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A nucleic acid probe labeled with desmethyl thiazole orange: a new type of hybridization-sensitive fluorescent oligonucleotide for live-cell RNA imaging†‡

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A new fluorescent nucleotide with desmethyl thiazole orange dyes, D'_{505}, has been developed for expansion of the function of fluorescent probes for live-cell RNA imaging. The nucleoside unit of D'_{505} for DNA autosynthesis was soluble in organic solvents, which made the preparation of nucleoside units and the reactions in the cycles of DNA synthesis more efficient. The dyes of D'_{505}-containing oligodeoxynucleotide were protonated below pH 7 and the oligodeoxynucleotide exhibited hybridization-sensitive fluorescence emission through the control of excitonic interactions of the dyes of D'_{505}. The simplified procedure and effective hybridization-sensitive fluorescence emission produced multicolored hybridization-sensitive fluorescent probes, which were useful for live-cell RNA imaging. The acceptor-bleaching method gave us information on RNA in a specific cell among many living cells.

Introduction

Fluorescence-labeled nucleic acids are important bioconjugate molecules because they provide a lot of important information on DNA and RNA sequences and structures of interest through their hybridization.[1] They are prepared through nucleic acid autosynthesis: oligodeoxynucleotides (ODNs) are synthesized on solid supports through successive incorporation of each nucleoside, after conversion into its phosphoramidite form in organic solvents. However, fluorescent dyes are often unsuitable for the nucleic acid autosynthesis system because they are only slightly soluble in organic solvents when they are ionic (*e.g.*, fluorescein, Cy5 and thiazole orange). In this case, either of the dye incorporation methods (1) postsynthetic modification of synthetic ODNs with fluorescent dyes through amide bond formation or click-type chemistry[2] or (2) improvement of solubility of dye-labeled nucleosides in organic solvents by extra protection[3] is selected.

Exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probes possess a thymidine derivative labeled with a positively charged dye, such as D_{514} or D_{600} (Fig. 1).[4] ECHO probes, which have a fluorescence-labeled nucleotide in which two molecules of thiazole orange or its

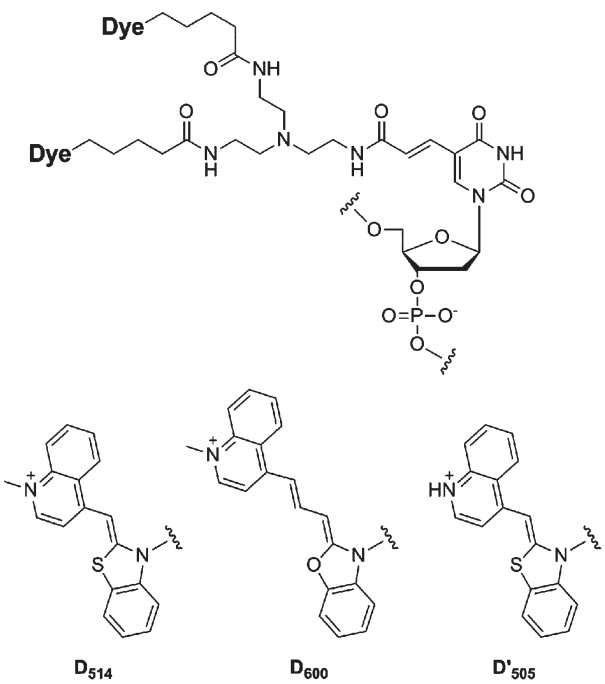

Fig. 1 ECHO probes containing nucleotides D_{514}, D_{600}, and D'_{505}.

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derivatives are covalently linked, use an excitonic interaction caused by the H-aggregation of fluorescent dyes as a new type of useful photophysical process for fluorescence-controlled nucleic acid sensing.[5,6] The hybridization-sensitive fluorescence emission of ECHO probes and the further modification of probes have made possible a variety of practical applications, such as RNA imaging in living cells[7] and facile detection of gene polymorphism.[8] Although several dye-labeled nucleotides for ECHO probes having different colors and functions have been developed,[9] their simultaneous incorporation into one probe molecule is impossible, because a postsynthetic modification procedure is used in which positively charged dyes are attached to the extra amino groups of synthetic ODNs (shown above as a dye incorporation method (1)). The difficulty in incorporating differently colored nucleotides with the effective ECHO probe functions into one ODN strand prevents us from expanding the diversity of the functions and uses of ECHO probes. The key to solving this problem is to design a dye-tethering nucleoside unit that is soluble in several organic solvents for the alternative dye incorporation method (2). Development of the dye-tethering nucleoside unit will facilitate the dye introduction step to the synthesized ODN and subsequent product purification step.

In this paper, we report an autosynthesis-friendly fluorescence-labeled nucleoside. Desmethyl thiazole orange has been tethered with the nucleoside unit of DNA autosynthesis to produce a new type of ECHO probe (D'_{505}, Fig. 1). Development of a 'ready-made' fluorescent nucleotide and its incorporation into an ODN by adoption of an uncharged dye enable the further incorporation of different fluorescence colors by combination with a conventional ECHO probe procedure. Double-colored ECHO probes give us much information on the target RNA molecules in a living cell through hybridization and energy transfer between dyes.

Results and discussion

Synthesis of nucleosides and oligonucleotides including desmethyl thiazole orange (D'_{505})

A nucleoside doubly modified with the dye appropriate for excitation at 505 nm, D'_{505}, in which a derivative is substituted for the thiazole orange moiety of D_{514}, was synthesized (Scheme 1). The desmethyl thiazole orange dye unit **5** of D'_{505} was obtained by coupling a quinolinium salt **2**, given by addition of a carboxyethyl group to the nitrogen atom of quinoline (**1**), with a methylbenzothiazolium salt containing an alkyl chain with a carboxyl end **3**,[9] followed by removal of the carboxyethyl group attached to the quinoline.[10,11] Two molecules of **5** were reacted with two of the three amino groups of tris(aminoethyl)amine to give **6**. The doubly dye-modified linker **6** was tethered to the thymidine derivative with a carboxyl linker **7**[5] to give a D'_{505} nucleoside form **8**.

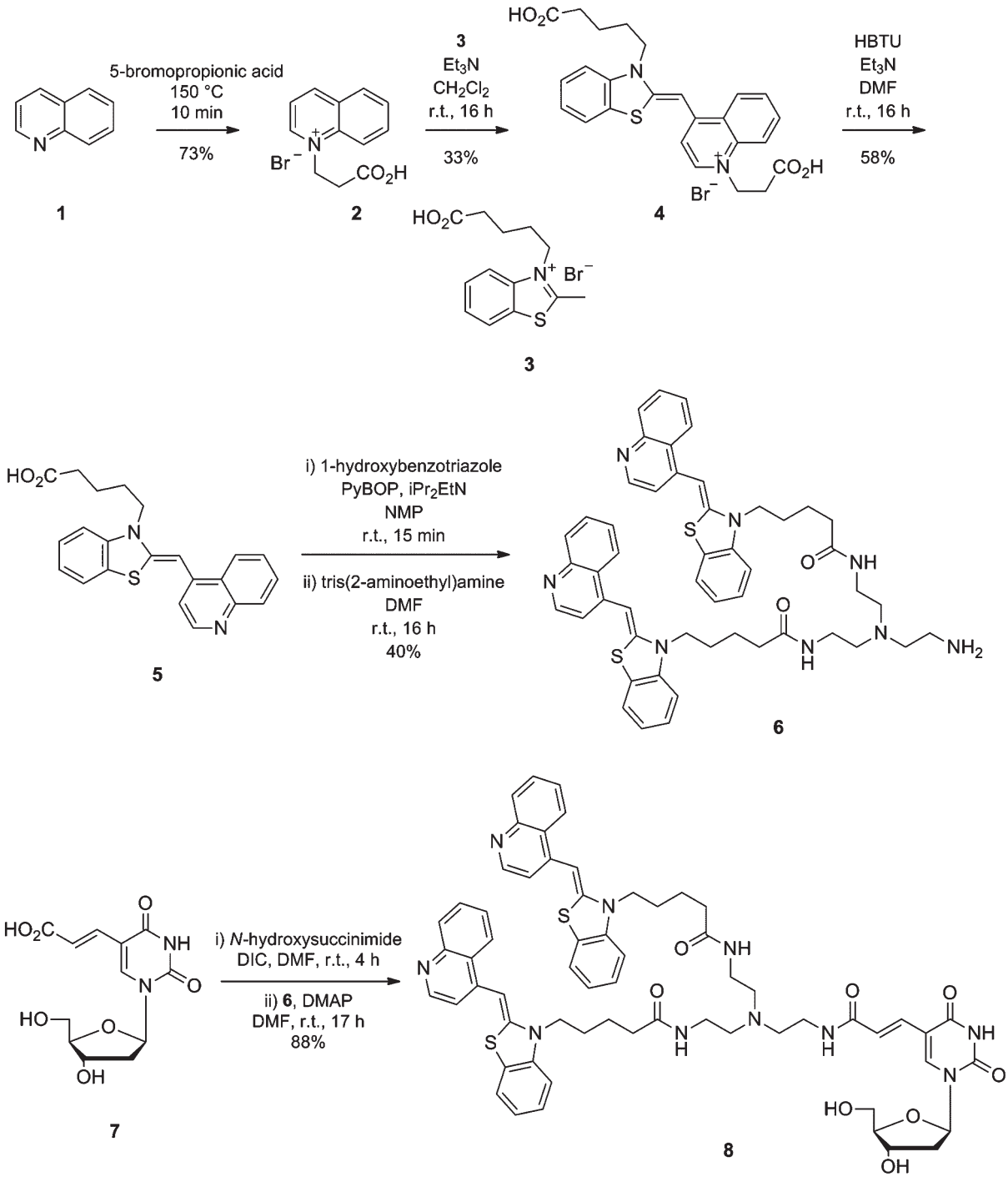
The uncharged nucleoside **8** is soluble in several organic solvents, *e.g.*, chloroform and DMF. The solubility of **8** in organic solvents made handling of the nucleoside unit in organic synthesis and preparation of the ODN with fluorescent dyes easier. The dye-labeled nucleoside **8** was protected with a dimethoxytrityl group to give a synthetic unit **9** for DNA autosynthesis (Scheme 2). The unit **9** was incorporated into ODNs through DNA autosynthesis using acetonitrile and DMF after conversion into the phosphoramidite form (coupling yield, >99%; ESI Fig. S1‡), and the ODN containing a fluorescent nucleotide D'_{505} was obtained after deprotection with ammonia. The synthesized D'_{505}-containing oligonucleotides can be used for RNA imaging after purification without any of the further modifications required in conventional ECHO probes.

Photophysical properties of D'_{505}-containing ODNs

The absorption and emission of a D'_{505}-containing ODN ODN1 (D'_{505}), CGCAATD′$_{505}$TAACGC, were investigated before and after hybridization with the complementary RNA strand at pH 7.0. Fluorescence emission at 522 nm was observed on excitation at 505 nm after ODN1(D'_{505}) (0.4 μM) was added to a solution of the A_{13} RNA (Φ_f 0.25), whereas the emission was suppressed in the unhybridized state (Φ_f 0.09) (Fig. 2a). This emission behavior is the same as the typical behavior of the fluorescence emitted by conventional ECHO probes.[4,12] The source of the fluorescence behavior is confirmed in the absorption spectra. The absorption band of the unhybridized probe (λ_{max} = 468 nm, ε = 42 750) appears at a shorter wavelength than that of the hybrid (λ_{max} = 497 nm, ε = 40 500) (Fig. 2b). This blue shift suggests a splitting of the excited state because of H-aggregation of the dyes. H-aggregation allows only transitions to the upper excitonic level.[13] The excited state is rapidly transferred to the lower level, but the path from this energy level to the ground state is not emissive. The emission and absorption spectra suggest that the fluorescence from the D'_{505}-containing ODN was suppressed by interdye excitonic interaction in the unhybridized state. The CD spectrum of the hybrid shows the induced CD signal at 450–550 nm, as observed for conventional ECHO probes (Fig. 2c). This suggests that the dyes of D'_{505} interact with the duplex structure of the hybrid to lose interdye interaction and emit fluorescence.[5] These photophysical phenomena were also observed for the D'_{505}-containing ODN with a different sequence, ODN2(D'_{505}), TTTTTTD′$_{505}$TTTTTT (ESI Fig. S2‡). ODN2(D'_{505}) showed a high fluorescence intensity at 525 nm at pH 7 in the presence of A_{13} RNA.

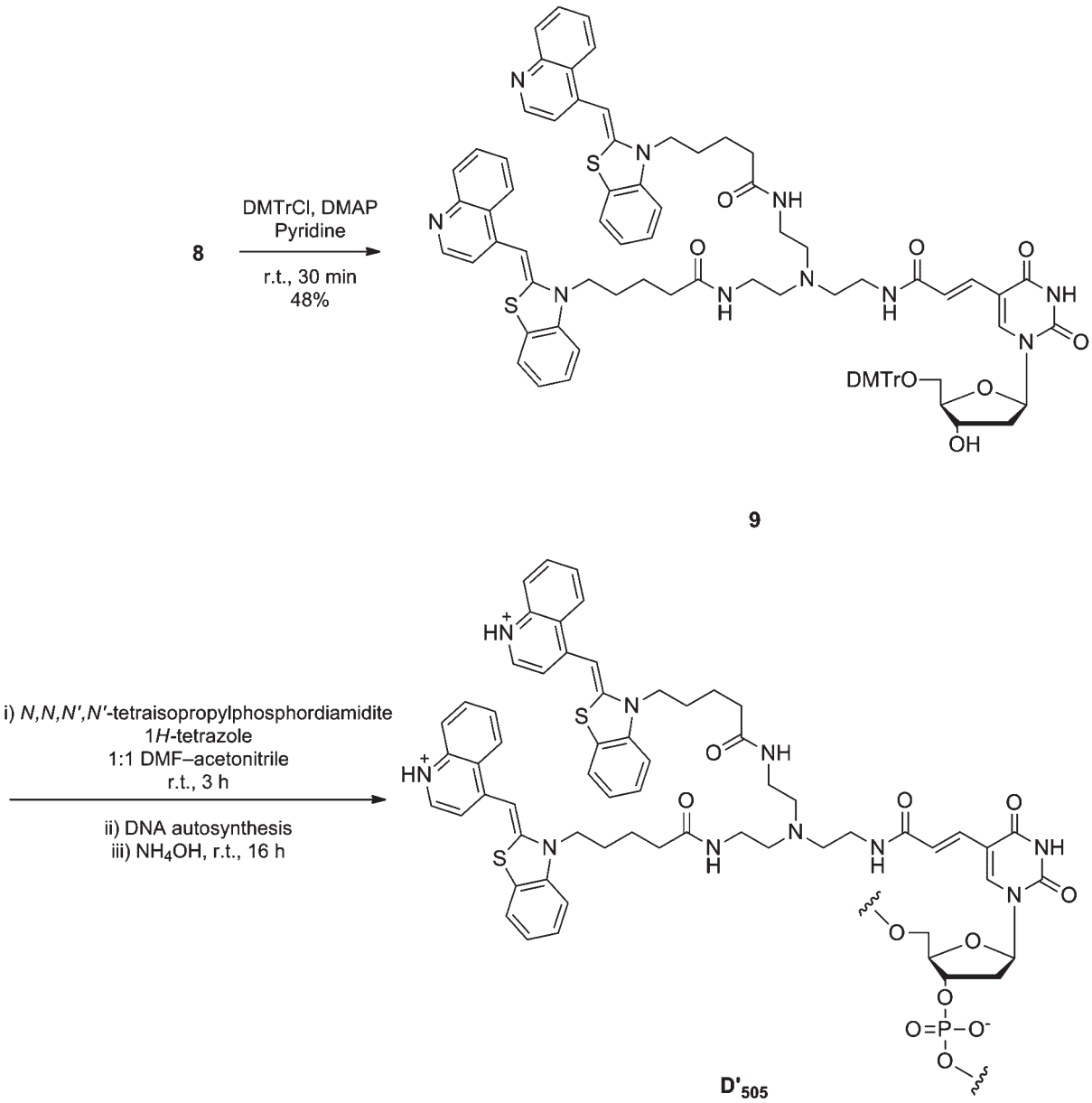
Protonation effect

Desmethyl thiazole orange, which is used for dyes of D'_{505}, is weakly emissive.[11] However, ODN1(D'_{505}) works as a good ECHO probe to emit fluorescence through recognition of the complementary RNA. The pK_a of desmethyl thiazole orange has been reported to be approximately 7.6,[11] and the protonation of D'_{505} may play an important role in hybridization-sensitive fluorescence emission. To investigate the effect of protonation of D'_{505}, the absorption and emission of ODN1 (D'_{505}) were monitored at various values of pH (Fig. 3). The measurements at pH 5–7 showed on–off switching of



Scheme 1 Synthesis of the D'_{505} nucleoside.

fluorescence and shift of absorption depending on addition of the complementary RNA strand, which is similar to conventional ECHO probes. In contrast, the fluorescence from ODN1 (D'_{505}) at higher pH (pH 8–10) was weak even in the presence of the complementary RNA strand. The emission spectrum of ODN1(D'_{505}) (0.4 μM) excited at 505 nm showed negligible fluorescence at pH 8.5, and the fluorescence intensity of a solution of ODN1(D'_{505}) was still low after addition of the complementary RNA. The pH at which the fluorescence intensity of the ODN1(D'_{505})–RNA duplex decreased is almost consistent with the pK_a of desmethyl thiazole orange, suggesting that protonation of desmethyl thiazole orange dye may be the key for D'_{505} to work as a fluorescent probe in which the emission is effectively controlled. The absorption spectra of ODN1(D'_{505}) at higher pH can explain the characteristic switching of fluorescence intensity depending on pH. The absorption band at pH 8.5 was predominantly observed at 500 nm (ε = 12 250) regardless of the presence of the



Scheme 2 Synthesis of the D'_{505}-containing ODN.

complementary RNA (Fig. 3c). The small shift in the absorption band suggests that the unprotonated desmethyl thiazole orange dyes of D'_{505} do not form H-aggregates even before hybridization. In addition, desmethyl thiazole orange is originally weakly emissive, as described above. ODN1(D'_{505}) lost the function of hybridization-sensitive fluorescence control at higher pH.

Multilabeled ECHO probes

The greatest advantage of incorporation of a 'ready-made' fluorescent nucleotide with the function of hybridization-sensitive fluorescence switching into ODNs is to make it possible to further incorporate a different fluorescence color by combination with the conventional ECHO probe procedure. Multicoloring of the probe can result in the simple simultaneous detection of multiple target nucleic acid sequences, and be useful in efforts to understand temporal correlations of gene expression and interactions between nucleic acids. For this purpose, multilabeled ECHO probes should be designed without loss of a high on–off performance as a hybridization-sensitive fluorescent probe based on excitonic interaction chemistry. In the conventional procedure for ECHO probes, it is difficult nucleotide-specifically to incorporate different fluorescent colors into one ODN in a conventional ECHO probe procedure, because dyes are incorporated into all deprotected amino groups after DNA autosynthesis. In contrast, by the effective incorporation of D'_{505}, the synthesis of a doubly colored ECHO probe was achieved as follows (Scheme 3): (i) an ODN containing both a D'_{505} nucleotide and an ECHO probe nucleoside unit with a branched diamino linker was prepared,

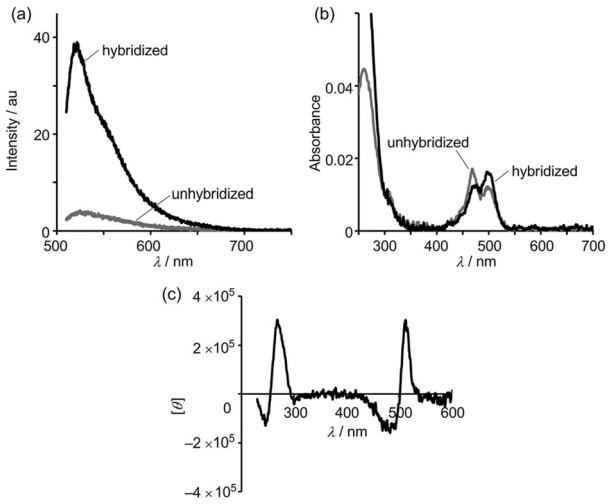


Fig. 2 Emission, absorption, and CD spectra of the D'_{505}-containing ODN ODN1(D'_{505}), CGCAATD'$_{505}$TAACGC. Emission and absorption spectra of the probes (0.4 μM) were measured in the presence or absence of the complementary RNA in 50 mM sodium phosphate (pH = 7.0) containing 100 mM sodium chloride. (a) Emission spectra on excitation at 505 nm; (b) absorption spectra; (c) CD spectrum of the hybrid.

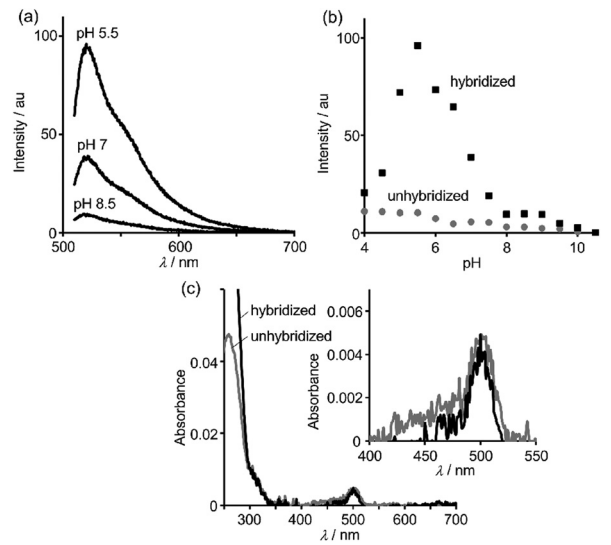


Fig. 3 Emission and absorption spectra at various pHs. Emission and absorption spectra of the ODN1(D'_{505}) (0.4 μM) were measured in the presence or absence of the complementary RNA in 50 mM sodium acetate (pH = 4.0–5.5), 50 mM sodium phosphate (pH = 6.0–8.5), or 50 mM glycine–sodium hydroxide (pH = 9.0–10.5), containing 100 mM sodium chloride. (a) Emission spectra of the ODN1(D'_{505})–RNA hybrid on excitation at 505 nm; (b) plots of fluorescence intensities of the ODN1(D'_{505})–RNA hybrid at 522 nm against pH; (c) absorption spectra at pH 8.5, (inset) enlargement of the absorption of the dye.

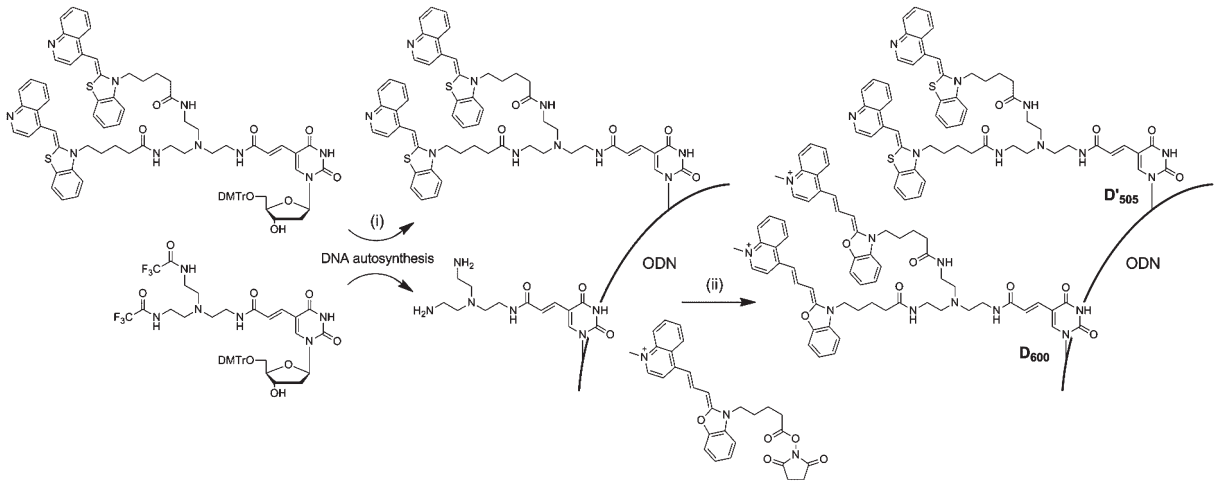
and then (ii) the dye with appropriate wavelength was incorporated by the conventional ECHO probe procedure.

A double-colored ECHO probe, ODN3(D'_{505},D_{600}) CAATD'$_{505}$-TAACGCCGCTTCGCCAATCCD$_{600}$CTCA, exhibited on–off switching of fluorescence at both emission wavelengths of D'_{505} and D_{600} depending on addition of the complementary RNA strand. The emission spectrum of single-stranded ODN3-(D'_{505},D_{600}) (0.4 μM) excited at 514 or 600 nm showed a weak fluorescence, whereas ODN3(D'_{505},D_{600}) hybridized with the fully complementary RNA, UGAGAGGAUUGGCGAAGCGGC-GUUAAAUUG, displayed stronger D'_{505} and D_{600} fluorescence (Fig. 4a). Both of the fluorescent nucleotides of ODN3(D'_{505}, D_{600}) exhibited the characteristic absorption band shift controlled by hybridization with RNA (Fig. 4b). The partially hybridized ODN3(D'_{505},D_{600}) emitted fluorescence corresponding to the hybridized region. ODN3(D'_{505},D_{600}) exhibited a hybridization-sensitive D'_{505} fluorescence on excitation at 514 nm and only a background fluorescence of D_{600} on excitation at 600 nm when only the region containing D'_{505} was hybridized using AGCGGCGUUAAAUUG and the region containing D_{600} was not hybridized (Fig. 4c), or *vice versa* when the use of UGA-GAGGAUUGGCGA covered only the D_{600} region (Fig. 4d).

Förster resonance energy transfer (FRET)[14] between D'_{505} and D_{600} can be observed when the distance between two nucleotides is short. Another D'_{505}/D_{600} system, ODN4(D'_{505}, D_{600}), TTTTTTD'$_{505}$TTTTTTD$_{600}$TTTTTT, which has fewer nucleotides between the fluorescent nucleotides, was prepared and the donor nucleotide D'_{505} was excited by irradiation at 505 nm. Emission from the FRET acceptor D_{600} (621 nm) as well as emission from D'_{505} (524 nm) was observed (Fig. 5). This FRET efficiently occurred only when ODN4(D'_{505},D_{600}) was hybridized with the complementary RNA. Before hybridization, only background fluorescence was observed.

Intracellular RNA imaging and acceptor bleaching assay

ECHO probes are suitable for imaging the dynamic and static behavior of RNA in a living cell because they have low background fluorescence before hybridization and the fluorescence-controlling system provides effective bioimaging without any washing process or nonspecific emission.[7] The FRET system of a multicolored ECHO probe may be applicable to RNA imaging and may provide a way to simplify monitoring of specific cells and specifically locating RNA in a cell through acceptor bleaching assay.[15] A poly(A)$^+$ RNA-targeting D'_{505}/D_{600} probe, ODN4(D'_{505},D_{600}), which was shown to have high FRET efficiency in an *in vitro* assay as described above, was transfected into living HeLa cells. The cells (mainly nuclei) emitted strong fluorescence through a 600–735 nm filter when excited with a 488 nm Ar laser (red in Fig. 6a). The fluorescence observed through a 500–590 nm filter (green) was weak, suggesting that the FRET process of ODN4(D'_{505},D_{600}) was active even in living cells. One of the strongly emitting cells was selected and then scanned repeatedly with a 561 nm diode-pumped solid-state laser. The fluorescence observed through a red filter was reduced to 16% only in the irradiated cell, suggesting that the D_{600} dyes in the probe were photobleached and were no longer able to work as a FRET acceptor (Fig. 6b). Upon photobleaching of D_{600} dyes, the strong fluorescence of D_{514}, the FRET donor, was recovered through a green filter (490% of the fluorescence before photobleaching). This acceptor-bleaching method for ODN4(D'_{505},D_{600}) facilitated monitoring of the target poly(A)$^+$ RNA in one living cell of interest.



Scheme 3 Synthesis of doubly labeled ECHO probes.

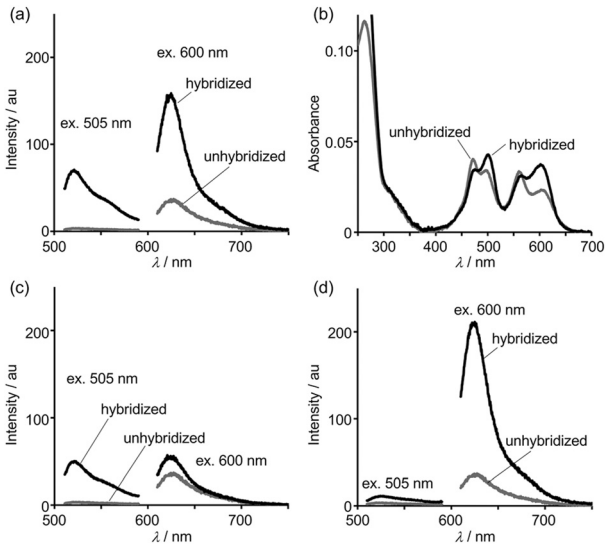


Fig. 4 Emission and absorption spectra of the double-colored ECHO probe ODN3(D'_{505},D_{600}). Emission and absorption spectra of the probes (0.4 μM) were measured in the presence or absence of the complementary RNA in 50 mM sodium phosphate (pH = 7.0) containing 100 mM sodium chloride. (a) Emission spectra of unhybridized ODN3(D'_{505},D_{600}) and a full duplex of ODN3(D'_{505},D_{600}) and the complementary RNA on excitation at 505 and 600 nm; (b) absorption spectra of unhybridized ODN3(D'_{505},D_{600}) and a full duplex of ODN3(D'_{505},D_{600}) and the complementary RNA; (c) emission spectra of ODN3(D'_{505},D_{600})–AGCGGCGUUAAAUUG on excitation at 505 and 600 nm; (d) emission spectra of ODN3(D'_{505},D_{600})–UGAGAGGAUUGGCGA on excitation at 505 and 600 nm.

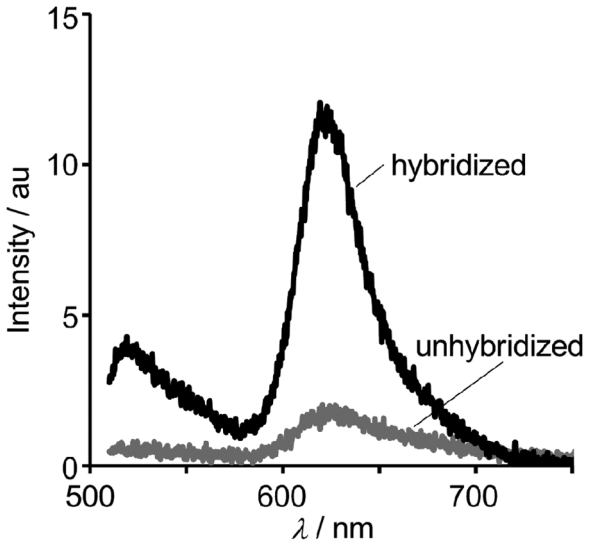


Fig. 5 FRET of the multicolored ECHO probe ODN4(D'_{505},D_{600}). Emission spectra of the ODN4(D'_{505},D_{600}) were measured in the presence or absence of the complementary RNA in 50 mM sodium phosphate (pH = 7.0) containing 100 mM sodium chloride on excitation at 505 nm.

Experimental

General

^{1}H and ^{13}C NMR spectra were measured using a JEOL JNM-Alpha400. The chemical shifts are shown in ppm, using residual dimethylsulfoxide (δ = 2.49 in ^{1}H NMR, δ = 39.5 in ^{13}C NMR) and methanol (δ = 3.30 in ^{1}H NMR, δ = 49.0 in ^{13}C NMR) as internal standards. DNA was synthesized on an NTS H-6 DNA/RNA synthesizer. RNA was synthesized by Japan Bio Service. Reversed-phase HPLC was performed on CHEMCOBOND 5-ODS-H columns (10 mm × 150 mm) with a Gilson Chromatograph, Model 305, using a JASCO multiwavelength detector, model MD-2015Plus. ESI mass spectra were recorded on a JEOL T100LC, AccuTOF. MALDI-TOF mass spectra were measured using a Bruker Daltonics Reflex. UV and fluorescence spectra were recorded on a Shimadzu UV-2550 spectrophotometer and an RF-5300PC spectrofluorophotometer, respectively.

1-(2-Carboxyethyl)quinolinium bromide (2)

Quinoline **1** (11.8 mL, 100 mmol) and 3-bromopropionic acid (15.3 g, 100 mmol) were stirred at 150 °C for 10 min. After cooling to room temperature, dichloromethane (500 mL) was added to the mixture. The resultant white solid was crushed to powder. The precipitate was filtered, washed with

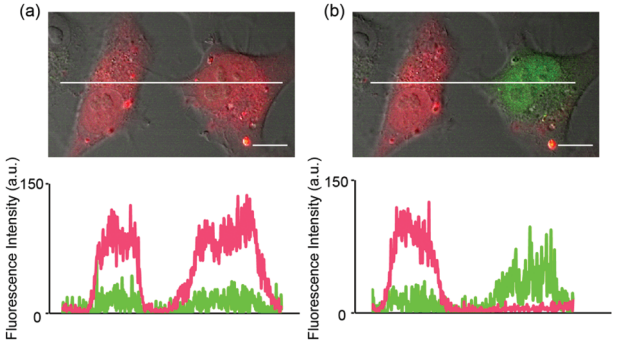


Fig. 6 Live-cell RNA imaging and acceptor bleaching. (a) An image of ODN4 (D'_{505},D_{600})-injected HeLa cells before bleaching. Green, fluorescence through a 500–590 nm filter; red, fluorescence through a 600–735 nm filter. Fluorescence intensities along the long white lines drawn in the images were plotted. Short scale bars, 10 μm. (b) An image after only the nucleus of the right cell was scanned repeatedly at 561 nm.

dichloromethane, and dried under reduced pressure to obtain **2** as a white powder (20.7 g, 73.4 mmol, 73%): ^{1}H NMR (DMSO-d_6):δ 12.69 (br, 1H), 9.62 (dd, J = 5.9, 1.5 Hz, 1H), 9.33 (d, J = 8.3 Hz, 1H), 8.63 (d, J = 8.8 Hz, 1H), 8.50 (dd, J = 8.3, 1.5 Hz, 1H), 8.28–8.24 (m, 1H), 8.20 (dd, J = 8.3, 5.9 Hz, 1H), 8.06–8.02 (m, 1H), 5.27 (t, J = 7.0 Hz, 2H), 3.09 (t, J = 7.0 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 171.4, 150.7, 147.7, 137.3, 135.7, 130.8, 129.8, 129.6, 121.9, 118.9, 53.2, 33.3; HRMS (FAB) calcd for $C_{12}H_{12}NO_2$ ($[M - Br]^+$) 202.0868, found 202.0872.

4-{[3-(4-Carboxybutyl)benzothiazol-2-ylidene]methyl}-1-(2-carboxyethyl)quinolinium bromide (4)

Triethylamine (25.4 mL, 182 mmol) was added to a suspension of **2** (6.0 g, 18.2 mmol) and benzothiazolium salt **3** (5.1 g, 18.2 mmol) in dichloromethane (70 mL). The mixture was stirred at room temperature for 16 h. The mixture was concentrated under reduced pressure, and then water (90 mL) was added. The precipitate was filtered, washed with water, and then dried under reduced pressure to obtain **4** as a red powder (3.18 g, 6.0 mmol, 33%): ^{1}H NMR (CD_3OD):δ 8.53 (d, J = 7.8 Hz, 1H), 8.42 (d, J = 7.3 Hz, 1H), 8.01 (d, J = 8.6 Hz, 1H), 7.91–7.88 (m, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.71 (t, J = 7.7 Hz, 1H), 7.59 (d, J = 8.1 Hz, 1H), 7.55–7.52 (m, 1H), 7.34–7.30 (m, 2H), 6.80 (s, 1H), 4.75 (t, J = 6.6 Hz, 2H), 4.48 (t, J = 7.7 Hz, 2H), 2.81 (t, J = 6.6 Hz, 2H), 2.41 (t, J = 7.0 Hz, 2H), 1.97–1.91 (m, 2H), 1.87–1.81 (m, 2H); ^{13}C NMR (CD_3OD) δ 178.0, 176.4, 161.1, 150.7, 145.6, 141.2, 138.5, 134.4, 129.4, 128.2, 126.6, 126.0, 125.8, 125.7, 123.7, 118.7, 113.7, 109.5, 88.8, 53.2, 47.3, 37.2, 35.1, 27.7, 23.5; HRMS (FAB) calcd for $C_{25}H_{25}N_2O_4S$ ($[M - Br]^+$) 449.1535, found 449.1537.

4-{[3-(4-Carboxybutyl)benzothiazol-2-ylidene]methyl}quinoline (5)

Triethylamine (21 g, 203 mmol) was added to a suspension of **4** (17.9 g, 33.8 mmol) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU, 25.6 g, 67.6 mmol) in *N*,*N*-dimethylformamide (35 mL). The mixture was stirred at room temperature for 16 h. After addition of water (10 mL), the mixture was stirred for 30 min, and then 2 M aqueous acetic acid (1 L) was added. The precipitate was collected and washed with 1 M acetic acid and then methanol to obtain **5** as a red powder (7.44 g, 19.8 mmol, 58%): ^{1}H NMR (DMSO-d_6):δ 12.18 (br, 1H), 8.65 (d, J = 5.9 Hz, 1H), 8.51 (d, J = 8.3 Hz, 1H), 7.89 (dd, J = 8.3, 1.0 Hz, 1H), 7.83–7.79 (m, 2H), 7.66–7.62 (m, 1H), 7.51–7.41 (m, 3H), 7.23–7.20 (m, 1H), 6.68 (s, 1H), 4.41 (t, J = 7.3 Hz, 2H), 2.32 (t, J = 7.2 Hz, 2H), 1.83–1.77 (m, 2H), 1.73–1.81 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 174.2, 153.5, 145.8, 144.9, 142.6, 140.5, 130.8, 127.4, 126.1, 124.7, 124.24, 124.19, 123.0, 122.8, 122.2, 111.0, 110.6, 85.8, 44.6, 33.2, 25.8, 21.7; HRMS (FAB) calcd for $C_{22}H_{21}N_2O_2S$ ($[M + H]^+$) 377.1324, found 377.1327.

N,*N*-Bis({[3-(4-valeryl)benzoxazol-2-ylidene]methyl}quinoline)-2-amidoethyl)ethylamine (6)

PyBOP (2.0 g, 3.8 mmol) and *N*,*N*-diisopropylethylamine (129 mg, 1.0 mmol) were added to a solution of **5** (753 mg, 2.0 mmol) and 1-hydroxybenzotriazole (270 mg, 2.0 mmol) in *N*-methylpyrrolidone (5 mL). The mixture was stirred at room temperature for 15 min. The reaction mixture was added dropwise to a solution of tris(2-aminoethyl)amine (146 mg, 1.0 mmol) in *N*-methylpyrrolidone (5 mL) at room temperature, and stirred at room temperature for 16 h. The resultant mixture was poured into diethyl ether, and then the precipitate was collected and dried under reduced pressure. The crude product was purified by silica gel column chromatography (7–20% methanol, 1% triethylamine–dichloromethane) to give **6** as an orange powder (348 mg, 0.40 mmol, 40%): ^{1}H NMR (DMSO-d_6): δ 8.72 (d, J = 5.1 Hz, 2H), 8.35 (d, J = 7.8 Hz, 2H), 7.92 (dd, J = 8.3, 1.2 Hz, 2H), 7.84 (t, J = 5.8 Hz, 2H), 7.69 (m, 2H), 7.61 (dd, J = 7.7, 1.1 Hz, 2H), 7.55 (m, 2H), 7.41 (d, J = 4.9 Hz, 2H), 7.30 (m, 2H), 7.20 (d, J = 8.3 Hz, 2H), 7.03 (td, J = 7.6, 0.90 Hz, 2H), 6.44 (s, 2H), 4.17 (t, J = 6.6 Hz, 4H), 3.05 (dd, J = 12.5, 6.6 Hz, 4H), 2.73 (t, J = 5.5 Hz, 2H), 2.38 (t, J = 6.5 Hz, 4H), 2.17 (t, J = 6.6 Hz, 4H), 1.70 (m, 8H); ^{13}C NMR (DMSO-d_6): δ 172.1, 149.7, 148.5, 148.1, 142.5, 141.2, 129.3, 129.0, 126.9, 125.4, 125.3, 123.9, 122.4, 121.9, 121.4, 113.2, 109.5, 84.4, 54.9, 53.6, 43.9, 37.0, 36.7, 35.0, 25.5, 22.7, 7.4; HRMS (ESI) calcd for $C_{50}H_{54}N_8O_2S_2$ ($[M + H]^+$) 863.3889, found 863.3907.

5-(2-[2-{*N*,*N*-Bis({[3-(4-valeryl)benzoxazol-2-ylidene]methyl}-quinoline)-2-amidoethyl}aminoethyl]carbamoyl-(*E*)-vinyl)-2-deoxyuridine (8)

N,*N*′-Diisopropyl carbodiimide (1.24 g, 9.8 mmol) was added to a solution of 7[5] (1.49 g, 5.0 mmol) and *N*-hydroxysuccinimide (1.15 g, 10.0 mmol) in *N*,*N*-dimethylformamide (16 mL). The mixture was stirred at room temperature for 4 h. Acetic acid (0.83 mL) was added to the mixture and stirred at room temperature for 1 h. The mixture was poured into the mixed solvent of dichloromethane (250 mL) and water (250 mL) with vigorous stirring. The precipitate was washed with water to give the succinimidyl ester as a white powder (1.75 g, 4.4 mmol).

The succinimidyl ester (297 mg, 0.75 mmol) was added to a solution of **6** (432 mg, 0.50 mmol) and *N*,*N*-dimethylaminopyridine (6.1 mg, 0.50 mmol) in *N*,*N*-dimethylformamide (6 mL), and the mixture was stirred at room temperature for 17 h. The mixture was poured into diethyl ether (150 mL) with stirring. The precipitate was washed with diethyl ether. The crude product was purified by silica gel column chromatography (5–10% methanol, 1% triethylamine–dichloromethane) to give **8** (594 mg, 0.50 mmol, 88% in two steps): ^{1}H NMR (DMSO-d_6): δ 11.78 (s, 1H), 8.67 (d, J = 5.2 Hz, 2H), 8.38 (d, J = 8.4 Hz, 2H), 8.26 (s, 1H), 7.99 (m, 1H), 7.90–7.89 (m, 2H), 7.76 (t, J = 5.2 Hz, 2H), 7.73–7.70 (m, 2H), 7.65 (d, J = 7.6 Hz, 2H), 8.56 (m, 2H), 7.38 (d, J = 5.2 Hz, 2H), 7.33–7.31 (m, 2H), 7.25 (d, J = 8.2 Hz, 2H), 7.13–7.06 (m, 4H), 6.49 (s, 2H), 6.13 (t, J = 6.6 Hz, 1H), 5.29 (m, 1H), 5.20 (m, 1H), 4.25 (m, 1H), 4.21 (m, 4H), 3.80 (t, J = 3.5 Hz, 1H), 3.65–3.56 (m, 2H), 3.05 (d, J = 5.3 Hz, 4H), 2.44–2.40 (m, 5H), 2.19–2.10 (m, 5H), 1.71 (m, 8H); ^{13}C NMR (DMSO-d_6): δ 172.4, 166.2, 162.3, 150.0, 149.7, 149.2, 147.6, 147.4, 143.6, 142.9, 141.6, 132.7, 129.8, 128.8, 127.4, 126.0, 125.5, 124.4, 123.0, 122.4, 122.1, 121.8, 113.1, 110.2, 109.5, 88.1, 85.2, 85.1, 70.4, 65.4, 61.4, 53.8, 44.6, 37.3, 35.4, 26.0, 23.1, 15.6. LRMS (FAB) calcd for $C_{62}H_{66}N_{10}O_8S_2$ ($[M + H]^+$) 1143.5, found 1143.5.

5′-*O*-Dimethoxytrityl-5-(2-[2-{*N*,*N*-bis({[3-(4-valeryl)benzoxazol-2-ylidene]methyl}quinoline)-2-amidoethyl}aminoethyl]-carbamoyl-(*E*)-vinyl)-2-deoxyuridine (9)

A solution of **8** (595 mg, 0.52 mmol), *N*,*N*-dimethylaminopyridine (6.1 mg, 0.05 mmol), and 4,4′-dimethoxytrityl chloride (457 mg, 1.35 mmol) in pyridine (5 mL) was stirred at room temperature for 2 h. After the reaction was quenched by methanol (5 mL), the mixture was concentrated and the residue was dried under reduced pressure. The crude product was purified by silica gel column chromatography (7–15% methanol, 1% triethylamine–dichloromethane) to give **9** (360 mg, 0.25 mmol, 48%) as a red powder: ^{1}H NMR (DMSO-d_6): δ 8.57 (d, J = 6.1 Hz, 2H), 8.48 (d, J = 8.3 Hz, 2H), 7.81 (m, 9H), 7.58 (t, J = 7.5 Hz, 2H), 7.45–7.15 (m, 16H), 7.08 (s, 2H), 6.85 (dd, J = 8.9, 6.0 Hz, 4H), 6.64 (s, 2H), 6.11 (t, J = 6.6 Hz, 1H), 5.28 (s, 1H), 4.35 (m, 4H), 4.20 (m, 1H), 3.87 (dd, J = 9.5, 4.2 Hz, 1H), 3.69 (s, 3H), 6.68 (s, 3H), 3.19–3.06 (m, 10H), 2.44 (m, 4H), 2.26 (qui, J = 6.7 Hz, 2H), 2.17 (t, J = 6.5 Hz, 4H), 1.71 (m, 8H); ^{13}C NMR (DMSO-d_6): δ 172.0, 165.7, 161.7, 158.0, 154.4, 149.2, 146.3, 144.8, 143.9, 142.7, 141.5, 140.4, 135.55, 135.46, 132.3, 131.1, 129.6, 127.8, 127.6, 127.5, 126.6, 126.2, 124.4, 123.9, 123.8, 123.1, 123.0, 122.4, 121.8, 113.2, 111.3, 110.1, 109.3, 86.0, 85.6, 84.7, 70.2, 63.9, 55.0, 54.9, 53.4, 53.2, 44.8, 34.8, 25.9, 22.5; HRMS (ESI) calcd for $C_{83}H_{85}N_{10}O_{10}S_2$ ($[M + H]^+$) 1445.5892, found 1445.5930.

D′$_{505}$-containing ECHO probes

2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (85.8 mg, 0.28 mmol) was added to a solution of **9** (144.5 mg, 0.055 mmol) and 1*H*-tetrazole (28 mg, 0.40 mmol) in 1 : 1 *N*,*N*-dimethylformamide–acetonitrile (800 μL). The mixture was stirred at room temperature for 3 h, and then filtered through a 0.45 μm filter. The mixture was used for DNA autosynthesis as a phosphoramidite unit of D′$_{505}$, with commercially available phosphoramidites for dA, dG, dC, and dT. The synthesized DNA oligomers were cleaved from the support and deprotected with 28% aqueous ammonia through incubation at room temperature for 16 h. After removal of ammonia under reduced pressure, the DNA oligomers were purified by HPLC (elution with a solvent mixture of 0.1 M triethylamine acetate (TEAA), pH 7.0, linear gradient over 30 min from 5% to 42.5% acetonitrile at a flow rate of 3.0 mL min^{-1}). The synthesized D′$_{505}$-containing ECHO probes were identified by MALDI-TOF mass spectrometry: ODN1(D′$_{505}$) CGCAATD′$_{505}$-TAACGC, calcd for $C_{178}H_{214}N_{56}O_{78}P_{12}S_2$ 4820.8 $[M + H]^+$; found 4820.7; ODN2(D′$_{505}$) TTTTTTD′$_{505}$TTTTTT, calcd for $C_{182}H_{224}$-$N_{34}O_{92}P_{12}S_2$ 4794.8 $[M + H]^+$; found 4795.8; ODN3(D′$_{505}$,D$_{600}$) CAATD′$_{505}$TAACGCCGCTTCGCCAATCCD$_{600}$CTCA, calcd for $C_{398}H_{482}N_{118}O_{187}P_{29}S_2$ 10 873.2 $[M - H]^+$; found 10 872.0; ODN4(D′$_{505}$,D$_{600}$) TTTTTTD′$_{505}$TTTTTTD$_{600}$TTTTTT, calcd for $C_{310}H_{366}N_{56}O_{146}P_{19}S_2$ 1122.96 $[M - 9H]^{7-}$; found 1122.96.

Absorption and fluorescence measurements

Absorption and fluorescence spectra of the 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.

CD measurements

CD spectra of the fluorescent probes (2.5 μM) were measured in 50 mM sodium phosphate buffer solution (pH = 7.0) containing 100 mM sodium chloride using a cell with a 0.1 cm path length.

Melting-temperature (T_m) measurements

The T_m values of the duplexes (2.0 μM) were measured in 50 mM sodium phosphate buffer solution (pH = 7.0) containing 100 mM sodium chloride. The absorbance of the samples was monitored at 260 nm from 5 °C to 80 °C with a heating rate of 1.0 °C min^{-1}. First derivatives were calculated from these profiles to determine the value of T_m.

Cell culture

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO. Reagents for culturing were from Sigma. 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 (Matsunami). Before microscope observation, the culture medium was washed and exchanged to phenol red-free DMEM. Cells were maintained in culture using an incubation system (INU; Tokai Hit) during observation.

Transfection and acceptor bleaching

HeLa cells were transfected with TTTTTTD′$_{505}$TTTTTTD$_{600}$-TTTTTT (25 μM) using lipofectamine 2000 (Invitrogen). After incubation for 1 h in probe/lipofectamine solution, cells were rinsed twice with PBS and observed in phenol red-free DMEM. During observation and imaging, cells were maintained under culturing conditions using a live-cell observation chamber system. Images were acquired on an Axio Observer Z1 (Zeiss) with a PlanApochromat 63×/1.40 oil DIC M27 objective. Fluorescence of D′$_{505}$ was recorded using 488 nm excitation (Ar laser) and 500–590 nm detection; the FRET signal of D$_{600}$ was recorded with 600–735 nm detection. Focal acceptor bleaching was performed using a 561-10 diode-pumped solid-state laser (15.0 mW, 177.32 ms dwell time per pixel, 10 times). Acquired images were processed and analyzed with ZEN 2008 (Zeiss).

Conclusions

A new fluorescent nucleotide with desmethyl thiazole orange dyes, D′$_{505}$, has been developed for expansion of the function of ECHO probes. D′$_{505}$, which was obtained by demethylation of thiazole orange dye, achieved three aims. (i) A nucleoside unit containing dyes has been developed for DNA autosynthesis. Uncharged desmethyl thiazole orange dye has made the preparation of the nucleoside unit and the reactions in the cycles of DNA synthesis more efficient because both the dye and the dye-tethering nucleoside are soluble in the organic solvents used in the synthetic processes. The dye introduction step to the synthesized ODN and subsequent product purification step, required in a conventional ECHO probe, were omitted. (ii) Incorporation of a 'ready-made' fluorescent nucleotide into ODNs by adoption of an uncharged dye enabled the further incorporation of a different fluorescence color by combination with the conventional ECHO probe procedure. Double-colored ECHO probes facilitated fluorometric observation in the diverse aspects of the physical and chemical status of the labeled ODN. (iii) The protonation of desmethyl thiazole orange showed stronger fluorescence in the disaggregated state. Desmethyl thiazole orange, which shows fluorescence at a lower pH, will be a useful dye unit for the design of pH-sensitive ECHO probes. The controlled emission provided valuable information on the hybridization of the probe to RNA both *in vitro* and in living cells. This new concept of hybridization-sensitive probes supported by a photochemical basis would be a starting point for the development of an effective method for detection of nucleic acids. It serves a variety of practical applications for multicolor RNA imaging in living cells.

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