*Award Accounts*

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# Design of Functional Nucleic Acid Systems for Biomolecular Analysis

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With the aim of realizing an in situ nucleic acid analysis, we have developed functional nucleic acid probes that can autonomously detect and analyze nucleic acid sequences of interest. Fluorophore-labeled artificial oligonucleotide probes with target-dependent self-ligation or self-digestion functionalities can be used to sense nucleic acids in solution, on solid supports, or even in cells. In addition, we have successfully established a new sensing system using nonmodified nucleic acids as a probe. These probes and sensors are all composed of nonmodified natural-type nucleic acids, and thus can be transcribed directly from dsDNA, giving a high potency for in situ or in-cell application.

## 1. Introduction

Nucleic acids play an essential role in the "Central dogma of molecular biology," and there is an increasing demand for rapid and accurate methods for nucleic acid analysis.[1] Although a number of methods have been reported, most of these are only applicable to isolated/purified target nucleic acids. Such isolation/purification processes require the destruction of the cell membrane, resulting in a loss of individual information for several types of cells. The information that can be obtained from such conventional techniques is the mean information from the cell assembly. However, recent reports have indicated that cellular nucleic acid activities are not uniform. For example, the timing of RNA transcription, and the amount and localization of transcripts vary among cells, and such differences may be a critical factor in cellular function.[2] To elucidate the biological significance of such nonuniformity on nucleic acid activity, a nucleic acid sensing method that can work in situ shows high promise, especially when applied in-cell without losing cellular individuality. We have taken up the challenge to develop an autonomous nucleic acid sensing probe aimed at in situ applications, and this paper provides an overview of our effort toward this goal.

## 2. Artificial Nucleic Acid Probes for Nucleic Acid Sensing

Nucleic acids can form a double-stranded structure by G–C and A–T (DNA) or A–U (RNA) complementary base pairing, so that nucleic acid sensing typically relies on target–oligonucleotide probe hybridization in combination with a reporter system. Fluorophore-modified oligodeoxynucleotides (ODNs) are particularly appropriate probes, as the fluorescence-based sensing approach is sensitive, and can be performed in real time, in a multiplex manner, and in situ.[3–5]

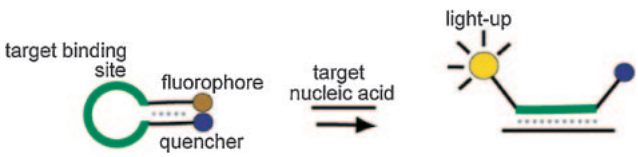


**Figure 1.** A schematic illustration showing how a molecular beacon works as a fluorescence-switching probe for sensing of a target nucleic acid.

To date, many fluorophore-labeled ODN probes have been reported, and a representative probe among these is the molecular beacon (MB) probe (Figure 1).[6–13] MB is a hairpin-shaped ODN that has a fluorophore (denoted by the brown circle in Figure 1) and a quencher (denoted by the blue circle in Figure 1) at both ends. In the absence of a target nucleic acid, MB forms a hairpin structure, in which the quencher molecule is in close proximity to the fluorophore molecule, and so quenches the fluorescence. On the other hand, in the presence of a target nucleic acid, the target binds to the loop region of MB (colored green in Figure 1) to form a rigid double-stranded structure, and thus the quencher/fluorophore pair separate from each other and the molecule "lights up." This is a simple but efficient way to turn on fluorescence in a target-nucleic-acid-dependent manner. The fluorescence-switching mechanism requires no washing steps and no additional reagents. Thus, MB can work as a mix-and-read probe, allowing in situ or even in-cell[11–13] detection of target nucleic acids. However, some shortcomings are also shown on MB probes. For example, the change in fluorescence of MB tends to be affected by the solution conditions; moreover, methods that rely on hybridization alone are less sequence selective. Therefore, we rose to the challenge of developing artificial ODN probes with

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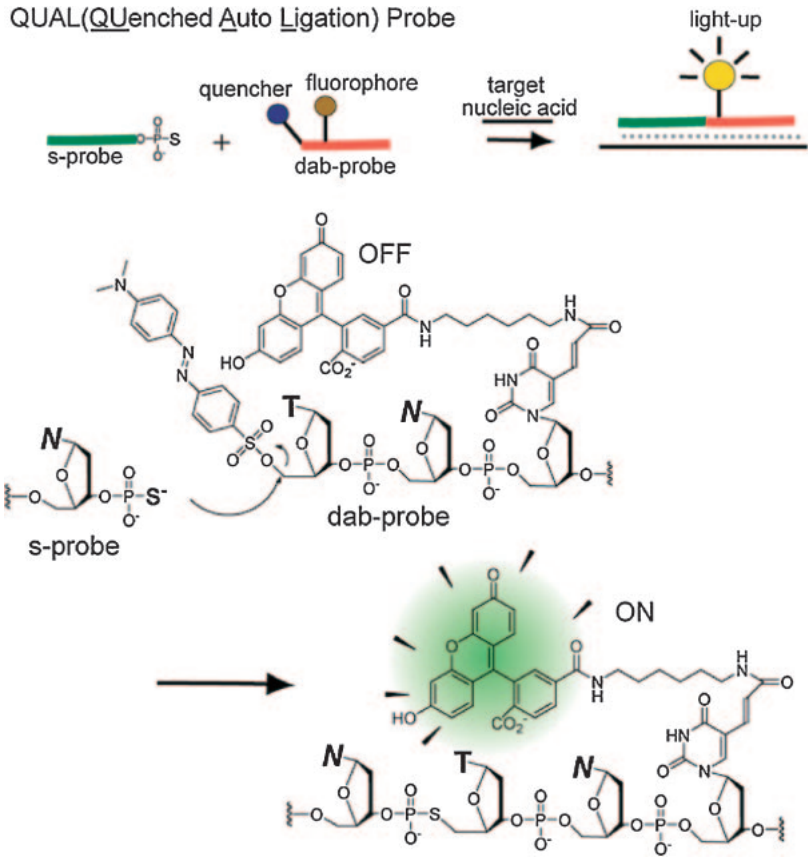


**Figure 2.** A QUAL probe for the light-up detection of a target nucleic acid.

improved robustness and sequence selectivity, aimed at the in-cell sensing of nucleic acids.

**2.1 QUAL Probe.** To realize the sequence-selective detection of target nucleic acids, the quenched auto ligation (QUAL) system was developed in the laboratory of Professor E. T. Kool.[14–16] The concept of the QUAL system is unique (Figure 2).[14–21] The two probes used in this system are complementary to the target nucleic acid, and hybridize on the target in a next-to-next fashion. The 5′-hydroxy group of the fluorophore-labeled probe (the dab-probe, colored red in Figure 2) is activated by a dabsyl quencher as a leaving group. The 3′-hydroxy group of the other probe (the s-probe, colored green in Figure 2) is modified with nucleophilic phosphorothioate. In the absence of a target nucleic acid, these ODN probes do not react. On the other hand, in the presence of a target nucleic acid, these probes bind to adjacent sites on the target, and undergo DNA-joining reactions via the nucleophilic displacement of the dab-probe by the nucleophilic s-probe. During the nucleophilic displacement reaction, the dabsyl group moves away from the dab-probe. The dabsyl activator doubles as a quencher of the adjacent fluorophore. Therefore, the loss of the dabsyl group results in a loss in the quenching capability of the fluorophore, and thus the molecule lights up. Since the DNA-joining reaction proceeds using the target nucleic acid as a template, the recovered fluorescence is indicative of the presence of the target.

When fluorescein-labeled QUAL probes were applied, this allowed a clear fluorescence-on detection of a target nucleic acid sequence in solution and on a solid support at the resolution of a single nucleotide.[14] These probes require no washing steps and no additional reagents, invoking the possibility of their application to cells. Fluorescein-labeled QUAL probe targeting 16S ribosomal RNAs worked in fixed[15,16] and nonfixed *Escherichia coli* (*E. coli*) cells[15] without showing a significant false-positive signal. To the best of our knowledge, this is the first use of a light-up probe in a nonfixed microorganism. In addition, multicolor identification of nucleic acids was achieved using four fluorescent-dye-labeled QUAL sets.[16] In particular, green/red QUAL probes have realized two-color nucleic acid sensing at a single nucleotide resolution in *E. coli* cells.[16]

**2.2 Programmed Self-Cleaving DNAzyme Probe.** QUAL probes achieved the autonomous sensing of target nucleic acids at the resolution of a single nucleotide. This was a breakthrough for in-cell applications. However, the method still had room for improvement in terms of its *sensitivity*. The sensitivity is another factor of vital importance. Since the ligated QUAL probes can be hybridized to the target nucleic acids, the target cannot be used catalytically, except for when [probe] $\gg$ [target].[17] The next challenge is signal amplification, which could be achieved when a sensing reaction occurs catalytically. This was attempted by designing a self-cleaving DNAzyme probe as a new type of mix-and-read nucleic acid sensor with signal amplification.[22,23] The designed probe was composed of three domains:[24–26] a target-binding sequence (colored red in Figure 3a), a DNAzyme sequence (colored orange in Figure 3a),[27] and a cleavage site (colored green in Figure 3a). As shown in Figure 3b, hybridization with the target in Step 1

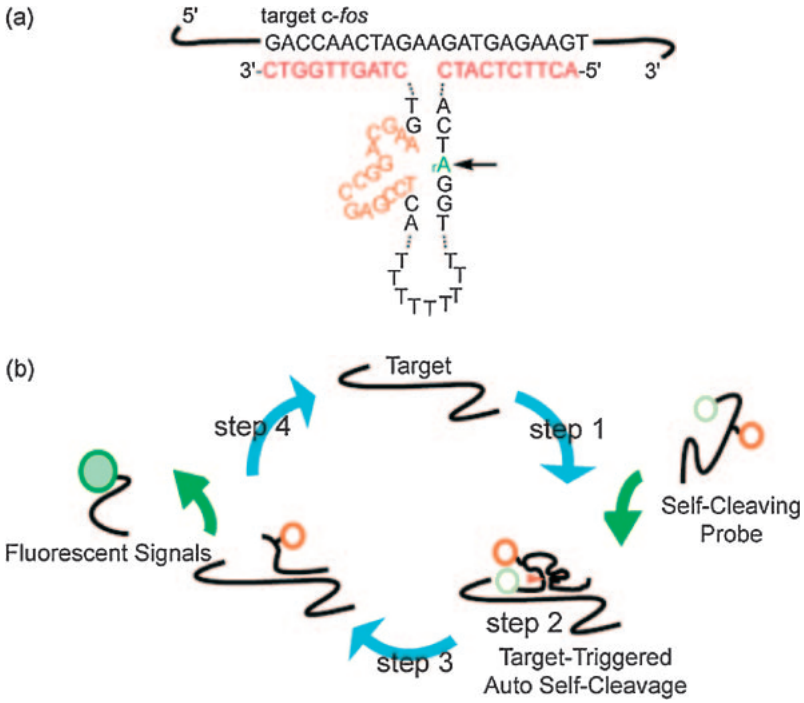


**Figure 3.** (a) Self-cleaving probe for the detection of a c-*fos* sequence. The probe is composed of a target-binding (red) and a DNAzyme (orange) component. Ribonucleotide rA (green) denoted by the black arrow is the site of self-cleavage. (b) A schematic illustration showing how a FRET-self-cleaving probe works as a fluorescence signaling sensor for a target nucleic acid.

stabilizes the internal hairpin structure of the probe to produce an active DNAzyme conformation, which in Step 2 cleaves the self-strand at the facing ribonucleotide moiety, denoted by the orange arrow in Figure 3b, and the two shortened fragments of the probe are released thermodynamically (Step 3) to form the probe-free target (Step 4), to which a new probe is bound (Step 1) to then drive a catalytic or amplification cycle.

Based on the design principle discussed above, we synthesized a fluorescence-reporting self-cleaving probe, targeting the c-*fos* sequence, having a fluorescein/dabsyl fluorescence resonance energy transfer (FRET) pair across the cleavage site.[22] The *mix-and-read* fluorescence assay using the FRET self-cleaving probe achieved a sensing of the targeted ODN having the c-*fos* sequence.[22] Although there are still improvements to be made in the catalytic activity, the above system is a promising prototype for in situ nucleic acid analysis.

## 3. Natural-Type Nucleic Acid Probes for Nucleic Acid Sensing

As described in Section **2**, artificial ODN probes, especially those with fluorescence-reporting functionalities, are the first choice for designing mix-and-read nucleic acid sensing probes. However, such artificial ODN probes have problems in in situ or in-cell applications because of their possible cytotoxicity and difficulty of delivery into cells. Therefore, we met the challenge of designing new nucleic acid sensing systems using a nonmodified natural-type nucleic acid as a probe, instead of using artificial probes.

**3.1 Molecular Beacon–mRNA.** We harnessed the power of cellular translation to achieve nucleic acid sensing using a nonmodified nucleic acid as a probe. The designed MB-type mRNA (MB–mRNA) probe contained a *cis*-acting MB-like RNA structure in the 5′-untranslated region (Figure 4a), wherein the loop (colored green in Figure 4a) was complementary to the target nucleic acid, and the stem consisted of sequences for a ribosome-binding site (RBS, colored red in Figure 4a) and the anti-RBS domain complementary to it (colored pink in Figure 4a).[28–31] The RBS was followed by a reporter gene sequence starting with an AUG start codon (colored blue in Figure 4a). In the absence of a target, the probe forms an MB-type hairpin structure with an RBS–antiRBS base pairing. Access of the ribosome to the RBS is essential for translation initiation, so that the formation of a hairpin switches off the translation activity.[32] On the other hand, the binding of a target at the loop opens the MB structure, thereby making the RBS domain accessible to the ribosome, and hence initiating the translation of the reporter protein, e.g., luciferase. In this system, we could detect the target nucleic acid by measuring the activity of the translated reporter protein.

First, we prepared a luciferase reporter-linked MB–mRNA targeting 16-nucleotide ODN sequence of the human CC chemokine receptor 5.[28] The MB–mRNA probes achieved sensing of the target ODN in a prokaryotic in vitro translation system by measuring the activity of the translated luciferase. The presence of a target ODN could be visualized using a chemiluminescence imager (Figure 4b). This is the first demonstration of an mRNA-based nucleic acid sensing system. The significance of the present approach is at least dual. First, this allows double amplification, i.e., not only in the enzymatic process of the translated reporter protein, but also in the translation/transcription processes. Second, the sensing is achieved using natural-type RNA. The probe requires no modification with fluorophore/quencher molecules or inorgan-

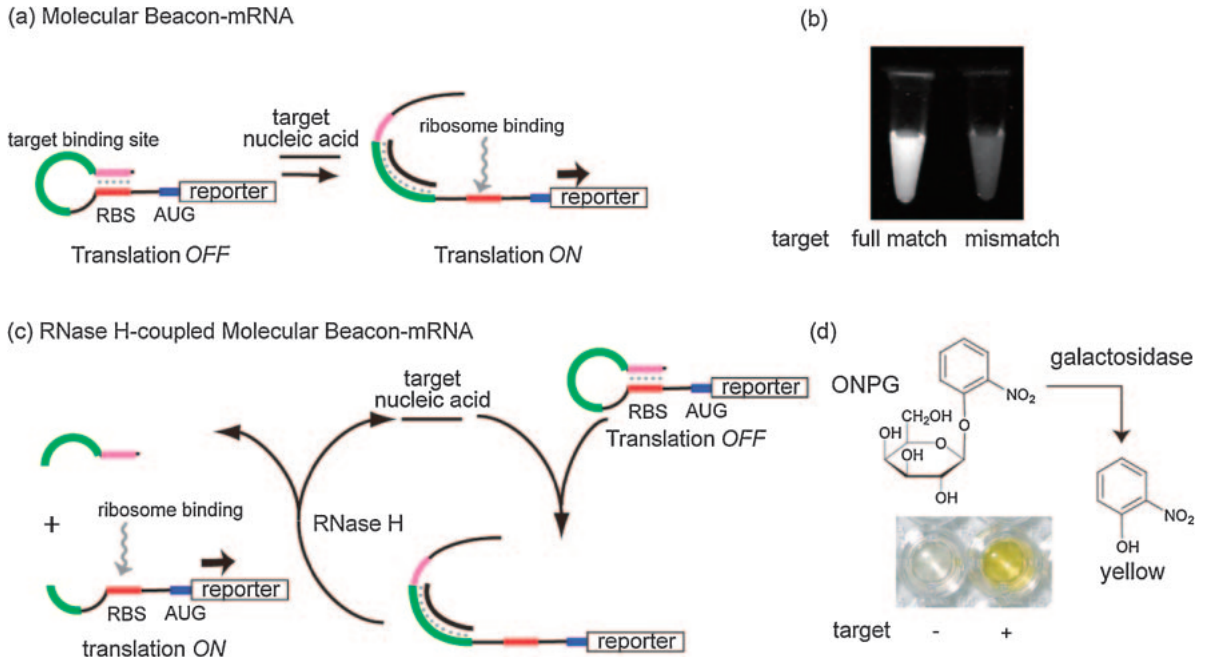


**Figure 4.** (a) A designed MB–mRNA probe. The ribosome-binding site (RBS), anti-RBS, start codon, and target-binding site are denoted in red, pink, blue, and green, respectively. (b) Chemiluminescence image of an MB–mRNA system (reporter protein: luciferase, substrate: luciferin) in the presence of a matched or two-base mismatched target nucleic acid. (c) An RNase H-coupled MB–mRNA system. The ribosome-binding site (RBS), anti-RBS, start codon, and target-binding domain are denoted in red, pink, blue, and green, respectively. (d) Visible sensing of a target nucleic acid using an RNase H-coupled MB–mRNA system (reporter protein: $\beta$-galactosidase, substrate: ONPG).

ic nanoparticles, such as quantum dots, and is thus directly transcribed from the dsDNA template. Therefore, it is friendly toward cells, and is suitable for in-cell application, since one can take advantage of the cellular transcription/translation system directly.

**3.2 Expanded Molecular Beacon–mRNA.** Although the MB–mRNA system was originally designed for in-cell nucleic acid sensing, the versatility of this system opened up applications for in vitro use. One example is an RNase H activity-coupled MB–mRNA system (Figure 4c).[29,30] RNase H is an endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA in a DNA/RNA heteroduplex. Therefore, the MB–mRNA system, coupled with an RNase H activity, could drive an additional process for the catalytic production of a translation-activated mRNA probe, wherein the target DNA-bound loop region is cleaved by the RNase H to release an anti-RBS stem domain away from the mRNA body irreversibly, thus allowing the catalytic use of a target DNA (Figure 4c). The RNase H-coupled system improved the sensitivity (i.e., fmol level) and selectivity (i.e., single nucleotide resolution).[29] In addition, since the MB–mRNA system can use any type of reporter protein, the system can detect target nucleic acids using a variety of output signals. When the RNase H-coupled MB–mRNA system was linked with multicolor luciferase reporters, it enabled multicolor sensing of single nucleotide differences (SNPs) in a target nucleic acid sequence.[30] Moreover, when coupled with $\beta$-galactosidase as a reporter in combination with an *ortho*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) substrate, the detection of target nucleic acid could be readily achieved with the naked eye (Figure 4d).[29]

## 4. Fluorescent Nucleic Acid

Fluorescent nucleic acid is another type of nucleic acid analysis probe that has a nonmodified nucleic acid structure. The Nobel Prize in Chemistry for 2008 was awarded to Profs Shimomura, Chalfie, and Tsien for their discovery and development of green fluorescent protein (GFP). GFP is an indispensable tool for protein analysis in cells. Proteins of interest can be fused to GFP at the genome level and translated in cells as a fusion protein with GFP, wherein such fused GFP is used as a fluorescent marker, typically called a fluorescent tag, to analyze the localization or movement of a target protein.[33] One can imagine that if we have a fluorescent nucleic acid, which is the DNA/RNA counterpart of the fluorescent protein, this could be an innovative tool for in-cell analysis of nucleic acids.[34] From an analogy with GFP technology, one can analyze nucleic acids of interest by fusing them with such a fluorescent nucleic acid as a tag. Ideal fluorescent nucleic acids are those that emit fluorescence by themselves. Unfortunately, the greatest obstacle is that such fluorescent nucleic acids have not been discovered yet. An alternative method would be to use a nucleic acid sequence/structure that binds specifically to, and thereby lights up, an otherwise nonfluorescent dye as a chromophore.[35–41] However, despite the high potential of such a light-up pair, no successful demonstration had so far been reported on the monitoring of the mRNA transcription processes, even under in vitro conditions.

Our challenge was to produce a light-up fluorophore–nucleic acid tag pair for such a biological application. However, the generation of a new pair was difficult, so we designed an

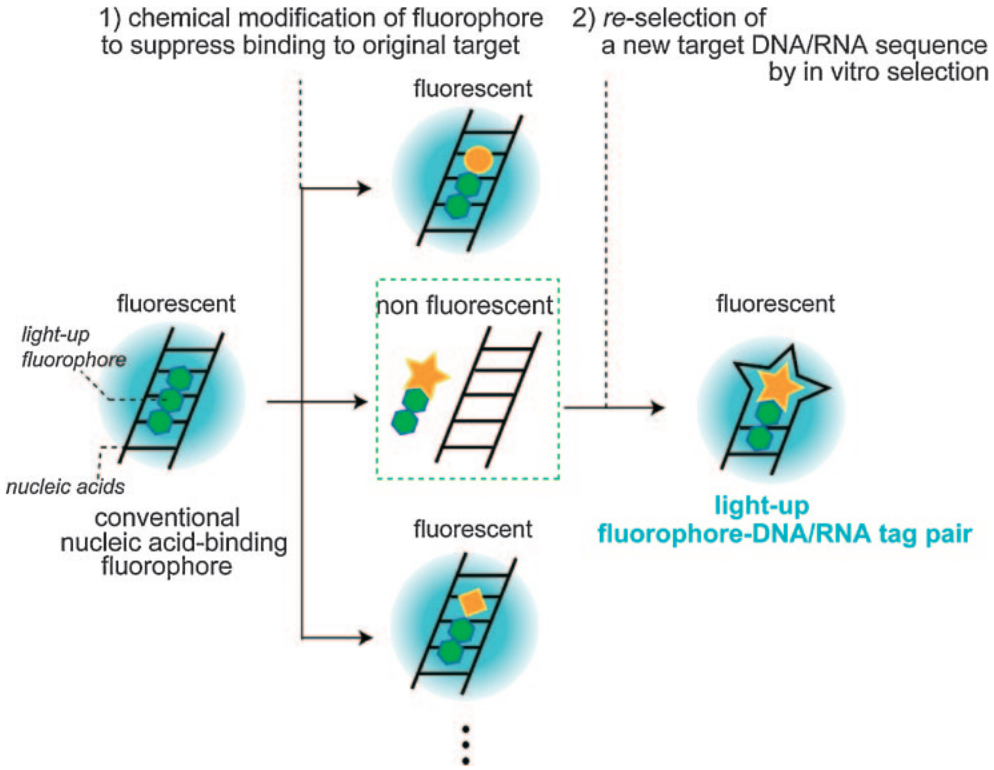


**Figure 5.** A strategy for developing a new light-up fluorophore–tag pair from a conventional nucleic acid-binding fluorophore.

unprecedented strategy for the generation of a new light-up pair starting from conventional cell-staining fluorescent dyes, which had already been verified as satisfying the prerequisites for in-cell use, i.e., good solubility in aqueous solutions, cell permeability, substantial fluorescence enhancement/shift on binding to a target, and a high degree of bio-orthogonality. Our approach was based on an improvement of such known cell-applicable nucleic acid-selective fluorescent dyes to a nucleic-acid-structure-specific dye by narrowing the range of adaptable target sites using the following steps (Figure 5). The first step was the chemical modification of a conventional nucleic acid-binding fluorophore to suppress its binding to the original target. The second step was *re*-selection using an in vitro selection method[42–44] of a new target (i.e., DNA or RNA tag) for the down-modified fluorophore. We thought that the fluorophore tag pairs thus obtained could work as fluorescent nucleic acids.

**4.1 Blue Fluorescent DNA.** We focused on the well-known Hoechst dye, a conventional dsDNA-imaging probe.[45,46] Following the evolution process shown in Figure 5, we successfully obtained a new fluorescent DNA composed of a modified Hoechst dye and an optimized 25-mer DNA-tag structure (Figure 6a).[40] The otherwise almost nonfluorescent-modified Hoechst dye, having *tert*-butyl groups on its benzene ring to suppress binding to the original dsDNA target, enhanced its fluorescence by 191 times on binding with the optimized DNA sequence ($K_d = 878$ nM). It is interesting, and even surprising, that the simple in vitro selected DNA sequence can work as a trigger to light up the otherwise nonfluorescent fluorophores on binding. This is the first blue fluorescent DNA reported. Then, we engineered the fluorescence DNA to a

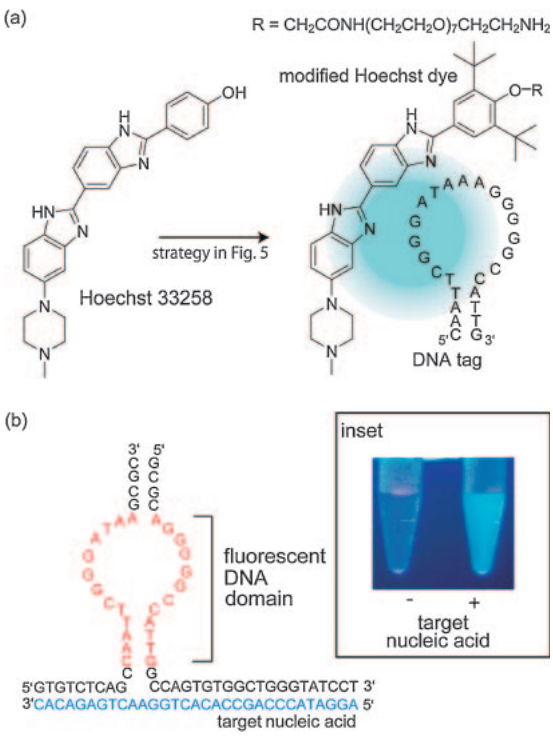


**Figure 6.** (a) A Hoechst 33258 (left-hand side) and a modified Hoechst dye-optimized DNA tag pair (right-hand side). (b) Binary probes for nucleic acid sensing. The inset shows a fluorescence image (excitation using a 366 nm transilluminator) of a solution containing the binary probes and the modified Hoechst dye in the presence (right-hand side) and absence (left-hand side) of the target nucleic acid.

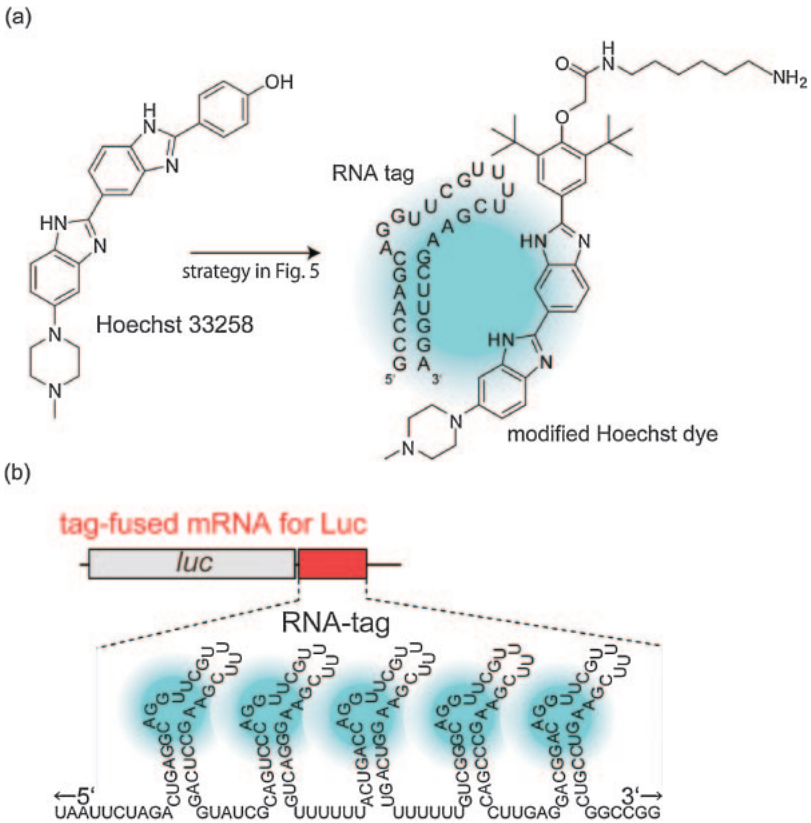


**Figure 7.** (a) A Hoechst 33258 (left-hand side) and a modified Hoechst dye-optimized RNA tag pair (right-hand side). (b) Tag-fused mRNA for luciferase. The fused mRNA contains a tag domain composed of five successive RNA tag sequences with slight modification.

nucleic acid sensing probe by dividing it into binary type,[47] designed to form the blue fluorescent DNA complex only in the presence of a target nucleic acid (Figure 6b). The binary blue fluorescent DNA sensor was capable of fluorescent-on detection of a target nucleic acid with single nucleotide resolution. Unlike typical FRET-based ODN probes, such as MB, the sensor was free from covalent modification with fluorophore and quencher dyes. Therefore, these unmodified probes can be prepared easily and cost effectively, and also have an advantage in that an unexpectedly cleaved probe does not generate an unfavorable false-positive signal, which sometimes causes problems in sensing experiments using FRET–ODN probes.

**4.2 Blue Fluorescent RNA.** The strategy shown in Figure 5 can also be applied to the generation of fluorescent RNAs. A blue fluorescent RNA was successfully obtained by in vitro selection of an RNA sequence against a modified Hoechst chromophore (Figure 7a).[41] The modified Hoechst dye bound to an optimized 29-nt RNA tag sequence with a dissociation constant of 35 nM enhanced its fluorescence in the blue region by approximately 50 times, indicating that the RNA tag–fluorophore complex was a blue fluorescent RNA. We applied the blue fluorescent RNA as a fluorescent tag for mRNA transcription monitoring (Figure 7b). During the T7 RNA transcription of luciferase mRNA, a clear enhancement of the fluorescence was observed only for the mRNA fused with the RNA tag. This indicates that the selected pair can be used as a light-up blue fluorescent RNA tag when fused with a given RNA.

**4.3 Blue Fluorescent RNA Sensors.** The blue fluorescent RNA can be used as a fluorescent RNA tag, and also as a fluorescent-signaling module of an aptamer sensor.[34] Aptamers specifically recognize targets, so there is much interest in applying aptamer technology to sensors.[48,49] The aptamer itself has no reporting properties, and thus needs to be coupled with a signaling module, i.e., covalent modification of an aptamer with a fluorophore/quencher.[50] However, these processes require considerable time and expertise, limiting their use in a variety of fields. One promising approach is to use fluorescent nucleic acids as a fluorescence-signaling module of the aptamer sensor.[51,52] Since the fluorescence RNA is composed of nonmodified RNA, the resulting aptamer sensor does not require covalent modification.

When producing such a blue fluorescence-signaling aptamer sensor, the critical issue is how to switch the fluorescence *ON* or *OFF* of an otherwise constitutively fluorescent active-blue fluorescence RNA using a target molecule–aptamer binding event as the trigger. Therefore, we designed a blue fluorescent RNA sensor based on the competitive structure-switching model (Figure 8),[53] wherein blue fluorescent RNA (signaling module, colored blue in Figure 8) and aptamer (recognition module, colored red in Figure 8) sequences compete for a UUGG sequence (communication module, colored green in Figure 8). In the absence of a target, the fluorescent signaling module hybridizes with the UUGG communication module to switch off the fluorescence of the blue fluorescence RNA. On the other hand, the presence of a target is designed to switch the

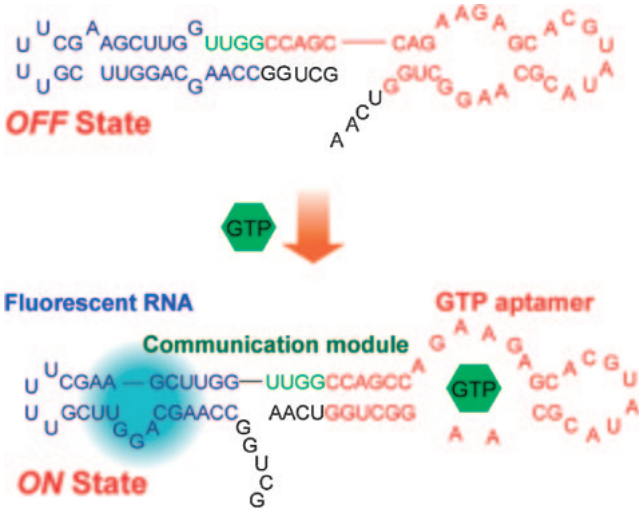


**Figure 8.** A designed blue fluorescent RNA sensor targeting GTP and the predicted conformational change on binding of the GTP. The fluorescent RNA, communication module, and GTP aptamer module are denoted in blue, green, and red, respectively.

RNA structure to light up the fluorescence of the blue fluorescent RNA module. Following this idea, initially we prepared a blue fluorescence aptamer sensor targeting adenosine diphosphate (ADP) by combination of an ADP aptamer[54] and our blue fluorescent RNA motif. The sensor enhanced the fluorescence in the presence of the target ADP.[52] The selectivity was high enough to discriminate simple differences in the phosphate structure among ADP, adenosine monophosphate (AMP), and cyclic AMP. An advantage of the present design strategy is its wide applicability to a variety of targets. Because the sensor can be divided into three structurally independent modules, "recognition," "communication," and fluorescence "signaling," the sensor can be applied to new sensors that can target different molecules by simply replacing the recognition module with an appropriate aptamer. When the ADP aptamer domain was replaced by a guanosine triphosphate (GTP) aptamer,[55] the sensor was successfully converted to a GTP aptamer sensor signaling with a blue fluorescence (Figure 8).[52] This versatility is a unique advantage of the fluorescent RNA sensor.

## 5. Conclusion

Several examples of our research have been described aimed at in situ nucleic acid sensing. Toward this objective, we have successfully developed nucleic acid sensing probes, including artificial and natural-type (i.e., chemically nonmodified) nucleic acid probes. In particular, nonmodified nucleic acid probes are a very promising approach. The MB–mRNAs, fluorescent RNA tags, and fluorescent RNA sensors discussed here are all composed of nonmodified RNA, and thus can be transcribed directly from a plasmid or genome. This biocompatibility is a critical difference from the conventional artificial probes, and thus gives high potency to in-cell applications. The continuing development of such probes could open up a new field of in-cell nucleic acid chemistry.

I would like to express my sincere gratitude to Professor Yasuhiro Aoyama and Professor Eric T. Kool for their kind guidance and discussion, and to all collaborators for their efforts in contributing to these studies.

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