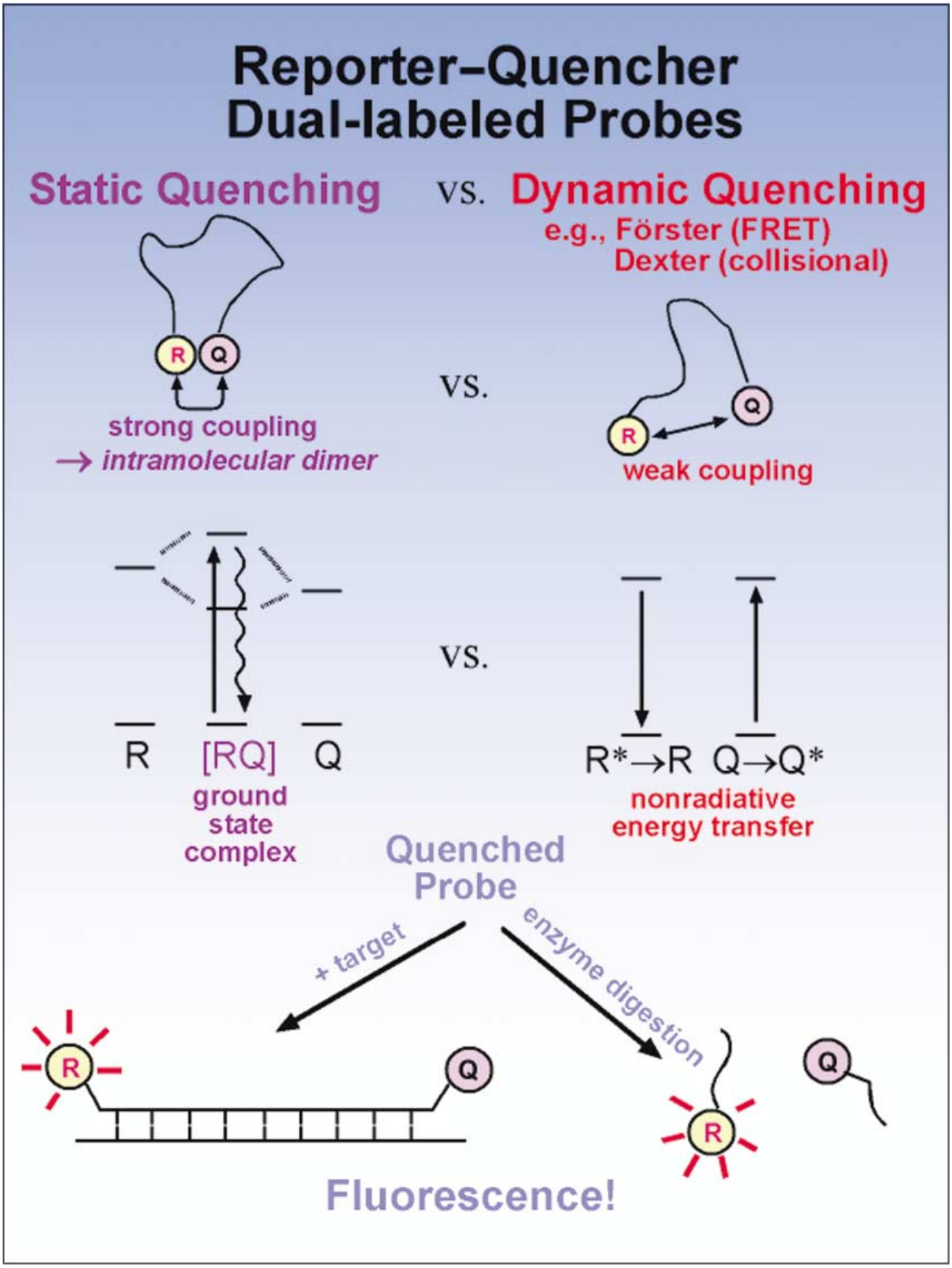

DOI: 10.1002/chem.200304941 *Chem. Eur. J.* **2003**, *9*, 3466 – 3471

Intramolecular Dimers: A New Design Strategy for Fluorescence-Quenched Probes

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Abstract: Fluorogenic probes dual-labeled with reporter and quencher dyes use a change in fluorescence to monitor biochemical events (e.g., substrate binding or enzyme digestion). Such events change the reporter–quencher distance, which affects fluorescence. Recently, it is has been shown that static quenching through intramolecular dimers is an important mechanism that can sometimes be more efficient than Förster resonance energy transfer (FRET).

Keywords: biosensors · fluorescent probes · FRET (fluoresence resonance energy transfer) · intramolecular dimer · stacking interactions

Introduction

Fluorescent labels are widely used as probes in biochemical assays.[1] In the simplest assays, a fluorescent dye noncovalently binds to a specific target and a change in fluorescence intensity or wavelength indicates that a biochemical event has taken place. However, it is often necessary to be more specific; in order to detect the binding of a specific moiety, a fluorescent dye must be covalently linked to a biomolecule. It is possible to achieve changes in fluorescence with singly-labeled biomolecules though a choice of dye that is affected by factors such as pH, hydrophobicity, or ionic strength.[2] For instance, singly-labeled oligonucleotide probes can be designed to take advantage of modest quenching between fluorophores and nucleobases.[3, 4] Upon binding to a complementary oligonucleotide, the distance between the fluorophore and neighboring nucleobases increases. This type of approach has also been used for detection of specific double-strand DNA sequences.[5] However, the sensitivity of assays with such probes is limited and can vary because nucleobase–fluorophore quenching is sequence-dependent and gives only modest increases of fluorescence intensity.[6] Some reporters for oligonucleotides[7, 8] and peptides[9] undergo more dramatic changes in fluorescence in hybridization and enzyme assays.

To improve upon the strategy of using singly-labeled probes, quencher dyes have been developed that can be paired with reporter dyes to augment the observable change in fluorescence. Dual-labeled probes with both covalently linked reporter and quencher moieties can have significant advantages over singly-labeled probes. Using a dual-labeled probe allows a greater selection of reporter dyes, including infra-red-emitting fluorophores, and in many cases improves sensitivity and specificity. In a typical biomolecular probe, the closed (i.e., quenched) form has the reporter and quencher close to each other in space, while the open (i.e., fluorescent) form has the reporter and quencher spatially separated. Förster resonance energy transfer (FRET) is the mechanism that is commonly cited as controlling fluorescence quenching in such systems.[10] According to Förster theory, the reporter and quencher should be chosen such that the spectral overlap between reporter fluorescence and quencher absorption is maximized (Figure 1).

Until recently, quenchers have typically been a second fluorescent dye, for example, fluorescein as the reporter and rhodamine as the quencher. The fluorescence of the reporter

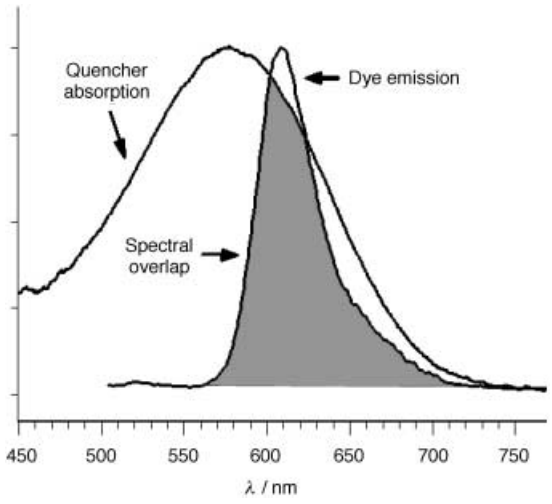

Figure 1. Reporter emission and quencher absorption with large spectral overlap.

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 DOI: 10.1002/chem.200304941

can be monitored alone, or ratiometric measurements can be made whereby both the increase in fluorescence of the quencher and decrease in fluorescence of the reporter are observed. Although FRET quenching may be very efficient in these systems, there can be high background noise due to overlap between quencher and reporter fluorescence spectra. This limitation often necessitates the use of optical filters and complex data analysis. Dark quenchers, that is, dyes with no native fluorescence, offer a solution to this problem because they do not occupy an emission bandwidth. Dark quenchers also enable multiplexing, whereby two or more fluorophore–quencher probes are used in situ.[11]

Our initial concept for developing a series of dark quenchers was that their utility would depend on their ability to quench common reporter dyes through the FRET mechanism. Our quenchers, which we have whimsically named "black hole quenchers" (BHQs), have a polyaromatic-azo backbone, which makes the dyes nonfluorescent (Figure 2).

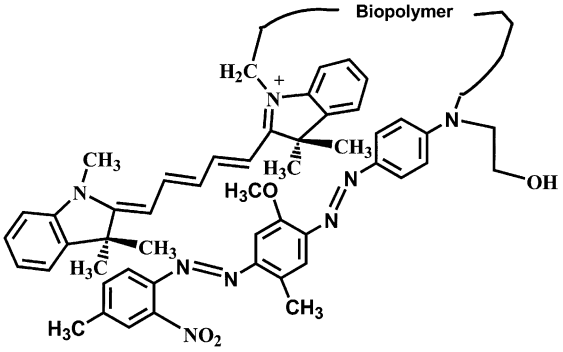

Figure 2. Hypothetical representation of an intramolecular Cy5–BHQ1 heterodimer.

The absorption maxima are tuned through appropriate choice of electron-donating and -withdrawing substituents on the aromatic rings. This methodology resulted in a series of nonfluorescing dyes with absorption spectra that overlap with the emission spectra of common reporter dyes and thereby maximize FRET quenching. However, we found that an alternative quenching mechanism can be extremely effective in dual-labeled probes: dye–quencher ground state complex formation to form an intramolecular dimer.

Quenching Mechanisms

Energy can be nonradiatively transferred between two dyes, a donor and an acceptor (quencher), through either dynamic or static quenching.[1] Förster[10] and Dexter[12] are dynamic quenching mechanisms, because energy transfer occurs while the donor is in the excited state (Figure 3). The Förster (also known as the Coulomb) mechanism is based on classical dipole–dipole interactions between the transition dipoles of the donor and acceptor. Förster quenching is extremely dependent on the donor–acceptor distance (R), falling off at a rate of $1/R^6$. FRET, which can occur up to distances of 100 Å, also depends on the spectral overlap of the donor fluorescence and quencher absorption, and the relative

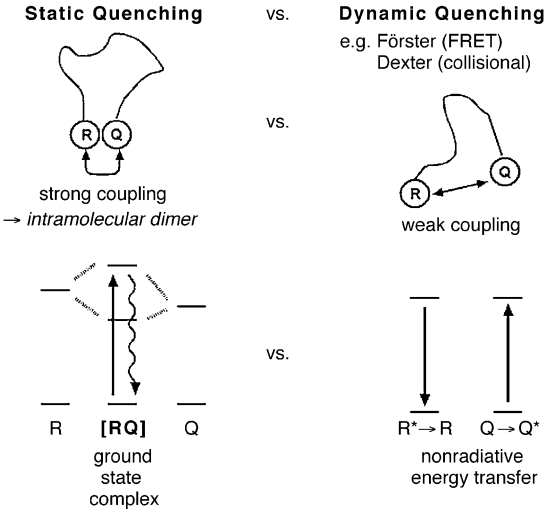

Figure 3. Comparison of static and dynamic quenching mechanisms.

orientation of the donor and acceptor transition dipole moments.[13] Dexter (also known as exchange or collisional energy transfer) is a short-range phenomenon that depends on the spatial overlap of donor and quencher molecular orbitals. The efficiency of Dexter transfer decreases with e^{-R} (R = donor–quencher distance). Because singlet–singlet transitions are involved in the vast majority of donor–quencher pairs, the Förster mechanism will be more important than the Dexter mechanism. The intrinsic properties of the donor and quencher dyes, such as the shape of their absorption and fluorescence spectra, are retained when quenching occurs with both the Förster and Dexter mechanisms.

Static quenching occurs through formation of a ground state complex. The donor and quencher moieties bind together to form a ground state complex, an intramolecular dimer, that has its own unique properties. Dye aggregation is well-known and is often attributed to hydrophobic effects—the dyes stack together to minimize contact with water. Steric and electrostatic forces may also determine if, and how, dyes aggregate.[14] In a ground-state complex, the excited-state energy levels of the dyes couple. The electronic properties of the dimer depend on the dipolar interaction and the relative orientation of the reporter and quencher transition dipole moments. In H-aggregates, absorption is allowed only to the top area of the exciton band, absorption is blue-shifted, and fluorescence is diminished. In J-aggregates, absorption is allowed to the bottom area of the exciton band, absorption is red-shifted, and fluorescence quantum yields are enhanced.[15, 16] In homodimers, in which two of the same dye form a ground-state complex, it is possible for an H-type dimer to be totally nonfluorescent if the dyes align such that their identical transition dipole moments completely cancel. However, in heterodimers, when both individual dyes are fluorescent (e.g., rhodamine and fluorescein), the H-type dimer will only have diminished fluorescence because it is very unlikely for the transition dipole moments to completely cancel. Nevertheless, in the case of a heterodimer between a fluorophore and a dark quencher, coupling of the fluoro-

phore's excitation to the quencher's dark channel can make the ground-state complex completely nonfluorescent.[17]

Other quenching mechanisms include exciplex (excited-state complex) formation and electron transfer. Exciplex formation involves aggregation after the reporter is in an excited state. Therefore, quenching due to exciplex formation occurs only if the reporter and quencher are in close proximity or if the reporter has a long-lived excited state, which increases the probability of reporter–quencher encounters.[18] Fluorescence quenching by electron transfer, rather than energy transfer, often causes photobleaching, that is, irreversible oxidation or reduction of the reporter dye.

Peptide Probes

Singly-labeled peptides can be effectively used in capture assays, for example, receptor-targeted optical imaging uses dye–peptide conjugates that accumulate in tumor tissue.[19] Fluorogenic peptides with fluorescent leaving groups have limited utility, because the cleavage site often has diminished substrate activity compared to the native peptide. Dual-labeled peptides can improve sensitivity in a wide variety of in vivo and in vitro assays. Depending on geometric determinants and the physical properties of the dyes, they can interact intramolecularly through static quenching, energy transfer, dark quenching, or electron transfer.[20] Fluorescence increases when the dual-labeled peptide is cleaved by a protease (which has a cleavage site between the dyes), or when the peptide is bound to an antibody (or other receptor site) causing the dyes to separate. Dual-labeled peptides have also been used to study secondary structure, for example, through intramolecular end-to-end contact between the dyes. Fluorescence lifetime measurements of dual-labeled peptides can track both the kinetics of folding and the distribution of differently folded peptides.[21]

There are many assays based on intramolecular resonance energy transfer within profluorescent dual-labeled peptides, including those with serine proteases (e.g., HIV-1),[22] signal peptidases (e.g., streptococcus pneumaniae),[23] and caspases.[24] Some assays that have been developed use dark quenchers.[25] Other studies have noted that efficient quenching can occur despite the fact that the absorption band of the quencher does not overlap with the emission band of the fluorophore—this indicates that a mechanism besides FRET occurs.[26, 27] Packard and co-workers have systematically investigated some aspects of quenching in homo-dual-labeled peptides. They describe intramolecular homodimers in profluorescent protease substrates, structural characteristics of fluorophores that promote homodimer formation (surprisingly, hydrophobicity was not very important),[28] and how length and conformation of the peptide plus linker controls dimer formation.[29] However, systematic studies of intramolecular heterodimers within dual-labeled peptides, and most notably, fluorophore–dark-quencher heterodimers, have not been found in the literature. Table 1 summarizes three different studies with dual-labeled peptides that form ground state complexes, including results with a rhodamine–dark-quencher heterodimer.[30]

Table 1. Fluorescence assays with dual-labeled peptides. Changes in absorption spectra show that static quenching through intramolecular dimers occurs in these systems.

Ref.	Peptide	Labels	Assay	Fluorescence increase
[28]	NorFES undecapeptide	rhodamine–rhodamine	serine protease	10:1
[27]	decapeptide	rhodamine–rhodamine	malarial protease	30:1
[26]	13-residue peptide	fluorescein–rhodamine	monoclonal antibody	8:1 increase for rhodamine
[30]	octapeptide	rhodamine– BHQ2	Trypsin	100:1

A potential limitation with static quenching especially applies to peptide probes. Enzyme activity and substrate recognition are usually extremely sensitive to peptide conformation; if formation of the intramolecular dimer were to change the shape of the peptide's active site, the activity rate could be dramatically decreased. If this is a problem, it may be necessary to re-engineer the placement of dye labels and their attachment, such that dimer formation will not alter peptide secondary structure.

Oligonucleotide Probes

Fluorescent oligonucleotide probes have become indispensable tools for genetic analysis, and have enabled new pathways for modern drug discovery and pharmacogenetic-based medicine.[31, 32] In particular, dual-labeled fluorescence-quenched oligonucleotide probes have become fundamentally important reagents in several commercial genetic assays, most notably in quantitative PCR (polymerase chain reaction), which measures the presence and copy number of specific genes or expressed m-RNA.[33–35] Numerous assays with dual-labeled oligomers that do not require PCR thermocycling have also been developed by using oligomer hybridization and/or cleavage to change the reporter–quencher distance.[36] Stem-loop structures, known as molecular beacons, decrease background fluorescence by holding the dye and quencher close together.[37] As a result, molecular beacons typically have higher signal/noise ratios in FRET assays than "linear" probes, but there are some disadvantages to molecular beacons; these include cost and difficulty in purification due to secondary structure.

Quenching due to aggregation of dye labels is an unwanted effect when multiple dye labels are used in order to amplify fluorescence signal.[38] There have been a few references to quenching in dual-labeled probes through non-FRET quenching mechanisms, especially in situations whereby the dyes are held close together through hybridization.[39, 40] Marras et al. compared static and FRET quenching efficiencies for a wide range of reporter–quencher pairs by placing the dyes on complementary oligonucleotides at 0, 5, or 10 bases apart.[41] They found that melting temperatures of blunt-end hybrids of the fluorophore–quencher pairs correlated well with percentage quenching, showing that the dyes that bind more strongly together in a dimer have higher quenching efficiencies.

Recently, we reported that dual-labeled oligonucleotides that do not have a stem structure can form intramolecular dimers; the oligonucleotide presumably acts as a tether, effectively increasing the relative dye concentration. In effect, this is a stemless molecular beacon in which dye–quencher aggregation brings the 3′ and 5′ ends together.[17] Figure 4 shows the spectral changes before and after complementary sequence is added to a Cy5–BHQ1 dual-labeled 25-mer oligonucleotide probe without defined secondary structure. The change in the shape of the absorption spectrum is indicative of Cy5–BHQ1 intramolecular dimer formation (Figure 4).

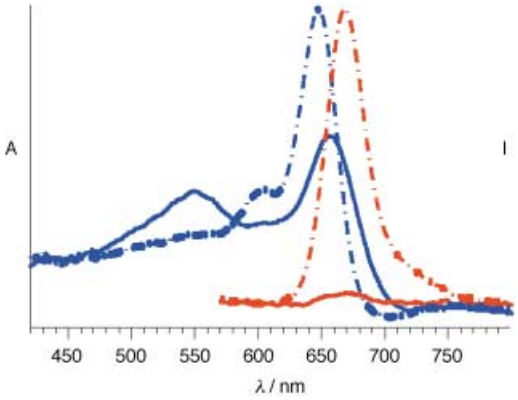

Figure 4. Hybridization assay with a 5′Cy5–β-actin–3′BHQ1 oligonucleotide probe. The blue curves are absorption spectra, the red curves fluorescence spectra. Solid lines are the probe alone, dashed lines are for probe with excess complement. Cy5 and BHQ1 have limited spectral overlap for FRET. Changes in fluorescence intensity and shape of the absorption curves indicate quenching by means of an intramolecular heterodimer.

A possible limitation to consider is that stability of fluorophore–quencher ground-state complexes is very temperature dependent. It is reasonable to assume that intramolecular dimer formation is governed by an association constant and a temperature-dependent equilibrium. Static quenching within dual-labeled oligonucleotides is most likely to be significant only in room temperature assays, or perhaps at moderately elevated temperatures. Thus, in real-time PCR oligonucleotide probes, for which the fluorescence intensity is typically read at 60 °C, quenching through intramolecular dimers may be less effective.

Outlook for Probes that use Intramolecular Dimers

Both for dual-labeled peptides and oligonucleotides, Förster energy transfer is the most often cited mechanism for quenching. However, there have been a growing number of references to static quenching. Static quenching between a fluorophore and a dark quencher can virtually eliminate fluorescence.[17, 41] To date, there have been some reports of quenching by means of intramolecular homodimers, and a few accounts of intramolecular heterodimers, most involving two fluorophores.

Static quenching, as an alternative to FRET, has several potential benefits. Pairing of dyes and quenchers for FRET is dictated by reporter-emission/quencher-absorption spectral overlap. Static quenching, on the other hand, depends on the overlap of dye and quencher absorption spectra. The ability to construct fluorescence-quenched probes without regard to emission–absorption spectral overlap reduces the constraints on quenched probe design and expands the breadth of their application and ease of their construction. Even existing assays employing FRET might be significantly improved by application of intramolecular dimer probe strategy.

For example, the application of fluorescence-quenched probes as agents for medical imaging is an exciting new prospect that could perhaps be further enabled by the understanding of intramolecular dimers. The concept of cell-specific-targeted fluorescent probes may be applied to a wide range of biological targets specific to virtually any disease state or cell type. For instance, recently a self-quenched cyanine-labeled somatostatin analogue was employed as a contrast media probe for optical imaging of mouse tumors that over express a somatostatin protease.[42] Furthermore, it may be possible to construct probes that enter targeted cells within an organism and elicit signals based on the specific internal biochemistry of the cell, a concept now known as "molecular imaging".[43] For instance, a fluorescence-quenched oligonucleotide probe could be designed to detect the presence of expressed m-RNA specific to a disease protein, such as HIV related protease.

Fluorescent reporters utilized for "in vivo" imaging are necessarily emitters in the near-infra red region (NIR, 750–850 nm), because this band is absent of spurious biofluorescence. Furthermore, NIR can penetrate tissue to 5–6 cm, allowing efficient excitation and imaging. To date, cyanine NIR dyes have been employed both as reporters and quenchers, an arrangement that inherently limits performance due to non-optimal FRET quenching and consequent high background noise. We believe the field of optical contrast media may have much to gain by investigating NIR-dye/dark-quencher pairs designed by using static quenching principles.

The future of fluorescent probes based on static quenched intramolecular fluorophore–quencher strategies holds great promise. Clearly, further investigation into the molecular parameters that modulate heterodimer formation would seem to be a ripe area for further research.

Acknowledgements

Research on intramolecular dimers in dual-labeled oligonucleotides and the development of black hole quenchers was supported in part by SBIR grant 1R43GM60848. We thank Drs. Derek Hudson and Mark Reddington for helpful discussions.

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