Dual-labeled oligonucleotide probe for sensing adenosine *via* FRET: A novel alternative to SNPs genotyping†

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A novel FRET based strategy for DNA sequence analysis utilising base-discriminating fluorescence (BDF) nucleoside, ^{Py}U/$^{2\text{-}Ant}$U, as donor in the dual-labelled oligonucleotide probe is reported; a selective/specific emission from acceptor, was observed upon excitation at the donor, only when the opposite base of the "smart" fluorescently labeled BDF nucleoside, ^{Py}U/$^{2\text{-}Ant}$U, is adenine on the complementary target sequence.

The alteration of DNA sequence by a single base, known as single nucleotide polymorphisms (SNPs),[1] is of immense interest to the scientific community of every sphere as SNPs are nowadays an attractive target for better understanding the genetic basis of complex diseases, and to realize the potential of pharmacogenetics. A great deal of effort has been paid by various research groups towards the development of SNP genotyping technologies for several years. While a lot of different SNP typing protocols, chemistry and platforms have become available, there is no single protocol available that meets all research needs.

Fluorescence sensing is one of the most promising research tools for SNP genotyping and has provided novel and unique detection systems, such as the TaqMan assay,[2] molecular beacons,[3] template-directed dye-terminator incorporation (TDI),[4] allele-specific hybridization,[5] hybridization probe assay,[6] invader assay[7a] and allele-specific PCR[7b] with universal energy-transfer-labeled primer. Fluorescence resonance energy transfer (FRET) is a powerful tool for elucidating DNA structure and dynamics by probing both distance and orientation between donor and acceptor fluorophores.[8] Hence, it is widely used as a detection method for genotyping on a large scale mainly because of its simplicity.

Our effort in designing base-discriminating fluorescence nucleosides (BDF),[9] leads us to develop a dual-labeled oligonucleotide probe in which the BDF nucleoside, ^{Py}U act as the donor separated by a defined base pair distance from the acceptor, fluorescein, attached to 5′-end of the probe. The concept of our design was as follows: (a) to observe the FRET process between the donor and the acceptor fluorophore if at all and (b) to explore the specificity/selectivity in sensing a single base alteration from the target ODNs *via* drastic enhancement of fluorescence intensity of the acceptor fluorophore at longer wavelength upon excitation of the donor. Thus, we have incorporated fluorescein at 5′-position of pyrene labeled BDF oligonucleotide probes in which the position of the BDF nucleoside, ^{Py}U, differs from the acceptor by defined base pair distances. In doing so, we observed a strong fluorescence resonance energy transfer from ^{Py}U to fluorescein when the complementary base opposite to BDF nucleoside, is adenine.

To achieve our goal, we have synthesized the BDF nucleoside ^{Py}U, (Fig. 1) in usual route adopted in our laboratory.[9a] We have previously reported the fluorescence properties of a ^{Py}U labeled oligonucleotide ODN (^{Py}U) probe and shown that it is extremely useful for typing single nucleotide polymorphisms (SNPs) where the opposite base is adenine.[9a] However, the problem is its shorter wavelength emission that limits its utility in DNA chip based detection techniques or in a cell. To solve this problem, we thought that if we could attach an acceptor fluorophore at the 5′-end and place the BDF nucleoside, ^{Py}U, separated by a certain defined number of base pair distances from the acceptor within the probe sequences, then there is a possibility of a FRET process occurring, and as a result the fluorescence emission from the acceptor at a longer wavelength is observable. For this purpose we chose fluorescein as the energy acceptor.

To check whether there is a possibility to observe FRET process, we have taken the emission spectrum of ^{Py}U and the excitation spectrum of fluorescein (**F**). We observed that the fluorescence emission spectrum of ^{Py}U ($\lambda_{max,em}$ = 405 nm) overlapped with the excitation spectrum of fluorescein ($\lambda_{max,ex}$ = 501 nm) in the wavelength range 400–500 nm (Fig. 2a). Thus, it is clear that the interaction between these two fluorophores separated by defined base pairs, might allow an energy transfer from ^{Py}U to fluorescein that results in a dominant fluorescence emission of fluorescein at 520 nm when excited at 340 nm and hence these hybrid probes can be used for SNP typing at longer wavelength. To explore our strategy, we have designed a series of such hybrid

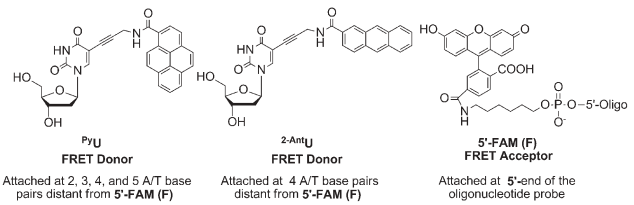

Fig. 1 Chemical structures of the two BDF nucleosides, ^{Py}U and $^{2\text{-}Ant}$U, as donors and the FAM (**F**) as the acceptor fluorophore.

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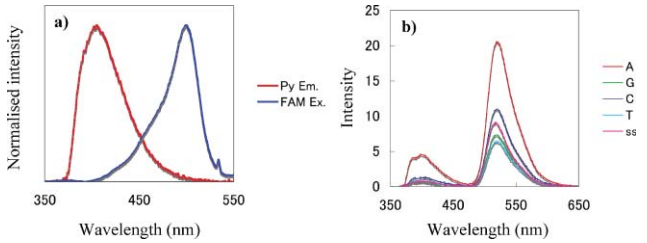

Fig. 2 (a) Overlap of the emission spectrum of ODN **3a** [ODN (2-^{Py}U)] (red) and the excitation spectrum of FAM (blue). (b) Fluorescence spectra of single stranded ODN **5** [ODN (**F**4-^{Py}U)] (2.5 μM) and the duplexes formed by hybridization with ODN **5b** {[ODN (4-**N**)] (**N** = A, C, G, T)} (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, RT). Excitation wavelength was 340 nm. "ss"denotes single-stranded ODN **5**.

probes containing ^{Py}U as the FRET donor and fluorescein (**F**) as the acceptor separated by two [ODN (**F**2-^{Py}U)], three [ODN (**F**3-^{Py}U)], four [ODN (**F**4-^{Py}U)], five [ODN (**F**5-^{Py}U)] and eight [ODN (**F**8-^{Py}U)] A/T base pairs (Table 1). The basis of such design was to know the dependency of base pair separation on the efficiency of FRET process and thereby the selectivity/sensitivity in recognizing a single matched base opposite to the BDF base (^{Py}U) from the target complementary sequence.

After synthesizing and characterizing the ODNs by MALDI-TOF mass spectrometry, we have investigated the fluorescence properties of all the ODNs in the presence of their complementary target ODNs. In all cases we found an enhanced fluorescence emission from the acceptor fluorophore when the base opposite to BDF base, ^{Py}U, is adenine in its target sequence. But the selectivity in sensing the opposite base from their target sequences differs as the distance between the acceptor and donor fluorophores changes. The maximum selectivity in sensing the opposite matched base, adenine, from its target sequence was observed for probe ODN **5** [ODN (**F**4-^{Py}U)] where the ^{Py}U and fluorescein are separated by four A/T base pairs. For better understanding we have calculated the quenching efficiency (Q_f) of ^{Py}U in the duplex ODN **5/5b** [ODN (**F**4-^{Py}U)/ODN (4-**A**)] that was 88%. The fluorescence lifetime of ^{Py}U in the matched duplex, ODN **5/5b** [ODN (**F**4-^{Py}U)/ODN (4-**A**)] obtained after excitation at 352 nm is

Table 2 Melting temperature and fluorescence properties of duplexes

Duplexes	T_m/°C	τ_1/ns (%)[a]	τ_2/ns (%)[b]	τ_M[c]	Q_f[d]	τ_{rise}/ns
ODN 3/3b	54.74 (**N** = **A**)	0.599 (69)	4.363 (31)	1.77	95%	0.359
ODN 3a/3b	59.50 (**N** = **A**)		7.913			
ODN 4/4b	54.76 (**N** = **A**)	0.700 (73)	2.248 (27)	1.12	93%	0.799
ODN 4a/4b	59.65 (**N** = **A**)		8.920			
ODN 5/5b	56.48 (**N** = **A**)	0.995 (66)	3.800 (34)	1.95	88%	1.023
ODN 5a/5b	57.46 (**N** = **A**)		8.458			
ODN 5c/5b	61.52 (**N** = **A**)	0.843 (81)	4.866 (19)	1.61	99.9%	
ODN 5d/5b	57.54 (**N** = **A**)		22.615			
ODN 6/6b	59.50 (**N** = **A**)	1.758 (88)	7.190 (12)	2.41	85%	1.563
ODN 6a/6b	59.96 (**N** = **A**)		8.788			
ODN 7/7b	62.34 (**N** = **A**)	1.638 (26)	5.308 (74)	4.35	64%	2.695
ODN 7a/7b	61.86 (**N** = **A**)		7.051			

[a] τ_1 is the decay time of ^{Py}U which is in interaction with the acceptor *via* FRET. [b] τ_2 is the decay time of ^{Py}U which is free from interaction *via* FRET with the acceptor. [c] τ_M is the mean decay time of ^{Py}U. [d] Q_f is the FRET quenching efficiency = $[(1 - F_{DA}/F_D) \times 100]$; where F_{DA} is the fluorescence intensity of the duplexes in which the probe ODN contains both the donor and the acceptor and F_D is the same without acceptor.

0.995 ns, which is much shorter than that of the duplex ODN **5a/5b** [ODN (4-^{Py}U)/ODN (4-**A**)] (8.458 ns), in which the probe does not possess a FRET acceptor fluorescein (Table 2). These observations suggest that effective energy transfer from ^{Py}U to fluorescein occurs in matched duplex ODN **5/5b** [ODN (**F**4-^{Py}U)/ODN (4-**A**)]. In contrast, the hybridization of ODN **5** [ODN (**F**4-^{Py}U)] with ODN **5b** [ODN (**N**-4)], where **N** is T, G, or C, resulted in a weaker emission (Fig. 2b).

From the fluorescence behavior it is clear that the selective fluorescence emission from the probe ODN **5** [ODN (**F**4-^{Py}U)] *via* FRET occurs when the opposite base of ^{Py}U is adenine in its complementary target sequence. In addition, it is notable that the fluorescence of pyrene in the dual-labeled single-stranded oligonucleotide probe ODN **5** [ODN (**F**4-^{Py}U)] is ten times suppressed in comparison with the strong fluorescence emission from pyrene in the single-stranded ODN **5a** [ODN (4-^{Py}U)] in which there is no fluorescence acceptor; thus indicating a FRET is occuring. From the difference in fluorescence intensities, it is clear that the fluorescence emission from the matched duplex ODN **5/5b** [ODN (**F**4-^{Py}U)/ODN (4-**A**)] was very strong and clearly distinguishable

Table 1 Oligonucleotide sequences used in this study

ODNs		Sequences	
1	[ODN (^{Py}U)]	5′-d(CGCAAT^{Py}UTAACGC)-3′	
2	[ODN (N)]	5′-d(GCGTTANATTGCG)-3′	[**N** = A, T, G, C]
3	[ODN (**F**2-^{Py}U)]	5′-d(**F**-AT^{Py}UTAACGCACACG)-3′	
3a	[ODN (2-^{Py}U)]	5′-d(AT^{Py}UTAACGCACACG)-3′	
3b	[ODN (2-N)]	5′-d(CGTGTGCGTTANAT)-3′	[**N** = A, T, G, C]
4	[ODN (**F**3-^{Py}U)]	5′-d(**F**-AAT^{Py}UTAACGCACACG)-3′	
4a	[ODN (3-^{Py}U)]	5′-d(AAT^{Py}UTAACGCACACG)-3′	
4b	[ODN (3-N)]	5′-d(CGTGTGCGTTANATT)-3′	[**N** = A, T, G, C]
5	[ODN (**F**4-^{Py}U)]	5′-d(**F**-AAAT^{Py}UTAACGCACACG)-3′	
5a	[ODN (4-^{Py}U)]	5′-d(AAAT^{Py}UTAACGCACACG)-3′	
5b	[ODN (4-N)]	5′-d(CGTGTGCGTTANATTT)-3′	[**N** = A, T, G, C]
5c	[ODN (**F**4-$^{2\text{-Ant}}$U)]	5′-d(**F**-AAAT$^{2\text{-Ant}}$UTAACGCACACG)-3′	
5d	[ODN (4-$^{2\text{-Ant}}$U)]	5′-d(AAAT$^{2\text{-Ant}}$UTAACGCACACG)-3′	
6	[ODN (**F**5-^{Py}U)]	5′-d(**F**-AAAAT^{Py}UTAACGCACACG)-3′	
6a	[ODN (5-^{Py}U)]	5′-d(AAAAT^{Py}UTAACGCACACG)-3′	
6b	[ODN (5-N)]	5′-d(CGTGTGCGTTANATTTT)-3′	[**N** = A, T, G, C]
7	[ODN (**F**8-^{Py}U)]	5′-d(**F**-AAAAAAAT^{Py}UTAACGCACACG-3′	
7a	[ODN (8-^{Py}U)]	5′-d(AAAAAAAT^{Py}UTAACGCACACG)-3′	
7b	[ODN(8-N)]	5′-d(CGTGTGCGTTANATTTTTTT)-3′	[**N** = A, T, G, C]

from the weak fluorescence arises from the other mismatched duplexes (Fig. 2b). Therefore, the dual labeled probe, ODN **5** [ODN (**F**4-^{Py}**U**)] is capable of sensing the opposite base adenine selectively. Thus, the method that makes use of the interaction between ^{Py}**U** and fluorescein constitutes a useful tool for typing single nucleotide alterations where adenine base is concerned.

To correlate the distance in terms of base pair separation and the efficiency of FRET process in detail, we compared the fluorescence properties of matched duplex ODN **5/5b** [ODN (**F**4-^{Py}**U**)/ODN (4-**A**)] with other matched duplexes where the two fluorophores are separated by two, three, five, or eight A/T base pairs in the corresponding probes (Table 2). In all the cases, fluorescence quenching efficiency in dual labeled ODNs decreases as the distance between donor and acceptor increases from two to five and to eight A/T base pairs and the quenching efficiencies lie in the range 85–95% (from five to two A/T base pairs) and drop drastically to 64% as the distance increased to eight A/T base pairs. The decrease in FRET efficiency was also reflected in the fluorescence decay profile of all the matched duplex ODNs. For example, the fluorescence decay profile of duplex ODN **5/5b** [ODN (**F**4-^{Py}**U**)/ODN (4-**A**)] was fitted to a bi-exponential function (ESI, Fig. S7†). The major short-lived component corresponds to the ^{Py}**U** molecules which are in interaction with the acceptor *via* FRET, had an extended lifetime [(τ_1 = 0.995 ns (66%)], and a new longer lifetime component [(τ_2 = 3.800 ns (34%)], corresponding to the ^{Py}**U** molecules which are free from interaction *via* FRET with the acceptor, appeared. From the fluorescence emission it is clear that the sensitivity in recognizing the opposite complementary base is maximum when the donor and the acceptor remain in an intermediate distance (four A/T base pairs), and the FRET quenching efficiency is of intermediate value, *i.e.*, in case of probe ODN **5** [ODN (**F**4-^{Py}**U**)]. When the donor and the acceptor are separated by too short or too long distance, *i.e.*, two, three, and five, or eight A/T base pairs, a remarkable decrease in the selectivity of opposite base, adenine, from their target sequences in the fluorescence emission was observed (ESI, Fig. S5, S6†). The decrease in FRET efficiency and thereby the selectivity was more marked in the duplex in which the two fluorophores are separated by five and eight A/T base pairs, *i.e.*, in case of duplex ODNs **6/6b** [ODN (**F**5-^{Py}**U**)/ODN (5-**A**)] and ODN **7/7b** [ODN (**F**8-^{Py}**U**)/ODN (8-**A**)]. Although the FRET efficiency is maximum for the duplex, ODN **3/3b** [ODN (**F**2-^{Py}**U**)/ODN (2-**A**)], the selectivity in sensing opposite base adenine was very low when the two fluorophores are separated by very short distance, *i.e.*, separated by two A/T base pairs.

For a quantitative understanding of FRET process in the present system, we examined the rate constant of resonance energy transfer, k_{FRET}, and Förster distance, R_0, between pyrene and fluorescein. Thus, we obtain the R_0 value as about 33 Å (ESI,† Fig. S1).[8a,8b]

As we found the maximum selectivity for the probe containing ^{Py}**U** as the donor separated by four A/T base pairs from the 5′-acceptor fluorescein, we were then interested to investigate the selectivity in sensing the opposite base utilizing our previously reported BDF nucleoside $^{2\text{-Ant}}$**U** as the donor (Fig. 1).[9b] Thus, we examined the possibility of occurring FRET from $^{2\text{-Ant}}$**U** to fluorescein (ESI,†Fig. S2). We have synthesized the probe ODN **5c** [ODN (**F**4-$^{2\text{-Ant}}$**U**)] and ODN **5d** [ODN (4-$^{2\text{-Ant}}$**U**)] and the fluorescence property was compared with that of the probe ODN **5** [ODN (**F**4-^{Py}**U**)] which contains ^{Py}**U** as the donor. The selectivity of ODN **5c** [ODN (**F**4-$^{2\text{-Ant}}$**U**)] in sensing the opposite base adenine from its target sequence is almost similar as in the case of probe ODN **5** [ODN (**F**4-^{Py}**U**)] but in this case the fluorescence of $^{2\text{-Ant}}$**U** is completely quenched by fluorescein indicating almost 100% quenching efficiency (ESI,†Fig. S2). Thus, from the above observation it is clear that base selectivity is maximum when the donor (^{Py}**U**) and the acceptor fluorophore (fluorescein) were separated by four A/T base pairs and the quenching efficiency was of intermediate value. We observed very high FRET efficiency and decreased selectivity if the distance between the two fluorophores is less than four A/T base pairs or both the FRET efficiency and the selectivity decreased as the distance increased by more than four A/T base pairs. The probe ODN **5c** [ODN (**F**4-$^{2\text{-Ant}}$**U**)] show a similar behavior but with a lower selectivity in sensing the opposite base adenine with high quenching efficiency. Therefore, the results of these fluorescence measurements for a series of dual-labeled probe ODNs imply that the FRET efficiency between ^{Py}**U** or $^{2\text{-Ant}}$**U** as a FRET donor and fluorescein as an acceptor has a prominent effect in sensing the opposite base, adenine, from their target complementary ODNs *via* the increased fluorescence intensity from the acceptor fluorophore, fluorescein. Thus our present method can be useful as an alternative to the available SNP typing protocols.

In conclusion, we have synthesized several dual-labeled probe ODNs capable of sensing adenine base opposite to the BDF base, ^{Py}**U** or $^{2\text{-Ant}}$**U** in their target ODNs by an enhancement of fluorescene intensity from the acceptor fluorophore *via* FRET. Therefore, the probe containing ^{Py}**U** or $^{2\text{-Ant}}$**U** as donor and fluorescein as acceptor separated by four base pairs distances, facilitates the detection of a single nucleotide alteration in a target sequence at the wavelength of fluorescein emission and thus can potentially be useful in DNA chip based detection method or in the detection of SNPs in a cell. Current research focuses on the design of more efficient FRET pairs and their application to chip base detection technology.

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