Insertion of two pyrene moieties into oligodeoxyribonucleotides for the efficient detection of deletion polymorphisms†

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For the detection of deletion polymorphisms, two pyrene moieties are tethered to an oligodeoxyribonucleotide (ODN) on both sides of the intervening base; one- and two-base deletions can be selectively detected by the strength of the excimer emission.

After the decoding of the human genome, more than 300,000 single nucleotide polymorphisms (SNPs) are estimated to be in the genome.[1] In addition, there are also insertion/deletion (*indel*) polymorphisms related to genetic diseases.[2] The relationship between these differences and genetic diseases has been elucidated. To develop an effective method for gene diagnosis, various kinds of fluorescent probes have been synthesized for SNPs detection.[3] However, there are only a few reports on the methods to detect *indel* polymorphisms.[4,5] Furthermore, a fluorescent probe that detects deletion or insertion of two bases or more has not yet been reported. Here, we propose a new method for the effective detection of a deletion mutant.

For detection of *indel* polymorphism, we designed a probe DNA as depicted in Scheme 1a by use of pyrene as a fluorophore.[6] Two pyrene moieties are tethered to an oligodeoxyribonucleotide (ODN) on both sides of the nucleotide of which we want to detect the deletion (such as **PAP** in Scheme 1b). When wild type ODN (**T1** in Scheme 1b) is hybridized, both pyrene moieties intercalate and thus interaction between the two pyrenes is suppressed by the intervening base-pair. As a result, only monomer emission should be observed from the duplex with wild type (see Wild type of Scheme 1a). On the other hand, on hybridization of this probe ODN with a deletion mutant that lacks one nucleotide (**N** in Scheme 1b), a three-base bulge (including two pyrene moieties) will be formed. In this case, two pyrene moieties should be close enough to exhibit excimer emission (see Deletion mutant of Scheme 1a). Therefore, one-base deletion should easily be distinguished by monitoring excimer emission. Furthermore, this strategy can be expanded to the detection of "more than one-base deletion" by increasing the number of intervening bases.

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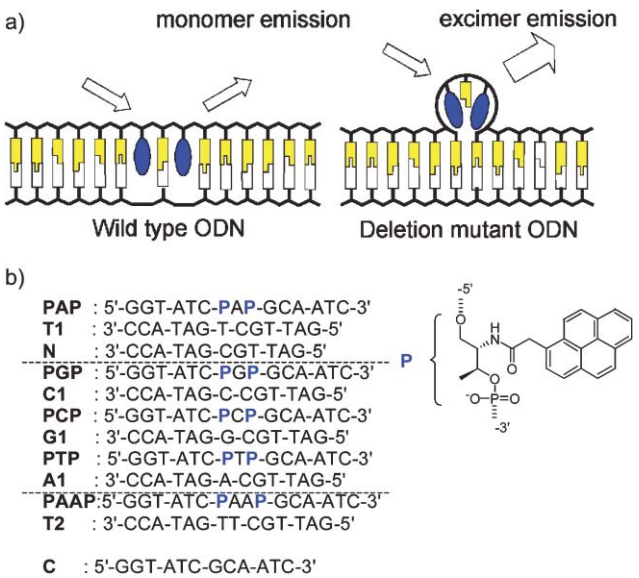
† Electronic supplementary information (ESI) available: Experimental procedures for synthesis of pyrene-modified oligodeoxyribonucleotides, emission spectra of ODN containing one pyrene moiety, fluorescent excitation spectra of **PAP/N** and detection of three-base deletion. See DOI: 10.1039/b604776a

In order to tether pyrene moieties to ODN, we used a D-threoninol as a linker because it promotes the intercalation of a chromophore as demonstrated in our previous papers.[7] A methylene group was inserted between the pyrene and the amide bond to facilitate the excimer formation in the bulge. Modified ODNs, listed in Scheme 1b, were synthesized using standard phosphoramidite chemistry.‡

Fig. 1 depicts emission spectra of **PAP**, **PAP/N**, and **PAP/T1** at 0 °C where the duplexes are formed.§ In the single-stranded state without **N** or **T1** (dotted line in Fig. 1), **PAP** showed strong monomer emission at around 380 nm and 400 nm. This strong emission indicates the pyrenes were in a hydrophilic environment because monomer emission of pyrene is usually enhanced in a polar solvent. In addition, excimer emission at around 480 nm from **PAP** was very low, indicating that, even in the single-stranded state, a nucleotide between the two **P** residues efficiently inhibited the excimer formation.[8]

When the wild type ODN (**T1**) was added to **PAP**, monomer emission decreased and excimer emission was completely suppressed (compare broken line with dotted line in Fig. 1). These results indicate that two pyrene moieties intercalate between each neighboring base-pair and are detached from each other by the intervening base-pair.[9]



Scheme 1 (a) Schematic illustration of the discrimination between wild type and one-base deleted sequence. (b) Modified DNA synthesized in this study.

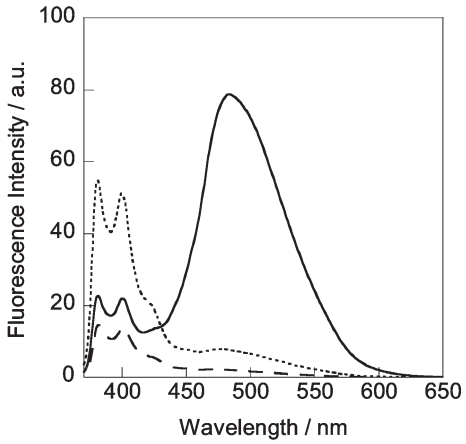


Fig. 1 Fluorescent emission spectra of **PAP/N** (solid line), single-stranded **PAP** (dotted line) and **PAP/T1** (broken line) at 0 °C. Excitation wavelength was 345 nm. Melting temperatures (T_ms) were **PAP/N**: 40.8 °C, **PAP/T1**: 45.5 °C. T_m of native duplex **C/N** was 47.7 °C.

In contrast, hybridization with a one-base deletion mutant (**N**) generated strong excimer emission at around 480 nm as we designed (see solid line in Fig. 1). These results demonstrate that two pyrenes were located in close proximity in the bulge structure, which facilitated the excimer formation. The red shift observed in the excitation spectrum also indicates that two pyrenes formed a dimer in the ground state.[10,11] The intensity of **PAP/N** at 500 nm was about 45-fold higher than that of **PAP/T1**. Thus, one-base deletion was easily distinguished by monitoring the strength of the excimer emission.

This probe design is also applicable to the detection of other nucleotides as depicted in Fig. 2. In **PGP/C1**, where an AT pair in **PAP/T1** was replaced with a GC pair, excimer emission was almost suppressed. As expected, very intense excimer emission was observed for the **PGP/N** duplex in which one cytosine was deleted (see red lines in Fig. 2). In the case of **PCP** or **PTP** in which a pyrimidine base was sandwiched with two pyrenes, one-base deletion was also detectable (see green and blue lines in Fig. 2),[12]

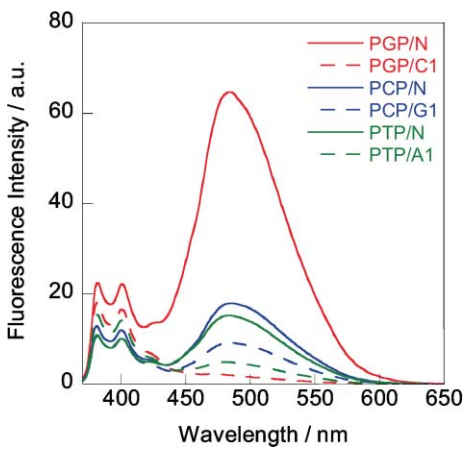


Fig. 2 Effect of the kind of intervening nucleotide on the fluorescent properties by hybridization with either one-base deletion mutants (solid lines) or wild types (broken lines) at 0 °C. Excitation wavelength was 345 nm. T_ms were **PGP/N**: 39.9 °C, **PGP/C1**: 46.8 °C, **PCP/N**: 39.8 °C, **PCP/G1**: 45.0 °C, **PTP/N**: 41.1 °C and **PTP/A1**: 41.4 °C.

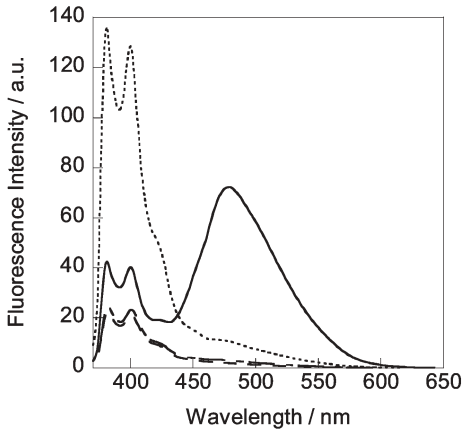


Fig. 3 Fluorescent emission spectra of **PAAP/N** (solid line), single-stranded **PAAP** (dotted line), **PAAP/T1** (dash-dotted line) and **PAAP/T2** (broken line) at 0 °C. Excitation wavelength was 345 nm. T_ms were **PAAP/N**: 36.6 °C, **PAAP/T1**: 44.2 °C and **PAAP/T2**: 48.4 °C.

although the difference in excimer emission between wild type and one-base deletion mutant was not as large as for **PAP** or **PGP**.[13,14]

An advantage of this method is that we can design a probe that detects the deletion of two bases (or even more). For example, **PAAP**, which has two intervening adenines between the two pyrenes, can detect two-base deletion in a similar manner to the **PAP** probe. When **PAAP** was hybridized with wild type ODN (**T2**), excimer emission was completely suppressed (see broken line in Fig. 3). Interestingly, strong excimer emission was observed only when **PAAP** was hybridized with **N** in which two thymidines were deleted. A hybrid of **PAAP** with **T1** (one-base deletion mutant) did not exhibit such excimer emission at all (see dash-dotted line in Fig. 3). Therefore, two-base deletion is selectively detectable by this probe.[15]

In conclusion, we have developed a simple but efficient probe for the detection of deletion polymorphisms. In the present paper, excimer formation was utilized for the probe design. But an exciplex or fluorophore-quencher combination should also be available for this probe design and much more distinct discrimination could be expected.

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Notes and references

‡ See Supplementary Information for synthesis of pyrene phosphoramidite monomer. All the modified DNAs listed in Scheme 1b were purified by reversed-phase HPLC and characterized by MALDI-TOFMS. MALDI-TOFMS for **PAP**: obsd. 4776 (calcd. for [**PAP** + H^+]: 4776), **PGP**: obsd. 4792 (calcd. for [**PGP** + H^+]: 4792), **PCP**: obsd. 4752 (calcd. for [**PCP** + H^+]: 4752), **PTP**: obsd. 4767 (calcd. for [**PTP** + H^+]: 4767), **PAAP**: obsd. 5089 (calcd. for [**PAAP** + H^+]: 5089).

§ Conditions of the sample solutions were as follows: [NaCl] = 0.1 M, pH 7.0 (10 mM phosphate buffer), [DNA] = 5 μM. The T_m value was determined from the maximum in the first derivative of the melting curve, which was obtained by measuring the absorbance at 260 nm as a function of temperature. The temperature ramp was 1 °C min^{-1}.

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8 We attributed low excimer emission to the effective separation of two pyrenes by the intervening base. But quenching of excimer by water would also contribute to this low emission.
9 The decrease in the monomer emission can also be interpreted as the intercalation of two pyrenes into the duplex. The intensity of monomer emission from **PAP/T1** was even lower than that from one pyrene moiety in a single-stranded ODN. See Supplemental Figure 1† for the emission spectra of this ODN containing one pyrene.
10 The peak of the excitation spectrum monitored at 483 nm (excimer emission) showed a red shift of 4 nm compared with the spectrum monitored at 377 nm (monomer emission), indicating that two pyrenes formed a "static excimer". See Supplemental Figure 2† for actual excitation spectra and Ref. 11.
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12 This small difference observed for **PCP** or **PTP** does not cause a serious problem in the practical application, because we just switch the target to its complementary strand. For example, we can also use 3′-CCA-TAG-**PGP**-CGT-TAG-5′ instead of **PCP** in Scheme 1b to detect G–C pair deletion.
13 Stronger quenching of excimer emission by pyrimidines (**PCP/N** and **PTP/N**) than by purines (**PAP/N** and **PGP/N**) may be attributed to the kinds of intervening nucleic acid bases. The order of pyrene-quenching activity is reported as A < G < T < C. In addition, low excimer emission was observed in **PCP/G1** and **PTP/A1**. This emission will be due to the smaller steric hindrance of pyrimidine bases. See Ref. 14 for the quenching activity of natural bases.
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15 Three-base deletion can also be detected by changing the pyrene monomer from 1-pyreneacetic acid to 1-pyrenebutyric acid. See Supplemental Figure 3† for the actual emission spectra.

