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PAPER

Hybridization-sensitive fluorescence control in the near-infrared wavelength range

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A series of near-infrared fluorescent probes were designed based on the concept of emission control caused by interdye excitonic interaction. The fluorescent probes showed very weak emission in the unhybridized state, whereas they emitted near-infrared fluorescence after hybridization with the complementary nucleic acid. The hybridization-dependent switching of fluorescence emission made it possible to monitor mRNA in human cells in the range of near-infrared wavelengths.

Introduction

Fluorescent optical imaging is a rapidly expanding methodology for the non-invasive evaluation of disease and tumor progression, with applications in drug and biomarker development.[1] The use of near-infrared (NIR) photons is a promising approach for biomedical imaging in living tissue.[2,3] NIR dyes emit over the range 650–900 nm, a range in which the absorption coefficients of tissues are relatively low, so that the autofluorescence of biological species is minimized. These dyes also have merits, such as avoiding photobleaching of the fluorophores and using inexpensive excitation light sources.[4,5] In addition, NIR dyes show a deeper penetration ability than visible wavelength dyes. The unique character of NIR dyes is directly reflected in improved image quality,[6,7] and NIR fluorescence imaging has emerged as a powerful tool for cell and tissue imaging.

It is also important for the establishment of effective nucleic acid imaging in cells to design an NIR fluorescent probe that has the feature that the fluorescence is turned off when the probe does not recognize the target nucleic acid and turned on when the probe has hybridized the target nucleic acid. The requirements for NIR fluorescent probes for nucleic acid detection are not only sequence-selective NIR emission and the avoidance of non-specific emission, but also expansion of the observable region of wavelength operated by the new fluorescent probes. The emission must be polychromatic for the simultaneous monitoring of different targets.

Herein, we report the synthesis and photophysics of hybridization-sensitive fluorescence probes with absorption and emission in the NIR wavelength region. The probes showed switching of fluorescence emission depending on hybridization with the complementary nucleic acids. The probes made it possible to monitor mRNA in a human cell in the range of NIR wavelengths.

Results and discussion

We focused on the system used in an exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probe. The fluorescence of the probe, in which two fluorescent dyes are attached to a thymine base, is well controlled by excitonic interaction (Fig. 1).[8–10] An excitonic interaction is produced by

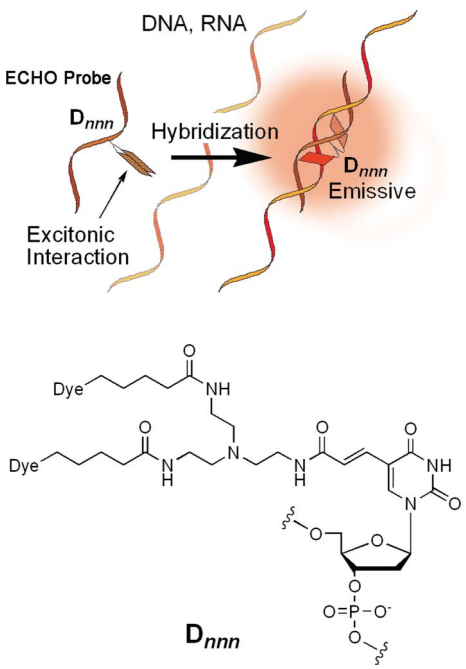

Fig. 1 An exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probe.

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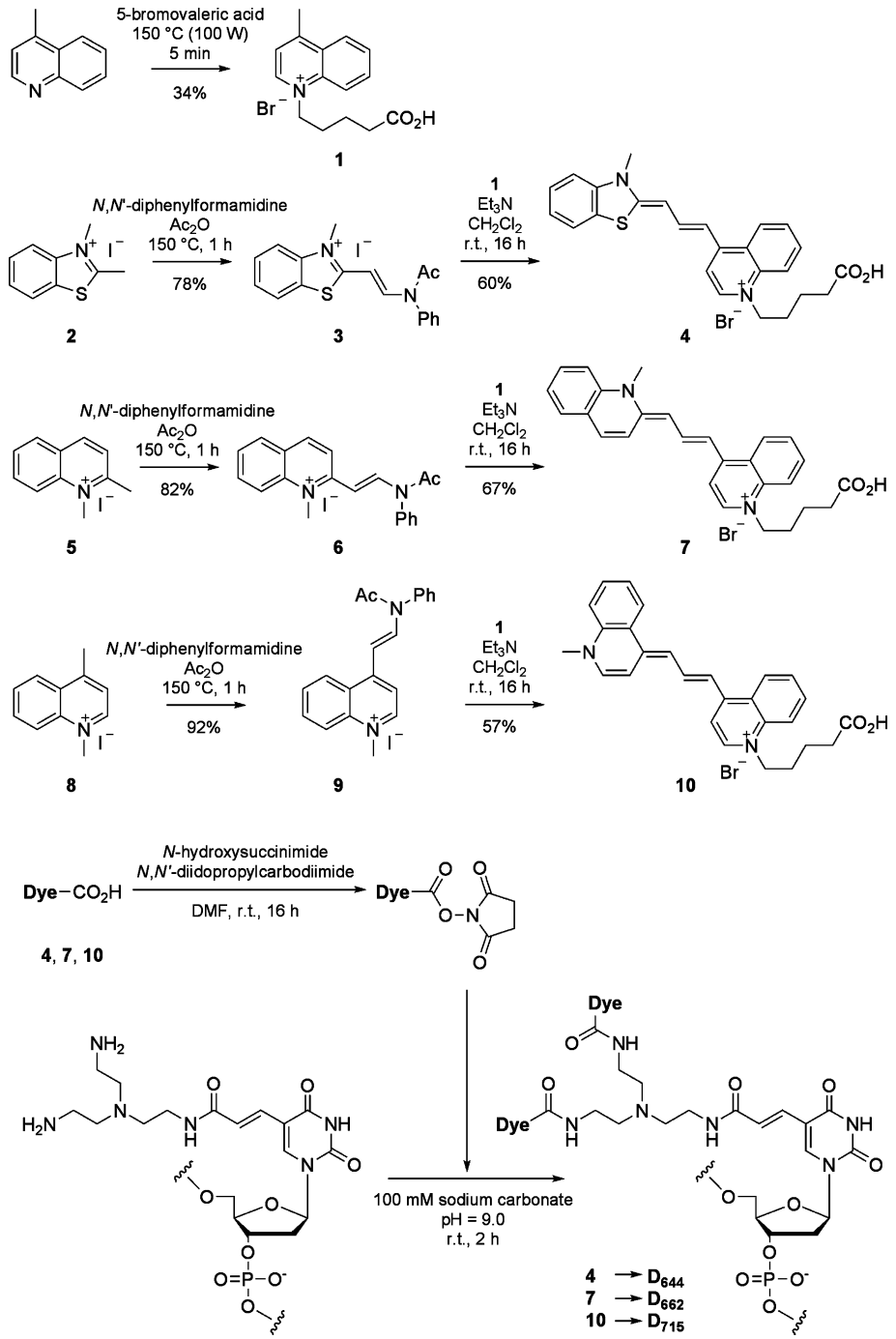

Scheme 1 Synthesis of NIR-ECHO probes.

the formation of an H-aggregate between dyes in the probe and, as a result, emission from the probe before hybridization is suppressed.[11–14] In contrast, dissociation of aggregates by hybridization with the complementary strand results in disruption of the excitonic interaction and strong emission from the hybrid. Multicoloring of the ECHO probe results in the simple simultaneous detection of multiple target nucleic acid sequences, and is useful in efforts to understand temporal correlations of gene expression and interaction between nucleic acids. However, there is no ECHO probe in which the fluorescence is controlled in the range of NIR wavelengths. For this context, new nucleotides doubly labelled with an NIR dye should be designed without loss of a high on–off performance as a hybridization-sensitive fluorescent probe based on excitonic interaction chemistry.

A series of new fluorescent nucleotides was synthesized for NIR-ECHO probes. The coupling of a quinolinium derivative with the carboxylate-ended alkyl linker **1**, prepared by the addition of 4-methylquinoline to 5-bromovaleric acid, with benzothiazole or methylquinoline subunits **3**, **6** and **9**, which were prepared by extension of the π-conjugate systems of **2**, **5** and **8** by

N,*N*′-diphenylformamidine and acetic anhydride, provided fluorescent dyes **4**, **7** and **10**, respectively (Scheme 1). The carboxylate end of the alkyl linker of the dyes was activated by succinimidylation, and subsequently two molecules of the activated dyes were incorporated into an oligodeoxyribonucleotide containing 2′-deoxyuridine with two amino ends.[7] The **4**-, **7**- and **10**-labeled nucleotides in the synthesized NIR probes were named D_{644}, D_{662} and D_{715}, respectively.

The absorption and emission properties of the synthesized NIR probes, **Probe1(D_{nnn})** 5′-d(CGCAATD$_{nnn}$TAACGC)-3′, were investigated before and after hybridization with the complementary nucleic acids. The fluorescence emission of these NIR probes was suppressed in their unhybridized states, but emission appeared immediately after the probe was added to a solution of the complementary nucleic acid (Fig. 2). Fluorescence of the hybrid with the complementary DNA was observed at 655 (Φ_f 0.29), 674 (Φ_f 0.073) and 727 nm (Φ_f 0.061) for **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, respectively. The fluorescence intensity of the hybrid was weaker when the probes were hybridized with the complementary RNA (Φ_f 0.10, 0.034 and 0.031 for **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, respectively), compared with those of the hybrid with DNA, although the fluorescence was distinguishable from the very weak fluorescence of unhybridized probes (Φ_f 0.07, 0.001 and 0.015 for **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, respectively). **Probe1(D_{662})** and **Probe1(D_{715})**, which have the dyes consisting of only quinoline moieties, showed a lower fluorescence intensity but a longer emission wavelength than **Probe1(D_{644})**, which contains a benzothiazole moiety in the dye. The use of quinoline-based fluorescent dyes may be effective for the design of the functional dyes with a longer fluorescence wavelength. The hybridization-sensitive fluorescence switching is supported by the absorption spectra. The absorption band of the unhybridized probe appears at about 50, 58 and 70 nm shorter wavelengths for **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, respectively, than that of the hybrid. This blue shift suggested a splitting of the excited state because of the formation of a bichromophoric H-aggregate. Therefore, the interdye excitonic interaction in a bichromophoric H-aggregate in the unhybridized state of the probes suppressed the fluorescence emission of the probes. This exciton-controlled fluorescence behavior was also observed for the probes with a polythymidine sequence, **Probe2(D_{nnn})** 5′-d(TTTTTTD$_{nnn}$TTTTTT)-3′. These NIR probes are ECHO probes that have the characteristic function that hybridization with the target nucleic acid resulted in a large enhancement of emission.

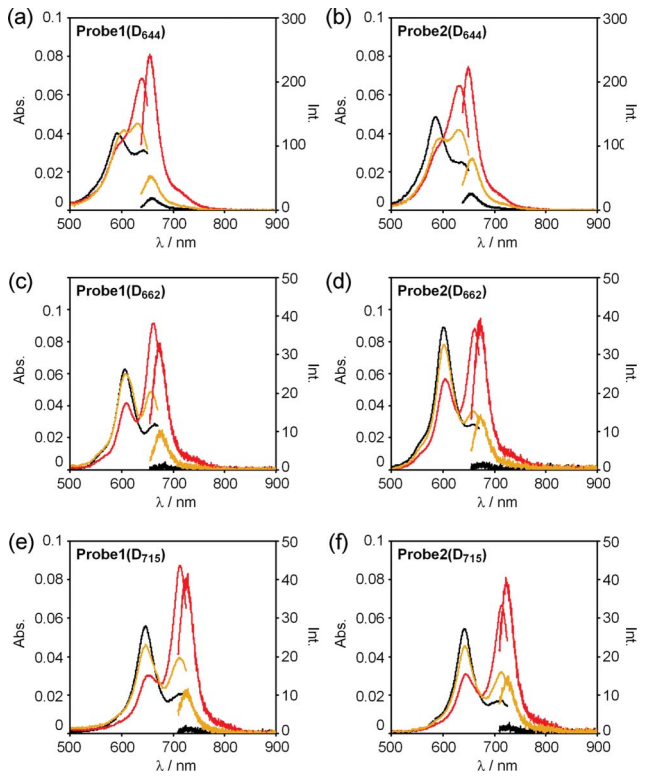

Fig. 2 Absorption and emission spectra of NIR-ECHO probes. Absorption and emission spectra of **Probe1(D_{nnn})** and **Probe2(D_{nnn})** (0.4 μM) were measured in 50 mM sodium phosphate (pH = 7.0) and 100 mM sodium chloride. λ_{ex} = 633 nm for D_{644} and D_{662}, and 694 nm for D_{715}. Black: unhybridized probes; red: probes hybridized with the complementary DNA; orange: probes hybridized with the complementary RNA.

The probes formed thermally stable duplexes with the complementary nucleic acids sufficient to detect the fluorescence emission. For example, the melting temperatures of the duplex of **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, and the complementary DNA were 58, 66 and 54 °C, respectively, which were the same as or higher than that with a natural DNA duplex (54 °C), whereas the melting temperatures of the duplex of **Probe1(D_{644})**, **Probe1(D_{662})** and **Probe1(D_{715})**, and the complementary RNA were 41, 38 and 43 °C, respectively, which were lower than that with a natural RNA duplex (43 °C). The data on duplex stability suggests that they reflect the higher fluorescence intensity from the hybrid with DNA and lower fluorescence intensity from the hybrid with RNA, as shown in Fig. 2. The duplexes formed by the probes and their complementary strands showed induced CD signals with broad negative signals and a sharp positive signal. For example, the induced CD signals were observed at 550–750 nm for the duplexes formed by **Probe1(D_{715})** and its complementary DNA (Fig. 3). These signals might result from mixed Cotton effects derived from several different binding modes of dyes to DNA, which consist of broad negative Cotton effects at 590 and 640 nm, and a sharp Cotton effect at 665 nm. The results of T_m and CD measurements suggest that the dyes of D_{nnn} strongly interact with the duplex structures after hybridization with the complementary nucleic acids. The interaction between the dye and the duplex induces dissociation of a bichromophoric aggregate in the probe, which contributes to enhancement of the fluorescence emission.

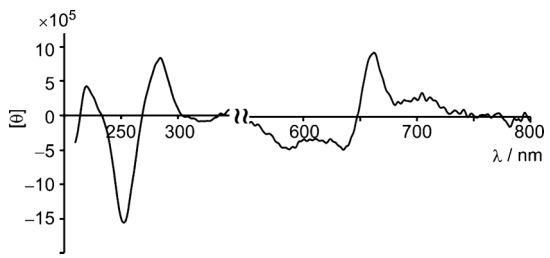

Fig. 3 CD spectrum of the duplex of **Probe1(D_{715})** and the complementary DNA. The spectrum of the duplex (10 μM) was measured in 50 mM sodium phosphate (pH = 7.0) and 100 mM sodium chloride.

Development of chemical probes for RNA imaging in living cells is essential for understanding cell life, which is greatly desired by life scientists. Having established that the NIR-ECHO probes are effective for nucleic acid detection, the fluorescence behavior of the probe was investigated in living cells. The NIR-ECHO probe capable of binding to the polyA tail of mRNA, **Probe2(D_{715})**, was transfected to HeLa cells using a common transfection reagent, Lipofectamine™ 2000, according to the protocol reported for mRNA imaging using the green-colored ECHO probe **Probe2(D_{514})**.[10,15,16] After incubation for 1 h and washing, fluorescence emission was observed through a filter at about 730 nm. The NIR fluorescence appeared clearly in transfected cells (Fig. 4). The distribution of NIR fluorescence of **Probe2(D_{715})** in cells was very similar to that of **Probe2(D_{514})** bound to mRNA in cells, reported earlier.[10,15,16]

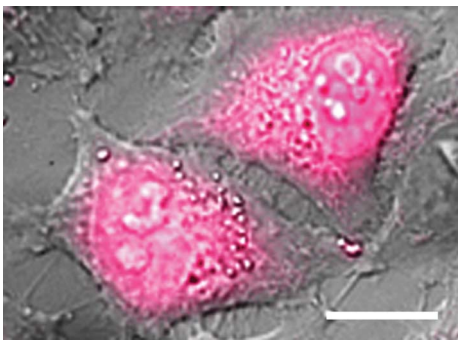

Fig. 4 Differential interference contrast and epifluorescence image of **Probe2(D_{715})**-transfected HeLa cells. The sample was excited through a 685 ± 15 nm band-pass filter and the fluorescence was collected through a 730 ± 15 nm band-pass filter. Bar: 20 μm.

Conclusions

Functional NIR probes for the detection of nucleic acids are reported in this paper. A series of NIR fluorescent probes were designed based on the concept of emission control caused by interdye excitonic interaction, and they contribute to extend the observable wavelength range operated by ECHO probes. They are the first NIR probes possessing the function that the absorption and emission were controlled at various NIR wavelengths, and this function facilitates the imaging of intracellular mRNA. Although further aspects remain to be examined in progressing toward an easier-to-use analysis, such as higher emission intensity for RNA detection, we anticipate that this new concept of hybridization-sensitive NIR probes, supported by its photochemical basis, will be the starting point for the development of a practical NIR detection in nucleic acid imaging in living organisms.

Experimental

General

^{1}H and ^{13}C NMR spectra were measured with a Varian NMR system 500. Coupling constants (J values) are reported in Hz. The chemical shifts are shown in ppm, using residual dimethyl sulfoxide (DMSO; δ = 2.49 in ^{1}H NMR, δ = 39.5 in ^{13}C NMR) as an internal standard. ESI mass spectra were recorded on a JEOL T100LC instrument. DNA was synthesized on an NTS H-6 DNA/RNA synthesizer. Reversed-phase HPLC was performed on CHEMCOBOND 5-ODS-H columns (10 × 150 mm) with a Gilson Chromatograph Model 305 using a UV detector Model 118 at 260 nm. MALDI-TOF mass spectra were measured with a Bruker Daltonics Reflex instrument. UV and fluorescence spectra were recorded on a Shimadzu UV-2550 spectrophotometer and a RF-5300PC spectrofluorophotometer, respectively.

1-(4-Carboxybutyl)-4-methylquinolinium bromide (1)

4-Methylquinoline (15.9 mL, 120 mmol) and 5-bromovaleric acid (18.1 g, 100 mmol) were stirred at 150 °C for 5 min under microwave irradiation (100 W). The reaction mixture was added to dichloromethane (1 L) with vigorous stirring. After cooling to room temperature, the solution was sonicated to obtain a white precipitate. The precipitate was filtered and dried *in vacuo*. The white powder was washed with diethyl ether (100 mL), filtered and dried *in vacuo* to give **1** as a white powder (11.1 g, 34.2 mmol, 34%): ^{1}H NMR (DMSO-d_6) δ 12.07 (br, 1H), 9.42 (d, J = 6.1, 1H), 8.60–8.53 (m, 2H), 8.26–8.23 (m, 1H), 8.08–8.03 (m, 2H), 5.02 (t, J = 7.3, 2H), 3.00 (s, 3H), 2.28 (t, J = 7.3, 2H), 1.99–1.93 (m, 2H), 1.62–1.56 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 173.9, 158.5, 148.3, 136.7, 135.0, 129.5, 128.9, 127.1, 122.6, 119.3, 56.5, 32.9, 28.8, 21.2, 19.7; HRMS (ESI) calc. for $C_{15}H_{18}NO_2$ ([M − Br]$^+$) 244.1338, found 244.1334.

2-[(*E*)-2-(*N*-Acetyl-*N*-phenylamino)ethenyl]-3-methylbenzothiazolium iodide (3)

2,3-Dimethylbenzothiazolium iodide (**2**, 2.91 g, 10 mmol) and *N*,*N*′-diphenylformamidine (3.92 g, 20 mmol) were suspended in acetic anhydride (20 mL). The suspension was stirred at 150 °C for 1 h. The reaction mixture was cooled to room temperature. Diethyl ether (150 mL) was added to the reaction mixture with stirring. The supernatant was discarded by decantation and the residue was washed with diethyl ether twice and ethyl acetate twice. Ethyl acetate was added to the residue, and the precipitate was filtered and dried *in vacuo* to give **3** as a dark red powder (3.39 g, 7.76 mmol, 78%): ^{1}H NMR (DMSO-d_6) δ 8.79 (d, J = 14.2, 1H), 8.31 (d, J = 22.0, 1H), 8.09 (d, J = 8.3, 1H), 7.80–7.52 (m, 7H), 5.68 (d, J = 13.7, 1H), 3.87 (s, 3H), 2.05 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 171.8, 170.0, 144.9, 141.6, 136.8, 130.6, 130.1, 129.0, 128.2, 127.8, 126.5, 124.0, 116.2, 96.4, 35.7, 23.2; HRMS (ESI) calc. for $C_{18}H_{17}N_2OS$ ([M − I]$^+$) 309.1062, found 309.1073.

Fluorescent dye 4

Carboxylic acid **1** (324 mg, 1.0 mmol) and **3** (436 mg, 1.0 mmol) were suspended in dichloromethane (20 mL). Triethylamine (1.4 mL, 10 mmol) was added to the suspension and the resultant solution was stirred at room temperature for 16 h. After the mixture was concentrated *in vacuo*, the residue was purified by silica gel column chromatography (1% acetic acid, 0–20% methanol–dichloromethane) to give **4** as a dark purple powder (299 mg, 0.60 mmol, 60%): ^{1}H NMR (DMSO-d_6) δ 8.39–8.38 (m, 2H), 8.10–8.00 (m, 2H), 7.90 (t, J = 7.8, 1H), 7.82 (d, J = 7.8, 1H), 7.76 (d, J = 7.1, 1H), 7.66 (t, J = 7.7, 1H), 7.50 (d, J = 8.1, 1H), 7.41 (t, J = 7.5, 1H), 7.25 (t, J = 7.5, 1H), 7.03 (d, J = 13.2, 1H), 6.47 (d, J = 12.2, 1H), 4.52 (t, J = 6.7, 2H), 3.70 (s, 3H),

2.29 (t, J = 7.3, 2H), 1.86–1.80 (m, 2H), 1.61–1.55 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 174.4, 161.5, 150.1, 143.7, 142.3, 141.8, 137.7, 133.2, 127.5, 126.6, 125.1, 124.6, 124.1, 124.0, 122.4, 117.8, 112.4, 109.4, 109.2, 98.9, 53.6, 33.4, 32.9, 28.3, 21.6; HRMS (ESI) calc. for $C_{25}H_{25}N_2O_2S$ ([M – Br]$^+$) 417.1637, found 417.1643.

2-[(*E*)-2-(*N*-Acetyl-*N*-phenylamino)ethenyl]quinoline methiodide (6)

1,2-Dimethylquinolinium iodide (**5**, 2.85 g, 10 mmol) and *N*,*N*′-diphenylformamidine (3.92 g, 20 mmol) were suspended in acetic anhydride (20 mL). The suspension was stirred at 150 °C for 1 h. The reaction mixture was cooled to room temperature. Diethyl ether (150 mL) was added to the reaction mixture with stirring. The supernatant was discarded by decantation and the residue was washed with diethyl ether twice. The residue was dissolved in a small amount of dichloromethane. The solution was added to ethyl acetate (500 mL) with stirring. The supernatant was discarded and the residue was washed with ethyl acetate twice. Ethyl acetate was added to the residue, and the precipitate was filtered and dried *in vacuo* to give **6** as a dark green powder (3.54 g, 8.22 mmol, 82%): ^{1}H NMR (CD_3OD) δ 8.98 (d, J = 13.9, 1H), 8.80 (d, J = 9.1, 1H), 8.30 (dd, J = 9.1, 0.5, 1H), 8.27 (d, J = 9.1, 1H), 8.21 (dd, J = 8.1, 1.5, 1H), 8.08 (m, 1H), 7.84 (m, 1H), 7.72–7.63 (m, 3H), 7.53–7.51 (m, 2H), 5.75 (d, J = 14.2, 1H), 4.13 (s, 3H), 2.09 (s, 3H); ^{13}C NMR (CD_3OD) δ 171.7, 158.6, 145.3, 145.1, 140.9, 139.2, 136.0, 132.0, 131.3, 129.8, 129.7, 128.9, 121.7, 119.6, 104.1, 40.0, 23.5; HRMS (ESI) calc. for $C_{20}H_{19}N_2O$ ([M – I]$^+$) 303.1497, found 303.1503.

Fluorescent dye 7

Carboxylic acid **1** (324 mg, 1.0 mmol) and **6** (430 mg, 1.0 mmol) were suspended in dichloromethane (20 mL). Triethylamine (1.4 mL, 10 mmol) was added to the suspension and the resultant solution was stirred at room temperature for 16 h. The solvent was evaporated *in vacuo*. The residue was suspended in dichloromethane (30 mL) and acetone (300 mL). The precipitate was filtered, washed with acetone and dried *in vacuo*. The residue was washed with dichloromethane (50 mL), filtered and dried *in vacuo* to give **7** as a dark green powder (327 mg, 0.67 mmol, 67%): ^{1}H NMR (DMSO-d_6) δ 8.69–8.64 (m, 1H), 8.41 (d, J = 8.3, 1H), 8.27–8.23 (m, 2H), 7.98–7.77 (m, 6H), 7.71–7.61 (m, 2H), 7.41 (t, J = 7.2, 1H), 7.10 (d, J = 14.0, 1H), 6.47 (d, J = 13.0, 1H), 4.46 (t, J = 7.2, 2H), 3.92 (s, 3H), 2.20 (t, J = 7.3, 2H), 1.84–1.78 (m, 2H), 1.59–1.53 (m, 2H); ^{13}C NMR (2 : 1 DMSO-d_6:CD_3COOD) δ 175.1, 153.2, 150.4, 145.0, 141.8, 140.7, 138.7, 135.6, 133.6, 132.8, 129.5, 126.7, 125.8, 125.2, 124.9, 120.8, 118.0, 116.7, 110.5, 109.3, 107.2, 54.1, 36.6, 33.7, 28.9, 22.3; HRMS (ESI) calc. for $C_{27}H_{27}N_2O_2$ ([M – Br]$^+$) 411.2073, found 411.2079.

4-[(*E*)-2-(*N*-Acetyl-*N*-phenylamino)ethenyl]quinoline methiodide (9)

1,4-Dimethylquinolinium iodide (**8**, 2.85 g, 10 mmol) and *N*,*N*′-diphenylformamidine (3.92 g, 20 mmol) were suspended in acetic anhydride (20 mL). The suspension was stirred at 150 °C for 1 h. The reaction mixture was cooled to room temperature. Diethyl ether (150 mL) was added to the reaction mixture with stirring. The supernatant was discarded by decantation and the residue was washed with diethyl ether twice. The residue was dissolved in a small amount of dichloromethane. The solution was added to ethyl acetate (500 mL) with stirring. The supernatant was discarded and the residue was washed with ethyl acetate twice. Ethyl acetate was added to the residue and the precipitate was filtered and dried *in vacuo* to give **9** as a brown powder (3.94 g, 9.16 mmol, 92%): ^{1}H NMR (DMSO-d_6) δ 9.14 (d, J = 6.3, 1H), 8.88 (d, J = 14.2, 1H), 8.36–8.33 (m, 2H), 8.17–8.14 (m, 1H), 7.91–7.84 (m, 2H), 7.71–7.52 (m, 5H), 6.01 (d, J = 13.7, 1H), 4.45 (s, 3H), 2.07 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 169.8, 152.6, 147.5, 140.8, 138.3, 137.9, 134.7, 130.6, 129.7, 129.2, 128.6, 125.2, 124.7, 119.5, 114.7, 102.6, 44.4, 23.3; HRMS (ESI) calc. for $C_{20}H_{19}N_2O$ ([M – I]$^+$) 303.1497, found 303.1503.

Fluorescent dye 10

Carboxylic acid **1** (324 mg, 1.0 mmol) and **9** (430 mg, 1.0 mmol) were suspended in dichloromethane (20 mL). Triethylamine (1.4 mL, 10 mmol) was added to the suspension and the resultant solution was stirred at room temperature for 16 h. The solvent was removed by evaporation. Dichloromethane (30 mL) and a few drops of MeOH were added to dissolve the residue and then acetone (300 mL) was added. The precipitate was filtered, washed with acetone and dried *in vacuo* to give **10** as a dark red-purple solid (278 mg, 0.565 mmol, 57%): ^{1}H NMR (DMSO-d_6) δ 8.59 (t, J = 12.8, 1H), 8.29 (t, J = 8.6, 2H), 8.06–8.04 (m, 2H), 7.85–7.76 (m, 4H), 7.69–7.64 (m, 2H), 7.58–7.50 (m, 2H), 7.02 (t, J = 12.1, 2H), 4.35 (t, J = 7.3, 2H), 3.95 (s, 3H), 2.21 (t, J = 7.2, 2H), 1.80–1.74 (m, 2H), 1.59–1.53 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 174.9, 148.3, 147.9, 142.3, 141.2, 140.3, 138.7, 137.8, 132.43, 132.37, 125.8, 125.5, 124.7, 124.5, 124.0, 123.8, 117.2, 117.1, 110.5, 110.3, 108.4, 108.2, 52.8, 41.3, 34.3, 28.2, 22.0; HRMS (ESI) calc. for $C_{27}H_{27}N_2O_2$ ([M – Br]$^+$) 411.2073, found 411.2081.

N-Hydroxysuccinimidyl ester of fluorescent dyes

Fluorescent dye **4**, **7** or **10** (80 μmol) and *N*-hydroxysuccinimide (18.4 mg, 160 μmol) were suspended in DMF (2 mL). After the addition of *N*,*N*′-diisopropylcarbodiimide (50 μL, 160 μmol), the reaction mixture was stirred at room temperature for 16 h.

Probe synthesis

DNA oligomers containing diamino-modified nucleotide were prepared by a standard phosphoramidite method on a DNA synthesizer. Diamino-modified nucleoside phosphoramidites were synthesized according to our previous report.[7] The synthesized DNA oligomer was cleaved from the CPG support with 28% aqueous ammonia and deprotected at 25 °C for 16 h. After removal of the ammonia from the solution under reduced pressure, the DNA was purified by reversed-phase HPLC elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA) (pH = 7.0) and a linear gradient over 20 min from 5 to 30% acetonitrile at a flow rate of 3.0 mL min^{-1}. The purified DNA was de-salted and dissolved in distilled water. A solution of the succinimidyl ester of the fluorescent dyes (40 mM) in DMF was added to a solution of purified DNA in 100 mM sodium carbonate buffer (pH = 9.0) and incubated at 25 °C for 2 h. The reaction mixture was diluted with ethanol. After centrifuging at 4 °C for 20 min, the supernatant was removed. The residue was dissolved in a small amount of water and then the solution was passed through a

0.45 μm filter. The product was purified by reversed-phase HPLC on a 5-ODS-H column, elution with a solvent mixture of 0.1 M TEAA (pH = 7.0) and a linear gradient over 28 min from 5 to 40% acetonitrile at a flow rate of 3.0 mL min^{-1}. After desalination, the probe was dissolved in a small amount of 10 mM sodium carbonate (pH = 9.0). The fluorescent DNA was identified by MALDI-TOF mass spectrometry (here, the molecular weight of the counter anions of the dyes is not included in the value of M): CGCAAT$\mathbf{D_{644}}$TAACGC, calc. for $C_{184}H_{221}N_{56}O_{78}P_{12}S_2$ ($[M - H]^+$) 4900.9, found 4901.2; CGCAAT$\mathbf{D_{662}}$TAACGC, calc. for $C_{188}H_{225}N_{56}O_{78}P_{12}$ ($[M - H]^+$) 4888.9, found 4891.8; CGCAAT$\mathbf{D_{715}}$TAACGC, calc. for $C_{188}H_{225}N_{56}O_{78}P_{12}$ ($[M - H]^+$) 4888.9, found 4888.7; TTTTTT$\mathbf{D_{644}}$TTTTTT, calc. for $C_{188}H_{231}N_{34}O_{92}P_{12}S_2$ ($[M - H]^+$) 4874.9, found 4876.5; TTTTTT$\mathbf{D_{662}}$TTTTTT, calc. for $C_{192}H_{235}N_{34}O_{92}P_{12}$ ($[M - H]^+$) 4862.9, found 4863.4; TTTTTT$\mathbf{D_{715}}$TTTTTT, calc. for $C_{192}H_{235}N_{34}O_{92}P_{12}$ ($[M - H]^+$) 4862.9, found 4863.6.

Absorption and fluorescence measurements

Absorption and fluorescence spectra of the fluorescent probes (0.4 μM) were measured in 50 mM sodium phosphate buffer (pH = 7.0) containing 100 mM sodium chloride using a cell with a 1 cm path-length. The excitation and emission bandwidths were 1.5 nm.

Melting temperature measurements

The T_m values of the duplexes (2.0 μM) were measured in 50 mM sodium phosphate buffer (pH = 7.0) containing 100 mM sodium chloride. The absorbance of the samples was monitored at 260 nm from 10 to 90 °C at a heating rate of 0.5 °C min^{-1}. From these profiles, first derivatives were calculated to determine the value of T_m.

CD spectroscopy

CD spectra of the duplex of **Probe1($\mathbf{D_{715}}$)** and the complementary DNA (10 μM) were obtained using a Jasco J-720 instrument. Measurements were carried out in 50 mM sodium phosphate buffer (pH = 7.0) containing 100 mM sodium chloride in a capped 1 mm path-length cell under nitrogen.

Cell culture

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO. HeLa cells were cultured at 37 °C in DMEM containing 10% heat-inactivated FBS, 25 U mL^{-1} penicillin and 25 mg mL^{-1} streptomycin under a humidified atmosphere with 5% CO_2. For experimental use, cells (passage numbers 5–9) were cultured in glass-based dishes. Prior to microscope observation, the culture medium was washed and exchanged in phenol red-free DMEM.

Transfection and cell imaging

A solution of **Probe1($\mathbf{D_{715}}$)** in water (50 μM, 2 μL) was introduced using 2 μL of Lipofectamine™ 2000 (Invitrogen). After incubation for 1 h in a solution of Lipofectamine™ 2000 and probe, the cells were washed twice with PBS and observed in phenol red-free DMEM. Cells were maintained under culturing conditions using an incubation system during the observation. Images were acquired with a motorized inverted microscope (Axio Observer Z1; Zeiss) equipped with a 20× objective and an XF142-2 filter set (Optoscience, ex 685AF30, dm 708DRLP, em 730AF30). The acquired images were analyzed and processed with AxioVision 4.2.

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