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An Efficient Fluorescence Resonance Energy Transfer (FRET) between Pyrene and Perylene Assembled in a DNA Duplex and Its Potential for Discriminating Single-Base Changes

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Abstract: To increase the apparent Stokes' shift of perylene, pyrene (donor) and perylene (acceptor) were assembled in a DNA duplex to achieve the efficient fluorescence resonance energy transfer (FRET) from pyrene to perylene. Multiple donors were introduced in the vicinity of acceptors through D-threoninol and natural base pairs were inserted between the dyes. Accordingly, donors and acceptors could be accumulated inside the DNA without forming an undesired excimer/exciplex. When two pyrene moieties were located in proximity to one perylene with one base pair inserted between them, efficient FRET occurred within the duplex. Thus, strong emission at 460 nm was observed from perylene when excited at 345 nm at which pyrene has its absorption. The apparent Stokes' shift became as large as 115 nm with a high apparent FRET efficiency ($\Phi > 1$). However, the introduction of more than two pyrenes did not enhance the fluorescence intensity of perylene, due to the short Förster radius (R_0) of the donor pyrene. Next, this FRET system was used to enlarge the Stokes' shift of the DNA probe, which can discriminate a one-base deletion mutant from wild type with a model system by incorporation of multiple donors into DNA. Two perylene moieties were tethered to the DNA on both sides of the intervening base, and two pyrenes were further inserted in the vicinity of the perylenes as an antenna. Hybridization of this FRET probe with a fully matched DNA allowed monomer emission of perylene when the pyrenes were excited. In contrast, excimer emission was generated by hybridization with a one-base deletion mutant. Thus, the apparent Stokes' shift was enhanced without loss of efficiency in the detection of the deletion mutant.

Keywords: DNA · energy transfer · fluorescent probes · perylene · pyrene

Introduction

Fluorescent dyes are commonly used for the detection of oligodeoxyribonucleotides (ODN) due to their high sensitivity.[1] While the quantum yield of the fluorescent dye is the dominant factor that determines its sensitivity, the Stokes' shift, that is, the difference between the band maxima of absorption and emission of identical dyes, also significantly affects sensitivity because the scattered light of excitation inhibits the detection of emission. Although conventional fluorescent dyes, such as Cy3 and Cy5, have a high quantum yield and can be applied to the labeling of ODNs, their Stokes' shifts are only 15 and 17 nm, respectively.[2,3] Since such a small Stokes' shift lowers the sensitivity, efforts have been made to expand the difference between the excitation and emission wavelengths.[4] One promising strategy for this purpose is the utilization of fluorescence resonance energy transfer (FRET).[5] Several studies on the application of FRET to DNA duplexes have reported an increase in the "apparent" Stokes' shift.[6–12] For example, pyrene and perylene, which are known as a FRET pair,[13] were introduced into an ODN to increase the apparent Stokes' shift. The emission band of pyrene appeared at 370–430 nm, whereas the absorption band of perylene appeared at 390–470 nm. Previously, these donor–acceptor pairs were tethered at the termini of ODNs,[14] or at the 2′-position of a locked nucleic acid (LNA).[15] Thus, pyrene and perylene were located at the end of the ODN, or in the minor groove, so that the flu-

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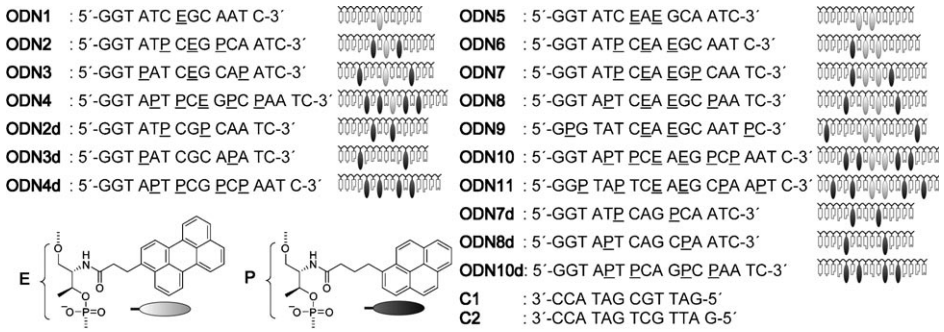
orophores were not rigidly fixed but could move to some extent. However, it might be difficult to introduce multiple donors as an antenna in close proximity to an acceptor dye by using these molecular designs, because fluorescence will be quenched upon dimer formation of donors or following donor–acceptor dimerization.

Here, we describe another FRET system in which donors are accumulated in close proximity to acceptors within the duplex. In this study, pyrene and perylene were introduced into DNA via a D-threoninol linker to facilitate the intercalation of this FRET pair between base pairs:[16] pyrene and perylene are intercalated inside the duplex and intervening base pairs separate these chromophores as depicted in Scheme 1. Consequently, fluorophores can be accumulated in close proximity without forming undesired excimers/exciplexes or quenching their fluorescence. Molecular cluster motifs reported by us and other research groups have difficulty in suppressing these interactions. [17–22] The design presented herein makes it possible to introduce more than one donor (pyrene) near the acceptor (perylene), which facilitates energy transfer from pyrene to perylene. In the present study, we investigated the effect of the position and number of donors on FRET efficiencies. We then applied this FRET system to enlarge the Stokes' shift of the fluorescent probe for potential application to the one-base deletion polymorphisms. Recently, much attention has been paid to non-single-nucleotide polymorphism (SNP) variations, such as small deletions, due to their frequent occurrence.[23] We have developed ODN probes detecting base-deletion polymorphisms.[24,25] Here, we demonstrate that the Stokes' shift of the fluorescent probe can be extended by locating donors in proximity to the acceptor. This strategy has a potential to be used for other fluorescent probes tethering intercalators.[26–30]



Scheme 1. Sequences of the ODNs synthesized in this study.

Results

Design of modified ODNs by using pyrenes and perylenes as donors and acceptors, respectively, and their fluorescent behavior: All the modified ODNs were synthesized by the standard phosphoramidite method. Scheme 1 shows the sequences of the ODNs used in this study. Both pyrenes (**P** in Scheme 1) and perylenes (**E**) were tethered on D-threoninol in the middle of the ODN and one to three natural nucleotides were inserted between the pyrenes and perylenes to avoid undesired excimer or exciplex formation. The complementary strands used were **C1** (for **ODN1**–**ODN4**) and **C2** (for **ODN5**–**ODN11**). In these strands, pyrene and perylene residues were inserted into the ODN as bulges (i.e. not in place of a natural nucleotide). By this sequence design, natural base pairs were kept intact in the duplex state. To evaluate the effect of distance between donors and acceptors, ODNs, which have one and three bases between pyrene and perylene, were synthesized (**ODN2** and **ODN3**). In addition, ODN with four pyrenes (**ODN4**) was also synthesized to investigate the effect of the number of donors. One acceptor and two or four donors were introduced into the ODNs (**ODN2**–**ODN4**) for the calculation of FRET efficiencies. In addition, ODNs without a donor (**ODN1**) or acceptor (**ODN2d**–**ODN4d**) were also synthesized as controls. As shown on the right-hand side of Scheme 1, probe-type sequences, which tethered two perylenes, were also prepared (**ODN6**–**ODN11**). To calculate FRET efficiencies, **ODN7d**, **ODN8d**, and **ODN10d** were synthesized as controls for **ODN7**, **ODN8**, and **ODN10**, respectively. Most of the spectroscopic measurements were carried out at 0 °C in which full duplex hybridization was ascertained (unless otherwise noted).

Fluorescent behavior of ODNs tethering one perylene: First, effect of the distance between the donor pyrene and acceptor perylene on the FRET efficiency was investigated by changing the number of base pairs between the pyrene and perylene. Figure 1 shows the fluorescence emission spectra of **ODN1**–**ODN4** hybridized with their complementary strand (**C1**) excited at 345 nm, a wavelength at which pyrene has its absorption.[31] The corresponding UV/Vis spectra are depicted in Figure S1 in the Supporting Information. The emission spectra of **ODN1/C1**, which has single perylene (**E** residue) and no pyrene, also showed a small emission at 460 and 491 nm because perylene still has very weak absorption at 345 nm (see Figure S1 in Supporting information). In contrast, other sequences exhibited much stronger emission at 450–550 nm than did **ODN1/C1**. In particular, the emission intensity of **ODN2/C1**, which has only one base pair between pyrene and perylene, was much higher than that of **ODN1/C1**, which demonstrates efficient energy transfer between pyrene and perylene. Similarly, insertion of three natural base pairs between perylene and pyrene (**ODN3/C1**) also showed a higher emission at 461 and 491 nm than **ODN1/C1**, although its intensity was lower than that of

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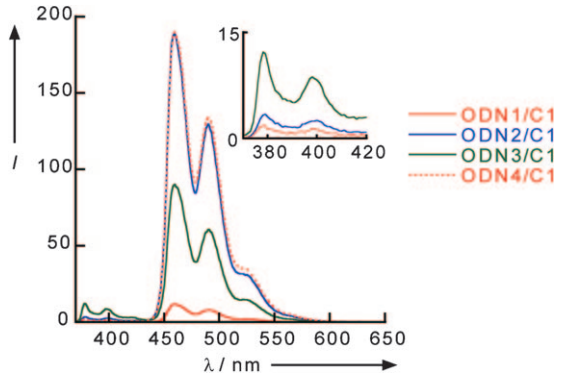

Figure 1. Fluorescence emission spectra of **ODN1/C1–ODN4/C1** at 0 °C. Spectra at 370–420 nm are magnified in the inset. The excitation wavelength was 345 nm. The solution conditions are as follows: [**ODN*n***] = 1.0 μM, [**C1**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

ODN2/C1 (compare the solid blue line with the green one in Figure 1). Concurrent with these effects, the emission peaks of pyrene at 370–430 nm were increased by the insertion of base pairs (see the inset in Figure 1). Accordingly, the FRET efficiency of **ODN2/C1** is higher than that of **ODN3/C1**.

Next, FRET efficiency of the accumulated donor pyrenes was evaluated by comparing **ODN2/C1** with **ODN4/C1**, which involves four donor pyrenes. An increase in the number of donors did not enhance the emission of the acceptor and the emission intensities of **ODN2/C1** and **ODN4/C1** were almost identical. Note that no excimer emission of pyrene was observed in **ODN4/C1** although five chromophores (one perylene and four pyrenes) were introduced and thus absorption at 345 nm was increased (see Figure S1 in the Supporting Information). This result demonstrates that each donor and acceptor is separated by an intervening base pair according to the design. We also calculated the FRET efficiencies (Φ_T) of these duplexes by using **ODN2d/C1–ODN4d/C1** as controls (see Table 1). The apparent Φ_T of **ODN2/C1** was greater than 1 (1.18), which may have been caused by a structural disturbance (see the Discussion section). As expected from the fluorescence emission spectra, Φ_T of **ODN3/C1** was lower than that of **ODN2/C1**. Moreover, **ODN4/C1**, containing twice as many pyrenes as **ODN2/C1**, showed a similar Φ_T to that of **ODN3/C1**. Thus, the Φ_T of **ODN4/C1** was lower than that of **ODN2/C1**, although the emission intensity was almost the same.

Table 1. Spectroscopic properties of modified ODNs involving an acceptor.

Sequence		T_m [°C][a]	Relative intensity[b,c]	Φ_T[b,d]
ODN1/C1		45.0	1.0	–
ODN2/C1		34.8	15.9	>1[e]
ODN3/C1		54.6	7.6	0.53
ODN4/C1		24.9	16.1	0.61

[a] [**ODN*n***] = 5.0 μM, [**C1**] = 5.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). [b] [**ODN*n***] = 1.0 μM, [**C1**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 0 °C. Excitation wavelength: 345 nm. [c] Relative intensity at 460 nm relative to that of **ODN1/C1**. [d] Efficiencies of FRET were calculated according to the method described in the Experimental Section. [e] Apparent FRET efficiency was calculated as 1.18.

The melting temperatures (T_ms) of these modified ODNs are listed in Table 1. The T_m of **ODN3/C1** was 54.6 °C, which was 9.6 °C higher than that of **ODN1/C1**, which indicates that the introduced pyrene stabilized the duplex by a stacking interaction. However, the T_m of **ODN2/C1** was about 20 °C lower than that of **ODN3/C1**. Similarly, the T_m of **ODN4/C1** was about 10 °C lower than that of **ODN2/C1**. These low T_m values are attributed to distortion of the duplex by alternately introduced chromophores and natural nucleobases. Despite this destabilization, pyrenes were efficiently separated by the intervening base pairs because no excimer or exciplex emission was observed (see Figure 1).[32]

Spectroscopic behavior of probe-type ODNs tethering two perylenes: We also investigated the spectroscopic behavior of probe-type sequences (**ODN5–ODN11**, see the next section for details), which tether two perylenes in the middle of the sequence to evaluate the FRET efficiency of accumulated donor pyrenes near the perylenes, and effect of the distance between the fluorophores.[25] Figure 2 shows the fluorescence emission spectra of **ODN5/C2–ODN11/C2** excited at 345 nm. The emission intensity of **ODN6/C2**, which has one pyrene, was almost double that of **ODN5/C2**. Moreover, **ODN7/C2**, which has two pyrenes showed much more intense peaks than **ODN5/C2**. As the number of base pairs between pyrene and perylene increased, (**ODN8/C2** and **ODN9/C2**), the emission intensity at 450–550 nm decreased. In particular, the emission of an **ODN9/C2** duplex with five base pairs between pyrene and perylene was as weak as that of **ODN5/C2**, which has no pyrenes (compare the red solid

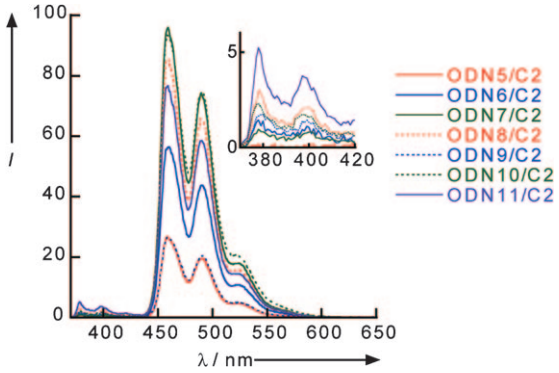

Figure 2. Fluorescence emission spectra of **ODN5/C2–ODN11/C2** at 0 °C. The excitation wavelength was 345 nm. Spectra at 370–420 nm are magnified in the inset. Solution conditions are as follows: [**ODN*n***] = 1.0 μM, [**C2**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

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line with the dotted blue line in Figure 2); this indicates that the antenna effect of the pyrenes was almost nil. On the other hand, emission at 370–430 nm showed a slightly complicated behavior; the emission intensity of **ODN8/C2** was higher than **ODN9/C2**, although that of **ODN7/C2** was lower than that of **ODN9/C2**. Electron transfer between pyrene and natural bases might affect emission intensity of pyrene in these duplexes. Incorporation of four pyrenes (**ODN10/C2** and **ODN11/C2**) did not enhance the emission of perylene (compare the green solid line with the green dotted and purple solid lines in Figure 2). As the distance between donors and acceptors decreased, more intense emission of perylene was observed, which shows that energy transfer from pyrene to perylene occurred.[33] The FRET efficiencies of **ODN7/C2**, **ODN8/C2** and **ODN10/C2** were calculated by using **ODN7d/C2**, **ODN8d/C2** and **ODN10d/C2**, respectively, as listed in Table 2. The Φ_T of **ODN7/C2** was 0.36, which was much lower than that of **ODN2/C1**. Insertion of one more base pair between pyrene and perylene decreased Φ_T (**ODN8/C2**). **ODN10/C2**, with four pyrenes exhibited a much lower Φ_T than **ODN7/C2** with two pyrenes, although their emission intensities were almost the same.

Table 2. Spectroscopic properties of modified ODNs involving two acceptors.

Sequence		Relative intensity[a,b]	Φ_T[a,c]
ODN5/C2		1.0	–
ODN6/C2		2.2	nd[d]
ODN7/C2		3.7	0.36
ODN8/C2		3.3	0.27
ODN9/C2		1.0	nd[d]
ODN10/C2		3.6	0.14
ODN11/C2		2.9	nd[d]

[a] [**ODN*n***] = 1.0 μM, [**C2**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 0 °C. Excitation wavelength: 345 nm. [b] Relative intensity at 460 nm relative to that of **ODN5/C2**. [c] FRET efficiencies were calculated according to the method described in the Experimental Section. [d] Not determined.

To investigate the orientation and position of chromophores, circular dichroism (CD) spectra of **ODN7/C2** at various temperatures were measured (Figure 3). At a temperature higher than the T_m, no obvious CD was observed above 350 nm (The T_m values are listed in Table 3). On the other hand, as the temperature decreased below 20 °C, an induced CD was observed at 400–460 nm in which perylene has its absorption. There are two positive peaks and two negative peaks at this region, which indicates that the two perylenes were oriented in a right-handed manner. A positive couplet, characteristic of B-form DNA, was observed at around 260 nm, although the couplet was not symmetrical, probably due to the CD induced by perylene at this region. Although **ODN7/C2** involves two pyrenes, no CD was observed at around 350 nm at which pyrene has its absorption.[34]

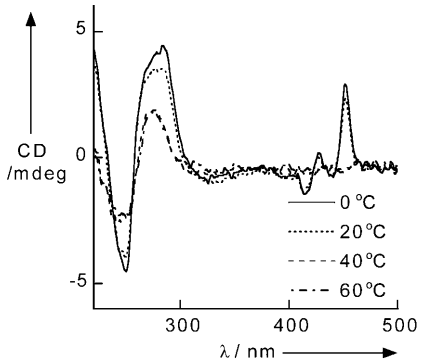


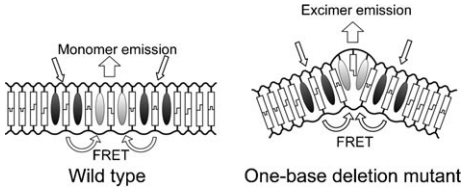
Figure 3. CD spectra of **ODN7/C2** at various temperatures. Solution conditions are as follows: [**ODN7**] = 1.0 μM, [**C2**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

Table 3. Effect of the donor position on polymorphism-discrimination ability.

	Sequence	I_{550}/I_{461}[a]		T_m [°C][b]	
		C1[c]	**C2**[d]	**C1**[c]	**C2**[d]
ODN5		0.84[e]	0.05[e]	46.7	49.9
ODN6		0.39[f]	0.06[f]	40.3	45.3
ODN7		0.61[f]	0.07[f]	33.0	36.3
ODN8		0.86[f]	0.05[f]	42.7	46.4
ODN9		0.21[f]	0.05[f]	50.9	54.6
ODN10		0.47[f]	0.19[f]	21.0	25.7
ODN11		0.94[f]	0.10[f]	33.9	38.8

[a] [**ODN*n***] = 1.0 μM, [**C1**] = [**C2**] = 1.2 μM, [NaCl] = 100 mM, pH 7.0, 20 °C (10 mM phosphate buffer). [b] [**ODN*n***] = 5.0 μM, [**C1**] = [**C2**] = 5.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). [c] One-base deletion mutant. [d] Full match. [e] Excited at 425 nm. [f] Excited at 345 nm.

Potential application to the detection of a one-base deletion with a FRET probe: We next used **ODN5**– **ODN11** to evaluate the discrimination ability of deletion polymorphisms from full match. The probe design for a one-base deletion is depicted in Scheme 2. Two perylenes and several donors were introduced into ODN. When this ODN is hybridized



Scheme 2. Schematic illustration of the design of the FRET probe.

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with a fully complementary strand (full match), all the chromophores are, most likely, intercalated between base pairs and monomer emission of perylene should be observed by excitation at 345 nm at which the donor pyrene has its absorption due to efficient FRET between perylene and pyrene. On the other hand, in a one-base-deletion mutant, two perylenes should come close to each other by forming a bulge-like structure so that an excimer of two perylenes should be formed.[25]

The fluorescence emission spectra of **ODN7/C1** and **ODN7/C2**, excited at 345 nm, are shown in Figure 4. When **ODN7** was hybridized with full-matched target (**C2**), strong

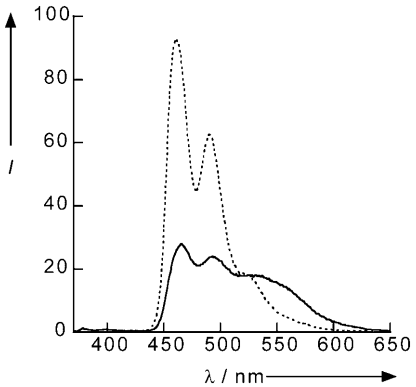

Figure 4. Fluorescence emission spectra of **ODN7/C1** (——) and **ODN7/C2** (·····) at 20 °C. The excitation wavelength was 345 nm. Solution conditions are as follows: [**ODN7**] = 1.0 μM, [**C*n***] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

monomer emission of perylene was observed. In contrast, monomer emission was significantly quenched upon hybridization with the deletion mutant **C1**. Concurrently, a new shoulder peak appeared at 550 nm, which indicated that an excimer of two perylenes was formed. The ratio of excimer to monomer emission (I_{550}/I_{461}) at 20 °C was as high as 0.61 for **ODN7/C1** corresponding to the deletion mutant due to excimer emission and strong quenching of monomer emission. This value was eight-times larger than that of **ODN7/C2** (0.07) corresponding to full match.[35,36] **ODN5**, a probe without pyrene as an antenna,[25] showed similar results: I_{550}/I_{461} was 0.84 for the deletion mutant **C1**, whereas it was 0.05 for full-match **C2** when excited at 425 nm at which perylene has its absorption maximum (see Table 3 and Figure S6 in the Supporting Information for the actual fluorescence spectra of **ODN5/C1** and **ODN5/C2**). Note that the Stokes' shift of **ODN5** was as small as 35 nm, whereas that of **ODN7**, which has pyrenes as an antenna was as large as 115 nm. Thus, incorporation of pyrenes into the appropriate positions, such as those in **ODN7**, could efficiently enhance the apparent Stokes' shift without losing the ability to discriminate between the wild type and the deletion mutant.[37] We also investigated sequence dependence on the discrimination ability by changing the intervening base. It was found that base-deletions of other nucleotides (not only A but C, G and T) could be detected by this method, although discrimination abilities varied depending on the sequence (see Tables S1 and S2 in the Supporting Information).[38]

The I_{550}/I_{461} ratios of other probes are summarized in Table 3. The ratios of the **ODN8** and **ODN9** probes were almost the same as that of **ODN7**. These results demonstrate that incorporation of two pyrenes does not affect excimer formation as long as the chromophores are separated by intervening base pairs. Furthermore, an increase in the number of pyrenes did not affect the ratio. The I_{550}/I_{461} of **ODN11** was evaluated as 0.94 for **C1**, which was nine-times larger than that for **C2** (0.10). On the other hand, the difference in I_{550}/I_{461} between **ODN10/C1** and **ODN10/C2** was smaller, because of the low melting temperatures of these duplexes (the T_m values of **ODN10/C1** and **ODN10/C2** were 21.0 and 25.7 °C, respectively (see Table 3)). Accordingly, at 0 °C, which is far below the T_m of **ODN10/C1** or **ODN10/C2**, I_{550}/I_{461} of **ODN10/C2** decreased to 0.06, whereas that of **ODN10/C1** was 0.38 (data not shown), demonstrating that incorporation of multiple donors did not lower the polymorphism-discriminating ability. Thus, all the results presented in this section show that the apparent Stokes' shift was successfully enlarged by the introduction of multiple donors without disturbing the polymorphism-discriminating ability.

Discussion

Efficiency of energy transfer from pyrene to perylene: The FRET efficiencies (Φ_T) of various duplexes were evaluated as listed in Table 1. The apparent Φ_T was estimated as >1 (1.18) for **ODN2/C1**, which indicated that efficient energy transfer occurred in this duplex. Sufficient proximity of the donor to the acceptor contributed to this highly efficient energy transfer. Thus, the separation of dyes by intervening base pairs is an effective strategy for the accumulation of chromophores in ODNs. However, a Φ_T value that is larger than 1 is not a reasonable value. One of the causes of this discrepancy is a structural disturbance in **ODN2/C1**. Because three fluorophores and four natural base pairs were alternately introduced into the centre of the duplex, the double-helical structure is expected to be disturbed to some extent. Thus, quenching by neighbouring bases may be reduced in the case of **ODN2/C1** as compared with **ODN1/C1**, which raised the emission from perylene (I_{AD}) and gave a large Φ_T. Indeed, the melting temperature of **ODN2/C1** was only 34.8 °C, which was 10.2 °C lower than that of **ODN1/C1**. These structural differences might affect the fluorescence intensity of perylene.[39]

In our system, FRET efficiency strongly depended on the distance between the acceptor and the donors. For example, the Φ_T value of **ODN3/C1** was as low as 0.53, although there are only three base pairs between pyrene and perylene. The dependency of Φ_T on the distance (r) is expressed by using Equations (1) and (2):

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$$\Phi_T = R_0^6/(R_0^6 + r^6) \quad (1)$$

$$R_0 = 0.2108[J(\lambda)\kappa^2 n^{-4}\Phi_D]^{1/6} \quad (2)$$

in which R_0 is the Förster radius, $J(\lambda)$ is the integral of spectral overlap between the donor and acceptor, κ is the orientational factor, n is the refractive index of the solution and Φ_D is the quantum yield of the donor.[40] Masuko et al. estimated R_0 as 22.3 Å assuming κ^2 to be 2/3.[14] In the present study, R_0 should be shortened because a three-base separation strongly decreased the FRET efficiency as observed with **ODN3/C1**.[41] Energy-minimized structures of **ODN2/C1** and **ODN3/C1**, calculated by using InsightII/Discover3, are shown in Figure 5. In both duplexes, pyrenes and perylenes were stacked within the duplex.[42] The relative orientations of the chromophores are also shown at the bottom of Figure 5. The angles between the transition dipoles of pyrene and perylene were 13 and 70° in **ODN2/C1**, but were 2 and 55° in **ODN3/C1**, which indicated that κ^2 was not very low in these duplexes.[43] From these results, the relatively short R_0 may be attributed to the low quantum yield of donor (pyrene) emission, although the quantum yield of pyrene was not determined directly.[44,45]

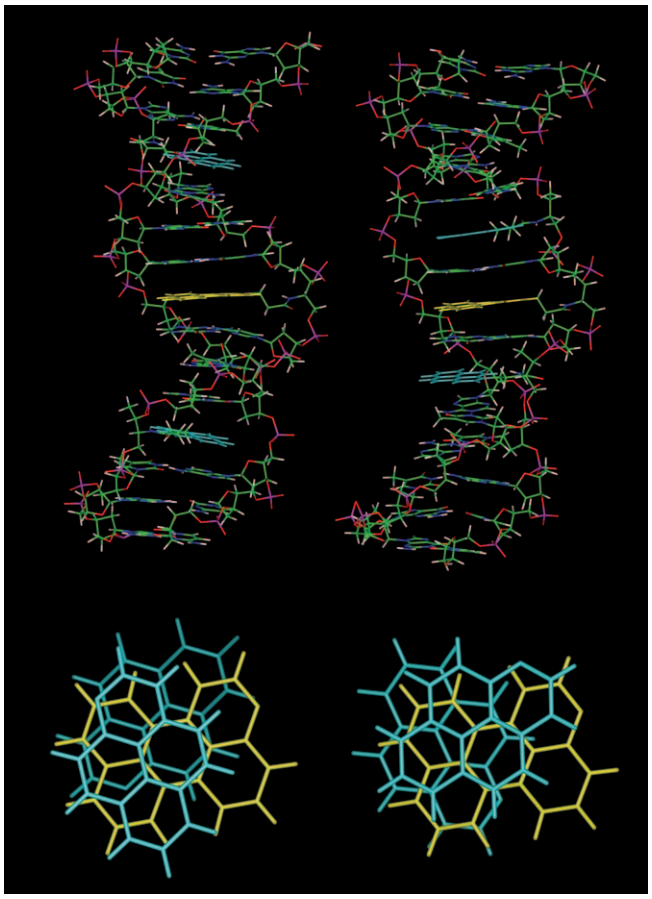

Figure 5. Energy-minimized structures of **ODN3/C1** (left side) and **ODN2/C1** (right side). The relative orientations of the chromophores are shown at the bottom. Pyrene and perylene moieties are coloured in blue and yellow, respectively.

Since the ODNs in the series **ODN5–ODN11** have two acceptors, we expected that FRET efficiencies should decrease to one-half of the value of the ODNs of the series **ODN1– ODN4** that have a single acceptor. However, Φ_T of **ODN7/C2** was as small as 0.36, whereas that of **ODN2/C1** was greater than 1. The relative intensity of **ODN7/C2** with respect to that of **ODN5/C2** was only 3.7. Although the reasons for this result are still not clear, the structural differences between **ODN2/C1** and **ODN7/C2** might affect the fluorescence intensity. A comparison of the fluorescence intensity of **ODN6/C2** with that of **ODN7/C2** indicates that two acceptors are required for efficient energy transfer. Since the R_0 of pyrene is rather short, excited energy transfers only to the nearest perylene and not to the distant perylene.[46] Such a short R_0 also inhibits the enhancement of fluorescence intensity even if the number of donors increases from two to four. Thus, the intensity of **ODN10/C2** was almost the same as that of **ODN7/C2**. Similarly, the intensity of **ODN11/C2** was even lower than that of **ODN8/C2**. On the basis of these results, we concluded that two pyrenes are sufficient for efficient energy transfer as long as one base pair is inserted between the donor (pyrene) and acceptor (perylene) due to the short R_0 of the intercalated pyrene.

The ODN series of **ODN6–ODN11** successfully discriminated the deletion mutant (**C1**) from the full match (**C2**) based on the change in I_{550}/I_{461}. By use of a FRET pair, the apparent Stokes' shift of these new probes was much larger (115 nm) than that of the previous probe composed of only perylene (35 nm).[25] As shown in Table 3, the I_{550}/I_{461} ratios of **ODN5/C2–ODN9/C2** did not show much change, which demonstrated that the polymorphism-discrimination ability did not decrease despite the fact that many donors were inserted. Even exciplex emission, which should be observed when a pyrene–perylene heterodimer is formed,[47,48] was not observed at all. These results demonstrate that one base pair is enough to separate donor and acceptor or multiple donors.

Duplex stabilities: The melting temperatures were strongly influenced by the position of pyrene. As shown in Table 1, insertion of pyrene in the nearest-neighbour base pairs largely destabilized the duplex (**ODN2/C1**). On the other hand, **ODN3/C1**, which has three base pairs between pyrene and perylene, stabilized the duplex. The same tendency was observed with **ODN7–ODN9**, in which, as the number of intervening base pairs increased, the T_m value increased. Note that the T_m of **ODN7/C2**, which has one base pair between pyrene and perylene, was much lower than that of **ODN8/C2**, which has two base pairs. This phenomenon is consistent with the nearest-neighbour exclusion principle: free intercalators cannot be intercalated between the nearest-neighbour base pairs.[49,50] The same is true for **ODN10** and **ODN11**, both of which have four pyrenes. The T_m value of **ODN10/C2** was 25.7 °C, which was 13.1 °C lower than that of **ODN11/C2** (38.8 °C).

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Conclusion

An efficient FRET system of pyrene and perylene intercalated inside a duplex was constructed. Chromophores can be accumulated in close proximity by this design because base pairs completely suppress their interactions. The apparent Stokes' shift of the FRET probe was successfully increased by the introduction of multiple donors close to acceptors without disturbing the excimer formation. In these probes, chromophores were strictly arranged in a predetermined position and order in the duplex without undesirable interactions so that energy transfer and excimer formation were strictly controlled. This strategy is especially useful for the monitoring of biological samples because autofluorescence from the sample that often interferes with the detection is minimized.

Experimental Section

Materials: All the conventional phosphoramidite monomers, CPG columns, reagents for DNA synthesis and Poly-Pak II cartridges were purchased from Glen Research. Other reagents for the synthesis of phosphoramidite monomers were purchased from Tokyo Kasei Co and Aldrich.

Synthesis of the DNA modified with pyrene and perylene: All the modified ODNs were synthesized on an automated DNA synthesizer (ABI-3400 DNA synthesizer, Applied Biosystems) by using phosphoramidite monomers bearing dye molecules. Synthetic procedures for the perylene monomer were reported previously.[25] See Scheme S1 in the Supporting Information for synthetic procedures and NMR spectroscopic assignments of the pyrene monomer. The coupling efficiency of the monomers corresponding to the modified residues was as high as the conventional ones as judged from the coloration of the released trityl cation. After the recommended workup, they were purified by reversed phase (RP)-HPLC and characterized by MALDI-TOFMS (Autoflex II, BRUKER DALTONICS). Purities of all the modified ODNs were >99% estimated by RP-HPLC analysis.

MALDI-TOFMS data for the ODNs: **ODN1**: calcd for [**ODN1**+H$^+$]: 4118; found: 4118; **ODN2**: calcd for [**ODN2**+H$^+$]: 4992; found: 4992; **ODN3**: calcd for [**ODN3**+H$^+$]: 4992; found: 4992; **ODN4**: calcd for [**ODN4**+H$^+$]: 5866; found: 5866; **ODN5**: calcd for [**ODN5**+H$^+$]: 4904; found: 4904; **ODN6**: calcd for [**ODN6**+H$^+$]: 5341; found: 5341; **ODN7**: calcd for [**ODN7**+H$^+$]: 5778; found: 5778; **ODN8**: calcd for [**ODN8**+H$^+$]: 5778; found: 5778; **ODN9**: calcd for [**ODN9**+H$^+$]: 5778; found: 5779; **ODN10**: calcd for [**ODN10**+H$^+$]: 6653; found: 6654; **ODN11**: calcd for [**ODN11**+H$^+$]: 6653; found: 6652; **ODN2d**: calcd for [**ODN2d**+H$^+$]: 4519; found: 4518; **ODN3d**: calcd for [**ODN3d**+H$^+$]: 4519; found: 4518; **ODN4d**: calcd for [**ODN4d**+H$^+$]: 5393; found: 5394; **ODN7d**: calcd for [**ODN7d**+H$^+$]: 4832; found: 4832; **ODN8d**: calcd for [**ODN8d**+H$^+$]: 4832; found: 4832; **ODN10d**: calcd for [**ODN10d**+H$^+$]: 5706; found: 5705.

Spectroscopic measurements: CD spectra were measured on a JASCO model J-820 with a 10 mm quartz cell that was equipped with programmed temperature controllers. A slight excess of complementary strand was used to ensure that all the probe ODN formed duplex. The sample solutions were as follows: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [**ODN7**] = 1.0 μM, [**C2**] = 1.2 μM.

Fluorescence spectra were measured on a JASCO model FP-6500 with a microcell. The excitation wavelength was 345 nm. For FRET analysis, experiments were conducted at 0°C to ensure sufficient hybridization. The sample solutions were as follows (unless otherwise noted): [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [**ODN*n***] = 1.0 μM, [**C*n***] = 1.2 μM.

Measurement of the melting temperature: The melting curve of duplex DNA was obtained with a Shimadzu UV-1800 by measurement of the change in absorbance at 260 nm versus temperature. The melting temperature (T_m) was determined from the maximum in the first derivative of the melting curve. Both the heating and cooling curves were measured, and the calculated T_m values agreed to within 2.0°C. The temperature ramp was 0.5°C min^{-1}. The sample solutions were as follows (unless otherwise noted): [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [**ODN*n***] = [**C*n***] = 5.0 μM.

Calculation of FRET efficiencies: FRET efficiencies (Φ_T) were calculated according to Equation (3):[40]

$$\Phi_T = \frac{A_A}{A_D}\left(\frac{I_{AD}}{I_A} - 1\right) \quad (3)$$

in which A_A is the absorbance at 345 nm of sequences which have only acceptor(s) (**ODN1/C1** or **ODN5/C2**). A_D is the absorbance at 345 nm of duplexes without acceptors (**ODN*n*d/C1** or **C2**). I_{AD} and I_A are the fluorescence intensities at the acceptor emission wavelength (460 nm) of sequences in the presence (**ODN2–4/C1** or **ODN7**, **8** or **10**/**C2**) or absence (**ODN1/C1** or **ODN5/C2**) of donors, respectively.

Molecular modeling: The Insight II/Discover 98.0 program package was used for conformational energy minimization. ODNs modified by perylene and pyrene were built from canonical B-form DNA by a graphical program. The AMBER95 force field was used for the calculation. All the structures were energy-minimized to an RMS derivative of <0.001 kcal Å^{-1}. Computations were carried out on a Silicon Graphics O2+ workstation with the operating system IRIX64 Release 6.5.

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[31] The wavelength of UV light for excitation ($\lambda = 345$ nm) might be too short especially for in vivo analysis because it potentially damages other biomolecules.
[32] Incorporation of a mismatch into **ODN4**/**C1** lowered the melting temperature although the degree of destabilization was dependent on the position of the mismatch. See Table S3 in the Supporting Information for melting temperatures.
[33] Electron transfer between pyrene and perylene is rather unlikely because location of pyrene near the perylene did not quench the fluorescence from perylene. Furthermore, quenching of pyrene was not necessarily facilitated by approximating pyrene to perylene (compare the fluorescence from pyrene ($\lambda = 370$–430 nm) of **ODN8/C2** and **ODN9/C2** in Figure 2).
[34] When **ODN7** was hybridized with full-matched **C2**, peaks of pyrene at 330–360 nm showed bathochromicity in absorption spectra, which indicated that pyrenes were intercalated between base pairs. On the other hand, peaks of perylene slightly shifted to shorter wavelength. This shift can be attributed to the change of dimeric perylene in the single-stranded state to monomeric state in the duplex with **C2**. See Figures S2 and S3 in the Supporting Information for UV/Vis and emission spectra. For the relationship between bathochromicity and intercalation, see: W. Müller, D. M. Crothers, *J. Mol. Biol.* **1968**, *35*, 251–290.
[35] Although the change in I_{550}/I_{461} was not so large, the difference in fluorescence can be discriminated by the naked eye. See Figure S4 in the Supporting Information for the photograph.
[36] The difference in I_{550}/I_{461} was enhanced with increasing ionic strength. See Figure S5 in the Supporting Information.
[37] The probe presented in this paper also emits fluorescence even in the single-stranded state. This emission should disturb the detection of the target especially when the amount of the target is small.
[38] The I_{550}/I_{461} ratio of **ODN7/C7** (5′-GGTAT**P**C**E**A**E**G**P**CAATC-3′/3′-CCATAG***G***CGTTAG-5′) containing an A–G mismatch was larger than that of full-matched **ODN7/C2**, but smaller than **ODN7/C1** containing one-base deletion. The increase in the I_{550}/I_{461} ratio by introducing mismatch is attributable to the disordering around the intervening bases that facilitated the dimerization of perylene. These results also support proper base pairing of "full match" even though multiple perylenes and pyrenes were incorporated in the nearest neighboring positions. See Table S4 in the Supporting Information.
[39] In other reports, chromophores were mainly introduced into grooves or outside duplex. Consequently, this structural problem could be avoided in those reports.
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[41] The distance between the donor and acceptor was about 13 Å, estimated from the energy-minimized structure shown in Figure 5.
[42] CD spectra indicated that both **ODN2**/**C1** and **ODN3**/**C1** took similar B-DNA structures. See Figure S7 in the Supporting Information.
[43] When the chromophores are stacked in the parallel plane, the orientation factor, κ, is determined from the following equation: $\kappa^2 = \cos^2\theta$, in which θ is the angle between the transition moments of the dyes. Since R_0 is proportional to sixth root of κ^2, the κ contributes to R_0 significantly only when θ is close to 90°. For example, in the case of $\theta = 13°$, R_0 increases only 6% relative to that calculated by assuming κ^2 as 2/3. On the other hand, R_0 decreased 25% in the case of $\theta = 70°$.
[44] In our study, peaks of pyrene were substantially weaker than those of perylene.
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[50] We believe that all the chromophores were intercalated within the duplex because of the following reasons. No obvious excimer or exciplex emission was observed even with **ODN10**/**C2**, which has six chromophores in the nearest-neighboring positions. If the chromophores were flipped out from the duplex, they should form an excimer or exciplex, because flipped out hydrophobic chromophores with flexible ethylene or propylene linkers prefer to be stacked against each other in the aqueous environment. Similar results were also reported by Murakami and Yamana (M. Nakamura, Y. Shimomura, Y. Ohtoshi, K. Sasa, H. Hayashi, H. Nakano, K. Yamana, *Org. Biomol. Chem.* **2007**, *5*, 1945–1951, A. Mahara, R. Iwase, S. Sakamoto, K. Yamana, T. Yamaoka, A. Murakami, *Angew. Chem.* **2002**, *114*, 3800–3802; *Angew. Chem. Int. Ed.* **2002**, *41*, 3648–3650). They demonstrated that bispyrene flipped out from the duplex was assembled to form an excimer, whereas intercalated bispyrene showed only monomer emission as observed in our case. However, we cannot completely rule out the possibility that some chromophores were flipped out without forming an excimer and exciplex because of the lack of direct evidence of intercalated structure.

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