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Intramolecular Dimers: A New Strategy to Fluorescence Quenching in Dual-Labeled Oligonucleotide Probes

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Abstract: Many genomics assays use profluorescent oligonucleotide probes that are covalently labeled at the 5′ end with a fluorophore and at the 3′ end with a quencher. It is generally accepted that quenching in such probes without a stem structure occurs through Förster resonance energy transfer (FRET or FET) and that the fluorophore and quencher should be chosen to maximize their spectral overlap. We have studied two dual-labeled probes with two different fluorophores, the same sequence and quencher, and with no stem structure: 5′Cy3.5−β-actin−3′BHQ1 and 5′FAM−β-actin−3′BHQ1. Analysis of their absorption spectra, relative fluorescence quantum yields, and fluorescence lifetimes shows that static quenching occurs in both of these dual-labeled probes and that it is the dominant quenching mechanism in the Cy3.5−BHQ1 probe. Absorption spectra are consistent with the formation of an excitonic dimer, an intramolecular heterodimer between the Cy3.5 fluorophore and the BHQ1 quencher.

Introduction

Oligonucleotides covalently labeled with fluorescent molecules are widely used in a variety of genomic assays including gene quantitation, allelic discrimination, expression analysis, and SNP typing.[1] Many assays use profluorescent probes in which a biochemical event (e.g., hybridization) causes an increase in fluorescence. For example, in a real-time polymerase chain reaction (RT-PCR), a dual-labeled oligonucleotide probe is modified with both a fluorophore and a quencher.[2] The fluorescence intensity of the dual-labeled probe is controlled by the fluorophore−quencher distance (Figure 1).

Complementary bases can be added to both the 5′ and 3′ ends of the probe to form a stem structure that brings the reporter and quencher dyes closer together. Probes with such a stem structure are referred to as molecular beacons.[3] It is commonly accepted that Förster resonance energy transfer (commonly referred to as FRET or FET)[4,5] is the mechanism that controls quenching in dual-labeled probes without a stem structure.[6] As a result, fluorophores and quenchers are matched according to their spectral overlap, i.e., they are chosen so that the fluorophore emission and quencher absorption curves overlap. Förster energy transfer[4] typically occurs over distances up to 20−60 Å. Quenching via the Dexter mechanism, also known as the exchange mechanism,[7] can occur only when the fluorophore and quencher are close enough to allow orbital overlap. In general, when the involved transitions on both molecules are singlet−singlet transitions, Förster quenching should dominate over Dexter quenching. Both the Dexter and Förster mechanisms are types of dynamic quenching, and the fluorophore and quencher molecules retain their intrinsic properties.

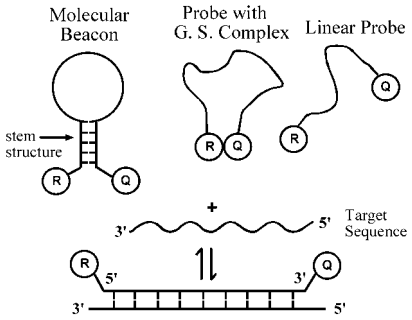

Figure 1. In molecular beacons and probes with a ground-state complex, the reporter dye and quencher are close together, preventing fluorescence. Linear probes have an uncontrolled dye−quencher distance. All three structures extend and fluoresce when complementary sequence is present. In RT-PCR, the sequence connecting the dye and quencher is broken.

Alternatively, we propose here that pairs of dyes can also be chosen such that their absorption bands overlap in order to promote a resonance dipole−dipole interaction mechanism within a ground-state complex. This report shows that such static quenching can be a dominant mechanism with certain fluorophore−quencher pairs in dual-labeled oligonucleotides, producing nonfluorescent heterodimers with their own distinct absorption spectra. There are reports of spectral changes in dual-labeled

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peptides due to intramolecular dye−dye interactions.[8,9] Nevertheless, an analogous, detailed report of such dye−dye interactions within a dual-labeled oligonucleotide has not been found.[10] There are a few reports of dye−dye interactions that cause changes in the absorption spectra of dual-labeled molecular beacons.[11] These dye−dye interactions, however, are induced by the oligonucleotide stem structure holding the dyes close together, and not so much by an inherent affinity of the dyes for each other.

The aggregation of unconjugated fluorescent dyes, such as fluorescein, rhodamines, and cyanines, to form ground-state complexes has been the subject of numerous studies. Dye aggregation often occurs in aqueous solvents and is controlled by electrostatic, steric, and hydrophobic forces.[12,13] Formation of homo- and heterodimers can cause distinct changes in the absorption spectrum due to coupling of the excited-state energy levels. Depending on the sign and size of the dipolar interaction and the relative orientation of the two transition dipole moments, the absorption bands and oscillator strengths will be modified for the dimer. One-dimensional aggregates, with one molecule per unit cell, are referred to as H-aggregates or J-aggregates, depending on whether absorption is allowed to only the top (H-aggregates) or the bottom (J-aggregates) area of the exciton band. As a result, H-aggregates have blue-shifted absorption while J-aggregated have red-shifted absorption.[14] Furthermore, H-aggregates often have significantly diminished fluorescence.[15] This can easily be understood because the radiant decay has to compete with ultrafast downward relaxation to all other nonradiating exciton states. J-aggregates, on the other hand, typically exhibit strongly enhanced radiative decay with high quantum yields.[16,17]

Here we propose an alternative mechanism of static quenching that can be important for oligonucleotide probes with certain fluorophore−quencher pairs. The proposed quenching mechanism differs from both the Förster and the Dexter mechanisms. We illustrate that efficient quenching can occur with minimal spectral overlap of fluorophore fluorescence and quencher absorption. Comparison of absorption spectra, relative fluorescence quantum yields, and fluorescence lifetimes of probes labeled only with a fluorophore versus measurements with dual-labeled probes modified with both the fluorophore and a quencher supports this hypothesis. In this study, we used Cy3.5 and FAM[18] as fluorophores, BHQ1 as a dark quencher,[19] and the β-actin sequence (without any stem structure) as the oligonucleotide (Figure 2). We propose that static quenching occurs by formation of a nonfluorescent intramolecular dimer between the fluorophore and quencher in a 5′fluorophore−3′quencher dual-labeled probe.

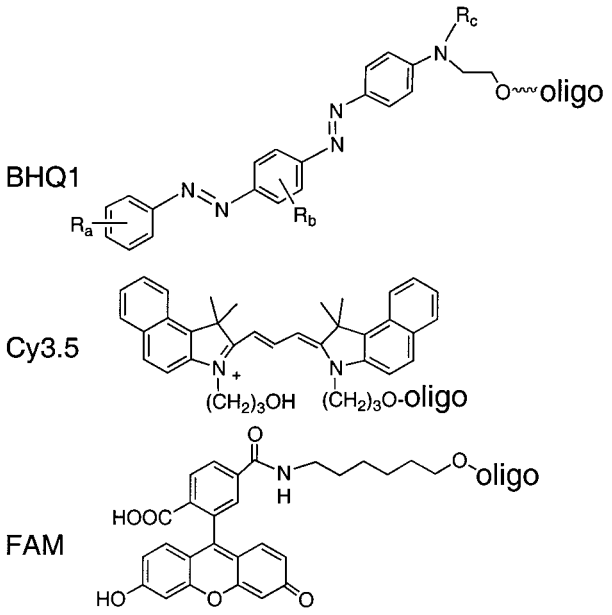

Figure 2. Quencher and fluorophores used in this study.

Experimental Section

DNA sequences were made with a Biosearch 8700 DNA synthesizer using standard phosphoramidite reagents. The β-actin sequence is 5′-d-ATG-CCC-TCC-CCC-ATG-CCA-TCC-TGC-G-3′. Complementary β-actin was made with three extra T bases on each end: 5′-d-TTT-CGC-AGG-ATG-GCA-TGG-GGG-AGG-GCA-TTT-T-3′. To make dual-labeled probes, BHQ1 CPG solid support (Biosearch Technologies) was used. Cy3.5 (Amersham Pharmacia) and FAM (Biosearch Technologies) were added to the 5′ end as phosphoramidites. All synthetic DNA samples were dual-HPLC purified by anion exchange (Dionex DNA Pac PA-100 column; solvent A=0.038 M tris[hydroxymethyl]-aminomethane with 15% acetonitrile; solvent B=solvent A with 1 M NaBr) followed by reversed-phase HPLC (Hamilton PRP-1 column; solvent A=0.1 N TEAA; solvent B=acetonitrile). Sample purity was confirmed by analytical anion exchange and reversed-phase HPLC.

All spectroscopic measurements were made in a buffer solution of 10 mM trizma hydrochloride, 50 mM KCl, and 3.5 mM $MgCl_2$. Absorption spectra were recorded using a HP 8452 diode array spectrophotometer. Additional absorption and fluorescence measurements were made using an S2000 Ocean Optics spectrometer with a CCD array detector, PX-2 pulsed Xenon lamp, and DT-1000 deuterium tungsten halogen light source. Additional fluorescence measurements were obtained with a Perkin-Elmer LS50b luminescence spectrometer and a Molecular Devices SpectraMax Gemini spectrofluorometer.

Time-correlated single photon counting measurements were performed with excitation by the output of a Coherent 9400 optical parametric amplifier (OPA), which was pumped by amplified pulses from a Coherent RegA 9000, which in turn is seeded by the output of a Mira system. A typical pulse duration from this system is in the order of 150 fs, and the repetition rate is ~190 kHz. The excitation wavelength was 481 nm for the FAM-containing samples and 551 nm for the Cy3.5-containing samples. Remaining white light was eliminated as much as possible by combinations of color filters, typically leaving a 30-nm transmission window around the excitation wavelength. Total excitation power did not exceed 1 μW. Cutoff filters were used before the detection (FAM samples,515 nm plus 530 nm; Cy3.5 samples, 570 nm plus 590 nm) to guarantee that no stray light from the excitation source reached the microchannel plate (MCP) detector. Time resolution

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(15) DNA-templated aggregation: Wang, M.; Silva, G. L.; Armitage, B. A. *J. Am. Chem. Soc.* **2000**, *122*, 9977−9986.
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(17) Fidder, H.; Knoester, J.; Wiersma, D. A. *J. Chem. Phys.* **1991**, *95*, 7880.
(18) FAM is a colloquial name for 6-carboxyfluorescein.
(19) A manuscript fully describing the synthesis of black hole quenchers (BHQs) from Biosearch Technologies is currently in preparation.

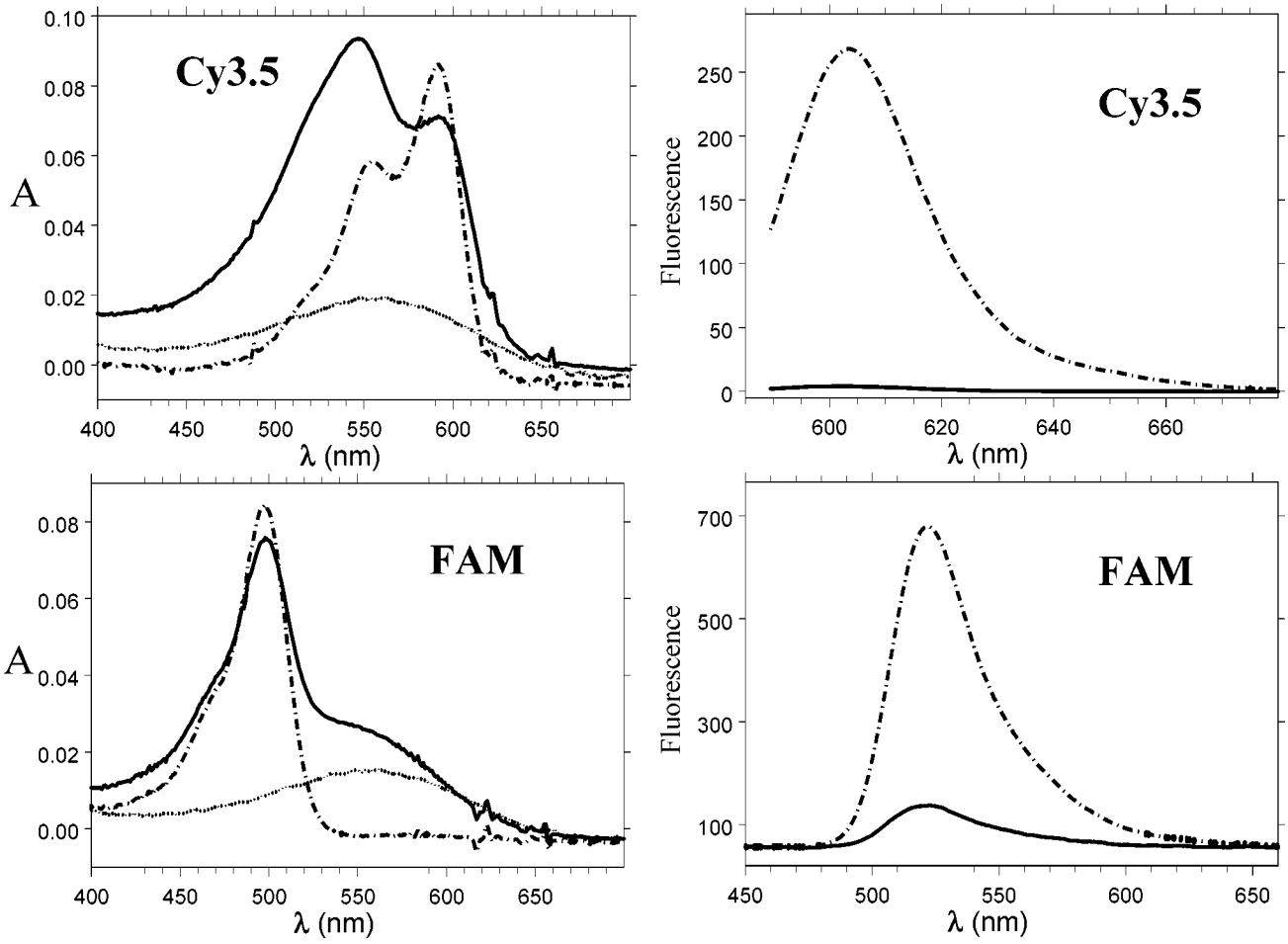

Figure 3. Absorption and fluorescence spectra of 5′Cy3.5- (top) and 5′FAM- (bottom) labeled β-actin oligonucleotides. Solid lines are 5′fluorophore−β-actin−3′quencher (BHQ1) dual-labeled probes. Dashed lines are singly labeled 5′fluorophore−β-actin probes. The same solutions were used for both absorption and emission measurements in order to compare intensities. The unscaled dotted absorption spectra are those of a T_{10}−3′BHQ1 solution.

in the experiments is limited by the response of the MCP (Hamamatsu 1564U). Fluorescence is collected on the MCP using an $f = 5$ cm lens. The output of the MCP is sent through an Ortec 9307 constant fraction discriminator before being fed to an Ortec 566 time-to-amplitude converter (TAC) as stop pulses. Start pulses were made from the electrical output of a silicon photodiode, which is illuminated by part of the OPA output. The output from the TAC is digitized and stored in a multichannel pulse-height analyzer (Canberra, Accuspec). Instrumental response functions were recorded by collecting scattering from a cell containing only buffer solution, without cutoff filters before the detector.

Results

Absorption Spectra of the Probes. The absorption spectrum of singly labeled oligonucleotide-3′BHQ1 has an unstructured peak with a maximum at 560 nm and ϵ = ca. 33 000 M^{-1} cm^{-1}. The absorption spectra of Cy3.5 and BHQ1 overlap more than the absorption spectra of FAM and BHQ1 do. This means that in a heterodimer, the dipolar coupling is more likely to lead to delocalized excitonic states for the Cy3.5−BHQ1 pair than for the FAM−BHQ1 pair, because the conditions for a resonant coupling are better met.

As shown in the left panels of Figure 3, neither of the dual-labeled probes, FAM−BHQ1 nor Cy3.5−BHQ1, has an absorption spectrum that is additive; i.e., the absorption spectra of the dual-labeled probes cannot be reconstructed by summing the absorption spectra of singly labeled BHQ1- and FAM- or Cy3.5-labeled oligonucleotides. For Cy3.5−BHQ1, the shoulder at 545 nm is noticeably blue-shifted, is significantly distorted, and has clearly more intensity than would be expected if the absorption spectrum were merely additive. (The extinction coefficient of BHQ1 at 550 nm is at least 3.5 times less than the extinction coefficient of Cy3.5 at 592 nm).[20] For FAM−BHQ1, the intensity around 550 nm is increased. These distortions in the dual-labeled probe absorption spectra indicate an interaction between the fluorophores (Cy3.5, FAM) and BHQ1. The blue-shift of the Cy3.5 absorption spectrum when BHQ1 is at the 5′ end is consistent with formation of an H-type[21] heterodimer.

Fluorescence Spectra of the Probes. BHQ1 has no detectable fluorescence and is therefore a dark quencher.[22] In the dual-labeled probes, linking BHQ1 at the other end of the oligonucleotide does not affect the spectral shapes of Cy3.5 and FAM emission. The fluorescence intensities, however, do change. To calculate the changes in the FAM and Cy3.5 quantum yields between the fluorophore and fluorophore−BHQ1 probes, the concentrations of the fluorophore and fluorophore−BHQ1 probe

(20) For free Cy3.5, $\epsilon = 120\,000$ M^{-1} cm^{-1} and $\Phi_F = 0.14$ (Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. *Bioconjugate Chem.* **1996**, *7*, 356). $\epsilon = 150\,000$ M^{-1} cm^{-1} has been reported for conjugated Cy3.5 (Amersham Pharmacia Biotech product literature).

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(22) Aromatic azo compounds usually do not fluoresce. See, for example: Luňák, S.; Nepraš, M.; Hrdina, R.; Mustroph, H. *Chem. Phys.* **1994**, *184*, 255.

Table 1. Absorbance Intensities of the Sample Solutions at 260 nm (Where Absorption Is Mostly Due to the Oligonucleotide) and at the Fluorophore Absorption Maxima

probe	abs at 260 nm	abs at 498 nm	abs at 592 nm
FAM−BHQ1	0.19	0.076	
FAM	0.34	0.085	
Cy3.5−BHQ1	0.21		0.071
Cy3.5	0.17		0.086

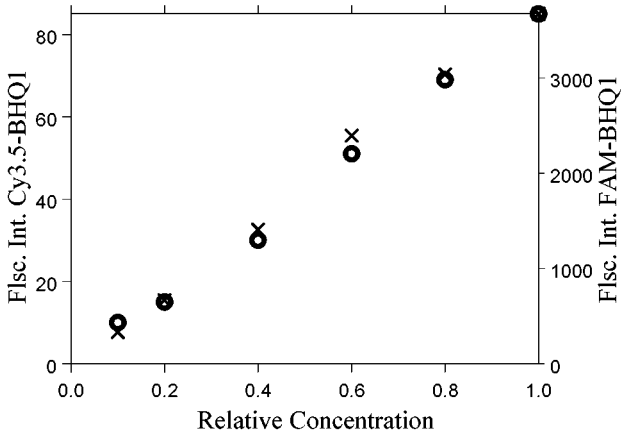

Figure 4. Fluorescence intensity vs concentration for Cy3.5−BHQ1 (○) and FAM−BHQ1 (×) dual-labeled probes.

solutions must be the same. It is difficult to accurately determine the concentrations because of the changes in the absorption spectra. From the absorbance readings in Table 1, it is clear that the four sample solutions have concentrations that do not differ by more than a factor of 2, and the concentrations are around 5×10^{-7} M.

The emission of the FAM oligonucleotide sample is 7.9 times more intense than the emission of the FAM−BHQ1 sample, while the emission of the Cy3.5 oligonucleotide sample is 67 times more intense than that of the Cy3.5−BHQ1 sample. This is counterintuitive according to Förster theory because the BHQ1 absorption spectrum overlaps more with FAM emission than with Cy3.5 emission. The fluorescence intensities of both dual-labeled probes were measured over a range of concentrations (Figure 4). For both probes, there is a linear relationship between fluorescence intensity and concentration, and the *y*-intercept is zero. This indicates that the quenching in both dual-labeled probes is intra- rather than intermolecular.

Fluorescence Excitation. The fluorescence excitation spectra of the Cy3.5−BHQ1 and FAM−BHQ1 probes are shown in Figure 5, together with their respective absorption spectra. For both probes, the excitation curve encompasses only a fraction of the absorption curve. This is partly due to the fact that BHQ1 has no emission. However, the difference between the absorption and excitation spectra of Cy3.5−BHQ1 around 550 nm and of FAM−BHQ1 around 430−470 nm cannot be entirely attributed to the absorption of BHQ1.

Interestingly, the fluorescence excitation spectra of the dual-labeled probes are very similar in shape to the absorption spectra of the singly labeled Cy3.5 and FAM oligomers. This suggests that the residual fluorescence from the dual-labeled probes probably originates from a minor fraction that does not form heterodimers. Comparison of the absorption, fluorescence, and excitation spectra strongly suggests that the Cy3.5−BHQ1 and FAM−BHQ1 heterodimers are nonfluorescent and that the weak

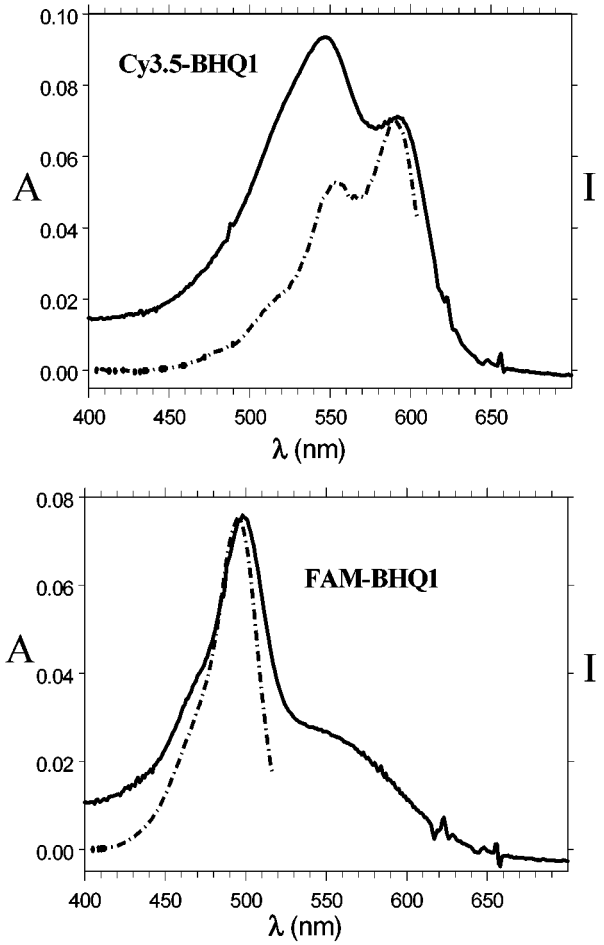

Figure 5. Fluorescence excitation (dashed) and absorption spectra (solid) of the Cy3.5−BHQ1 (Em λ = 614 nm) and FAM−BHQ1 (Em λ = 525 nm) probes.

fluorescence from the probes is from Cy3.5 and FAM monomers that are not spatially close to or interacting with BHQ1 (see Discussion section).

Fluorescence Lifetime Measurements. In a donor−quencher system where only dynamic quenching occurs and the population of fluorophores in the excited state is depleted, $\tau_{donor}/\tau_{donor-quencher}$ should be equal to $\Phi_{donor}/\Phi_{donor-quencher}$. In a case of pure static quenching, a nonfluorescent ground-state complex forms between the donor and quencher. In this case, the $\tau_{donor}/\tau_{donor-quencher}$ ratio is unity, while $\Phi_{donor}/\Phi_{donor-quencher}$ increases.[23,24] Figure 6 compares the fluorescence decays of the singly and doubly labeled β-actin oligonucleotides. The fluorescence decay of the singly labeled Cy3.5 oligonucleotide was fitted with a single exponential giving a lifetime of 2.31 ns. The decay of the Cy3.5−BHQ1 probe was fitted with a biexponential giving decay times of 2.1 ns (fraction: 0.83) and 0.6 ns (fraction: 0.17). From this an average lifetime of 1.8 ns can be extracted, yielding a ratio for $\tau_{donor}/\tau_{donor-quencher}$ of 1.28 for Cy3.5. From Figure 3, it was estimated that the ratio of the quantum yields is 67, which is significantly larger than 1.28. This discrepancy between ratios of fluorescence lifetimes and quantum yields indicates that a majority of the quenching that occurs between Cy3.5 and BHQ1 is static quenching.

(23) Lakowicz, J. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Plenum Press: New York, 1999.
(24) Webber, S. E. *Photochem. Photobiol.* **1997**, *65*, 33−38.

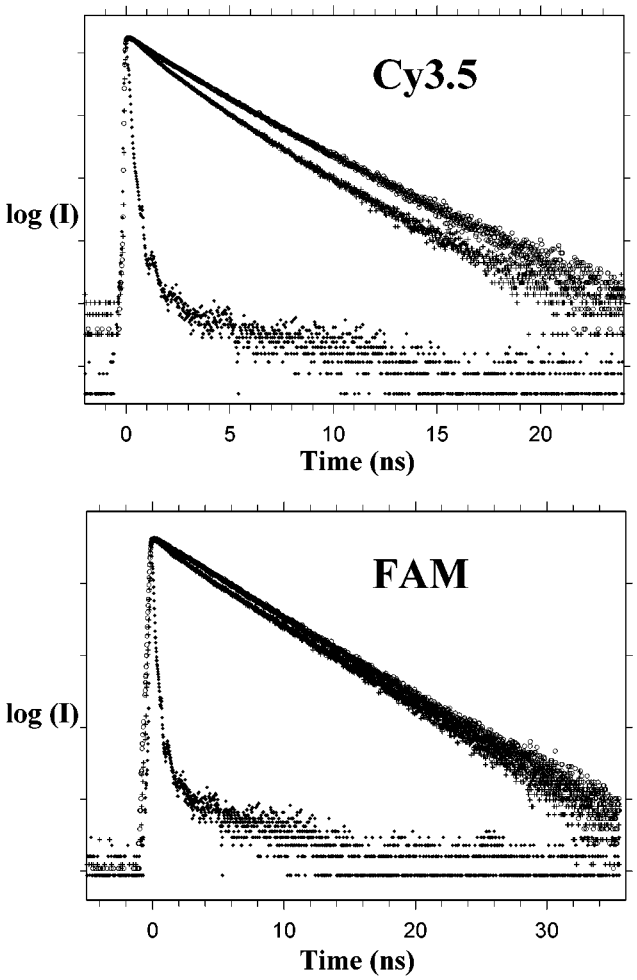

Figure 6. Time-correlated single photon counting data. Top panel (top to bottom): singly labeled Cy3.5−β-actin fluorescence decay, dual-labeled Cy3.5−BHQ1 fluorescence decay, and the instrumental response curve. Bottom panel (top to bottom): singly labeled FAM−β-actin fluorescence decay, dual-labeled FAM− BHQ1 fluorescence decay, and the instrumental response curve. The maximum intensities of each curve were normalized.

The fluorescence decay of the singly labeled FAM oligonucleotide gave a lifetime of 4.5 ns. The biexponential fit for the decay for the FAM−BHQ1 dual-labeled probe gave the time constants 4.5 ns (fraction: 0.90) and 1.6 ns (fraction: 0.10), resulting in an average lifetime of 4.2 ns. Thus the $\tau_{donor}/\tau_{donor-quencher}$ ratio for FAM−BHQ1 is 1.07. This ratio is smaller than $\Phi_{FAM}/\Phi_{FAM-BHQ1}$ ($\approx$7.9), which suggests that static quenching also occurs to a large extent with the FAM−BHQ1 pair.

Spectral Changes upon Addition of Complementary DNA. Figure 7 shows absorption spectra of the four probes, Cy3.5−β-actin−BHQ1, Cy3.5−β-actin, FAM−β-actin−BHQ1 and FAM−β-actin, before and after the addition of complementary DNA. The Cy3.5−β-actin−BHQ1 spectrum is the only one that changes dramatically, with the peaks moving closer together and the Cy3.5 peak around 591 nm gaining intensity when complement is present. The absorption spectra of both the singly labeled 5′Cy3.5−β-actin and the 5′FAM−β-actin−3′BHQ1 samples do not change appreciably when complementary sequence is added. This comparison demonstrates that addition of complement to the 5′Cy3.5−β-actin−3′BHQ1 sample changes the absorption spectrum because of an interaction between BHQ1 and Cy3.5.

The addition of complement causes the Cy3.5−BHQ1 probe absorption to change from a distorted to an additive shape (see Figure 3). This indicates that excitonic coupling between the Cy3.5 and BHQ1 chromophores within the dual-labeled probe is disrupted by the addition of complement. Presumably the distance between the Cy3.5 and BHQ1 subunits increases when duplex DNA forms, thereby nullifying the dipolar coupling.

Tables 2 and 3 summarize the ratios of fluorescence intensities between different samples. The addition of complementary sequence to the singly labeled 5′Cy3.5−β-actin and 5′FAM−β-actin samples did not significantly change their absorption spectra and did not dramatically change their fluorescence quantum yields. This indicates that the FAM and Cy3.5 dyes do not interact appreciably with the oligonucleotide bases. It is not clear why the Cy3.5 fluorescence decreases by 0.56 upon the addition of complement. The decrease suggests, however, that the photophysical properties of the Cy3.5 chromophore are slightly changed due to interactions with double-stranded DNA (as has been reported with fluorescein[25]). Nevertheless, these changes are very small compared to the dramatic ones found for the dual-labeled Cy3.5−BHQ1, and therefore they do not affect our main conclusions.

The correlation is quite good between the changes in fluorescence intensity starting with the dual-labeled probe and then either adding complement or comparing with the singly labeled sample (45 vs 67 for Cy3.5 and 5.7 vs 7.9 for FAM). The discrepancy in fluorescence changes for Cy3.5 (45 vs 67, giving a ratio of 0.67) is partly accounted for by the effect that adding complement has on the fluorescence intensity of Cy3.5 (decrease of 0.56). The analogous discrepancy for the changes in the fluorescence intensity of FAM (7.9 vs 5.7) may indicate that the concentrations of the singly labeled FAM sample and the dual-labeled FAM−BHQ1 sample were only approximately equal. Nevertheless, it is clear that BHQ1 quenches Cy3.5 much more effectively than it quenches FAM, despite the fact that BHQ1 has better spectral overlap for Förster energy transfer with FAM than with Cy3.5.

Discussion

Intramolecular aggregation is consistent with the notion that the flexible oligonucleotide functions as a tether that increases the relative concentration of the fluorophore and quencher. A 25-mer in a double helix will have a length of around 8.5 nm. Using 10 nm as a crude estimate of the average distance between the fluorophore and quencher on the 5′ and 3′ ends and assuming the oligonucleotide link is very flexible, the effective concentration between the fluorophore and quencher is 2×10^{-3} M. For dyes that spontaneously form aggregates, such a concentration would typically be sufficient to promote aggregation. Therefore, attaching dyes at the 5′ and 3′ ends of a dual-labeled probe could very well favor formation of a heterodimer. Of course, salt concentration, electrostatics, and steric effects are all possibly very important to this intramolecular dimer formation.

From comparison of absorption and excitation spectra, it is clear that there is either no or minimal fluorescence from fluorophore−BHQ1 heterodimers. The Cy3.5 and FAM emis-

(25) Sjöback, R.; Nygren, J.; Kubista, M. *Biopolymers* **1998**, *46*, 445−453.

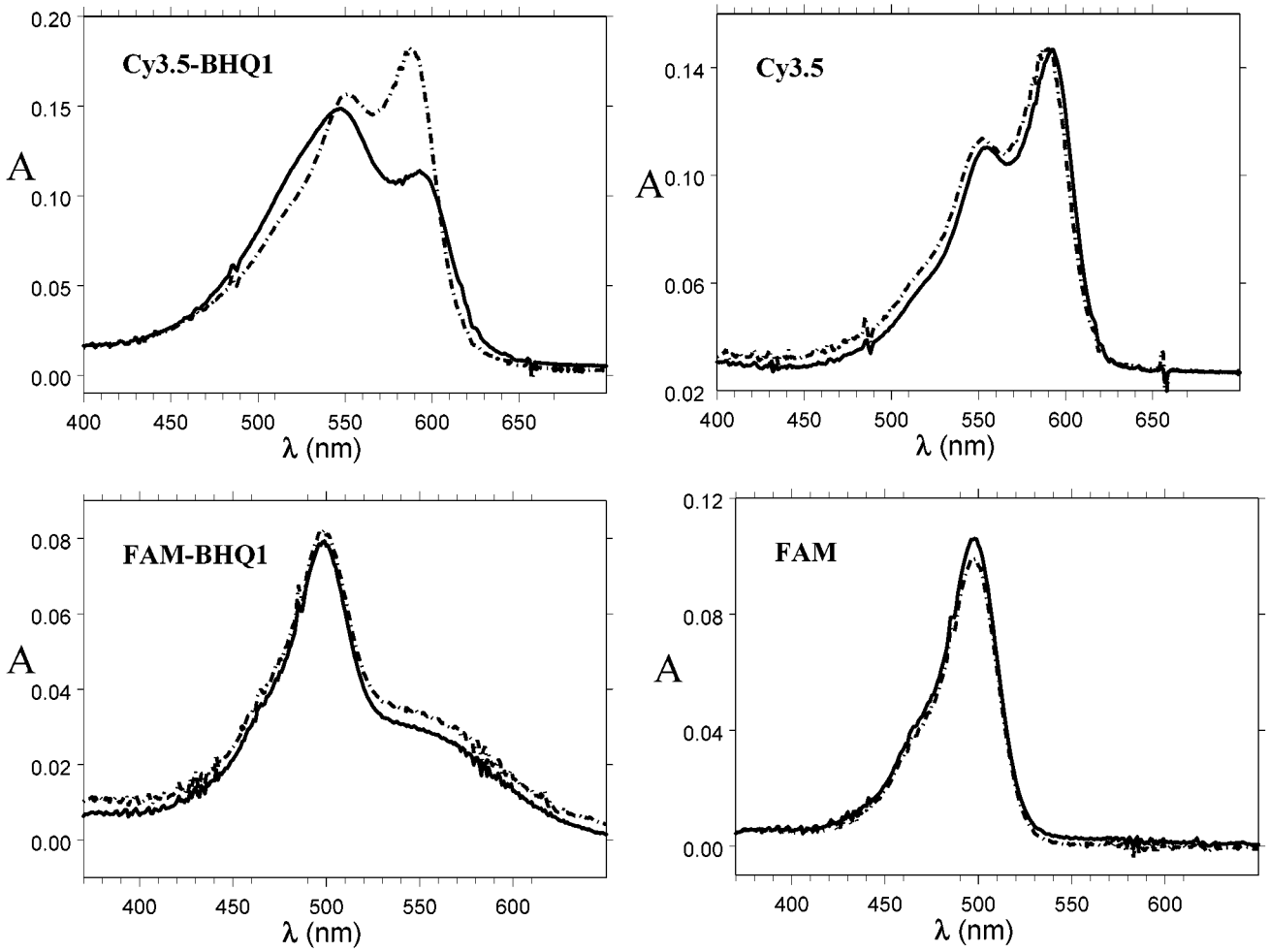

Figure 7. Absorption spectra of all four samples alone (solid lines) and after the addition of two equivalents of complementary DNA (dashed lines).

Table 2. Ratios of Fluorescence Intensities: Adding Complement to Singly Labeled Probes

samples	ratio of fluorescence intensities
Cy3.5 + comp.[a]/Cy3.5	0.56
FAM + comp.[a]/FAM	1.1

[a] Complementary sequence that has three extra T bases on each end (see Experimental Section).

Table 3. Ratios of Fluorescence Intensities: Adding Complement vs Removing Quencher

samples	ratio of fluorescence intensities
Cy3.5/Cy3.5−BHQ1[a]	67
Cy3.5−BHQ1 + comp.[b]/Cy3.5−BHQ1	45
FAM/FAM−BHQ1[a]	7.9
FAM−BHQ1 + comp.[b]/FAM−BHQ1	5.7

[a] Probe concentrations estimated to be the same within a factor of 2. [b] Complementary sequence that has three extra T bases on each end.

sion spectra have the same shape with or without BHQ1 at the 3′ end. If the heterodimer were fluorescent, its emission would very likely differ from the monomer's emission. It is reasonable to assume that there would be an association constant and a temperature-dependent equilibrium for heterodimer formation, so that some of the dual-labeled probes are not in the heterodimer form. We stress that this is a natural consequence of the intrinsic connection between the self-assembling process and the solubility product. Therefore, we propose that (1) a nonfluorescent, intramolecular, fluorophore−BHQ1 heterodimer is formed and dominates the absorption spectrum, particularly for Cy3.5−BHQ1, and (2) the residual fluorescence observed from the dual-labeled probes is dominated by a tiny fraction of probes that are not in the excitonic heterodimer form. Nevertheless, the strong increase in Cy3.5−BHQ1 probe fluorescence upon addition of complementary DNA demonstrates that intramolecular static quenching is substantial and of practical importance. We are currently exploring a variety of dye−quencher pairs in order to optimize intramolecular heterodimer formation.[26]

Clearly, the Cy3.5−BHQ1 heterodimers are virtually nonfluorescent not just because they form H-type dimers.[27] In homodimers, it is possible for an H-type aggregate to have no fluorescence because identical transition dipoles are coupled which can completely cancel each other if properly aligned. Such complete cancellation is very unlikely in a heterodimer. In the present case, dipolar coupling leads to formation of excitonic states delocalized over both the fluorophore and BHQ1. The optical excitation of the BHQ1 monomer clearly has an

(26) The structural characteristics of fluorophores that form intramolecular homodimers in labeled peptides have been studied: Packard, B. Z.; Komoriya, A.; Toptygin, D. D.; Brand, L. *J. Phys. Chem. B* **1997**, *101*, 5070.

(27) Packard, B. Z.; Toptygin, D. D.; Komoriya, A.; Brand. L. *J. Phys. Chem. B* **1998**, *102*, 752.

extremely efficient coupling to its nonradiative manifold, which makes it a dark quencher. The new, delocalized Cy3.5−BHQ1 eigenstates are, to a first approximation, linear combinations of the direct product states, $\Psi^*_{Cy3.5}\Psi_{BHQ1}$ and $\Psi^*_{BHQ1}\Psi_{Cy3.5}$ (the asterisk denotes excited electronic state). Obviously, due to the amplitude of $\Psi^*_{BHQ1}\Psi_{Cy3.5}$, these delocalized dimer states will also couple strongly to the BHQ1 nonradiative manifold. Thus, we conclude that quenching in the Cy3.5−BHQ1 heterodimers is strongly enhanced because the Cy3.5 fluorophore obtains an efficient direct channel into the BHQ1 nonradiative manifold.

Furthermore, coupling of Cy3.5, and to a lesser extent the FAM excitation, to a dark channel in the BHQ1 moiety can explain the discrepancy between changes in fluorescence quantum yields and lifetimes between singly and doubly labeled probes. Exciton coupling within the fluorophore−BHQ1 heterodimers presumably causes an extremely short-lived excited state, which cannot be distinguished from the instrumental response in Figure 6. There is, however, a slight decrease in average fluorescence lifetime going from the fluorophore to the fluorophore−BHQ1 samples. We believe that the residual emission from the dual-labeled probes originates from a small fraction of fluorophores that are not forming a strongly coupled ground-state complex. These fluorophores, while not close enough to BHQ1 to have significant dipolar coupling, could still be quenched via dynamic energy transfer, which would be expected to give multiexponential fluorescence decay kinetics due to a distribution of fluorophore−BHQ1 distances.

Conclusions

In summary, we propose that some dye−quencher pairs in dual-labeled oligonucleotides can have a strong enough affinity for each other that they form an intramolecular, nonfluorescent complex. This hypothesis is supported by spectral changes and fluorescence lifetime measurements of a series of singly labeled and dual-labeled oligonucleotides. Intramolecular heterodimers are formed between the 5′fluorophore and 3′quencher on a β-actin probe without a stem structure. This suggests that the oligonucleotide acts as a flexible tether that effectively increases the fluorophore−quencher concentration, promoting the formation of the heterodimers.

In the current literature, there is a strong focus on developing dual-labeled probes by matching quenchers and dyes for dynamic energy transfer. Probes with intramolecular heterodimers, as discussed here, present a major advantage in that efficient quenching can be obtained without the use of molecular beacon structures. This report demonstrates that static quenching is an alternative mechanism that allows new possibilities and freedom in matching dye−quencher pairs for profluorescent assays with dual-labeled oligonucleotides.

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