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“Second-generation” fluorogenic RNA-based sensors

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Abstract

A fluorogenic aptamer can specifically interact with a fluorophore to activate its fluorescence. These nucleic acid-based fluorogenic modules have been dramatically developed over the past decade, and have been used as versatile reporters in the sensor development and for intracellular imaging. In this review, we summarize the design principles, applications, and challenges of the first-generation fluorogenic RNA-based sensors. Moreover, we discuss some strategies to develop next-generation biosensors with improved sensitivity, selectivity, quantification property, and eukaryotic robustness. Using genetically encoded catalytic hairpin assembly strategy as an example, we further introduce a standard protocol to design, characterize, and apply these fluorogenic RNA-based sensors for *in vitro* detection and cellular imaging of target biomolecules. By incorporating natural RNA machineries, nucleic acid nanotechnology, and systematic evolution approaches, next-generation fluorogenic RNA-based devices can be potentially engineered to be widely applied in cell biology and biomedicine.

Keywords

Light-up aptamer; RNA; Fluorogenic dye; Biosensors; Live-cell imaging

1. Introduction

Imaging and detection of proteins, nucleic acids, and metabolites is crucial for the in-depth understanding of various molecular pathways occurring within cells. Cellular signal transduction require specific interaction patterns among biomolecules. These biomolecules interact with each other through their conformational changes, colocalizations, self-assemblies into macromolecular complexes, and dissociations. To elucidate and regulate these cellular interaction networks, it is important to develop effective tools to detect the intracellular levels and the spatial and dynamic distributions of biomolecules of interest [1,2].

Traditional methods to measure the cellular levels of target biomolecules include mass spectrometry, liquid chromatography, gel electrophoresis, absorbance and NMR

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spectroscopy, etc. [3]. However, these approaches cannot be conducted in live cells or in continuous assays. In addition, limited information is provided about the target distribution heterogeneity over time or from one cell to another. As a result, it is challenging to apply these traditional approaches to access the micro-environmental profiles of target biomolecules.

Fluorescence microscopy has been an attractive alternative approach. Fluorescence-based qualitative and quantitative tools have been widely used for imaging biological targets in live cells and organelles [4–6]. Powerful fluorescent probes have been developed for diverse applications from visualizing subcellular compartments, detecting proteins and RNAs, to sensing environmental parameters such as pH or oxygen level variations [7–9]. These fluorescent tools are sensitive, selective, accurate, safe-to-handle, and capable of analyzing targets in real time and in multiple colors for simultaneous detection [10].

Fluorescent probes play a central role in the advancement of imaging techniques in cell biology and biomedicine. As of now, a large number of intensity- or ratiometric-based small-molecule fluorescent compounds have been constructed into sensors [11,12]. Various dyes, such as fluorescein, rhodamine, BODIPY and cyanine, have been used as the reporters in these small-molecule sensors [13–19]. However, the real biological applications of these organic probes have been largely limited due to concerns over their influence on natural cellular environment, toxicities, nonspecific interactions, rapid diffusions, and less controllable localizations [20–22].

The development of fluorescent protein (FP)-based genetically encoded sensors has revolutionized the field of bioimaging. Versatile FPs are now available for target imaging with high spatiotemporal resolution and good sensitivity in live cells and organelles [23]. Compared to synthetic small-molecule probes, these genetically encoded sensors can be expressed within specific cell types, at particular intracellular locations, and can be incorporated for long-term imaging. Especially, FP-based Förster resonance energy transfer (FRET) sensors have been widely used to measure target levels and to study molecular interactions inside cells [24–28]. The function of these FRET sensors requires appropriate target-binding protein domains to induce FP conformational changes. Unfortunately, for many physiologically important targets, the protein domains that can selectively bind to these target analytes are not readily identified, not to mention the requisite target-induced conformational changes [17]. These FP-based sensors normally require a large amount of encoded material to be added to the genome, and as a result, may induce high pressure for the expression systems as well as the target cells or organelles. Biosensors with such a large molecular weight may also interfere with the cellular location and biological functions of target molecules. Moreover, the application of FP is still limited in imaging RNA, especially non-coding RNA targets at the transcriptional level. In addition, the limited dynamic range and signal-to-noise ratio of existing FP-based sensors have further prevented their application in imaging a large number of critical biomolecules in living systems [29,30].

We and others recently developed another type of genetically encoded sensor based on fluorogenic RNA aptamers, such as Spinach [31]. Aptamers are short oligonucleotides that can fold and specifically recognize target molecules [32,33]. Aptamers that can bind with

fluorophores, such as sulforhodamine, fluorescein, and cyanine, have been used for cellular imaging [34,35]. However, these traditional fluorophore-binding aptamers suffer from high background signals because fluorophores emit strongly even without the presence of aptamers. Malachite green fluorogenic aptamer is one of the first probe aims to solve this problem [36–38]. However, due to the intrinsic toxicity of the malachite green aptamer [39], as well as the limited performance of other early version fluorogenic RNAs [39,40], genetically encoded RNA-based sensors did not achieve a breakthrough until the development of Spinach.

Spinach, also known as the RNA mimic of green fluorescent proteins, is a 98-nucleotide-long RNA aptamer that can specifically bind and switch on the fluorescence of a chromophore, 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) [31]. DFHBI is derived from 4-hydroxy-benzylidene-imidazolinone (HBI), the natural fluorophore existing in green fluorescent proteins through an autocatalytic cyclization reaction of a Ser-Tyr-Gly tripeptide [41]. Similar to HBI, DFHBI is non-fluorescent in its free form, but binding with Spinach induces the activation of DFHBI fluorescence to a level comparable to that of enhanced green fluorescent proteins [31]. Importantly, DFHBI is cell permeable and induces no cytotoxicity or phototoxicity. As a result, Spinach has been applied as a genetically encoded fluorescent tag for various intracellular RNA imaging in live cells [31,42].

To further improve the spectral properties and thermal stabilities of Spinach, several Spinach RNA mutations have been developed, including Spinach 2 [43], Baby Spinach [38,44–46], iSpinach [47], etc. These Spinach derivatives have shown enhanced folding efficiency and brightness both *in vitro* and in living cells. Currently, one of the most popular version of fluorogenic aptamer is known as Broccoli [48]. Broccoli consists of 49 nucleotides and does not require a tRNA scaffold or high magnesium concentration for its cellular folding. These enhanced spectral and biophysical properties have made Broccoli a more favorable fluorogenic aptamer than Spinach in cellular imaging.

In addition to Spinach and Broccoli, there are other groups of fluorogenic aptamers. These aptamers can bind and activate the fluorescence of different classes of fluorophores, and as a result, target detection at different wavelengths is now feasible. Mango [49], Corn [50], SRB-2 [51], and DNB [52] are some examples of non-Spinach fluorogenic RNA aptamers that emerged for cellular imaging during the past decade.

In this review, we focus on the general design principles and applications of fluorogenic RNA-based biosensors. Starting with the traditional design of allosteric Spinach sensors and Spinach riboswitches, we will further introduce the recent progress in generating “second-generation” advanced fluorogenic RNA-based sensors for more sensitive and quantitative imaging of biomolecules.

2. “First-generation” fluorogenic RNA-based sensors – allosteric Spinach sensors

Fluorogenic RNAs have been used to measure the cellular distribution, abundance, and flux of metabolites and proteins [39,53]. Allosteric activation of Spinach is one of the first and

still popular strategy to engineer these RNA-based sensors. In this design principle, a transducer module is used to connect a target-binding aptamer to the structurally critical stem of Spinach (Fig. 1) [54]. In the absence of target, both Spinach and target-binding aptamer are unfolded. When the target is present, target-binding aptamer folds and induces the correct folding of Spinach via the transducer module, which finally results in the binding and fluorescence activation of DFHBI. Target-binding aptamers can be identified using a Systematic Evolution of Ligands by EXponential enrichment (SELEX) strategy [55,56], which allows the identification of aptamers with high binding affinity and specificity towards different target compounds of interest. Once identified, these target-binding aptamers can be modularly connected to Spinach for the development of RNA-based sensors. One major advantage of allosteric Spinach-based sensors is their modular design, which enables customized sensor development for a diverse range of target molecules [57].

Allosteric Spinach-based sensors have been used for intracellular imaging of various metabolites, signaling molecules and proteins. For example, Paige et al. have applied these RNA-based sensors to monitor the cellular dynamics of adenosine 5-diphosphate (ADP) and S-adenosylmethionine (SAM) in individual *E. coli* cells [58]. In the absence of methionine, a precursor for the cellular synthesis of SAM, minimal fluorescence was shown in allosteric Spinach sensor-expressing cells. In contrast, the cellular fluorescence increased by ~6-fold over 3 h after adding 25 μ M methionine. Importantly, cell-to-cell variations in the SAM levels can be assessed using these fluorogenic RNA-based sensors. In the same study, an ~18-fold ADP-induced fluorescence activation was observed within ADP Spinach sensor-expressing cells [58].

Using a similar strategy, cyclic diguanylate (c-di-GMP)-targeting allosteric Spinach sensors have been developed by fusing Vc2 GEMM-I class c-di-GMP riboswitch with Spinach [59–61]. Cyclic dinucleotides are important secondary messengers in bacteria, and more recently in mammalian cells, to regulate diverse physiological processes such as biofilm formation, sporulation, cell division, and immune cell stimulation [62–65]. In these sensor design, a natural P1 stem in the riboswitch was used as the transducer module. More recently, the same group have also engineered allosteric Spinach sensors for other cyclic dinucleotides, including cyclic AMP-GMP and cyclic-di-AMP [59,66–68]. These sensors have been used to image target cyclic dinucleotides in various Gram-negative and Gram-positive bacteria.

In another study, a set of aptamers were identified to selectively bind 5-hydroxytryptophan or 3, 4-dihydroxy phenylalanine [69]. To create functional biosensors, the identified aptamers were linked to Broccoli RNAs via short helical transducer modules. Cell imaging data demonstrated the potential of these fluorogenic Broccoli-based sensors for target detection in living systems. Here, the sensor design is quite similar to that of allosteric Spinach sensor, while this study further demonstrates that different fluorogenic RNA aptamers can be involved in the development of allosteric RNA-based sensors.

Allosteric Spinach sensors have also been used to measure cellular protein expressions. For example, Song et al. have generated Spinach-based sensors targeting streptavidin, thrombin, and MS2 phage coat protein (MCP), respectively [70]. These RNA-based sensors can provide up to 41-fold signal enhancement *in vitro* and be used to quantitatively measure

protein concentrations. Streptavidin- and MCP-targeting sensors have been further applied to monitor protein levels in living *E. coli* cells [70]. Based on the western blot data, a linear correlation between the protein expression and cellular fluorescence level was observed, indicating that these RNA-based sensors can reliably measure cellular protein expressions.

Altogether, allosteric Spinach/Broccoli sensors have been developed for the cellular imaging of diverse targets, including metabolites, signaling molecules, and proteins. These genetically encoded RNA-based sensors can be potentially used to monitor metabolic pathways and enzymatic functions, as well as to facilitate gene discoveries.

3. “First-generation” fluorogenic RNA-based sensors – Spinach riboswitches

Allosteric Spinach/Broccoli sensors are capable of modularly detecting cellular targets. However, their performance is largely dependent on the identification of proper target-binding aptamers: these aptamers should be highly selective towards the target. Generally, target-binding aptamers that identified through *in vitro* SELEX often should be further tested and optimized within the cellular environment. For example, a widely used ATP aptamer selected *in vitro* cannot efficiently discriminate ATP from AMP or ADP molecules, and thus it is challenging to be directly used for intracellular imaging of ATP [71–73]. Indeed, it is still difficult to perform negative or counter selection in regular SELEX against all the diverse molecules found in living systems that are structurally related with the target.

Additionally, aptamer affinity is crucial for effective target binding as well as the sensitivity of the RNA-based sensors [57]. Aptamers that bind the target too strongly will result in high occupancy sensors that are always on and are not able to detect concentration changes in the cells. In addition, sensors based on these high-affinity aptamers may bind the target so tightly that they limit target availability for normal cellular functions. On the other hand, if the aptamer affinity is too low, sensors will produce very little fluorescence in response to cellular target concentrations. Thus, it is critical to apply a target-binding aptamer of precise affinity that is compatible with target levels in the real cellular environment.

To obtain RNA-based sensors with high target selectivity and suitable binding affinity, we have recently engineered another type of genetically encoded fluorogenic sensor, named Spinach riboswitches [74]. Riboswitches are regulatory elements normally within messenger RNAs that can selectively bind with a diverse group of metabolites and signaling molecules. Upon target binding, riboswitches can change their conformations to activate, inhibit, or pause protein synthesis or other mRNA metabolism processes [75,76]. Thus, riboswitches are naturally evolved RNA devices that function in a highly precise manner responding to dynamic changes of the target cellular concentrations [77].

In general, riboswitches consist of two domains, an evolutionarily conserved target-binding domain (aptamer domain) and an expression platform that regulates the downstream gene expression events [78]. In order to develop a fluorescent sensor, we take advantage of the structural similarity between Spinach and the expression platform [74,79], and swap the expression platform of riboswitches with Spinach (Fig. 2). In this way, highly specific target

binding with the aptamer domain will induce the fluorescence activation of Spinach, rather than the regulation of gene expression. After optimizing the linkers and transducers, the resulting RNA sensor can switch between two conformations where a switching sequence binds with Spinach or the aptamer domain depending on the target concentration (Fig. 2).

We have successfully developed Spinach riboswitches that can detect thiamine-5-pyrophosphate (TPP) in live bacterial cells (Fig. 3). In this case, the structural similarity between a ribosomal binding portion of *thiM* TPP riboswitches and Spinach supported the design of a TPP-targeting Spinach riboswitch (Fig. 2) [74]. Up to ~16-fold TPP-induced enhancement in fluorescence was observed *in vitro*. Importantly, the target selectivity and dynamic range of the Spinach riboswitch is similar as that of natural *thiM* TPP riboswitches. Intracellular data further revealed the function of these genetically encoded sensors for selective TPP imaging. Using Spinach riboswitches, TPP-induced fluorescence activation, as well as cell-to-cell variations in TPP synthesis, could be successfully measured in live *E. coli* cells [74].

The same design strategy has been further used to develop Spinach riboswitches for different targets. Inspired by naturally evolved riboswitches, RNA-based sensors have been engineered to selectively detect guanine, adenine, and SAM [74]. Considering the broad choice of riboswitches (~100 classes), novel genetically encoded sensors can be potentially developed to detect diverse metabolites and signaling molecules. Indeed, currently, tertiary structures for most of the commonly known riboswitches have been identified and are readily available [76,80–83] for the development and optimization of Spinach riboswitch-based metabolite sensors.

4. “Second-generation” RNA-based sensors – general considerations

There are still some limitations in the current design of Spinach riboswitches and allosteric Spinach/Broccoli sensors. Most of these existing sensors are not sensitive enough for the cellular imaging of low-abundance targets. Current RNA-based sensors are limited to detect cellular targets that are present in the high micromolar to millimolar range. This is mainly due to moderate brightness and photostability of Spinach and Broccoli that should be further optimized [39,57]. On the other hand, in almost all currently existing sensors, one target molecule can maximally activate one Spinach or Broccoli, and exhibit only one equivalent fluorescence signal. In order to improve the sensitivity of these RNA-based sensors, it will be useful to evolve brighter, more photostable, and better folded fluorogenic RNAs, as well as to develop new approaches to realize signal amplifications.

Secondly, fluorogenic RNA-based sensors have so far only been developed based on a green-colored Spinach/DFHBI or Broccoli/DFHBI complex. Quantitative and multiplexed imaging of cellular analytes is still challenging. Extra care is required to apply the obtained fluorescence signal for the quantification of target cellular levels. Indeed, variations in RNA expression levels, spectral properties of RNA/dye complexes, and the subcellular structures and physical dimensions of the specimen (e.g., cell shape and thickness) can be problematic.

In addition, almost all existing fluorogenic aptamer-based sensors are limited in use to prokaryotic cells. Expression systems that enable accumulation of high levels of RNA sensors inside eukaryotic cells are critically needed. Indeed, compared to proteins (often in micromolar level), the low expression of RNAs (normally in nanomolar level) is related to the rapid degrading short RNA constructs in eukaryotic cells. This issue may also explain why riboswitches that widely exist in bacteria and fungi are not usually observed in mammalian cells. The direct incorporation of bacterial riboswitch-based sensors within mammalian cells may not be that ideal. Approaches to develop reliable RNA-based sensors for eukaryotic cell imaging are highly desired.

In the following discussions, we will introduce current progress in improving the performance of fluorogenic RNA-based sensors. Distinguishing from existing allosteric Spinach/Broccoli sensors and Spinach riboswitches in their design principle, sensitivity, selectivity, quantification property, and eukaryotic robustness, these new powerful sensor platforms may potentially be applied as the “second-generation” fluorogenic RNA-based sensors.

5. Potential “second-generation” RNA-based sensors – CHARGE circuits

As an example to improve the sensitivity of fluorogenic RNA-based sensors, we have recently developed an RNA circuit, termed CHARGE, Catalytic Hairpin Assembly RNA circuit that is Genetically Encoded [84]. Catalytic hairpin assembly (CHA) has been widely used for *in vitro* bioanalysis [85–88]. In the CHA circuit, two complementary DNA or RNA hairpins are designed in such a way that their spontaneous hybridization is kinetically trapped by embedding the complementary regions within the hairpin stems (Fig. 4). Only in the presence of the target, the H1 hairpin opens up based on a toehold-mediated strand displacement reaction to generate subsequent hybridization of both hairpins [85]. One recent study demonstrated that Spinach can function as a reporter in the CHA circuit [89]. However, this system can hardly be used for intracellular applications due to the poor signal-to-noise ratio and the slow kinetics.

There are several reasons that we believe CHA is suitable for the development of next-generation RNA-based sensors. First, CHA is a highly efficient, enzyme-free, and isothermal circuit. One target can induce the self-assembly of hundreds of hairpin strands. In addition, only self-folded hairpins are needed in this system. During the intracellular co-transcriptional folding process, hairpins are more accurately folded than inter-strand hybridizations. Therefore, CHA can minimize the background signal that are usually observed in other types of inter-strand hybridization-based RNA circuits.

In our CHARGE design, instead of Spinach, we chose to use Broccoli as the reporter, considering its improved folding and brighter cellular signal. We divided Broccoli into two non-fluorescent parts, called “Broccoli” and “Coli”, which were respectively fused to the 3’ and 5’ ends of two CHA hairpins (H1 and H2). Target binding induces the hybridization of H1 and H2, which further results in the reformation of Broccoli to turn on the fluorescence of DFHBI. Importantly, binding of one target can catalytically activate tens-to-hundreds of

Broccoli fluorescence events (Fig. 4) [84,90]. This catalytic system can provide robust fluorescence signals and be used for sensitive RNA imaging within living bacterial cells.

Here, using the CHARGE circuit as an example, we provide a detailed protocol on how to optimize and apply these fluorogenic RNA-based sensors. We start with the procedure for the design and *in vitro* test of CHARGE:

1. First, some free online software, including NUPACK and mfold, can be used to design and *in silico* simulate the folding and secondary structure of Broc-H1 and Coli-H2 strands in the presence or absence of the target RNA sequence.
2. Once the hairpin sequences are selected, a T7 promoter sequence (5'-TAATACGACTCACTATAG-3') should be added to the 5'-end of the DNA non-template strand for successful *in vitro* transcription. DNA oligonucleotides can be commercially synthesized and purified, for example, by Integrated DNA Technologies (Coralville, IA) or the W. M. Keck Oligonucleotide Synthesis Facility (Yale University School of Medicine).
3. Commercially synthesized oligonucleotides can then be dissolved at ~100 μ M concentration using a 10 mM Tris-HCl, 0.1 mM EDTA buffer at pH= 7.5, and stored at -20°C. Double-stranded DNA templates for the *in vitro* transcription are prepared following a standard PCR protocol. The PCR products are verified in a 2% agarose gel and purified using a PCR clean-up kit, such as a QIAquick PCR purification kit (Qiagen, Germantown, MD). If the PCR products are not sufficiently pure, a gel purification is needed. Afterwards, the DNA concentrations are measured using a NanoDrop One UV-Vis spectrophotometer.
4. Following the manufacturer's protocol, RNAs for the *in vitro* test can be transcribed from the double-stranded DNA templates using, for example, a HiScribe™ T7 high yield RNA synthesis kit (New England BioLabs, Ipswich, MA). It is critical to gel purify the desired RNA product with 10% denaturing PAGE using established protocols. After determining the concentration of RNAs by UV absorbance, all the RNA molecules are dispensed into aliquots with RNase-free water or TE buffer and stored at -20°C for immediate usage or at -80°C for long-term storage.
5. To ensure the proper folding of each RNA strand, before each test, RNA molecules are pre-heated at 95°C for 3 min and then slowly cooled to 25°C at the rate of -3°C/min.
6. To test the *in vitro* performance of CHARGE, fluorescence assays can be conducted in a buffer consisting of 10 mM Tris, 5 mM MgCl₂, 100 mM KCl, and 10 mM NaCl at pH= 7.5. In these measurements, 100 – 500 nM H1 and H2 can be used with the addition of 0.1 – 250 nM target RNA. All these reactions can be initiated by adding either target or H1 to the mixture of other RNA strands and dyes. For the dose response test, well-mixed RNA samples can be incubated for ~2 h before taking the fluorescence measurements. Similarly, the kinetics can be tested by monitoring the signal increase over 2 h after adding the target RNA.

The optimal sensor should exhibit a large increase in fluorescence and faster kinetics.

7. In general, 500 – 600 nm emission spectra should be collected by exciting at 480 nm. The kinetic assays can be conducted using 480 nm excitation and 503 nm emission. The obtained fluorescence results should be compared to the fluorescence of Broccoli (positive control) measured at the same concentration level under the same experimental conditions.

Following this protocol, our *in vitro* data revealed that CHARGE is highly sensitive. For example, using 250 nM H1 and H2, as low as 2.5 nM target RNA can induce high fluorescence with intensity similar to that observed for 250 nM targets [84]. Hence, CHARGE can be applied to detect low-abundance RNAs. Moreover, based on the *in vitro* kinetic data, CHARGE provides fast response in the presence of even small concentration of target RNAs [84].

To make CHARGE more easily tuned towards the detection of various target sequences, we have further introduced a modular molecular beacon unit (Fig. 4b). Here, a catalyst sequence, which had previously been optimized as a “target” through the above-mentioned protocol, was embedded in the stem region of a molecular beacon. The loop region of the beacon can be easily adjusted to hybridize with different cellular RNA targets. Upon target binding, the molecular beacon opens and releases the designer catalyst sequence to activate CHARGE and generate the fluorescence signal. Using the same H1 and H2 strands, by modifying the loop of molecular beacon, we have successfully detected miRNA 21 and a bacterial sugar transport related small RNA, SgrS [91–93].

Next, to study the efficiency of CHARGE for target detection in live bacterial cells, we expressed H1 and H2 in a dual expression vector, pETDuet, where two hairpins were expressed simultaneously at a similar cellular level. The target RNA was co-expressed in a pCDFDuet vector. Both vectors have a similar copy number, use a common *lac* promoter and undergo a T7-driven transcription. Here we illustrate a procedure that can be used for intracellular imaging with CHARGE (Fig. 4):

1. First, H1 and H2 hairpins are cloned into a dual-expression vector, such as pETDuet. Traditional restriction site cloning protocol can be followed here. For example, in our case, a pETDuet vector was first digested with the *NdeI* and *PacI* restriction enzymes (New England BioLabs). After gel purification, the digested vector was ligated with a similarly digested H2 insert using T4 DNA ligase (New England BioLabs). In order to prepare such digested H2, primers containing either *NdeI* or *PacI* restriction sites on the 5'- and 3'-ends were used to PCR amplify the H2 DNA template.
2. If traditional restriction site cloning is difficult to achieve, H1 or H2 strand can also be cloned using a Gibson Assembly® cloning kit (New England BioLabs). After preparing H1- and H2-expressing vectors, the same vector backbone with only H1 or H2 insert can be prepared as a negative control.

3. Afterwards, three tubes of chemical competent cells, such as BL21 (DE3)* *E. coli* cells (New England BioLabs) are thawed on ice. Then ~40 ng of the ligation plasmid product containing both H1 and H2 (positive control), H1 only (negative control), and H2 only (negative control), is added to each tube respectively. After transformation, 50 μ L incubated cells are plated on an LB agar plate supplemented with 150 μ g/mL ampicillin, and several colonies are chosen to isolate plasmids. This should be followed by sequencing to confirm the correct transformation.
4. The catalyst and molecular beacon constructs can be cloned into a compatible dual-expressing vector, such as pCDFDuet (EMD Millipore, Burlington, MA). After digestion with *Eco*NI and *Hind*III restriction enzymes (New England BioLabs) and gel purification, the digested vector is ligated with a similarly digested insert. The ligated product is then transformed into BL21 (DE3)* cells based on the streptomycin resistance. All these plasmids should be isolated and confirmed by Sanger sequencing, for example with Genewiz, NJ. H1- and H2-expressing vector and catalyst-expressing vector can be co-transformed into above prepared positive and negative BL21 (DE3)* cells based on their resistance towards both ampicillin and streptomycin.
5. Cellular imaging can be performed according to a previously established protocol [54]. Briefly, a single colony of plated cells is inoculated with 5 mL LB medium and 150 μ g/mL ampicillin and 80 μ g/mL streptomycin and are grown overnight at 37°C with shaking.
6. The overnight culture is diluted and re-grown in LB media at 37°C until the optical density at 600 nm reaches 0.4 – 0.5. Then 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) is added for a 2 h induction. In the SgrS detection assay, cells can be IPTG induced for 1.5 h and then treated with glucose (1 g/L and 20 g/L) for an additional 30 min.
7. A 200 μ L aliquot of an IPTG-induced culture is spun at 5,000 g for 2 min at room temperature to pellet the culture followed by resuspension in 1 mL of PBS medium. Add 200 μ L aliquot of the resuspended culture to a poly-D-lysine-coated plate and incubate at 37°C for 45 min to allow the cell adherence. Afterwards, the adherent cells are washed twice with 500 μ L PBS to remove unattached cells. Then, 200 μ L of PBS supplemented with 200 μ M DFHBI is added followed by incubation at 25°C or 37°C for 1 h before imaging.
8. The cells are imaged by exciting with a 488 nm laser through a 60 $\times$ oil immersion objective. An example filter set includes a 470/40 nm excitation filter, a dichroic mirror 495 nm (long pass), and an emission filter 525/50 nm. Imaging should be started with the cells expressing pETDuet-Broccoli (positive control) by looking for a field containing 50–100 evenly adhered cells. Proper exposure time is normally in the range of 100 ms to 2 s, which allows the highest signal without saturated pixels.

9. Finally, data analysis can be performed with a NiS-Elements AR Analysis software. More detailed data analysis can be obtained by a previously established protocol [54]. Data calculation and fitting can be done using the Origin software.

Based on our *in vitro* and intracellular data, CHARGE, as a novel genetically encoded RNA circuit, can detect targets with high sensitivity in live bacterial cells. This circuit can be easily programmed to detect multiple targets. Based on a similar approach, CHARGE can be potentially engineered to detect other proteins and small molecules, as well as to function as a switch to regulate gene expression. Indeed, many of these features have been demonstrated with the DNA-based CHA system *in vitro* [94]. CHARGE is currently limited for target imaging in prokaryotic cells (Fig 5). With promising properties for target imaging in bacterial cells, CHARGE could also potentially be further improved to apply for eukaryotic cell imaging of RNAs and other biomolecules.

6. Other potential “second-generation” fluorogenic RNA-based sensors

As shown in CHARGE, nucleic acid-based circuits are advantageous in their programmability, biocompatibility, and versatile in design and functions. Indeed, DNA-based circuits have been widely used to develop sensors and regulators *in vitro* [95,96]. However, the intracellular applications of these DNA-based circuit sensors are still limited due to difficulties in the biological delivery and degradation [97,98]. In contrast, RNAs can be genetically encoded and have similar potentials as DNAs to develop circuit-based biosensors. In addition to CHARGE, some other RNA-based circuits, e.g., hybridization chain reaction (HCR) and entropy-driven catalysis (EDC), may be potentially engineered into fluorogenic aptamer-based sensors.

Similar as CHA, HCR is comprised of two kinetically trapped hairpins that can undergo a succession of polymerization and hybridization events upon binding to a target strand [99]. This polymerization process continues as long as hairpins remain available, which results in a nicked nucleic acid nanostructure. HCR is an isothermal and enzyme-free method. Its design is straightforward and can map multiple targets simultaneously. Compared to CHA, HCR can provide distinct spatial information about the location of the target [100–102]. Recent development of *in situ* HCR has further reduced the background signal of HCR by carefully engineering the amplification component [103–105]. Even though traditionally DNAs have dominated the development of HCR, RNAs can be engineered into these circuits as well [106]. By conjugating the HCR hairpins with fluorogenic RNA aptamers, HCR could be a potentially ideal signaling cascade circuit for imaging and tracking low-abundance biomolecules.

EDC-based circuit is another potential candidate for this purpose [107]. EDC is driven by the overall entropy gain in the process, where an input strand can initiate the release of a large number of output strands. Here, with the help of a fuel strand, EDC has been used to achieve hundreds fold signal amplification in response to a 1 – 10 pM input [107,108]. Again, most current EDC circuits are designed based on DNAs, but with further engineering of RNA-based EDC and incorporating fluorogenic RNA aptamers as the output, it is

possible to develop genetically encoded EDC circuits for programmable and sensitive imaging in live cells.

Engineering more quantitative platform is another direction for the “second-generation” fluorogenic RNA-based sensors. One way to achieve this goal is by the ratiometric imaging with two (or multiple) fluorogenic aptamers that emit fluorescence at different wavelengths. For example, one fluorogenic RNA can be used to normalize individual cell expression variations of the RNA sensors, while a secondary fluorogenic RNA is used to give accurate values in the cellular concentrations of the target. In another approach, if two fluorogenic RNAs can form a FRET pair, quantitative imaging can be followed through target-induced FRET efficiency changes.

In addition to green fluorescent Spinach and Broccoli, fluorogenic RNAs emitting at longer wavelengths have also been evolved (Table 1). For example, an RNA aptamer named Corn has been evolved to bind and activate another HBI derivative, 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime (DFHO) (ex/em: 505/545 nm) [50]. As another example, Mango is an RNA aptamer that can tightly bind ($K_d \sim 3$ nM) and activate the fluorescence ($\sim 1,000$ fold) of thiazole orange (TO) dyes, including TO1-Biotin (ex/em: 510/535 nm) and TO3-Biotin (ex/em: 637/658 nm) [49,109]. Mango can be used to image low-abundance RNAs with high sensitivity in bacterial and mammalian cells. Recently evolved Mango II, III, and IV have further improved biophysical properties than Mango, such as the larger binding affinity and less salt dependency [110]. Contact-quenched fluorogenic probes can also be used to achieve multi-color and quantitative imaging. RNA aptamers that can either target dinitroaniline quencher (DNB) or sulforhodamine B fluorophore (SRB-2) have been used for this purpose [51,52]. Dual-color imaging of two fluorogenic RNA tags is now feasible in living cells [52].

CHARGE may be also potentially engineered into a quantitative ratiometric system. For example, a second aptamer that detects at a different wavelength (e.g., Mango) can be added to the domain 1 end of the H1 hairpin. As a result, the fluorescence signal of Mango functions as a reference that remains constant during the activation of CHARGE. In another potential design, the second aptamer can replace the loop region (4*) of H1. In this case, the target binding with H1 will decrease the signal of Mango due to the disruption of the loop structure. Together with the fluorescence enhancement of Broccoli upon target binding, some quantitative imaging can be obtained based on the normalized Broccoli-to-Mango fluorescence ratio.

RNA origami has been recently used as a scaffold to generate genetically encoded RNA-based FRET sensors [111]. Here, single-stranded RNA nanostructures were rationally incorporated with Spinach and Mango (Fig. 6). These fluorogenic RNAs were placed at a close proximity such that they provide a FRET read out. To obtain an optimal FRET efficiency, the relative orientation and positioning of Spinach and Mango were carefully tuned by adjusting the RNA origami scaffold. To develop a metabolite sensor based on this design, the authors have fused a partially structured SAM riboswitch into the scaffold (Fig. 6). This whole construct was then successfully applied to image intracellular SAM levels in *E. coli*. As shown in this example, the combination of fluorogenic RNAs with rationally

designed RNA nanostructures can be potentially used for the development of advanced sensory systems with more quantitative features.

Lastly, to engineer RNA-based sensors for eukaryotic cell imaging, it is important to develop expression systems that can accumulate high-level RNA sensors inside eukaryotic cells. Two complementary approaches can be useful here. First, fluorogenic RNAs of large quantum yield, extinction coefficient and photostability should be further evolved to increase the overall cellular brightness of RNA sensors. The second approach may rely on highly stable cellular RNA constructs, such as circular RNAs. Recent studies have shown these circular RNAs to be stable (days-to-weeks) and capable of accumulating at high levels in diverse eukaryotic cells and organisms [112–114]. Because these RNAs lack both free 5'- and 3'-end, they are expected to be resistant to all exonucleases in cells. Thus, systems that enable the high-level expression of circular RNAs will be a major advance for RNA sensor applications in eukaryotic cells.

In conclusion, fluorogenic RNA aptamers have been used as versatile reporters in sensor development. Without covalently attaching non-natural compounds, the entire RNA construct can be genetically programmed for intracellular imaging. By conjugating Spinach or Broccoli with a target recognition unit and a transducer module, the first-generation fluorogenic RNA-based sensors have been used for the cellular detection of RNAs, metabolites, and proteins. With the goal of dramatically improving the sensitivity, selectivity, quantification and eukaryotic robustness, second-generation RNA-based sensors have recently emerged by approaches incorporating natural RNA machineries, nucleic acid nanotechnology, and systematic evolution. The number of designs for more effective, sensitive and precise biosensing and bioimaging tools will surely continue to increase over the coming years. Next-generation fluorogenic RNA-based devices will further allow target detection in a wide variety of analytical platforms for cell biology and biomedical applications.

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Highlights

- Introduce a type of novel biosensors based on fluorogenic RNA aptamers
- Summarize different design principles of fluorogenic RNA-based sensors
- Discuss about the current applications and challenges of fluorogenic RNAs for cellular imaging and bioanalysis
- Provide several potential pathways to improve the sensitivity, efficiency, and robustness of RNA-based sensors
- Illustrate a guideline and protocol to develop and apply a CHARGE circuit

Fig. 1. Schematic of allosteric Spinach sensors. In the presence of target (e.g., metabolites), target-binding aptamer folds and induces the correct folding of Spinach through a transducer module. Finally, DFHBI binds with Spinach to produce a fluorescent complex.

Fig. 2. Schematic of Spinach riboswitches. Similar as natural riboswitches, the binding of target induces the conformational changes in the interactions between the switching sequence and transducer sequence. The resultant folding of Spinach binds with DFHBI to give out a strong fluorescence.

Fig. 3.

Imaging cellular TPP biosynthesis with Spinach riboswitches. Images were taken after addition of 10 μ M thiamine to the *E. coli* cells expressing Spinach riboswitch. Cells were grown briefly in thiamine-free medium for 2 h and then images were taken from 15 min to 3 h after the addition of thiamine. Images are pseudocolored to show the fold increase in fluorescence at each time point relative to 0 min. The color scale represents 0- to 10.0-fold changes (black to yellow) in fluorescence signal. Scale bar, 5 μ m [74].

Fig. 4. Schematic of the CHARGE circuit. **a)** The H1 and H2 strand is modified with split Broccoli, termed Broc and Coli, respectively. Catalyst (C) will initiate and catalyze the assembly of H1 and H2 into an H1+H2 duplex. Recombined Broccoli in the H1+H2 duplex activate the fluorescence of DFHBI dye. **b)** A modular CHARGE system to detect various RNA targets. Here, the binding of target RNA induces the opening of the molecular beacon, which further activates the catalyst to initiate the assembly of H1 and H2, finally resulting in Broccoli fluorescence.

<u>First generation fluorogenic RNA sensors</u>		<u>Characteristics</u>	<u>Limitations</u>
Allosteric Spinach/Broccoli sensors		Modularity Specificity	Sensitivity Multiplexing Quantitative imaging Eukaryotic application
		Specificity Modularity Precise affinity	Sensitivity Multiplexing Quantitative imaging Eukaryotic application
Spinach riboswitches
<u>Second generation fluorogenic RNA sensors</u>		<u>Characteristics</u>	<u>Limitations</u>
CHARGE		Sensitivity Specificity Modularity	(Eukaryotic application) (Multiplexing) (Quantitative imaging)

Fig. 5. Summary of the characteristics and limitations associated with the current first-generation (allosteric sensors and Spinach riboswitches) and second-generation (CHARGE) fluorogenic RNA sensors. Parenthetical (Eukaryotic application), (Multiplexing) and (Quantitative imaging) indicated the potential of CHARGE to be engineered with the properties of multiplexing and quantitative imaging for eukaryotic applications.

Fig. 6. Schematic of an RNA-based FRET sensor construct. In the absence of the target, partially structured aptamer domain (blue line) separates apart the Mango from the Spinach/Broccoli aptamer, and gives low FRET signal. Target binding induces the refolding of the aptamer domain and restores an efficient FRET signal.

Table 1.

A list of commonly used fluorogenic RNA aptamers and their spectral properties

Fluorogenic aptamer	Fluorophore	K_D (nM)	Ex / Em (nm)	ϵ (M ⁻¹ cm ⁻¹)	ϕ	Ref.
	GFP		395 / 508	21,000	0.77	[115]
Spinach	DFHBI	540	469 / 501	24,300	0.72	[31]
Spinach-2	DFHBI-1T	560	482 / 505	31,000	0.94	[116]
Spinach-2	DFHBI-2T	1,300	500 / 523	29,000	0.12	[116]
Broccoli	DFHBI-1T	360	472 / 507	29,600	0.94	[48]
Red-Broccoli	DFHO	206	518 / 582	35,000	0.34	[50]
Corn	DFHO	70	505 / 545	29,000	0.25	[50]
Mango	TO1-Biotin	3.2	510 / 535	77,500	0.14	[49]
Mango II	TO1-Biotin	0.7	510 / 535	~77,000	~0.2	[110]
Mango III	TO1-Biotin	5.6	510 / 535	~77,000	~0.56	[110]
Mango IV	TO1-Biotin	11.1	510 / 535	~77,000	0.42	[110]
Mango	TO3-Biotin	5.1	637 / 658	9,300	N/A	[49]
Mango	YO3-Biotin	25.6	597 / 618	N/A	N/A	[111]
MG-aptamer	Malachite Green	117	630 / 650	150,000	0.187	[36]
SRB-2	SR-DN	1,400	579 / 596	85,200	0.65	[117]
SRB-2	TMR-DN	35	564 / 587	90,500	0.33	[51]
DNB	SR-DN	800	572 / 591	50,250	0.98	[52]
DNB	TMR-DN	350	555 / 582	47,150	0.90	[52]
BHQ apt (A1)	Cy3-BHQ1	4,700	520 / 565	N/A	N/A	[118]

N/A: not available; ϵ : absorption coefficient; ϕ : quantum yield; Ex / Em: excitation/emission wavelength peak value

Table 2:

Properties of the fluorogenic RNA-based sensors discussed in this review

Sensor	Target	EC_{50} (μ M)	Fold activation *	Tested in bacteria	Ref.
Allosteric Spinach	ADP	270	20	Yes	[58]
Allosteric Spinach	SAM	120	25	Yes	[58]
Allosteric Spinach	Adenosine	44	20	No	[58]
Allosteric Spinach	Guanine	1.5	32	No	[58]
Allosteric Spinach	GTP	7,700	15	No	[58]
Allosteric Spinach	c-di-GMP	0.23	(37°C) ~6	Yes	[59]
Allosteric Spinach	c-AMP-GMP	4.2	(37°C) ~8	Yes	[59]
Allosteric Spinach	c-di-GMP	~2	>30	No	[60]
Allosteric Spinach	c-di-GMP	0.005 – 0.4	(37°C) 5 – 7	Yes	[66]
Allosteric Spinach	c-di-AMP	3.4 & 29	2.4 & 9.1	Yes	[67]
Allosteric Spinach	Streptavidin	<0.2	10.3	Yes	[70]
Allosteric Spinach	MCP coat protein	~0.6	41.7	Yes	[70]
Allosteric Spinach	Thrombin	~0.2	6.9	No	[70]
Allosteric Broccoli	c-di-GMP	N/A	~7	No	[48]
Allosteric Broccoli	5-hydroxytryptophan	N/A	>5	Yes	[69]
Allosteric Broccoli	3, 4-dihydroxy phenylalanine	N/A	>5	Yes	[69]
Spinach riboswitch	TPP	9.0	15.9	Yes	[74]
Spinach riboswitch	SAM	1.2	8.8	No	[74]
Spinach riboswitch	Guanine	0.19	6.2	No	[74]
Spinach riboswitch	Adenine	10	4.3	No	[74]
CHARGE	RNA	~0.001	2.2	Yes	[84]
Spinach-Mango FRET	SAM	17	<2	No	[111]

N/A: not available;

* Fold activation indicates the *in vitro* sensor performance after adding target as measured at 25°C.