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Polyfluorophore Excimers and Exciplexes as FRET Donors in DNA

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Abstract

We describe studies aimed at testing whether oligomeric exciplex- and excimer fluorophores conjugated to DNA have the potential to act as donors for energy transfer by the Förster mechanism. Oligodeoxyfluorosides (ODFs) are composed of stacked, electronically interacting fluorophores replacing the bases on a DNA scaffold. The monomer chromophores in the twenty tetramer-length ODFs studied here include pyrene (Y), benzopyrene (B), perylene (E), dimethylaminostilbene (D), and a nonfluorescent spacer (S); these are conjugated in varied combinations at the 3' end of a 14mer DNA probe sequence. In the absence of an acceptor chromophore, many of the ODF-DNAs show broad, unstructured long-wavelength emission peaks characteristic of excimer and exciplex excited states, similar to what has been observed for unconjugated ODFs. Although such delocalized excited states have been widely studied, we know of no prior report of their use in FRET. We tested the ability of the twenty ODFs to donate energy to Cy5 and TAMRA dyes conjugated to a complementary strand of DNA, with these acceptors oriented either at the near or far end of the ODF-conjugated probes. Results showed that a number of the ODF fluorophores exhibited relatively efficient energy transfer characteristic of the Förster mechanism, as judged by drops in donor emission quantum yield and fluorescence lifetime, accompanied by increases in intensity of acceptor emission bands. Excimer/exciplex bands in the donors were selectively quenched while shorter-wavelength monomer emission stayed relatively constant, consistent with the notion that the delocalized excited states, rather than individual fluorophores, are the donors. Interestingly, only specific sequences of ODFs were able to act as donors, while others did not, even though their emission wavelengths were similar. The new FRET donors possess large Stokes shifts, which can be beneficial for multiple applications. In addition, all ODFs can be excited at a single wavelength; thus, ODFs may be candidates as “universal FRET donors”, thus allowing multicolor FRET of multiple species to be carried out with one excitation.

Keywords

excimer; exciplex; pyrene; perylene; benzopyrene; Förster; cyanine

Introduction

Multichromophoric systems have displayed tremendous utility in numerous applications, giving some useful advantages over their monomeric counterparts. Applications in light harvesting, material science, electronic, photonic and sensor devices have inspired the design of systems like conjugated polymers (1–3), dendrimers (4,5), photonic wires (6,7) and

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Supporting Information Available: Mass spectrometric data for ODF-containing oligonucleotides; fluorescence lifetimes measurements; emission spectra from hybridization experiments of all ODF-DNA duplexes with 5'-TAMRA, 5'-Cy5, 3'-TAMRA and 3'-Cy5 – labeled complementary strands; as well as the natural complementary strand. This material is available free of charge via the Internet at <http://pubs.acs.org>.

functionalized polypeptides (8). In recent years, the deoxyribose backbone of DNA has also been harnessed as a scaffold for the design of new multichromophoric systems. Oligodeoxyfluorosides (ODFs), an example of the latter, are a novel system of fluorophores built on the deoxyribose backbone. Aromatic fluorophores such as pyrene, perylene and benzopyrene are attached to consecutive anomeric positions of the deoxyribose backbone (9, 10). This backbone provides a rigid structure that brings the aromatic fluorophores into close proximity, allowing π -stacking interactions and various forms of photophysical interactions to occur (11,12).

Other systems of functionalizing the DNA scaffold with multiple chromophores have been developed recently. Häner and coworkers assembled a series of phenanthrenes and pyrenes as non-nucleosidic base surrogates (13,14). These aromatic compounds interact favorably through stacking interactions. In work by Wagenknecht, clusters of aromatic chromophores covalently attached to uridine were also shown to form a helical π -array along the major groove of the DNA duplex (15,16). Strongly enhanced emission from clusters of stacked pyrene-labeled uridines was observed. Nonfluorescent methyl red and naphthyl red dyes had been incorporated as base substitutes and assembled as clusters into duplex DNA (17,18). Bipyridyl and biphenyl moieties were employed by Leumann as base substitutes for C-nucleosides to synthesize a zipper-like interstrand stacking motif of DNA (19). Excimer formation has also been observed by Inouye in alkynyl C-nucleosides with pyrene, perylene and anthracene as base substitutes (20). Besides DNA, multi-labeling of other nucleic acids architectures, RNA (21,22) and LNA (23), were also carried out. Non-covalent assembly of chromophores on the DNA structure had been explored with cyanine dyes in the minor groove (24,25).

A number of laboratories have also explored multichromophore FRET (Förster resonance energy transfer) interactions in DNA using traditional monomeric fluorescent dyes (26,27). In addition, multi-fluorophore systems based on conjugated polymers have also been employed as FRET donors (28,29). FRET is widely employed in biological and biophysical studies because of the well-known distance dependence of the non-radiative energy transfer (30,31). This allows quantitative information about the association of biomolecules and their relative distance and orientation on length scales beyond what other methods can provide. Because of this broad utility, the addition of new classes of chromophores that can undergo FRET can positively affect the types of experiments that can be carried out.

Here we report studies in employing the multichromophore excimer and exciplex interactions of the stacked chromophores of ODFs to test a set of possible FRET donors that can be excited at a single wavelength. To the best of our knowledge, the use of excimers and exciplexes as FRET donors has not been explored. In this report, twenty different ODFs were attached to DNA sequences as potential donors, and common acceptor dyes TAMRA and Cy5 were placed on complementary DNA strands at either proximal or distal ends. Upon hybridization, Förster energy transfer was observed between several ODF donors and Cy5 or TAMRA acceptors. Since multiple ODFs can be excited at a single wavelength, this might ultimately lead to their use as “universal” FRET donors.

Experimental Section

Fluoroside Phosphoramidites Syntheses E, Y, D, S, B

Syntheses of the monomer 1'-alpha-2'-deoxyriboside 5'-dimethoxytrityl-3'-phosphoramidite derivatives of pyrene (Y), perylene (E), benzopyrene (B) and dimethylaminostilbene (D) were carried out as previously described (9,10,32). The abasic tetrahydrofuran spacer S was obtained commercially (dSpacer CE phosphoramidite from Glen Research Corporation).

Synthesis of ODF-labeled Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer on a 1 μ mol scale and possessed a 3'-phosphate group. Coupling employed standard β -cyanoethyl phosphoramidite chemistry, but with extended coupling time (900 s) for fluoroside phosphoramidites. ODFs were synthesized directly on the CPG beads and the DNA sequence was then built on its 5' end. All oligomers were deprotected using the Ultramild deprotection method with potassium carbonate solution (25 $^{\circ}$ C, 8 h), then purified by reverse phase HPLC. The recovered material was quantified by absorbance at 260 nm with molar extinction coefficients determined by the nearest-neighbor method. Molar extinction coefficients for ODFs were estimated by adding the measured value of the molar extinction coefficient of the fluoroside (at 260 nm) to the calculated value for the natural DNA fragments. ODF-labeled oligomers were characterized by MALDI-TOF mass spectrometry (data is given in the Supporting Information, Table S1). TAMRA-CPG, TAMRA-dT and Cy5 phosphoramidites were purchased from Glen Research Corporation. Phosphoramidites were attached to either the 3' or 5' end of the complementary DNA sequence using manufacturer's methods. Purification was achieved using reverse phase HPLC.

Optical Methods

Steady-state absorption measurements were carried out on a Varian Cary 100 UV-Visible spectrophotometer. Steady-state fluorescence measurements were carried out at 2 μ M concentration (each strand) on a HORIBA Jobin Yvon Fluorolog-3 fluorescence spectrometer (2 nm slit width) equipped with Lauda Brinkmann RM6 temperature controller at 25 $^{\circ}$ C. Samples were buffered at pH 7.0 with 100 mM NaCl, 10 mM MgCl_2 , and 10 mM Na-PIPES. Buffers were not deoxygenated. The excitation wavelength for ODF-labeled strands was 330 nm. To prevent aggregation and reabsorption of light during quantum yield measurements, samples were diluted to solutions with absorption of less than 0.1 at wavelengths longer than λ_{ex} . 9,10-Diphenylanthracene ($\Phi = 0.90$) (33) in cyclohexane was used as the reference for quantum yield calculations. Quantum yields were calculated using the following equation (34):

$$\frac{\Phi}{\Phi_R} = \frac{n^2}{n_R^2} \times \frac{A_R}{A} \times \frac{\int F}{\int F_R}$$

where Φ and Φ_R are the quantum yields of the unknown and reference compounds respectively. n and n_R are the refractive indices of water and cyclohexane respectively. A and A_R denote the absorbances at 330 nm for the unknown and reference samples and $\int F$ and $\int F_R$ are the integrals of fluorescence emission intensities of the unknown and the reference samples respectively.

Fluorescence lifetime measurements were carried out on a PTI EasyLife instrument using 325-nm LED for excitation of all ODFs. A bandpass filter with transmittance from 400–510 nm was used to ensure only emission from the donor ODF was monitored. Fitting and analysis was performed using Felix32 software. The goodness of the fit was evaluated based on χ^2 values and the randomness of the residuals. The instrument response function (IRF) was measured using a 2% Ludox scattering solution (Sigma Aldrich, MO). To prevent aggregation and reabsorption of light, samples were diluted to absorption at λ_{max} of less than 0.05.

Analysis of Energy Transfer

The efficiency of FRET, E_{FRET} , was calculated from the sensitized emission of the acceptors using eq. 1 (35,36):

$$E_{\text{FRET}} = \left(\frac{I_{AD}}{I_A} - 1 \right) \frac{\varepsilon_A}{\varepsilon_D} \quad (1)$$

where I_{AD} and I_A are the fluorescence emission intensities of the acceptor in the presence and absence of donor respectively. ε_A and ε_D are the molar extinction coefficients of the acceptor and donor respectively at the excitation wavelength. The excitation wavelength was 330 nm for all experiments. TAMRA emission was monitored at its emission maxima at 580 nm while Cy5 emission was monitored at its emission maxima at 662 nm. Where only a diminished donor emission signal was observed, and the acceptor emission remained the same or decreased, quenching was likely to be due to contact quenching. This quenching efficiency was calculated using eq. 2:

$$Q_{\text{eff}} = 1 - \frac{I_{DA}}{I_D} \quad (2)$$

where I_{DA} and I_D are the integrated emission intensities of the donor in the presence and absence of the acceptor, TAMRA, respectively. The efficiency of FRET computed from lifetime measurements, EL_{FRET} , was determined using eq. 3 (35).

$$EL_{\text{FRET}} = 1 - \frac{\langle \tau_D \rangle}{\langle \tau_D^0 \rangle} \quad (3)$$

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i}{\sum_i \alpha_i}$$

where $\langle \tau_D \rangle$ and $\langle \tau_D^0 \rangle$ are the amplitude-averaged donor lifetimes in the presence and absence of the acceptor respectively.

Results

A representative set of 20 tetrameric sequences of ODFs was chosen from a larger random library for this study; for hybridization experiments, they were appended to the 3' terminus of a 14-mer probe DNA sequence (Table 1). The ODF labels were synthesized from five monomeric 2'-deoxyfluorosides where the base-replacement fluorophore was a benzopyrene (B), pyrene (Y), perylene (E), dimethylaminostilbene (D) or an abasic spacer-tetrahydrofuran moiety (S) (see Figure 1A). The α -glycosidic anomers, which like beta anomers can stack with one another in a DNA context (11), were used for synthetic convenience in this study. ODFs were assembled using automated DNA synthesis chemistry from phosphoramidite derivatives of the monomeric fluorosides. Note that sequences of ODFs are listed in 5' to 3' order in analogy to DNA.

Fluorescence properties of ODFs

Absorption and emission spectra of the twenty ODF labels conjugated to single-stranded DNA were measured in pH 7.0 hybridization buffer containing 100 mM Na⁺ and 10 mM Mg²⁺ (see Experimental section). The primary absorption and emission bands are given in Table 2, and emission spectra are shown in Supporting Information. Quantum yields for fluorescence were measured in the same buffer (Table 2). Emission spectra of the ODF labels were in nearly all cases quite different from the combined monomer emissions, which were reported previously

(37). Most cases showed broad bands between 450–600 nm, consistent with excited state dimer (exciplex and excimer) states. The maxima of these bands varied from 460 nm to 530 nm, and some cases showed multiple maxima (as many as three) in this range. In general, the twenty DNA-conjugated ODFs were efficient fluorophores, with quantum yields for emission as high as 42% (Table 2). The quantum yields varied with chromophore composition and sequence; for an example of the latter, SSYY ($\Phi_{\text{FL}} = 19\%$) and YSSY ($\Phi_{\text{FL}} = 2.5\%$) have the same composition but substantially different fluorescence quantum yields.

ODFs as donors in Förster energy transfer

To test the possibility of Förster energy transfer between ODFs and common acceptor dyes, we hybridized the 3'-ODF-conjugated probes with their DNA complements carrying Cy5 or TAMRA dye (Fig. 1). To test the effect of distance between the putative donors and acceptors, we prepared dye-conjugates in which Cy5 or TAMRA was located at either the 5' end of the ODF complement (proximal to the ODF) or at the 3' end (distal to the ODF). Since the probes were 14 nt in length, the difference in distance at proximal and distal locations was approximately 56 Å based on a 3.4 Å rise per base pair of B-DNA. This distance was calculated from the middle of each ODF donor dye to either Cy5 or TAMRA. Hybridization and fluorescence experiments were performed using DNA strand concentrations of 2 μM each, and 10 mM PIPES buffer with 100 mM NaCl, 10 mM MgCl_2 , at a temperature of 25°C. An example is shown in Figure 1C.

The sequences of the ODFs in this initial labeled DNA probe set were chosen essentially at random from an earlier study of quenching behavior (38) (see sequences in Table 2) with some additional cases included based on early observations in this study. To initially screen the photophysical interactions of each ODF with the Cy5 and TAMRA acceptors, we measured the emission spectra of the duplexes containing both ODF putative donors and the acceptor dyes. In addition, we measured the emission spectra of the acceptor dyes (conjugated to their DNAs) alone and the ODF donor strands alone, again using this same excitation wavelength (330 nm; chosen for efficient excitation of the aromatic monomers in the ODFs). At this wavelength, the absorbances of TAMRA or Cy5-conjugated strands are minimal.

As a control for the effects of the acceptor dyes on ODF quenching, we also measured the effects of hybridizing the ODF DNAs to unlabeled DNA complement, D1 (see data in SI). The effects of hybridization to D1 varied from a small enhancement of fluorescence to a small degree of quenching, and several of the ODFs displayed no change. Thus in the majority of cases, the quenching effects observed in the hybridization experiments came entirely (or nearly so) from the presence of the acceptor fluorophore on the complement DNA strand.

Exciplexes as FRET donors

Emission data from selected experiments are shown in Figure 2 (TAMRA) and 3 (Cy5); see SI for spectra from all cases. Upon hybridization, the long wavelength emission band between 460 nm and 510 nm of the donor ODF was diminished in all cases, compared to the emission before hybridization. In addition, a significant enhancement of fluorescence intensity at 590 nm was observed when the ODF strands were hybridized to T3'. This corresponded to the sensitized emission of TAMRA upon hybridization. Since TAMRA cannot be significantly excited at 330 nm, the enhanced emission was likely due to energy transfer from the ODF donor, upon its excitation. Since there is significant overlap of the emission spectra of ODFs and the absorbance spectra of TAMRA, and both ODF and TAMRA are about 14 DNA bases apart (~ 56 Å), the energy transfer was tentatively characterized as a long range weak dipole-dipole interaction, or Förster resonance energy transfer (FRET).

The sensitized enhancement of the acceptor emission can be used to determine the efficiency of FRET (E_{FRET} , see Experimental Section). As shown in Table 2, the ODF sequence EYSS displayed the highest apparent efficiency of FRET to T3' at 0.752, followed by BEBY (0.244) and ESBS (0.159). The other ODF sequences had relatively low E_{FRET} , showing weak or borderline FRET behavior, possibly due to low quantum yields, weak dipoles or unfavorable dipolar orientation.

Contact quenching in proximally labeled ODF strands

When the 3' ODF-labeled strands were hybridized to 5' TAMRA-labeled complement or 5' Cy5-labeled complement, the ODF dyes were at a proximal distance to the acceptor fluorophores. Contact quenching can come into play when the two fluorophores are within close distance of each other (39). As observed from our experimental data, quenching of more than 30% was observed in 19 out of 20 ODF-labeled strands when hybridized to T5'. Only one strand, P20 (EEDE), experienced little (4%) quenching upon hybridization (see SI for complete emission spectra). Quenching efficiency, Q_{eff} , for each pair was determined (see Experimental Section) and is shown in Table 2. Q_{eff} values ranged from 0.038 to 0.739. The TAMRA emission band was also quenched in most cases, or experienced no change in emission intensity. Because quenching in these cases was not coupled to an associated increase in TAMRA emission, we assigned these cases to simple contact quenching and not to FRET. Additional evidence for contact quenching was obtained for one case (sequence P6; BYSS) by measurement of the lifetime of the acceptor (data not shown). Results showed a decrease in lifetime of the TAMRA acceptor fluorophore upon hybridization with donor ODF-labeled strand at the direct excitation wavelength of TAMRA.

Hybridization with Cy5-labeled complements

Hybridization between ODF-labeled strands and their Cy5-labeled complements, C3' and C5', also resulted in the sensitized emission of the acceptor Cy5 coupled with a decrease in donor emission in some of the strands. Selected examples are given in Fig. 3. The emission intensity of the hybrid at 660 nm was enhanced compared to the emission profile of the acceptor dye strand alone in 7 out of the 20 strands tested with distal cyanine dye (C3'), consistent with FRET behavior. Interestingly, hybridization of ODF-labeled strands with proximal C5' also resulted in the enhancement of acceptor emission in 9 cases, which was distinct from the results with TAMRA, where quenching dominated. The remaining cases with Cy5 exhibited quenching of both the donor emission and acceptor emission upon hybridization, most probably due to the phenomenon of contact quenching.

Excimers as FRET donors

The above examples document FRET behavior in exciplexes, which have inherent dipoles. To test if similar effects were observed in excimers (which in some cases do not have dipoles in the excited state; see Discussion), the pyrene excimer was used and three different ODF variants were synthesized. Two pyrene nucleosides were placed adjacent to each other in SSYY (P3), producing the highest efficiency of excimer formation. The efficiency of excimer formation was tested by placing spacer groups (S) between the pyrenes in YSSY (P4) or two dihydrothymidine nucleosides (H) between the pyrenes in YHHY (P5). Dihydrothymidine was shown in a previous study to possess excited-state "insulating" effects in oligodeoxyfluorosides (40).

The data for hybridization with TAMRA-containing strands are shown in Figure 4; results showed little if any evidence for FRET, displaying only a decrease in donor emission intensity, typical of contact quenching. In marked contrast, hybridization of the pyrene excimer-emitting ODFs to DNAs containing Cy5 (either proximal or distal, Figure 5) yielded significant decreases in donor emission intensity and strong increases in the acceptor emission intensity,

providing evidence for efficient FRET. Detailed emission spectra are shown in the SI. The most efficient FRET occurred in the case with adjacent pyrenes or pyrenes with dihydrothymidine monomers in between, while the addition of spacers lowered the efficiency substantially.

Time-resolved measurements

To seek additional evidence for a putative FRET energy transfer mechanism from donor ODF fluorophores to acceptor TAMRA or Cy5, we measured donor emission lifetimes in the absence and presence of the acceptor dye. For the Förster mechanism one expects a decrease in lifetime of the ODF donor in the presence of the acceptor. If this occurred, this would provide further evidence for FRET with these unusual donors, in addition to the above steady-state spectral observations. Time-resolved fluorescence decay profiles were measured for selected ODF dyes upon hybridization with TAMRA- and Cy5-labeled complement strands. Emission filter sets were selected to ensure that only the lifetime of the donor ODF was measured. Lifetime measurements were determined before and after the addition of the complement and results are shown in Table S2 (Supporting Information).

The results showed that ODF labels (P1–P8) that experienced sensitized acceptor emission and decreased donor emission as described above, also experienced shortened donor emission lifetimes. Hybridization with the acceptor-containing strands lowered excited-state lifetimes by 8–27%, with one exception (P5 + C5'). Combined with data from the steady-state studies, this provides further evidence for the occurrence of FRET between ODFs and acceptor TAMRA and Cy5 dyes. It is worth noting that the E_{FRET} and EL_{FRET} values calculated from the two methods were not especially well correlated; the most extreme examples are the P5 cases, where two dihydrothymine bases are placed between excimer-forming pyrenes in the ODF donor. We hypothesize that poor correlations for this ODF are due to a delay in formation of the excimer state by the intervening bases (see below). In addition, differences between efficiency values measured from different FRET methods can result from the presence of more than one species in the excited state, which results in lifetimes that vary from single exponential decay, and also by factors such as crosstalk in emission detection and bleedthrough in excitation (41).

Determination of Förster distance, R_0

To further characterize ODFs as FRET donors we measured the Förster distance, R_0 , with TAMRA and Cy5. Förster distance, R_0 , determined using eq. 4, is the distance between the donor and acceptor when the FRET efficiency is 50%. The orientation factor, κ^2 , in the determination R_0 can be approximated to be 2/3 if both donors and acceptors sample random orientations during the excited state lifetime, and an estimation of $\kappa^2=1/3$ is used for cases when either the FRET donor or acceptor experiences static orientation (36,42). Although the orientations of ODFs are not known to certainty, evidence suggests they are stacked with the adjacent DNA, which suggests the use of $\kappa^2=1/3$. Nevertheless, we calculated R_0 between each ODF-labeled strand and its complement T3' strand, using either $\kappa^2=1/3$ or $\kappa^2=2/3$; these are shown in Table 3 for comparison. R_0 values ranged from 25.3 Å (P4, YSSY) to 42.6 Å (P7, EYSS), and varying the value of κ^2 led to a ~10% difference in the value of R_0 . The R_0 values calculated for P1–P20 and T3' are typical of values of commonly used FRET dye pairs such as dansyl and FITC (33–41 Å), or pyrene and perylene (36 Å) (43).

$$R_0(\text{cm}) = [(8.79 \times 10^{-25}) \kappa^2 \eta^{-4} Q_D J(\lambda)]^{\frac{1}{6}} \quad (4)$$

where κ^2 is the orientation factor, η is the refractive index of water (1.33), Q_D is the quantum yield of the donor and $J(\lambda)$ is the spectral overlap integral between the emission spectrum of

the donor and absorption spectrum of the acceptor. The spectral overlap $J(\lambda)$ is calculated with eq. 5

$$J_{DA} = \int_0^{\infty} F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda \quad (5)$$

where F_D is the normalized donor emission and ϵ_A is the molar extinction coefficient of the acceptor.

Upon the determination of R_0 , the actual distance between the donor (ODF) and acceptor (TAMRA) of FRET, R_{DA} , can be determined by eq. 6. Using both E_{FRET} and EL_{FRET} , R_{DA} was calculated and shown in Table 3. The ODF fluorophore in P1–P20 and TAMRA in T3' were 14 base pairs apart. Based on canonical B-DNA structure, the distance between them approximated from the center of a tetrameric ODF to the TAMRA dye stacked on the end of the double strand was approximately 56.1 Å. As shown in Table 3, the calculated values of R when using $\kappa^2=2/3$ and E_{FRET} were within 25% of this approximated distance, except for three cases (P7, P10 and P16), where the calculated distance was considerably longer, reflecting very poor FRET efficiency. Interestingly, calculations of R_{DA} using $\kappa^2=1/3$ provided closer approximations to the theoretical distance of 56.1 Å, giving smaller average deviation per strand. This is however an approximation and deviations can occur due to the flexible tether of TAMRA on T3' and the varied distance of the excited donor of each ODF.

$$E = \frac{R_0^6}{R_0^6 + R_{DA}^6} \quad (6)$$

where E is the energy transfer efficiency, using either E_{FRET} or EL_{FRET} .

Discussion

Taking both the steady-state and time-resolved spectroscopy data together, our results establish that both exciplex and excimer ODFs can act as FRET donors to the common acceptor fluorophores TAMRA and Cy5. To our knowledge, neither of these delocalized excited-state species has been reported as a FRET donor before. Excimers and exciplexes are unusual as fluorophores in the sense that the excited state is delocalized over two adjacent chromophores. For exciplexes, where the two chromophores are different, this has the important consequence of creating an excited-state dipole that is expected to have a strong component perpendicular to the plane of the stacked chromophores. If (as expected) (11) they are stacked with the neighboring DNA, the dipole then has a strong directional component parallel to the helix axis. This is quite different from other DNA-stacked dyes (such as 2-aminopurine), where the chief dipole lies in the plane of the chromophore. Excimers are yet more unusual because the two chromophores are identical. In the absence of DNA, a pyrene excimer has no net excited-state dipole due to symmetry (44). As a result, it should not be able to participate in Förster energy transfer. However, by placing pyrenes in DNA the symmetry is broken, and our data establish that a net dipole clearly exists in the present structural context, since FRET from pyrene excimer donors appears to be among the most efficient cases observed here.

All of the ODF fluorophores studied here have emission bands between 440–540 nm. Most of them likely have substantial or dominant components of excimer and exciplex that account for this long-wavelength emission, since the monomers B, D, Y, and S do not emit above 430 nm. However, perylene (E) monomer emits at 440–470 nm, and a few of the perylene-containing ODF donors here are likely to emit as a mixture of perylene monomer and excimer or exciplex states. In those cases we cannot rule out FRET from the perylene monomer state rather than

(or in addition to) the delocalized states. However, some of the strongest FRET donors here are unambiguous. For example, P6 (BYSS) emits strongly at 450–550 nm, far redshifted from the monomer emissions of B or Y and strongly indicative of an exciplex. In the presence of a Cy5 acceptor, this emission drops by 60–80% in intensity, while the Cy5 acceptor emission increases by 25% to >100% (depending on acceptor distance). Likewise, the sequence P3 (SSYY) is an excimer donor, emitting strongly at 440–560 nm. In the presence of Cy5, the donor band is quenched by 25–30%, and the acceptor increases its emission intensity by strong factors of 5–10-fold. Exciplex and excimer FRET cases are also supported by the time-resolved data (Table S2). Interestingly, monomer emission bands of pyrene are also quenched in these excimer donor cases. Since the pyrene monomer emission does not overlap with the absorbance of Cy5 (with a nearly 100 nm gap separating them), it is unlikely that pyrene monomers contribute directly to the FRET donor properties of these cases. It seems possible that this quenching instead reflects the dynamic equilibrium between excimer and monomer excited states.

Of the two acceptor dyes examined here, a distal TAMRA (T3') was able to act as a more universal acceptor dye for FRET, which may be due to its greater spectral overlap of ODF donor emissions with TAMRA absorption. Thus, weak to moderate FRET occurred from all 20 of the ODF-labels to the distal T3' label. The most efficient donors for TAMRA were the exciplex ODF sequences BEBY and EYSS, which showed relatively large spectral changes in donor and acceptor emission (Fig. 2 and SI). At too close proximity, when P1–20 were hybridized near to TAMRA (T5'), this resulted in mutual quenching of the ODF emission and its own emission.

Cy5 was also shown here to be an acceptor dye for FRET with ODF donors, and indeed showed highest efficiency with some examples. FRET was observed in 7 and 9 cases of hybridization of P1–20 with C3' and C5' respectively. It is interesting to note that in these cases, the hybridization of ODF to a proximal Cy5 acceptor did not result in mutual quenching. The most efficient donors for the Cy5 acceptor were found to be the pyrene excimer cases SSYY and YHHY, which showed strong spectral changes due to FRET (Fig. 5). This occurred despite the non-optimal spectral overlap of the donor excimer emission (440–590 nm) with the Cy5 absorption band (band 548–689 nm; maximum at 646 nm). The results are consistent with a strong dipole in the ODF excimer and efficient dipolar interaction with Cy5.

Interestingly, although the emission bands of the present donor ODFs were mostly around the same range of wavelengths, FRET efficiencies varied considerably. One possible reason is that quantum yields vary to a substantial extent among ODF donors. Quantum yields of the donors affect the Förster distance, R_0 , directly, and thus plays a role in E_{FRET} . The varying excited state lifetimes of the donors could also affect the ability of the ODFs to sample favorable orientations and allow efficient FRET to occur. Thus, ODF donors with longer lifetimes typically displayed higher FRET efficiencies. In addition the dipole strength and orientation of the excimers and exciplexes in the various ODFs may vary significantly, resulting in different degrees of FRET efficiency.

As mentioned above, the lifetimes measurements for these unusual multichromophore FRET donors can be complicated by two factors that may not be seen in some common single-chromophore donors: namely, the presence of multiple excited-state species, and the competing kinetics of delayed formation of an excited state. The former can complicate evaluation of decay kinetics by presenting more than one overlapping decay curve. The latter can result when excimer- or exciplex-forming species are separated physically. For example, the P5 + C5' case, where two H residues separate two pyrenes, shows clear steady-state evidence of FRET (Fig. 5F) but no shortening of lifetime (Table S2). We suggest that in this case the kinetics most likely reflect the added time required for structural reorganization of the chromophores during

the long pyrene monomer excited state, until the favored conformation for excimer interaction can be reached. As a result of such effects, we consider the steady-state spectral changes (see Fig 2–Fig 5, Table 2) to be the most convincing measurement of FRET behavior for multichromophore ODF donors, and interpret the decrease in lifetimes as being consistent with, but not necessarily diagnostic of, FRET. More detailed time-resolved studies would be useful in investigating these phenomena.

The uncertainty of the orientation dipole factor (κ^2) in R_0 calculations is a known problem in the literature (45,46). In our study we used two estimations of κ^2 : a value of 1/3 (typically used when one member of the FRET pair is in a static orientation), and the value 2/3, assuming both donor and acceptor sample random orientations during the excited state. In our experiments, the former assumption yielded a better approximation to the theoretical distance between donor ODF and acceptor TAMRA. This would be consistent with the notion that the either ODF donor chromophores or TAMRA and Cy5 acceptor dyes were stacked at the end of the DNA with relatively fixed orientation. Previous studies have shown that aromatic hydrocarbons such as those in these ODFs stack with DNA with high affinity (11) and absorption spectra support the notion that the neighboring aromatic chromophores are stacked with one another in the ground state as well (37). NMR studies showed that a significant population of Cy5 dyes can stack onto the end of a DNA duplex (47) while some remain freely mobile as shown in single molecule experiments and simulations (48).

Not only is the observance of FRET from excimer and exciplex dyes of theoretical interest, but also the ODFs offer some potential advantages in practical use. Relative to common donors used with TAMRA and Cy5, the ODFs offer high quantum yields and a fixed orientation in the DNA. In addition, ODFs are directly incorporated into DNA using automated solid-phase synthesis, unlike some common dyes that require postsynthetic labeling. The ODFs are composed of combinations of a small set of monomers; for example, optimal donor ODFs for TAMRA and Cy5 are composed of only two fluorescent monomers (Y and E) along with a commercially available spacer (S).

In addition to the above, perhaps the most distinctive aspect of the current multichromophoric dyes as FRET donors is their unusual spectral characteristics. Since all the ODFs studied can be excited simultaneously at one wavelength (330 nm was used here), the ODFs can function as FRET donors potentially to a wide variety of acceptors using a simple filter set. Further experiments will be needed to confirm this. In addition, the ODFs have unusually large Stokes shifts (144–149 nm in the present cases), thus providing a large spectral separation between exciting light and emission. These properties can simplify experimental setups involving simultaneous analysis of multiple species. Thus these multichromophore labels present substantial potential for development and application in biomolecular analysis and imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A **B**

C

Figure 1.

Structures in this study. A. Monomeric 2'-deoxyfluorosides B, E, Y, D and S. B. Structures of two representative ODFs (BEBY and YSSY) that were studied as DNA conjugates. These were attached to the 3' end of a DNA probe sequence (R). C. An example of a hybridization experiment: hybridization of BEYY-labeled probe (P2) with an acceptor-labeled complement (C3').

Figure 2.

Representative evidence for FRET between ODF fluorophores and TAMRA in a DNA duplex (see full data in Supporting Information). Fluorescence emission spectra (excitation 330 nm) are shown for hybridization of P1 and P6 with T3' and T5' (red lines) and individual emission spectra of P1, P6 (blue), T3' and T5' (green). Note spectral changes characteristic of FRET for the distal-substituted TAMRA (A, C).

Figure 3.

Evidence for FRET between ODF donors and a Cy5 acceptor in duplex DNA. Fluorescence emission spectra are shown for hybridization of ODF conjugates P2 and P6 with C3' and C5' (red lines) ($\lambda_{\text{ex}} = 330$ nm) and individual emission spectra of P2, P6, C3' and C5' ($\lambda_{\text{ex}} = 330$ nm) (blue and green lines). Note quenching of excimer and exciplex bands (440–560 nm) concomitant with enhancement of Cy5 emission at ~670 nm.

Figure 4.

Testing possible FRET donation from pyrene excimers to TAMRA. Fluorescence emission spectra upon hybridization of A. P3; B. P4 and C. P5 with T3' respectively ($\lambda_{\text{ex}} = 330$ nm) and individual emission spectra of P3, P4, P5 and T3' ($\lambda_{\text{ex}} = 330$ nm).

Figure 5.

Evidence for strong FRET donation from pyrene excimers to Cy5 acceptor in DNA. Fluorescence emission spectra upon hybridization of P3, P4 and P5 with C3' and C5' respectively (red lines) ($\lambda_{\text{ex}} = 330 \text{ nm}$) and individual emission spectra of P3, P4, P5, C3' and C5' (blue and green lines) ($\lambda_{\text{ex}} = 330 \text{ nm}$).

Table 1

Sequences of DNA probes in this study.

Strand ID	Sequence ^[a]
P1 – P20	5'-TCC ACA GAA ACA TA – XXXX- 3'
C5'	3'- AGG TGT CTT TGT AT – Cy5 - 5'
T5'	3'- AGG TGT CTT TGT AT – TAMRA - 5'
C3'	3'- Cy5 - AGG TGT CTT TGT AT - 5'
T3'	3'- TAM - AGG TGT CTT TGT AT – 5'

^[a]XXXX denotes the sequence of ODF (listed in Table 2).

Sequences and photophysical data for 20 composition- and sequence-varied ODFs appended to the 3' end of the DNA probe sequences P1–P20.^{a, b}

Table 2

Strand ID	ODF Sequence (5'→3')			$\lambda_{\text{abs}}, \text{max}$ (nm)	$\lambda_{\text{em}}, \text{max}$ (nm)	Φ_D (fluor)	$E_{\text{FRET}}^{\text{T3'}}$	$Q_{\text{eff}}^{\text{T5'}}$	$E_{\text{FRET}}^{\text{C3'}}$	$E_{\text{FRET}}^{\text{C5'}}$	
P1	B	E	B	Y	258, 347, 401, 450	492	0.194	0.244	0.595	n.a.	0.125
P2	B	E	Y	Y	264, 344, 402, 452	487	0.266	0.037	0.325	0.056	0.105
P3	S	S	Y	Y	263, 331, 347	488	0.187	0.048	0.480	1.449	1.065
P4	Y	S	S	Y	264, 332, 350	378, 395, 490	0.025	0.045	0.315	0.463	0.646
P5	Y	H	H	Y	267, 333, 349	375, 395, 490	0.044	0.051	0.500	1.374	1.261
P6	B	Y	S	S	261, 349, 403	375, 395, 416, 470	0.205	0.075	0.689	0.129	0.503
P7	E	Y	S	S	257, 346, 451	461, 490	0.421	0.752	0.502	n.a.	0.053
P8	E	Y	E	Y	257, 349, 452	375, 465, 493	0.124	0.054	0.725	0.018	n.a.
P9	E	Y	Y	E	256, 347, 451	464, 490	0.130	0.037	0.487	0.004	n.a.
P10	E	E	Y	Y	257, 347, 451	490	0.141	0.001	0.384	n.a.	n.a.
P11	Y	Y	E	E	254, 349, 441	462, 489	0.110	0.017	0.663	n.a.	n.a.
P12	E	S	E	Y	256, 347, 451	375, 395, 494, 530	0.148	0.028	0.401	n.a.	0.038
P13	B	S	B	D	258, 361, 401	375, 492	0.044	0.070	0.536	n.a.	n.a.
P14	E	Y	B	E	257, 347, 401, 451	465, 493	0.092	0.037	0.615	n.a.	n.a.
P15	E	D	S	S	257, 355, 450	461, 490	0.161	0.057	0.739	n.a.	n.a.
P16	E	S	Y	D	257, 346, 349, 451	460, 488	0.191	0.014	0.630	n.a.	0.056
P17	D	S	E	Y	257, 343, 349, 451	375, 463, 490	0.303	0.038	0.435	n.a.	n.a.
P18	E	S	B	S	258, 402, 451	470, 500	0.219	0.159	0.712	n.a.	n.a.
P19	S	E	S	E	254, 449	449, 477	0.182	0.034	0.329	n.a.	n.a.
P20	E	E	D	E	254, 355, 449	374, 454, 478	0.062	0.077	0.038	n.a.	n.a.

^aT3' is 3'-conjugated TAMRA on the complementary strand; T5' is 5'-conjugated TAMRA. C3' is 3'-conjugated Cy5; C5' is 5'-conjugated Cy5. B is benzopyrene deoxyriboside; D is dimethylaminostilbene deoxyriboside; E is perylene deoxyriboside; Y is pyrene deoxyriboside and S is an abasic spacer.

^bEFRET values were calculated using eq. 1. n.a. indicates no FRET observed. Q_{eff} is quenching efficiency, given for T5', where no significant FRET was observed.

Table 3

Calculated R_0 values and R_{DA} values for ODF donors. Note that only a subset of cases exhibit efficient FRET (Table 2).

Strand ID	$R_0/\text{\AA}$ $\kappa^2=2/3$	$R_0/\text{\AA}$ $\kappa^2=1/3$	$R_{DA}/\text{\AA}$ $\kappa^2=2/3$	$R_{DA}/\text{\AA}$ $\kappa^2=1/3$	E_{FRET}	E_{FRET}	$E_{\text{L-FRET}}$	$R_{DA}/\text{\AA}$ $\kappa^2=1/3$
P1	39.4	35.1	47.6	42.4	46.5	41.4		
P2	38.5	34.3	66.2	59.0	52.0	46.3		
P3	37.9	33.8	62.4	55.6	46.0	41.0		
P4	25.3	22.5	42.1	37.5	36.2	32.3		
P5	27.8	24.8	45.2	40.3	23.2	20.7		
P6	33.7	30.0	51.1	45.6	39.8	35.5		
P7	42.6	38.0	35.4	31.6	57.4	51.2		
P8	37.5	33.4	60.4	53.8	48.8	43.5		
P9	38.1	34.0	65.6	58.4	n.d.	n.d.		
P10	38.3	34.2	118.5	105.6	n.d.	n.d.		
P11	34.3	30.5	67.3	59.9	n.d.	n.d.		
P12	36.2	32.2	65.4	58.2	n.d.	n.d.		
P13	28.8	25.6	44.3	39.4	n.d.	n.d.		
P14	34.5	30.8	59.4	52.9	n.d.	n.d.		
P15	34.3	30.6	54.8	48.9	n.d.	n.d.		
P16	38.2	34.0	77.7	69.2	n.d.	n.d.		
P17	40.0	35.7	68.5	61.0	n.d.	n.d.		
P18	39.1	34.8	51.6	46.0	n.d.	n.d.		
P19	37.2	33.1	64.8	57.7	n.d.	n.d.		
P20	29.4	26.2	44.5	39.6	n.d.	n.d.		

n.d. = not determined. R_0 = R value calculated from drop in donor emission; R_{DA} = value calculated from changes in both donor and acceptor.