Fluorescent Probes

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Exciton-Controlled Hybridization-Sensitive Fluorescent Probes: Multicolor Detection of Nucleic Acids**

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The design of a fluorescent probe in which the fluorescence is switched off when the probe does not recognize the target nucleic acid is very important for the establishment of a nucleic acid imaging method. The requirements for fluorescent probes for nucleic acid detection are not only sequence-selective emission and the avoidance of nonspecific emission, but it is also important to ensure that the emission is polychrome for the simultaneous monitoring of different targets. The following have been used to date for the molecular design of highly functional fluorescent probes containing an "on–off" switching system: photophysics and photochemistry (e.g. excimer formation,[1] photoinduced charge transfer,[2] photoinduced electron transfer,[3] and fluorescence resonance energy transfer).[4] In the design of these probes, the sensitivity of the dye to the environment, higher-ordered probe conformations, and quenching by electron transfer from/to nucleobases all have a strong influence on fluorescence intensity and sensitivity and often impair predictions. The multicoloring of probes is also limited by a fluorescence-switching mechanism, the probe conformation, or synthetic processes.

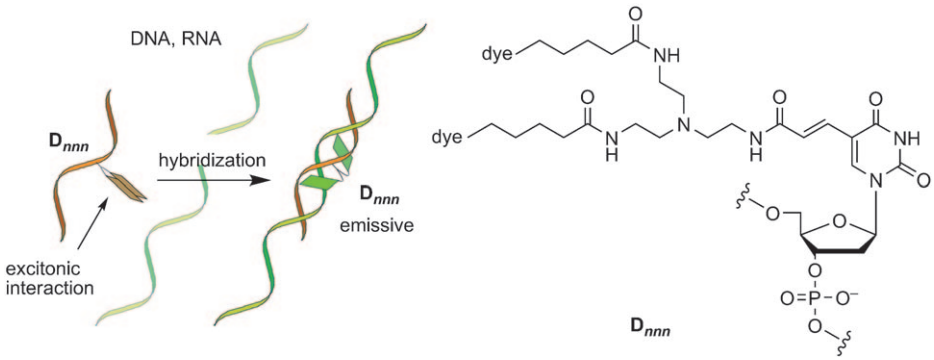

Figure 1. Structure of excitonic-interaction-controlled fluorescent probes for nucleic acid detection. Doubly fluorescent dye labeled nucleotides are shown as $\mathbf{D}_{nnn}$ ("*nnn*" denotes the best wavelength for excitation).

We have focused on the excitonic interaction observed for thiazole orange fluorescent dyes and designed a doubly fluorescence labeled nucleotide for a new efficient nucleic acid detection method.[5–7] The fluorescence of the probe in which two thiazole orange dye molecules are attached to a pyrimidine base is well-controlled by an excitonic interaction (Figure 1). An excitonic interaction is produced by the formation of an H aggregate between the dyes. As a result, emission from the probe before hybridization is suppressed. Dissociation of the aggregates by hybridization with the complementary strand results in disruption of the excitonic interaction and strong emission from the hybrid. This clear change in fluorescence intensity is rapid and reversible.[5,7] Multicoloring of this exciton-controlled probe could result in the simple simultaneous detection of plural target nucleic acid sequences and could be useful in efforts to elucidate the temporal correlation of gene expression and interactions between nucleic acids. For this purpose, new nucleotides doubly labeled with other dyes should be designed without loss of the high on–off performance as hybridization-sensitive fluorescent probes based on excitonic interactions.

We herein report a new concept for the effective design of hybridization-sensitive fluorescent DNA probes with different colors. Our designed probes showed strong emission upon hybridization with the target strand, whereas emission was suppressed in the single-stranded state. Their fluorescence is effective for the multicolor imaging of intracellular RNA, as it is controlled by an excitonic interaction.

We synthesized a series of new fluorescent nucleotides in which derivatives are substituted for the thiazole orange moiety (Figure 2). The fluorescent dyes for incorporation were obtained by coupling a methylquinoline, dimethylaniline, or benzothiazole subunit with a carboxylate-terminated alkyl linker containing benzothiazole, benzoxazole, or benzoselenazole derivatives.[8] The carboxylate end of the alkyl linker of the dyes was activated by succinimidylation, and two molecules of the activated dye were subsequently incorporated into an oligodeoxyribonucleotide (ODN) containing 2′-deoxyuridine with two amino ends.[5]

The absorption and emission of the ODN strands doubly labeled with twelve different colors, 5′-d(TAC-CAG$\mathbf{D}_{nnn}$CACCAT)-3′ (where doubly fluorescent dye

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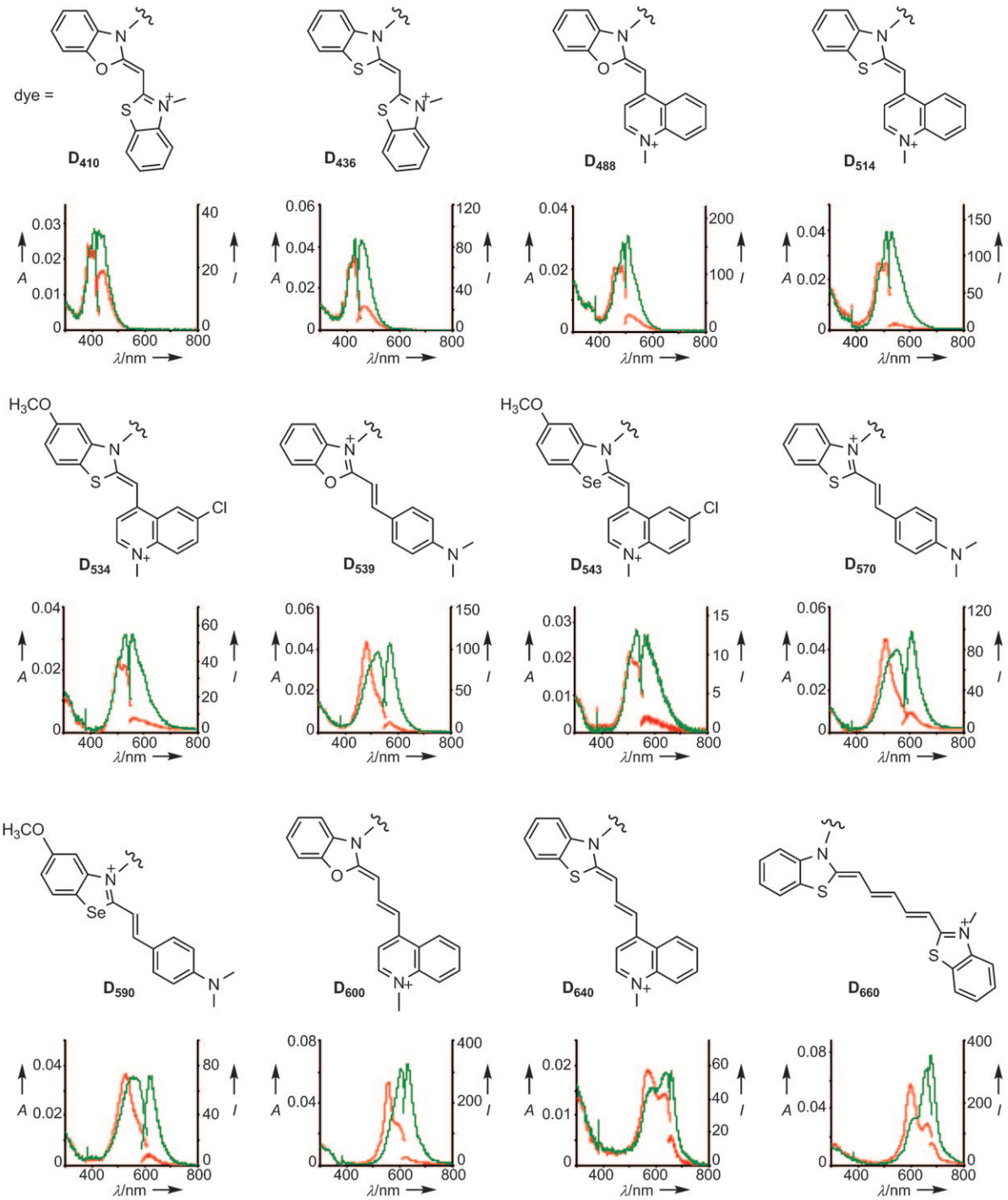

Figure 2. Absorption and emission of excitonic-interaction-controlled fluorescent probes before and after hybridization with the complementary DNA. Absorption (shorter wavelength) and emission (longer wavelength) spectra are indicated by red and green lines for the nonhybridized probe and the hybrid, respectively. The following nucleic acid sequences were used: 5′-d(TACCAG**D**$_{nnn}$CACCAT)-3′/5′-d(ATGGTGACTGGTA)-3′. Photophysical data are given in Table S1 in the Supporting Information.

labeled nucleotides are shown as $\mathbf{D}_{nnn}$), were investigated before and after hybridization with the complementary DNA strands (Figure 2; see Table S1 in the Supporting Information). Fluorescence emission was observed immediately after the addition of the labeled ODN to a solution of the complementary nucleic acid. The emission was suppressed in the nonhybridized state. The source of the fluorescence behavior was confirmed by the absorption spectra. The absorption band of the nonhybridized probe appears at a shorter wavelength than that of the hybrid. This blue shift suggested the splitting of the excited state because of H aggregation of the dyes.[5,9] H aggregation allowed only

transitions to the upper excitonic level. The excited state was rapidly transferred to the lower level, but the path from this energy level to the ground state was not emissive. The fluorescence from the fluorescent ODNs was suppressed by an interdye excitonic interaction in the nonhybridized state. Fluorescence quenching was much more effective than for probes labeled with one dye unit.[5,10] Hybridization with the target nucleic acid resulted in a great enhancement of emission with dissociation of the aggregate. This exciton-controlled fluorescence behavior was also observed for doubly labeled ODNs with other sequences (see Table S2 in the Supporting Information).

However, among the twelve colored nucleotides considered, only the D_{410}-containing ODN showed relatively strong emission, even when the ODN was in the nonhybridized state. In this case, the blue-shifted absorption spectrum suggested the existence of an interdye excitonic interaction. The excitation spectra revealed two peaks, and the absorption bands of the aggregate and the non-aggregate overlapped. These spectra are quite different from the excitation spectra of the $\mathbf{D_{514}}$ probe, for which only one signal corresponding to the non-aggregate was observed (Figure 3). Furthermore, the excitation and emission spectra showed that excitation at a shorter wavelength (aggregate) corresponded to emission at a longer wavelength, while excitation at a longer wavelength (non-aggregate) corresponded to emission at a shorter wavelength. This result suggested that the dyes of the $\mathbf{D_{410}}$ nucleotide were inclined with respect to one another, and that the emission from the lower excitonic state after excitation to the upper excitonic state was not completely forbidden.[9,11]

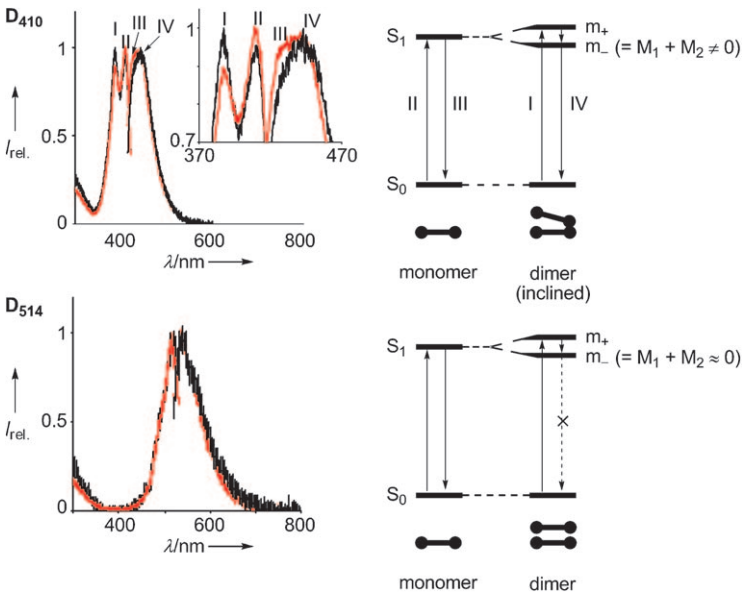

Figure 3. Excitation and emission spectra of the single-stranded $\mathbf{D_{410}}$ and $\mathbf{D_{514}}$ probes 5′-d(TACCAG$\mathbf{D}_{nnn}$CACCAT)-3′, and a model of molecule orientation and transition dipole interactions in H aggregates. $\mathbf{D_{410}}$: excitation spectra for emission at 426 (red) and 480 nm (black), and emission spectra at 410 (red) and 360 nm (black); $\mathbf{D_{514}}$: excitation spectra for emission at 537 (red) and 600 nm (black), and emission spectra at 516 (red) and 480 nm (black). M is the transition dipole moment; m_+ and m_- are the excitonic states.

Control of fluorescence emission by an excitonic interaction was also evident when the target was RNA. Several colored nucleotides exhibited a shift of the absorption bands and the switching of fluorescence intensity upon hybridization with the complementary RNA strands. However, inefficient exciton control was observed in some nucleotides. The shift of the absorption bands was minimal for nucleotides with hemicyanine dyes ($\mathbf{D_{539}}$, $\mathbf{D_{570}}$, and $\mathbf{D_{590}}$). The absorption bands occurred at relatively short wavelengths for both the hybrid and the nonhybrid form, in contrast to the absorption behavior of nucleotides in Figure 2. The DNA-binding ability of hemicyanines is known to be inherently lower than that of thiazole orange dyes.[12] The small shift in the absorption band suggests that the dissociation of the dye aggregate is inefficient in the hybrid with RNA, and that it is difficult for the dyes to bind independently to the hybrid structure. Similarly, $\mathbf{D_{640}}$ and $\mathbf{D_{660}}$, which have relatively larger conjugated systems, showed low efficiency of dye-aggregate dissociation upon hybrid formation with RNA. The ratio of the fluorescence intensity of the hybrid to that of the nonhybrid in these nucleotides was not as high as for other nucleotides that were controlled well by an excitonic interaction.

The appropriate design of dye aggregation in the nonhybridized state is the key point that determines the ability of doubly labeled ODNs to function as hybridization-sensitive fluorescent probes. We developed a series of hybridization-sensitive fluorescent probes that cover the excitation-wavelength range 400–700 nm. From this series, we can select the probe that is most suitable for the nature and number of nucleic acids, and the filter type of the detector.

We believed that these doubly labeled ODNs might be useful for multicolor RNA imaging. The development of chemical methods for imaging the dynamic and static behavior of RNA in a living cell is essential for increasing our understanding of cell life: a key goal of life scientists.[13] Although many methods for RNA detection have been developed, such as molecular beacons,[14] MS2-GFP fusion proteins,[15] GFP reconstitution,[16] quenched autoligation probes,[17] and dye-binding aptamers,[18] there are various associated problems, including the low availability of many color probes, limitations in sequence design, slow response in terms of reactivity or conformation change, high background fluorescence, or low fluorescence reversibility. Our new hybridization-sensitive probes may offer solutions to these issues and provide advantages.

We next designed a model experiment with a series of probes for RNA imaging in a living cell. The probes capable of binding to the polyA tail of mRNA, 5′-d($T_6\mathbf{D}_{nnn}T_6$)-3′, were transfected to HeLa cells with the common transfection reagent Lipofectamine 2000. After incubation for 1 h and washing, fluorescence emission from the cells was observed at the wavelength expected for each $\mathbf{D}_{nnn}$ (Figure 4a). The fluorescence from the cells decreased upon competitive hybridization of a dT 70-mer (dT_{70}) DNA molecule. This fluorescence

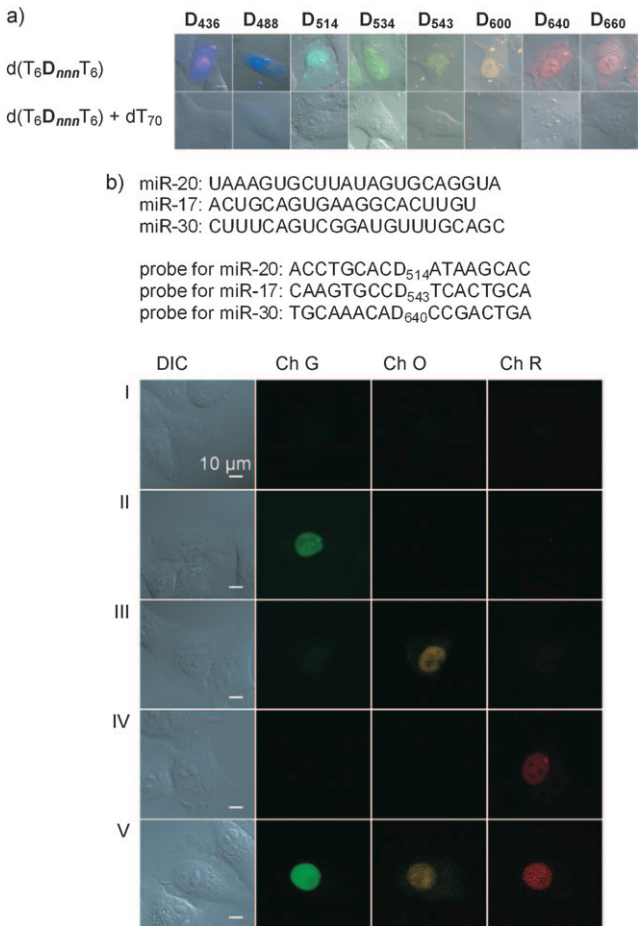

Figure 4. Multicolored living cells. a) Transfection of color probes 5′-d(T_6**D**$_{nnn}$$T_6$)-3′ to HeLa cells. Top: Fluorescence images of probe-transfected cells. Bottom: Fluorescence images of the cells in which probe binding to the mRNA polyA tail is blocked by dT_{70} DNA. Scale bar: 10 μm. b) Fluorescence images of miRNA-rich HeLa cells, into which the probe mixture was injected. DIC: differential interference contrast images; Ch G: λ_{ex} = 514 nm (Ar), λ_{em} = 520–555 nm; Ch O: λ_{ex} = 543 nm (He–Ne), λ_{em} = 560–615 nm; Ch R: λ_{ex} = 633 nm (He–Ne), λ_{em} = 656–731 nm. I) Control, II) miR-20-rich cell, III) miR-17-rich cell, IV) miR-30-rich cell, V) miR-17-, miR-20-, and miR-30-rich cell. Scale bar: 10 μm.

decrease suggests that the probe to be hybridized with the polyA tail of mRNA was replaced with a dT_{70} DNA unit to form a more stable duplex with polyA RNA (T_m = 61 °C).[7] The transfection efficiency of the probes labeled with hemicyanine dyes (**D**$_{539}$, **D**$_{570}$, and **D**$_{590}$) was very low (< 5 %), and fluorescence from the cells was not observed.

The model experiment of multicolor live-cell RNA imaging was also carried out with HeLa cells. Cells containing one or all of three different small noncoding microRNA strands (miRNA) were prepared by microinjection into nuclei (Figure 4b).[19] We synthesized three probes, each of which corresponded to a different miRNA strand. The probes each had one of three different fluorescence-labeled nucleotides (**D**$_{514}$, **D**$_{543}$, or **D**$_{640}$), which were selected from the series of **D**$_{nnn}$ because their fluorescence wavelengths were widely separated. The three probes were mixed and microinjected into the nuclei of living HeLa cells. The fluorescence appeared immediately in the nucleus of each cell, and the emission wavelength corresponded to the type of intracellular miRNA. The target-specific emission indicated that the probe recognized the target miRNA in the cell and emitted fluorescence with a characteristic color. The fluorescence in the cells was identified as emission from the dyes of the probes on the basis of emission spectra obtained with a multichannel spectrum detector. Three colors were observed simultaneously for the cell containing the three miRNA strands.

In conclusion, we have designed a series of fluorescent probes based on the concept of quenching caused by an excitonic interaction. The hybridization-sensitive, quencher-free fluorescent probes with various colors facilitate the imaging of intracellular RNA. Although further aspects need to be examined, such as the detection of a very small amount of RNA, we anticipate that this new concept of hybridization-sensitive probes based on photochemical techniques will be the starting point for the development of a practical assay for the simultaneous imaging of plural RNA molecules in living cells.

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[19] Approximately 100 fL of a solution of miRNA strands (6–48 μM), estimated to be 10^2–10^3 times the inherent amount of intracellular miRNA strands, was microinjected. Fluorescence from the original miRNA is therefore negligible in this model experiment.

