Chapter 4

Fluorescence Resonance Energy Transfer (FRET) by Minor Groove-Associated Cyanine-Polyamide Conjugates

The work described in this chapter was accomplished in collaboration with V. Rucker (Dervan group; Caltech).

Abstract

Fluorescence resonance energy transfer has been used for both qualitative and quantitative analysis of nucleic acid structure in biologically relevant contexts. The timescale of FRET and the range of distances (10-90 Å) over which it is useful are complementary to other techniques commonly applied to the analysis of DNA. Previous work in the Dervan group has attempted to use of FRET between fluorophore-polyamide conjugates as a means of sequence specific detection of DNA; however, these efforts were unsuccessful, possibly resulting from the choice of fluorophores and the design of polyamide conjugates.

This chapter describes FRET between cyanine-hairpin polyamide conjugates in a model system. The physical properties of cyanine conjugates, including their spectral characteristics, affinities for DNA, and the degree of quenching they exhibit in the absence of DNA, are summarized. Preliminary results using a Cy3 fluorophore as the donor and a Cy5 fluorophore as the acceptor are presented in detail. Alternative donors and acceptors, previously synthesized, are suggested for use in future experiments. Potential applications for polyamide-based FRET are also presented.

Background and Significance

Fluorescence resonance energy transfer (FRET) is a spectroscopic process by which a donor fluorophore transfers energy nonradiatively over long distances (10-90 Å) to an acceptor fluorophore. The relationship between fluorophore distance and energy transfer was first described by Förster in the 1940's,[1] and was later verified by Stryer and colleagues, in the late 1970's.[2] A number of advantages are inherent to this technique, including the sensitivity of fluorescence-based detection, the relatively short timescale of energy transfer, and the appreciable range of distances over which it can be applied.[3]

The molecular processes underlying FRET have been reviewed extensively and are illustrated in Figure 4.1.[4,5] The first step involves absorption of energy by the donor molecule, resulting in excitation from the ground state, S_0^D, to an excited singlet state, S_1^D. Several excited states are available to the donor; however, vibrational relaxation to S_1^D by internal conversion is rapid, ensuring that a majority of emission occurs from this state. Several fates are possible for the excited donor, including spontaneous emission and nonradiative processes. If a suitable acceptor fluorophore is nearby, then nonradiative energy transfer between the donor and acceptor can occur. This transfer involves a resonance between the singlet-singlet electronic transitions of the two fluorophores, generated by coupling of the emission transition dipole moment of the donor and the absorption transition dipole moment of the acceptor. Thus, the efficiency of FRET and the range of distances over which it can be observed are determined by the spectral properties of a given donor-acceptor pair.

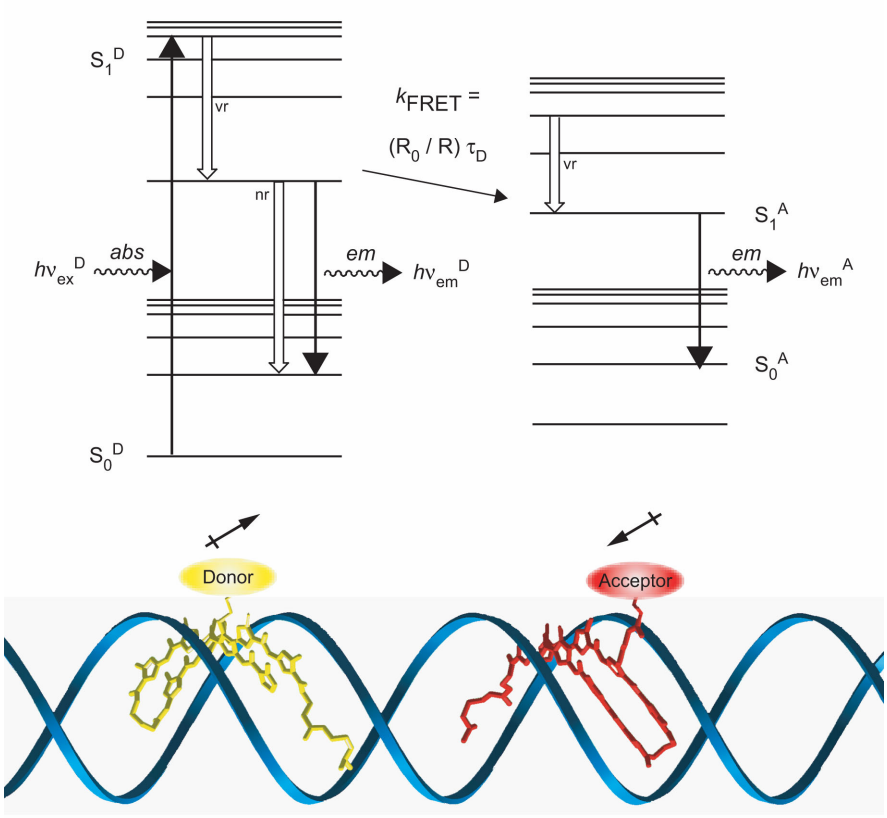


Figure 4.1 Molecular processes underlying FRET illustrated for fluorophore-polyamide conjugates.

FRET can be experimentally measured in a number of ways, using either time-resolved or steady-state techniques. The most commonly employed methods for quantifying FRET monitor either the reduction in the donor quantum yield in the presence of acceptor or the enhancement of acceptor emission in the presence of donor. Quantum mechanics dictates that the rate of energy transfer correlates with the inverse sixth power of the distance separating the fluorophores, R. In the context of steady-state experiments, this relationship allows the efficiency of energy transfer, E_T, to be translated into relative distances according to Equation 1, where

$$E_T = 1 \Big/ \left[1 + \left(\frac{R}{R_0} \right)^6 \right]$$

R_0 is the characteristic Förster radius for a given donor-acceptor pair. The Förster radius is defined by Equation 2, where Φ_D is the fluorescent quantum yield of the donor in the absence of acceptor, κ is an orientation factor that depends on relative dipole positions of the donor and acceptor, η is the refractive index of the medium, and $J(\lambda)$ is the spectral overlap of donor emission and acceptor absorption. Thus, when $R = R_0$ the efficiency of FRET is 50%. In practice, a more manageable version

$$R_0^6 = 8.8 \times 10^{-28} \bullet \Phi_D \bullet \kappa^2 \bullet \eta^{-4} \bullet J(\lambda)$$

of this relationship is desirable, using the definition of quantum yields to derive Equation 3 which correlates two experimentally determined values, E_T and Φ_D, with the distance between two donor and acceptor.

$$E_T = 1 - \left(\Phi_{DA} \Big/ \Phi_D \right)$$

FRET can be used as either a quantitative tool for determining absolute distances in macromolecular assemblies or for quantitative measure of relative distances.[6] In the context of polyamide-based FRET, quantitative techniques present several experimental difficulties and offer no real advantage to existing sequencing techniques. Qualitative applications of FRET, on the other hand, are well-suited to polyamides and can provide structural insight for DNA and DNA-protein complexes. Similar approaches to FRET have been used to measure DNA bending and kinking inherent to purine tracts or resulting from protein association.[7,8] FRET can also be combined with other analytical techniques, including HPLC, flow cytometry, or gel electrophoresis to allow greater S/N ratios.

Previous Work toward Polyamide-Based FRET (Figure 4.2)

Seminal efforts to demonstrate FRET between fluorophore-polyamide conjugates bound proximally in the minor groove were carried out by Scott Carter. Eight-ring hairpin polyamides, bearing coumarin or BODIPY moieties at the turn position, were synthesized and screened using a set of model oligonucleotide duplexes, without success. The relatively poor specificity of the polyamide scaffold selected for donor attachment, **1**, was discussed in Chapter 3 and must be considered at least a contributing factor to the experimental failure. The choice of the turn for covalent modification with fluorophores is also suspect as similar conjugates have shown either reduced affinity or variation in fluorescence with respect to the proximal DNA sequences. The fluorophores selected in these experiments are also characterized by lower quantum yields and molar extinction coefficients than their cyanine counterparts. Finally, the oligonucleotide duplexes employed to measure FRET examined extremely short inter-fluorophore distances which allows energy transfer to occur by alternate mechanisms (Dexter),[9] complicating the interpretation of results, if allowing association of both polyamides at all.

Subsequent research efforts, conducted by Victor Rucker, replaced the above fluorophores with the well characterized fluorescein-TMR donor-acceptor pair. A different covalent modification strategy, using the N-Me position of Py residues for fluorophore attachment, was also employed. Oligonucleotide duplexes were redesigned to provide greater fluorophore separations, however, the promiscuous donor-polyamide scaffold, **3**, was maintained and FRET was not observed. A third

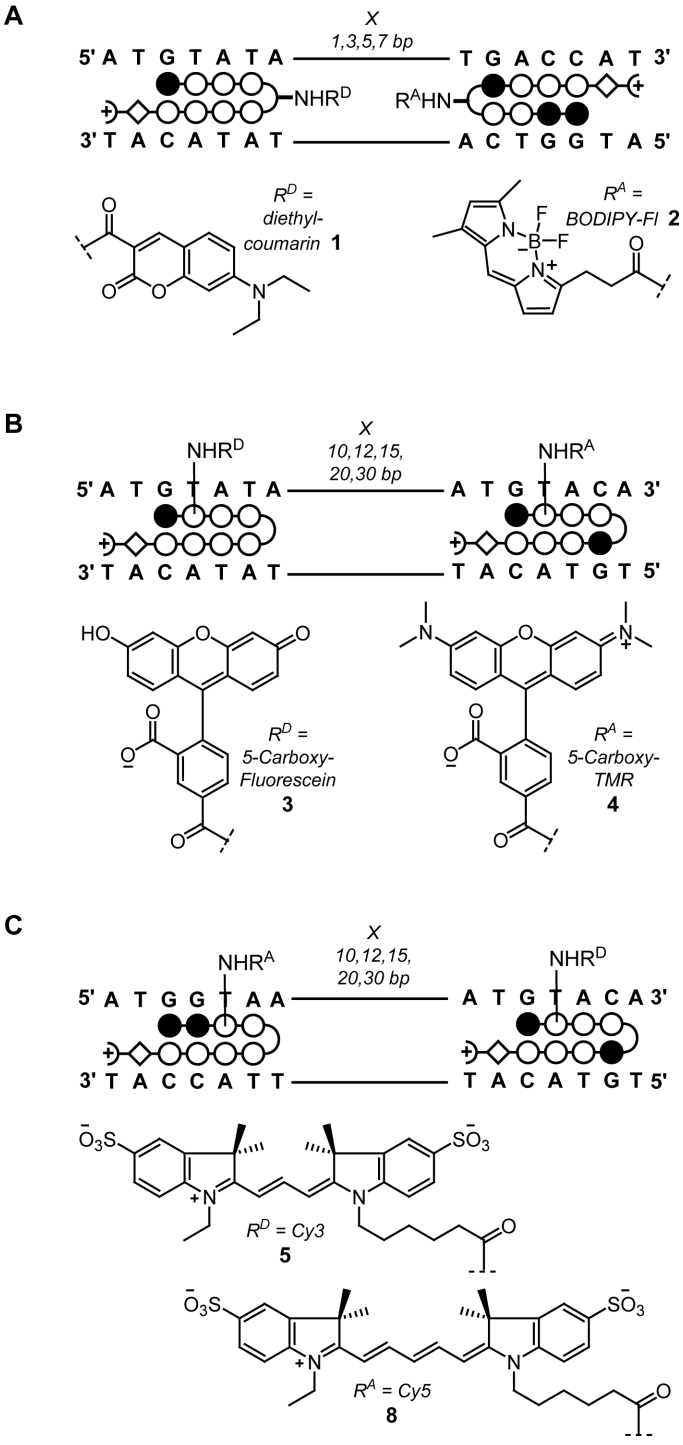


Figure 4.2 Previous efforts to demonstrate polyamide-based FRET. **(A)** First generation conjugates for FRET employed coumarin- and BODIPY-modified turn moieties. **(B)** Second generation conjugates contained Py ring-attached fluorescein and tetramethylrhodamine probes. **(C)** Current polyamide conjugates for FRET use Py ring-attached cyanine probes.

evolution of polyamide-based FRET was then undertaken, using polyamide scaffolds

with greater specificity in conjunction with brighter cyanine probes.

Model System for Polyamide-Based FRET

Oligonucleotide duplexes containing donor- and acceptor-polyamide binding sites separated by variable intervening sequences were carefully designed to avoid the presence multiple binding sites for either polyamide (Figure 4.3A). Fluorophore-fluorophore distances were then determined using the double helix as a spectroscopic ruler (10 bp ~ 34Å). In light of the findings in Chapter 3, hairpin oligonucleotides were used to assess the behavior of cyanine-polyamide conjugates in the presence and absence of DNA (Figure 4.3B). In particular, the affinity of the donor for the acceptor binding site and the affinity of the acceptor for the donor binding site were investigated. Specificity was also addressed in the design of polyamide scaffolds and the promiscuous scaffold used in previous experiments was replaced by a more specific polyamide whose match site is a double base pair mismatch for the other polyamide. Cyanine probes were attached to these polyamides using the Py ring as a point of attachment with the aim of minimizing the influence of fluorophores on the DNA recognition properties of the polyamides. A family of hairpin conjugates containing three different donors and two acceptors were synthesized, though FRET experiments to date have focused on the Cy3-Cy5 (**5-8**) donor-acceptor pair (Figure 4.4).

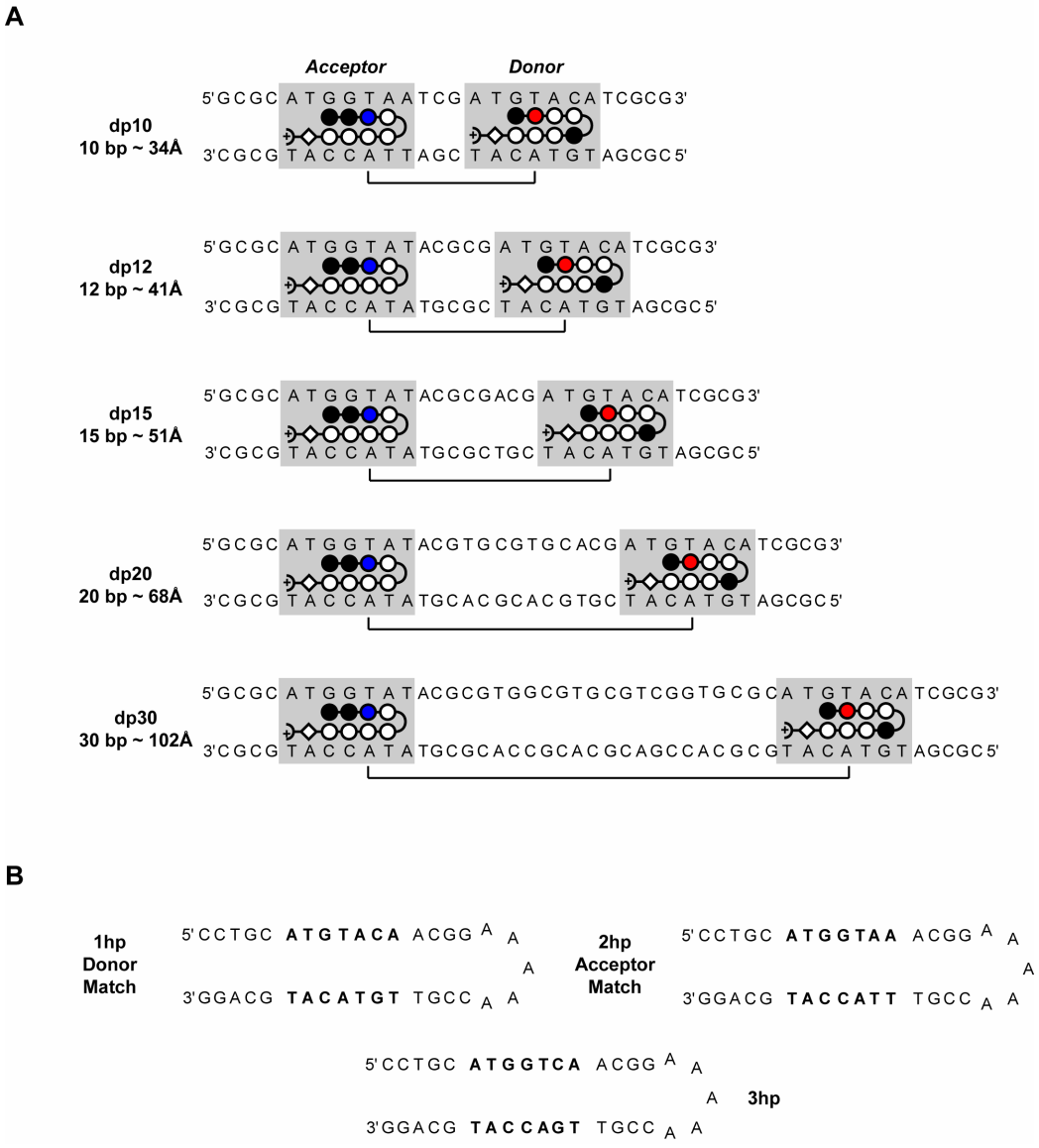


Figure 4.3 Model systems for examination of FRET by polyamide conjugates. **(A)** Duplex oligonucleotides for distance-dependent titrations. Circle shaded in blue represents pyrrole ring with attached Cy5 acceptor. Circle shaded in red represents a pyrrole ring with attached Cy3 donor. Inter-fluorophore distances determined using approximation that 10 bp ~ 34Å. **(B)** Hairpin duplex oligonucleotides used to determine quenching of cyanine-polyamide conjugates.

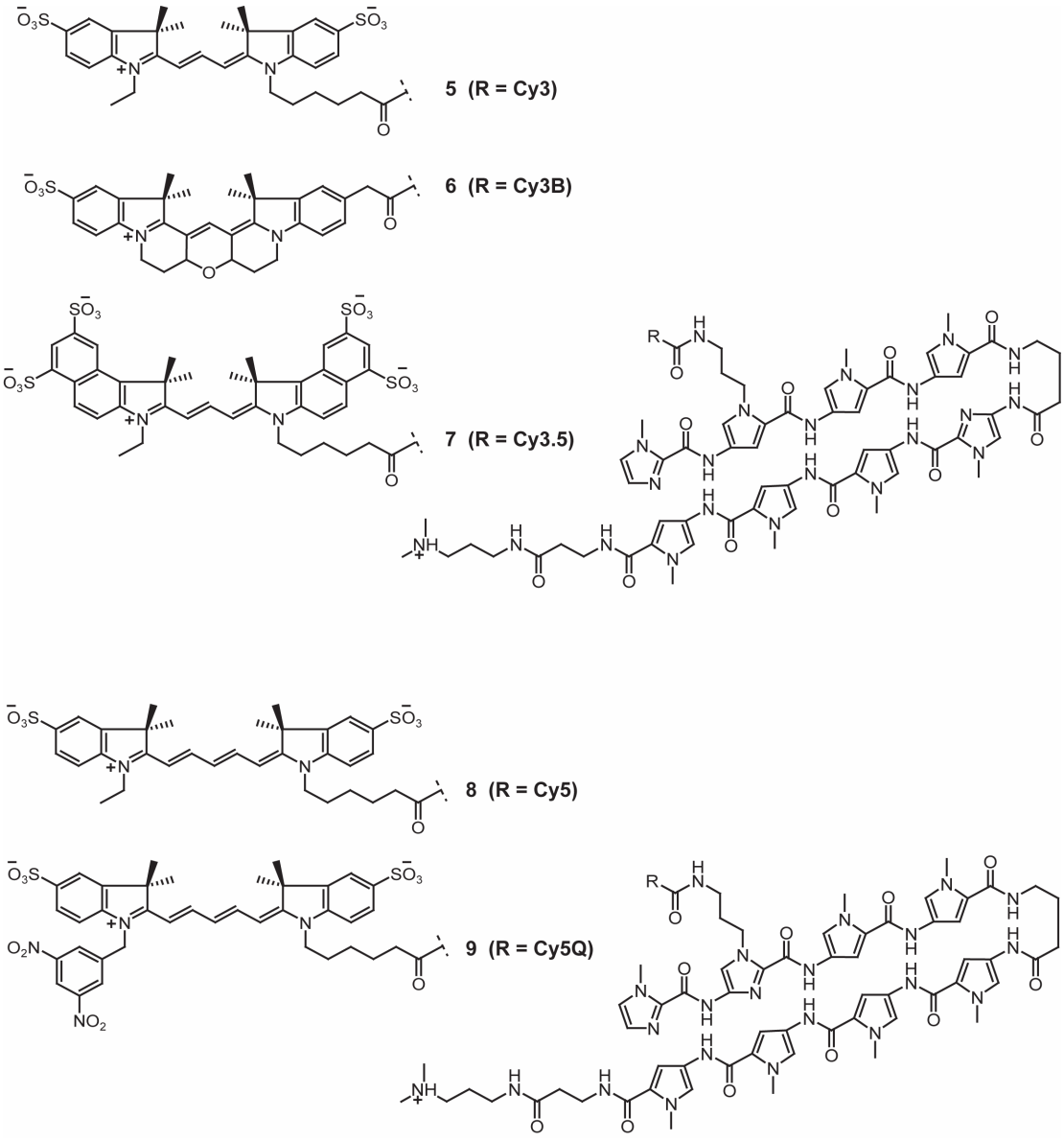


Figure 4.4 Chemical structures of cyanine-polyamide conjugates.

Physical Properties of Cyanine-Polyamide Conjugates

The cyanine donors employed, Cy3 (**5**), Cy3B (**6**), and Cy3.5 (**7**) span a range of excitation-emission wavelengths complementary to the Cy5 acceptor (**8**), as evidenced by their normalized absorption-emission spectra (Figure 4.5). These compounds have intrinsically higher molar extinction coefficients than TMR or

fluorescein and show no absorption in the 300 nm range, allowing more reliable determination of conjugate concentration by spectroscopic methods (Table 4.1). The affinities of cyanine conjugates for their match sites were determined by DNase I footprinting and, with the exception of **7**, show reasonable association constants (Table 4.1). The Cy3.5 chromophore contains four sulfate groups that could generate unfavorable electronic contacts with the phosphate backbone of the DNA upon polyamide association in the minor groove.

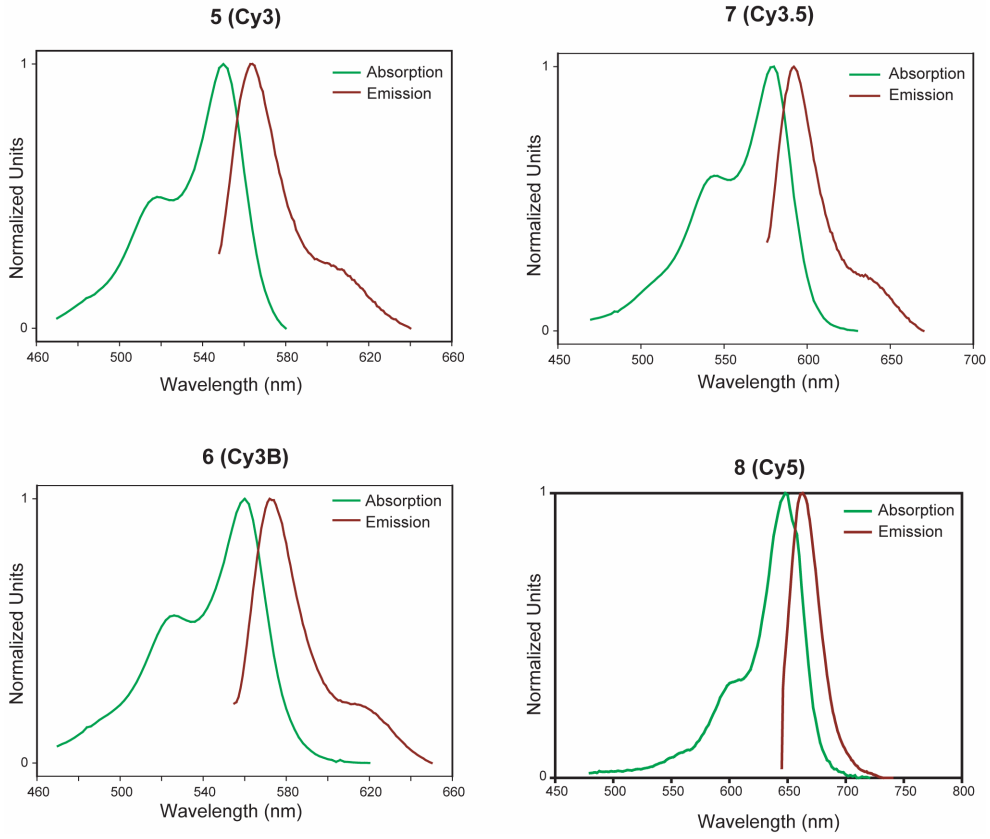


Figure 4.5 Normalized absorption emission spectra for cyanine-polyamide conjugates. All measurements taken in the presence of match duplex DNA.

Table 4.1 Physical properties of cyanine-hairpin polyamide conjugates.

polyamide	λ_{ex}	ε (M^{-1} cm^{-1})	λ_{em}	K_A (M^{-1})
5	555 nm	75, 000	564 nm	**1.0×10^9** *(5'-AGTACT-3')*
6	566 nm	113, 000	572 nm	**7.5×10^8** *(5'-AGTACT-3')*
7	585 nm	96, 000	592 nm	**$< 1.0 \times 10^7$** *(5'-AGTACT-3')*
8	653 nm	127, 000	660 nm	**1.0×10^8** *(5'-TGGTAA-3')*

The occurrence of fluorescence quenching in cyanine conjugates was investigated by titration with hairpin oligonucleotides containing the donor-polyamide binding site, the acceptor-polyamide binding site, and a third polyamide binding site. The conjugates showed different degrees of fluorescent enhancement in the presence of match duplex DNA, increasing in the order: Cy3 < Cy3.5 < Cy5 < Cy3B ~ 5-TMR (Figure 4.6). The Cy3B fluorophore is especially promising, exhibiting comparable fluorescent enhancement to 5-TMR with substantially higher overall intensities.

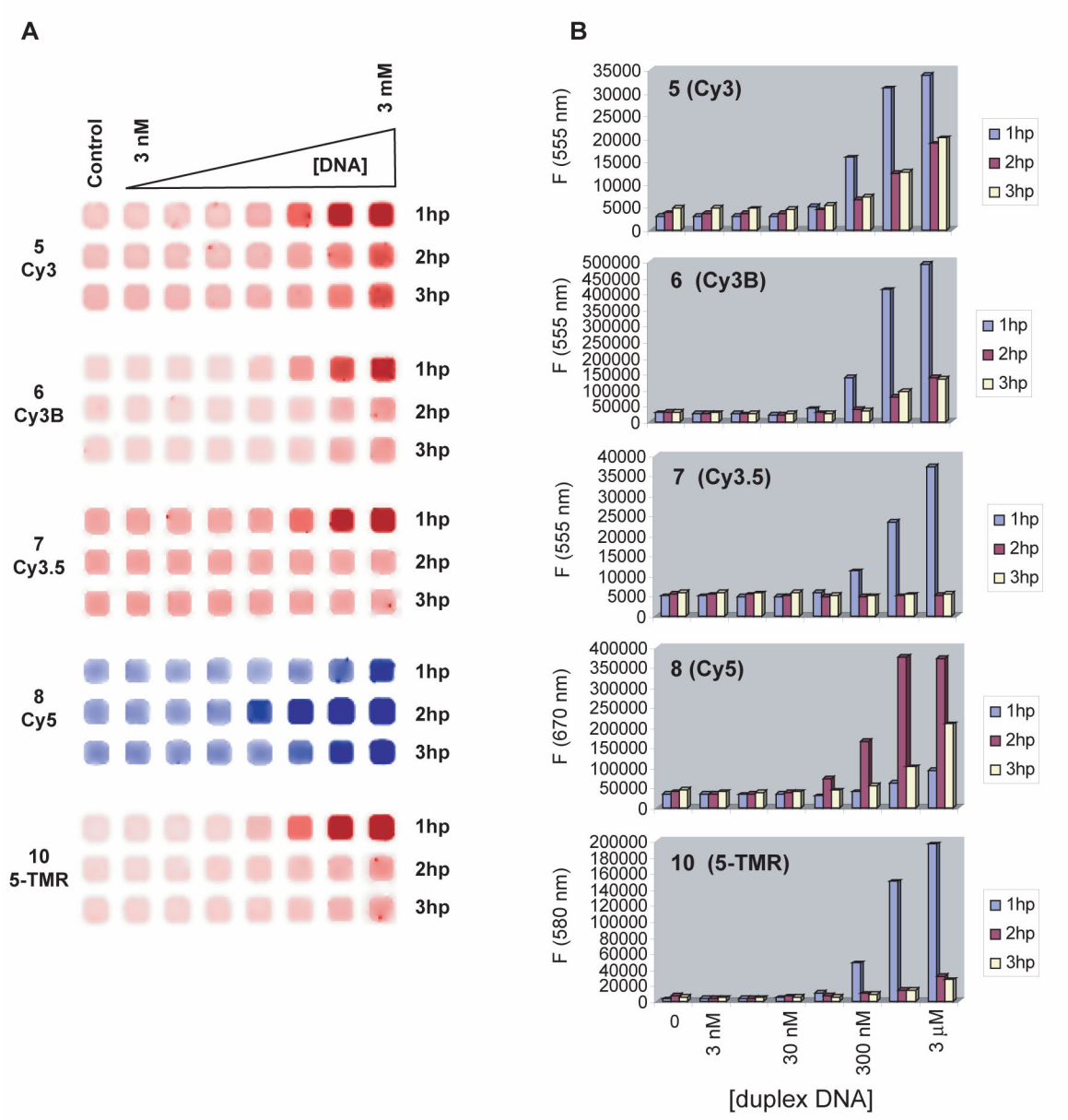


Figure 4.6 Cyanine-polyamide conjugates exhibit fluorescent enhancement when bound to DNA. **(A)** Microplate titration of polyamide conjugates with hairpin oligonucleotides. Cy3, Cy3.5, and Cy3B emission were monitored at 555 nm while 5-TMR fluorescence was monitored at 580 nm, both with excitation at 532 nm. Cy 5 emission was measured at 670 nm with excitation at 633 nm. **(B)** Averaged data from replicate microplate assays is illustrated by plotting fluorescent intensity as a function of duplex concentration.

FRET Experiments Using Cyanine-Hairpin Polyamide Conjugates

Resonance energy transfer by the Cy3-Cy5 (**5-8**) donor-acceptor pair was observed by steady-state techniques, using both fluorimeter- and microplate-based

assays. The well-separated emission and absorption wavelengths of **5** and **8** allow resonance energy transfer to be measured with respect to donor quenching or sensitized emission of the acceptor (Figure 4.7A). Titration of cyanine-polyamide conjugates **5** and **8** (2 μM) with model oligonucleotide duplexes (Figure 4.3A) resulted in a distance-dependent decrease in the quantum yield of donor **5**. Triplicate measurements in a standard fluorimeter were fitted using Equation 3, giving an observed R_0 of 35.9Å. Similar experiments using microplates gave comparable results.

Subsequent microplate assays with the optimal model duplex (**d10**) examined the influence of concentration and equilibration time on both donor quenching and sensitized emission (Figure 4.8). Polyamide concentrations ranging from 100 nM to 2 μM gave R_0 values comparable to those obtained previously. Higher concentrations (500 nM) were required to facilitate detection of sensitized emission as indicated by S/N ratios, determined relative to a control lacking duplex DNA. Decreasing equilibration times for polyamide conjugates with duplex DNA did not significantly influence transfer efficiencies and this finding was confirmed by DNase I footprinting studies of **8**, which exhibited comparable association constants at both short and long time points.

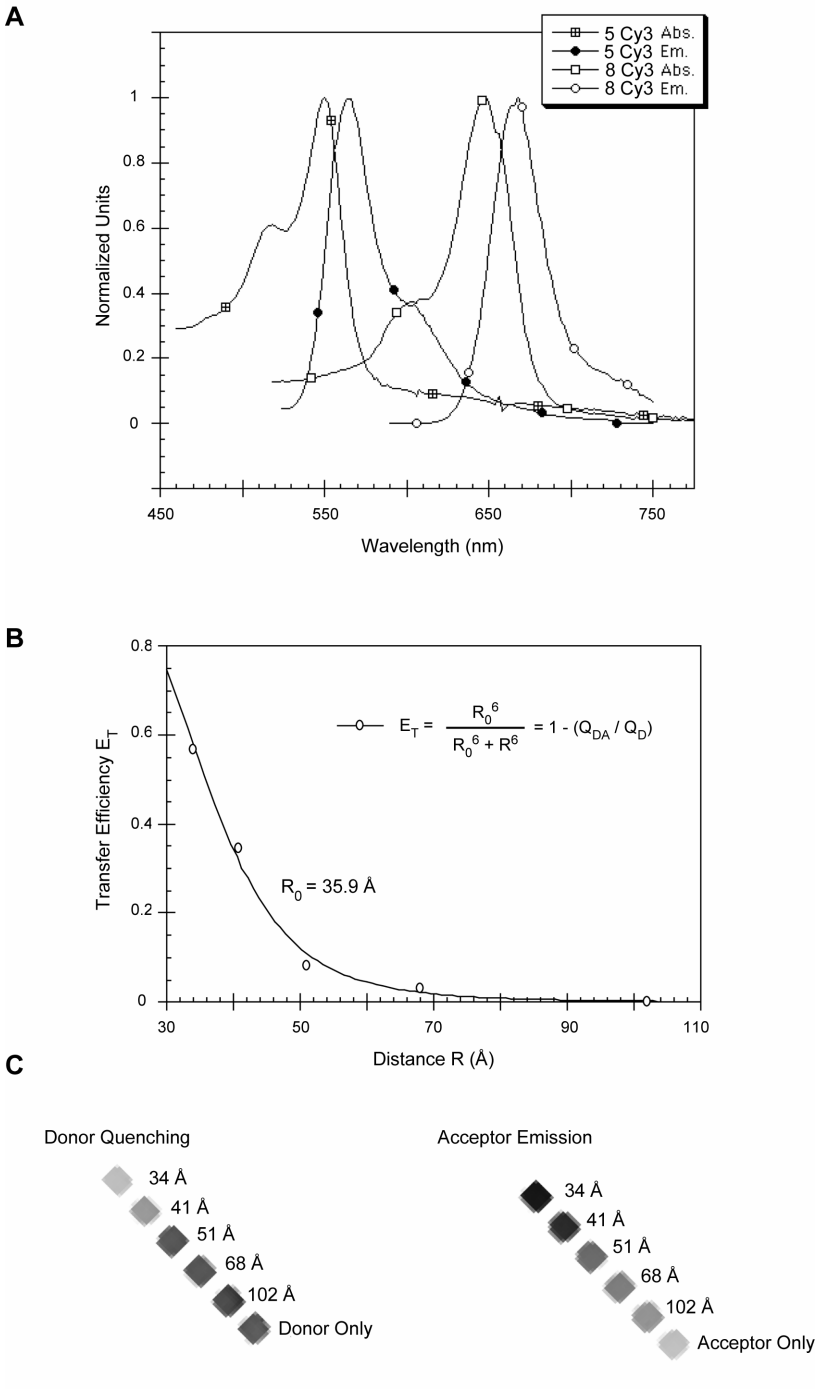
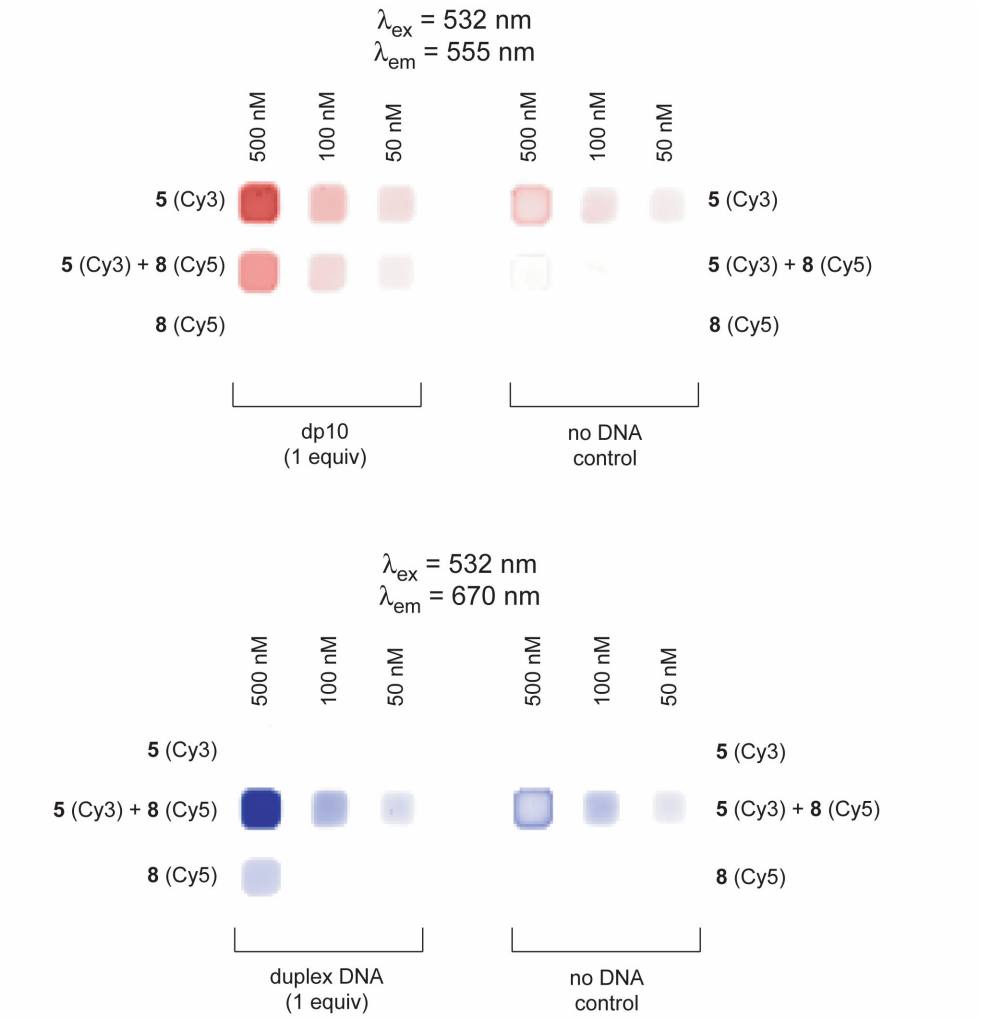


Figure 4.7 Demonstration of FRET by minor groove-associated cyanine-polyamide conjugates. **(A)** Conjugates 5 and 8 show significant spectral overlap. **(B)** Fit of data from duplex oligonucleotide titration shows distance-dependence inherent to resonance energy transfer. **(C)** Microplate assays can monitor FRET by quenching of donor (*left*) and by sensitized emission of acceptor (*right*).



	$t_{eq.}$ = 30 min.			$t_{eq.}$ = 10 hr		
	500 nM	100 nM	50 nM	500 nM	100 nM	50 nM
E	0.52	0.46	0.40	0.48	0.51	0.41
R_0	34 Å	33 Å	32 Å	34 Å	34 Å	32 Å
S/N	6.5	1.3	1.2	6.6	1.0	0.9

Figure 4.8 Optimization of condition for polyamide-based FRET. Microplate experimental layout and raw data is shown with emission at 555 nm indicated by red scale and emission at 670 nm shown in blue scale. Averaged data for replicate experiments is summarized in the table shown at bottom.

Future Directions for Polyamide-Based FRET

The Förster radii determined for the Cy3-Cy5 pair can be used to estimate the distance interval to which it can be applied in biological applications. Qualitative FRET measurements can usually be made in the range of $(0.2)R_0$ - $(0.8)R_0$, corresponding to 7-28 Å for **5** and **8**. This range does allow for FRET between proximal binding sites on the double helix, and could be used to enhance the specificity of DNA detection by polyamides in genomic contexts. The non-invasive nature of polyamide association might also allow the association of proteins proximal to a polyamide binding site to be measured. Mapping of condensed DNA structures with lower S/N is another possible application.

The range of applications available to polyamide-based FRET could easily be extended by using a donor-acceptor pair with a larger R_0. The Cy3B donor might prove useful to this end as it has even greater spectral overlap with Cy5 absorption, in addition to exhibiting a higher quantum yield and greater degree of quenching in the absence of DNA. The utility of the Cy3B-Cy5 pair can be determined using the same protocol discussed above. The Cy5Q acceptor is a quenched version of Cy5, such that FRET with Cy3 or Cy3B results in decreased quantum yield of donor without sensitized emission at the Cy5 wavelength. “Three-probe” experiments are readily adaptable single molecule experiments, where polyamide-based FRET could be measured at the donor wavelength and DNA position could be confirmed by fluorescence of an attached Cy5 fluorophore. The utility of Cy5Q can also be determined by the above protocol.

Cyanine-polyamide conjugates showed little deviation in fluorescent signal with variation in buffer conditions, suggesting applications with enzymes. Model duplexes containing an enzyme binding site flanked by polyamide binding sites could be designed to evaluate these assays. Enzymes known to cleave or bend DNA would be expected to reduce or increase FRET efficiency, respectively. The structural implications for repeat sequences or purine tracts could also be qualitatively assessed with polyamide-based FRET. Three-color experiments are also attractive as they would further enhance the specificity of detecting a given eight-ring binding site, without increasing the size of the ligand.

Conclusions

The preliminary data presented above demonstrates the potential of polyamide-based FRET as another tool for the sequence specific detection of DNA. Further work is needed to fully characterize this class of conjugates and to optimize the choice of donors and acceptors for biologically relevant applications. In this regard, the fluorescein-TMR donor-acceptor pair might prove applicable to polyamide-based experiments in live cells.

Experimental

The synthesis of cyanine-polyamide conjugates was described in detail in Chapter 2. Microplate experiments were performed as described in Chapter 3A using a Typhoon imaging system. Duplex oligonucleotides were annealed at 100 °C for 15 minutes and cooled to ambient temperature slowly before use. Concentrated stock solutions of cyanine conjugates were prepared by dissolving dry aliquots in a minimal volume of DMSO and diluting this stock with ultrapure water. Fluorescence quantum yield determinations were made relative to sulforhodamine, carboxyrhodamine, and nile blue standards obtained from Molecular Probes.

References

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