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RNA-Based Fluorescent Biosensors for Live Cell Imaging of Small Molecules and RNAs

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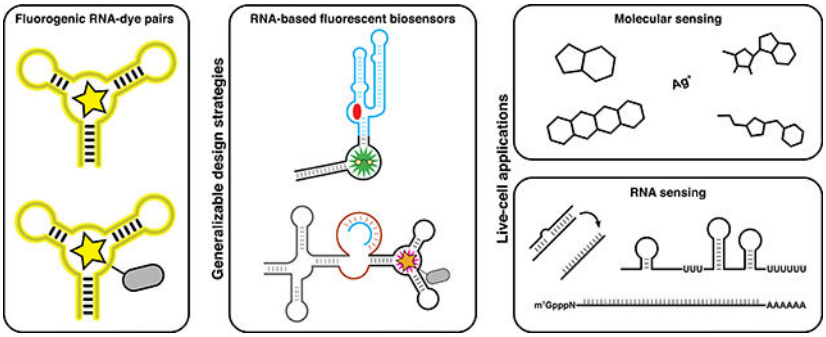
Abstract

Genetically encodable fluorescent biosensors provide spatiotemporal information on their target analytes in a label-free manner, which has enabled the study of cell biology and signaling in living cells. Over the past three decades, fueled by the development of a wide palette of fluorescent proteins, protein-based fluorescent biosensors against a broad array of targets have been developed. Recently, with the development of fluorogenic RNA aptamer-dye pairs that function in live cells, RNA-based fluorescent (RBF) biosensors have emerged as a complementary class of biosensors. Here we review the current state-of-the-art for fluorogenic RNA aptamers and RBF biosensors for imaging small molecules and RNAs, and highlight some emerging opportunities.

Graphical Abstract



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Author statement

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Declaration of interests

The authors have filed a patent application for a fluorescent biosensor for methyltransferase assays.

Keywords

Fluorogenic RNA aptamer; RBF biosensors; molecular imaging; RNA imaging

Introduction

To study cell signaling and other dynamic and spatiotemporally controlled processes in cells requires some method of molecular tracking. Whereas metabolic incorporation strategies and tagging strategies provide different means to label a molecule, a biosensor that binds to an endogenous molecule provides a means for label-free detection. Furthermore, genetically encodable biosensors that yield fluorescent or bioluminescent signals are amenable for live cell imaging experiments. In addition, the reversible nature of the binding event allows biosensors to capture dynamic and transitory processes, e.g. rise and fall of levels.

With the advent of natural and engineered fluorescent and bioluminescent proteins, protein-based biosensors utilizing fluorescence turn-on, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and other mechanisms have been developed that exploit protein domains that undergo conformational change in response to target analytes. Protein-based biosensors have been applied for real-time imaging of small molecules, redox factors, ions, pH, voltage, proteins, and protein modifications in live cells [1]. However, one general limitation for genetically encodable biosensors is the availability of suitable binding domains. Not only do the target binding domains have to retain stability, affinity, and specificity in the context of the constructed biosensor, but importantly they have to undergo ligand-dependent conformational change in order to alter signal. This latter property is a key feature that often is difficult to engineer.

An alternative class of biomolecules that is capable of molecular recognition and genetically encodable is RNA. For example, riboswitches are natural well-structured RNA folds that undergo conformational change in response to specific analytes, including small molecules, redox factors, and ions [2], and natural and engineered RNA regulatory elements undergo structure switching in response to specific RNAs [3]. Thus, with the recent advent of fluorogenic RNA aptamer-dye pairs that are amenable for application in live cells, these two RNA classes have become a rich and novel source of binding domains for genetically encoded RNA-based fluorescent (RBF) biosensors. This review focuses on recent and exciting progress on RBF biosensor development by first giving an overview of fluorogenic RNA aptamer-dye pairs, presenting notable design approaches and applications for RBF biosensors for small molecules, ions, and RNAs, and concluding with some potential opportunities and challenges for this field.

Fluorogenic RNA aptamer-dye pairs for live cell imaging applications

To our knowledge, the first RBF biosensors were developed based on the malachite green fluorescent dye and its aptamer [4]. Detection of small molecules [5] and nucleic acids [6] was shown *in vitro*, but application of this system to live cell imaging was limited by the cytotoxicity and nonspecific binding of malachite green. In order to be useful for live cell imaging, a fluorogenic dye compound should be highly soluble in water, low in molecular

weight, cell permeable, and not toxic to cells. Another critical property is the dye should specifically bind to its RNA aptamer and not to other cellular components, especially other high abundant nucleic acids.

Current fluorogenic RNA aptamer-dye pairs can be divided into two design categories, single dyes (Figure 1A) and fluorophore-quencher conjugates (Figure 1B). Single dye systems employ fluorescent compounds that exhibit low fluorescence quantum yield in their free states in water, but become highly fluorescent when bound by the RNA aptamer. Fluorophore-quencher conjugate systems use fluorescent dyes that exhibit high fluorescence quantum yield in their free states in aqueous solution, but exhibit low fluorescence when conjugated to a quencher. Binding of the RNA aptamer to either the fluorophore or quencher moiety restores high fluorescence by interfering with the quenching mechanism.

Most single dye systems have been developed and used as fusion tags for intracellular imaging of RNAs (Figure 1A). A notable exception is the DIR2s aptamer, an *in vitro* selected aptamer that binds and activates either dimethylinodole red (DIR) or oxazole thiazole blue (OTB) dyes, giving two well-resolved emission colors [7]. This system was fused to an aptamer that binds the cell-surface EGF receptor protein and enabled live cell imaging of the surface protein, presenting an alternative application when the dye is not cell-permeable. The key development goals for current fluorogenic RNA systems are to improve signal-to-background for reducing the number of copies of tag needed and imaging lower abundance transcripts, to enable multi-color imaging, and to enable advanced methods such as single-molecule and super-resolution fluorescence microscopy. These goals also are desirable for biosensing applications.

The first single dye system successfully developed for live cell imaging to our knowledge was the Spinach aptamer with 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), an analogue of the GFP chromophore [8]. This system has been improved with the development of DFHBI analogues with different spectral properties [9] and with next-generation variants of Spinach (e.g. Spinach2 [10], Broccoli [11], iSpinach [12], baby Spinach [13]) with increased brightness, intracellular stability, and folding fidelity, lower salt dependence, and smaller tag size. The same group recently developed an aptamer-dye pair with yellow fluorescence and slower photobleaching, the Corn aptamer with 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime (DFHO), an analogue of the DsRed chromophore [14,15]. Spinach and Corn systems have been employed in both RNA imaging and biosensor applications [8,15–17•].

Another well-developed single dye system is the Mango aptamer with the thiazole orange (TO1)-biotin conjugate [18]. Several Mango aptamer variants (e.g. Mango-II to Mango-IV) with improved intracellular brightness have been engineered, and this system is distinguished by very high affinities to the dye-biotin conjugate (K_d ~ 1–11 nM) [19•]. Low concentrations of the dye-biotin conjugate can be used in live cell imaging experiments, which reduces background fluorescence from nonspecific nucleic acid interactions. Other biotin conjugates to related dyes, red-shifted thiazole orange (TO3) and oxazole yellow (YO3), also were found to bind the Mango aptamer [18,20•]. The former enables far-red

fluorescent RNA imaging and the latter was applied to develop a FRET biosensor incorporating both Spinach and Mango aptamers.

Other promising single dye systems recently have been advanced for RNA tagging and are briefly summarized because they have not yet been applied to RBF biosensors. The silicon rhodamine aptamer (SiRA) binds to silicon rhodamines (SiR) and activates fluorescence through potentially stabilizing the fluorescent zwitterionic state over the nonfluorescent spirolactone [21•]. This system exhibited exceptional brightness in the far-red to near-infrared and enabled super-resolution imaging of mRNA in live *Escherichia coli.* The Pepper aptamer binds with high affinity to a novel family of fluorogenic dyes, (4-((2-hydroxyethyl)(methyl)amino)-benzylidene)-cyanophenyl-acetonitrile (HBC), that yield a broad range of fluorescence from cyan to red [22••]. This impressively bright system was applied to live cell imaging of RNA polymerase II transcripts and is compatible with advanced microscopy techniques such as two-photon imaging and super-resolution imaging.

Since fluorescence activation of single dyes can be difficult to predict or to engineer, fluorophore-quencher conjugates present a promising alternative (Figure 1B). One general benefit of this strategy is that existing bright, fluorescent dyes can be used, such as fluoresceins, rhodamines, cyanines, and Atto dyes. In cases where the RNA aptamer binds to the quencher moiety, there is an added advantage that the fluorophore can be easily swapped. It has been shown that fluorescence unquenching and overall signal brightness in these systems can be improved by altering the spectral overlap between fluorophore and quencher, the linker length, and the fluorophore group [23•–26]. One potential issue for these systems is the necessarily larger size of fluorophore-quencher conjugates, which affects cell permeability and may require cellular delivery.

The SRB-2 aptamer binds several structurally related fluorophores, including Sulforhodamine B (SR) and 5-carboxytetramethylrhodamine (TMR). Binding to the RNA was shown to disrupt quenching by the dinitroaniline (DN) or mononitroaniline (MN) group, resulting in fluorescence turn-on [25,26]. The black hole quencher (BHQ) aptamer and DN-binding aptamer bind to the respective quenchers, permitting potentially any fluorophore to be conjugated, although with variable turn-on efficiency. Each of these aptamers have been applied in dual-color imaging and to make different RBF biosensors [27•, 28, 29•, 30•].

Whereas development of all fluorogenic systems described above involved *in vitro* selection of the aptamer, the Riboglow system was engineered from a natural bacterial cobalamin (Cbl) riboswitch aptamer that binds to Cbl as the quencher [23•]. Cbl is shown to be an effective quencher of a wide variety of fluorophores, including the extremely bright Atto and cyanine class dyes. The fluorophore-quencher compounds are delivered via bead loading for live-cell imaging of mRNA translocation.

The expanded palette of fluorogenic RNA-dye pairs means that novel RBF biosensors can be made with different designs (e.g. FRET-based), tailored spectral properties, and multiplexed applications in mind. Some orthogonality has been demonstrated in live-cell imaging experiments for Spinach-Mango [20•], Spinach-DNB [30•], DNB-SRB-2 [25] and DNB-BHQ [29•] aptamer pairs. With the general biosensor design strategies discussed in the

following sections, we expect that most of the novel RNA-dye pairs can be engineered into functional biosensors for molecular and RNA imaging.

RNA-based fluorescent biosensors for molecular imaging

To date, almost all published examples utilize the Spinach-DFHBI family of dye-binding aptamers, which were the first developed and employ commercially available dyes. The first RBF biosensors were designed in analogous fashion to the (1) *split-GFP approach*, and this remains the most common and generalizable strategy (Figure 2A) [16,31]. The biosensor comprises a contiguous RNA sequence with the dye-binding aptamer "split" by the ligand binding domain, which acts through a transducer stem to reconstitute the dye-binding domain in the presence of target analyte. However, unlike split-GFP systems, which often remain reconstituted, it was shown that depletion of the analyte leads to loss of fluorescence, which enables dynamic monitoring of the small molecule target [16,31]. This type of biosensor also has been shown to accommodate circular permutations of the ligand-binding domain, which permits alternative stems to be used as the transducer [32].

Alternative strategies for designing RBF biosensors exploit the unique ease of designing RNA secondary structures and expand the ligand-binding domain structures that can be adapted into RBFs (Figure 2B–E): (2) *Split-binding domain approach*: the biosensor comprises a contiguous RNA sequence with the ligand binding domain "split" by a dye-binding aptamer, which inverts the classic strategy. In general, this approach enables biosensors to be developed using binding domains where the 5' and 3' ends are far apart, and requires using a circularly permuted dye-binding aptamer. The SAH biosensor was developed using this method with cpSpinach2 [33•]. (3) *Fluorogenic riboswitch approach*: the structure-switching mechanism of riboswitches can be exploited by replacing the gene regulatory expression platform in the natural riboswitch with a dye-binding aptamer. The TPP biosensor was developed using detailed mechanistic information about the riboswitch [34]. (4) *RNA origami approach*: The first FRET-based RBF biosensor (apta-FRET) was developed by integrating a SAM-III riboswitch aptamer domain to control the orientation of Spinach and Mango aptamers positioned on each arm of a single-stranded RNA origami called 2H-AE. Upon binding to SAM, the Mango-SAM-III arm is proposed to switch to a rigid conformation, leading to higher FRET efficiency between Spinach-DFHBI-1T and Mango-YO3-biotin [20•]. (5) *Allosteric ribozyme approach*: allosteric ribozymes are developed by fusing ligand-sensing aptamers to a ribozyme and have been applied to control expression of fluorescent proteins [35]. A recent report applied this principle to develop a cyclic di-GMP biosensor that controls expression of fluorogenic RNA aptamers as alternate reporters [36].

Current RBF biosensors have been developed that respond to cofactors (e.g. SAM, TPP, ATP, etc.) [16,34], metabolites (e.g. SAH, ADP, adenine, guanine, etc.) [16,34], signaling molecules (e.g. cyclic dinucleotides, neurotransmitters) [31,36–37,38•,39–41•], drugs (e.g. tetracycline) [30•], and ions (e.g. Ag^{+}) [42]. The majority of these biosensors use natural riboswitch binding domains, because of their extraordinary affinity and specificity, robust folding in the intracellular environment, and structure-switching properties. *In vitro* selected aptamers could be utilized, as exemplified by the biosensors for adenosine, ADP, and

tetracycline [16,30•]. However, most *in vitro* selected aptamers may not be suitable as binding domains for the same reason that antibodies are not used in protein-based biosensors: they are selected for binding, not conformational change.

To expand the scope of ligands while retaining the advantageous properties of natural riboswitches, novel ligand sensing domains have been engineered via either structure-based design [31,33•] or structure-based selection of riboswitch scaffolds [41•]. The former strategy was employed to develop an RBF biosensor capable of detecting an innate immune signal in mammalian cell lysates. The latter strategy was employed to develop RBF biosensors for 5-hydroxy-L-tryptophan (5HTP) and 3,4-dihydroxy-Lphenylalanine (L-DOPA), the precursors of neurotransmitter serotonin and dopamine.

RBF biosensors have enabled the detection of many small molecules for which no other biosensor tool exists to target, and the ease of producing them through *in vitro* transcription empowers both *in vitro* and *in vivo* applications. *In vitro* applications that have been demonstrated include: (1) *Discovery and characterization of riboswitch-ligand interactions*: RBF biosensors provide a convenient way to screen for ligands that bind to the riboswitch and to determine relative binding affinities. We discovered the 3',3'-cGAMP riboswitch using biosensors [37]. We also have characterized riboswitch mutants [43] and shown that 42 out of 53 (79%) bioinformatically predicted riboswitch sequences function in the same biosensor context [39]. (2) *High-throughput enzyme activity assays*: RBF biosensors serve as robust reagents for screening enzyme activity, exhibiting better sensitivity and selectivity than antibody-based assays in some cases. We showed that the SAH biosensor can be used to identify methyltransferase inhibitors [33•] and the 2',3'-cGAMP biosensor can be used to identify modulators of the human cGAS enzyme [40].

In vivo applications that have been demonstrate include: (1) *Discovery of novel enzyme activities*: RBF biosensors provide a way to implement cell-based screens for enzyme activity. This approach has been particularly effective to study overexpressed signaling enzymes, including membrane-bound ones. For example, we discovered enzyme classes that produce and degrade the cyclic dinucleotide signal 3',3'-cGAMP using biosensors [44,45•]. (2) *Validation of enzyme agonists or antagonists*: We also have shown that RBF biosensors can detect the modulation of endogenous enzyme activities in live cells. We have visualized chemical inhibition of quorum signal production and activation of cyclic di-GMP signal production [33,46••]. (3) *Biosensing under anaerobic conditions*: RBF biosensors bind synthetic dyes so do not require chromophore maturation like the majority of fluorescent protein-based biosensors. We have shown that the cyclic di-GMP biosensor works in cells grown under anaerobic conditions [39]. (4) *Evaluation of the efficacy of therapeutic agents:* Silver is a widely used disinfection reagent and an RBF biosensor for silver ion has been applied to measure the cellular flux and silver ion release of silver nanoparticles [42].

RNA-based fluorescent biosensors for RNA imaging

While fluorogenic aptamer-dye pairs are highly useful as fusion tags for tracking mRNAs in live cells, there are shorter RNA species whose function, processing, and translocation may be affected by tagging approaches. For example, microRNAs (miRNAs) are 21–24

nucleotides in size and play key roles in regulating development, epigenetics and immune response in both plants and metazoans [47]. Thus, for imaging native RNAs in a label-free manner, especially small RNAs such as miRNAs, there is a demand for trans-acting sensors.

RBF biosensors that target specific RNAs present a similar strategy to classic molecular beacon and RNA-FISH (fluorescence *in situ* hybridization) methods. However, a major difference is that RBF biosensors are genetically encodable, with an exogenously added dye or dye-quencher. This is advantageous for live cell imaging experiments, as cellular delivery of small molecule fluorophores is easier than for dye-conjugated RNA probes. CRISPR protein-based sensors also have been developed that permit imaging of native RNAs [48,49]. These systems are analogous to the MS2-fusion tagging strategy, except with guide RNA specified targeting of the CRISPR complex. In order to visualize cytoplasmic mRNA transcripts, background fluorescence must be reduced by confining unbound fluorescent proteins in the nucleus or keeping fluorescent protein expression low via negative feedback.

Again, one strategy to design RBF biosensors for imaging RNAs is analogous to the split-binding domain approach (Figure 3A). Rather than a ligand-binding aptamer, the sensing domain in this case is a complementary sequence to the target RNA of interest. The critical aspect for this design strategy is that there must be a structural change when the RNA-sensing domain binds its target to reconstitute the dye-binding domain. Secondary structure prediction is required but remains particularly challenging for structure-switching RNAs [50•].

An early example of a miRNA biosensor was called Pandan and based on stem-loop insertion into a circularly permuted Spinach aptamer [51]. Although Pandan functions *in vitro*, the *in vivo* performance was not reported. The FASTmiR sensors employed a similar design and were developed to target human miR122 and *Arabidopsis thaliana* miR171 [52•]. The miR122 sensor was multiplexed and applied to report miR122 expression level in mammalian cells. The miR171 sensor was applied as an *in vitro* assay for directly quantifying miR171 levels in *A. thaliana* total leaf and flower RNA extract. To achieve ratiometric imaging, a miRNA biosensor based on the SRB-2 aptamer was co-expressed with GFP encoded on the same transfected plasmid [27•]. Levels of miR-21 could be reliably quantified in different mammalian cell lines.

A simpler design was developed for imaging mRNAs (Figure 3A). The fluorogenic aptamer first is destabilized to achieve low background, so that target RNA binding to two complementary regions flanking the domain is required to reconstitute the dye-binding domain. A family of RNA targeting aptamers (RT-aptamers) was developed by flanking the BHQ aptamer with two targeting sequences of 9 nt each, and were applied to image mRNAs such as β-actin, ARFIP2, CTTN and CYFIP2 [28]. Using a similar design but based on the Spinach aptamer, biosensors for imaging mRNAs in *E. coli* have been developed against targets such as *mreB*, *dnaJ*, *dnaK* and *rpoH* [53].

More recently, a splitting sensor strategy was implemented and shown to lower fluorescence background and thus increase sensitivity of detection. The biosensor was designed by flanking the Broccoli aptamer with two targeting sequences and then splitting the biosensor

into separate RNA halves. Thus, target mRNA binding is required to reconstitute the dye-binding domain. mRNAs such as β-actin, CFL1 and GAPDH were visualized in live HeLa and HuMSC cells [54•]. Alternatively, a hairpin assembly circuit for detection of target RNA was developed that catalyzes the reconstitution of split Broccoli (Figure 3C), and was applied to detect changes in endogenous small RNA SgrS in live *E. coli* [55].

Conclusions

The emerging field of RNA-based fluorescent biosensors has seen rapid progress and creative contributions by many research groups in the development of fluorogenic aptamers and biosensor designs. Going forward, we consider that the greatest opportunity in the next stage of the field lies in establishing and disseminating these tools to address important questions in cell biology. Towards this goal of visualizing spatiotemporal dynamics of molecular targets, the field will have to tackle general issues such as increasing signal-to-noise, accounting for expression variability, and improving biosensor kinetics. Approaches to date include optimizing intracellular RNA stability through circularization [56••] and ratiometric systems employing two dye compounds [20•], but we look forward to many more advancements to come.

We consider that one of the grand challenges for the field is to enable other researchers to design their own biosensors. A practical aspect is that while RNA aptamer or biosensor constructs are readily shared by request and often made publicly available through Addgene, one limitation to non-chemists is the availability of synthetic dyes and dye-quencher compounds. RNA aptamer paired with degron-tagged fluorescent proteins could provide an "all encoded" solution [57•], but the potential of this system for biosensor engineering has yet to be explored. Another important aspect for enabling growth of the field is to explain the general design strategies, which we have presented in this review. In our opinion, it also is important to consider whether increasing the complexity of a given biosensor design is warranted and would improve utility for cell biology studies. Finally, we anticipate that there will be advances in the computational design of RNA targeting biosensors that will reduce experimental trial-and-error, and that the scope of binding domains will be greatly expanded through natural discovery and exciting advances to the *in vitro* / *in vivo* / *in silico* evolution of riboswitches [41,50,58,59••].

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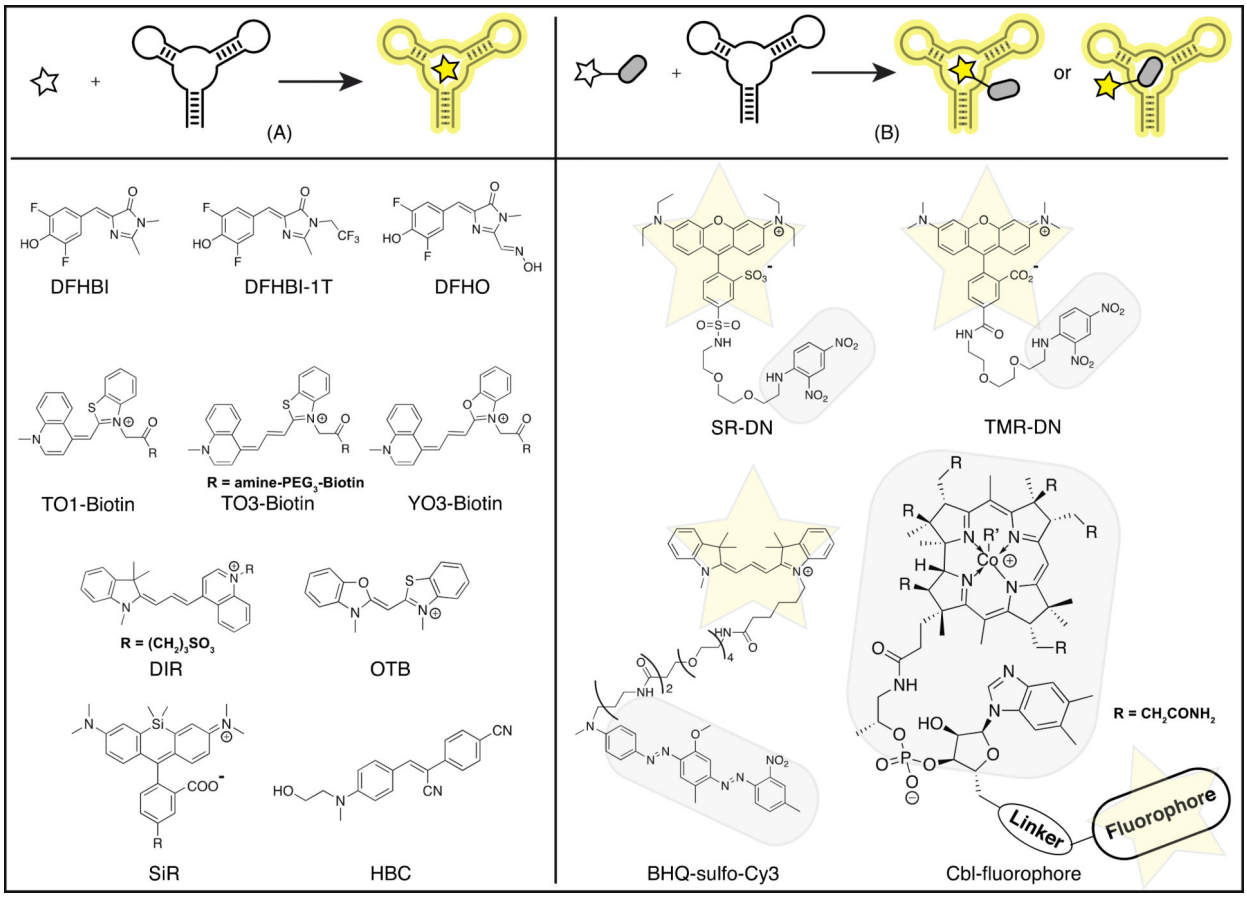

Figure 1.

Fluorogenic RNA aptamers-dye pairs. Above, schematic representations of two types of fluorogenic RNA aptamers based on their paired dyes: single dyes (A) and fluorophore-quencher conjugates (B). Yellow star, fluorophore; Gray rounded rectangle, quencher. Below, chemical structures of representative dyes. DFHBI, 3,5-difluoro-4-hydroxybenzylidene imidazolinone [8]; DFHBI-1T, DFHBI with a 1,1,1-trifluoroethyl substituent [9]; DFHO, 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime [15]; TO, thiazole orange [18]; YO, oxazole yellow [20]; DIR, dimethylindole red [7]; OTB, oxazole thiazole blue [7]; SiR, silicon rhodamine [21]; HBC, (4-((2-hydroxyethyl)(methyl)amino)-benzylidene)-cyanophenyl-acetonitrile [22]; SR, Sulforhodamine B; DN, dinitroaniline; TMR, 5-carboxytetramethylrhodamine; MN, mononitroaniline [25,26]; BHQ, black hole quencher; Cbl, cobalamin [23].

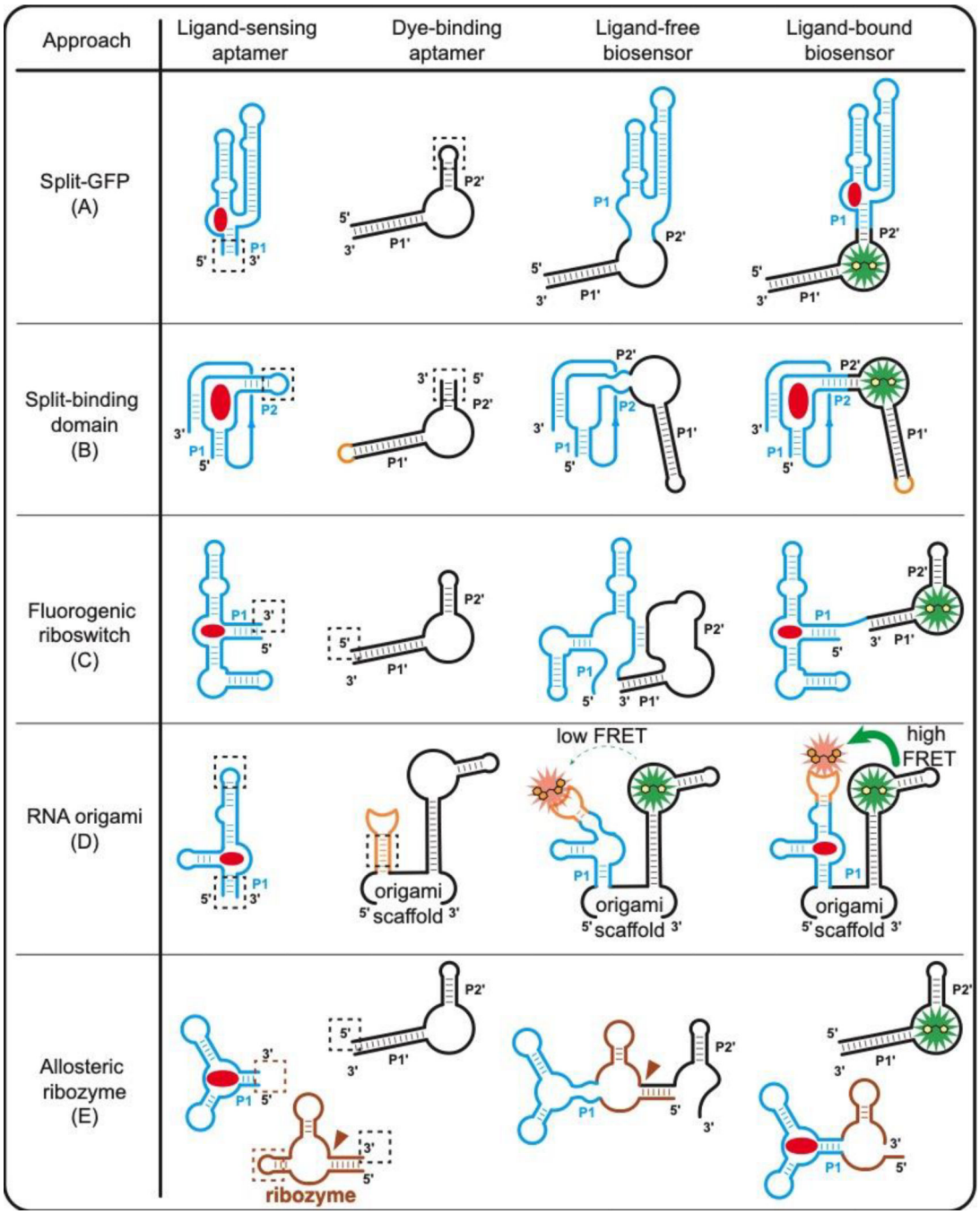

Figure 2.

Design strategies of RNA-based fluorescent biosensors for molecular sensing. Functional RBF biosensors can be generated either by (A) splitting the dye-binding aptamer [16] or (B) splitting the ligand-sensing aptamer [33•]. (C) Fluorogenic riboswitches can be generated by replacing the regulatory expression platform in natural riboswitches with a dye-binding aptamer [34]. (D) Ligand-sensing aptamer can be inserted into an established RNA origami scaffold for ligand-dependent FRET signal change [20•]. (E) Allosteric ribozymes can be fused to a dye-binding aptamer for ligand-dependent release of the fluorogenic aptamer [36].

Red circle, target ligand; dashed square, fusion section between the ligand-sensing domain and the signal reporter domain. Brown triangle, self-cleavage site of ribozyme.

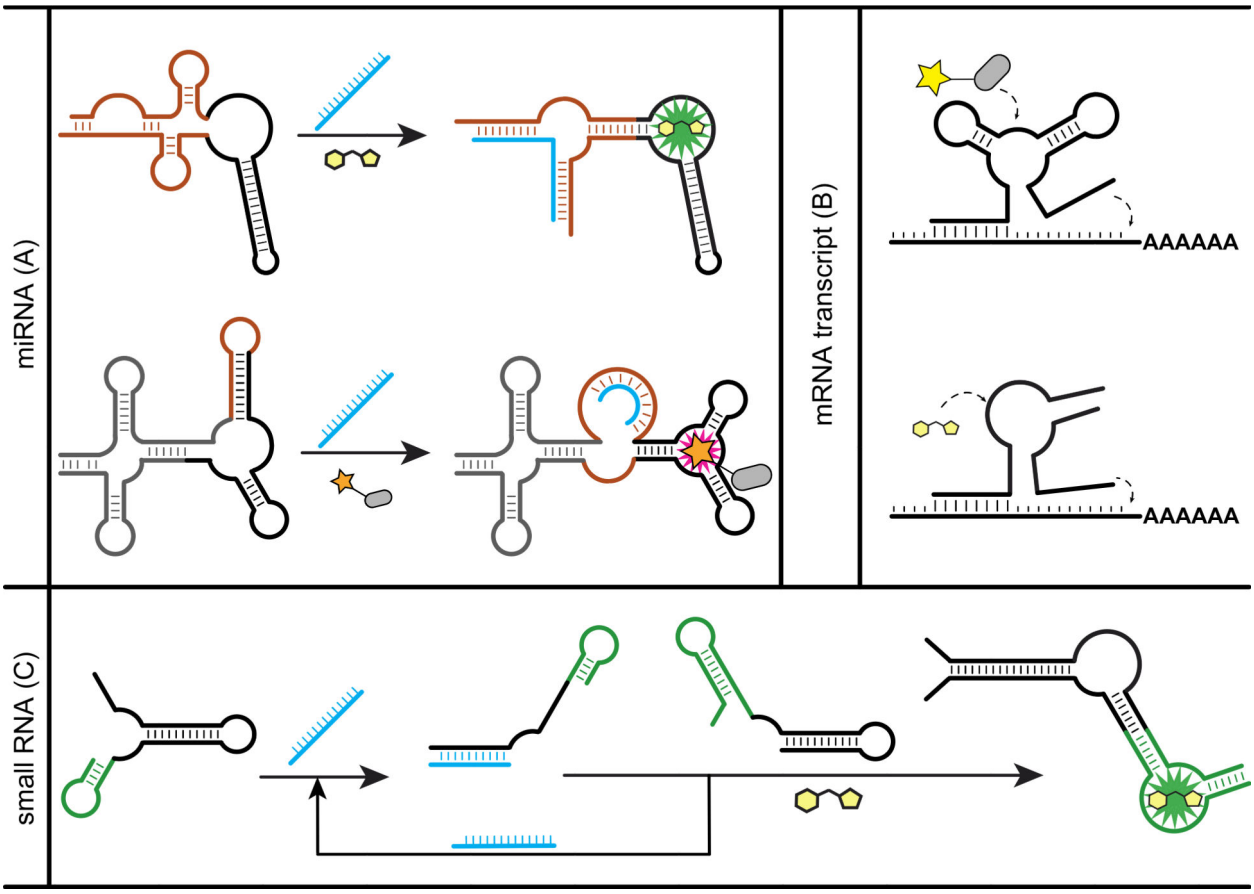

Figure 3.

Examples of RBF biosensors for RNA sensing. (A) Split-binding domain strategy was applied on Spinach and SRB-2 aptamer to generate biosensors for miRNA [52•,27•]. (B) Destabilizing the fluorogenic aptamer by shortening a stem or splitting led to functional biosensors for mRNA [28,53]. (C) For sensitive RNA detection, the catalytic hairpin assembly was used to amplify the signal triggered by target RNA [55].