorophore, and acceptor fluorophore, respectively. (B) A schematic example of donor and acceptor molecular beacons, with a probe length of 19 bases and a stem length of 5 bases, hybridized to the synthetic target and separated by a four-base gap. The underscores indicate the 38-base sequence of the target complementary to the molecular beacons.

detection, it is possible to selectively record fluorescence emission after the background fluorescence from organic dyes, scattering, and autofluorescence has decayed.[19,20] The only signals remaining in this long-time domain are those from the lanthanide chelates and the acceptor fluorophores that have participated in LRET. In this case, the narrow emission peaks of a lanthanide chelate render the background fluorescence close to zero at certain wavelengths, leading to extremely large signal-to-background ratio. The unique spectral properties of lanthanide chelates allow the donor probe in a LRET pair to be simply a linear probe, i.e., neither quencher nor hairpin structure is necessary.

EXPERIMENTAL SECTION

Oligonucleotide Synthesis. Oligonucleotide probes and targets were synthesized at Integrated DNA Technologies, Inc. (Coralville, IA). The details of the synthesis can be found in Tsourkas et al.[7]

Probe and Target Design. All oligonucleotide probes were designed to be complementary in antisense orientation to the human GAPDH gene, as illustrated in Figure 1B and Table 1. Specifically, a dabcyl quencher (4-(4′-dimethylaminophenylazo)-benzoic acid) was attached to the 5′-end and a 6-FAM fluorophore (6-carboxyfluorescein) was attached to the 3′-end of donor molecular beacons; a dabcyl quencher was attached to the 3′-end and either a cyanine 3 (Cy3), 6-carboxy-X-rhodamine (ROX), or Texas Red fluorophore was attached to the 5′-end of acceptor

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Table 1. Design of Probe and Target Oligonucleotides

Probe Sequence (5' – 3')	
FAM donor-MB[a]:	Dabcyl-ccacaTGATGGCATGGAC**TGTGG**-FAM
Tb donor LP[b]:	TGATGGCATGGACTGTGG-DTPA-cs124-(Tb)
Eu donor LP:	TGATGGCATGGACTGTGG-DTPA-cs124-(Eu)
Cy3 acceptor-MB:	Cy3- **GAGTC**CTTCCACGATACCgactc -Dabcyl
Cy5 acceptor LP:	Cy5- GAGTCCTTCCACGATACC
ROX acceptor-MB:	ROX- **GAGTC**CTTCCACGATACCgactc -Dabcyl
TR acceptor-MB:	Texas Red- **GAGTC**CTTCCACGATACCgactc -Dabcyl
Target[c] Sequence (5' – 3')	
ACTTTGGTATCGTGGAAGGACTCATACCACAGTCCATGCCATCACTGCC	
ACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCCATCACTGCC	
ACTTTGGTATCGTGGAAGGACTCATTGACCACAGTCCATGCCATCACTGCC	
ACTTTGGTATCGTGGAAGGACTCATTTGACCACAGTCCATGCCATCACTGCC	

[a] Molecular beacon (MB): lower case, bases added to create stem domains; upper case, probe−target hybridizing domains; upper case boldface type, bases participating in both stem formation and target binding. [b] LP: Linear probe. [c] Targets: underscore, 18-base sequence complementary to MB target binding domains.

molecular beacons. The stem sequence was designed to participate in both hairpin formation and target hybridization.[21] This molecular beacon design was chosen to help fix the relative distance between the donor and acceptor fluorophores and improve energy-transfer efficiency. Both the donor and acceptor molecular beacons were designed with a probe length of 18 bases and a stem length of 5 bases. Here the probe length is defined as the portion of the molecular beacon that is complementary to the target. The synthetic wild-type GAPDH target has a four-base gap between the donor dye and the acceptor dye. Gap spacing was adjusted to three, five, and six bases by either removing a guanine residue or adding one or two thymine residues, as shown in Table 1.

Lanthanide Chelate Synthesis. A linear oligonucleotide with a probe length of 18 bases was labeled at its 3′-end with a diethylenetriaminepentaacetic acid (DTPA) chelate covalently joined to a sensitizer, cs124.[22] As shown in Table 1, the sequence of this linear probe was identical to the probe domain of the donor molecular beacons specific for exon 7 of the human GAPDH gene. The lanthanide chelate was prepared by first dissolving DTPA (500 mg, 1.4 μmol) in 30 mL of DMF and 1 mL of triethylamine. Cs124 (240 mg, 1.4 μmol), dissolved in 4 mL of DMF, was then added dropwise and mixed for 30 min. To this mixture, 5 mL (75 μmol) of ethylenediamine (EDA) was added and the resultant mixture stirred at room temperature for 2 h. After stored in the refrigerator overnight, the DMF supernatant was removed from the mixture and the pellet was washed with 2-propanol several times, resulting in fine white powder, which was dried under vacuum for 2 h. The powder was resuspended in water and reversed-phase (RP) HPLC purified using a Hamilton PRP-1 column. The DTPA−cs124 product was dried and reconstituted to a concentration of 15 mM in 0.1 M borate buffer, pH 8.5. To form oligonucleotide−DTPA−cs124−EDA conjugates, 50 μL of 15 mM DTPA−cs124−EDA product in borate buffer was added to the oligonucleotide solution and mixed overnight. The conjugates were purified using RP-HPLC. The oligonucleotides were loaded on a PRP-1 column and eluted with a linear 5−50% acetonitrile gradient in 0.1 M TEAA, pH 7.2, over 40 min. The collected peak was lyophilized and reconstituted in distilled H_2O at 5 μM. $TbCl_3$ (terbium) dissolved in PBS was then added to the sample at a 10:1 molar ratio and the resultant mixture incubated at room temperature for 30 min. The europium chelates were synthesized following the same protocol.

Hybridization and Detection Assays. Hybridization experiments were conducted with 50 pmol of donor molecular beacon, 50 pmol of acceptor molecular beacon, and 50 pmol of complementary target in a total volume of 100 μL (0.5 μM). All experiments were conducted at 37 °C in HB buffer containing 10 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5, which was supplemented with 1% bovine serum albumin to block nonspecific interactions with the microplate. The samples were mixed and allowed to equilibrate at 37 °C for 20 min before performing fluorometry. A Safire microplate fluorometer (Tecan, Zurich, Switzerland) was used to excite the donor molecular beacons and detect resulting emission (500−650 nm) in FRET measurements. The excitation wavelength was varied from 395 to 495 nm to determine the wavelength that resulted in the maximal FRET between the donor and acceptor molecules. For LRET measurements, the terbium and europium donor probes were excited at a wavelength of 325 nm, and the emission was recorded from 500 to 650 nm for assays with terbium donors and from 550 to 750

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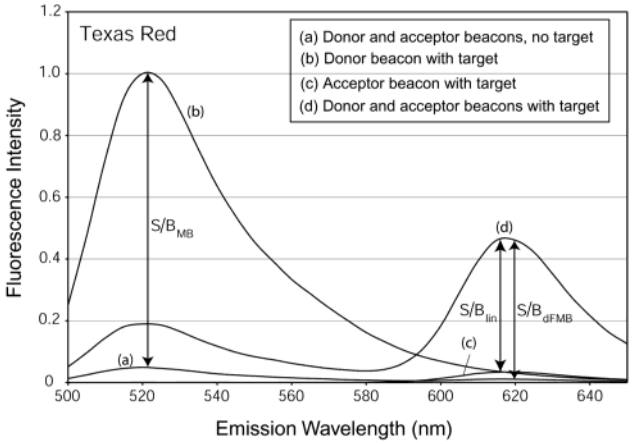

Figure 2. Emission spectra for dual FRET molecular beacons with a FAM−Texas Red FRET pair in the presence and absence of targets. Curve a is the fluorescence emission of donor and acceptor molecular beacons in the absence of targets. Curve b is the fluorescence emission of donor molecular beacons in the presence of targets. Curve c is the fluorescence emission of acceptor molecular beacons in the presence of target due to indirect excitation. Curve d is the FRET-induced fluorescence emission of donor and acceptor molecular beacons in the presence of target.

nm for assays involving europium donors. The emission detection had a lag time of 50 μs with an integration time of 1 ms.

RESULTS AND DISCUSSION

FRET Signal-to-Background Ratios and Fluorescence Intensities. A series of homogeneous solution-phase assays were conducted to determine the signal generated by a pair of molecular beacons labeled with a donor and acceptor fluorophore (dual FRET molecular beacons), respectively, in the presence and absence of target. To select a favorable FRET pair, the same donor molecular beacon (with 6-FAM as the donor dye) was used with three different acceptor molecular beacons (with Cy3, ROX, and Texas Red as the acceptor dye). The excitation wavelength was varied to determine optimal conditions. The molecular beacons were designed to hybridize to adjacent regions, separated by four bases, on the target molecule (Figure 1B). Analogous experiments were also conducted with a pair of linear probes labeled with donor and acceptor fluorophores, respectively, as well as single molecular beacons. The donor molecular beacon in the presence and absence of target molecules was used to determine the performance of individual molecular beacons.

To evaluate the effectiveness of the three different nucleic acid detection methodologies, fluorescence emission spectra were obtained as a quantitative measure for the probes in the presence and absence of target. The emission results were then evaluated in terms of both the signal-to-background ratio and the signal intensity. For individual (unpaired) molecular beacons labeled with 6-FAM and dabcyl, the signal-to-background ratio (S/B_{MB}) was defined as the peak emission of the molecular beacon in the presence of target divided by the peak emission of the molecular beacon in the absence of target, as illustrated in Figure 2 in which an example of the entire emission spectrum generated using the FAM−Texas Red FRET pair is given. The signal intensity was defined as the peak emission of the molecular beacon in the presence of target. For the two-probe FRET assays using either labeled linear oligonucleotides or molecular beacons, the sensitized fluorescence emission was used as the measure for comparison to unpaired molecular beacons. Specifically, for dual FRET molecular beacons, the S/B_{dFMB} was defined as the peak acceptor emission in the presence of target divided by the donor and acceptor emission at the same wavelength in the absence of target. For the linear FRET probes, the S/B_{lin} was defined as the peak acceptor emission in the presence of target divided by the donor or acceptor emission (whichever was higher) at the same wavelength in the absence of target. The signal intensities were defined as the peak acceptor emissions in the presence of target. Since the fluorescence of a fully opened molecular beacon (i.e., hybridized to a complementary target) is essentially the same as the corresponding linear probe, in this study, it was assumed that the fluorescence emission of unbound individual linear probes is equivalent to that of fully opened donor or acceptor molecular beacons, and the fluorescence emission of linear probe pairs participating in FRET is equivalent to that of dual FRET molecular beacons bound to target.

As shown in Figure 3A, when FAM and Cy3 were used respectively as the donor and acceptor fluorophores in a dual FRET molecular beacon assay, the S/B_{dFMB} did not vary much over the entire range of excitation wavelengths. Moreover, the dual FRET molecular beacons exhibited a signal-to-background ratio (ranging between 20 and 25) almost identical to that of individual FAM-labeled molecular beacons, as the excitation wavelength was increased. There was a rather large variation in the signal-to-background ratios in these two assays because of the low signal generated by molecular beacons in the hairpin conformation (i.e., in the absence of target): a small fluctuation in the fluorescence emission of unbound molecular beacons (possibly due to thermal or instrumentation fluctuations) could lead to a large variation in the calculated signal-to-background ratio.

The linear FRET probes only exhibited a S/B_{lin} of 2−3. This was relatively low compared with the duel FRET molecular beacon assay, because of the high background fluorescence due to the large spectral overlap of the donor and acceptor dyes. The S/B_{lin} has added importance in the evaluation of donor/acceptor pairs for dual FRET molecular beacons since it simulates the worst possible performance of the dual FRET molecular beacons in a harsh intracellular environment where a molecular beacon could be degraded or opened due to nonspecific interactions. When degraded, molecular beacons will emit a fluorescence signal similar to that of the unbound linear probes. The fact that S/B_{lin} was greater than unity demonstrated that the dual FRET molecular beacons can differentiate between degraded and hybridized probes even in the most unfavorable conditions.

In contrast to that with Cy3, when a ROX fluorophore was used as the acceptor dye, S/B_{dFMB} was found to be dependent on the excitation wavelength (Figure 3B). At low excitation wavelengths (395−425 nm), S/B_{dFMB} was only ~10, about half of S/B_{MB}. However, when the excitation wavelength was increased, S/B_{dFMB} also increased, reaching values above 30. Unexpectedly, the dual FRET molecular beacons exhibited an improved signal-to-background ratio over individual (unpaired) molecular beacons for all excitation wavelengths above 460 nm. The value of S/B_{lin} also increased with excitation wavelength, reaching values be-

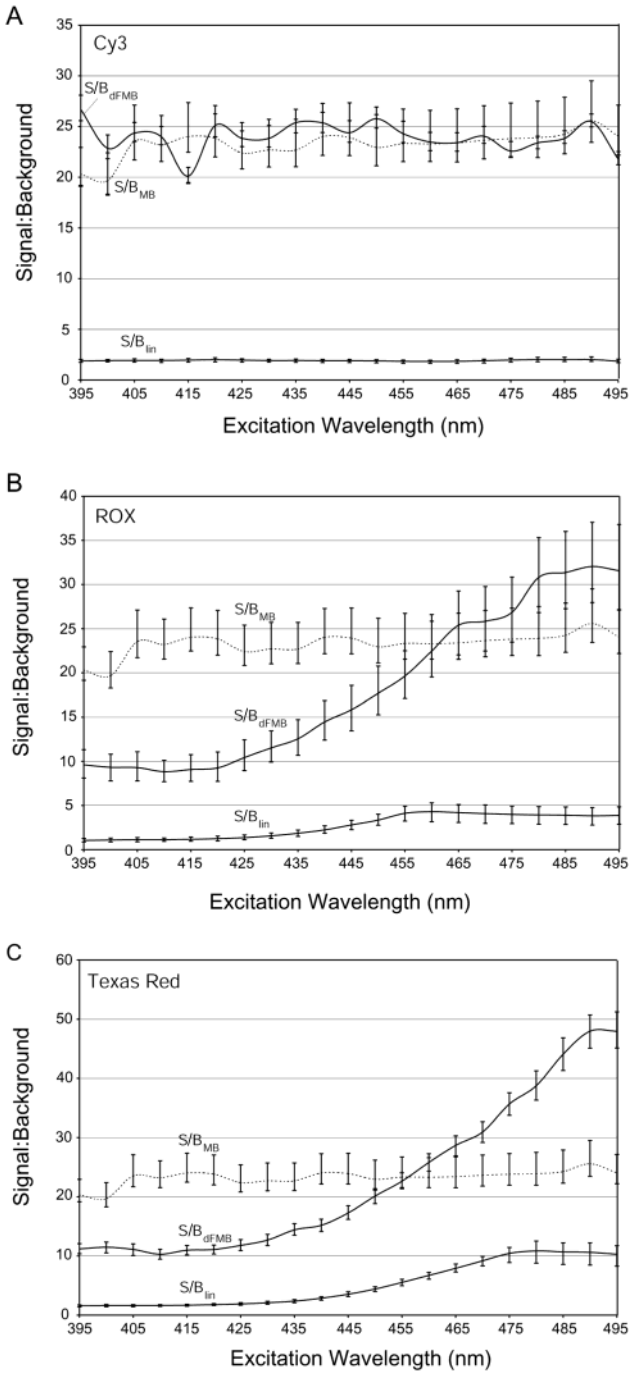

Figure 3. Signal-to-background ratios for FAM-labeled molecular beacons (S/B_{MB}), dual FRET-molecular beacons (S/B_{dFMB}) with (A) a FAM−Cy3 FRET pair, (B) a FAM−ROX FRET pair, and (C) a FAM−Texas Red FRET pair, and linear FRET probes (S/B_{lin}) with the same donor and acceptor pairs as the dual FRET molecular beacons. The error bars display the minimum and maximum ratios calculated for dual FRET molecular beacons separated by three to six bases.

tween 4 and 5 for excitation wavelengths greater than 455 nm. This was an improvement over the FAM−Cy3 FRET pair but was still substantially lower than the signal-to-background ratio obtained in the dual FRET molecular beacon assay.

Acceptor molecular beacons labeled with Texas Red performed the best among the three acceptor fluorophores considered. The value of S/B_{dFMB} increased from 10 to nearly 50 as the excitation wavelength was increased from 395 to 495 nm, and S/B_{lin} increased from ∼2 to over 10 (Figure 3C). The dual FRET molecular beacons even exhibited an improved signal-to-background ratio compared with individual molecular beacons for all excitation wavelengths greater than 455 nm. S/Bs at excitation wavelengths above 495 nm (peak excitation of FAM) were excluded from Figure 3 due to leaking of the excitation light into the emission spectrum of the FAM-labeled molecular beacon. For all three acceptor fluorophores, when the wavelength was increased beyond 495 nm, we found that the FRET signal decreased and the background increased due to the elevated direct excitation of the acceptor, resulting in a reduced S/B_{dFMB}.

To directly compare the fluorescence intensities generated by dual FRET molecular beacons with that of single molecular beacons, the peak emission of the donor−acceptor molecular beacon pairs in the presence of target were normalized by the peak emission of the FAM-labeled molecular beacon alone. When Cy3, Texas Red, and ROX were used as the acceptor dyes, the peak fluorescence intensity of the dual FRET molecular beacons was found to be respectively 0.48, 0.36, and 0.21 of the peak intensity of the FAM-labeled molecular beacon. Therefore, the increased specificity of the dual FRET molecular beacons method may lead to a slightly compromised sensitivity. It should be noted, however, that the detection sensitivity of a fluorescent probe assay not only depends on the signal intensity but also depends on the S/B as well as the fraction of probes bound to their target.

The fraction of targets bound by two probes can be estimated for low-abundance genes as follows. Assuming the concentration of molecular beacons is much greater than that of the target, at equilibrium, the fraction α of targets on which both the donor and acceptor molecular beacons are hybridized can be written as $\alpha = [1/(1 + K_{D1}/B_1)][1/(1 + K_{D2}/B_2)]$, where B_1 and B_2 are the initial concentrations of the donor and acceptor molecular beacons, respectively, and K_{D1} and K_{D2} represent the dissociation constants of the donor−target and acceptor−target hybrids, respectively. Consequently, when $B \gg K_D$, in homogeneous assays, the target molecules can be detected by the dual FRET probes with similar sensitivity as a single-probe system. Note that having an excessive amount of molecular beacons may increase the background signal and thus reduce the signal-to-background ratio.

Effect of Gap Size between Donor and Acceptor Molecular Beacons. In addition to the choice of donor and acceptor fluorophores, the distance between the donor and acceptor fluorophores can also influence the efficiency of FRET. Too large a gap size reduces the energy-transfer efficiency, while too small a gap size may result in steric interference between fluorophores or lead to other interactions between donor and acceptor (such as ground-state quenching), which are unfavorable.[23,24] Earlier studies performed on single-stranded dual-labeled oligonucleotides demonstrated that optimal energy transfer occurred when separation between donor and acceptor was eight bases.[25,26] However, for a mixed single-stranded and double-stranded DNA (two probe−target hybrids) the optimal spacing may be different. It

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has also been shown that the interaction between a fluorophore and the oligonucleotide in proximity can significantly influence fluorescence emission.[27,28] To minimize the possible inconsistency in fluorescence emission due to base composition near the fluorophores, the nucleotides closest to the probe-binding region on the target were kept identical for all the target sequences (Table 1). We found that, when the distance between the donor and acceptor molecular beacons was increased from three to six bases, there was a slight increase in the FRET signal intensity (data not show). This trend was found to be the same for all the acceptor fluorophores studied. It seems that a gap size of five or six bases is optimal whereas a three-base gap is less favorable due to possible interference between the donor and acceptor dyes.[29]

Time-Resolved LRET Exhibits Extremely Low Backgrounds. The signal-to-background ratio of FRET assays can be further improved by substituting a lanthanide donor for the FAM donor and modifying the detection system to employ time-resolved spectroscopy,[22] taking advantage of the unique properties of lanthanide chelates. Lanthanide chelates exhibit very sharp emission peaks separated by valleys of little or no fluorescence. For an LRET assay, these valleys are of particular interest in that the only detectable fluorescence emission in these valleys is due to the transfer of energy from the lanthanide chelate to another dye molecule. Hence, for two-probe LRET assays, the lanthanide chelate donor does not generate any background fluorescence at certain wavelengths (such as those corresponding to the valleys). It is worthy mentioning that no quencher is needed to reduce the signal of unbound lanthanide chelates because the signal level is inherently low at the emission valleys. There is also no need for the lanthanide-based oligonucleotide probe to have a hairpin structure if no detection of SNP is involved. If SNP detection is involved, the acceptor molecular beacon with an organic dye can be designed to target the SNP.

Another major advantage of using a lanthanide chelate donor is that it can significantly reduce the background signals generated by organic acceptor fluorophores, the experimental medium, and autofluorescence of cells. The long fluorescence lifetime exhibited by lanthanide chelates, combined with time-gated detection, allows for selective recording of emission after fluorescence from the direct excitation of the acceptor dye and autofluorescence has decayed; thus, the only acceptor fluorescence remaining is that resulting from LRET.[30]

The results of the LRET experiments with a lanthanide donor are displayed in Figure 4. As shown by curve 1 in Figure 4A, when just the terbium chelate-labeled donor probes were bound to complementary targets and excited with a wavelength of 325 nm, they exhibited several sharp emission peaks separated by valleys with fluorescence intensity close to zero. In the absence of target, the peak emissions were substantially lower, as demonstrated by curve 4. The increase in luminescence in the presence of target is likely due to the interaction between the terbium chelate and DNA target. Specifically, the nearby bases most likely serve as

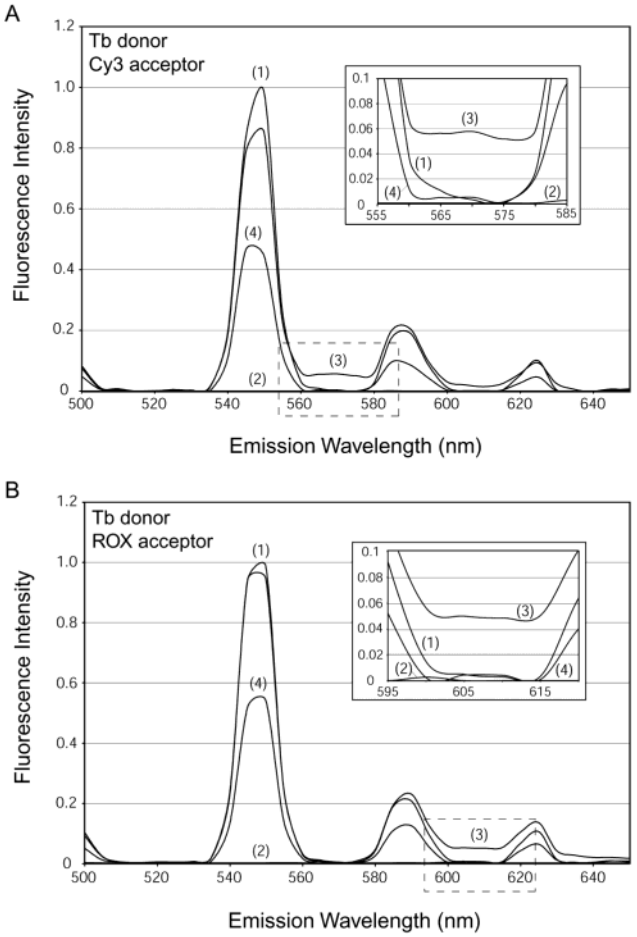

Figure 4. Time-resolved emission spectra obtained in a two-probe detection assay using a terbium chelate as a donor and either (A) Cy3 or (B) ROX as acceptors. All samples were excited at a wavelength of 325 nm. Different emission curves represent the following: (1) terbium chelate-labeled donor probe in the presence of target; (2) acceptor molecular beacon in the presence of target; (3) donor and acceptor probes in the presence of target; (4) donor and acceptor probes in the absence of target.

additional sensitizers for the lanthanide. In either case, fluorescence emission in the valleys was negligible. When just the acceptor molecular beacons were hybridized to targets, the fluorescence emission was not detectable, as shown by curve 2. Upon binding of both the donor probes with the terbium chelate and acceptor molecular beacons to target, a sensitized emission of the acceptor due to LRET was observed, as demonstrated by curve 3. As shown by the inset in Figure 4A, at certain emission wavelengths, the background from the lanthanide donor was near zero, resulting in an extremely high signal-to-background ratio. For the Cy3-labeled acceptor molecular beacon, the optimal detection wavelength was ~573 nm. Similar features are exhibited in Figure 4B, where the time-resolved emission spectra were obtained using a terbium chelate as a donor and ROX as an acceptor. It was again observed that at certain emission wavelengths the signal-to-background ratio approaches infinity. As can be seen from the inset of Figure 4B, for a ROX-labeled acceptor molecular beacon, the optimal detection wavelength is ~614 nm. Note that although the use of a terbium donor gave very high S/Bs, the actual LRET signal was relatively weak. One possible cause is that the DTPA−cs124 chelate employed in this study

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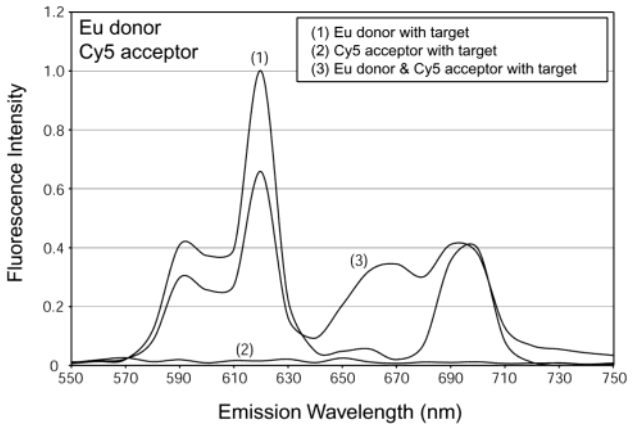

Figure 5. Time-resolved emission spectra generated by a two-probe detection assay utilizing a europium-labeled oligonucleotide as a donor probe and a Cy5-labeled oligonucleotide as an acceptor probe. (1) Europium chelate-labeled donor probe in the presence of target. (2) Acceptor molecular beacon in the presence of target. (3) Donor and acceptor probes in the presence of target.

allowed the terbium to be partially quenched by neighboring water molecules. It is expected that the use of alternative chelates, such as 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) and 4,4-bis(1,1,1,2,2,3,3-heptafluoro-4,6-hexanedion-6-yl)-chlorosulfo-o-terphenyl (BHHCT) will improve the energy-transfer efficiency.[16,17,20]

To assess any effect that gap size may have on LRET, the spacing between the terbium-labeled donor probe and the Cy3 or ROX-labeled acceptor molecular beacon was varied from three to nine bases. We found that the detected fluorescence intensity was not sensitive to the gap size tested, i.e., with a spacing of three to nine bases, the signal levels were similar (data not shown), suggesting that the steric effects were negligible when both probes hybridized to the target even with a relatively small spacing. Calculations of energy-transfer efficiency $E = 1/(1 + R^6/R_0^6)$ indicated that, as the gap spacing increases from three to nine bases (i.e., from 1 o 3 nm), the energy-transfer efficiency only decreases by 1.4%, since the Förster distance R_0 for the terbium−Cy3 LRET pair is large (~6.12 nm).[18]

As an alternative donor, europium chelates exhibited similar behavior compared with terbium chelates but with a red-shifted emission spectrum, as shown in Figure 5. Specifically, donor oligonucleotide probes labeled with a europium chelate at its 3′-end and acceptor oligonucleotide probes labeled with a Cy5 fluorophore at its 5′-end were synthesized, and in-solution hybridization and time-resolved emission detection assays were performed. Similar to the results obtained using terbium chelate donors, at 325-nm excitation, the emission spectrum of europium donor probe alone bound to target showed several peaks separated by valleys within the range of 550−750 nm, as demonstrated by curve 1 of Figure 5. When just the Cy5-labled acceptor probe was hybridized to the target, the fluorescence emission was almost zero (curve 2). When both donor and acceptor probes hybridized to the same target, there was a sensitized emission of the acceptor due to LRET, as shown by curve 3. Thus, at certain wavelengths (such as 670 nm), the background due to non-LRET donor and acceptor emission was very low, resulting in a high signal-to-background ratio. Overall, when europium chelate was used, the S/Bs were lower than that of the terbium system but the actual LRET signal detected was stronger. However, the emission curve of the donor was noisier, possibly due to the weak emission intensities of the donor at certain wavelengths.

CONCLUSIONS

As a new method for gene detection and quantification, dual FRET/LRET molecular beacons have been developed. Our spectroscopy studies demonstrated that dual FRET/LRET molecular beacon pairs not only can distinguish between bound and degraded probes but also have an improved signal-to-background ratio compared with single molecular beacons. Moreover, with a lanthanide chelate as the donor dye and using time-gated detection, the background signal can be reduced to zero at certain wavelengths. Although linear oligonucleotide probes can detect a particular nucleic acid target in a FRET assay, for the discrimination of targets with single-nucleotide polymorphism, stem−loop hairpin molecular beacons have a better specificity.[6,7] As demonstrated in this study, molecular beacons also have a lower background (i.e., fluorescence in the absence of target) and thus a higher signal-to-background ratio than two-probe FRET assays. We envision that, with two-photon imaging of dual FRET assays and with better lanthanide chelate donors for LRET assays, the signal-to-background ratio can be further increased. These features are especially important in the detection and quantification of gene expression in living cells where false-positive signals must be distinguished from the true signal that results from probe/target binding. Detailed living-cell studies of mRNA detection using dual FRET molecular beacons will be the subject of a separate publication.

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