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RNA imaging by chemical probes

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ABSTRACT

Sequence-specific detection of intracellular RNA is one of the most important approaches to understand life phenomena. However, it is difficult to detect RNA in living cells because of its variety and scarcity. In the last three decades, several chemical probes have been developed for RNA detection in living cells. These probes are composed of DNA or artificial nucleic acid and hybridize with the target RNA in a sequence-specific manner. This hybridization triggers a change of fluorescence or a chemical reaction. In this review, we classify the probes according to the associated fluorogenic mechanism, that is, interaction between fluorophore and quencher, environmental change of fluorophore, and template reaction with/without ligation. In addition, we introduce examples of RNA imaging in living cells.

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Contents

1. Introduction

RNA contributes to various cellular functions in the form of mRNA, rRNA, and microRNA. These RNA species differ markedly in their sequence, abundance, and function. Because the function of RNA is determined by its sequence, sequence-specific detection is required to understand life phenomena, such as gene expression and developmental biology. Fortunately, the complementary interaction of nucleic acids enables us to detect target RNA in a sequence-specific manner, although the variety and scarcity of RNA make the analysis difficult. A method for amplifying and detecting RNA *in vitro* has been established and refined. Specifically, RNA molecules can be amplified from cellular extracts by reverse transcription and polymerase chain reaction (PCR). In addition, methods of amplification using several different enzymes have been developed in combination with DNA strands [1,2], DNAzyme [3], helicase [4], and endonuclease [5]. However, the detection of intracellular RNA in living cells in particular is required to understand the changes of gene expression. Unfortunately, the above-mentioned enzyme-based amplifications require strict reaction conditions, making it difficult to amplify intracellular nucleic acids. In addition, some biological components inhibit these enzymes. Because of these limitations, enzymatic amplification is not available for intracellular RNA detection. To understand the localization of intracellular RNA, fluorescence *in situ* hybridization (FISH) has been developed. In canonical FISH, cells are fixed and treated with oligo-nucleotide probes labeled with fluorophore or enzymes. Unbound probes should be removed at the washing step because they provide false-positive signals. However, when signals depend on the interaction between the probe and its target, RNA in fixed cells can be detected without washing. In addition, some methods for

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RNA imaging in living cells has been developed by using interaction between nucleic acid and dye or protein. Some dyes emit fluorescence only when binding to RNA with specific sequence, spinach aptamer [6]. Because of this, RNA containing specific sequence can be detected by applied dyes. Similarly, specific interaction between the protein and nucleic acid with specific sequence can be utilized for nucleic acid detection. In MS2 method, GFP-fused MS2 protein, one of RNA binding proteins, is utilized as probe [7]. Though these methods have realized RNA imaging in living cells, the requirement of specific sequences implies that the sequence should be introduced by genetic modification. Because genetic modification can induce unpredictable effects in the cell, RNA imaging without genetic modification is desired for understanding natural behavior of RNA. Recently, several chemical probes have been developed for intracellular RNA imaging without genetic modifications. The characteristics of fluorophore and nucleic acid enable specific detection without washing and with high sensitivity. In this review, we summarize the chemical probes used for intracellular RNA imaging.

2. Molecular beacons

First, we summarize fluorescence probes without signal amplification. An example of these is molecular beacons, which were developed by Tyagi and Kramer in pioneering work [8]. The structure of a molecular beacon is shown in Fig. 1A. Its conformation changes in response to its targeted nucleic acid. The probe has sequence complementary to the target RNA and both a fluorophore and its quencher at each terminus. In the first design of such a probe, 5-((2-aminoethyl)amino) naphthalene-1-sulfonic acid (EDANS) and its quencher, 4-((4-(dimethylamino)phenyl)azo)benzoic acid (dabcyl), were utilized as fluorophore and quencher, respectively (Fig. 1B). Without target RNA, the probe strand forms a stem-loop structure. In this state, fluorescence of EDANS cannot be observed because the dabcyl group acts as a quencher for EDANS. Hybridization of the probe with target RNA induces opening of the stem-loop structure and separates EDANS from the quencher. In this hybridization state, the fluorescence signal of EDANS is detectable. This target-dependent fluorescence enables identification of a single-nucleotide mismatch. In 1998, *in situ* RNA visualization with a molecular beacon was reported [9]. Unfortunately, nucleic acids including a molecular beacon are spontaneously transported from the cytosol to the nucleus, which is thought to decrease the efficiency of the intracellular detection of mRNA. As one solution to this, it has been reported that localization of the beacon can be controlled by linking it to an artificial transcript of tRNA [10].

Other fluorophores have been utilized. For example, EDANS in the molecular beacon can be replaced with various fluorophores, such as coumarin, fluorescein, BODIPY, eosine, tetramethylrhodamine, and Texas red [11]. These fluorophores can be quenched by dabcyl with high efficiency. This character indicates that it is possible to develop a multi-color molecular beacon. In addition, molecular beacons can be utilized for real-time PCR [11–13]. Specifically, they are more useful for real-time PCR quantification of DNA containing tandem-repeat sequences than a fluorogenic intercalator.

To improve the specificity of molecular beacons, efforts have been made to evaluate the effects of modification of the sugar backbone [14]. 2′-OMe is one of the most common modifications of sugar backbones. Kinetic analysis indicated that 2′-OMe modification improved the stability of the probe with target RNA. In contrast, a molecular beacon containing a 2′-deoxy group is suitable for the recognition of DNA targets. Some groups also utilized artificial nucleic acids as molecular beacons. For example, D-threoninol nucleic acid is one of the nonribose artificial nucleic acids, which can hybridize with natural nucleic acids with high affinity, although the structure of its sugar backbone differs from the natural one. It has been reported that a molecular beacon composed of only this artificial nucleic acid achieved the specific detection of mRNA [15].

When target RNA has adopted a double-stranded structure, unwinding of the double strand is required. As one of the solutions for this, Frank-Kamenetskii and colleagues applied peptide nucleic acid (PNA) as a double-strand opener [16]. PNA interacts with complementary nucleobases with higher affinity than natural oligonucleotides and PNA strands possess high resistance to nuclease. When PNA containing a target sequence is added to a reaction solution, the PNA strand opens the double strand of the target gene [16]. However, PNA molecular beacons are less sensitive than molecular beacons, composed of natural DNA, because of PNA duplex stability. Interestingly, the specificity of molecular beacons can be improved without chemical modification. For example, Kolpashchikov's group reported that the specificity was improved by the combination of multiple DNA strands (Fig. 2A) [17]. The additional oligo-nucleotide strand for this can contain an analyte-

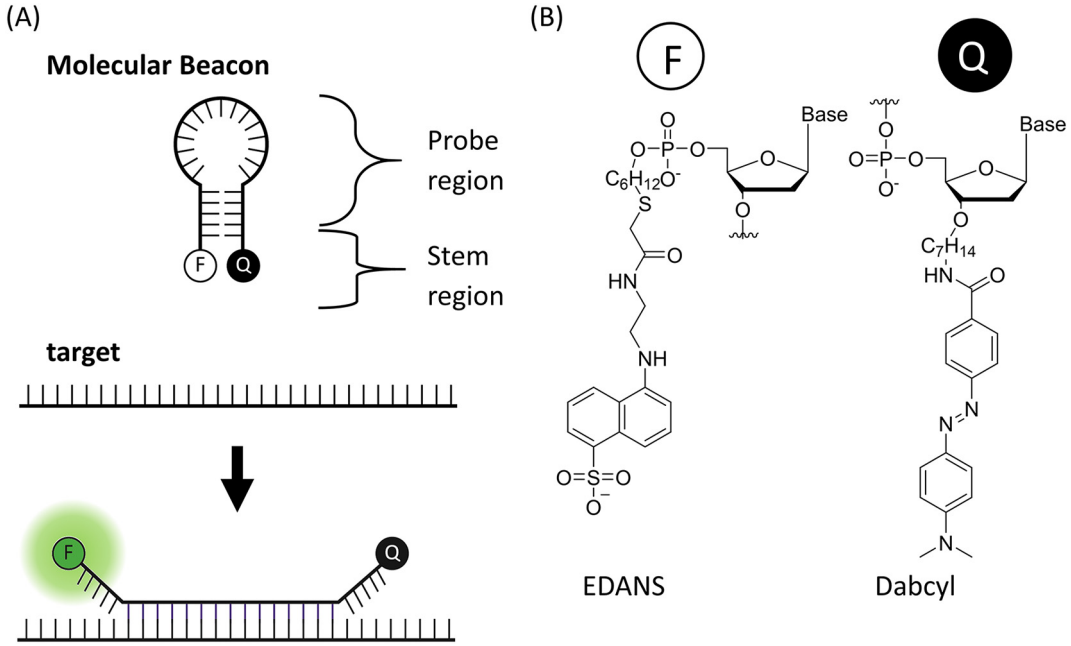


Fig. 1. Molecular beacon. Schematic diagram (A) and molecular structure (B) of a molecular beacon. F and Q indicate fluorophore and quencher, respectively.

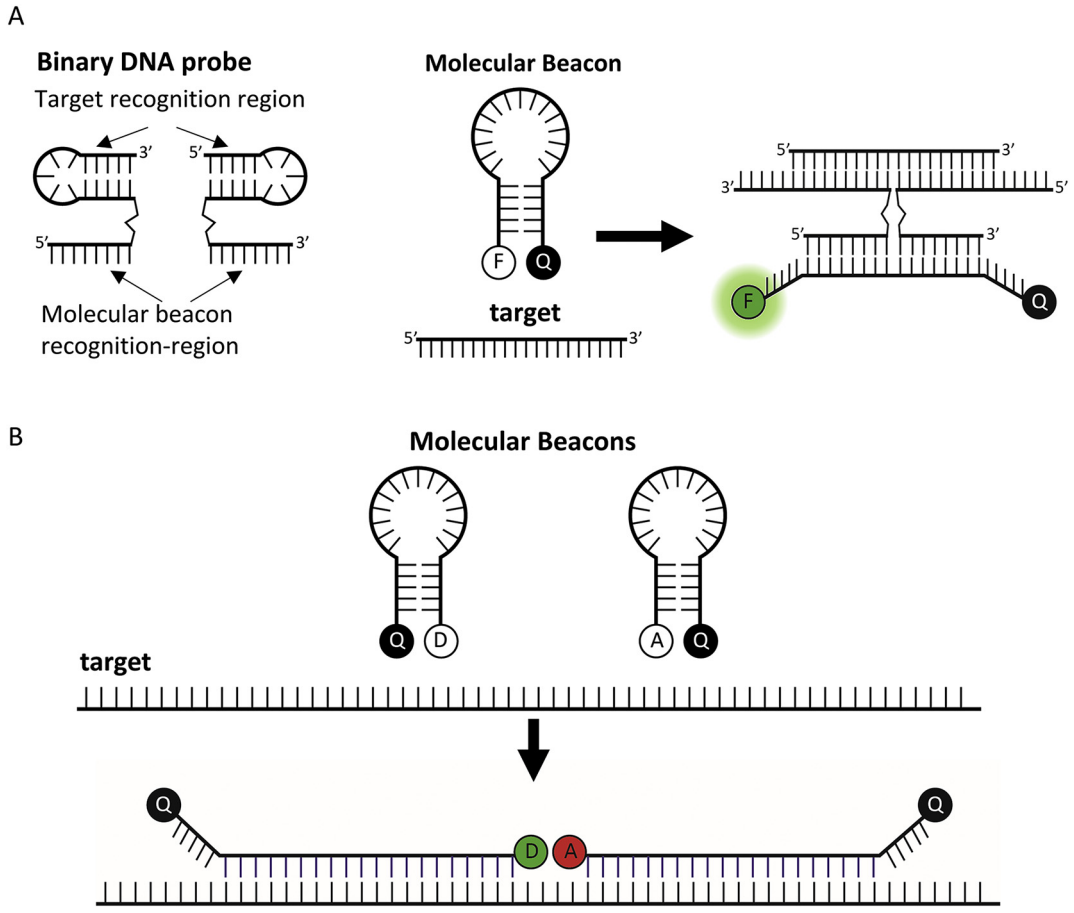


Fig. 2. Additional strand improves the specificity. (A) Binary DNA probe, (B) double FRET. D, A and Q indicate fluorescence donor, acceptor and quencher, respectively.

and beacon-binding arm. Only when the target strand hybridizes to the oligo-nucleotides does the molecular beacon hybridize to the oligo-nucleotides and open the stem region. This selection step increases the selectivity 200 times and enables the identification of bacterial species based on the sequence of ribosomal RNA [18]. The above-mentioned group developed a method to detect DNA using the combination of this strategy and DNAzyme [19]. In this method, additional oligo-nucleotide strands containing a DNAzyme catalytic core were used. When the target DNA strand hybridized to the additional oligo-nucleotide strands, the catalytic core was formed and catalyzed the cleavage of the molecular beacon. Because the cleaved molecular beacon was released and exhibited fluorescence, the sensitivity was improved. Recently, Katz and colleagues realized rapid and temporal control of nucleic acid detection with DNAzyme by introducing magnetic beads into probes [20,21]. In their improved strategy, magnetic field induces the aggregation of probes and promotes the DNAzyme-catalyzed reaction. Using this, target nucleic acids *in vitro* and in living cells can be detected only under magnetic field.

In addition, the function of a molecular beacon could be controlled by modifying its structure. By introducing a photo-cleavable linker into its terminus, the function of molecular beacons can be controlled by light [22]. This function is useful for the time- and location-specific detection of target RNA.

Further applications of molecular beacons have been reported. For example, recently, the detection of microRNA in exosomes was reported [23]. Although molecular beacons need to be specific probes for nucleic acid as described above, they have a shortcoming regarding a low signal-to-background ratio. To improve this ratio, a method of using a dual molecular beacon has been proposed (Fig. 2B) [24,25]. Specifically, when lanthanide chelate is utilized as a donor fluorophore in the dual system, the signal-to-background ratio is reported to be improved. Because background fluorescence occurs upon degradation of the stem region, the stability is increased by using artificial nucleotides such as phosphorothioate [26], 2′-OMe [14], PNA [16], and locked nucleic acid (LNA) [27,28]. Because LNA is one of the most stable artificial nucleic acids, the use of an LNA molecular beacon enables the detection of intracellular RNA [28]. In addition, it was reported that the molecular beacon–target complex was stabilized by photo-cross-linking [29].

As a procedure using another type of this probe, a displacement approach has been suggested [30]. In this method, a nucleic acid strand containing a fluorophore hybridizes with a strand containing a quencher. By displacing the quencher strand with the target strand, the quencher stand is released from the fluorophore strand. Furthermore, quencher was replaced with gold nanoparticles [31]. Fortunately, the quenching efficiency of the nanoparticle was equal to that of the dabcyl group. Furthermore, simultaneous detection of different cellular RNA species could be achieved by immobilizing multiple molecular beacons on gold nanoparticles [32–34].

For the improvement of fluorescence sensitivity, fluorophore and quencher can be localized at the stem region [35,36]; such probes are called in-stem molecular beacons. As specific examples, in-stem molecular beacons have been developed by introducing a fluorophore and its quencher into the D-threoninol backbone, one of the non-ribose nucleic acid backbones [36]. When perylene and anthraquinone were introduced into the D-threoninol backbone as a fluorophore and its quencher, respectively, perylene fluorescence could be quenched by pseudo-base-pair formation of these chemical groups (Fig. 3). In this beacon, multiple pairs of fluorophore and quencher could be introduced to improve the sensitivity and the signal-to-noise ratio [36–38]. When Cy3 derivative was utilized as a fluorophore, natural nucleotides could work as a quencher [35].

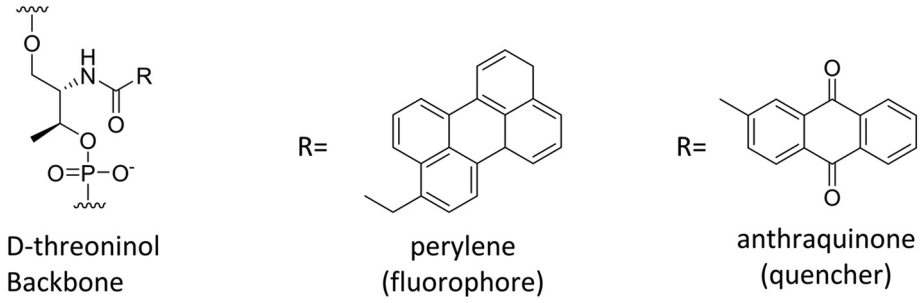


Fig. 3. Structures of perylene and anthraquinone.

Like molecular beacons, some DNA aptamers can also be utilized for RNA detection. When Cy3 is conjugated to BHQ, Cy3 fluorescence is quenched. The interaction of BHQ with BHQ aptamer activates Cy3 fluorescence. Uesugi and colleagues utilized this principle for RNA detection. They developed a DNA strand containing an RNA-targeting sequence and a BHQ aptamer. Interestingly, the aptamer structure formed only when the RNA–target moiety formed a double strand with the target strand in this probe. Because of this, Cy3 fluorescence was detected depending on the target strand [39].

3. Hybridization-sensitive probe

The fluorescence of some fluorophores is sensitive to the environment. For example, the fluorescence of thiazole orange changes dramatically when the fluorophore is intercalated between nucleobases. As such, when PNA possessing thiazole orange at the terminus *via* a flexible linker hybridized to the complementary strand, conjugated thiazole orange intercalated between the nucleobases and emitted a fluorescence signal [40]. Moreover, Seitz and colleagues developed a PNA probe containing thiazole orange within it. As shown in Fig. 4, this probe emits a fluorescence signal depending on the target RNA. This is referred to as the use of a forced intercalation (FIT) probe [41,42]. In FIT probes, thiazole orange is inserted at an internal region instead of the complementary nucleobase and behaves like canonical nucleobases [43]. Thiazole orange derivatives with different fluorescence wavelengths have also been developed. Seitz and colleagues achieved dual color imaging by using thiazole orange derivatives (Fig. 4B) [44]. Moreover, quinoline blue and BisQ exhibited emission maxima at wavelengths over 600 nm and suitable fluorophores for cellular imaging [45]. In another study, single-nucleotide alteration in mRNA could be detected by using two probes containing FRET acceptor and donor [46]. The combination of synthesized siRNA and PNA containing thiazole orange at the terminus enabled the visualization of intracellular siRNA [47]. As an intracellular application of FIT probe, the detection of mRNA and viral RNA was reported [44,48]. As another fluorophore, adenosine derivative and tricyclic base-linked acyclonucleoside have also been utilized [49-51].

As another design of a hybridization-sensitive probe, Asanuma and colleagues utilized multiple anthraquinone and perylene groups for a FIT probe as a quencher and a fluorophore, respectively [52,53]. In this probe, perylene quenched the fluorescence of anthraquinone in the absence of target RNA. In a state of hybridization with the target RNA, perylene intercalated with bases, resulting in a fluorescence signal. Moreover, by introducing multiple fluorophores, the group successfully increased the fluorescence signal and improved the signal-to-background ratio. Seitz and colleagues also increased the fluorescence intensity of FIT probe by using a combination of thiazole orange and its derivative [54]. This increase of fluorescence intensity could not be achieved by introducing multiple thiazole oranges into the probe strand. Recently, Seitz and colleagues also developed new fluorophores for FIT probes and achieved the simultaneous detection of multiple intracellular nucleic acids by FIT probes [55]. Hybridization-sensitive probes with other designs have also been reported. For example, Okamoto and colleagues developed an excitation-controlled hybridization-sensitive fluorescence oligonucleotide (ECHO) probe by introducing two thiazole oranges *via* a long flexible linker into the DNA strand [56]. The ECHO probe has a low background level because, in it, two thiazole orange moieties interact with each other and suppress fluorescence emission without a target strand. Fluorescence signals from two thiazole orange moieties were provided by the intercalation of thiazole orange moieties between nucleobases of the target strand. The ECHO probe has also been utilized for FISH [57] and RNA imaging in living cells [58].

Because the detection of RNA depends on hybridization of probe and target RNA, the detection of double-stranded RNA regions is challenging. Recently, it has been reported that dsRNA is detectable by utilizing its formation of a triplex with PNA [59,60]. In this method, a homopyrimidine PNA strand forms a Hoogsteen base pair with dsRNA. Nishizawa and colleagues reported that homopyrimidine PNA containing thiazole orange exhibits a fluorescence signal upon triplex

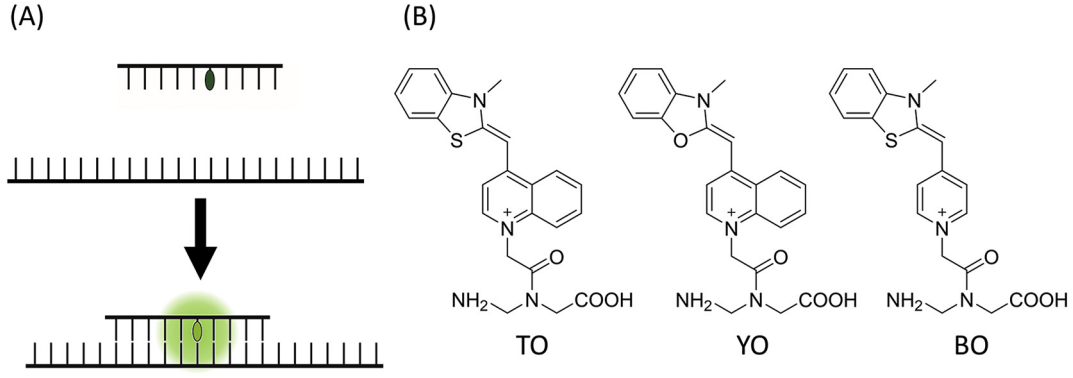


Fig. 4. Scheme of FIT probe. (A) Scheme of FIT probe, (B) structure of intercalators.

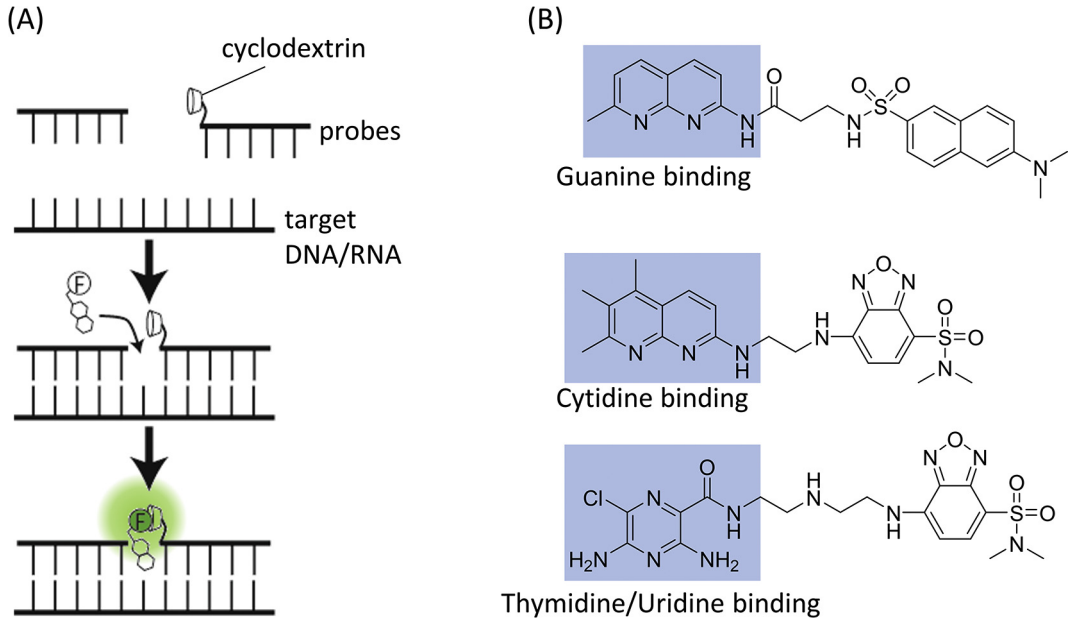


Fig. 5. Nucleobase detection using cyclodextrin-conjugated DNA. (A) Detection scheme, (B) structure of fluorophore-conjugated heterocycle.

formation [59]. In a further study, they succeeded in utilizing other fluorophores, quinoline blue and thiazole red, for triplex FIT probes [61]. Unfortunately, dsRNA can be detected with triplex-forming probes only at low pH, since the protonation of cytosine is indispensable for Hoogsteen base pairing. To overcome this bottleneck, Rozners and colleagues introduced 2-aminopyrimidine into PNA. They reported that the replacement of cytosine with 2-aminopyrimidine improved the binding affinity of PNA with dsRNA at pH 7.0 [62].

The interaction of fluorophore and probe–DNA complex has also been utilized for the detection of RNA. The combination of base-specific heterocycle and cyclodextrin-conjugated DNA enabled the reading of a single nucleotide at a specific position [63]. In this method, two DNA strands hybridize the target DNA or RNA strand and exhibit a gap. Cyclodextrin was conjugated to the 5′ terminus of one of the DNA strands and was located in the gap (Fig. 5). Fluorophore-conjugated heterocycle bound to this gap. The fluorophore exhibited emission by interaction with cyclodextrin, resulting in nucleobase-dependent fluorescence. Similarly, DNA strand detection could be achieved by using interaction between an abasic site and a fluorophore. Because some fluorophores interact with the specific nucleobase opposite an abasic site, the formation of target nucleic acid and DNA containing an abasic site enabled DNA detection [64–66] or RNA detection [67]. This method specifically enables the discrimination of polymorphisms in nucleobases opposite abasic sites. Furthermore, the signal to noise ratio could be improved by conjugation of an abasic-site binding ligand to an environmentally sensitive fluorophore [68,69]. In this method, the fluorophore moiety was intercalated between nucleobases [68] or interacted with the major groove [69] in the DNA and target RNA complex when an abasic site-binding ligand moiety of the probe ligand bound to an abasic site (Fig. 6). The conjugation to a fluorescent intercalator enabled the production of a fluorescence signal depending on the interaction with the abasic site [68]. On the other hand, the conjugation of abasic site-binding ligand with the minor groove-sensitive fluorophore enabled ratiometric analysis because the fluorescence of both ligand and fluorophore moiety was changed by the interaction with DNA [69].

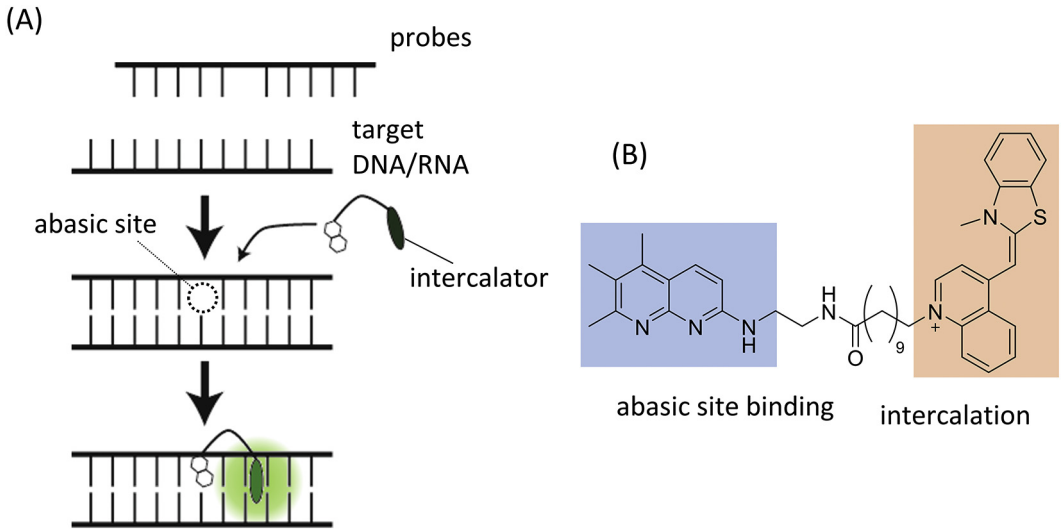


Fig. 6. Nucleobase detection using abasic site. (A) Interaction of DNA and probe, (B) structure of probe.

4. Chemical ligation for RNA detection

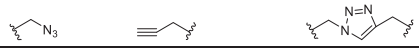
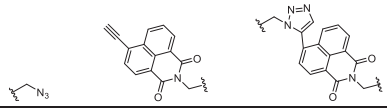
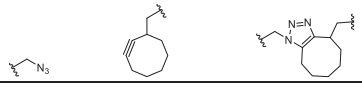
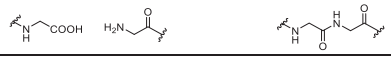
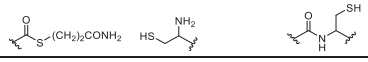
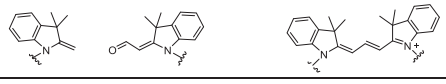
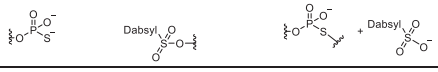
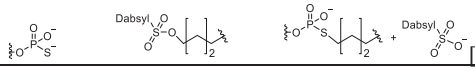
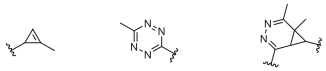
Generally, RNA species such as mRNA and microRNA play important roles in biological phenomena at low concentration. Because of this, signal detection requires improvements of the sensitivity of the detector and quantum yield of the fluorophore. One of the other solutions to this problem is to amplify the signal. Although various enzymatic amplifications have been suggested [70–75], the requirements regarding reaction conditions (*e.g.*, temperature, salt concentration, co-factor, and substrate concentration) make it difficult to amplify the signal from scarce target RNA in cells. This implies that a target-dependent chemical reaction is required. A nucleic acid templated reaction is a chemical reaction that occurs in a manner dependent on the complementary strand. In this reaction, hybridization of probes with target RNA induces the reaction between reactive moieties at probe termini. Some template reactions result in nonenzymatic chemical ligations. To ligate two DNA strands, such as in photo-ligation, click reaction [76,77] and native chemical ligation [78] were developed. These types of template-dependent chemical ligation contribute to the detection of nucleic acids.

Fujimoto and colleagues utilized template-dependent photo-ligation for nucleic acid detection. They activated 5′-carboxyvinyldeoxyuridine by using 366-nm light and triggered the ligation between this group and a cytidine base (Table 1.1) [79]. The specificity of this template reaction enables the detection of point mutations in DNA [80] and RNA [81]. Similarly, anthracene was utilized for photo-ligation [82].

Click chemistry, involving one of the biorthogonal reactions, has been utilized for template-dependent ligation in some studies. PNA or DNA strands could be ligated by introducing azido and alkyne groups into the strand terminus (Table 1.2) [76,77]. Besides simply ligating strands depending on the target strand, Mokhir and colleagues utilized click chemistry for fluorogenic ligation (Table 1.3) [83]. In this method, the formation of a ring structure by a click reaction activated the fluorescence. Even though the discrimination of single-nucleotide mutation could be achieved, these reactions requires copper. Because it is difficult for reactions with copper to occur in living cells, copper-free template-dependent ligation by click chemistry was reported (Table 1.4) [84].

Seitz and colleagues utilized chemical ligation between PNA strands for nucleic acid detection [78]. In the PNA ligation, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) activated the PNA terminus and triggered the ligation (Table 1.5). The condensing agent could be omitted by introducing a thioester group and a cysteine residue at the C– and N-termini of PNA probes, respectively (Table 1.6). Cysteine near the thioester triggered native chemical ligation. This group detected the ligation product *via* PCR amplification with biotinylated primer. The specificity of PNA ligation toward the target sequence required abasic ligation formation. When

Table 1
Ligation method.

Reaction name	Substrate	Product	Ref.
1 Photo-activate ligation			[79]
2. Click reaction			[76, 77]
3. Fluorogenic Click reaction			[83]
4. Copper-free Click reaction			[84]
5. EDC ligation			[78]
6. Native chemical ligation			[78]
7. Hemicyanine DPC reaction			[89]
8. Aldolization-elimination			[90]
9. QUAL			[91]
10. QUAL (universal linker)			[96]
11. Tetrazine ligation			[98]

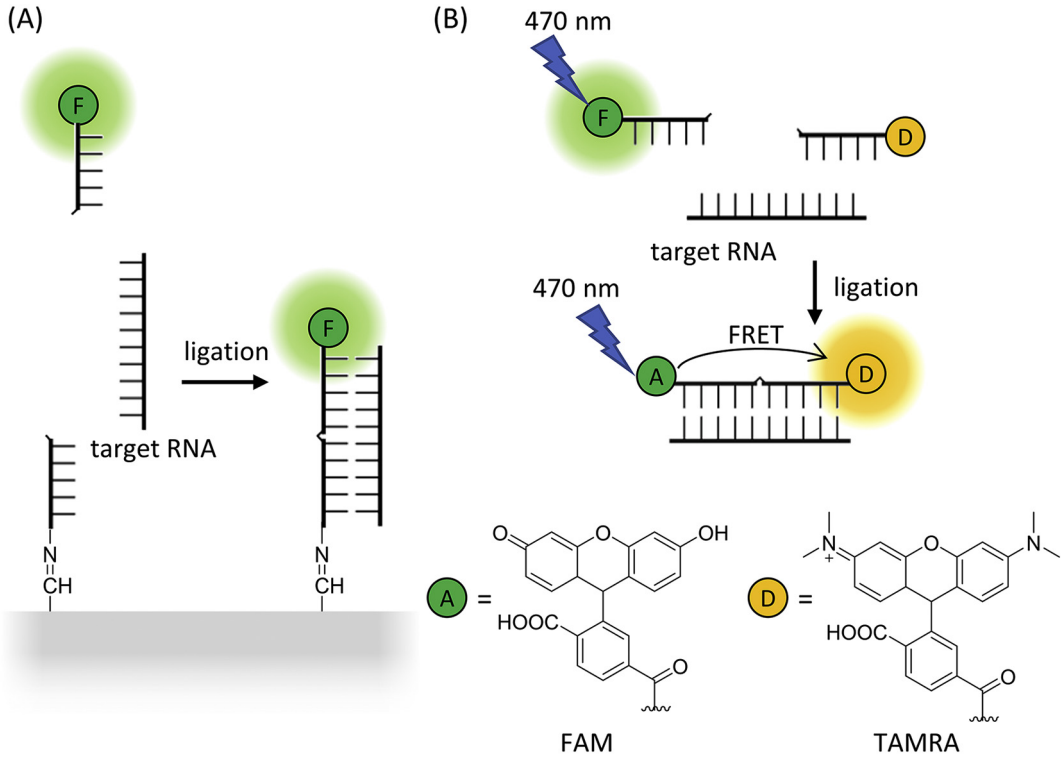


Fig. 7. Detection of ligation product. (A) Immobilization of probe, (B) FRET.

two PNA strands covered the target strand completely, the specificity was reduced [85].

Direct detection of the ligation product requires changes in fluorescence upon occurrence of the ligation reaction. The simple method for achieving this is the ligation of a fluorescently labeled DNA probe with an immobilized DNA probe (Fig. 7A) [81,86] or gel electrophoresis [77,87]. It has been reported that fluorescence changes are triggered by chemical ligation. One of the methods for this is the detection of ligation product by a FRET signal. When two probe strands contain a FRET donor or acceptor, the FRET signal is produced by the ligation of two probes, possessing a fluorescence donor or acceptor on the complementary nucleic acid (Fig. 7B) [88]. In this method, the FRET signal depends on the complementary strand and can be amplified by replacing the ligation product with unreacted probes. In addition, it has been reported that a fluorophore was formed between probes by a template reaction (Table 1.7 and 8) [89,90].

Kool and colleagues utilized a chemical ligation coupled with quencher release for chemical ligation, namely, a quenched autoligation (QUAL) method (Fig. 8 and Table 1.9) [91]. In their strategy, two nucleotides at the 5′ terminal region of one of two probe strands were modified. Thymidine at the 5′ terminus was conjugated with the dabcyl group *via* a sulfonic ester, and another modification was performed involving the conjugation of fluorescein to nucleobases. When probes hybridized with the target strand, phosphorothioate nucleophile at the 3′ terminus of another probe strand attacked the carbon linked with the leaving group. After this reaction, the dabcyl group was removed from the probe strand. This reaction occurred only when the target strand had probes located side by side, that is, involving a template-dependent reaction. Using this probe, target RNA-dependent fluorescence has been observed [92,93]. By replacing the fluorophore with FAM, TMR, AL350, and Cy5, multi-color QUAL was developed [94]. The evaluation *in vitro* indicated that multi-color QUAL can be utilized to identify single-nucleotide polymorphisms.

Although these chemical ligation methods exhibited target-dependent turnover, the ligation product inhibited the turnover rate. In fact, the reaction product exhibited higher affinity toward the target strand than the substrate because the length of the product was longer than that of substrates. To solve this problem, the ligation product was removed from the target strand by DNA polymerase at high temperature [95]. To reduce the product inhibition, the ligation product–template complex was destabilized by introducing an ethyl linker between the dabcyl group and the nucleic acid strand [96]. In this design, 5′ phosphorothioate attacks and forms a covalent bond with carbon next to the dabcyl group (Table 1.10). The structure of the ligated product differs from that of the natural DNA strand, meaning that the duplex of target and ligation product becomes unstable. When the PNA backbone is utilized for a probe, product inhibition is reported to be reduced by modification. The replacement of a cysteine moiety at the probe terminus with isocysteine reduced the stability of DNA–PNA and improved the reaction turnover rate [97]. Other reactions can also be utilized for this strategy. For example, Devaraja and colleagues utilized tetrazine ligation [98]. Tetrazine works as a quencher, conjugated to the 5′-terminus of the DNA strand, and quenches the fluorescence of fluorescein conjugated in the same DNA strand (Fig. 9 and Table 1.11). When the probe strand containing a tetrazine moiety hybridizes to the target RNA next to the probe containing methyl cyclopropene, probes are ligated by cycloaddition reaction. Because tetrazine's function of quenching the fluorescence is eliminated by this reaction, the fluorescence signal becomes detectable. This probe has benefits in terms of reaction velocity and stability.

5. Ligation-free method

As described above, ligation products have higher affinity to the template than to the substrate. A ligation-free template reaction is required for efficient signal amplification, which was successfully achieved by Seitz and colleagues. In this design, there are two PNA probes containing FAM or TAMRA. The probe containing FAM is linked to the dabcyl quencher *via* a cysteine residue. In contrast, the terminus of the probe containing TAMRA is cysteine. When probes are located side by side, the quencher is transferred to cysteine in another probe strand [99,100], which induces a change of fluorescence. In addition,

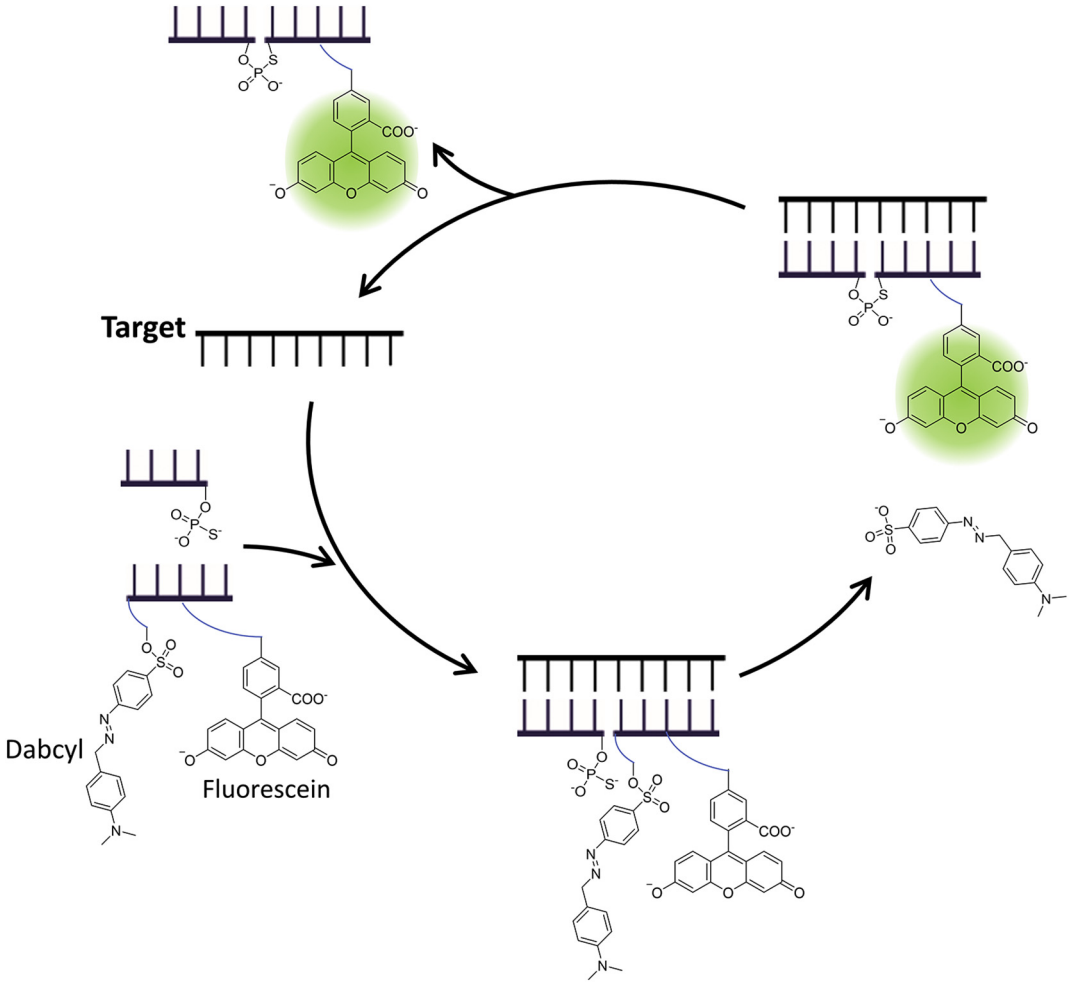


Fig. 8. Chemical ligation for fluorogenic reaction. Scheme of QUAL probe's reaction. Quencher leaves through ligation reaction.

they replaced the dabcyl group with biotin and trapped horseradish peroxidase (HRP) [101]. In this method, PNA as a biotin acceptor possessed a thioester group and a histidine tag at the N- and C-termini, respectively. After the template reaction, the biotin-acceptor strand was immobilized on a Ni-coated surface by interaction between the His tag and nickel. Following this, streptavidin-conjugated HRP was

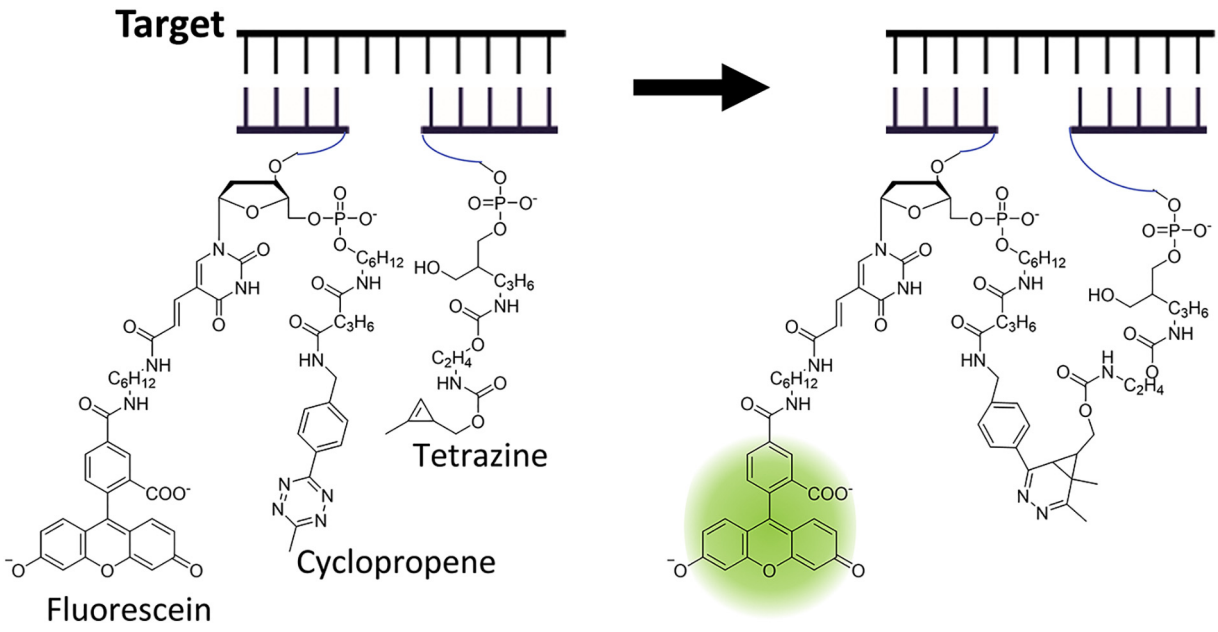


Fig. 9. Ligation with tetrazine reaction. Hybridization of probes to target RNA induces ligation between tetrazine and cyclopropane.

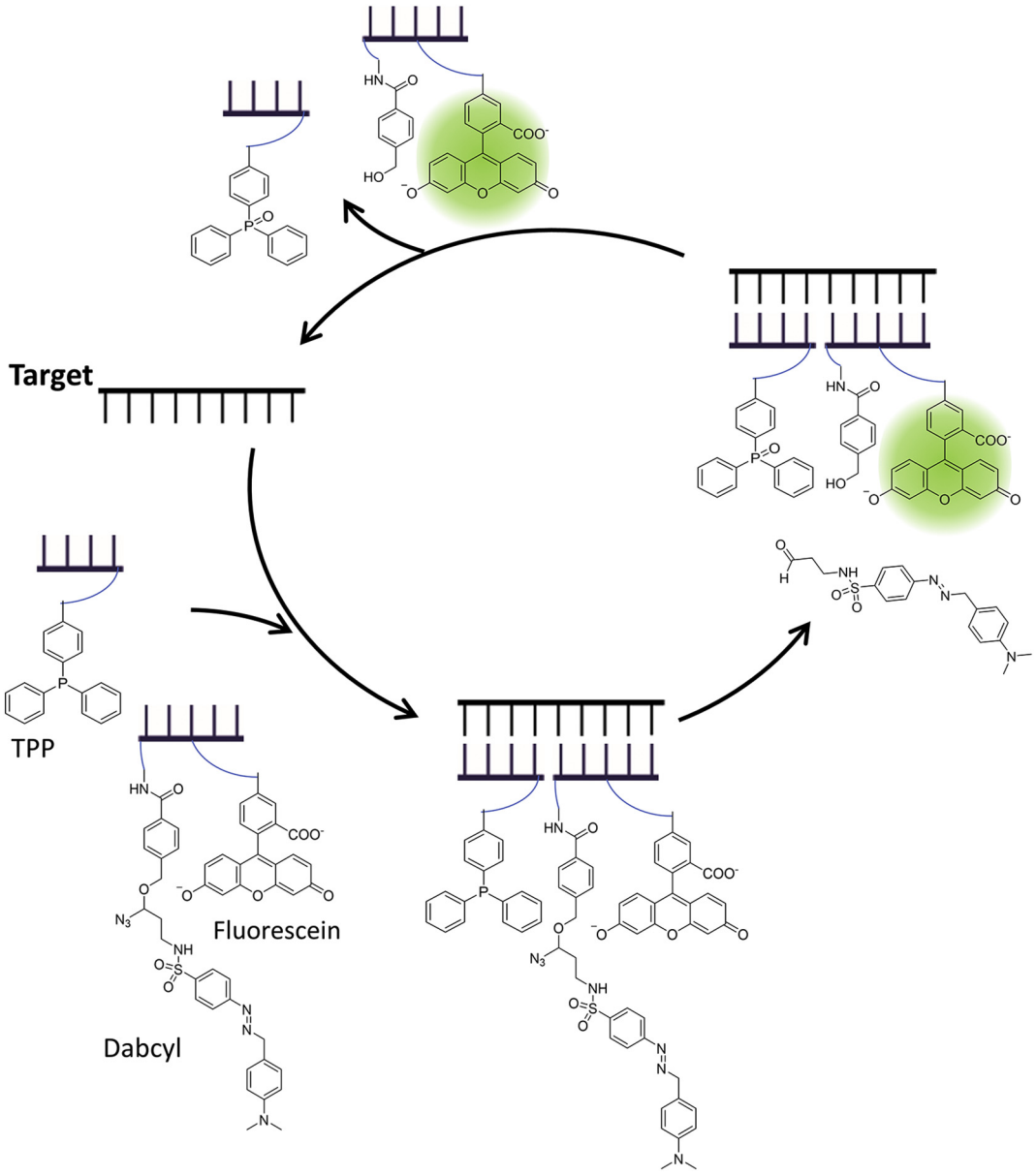


Fig. 10. Q-STAR. Reaction cycle of Q-STAR. Hybridization results in movement of the quencher away from the probe strand without ligation.

introduced onto the surface. When the template reaction occurred, HRP activity could be observed. Because this method involves two amplification procedures, a template reaction and HRP catalysis, high sensitivity, specifically, 500 attomoles of virus RNA, could be achieved.

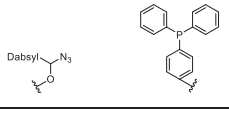
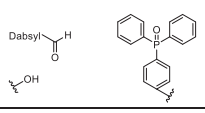
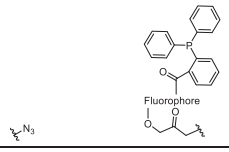
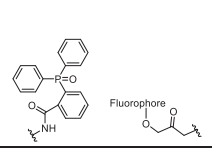
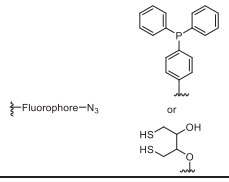
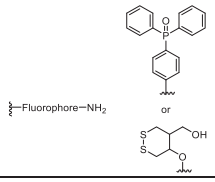
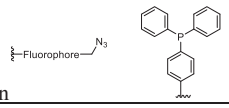
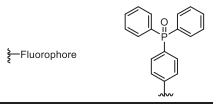
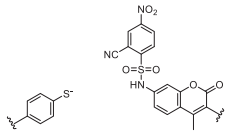
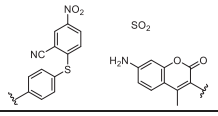
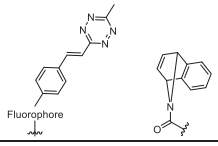
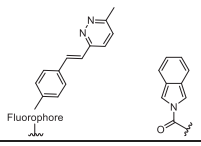
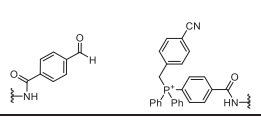
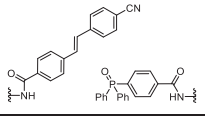
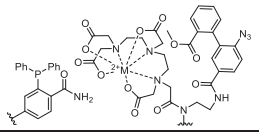
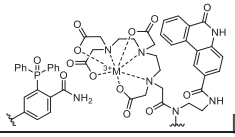
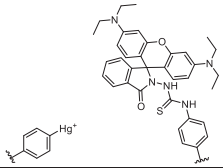
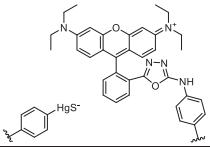
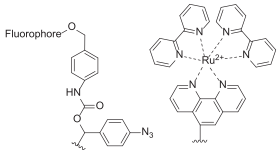
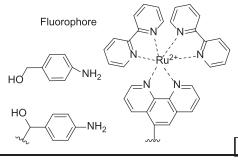
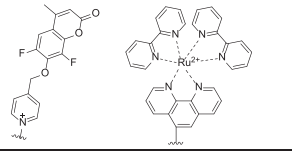
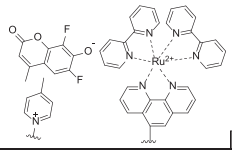
Kool and colleagues utilized the Staudinger reaction, the reaction between a phosphine and an azido group, for the template reaction for releasing the quencher, named Q-STAR (Fig. 10 and Table 2.1) [102]. The quencher in the Q-STAR probe is conjugated to the probe strand *via* an azido-linker, and phosphorothioate in QUAL is replaced with triphenylphosphine (TPP). In the ternary complex of probes and target, TPP reduces the azido-linker and induces a fluorescence signal without ligation. The background intensity can be reduced by utilizing double TPP groups or double quencher groups in a single probe [103]. Unfortunately, degradation of the probe strand containing a quencher and a fluorophore activates fluorescence. One of the solutions to this problem is 2′-OMe protection of the 3′ terminus of the strand [104].

In addition, it was reported that the sensitivity of mRNA could be improved by amplification with a combination of enzymes [104]. By this combination, the detection threshold could be achieved at 200 pM in solution.

The fluorescence wavelength can be shifted from green to red by using FRET [102]. In this probe, one of the oligo-nucleotides contains three chemical group, dabcyl, fluorescein, and TAMRA. The combination of this red-shifted probe and a normal probe enables the identification and differentiation of bacterial species simultaneously.

In 2004, Taylor and colleagues conjugated TPP on the fluorophore (Table 2.2) [105]. Because TPP conjugation masks the fluorescence of fluorescein, this transfer enables the activation of fluorescence. However, the instability of TPP constitutes a limitation in the method utilizing a TPP-conjugated fluorophore. Fortunately, an azide group, which is stable chemically, can act as a mask of the fluorophore. As one example of this, Winssinger and colleagues developed methods using PNA conjugated with azido-masked coumarin. In this method, TPP conjugated with another PNA strand removed an azide group from coumarin and activated fluorescence [106]. Moreover, Abe and colleagues conjugated azido-masked fluorescein to natural DNA and reduced the azido group by using DTT or TPP (Table 2.3) [107]. Like fluorescein and coumarin [106], azido-masked rhodamine [108,109] was also utilized (Fig. 11). Winssinger and colleagues introduced these two fluorogenic groups into the terminus of PNA.

Table 2
Ligation-free method.

Reaction name	Substrate	Product	Ref.
1.Q-STAR			[102]
2. TPP transfer			[105]
3. Azido group reduction			[106-109]
4. azidomethyl group reduction			[110, 111]
5. Aromatic substitution			[113]
6. Tetrazine mediate			[114]
7. Stilbene formation			[115]
8. Lanthanoid			[116]
9. Organometallic			[117]
10. Ru^{II}-catalysis			[119, 120]
11. Ru^{II}-catalysis			[121, 128]

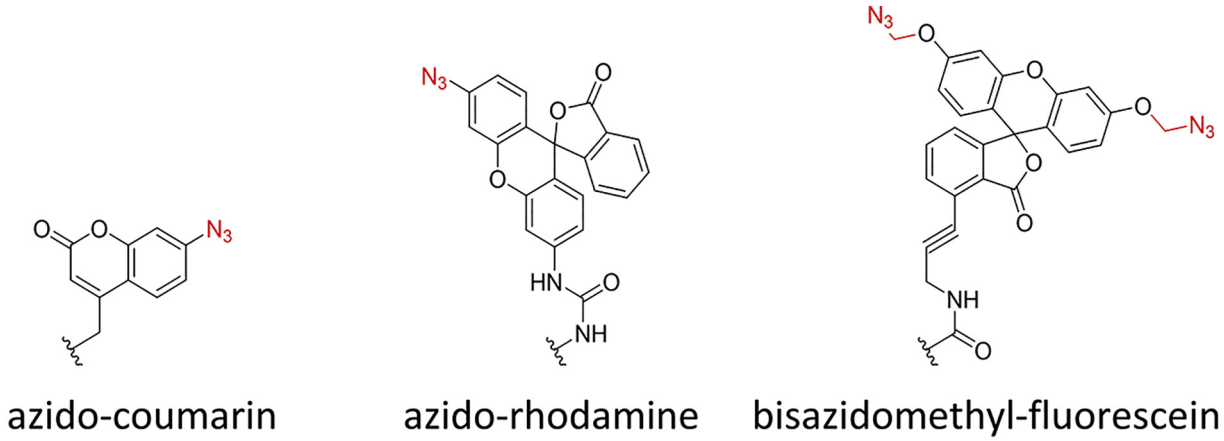


Fig. 11. Structures of azido-masked fluorophore. The moieties reduced by template reaction are shown in red.

Because sensitivity depends on the turnover rate of the template reaction, optimization of the reaction is one of the most important issues in chemical probe development. Abe and colleagues thus focused on the rate-limiting step of the turnover cycle in the template reaction. Kinetic analysis indicated that the chemical reaction in the template reaction is the rate-limiting step. Because of this, efforts were made to accelerate the chemical reaction by replacing the azido group with an azidomethyl group (Table 2.4 and Fig. 11) [110,111]. The promotion of strand exchange is one of the other solutions for improving sensitivity. Product inhibition is one of the major hindrances in template reactions. To reduce the production inhibition, Seitz and colleagues promoted template exchange by adding a polymer containing a poly(L-lysine) backbone and dextran side chain [112]. As another approach, a nucleophilic aromatic substitution reaction, one of the most rapid reactions, was utilized (Table 2.5) [113]. The turnover number reached 1500 times, indicating that a low concentration of the target became detectable by signal amplification. Similarly, Devaraj and colleagues utilized a tetrazine-mediated reaction to improve the turnover rate of a template reaction (Table 2.6) [114]. There are also other species for ligation-free template reactions for RNA detection. As one of the other species, *de novo* synthesis of fluorophore has been reported. The template reaction in this method catalyzes the transfer of a benzyl group to benzaldehyde, resulting in stilbene (Table 2.7) [115]. The fluorescence of this product is detectable with α-cyclodextrin. To achieve RNA detection in the intracellular environment, several approaches using other reactions are available. Auto-fluorescence of biological components increases the background signal and precludes fluorescence detection. When a fluorophore exhibits long-lived fluorescence, the signal from the probe and background fluorescence can be distinguished by time-gated detection. Lanthanoid is utilized for a template reaction for long-lived luminescence (Table 2.8) [116]. The long-lived fluorescence of lanthanoid enables reduction of the background signal. The biological environment can induce unmasking of the caged fluorophore in the absence of target RNA. To avoid this nonspecific reaction, organomercury is utilized for activating fluorescence (Table 2.9) [117]. However, in this context, the stability of the reaction moiety is one of the most important bottlenecks. For example, phosphine groups are easily oxidized. When a dinitrobenzenesulfonyl group is utilized as a caging group for a fluorophore, the fluorophore can be unmasked by the reaction with phosphorothioate, a stable moiety [118].

Temporal and locational control of probe function has been proposed. Winssinger and colleagues developed a photo-switchable probe by utilizing a Ru^{II}-catalyzing reaction (Table 2.10) [119]. The Ru^{II} group catalyzes the oxidization of an azido-benzyl moiety in a manner dependent on UV irradiation, and the oxidization induces fluorophore release. This method has the benefit of enabling controllable initiation of the reaction. The use of a chemical probe in developmental biology requires a switchable reaction for signal amplification because a chemical probe can be introduced only into an early embryo. The requirement of photo-exciting Ru^{II} to catalyze the reaction indicates that signal amplification can be initiated at a certain stage. In fact, the detection of microRNA in a live vertebrate has been reported (Fig. 12) [120]. Although the Ru^{II}-catalyzing probe enabled temporal and locational control, the reaction velocity was slow. Regarding further progress, the velocity of this photocatalytic reaction was improved by utilizing a pyridinium ring by a photoremovable protecting group (Table 2.11) [121]. This optimization resulted in a reaction rate approximately 200 times faster than the reaction with an azido-phenyl group.

6. Imaging of RNA by chemical probes

As described in the introduction, chemical probes can detect target RNA with high specificity and a high signal-to-background ratio without washing. Such probes have been utilized for intracellular RNA imaging since the 1990s [9,122]. In early research, probes were synthesized targeting ribosomal RNA, one of the most abundant types of RNA in cells, or mRNA of highly expressed genes. In addition, the intracellular dynamics of mRNA was analyzed using molecular beacons [123]. Recently, focus has shifted onto other RNA species. microRNA is one of the crucial targets. The maturation of this RNA species is induced by dicer and works as siRNA, resulting in the inhibition of gene expression. Recently, it has been shown that exosomes contain microRNA and play an important role in intercellular communication. Rhee and colleagues succeeded in detecting microRNA by introducing a molecular beacon into exosomes using streptolysin O [23]. Some groups focused on nucleic acids forming secondary structures such as G-quadruplexes and hairpin loops. It was reported that structured DNA, hairpin and G-quadruplexes, could be detected specifically by using chemical probes [124]. Moreover, Abe and colleagues focused on immature or mature mRNA. During the splicing step, part of the mRNA forms a lariat structure and is removed from mature mRNA. Lariat RNA and mature mRNA were distinguished successfully using a reduction-triggered probe [125,126]. Analysis of the epigenetic code is particularly important when using probes because this information cannot be obtained from canonical genetic sequences. One form of the epigenetic code involves the editing of adenosine to inosine after transcription. As one example of adenosine editing detection, Sugimoto and colleagues reported that edited and unedited RNA could be distinguished by the formation of a triplex with a PNA probe containing 2-aminopyrimidine [127]. Recently, the triplex formation was utilized for a template reaction. For example, Winssinger and colleagues utilized template and

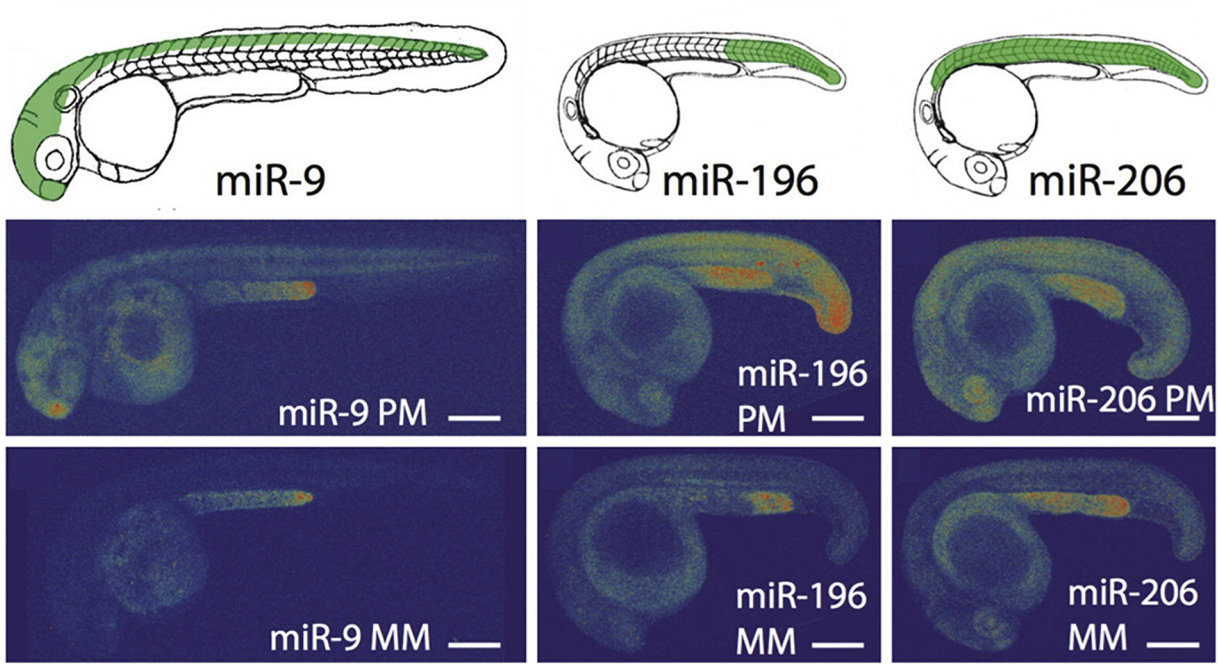


Fig. 12. RNA imaging in embryo. RNA imaging in zebrafish embryo. (Copyright 2016, ACS publication).

photo-exciting Ru^{II} catalysis for double-stranded RNA-specific detection [128].

Although various chemical probes have been successfully developed and utilized for RNA detection, intracellular imaging has several bottlenecks. First, the signal-to-background ratio should be high in cells. Although this ratio is high in *in vitro* evaluation, some cellular components reduce this ratio by exhibiting auto-fluorescence and inducing nonspecific signals. To reduce the effect of auto-fluorescence from cellular components, long-lived luminescence has been utilized [116,129]. Recently, the reduction of the background noise was achieved by limiting diffusion [130]. Second, probes should be stable in the cellular environment. Even though a Staudinger reaction has been widely utilized for the template reaction, the phosphine group tends to get spontaneously oxidized. For chemically stable probes, nucleophilic aromatic substitution has been utilized [118]. Recently, in the field of developmental biology, demands have emerged for the monitoring of RNA by chemical probes. As an example of this, Winssinger and colleagues achieved the detection of intracellular RNA at certain developmental stages of zebrafish embryo [120]. Moreover, there is a need to develop an efficient and biocompatible method to introduce probes into cells. For bacteria, probes have been introduced by treating cells with SDS [92,102,131] or paraformaldehyde [94,107]. In contrast, there are several methods for introducing probes into eukaryotic cells, such as lipofection, injection, and pore formation. The methods and probes introduced in this review are summarized in Table 3. Although streptolysin O treatment and lipofection are the most conventional methods, they are associated with high toxicity. Injection requires technical skill and is unsuitable for large numbers of cells. Because of this, there is an urgent need for the spontaneous cellular uptake of probes. There are several structures or chemical modifications providing probes with spontaneous cellular permeability. For example, the cellular permeability of nanoparticles enabled molecular beacons, conjugated to gold nanoparticles, to detect cellular mRNA spontaneously [32–34]. In addition, it is well known that the conjugation of cationic peptide enhances the spontaneous cellular uptake of PNA strands. A PNA probe was introduced into living cells spontaneously by the conjugation of four lysine residues at the PNA terminus [45,132–134]. As another solution to improve cellular permeability, guanidine modification in the PNA backbone improves cellular permeability [135,136], and guanidine was introduced into the PNA backbone to achieve spontaneous cellular uptake of PNA probe [108]. Although probes could be introduced without any lipofection agent, this required incubation for a long time and was associated with the observation of nonspecific fluorescence on the cellular membrane. Recently, Rozner and colleagues improved the cellular permeability of PNA probe only by utilizing 2-aminopyridine-modified PNA [62].

7. Conclusion

Monitoring intracellular RNA is indispensable for understanding life phenomena, and biochemical and molecular biological approaches have been developed. Because PCR, one of the most popular biochemical approaches, is unsuitable for detection of RNA localization, fluorescence imaging approaches have been proposed by using DNA probe containing fluorescence (FISH), aptamer for dye (spinach) and RNA-binding protein (MS2). However, these biochemical and molecular biological methods possess some limitations such as sensitivity and sequence dependency. Meanwhile, chemical probes enable to detect intracellular RNA without genetic modifications. Though it is impossible to amplify signals by molecular beacon and hybridization-sensitive probes, these probes provide the information about target location. On the other hand, probes with chemical ligation and ligation-free template reaction, other group of chemical probes, amplify signal. This imply that these

Table 3
Methods for introduction of probes.

	Probe type	Ref.
Lipofection	Molecular beacon	[10]
	Hybridization-sensitive probe	[52,58]
	Ligation-coupled template reaction	[98]
	Ligation-free template reaction	[119]
Pore-formation	Molecular beacon	[23,25]
	Hybridization-sensitive probe	[44,46,48]
	Ligation-coupled template reaction	[93]
	Ligation-free template reaction	[110,119]
Injection	Molecular beacon	[10,28,122,123]
	Hybridization-sensitive probe	[58]
	Ligation-free template reaction	[120]

probes are suitable for detection of target at low concentration though localization information of target can be unclarity because of the diffusion of the reaction products. As described above, these probes should contribute to boosting our understanding of life phenomena such as gene expression and developmental biology.

Acknowledgmnets

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