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Abstract

Fluorescent biosensors are powerful tools with which to detect biochemical events inside of cells with high spatiotemporal resolution. Biosensors based on fluorescent proteins often suffer from issues with photostability and brightness. On the other hand, hybrid, chemical-genetic systems present unique opportunities to combine the strengths of synthetic, organic chemistry with biological macromolecules to generate exquisitely tailored semisynthetic sensors.

Genetically encoded fluorescent biosensors have revolutionized the way that we measure biochemical activity. They permit non-invasive monitoring of the biochemistry that governs life in real-time in live specimens, giving us access to the dynamics and heterogeneity of the reactions that underpin important processes. Biomacromolecule-based tools are deliverable by standard genetic manipulation tools and can be targeted to specific cell types. This is possible due to the discovery and development of fluorescent proteins (FP) such as green fluorescent protein (GFP) [1] and their subsequent utilization in the earliest biosensors, which combine an FP and a protein domain that responds to a biochemical process [2,3]. However, despite many advances and improvements of the palette of FPs, they still suffer from drawbacks—namely, reduced photostability and brightness—in comparison to synthetic, organic fluorophores [4]. To address these issues while retaining the benefits of genetic targetability, many groups have proposed hybrid systems comprised of a biomacromolecule and a synthetic fluorophore. Initial efforts were focused on the development of tagging systems such as Halotag [5], SNAP-tag [6], CLIP-tag [7], the photoactive yellow protein (PYP)-tag [8], the Fluorescence-Activating and absorption-Shifting Tag (FAST) [9] and Fluorogen-Activating Proteins (FAPs) [10], which use proteins, as well as Spinach [11] and other RNA aptamers that bind fluorogenic dyes. More recently, developers have turned towards adapting these systems to fluorescent biosensing, coupling the detection of a biochemical event to a fluorescent read-out. Recent reviews have covered these hybrid tagging systems extensively [12-14]. This short review is thus focused on notable advances in the development of fluorescent chemical-genetic (or semisynthetic) biosensors from the last two years.

I. Sensing with fluorescent chemical-genetic hybrids: Functional Fluorophores

Functional fluorophores can be used to couple the detection of a biochemical process to a fluorescent read-out. These fluorophores can themselves be sensitive to a particular process or facilitate the detection of process through modulating the activity of a biomacromolecule.

Voltage indicators have been a major focus of biosensor research as they can provide a minimally invasive, yet highly sensitive readout of neurological activity. Dyes that respond directly to changes in membrane voltage have the potential to be powerful tools due to the kinetics of their response and their photostability. However, targeting the dye and reducing off-target labeling remain challenges in their implementation. Thus, a hybrid system using a voltage-sensitive derivative of Nile Red, an environmentally sensitive fluorogenic dye, and the acyl carrier protein (ACP)-tag [15], a small, 8 kDa protein tag, was developed to address these issues [16]. The protein tag was sufficiently small so as to allow the insertion of the dye in the membrane for voltage detection as well as providing a genetically encoded handle to target the dye to particular cell types. The resulting semisynthetic tethered voltage indicator 1 (STeVI1) was able to detect fast neuronal activity with high sensitivity using reduced light power.

Another strategy for biosensing relies on exploiting small molecule-binding proteins. Recent progress in the Johnsson lab building off of the SNAP-tag based indicators with a fluorescent intramolecular tether (Snifit) [17] and luciferase-based indicators of drugs (LUCID) [18] concepts resulted in fluorescent biosensors for measuring the NADPH/$NADP^+$ ratio [19] and for quantitative detection of NADPH in point-of-care applications [20]. These systems take advantage of self-labeling tags such as SNAP-tag or Halotag and their tolerance for a wide variety of ligands, as long as the reactive handle for the protein is incorporated. While the original Snifits were based on the competition between the natural ligand for a protein and a tethered ligand attached using a self-labeling tag [17], this new generation relies on the co-recruitment of a ligand in the presence of NAD(P) or NADPH (Figure 1A). A change in FRET efficiency was produced by the binding of $NADP^+$ and a sulfa drug, which induces a conformational change in a SNAP-Halo-sepiapterin reductase (SPR) chimera. $NADP^+$ and NADPH compete for the same binding site on SPR, but only $NADP^+$ is able to recruit the tethered ligand, meaning that the sensor can effectively measure the ratio of NADPH:$NADP^+$.

Coupling this strategy to a luciferase has proven to be a method to bring these techniques to point-of-care diagnostics. The Johnsson lab recently demonstrated a

technique to quantitatively detect metabolites that are metabolized by dehydrogenases [20]. A coupled dehydrogenase assay was developed to stoichiometrically produce NADPH in response to a particular metabolite, in this case, phenylalanine. In an analogous topology to the Snifits, the cooperative binding of NADPH and trimethoprim (TMP) to DHFR was used to quantify the amount of NADPH in complex mixtures by modulating the BRET efficiency.

Combining the molecular recognition of proteins with a functional fluorophore that recognizes particular biomolecules can facilitate high contrast detection of challenging targets. Recently, the Kikuchi group developed a novel PYP-tag based system for detecting epigenetic markers on DNA (Figure 1B) [21]. This system addresses drawbacks in previously developed techniques such as high background fluorescence through the use of a fluorogenic PYP-ligand based on oxazole yellow, whose fluorescence is enhanced upon binding with DNA. Targeting oxazole yellow to DNA is done through the addition of a methyl-CpG-binding domain to ensure that the PYP-tag-conjugate only accesses DNA where there are methylation sites. This conjugate enabled live cell imaging of DNA methylation during mitosis.

II. Sensing with fluorescent chemical-genetic hybrids: Protein-based Recognition

The strength of genetically-encoded biosensors lies not only in the fact that they can be targeted to particular subcellular localizations and cell types, but also in that they take advantage of the exquisite molecular recognition capabilities of proteins conferred through evolutionary pressure. Many of these recognition events result in a conformational change in the protein, which can then be coupled to a fluorescent read-out. While the systems detailed in Part I certainly take advantage of protein activity, the systems detailed here rely exclusively on protein activity to generate contrast. Here, we highlight new systems that use protein-based chemistry to detect changes in Ca^{2+} concentrations, membrane voltage, and protein-protein interactions.

Probes that emit further into the red are beneficial because these wavelengths are associated with better tissue penetration, lower background autofluorescence, and

lower phototoxicity. However, developing far-red and near-infrared (NIR)-emitting fluorescent proteins from the classic mFruits scaffold has proved challenging. Finally, this was addressed through massive protein engineering efforts to generate proteins that can use biliverdin as a fluorogenic substrate [22-24]. These proteins have resisted conversion into biosensors until a recent report from the Campbell group in which mIFP was used in a NIR-emitting genetically encoded Ca^{2+} indicator for optical imaging (NIR-GECO) [25]. After identification of an insertion site for the Ca^{2+} detection moiety, calmodulin-R20 peptide, extensive protein engineering was performed to optimize the response of the sensor. NIR-GECO1 is an inverse response sensor (meaning brighter in the absence of Ca^{2+}) with a similar contrast between the calcium-bound and unbound forms of the sensor as GCaMP3. It performs well in cultured neurons and, like other biliverdin-containing FPs, benefits from the addition of exogenous biliverdin and/or overexpression of heme-oxygenase 1. Furthermore, NIR-GECO1 can be used in brain slices or in mesoscale mice imaging and in multiplexed sensing experiments or in conjugation with an optogenetic system. Overall, this new system extends possibilities for highly multiplexed imaging experiments.

While Ca^{2+} imaging is widely used as an indicator of neurological activity, the kinetics and the threshold needed to trigger a Ca^{2+} response complicate analysis of action potentials or cause them to be missed entirely. On the other hand, genetically encoded voltage indicators (GEVIs) have been difficult to generate due to the kinetics of voltage dynamics as well as the brightness and photostability of fluorescent proteins. To address these issues, the Schreiter group employed chemical genetic tagging systems with microbial rhodopsin domains to sense voltage by electrochromic FRET from the Schiff base of the retinal cofactor to a synthetic dye (Figure 2A) [26]. While this was demonstrated for both SNAP-tag and Halotag and various rhodopsins, the best performing system, termed Voltron, uses Ace2 as the rhodopsin domain and Halotag-Janelia Fluor (JF) as the FRET donor. Voltron functions with multiple improved rhodamine dyes from the JF family and is significantly brighter and more photostable than GEVIs using FPs, with an approximate 10-fold increase in photon yield in neurons. Furthermore, this system works well in *in vivo* models including mice, zebrafish, and adult *Drosophila*. Voltron produces an inverse signal in response to positive changes in

voltage, typical of all previously developed GEVIs. This response is due to the increase in rhodopsin absorbance in response to increased membrane voltage via changes in the protonation equilibrium of the retinal Schiff base, which equilibrates with the cytoplasm. This model was used to engineer a positive-response GEVI [27]. In order to do, the proton-donating residue was mutated to a neutral residue, effectively blocking the access of protons from the cytoplasm. The rest of the proton transport pathway was reengineered to improve the efficiency of proton movement towards the outside of the cell: the canonical proton-accepting residue was mutated to proton donor and proton relays were subjected to saturation mutagenesis. The resulting construct, called Positron, displayed a positive response to changes in membrane voltage with responses on par with other state-of-the-art GEVIs. Furthermore, the authors demonstrated that this strategy is extendable to other rhodopsin domains and to other fluorophores, providing a general pathway to engineering positive response GEVIs.

Protein-protein interactions (PPIs) play an enormous number of roles in many cellular processes, including cellular signaling, respiration, and replication. However, they remain difficult to detect in space and time, largely due to a dearth of methods that are capable of detecting them with high spatiotemporal resolution. In particular, detection of PPIs is hindered by techniques that are difficult to implement (e.g. FRET-based approaches) [28] or that are unable to resolve important information such as the reversibility of an interaction (e.g. bimolecular fluorescent complementation based on split fluorescent proteins (splitFPs)) [29]. We recently developed a technique that preserves the ease of implementation of splitFPs while displaying rapid and reversible complementation (Figure 2B) [30]. Building off of previous work in our lab, we split FAST, which specifically and reversibly binds fluorogenic hydroxybenzylidene rhodanine (HBR) analogs, into two fragments at its circular permutation site resulting in a larger (~11 kDa) N-terminal fragment, NFAST, and an 11 amino acid peptide, CFAST. The affinity can be modulated by modification of the small fragment of FAST. We showed that this system is reversible and can detect a range of static and dynamic interactions in cells. Furthermore, this split topology was also successfully used for biosensing, to detect Ca^{2+} oscillations or caspase activity.

III. Detecting RNA using fluorescent chemical-genetic hybrids

RNAs play a key role in the cell and can exist in many different forms, including mRNA, non-coding RNA (ncRNA), micro RNAs (miRNA). The gold standard RNA detection method has been the MS2 system, comprised of a viral coat protein, MCP (or PCP), that interacts specifically with an RNA stem loop, MS2 (or PP7) [31,32]. Fusing MCP with GFP can then be used for live cell imaging of RNAs. However, this technique results in high background due to unbound GFP-labeled MCP proteins. Furthermore, many copies of the stem loops must be inserted into the RNA of interest. Thus, RNA aptamers that can interact specifically with a fluorogenic dye have attracted interest as a method to reduce the complexity of the system and to take advantage of bright and photostable fluorophores (Figure 3). Initial efforts in this direction leading to systems such as Spinach suffered from issues with photostability and brightness, such that only highly transcribed transcripts were detectable [33,34].

Recently, the Palmer group has addressed these issues through the development of a novel aptamer-based system called Riboglow (Figure 3A) [35]. This creative system relies on a short riboswitch sequence that recognizes cobalamin. Since cobalamin is an effective fluorescence quencher, fluorophores could be linked to the 5' hydroxyl group of cobalamin and be efficiently quenched. Binding to the riboswitch then forces a conformational switch, shielding the fluorophore from cobalamin, thus reducing the intramolecular quenching and resulting in high fluorescence contrast. These short (~100 nt) aptamers exhibit strong affinities (~nM) for the probe and good stability. Furthermore, Riboglow does not require a tRNA folding scaffold for proper folding in mammalian cells. The detection of small non-coding U1 recruitment to U-bodies was possible with 1 copy of the aptamer while mRNA recruitment to stress granules required 4 copies of the aptamer. This represents the first time that aptamer-based imaging systems have been used to detect RNA Pol II transcripts, which are less abundant than the RNA Pol III transcripts that have typically been imaged using aptamers.

Addressing the limitations of aptamer-based imaging systems in order to image lower abundance RNA Pol II transcripts through fluorophore engineering has been the

focus of several efforts. Ideally, these systems will exhibit extraordinary brightness and photostability in order to be able to detect single molecules transcribed by RNA Pol II, in addition to a high degree of fluorogenicity to give contrast. In addition to the cobalamin-quenched fluorophores detailed above, two new aptamer-based imaging systems have been published recently that rely on novel small molecules to improve the performance of RNA imaging systems. In one system, dimerized sulforhodamine B dyes that self-quench in solution were used in conjunction with a novel aptamer, o-Coral, developed through repetitive rounds of SELEX and microfluidic-assisted in vitro compartimentalization (Figure 3A) [36]. These dimer systems were capable of imaging both RNA Pol III and RNA Pol II transcripts with only a single copy of the aptamer (i.e. two fluorophores). Another particularly promising system relied on RNA aptamers called Peppers, which bind and activate the fluorescence of fluorogenic dyes composed of a rigid electron acceptor and strong electron donor (Figure 3B) [37]. SELEX selection followed by multiple rounds of optimization resulted in an RNA aptamer with reduced Mg^{2+} and K^{+} sensitivity, tight (nM) binding, and no requirement for RNA scaffolds to aid in folding. Peppers can be used with four copies of the aptamer to image RNA Pol II constructs, including mRNA and ncRNAs. Futhermore, the aptamer can be inserted into gRNA for CRISPR-based imaging. This system can also be used for more advanced imaging modalities, including 2 photon excitation and structured-illumination microscopy (SIM).

RNA is known to play many biological roles outside of the canonical messenger, ribosomal, and transfer RNA roles, most importantly as regulatory molecules, but also as genomic material in viruses. Elucidating the various roles of RNA requires methods to detect RNA in action; particularly in contexts where modifying the RNA by insertion of an aptamer can perturb function or is otherwise intractable. In order to target miRNAS, Zhong and Sczepanski used a heterochiral strand displacement mechanism to sequence-specifically target endogenous RNAs [38]. Small sequences of nucleic acids are often sensitive to nuclease digestion, while L-RNA is resistant to nuclease activity. Using this feature of L-RNA the authors developed a system consisting of the L-RNA aptamer, Mango III, which binds thiazole orange, and a peptide nucleic acid (PNA) to detect miRNA155. The PNA serves to block the folding of L-Mango III until miRNA155 is

detected through binding to a toehold residue and subsequent displacement of the aptamer, which is then free to bind thiazole orange. This system was delivered using a cholesterol conjugation self-delivery strategy to facilitate sensor entry into cells. Elevated levels of miRNA155 were detectable in a stable cell line overexpressing the miRNA using this strategy.

IV. Conclusion

Recent advances in fluorescent chemical-genetic biosensor development show the potential and versatility of hybrid approaches. High performing systems are now employable in a range of settings, from detecting fast processes *in vivo* to point-of-care diagnostics. We fully expect the advances in this field to continue to yield novel and powerful tools that expand our ability to visualize cryptic biochemical events in live cells.

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Conflicts of Interest: The authors declare the following competing financial interest: A.G. is co-founder and holds equity in Twinkle Bioscience/The Twinkle Factory, a company commercializing the FAST technology.

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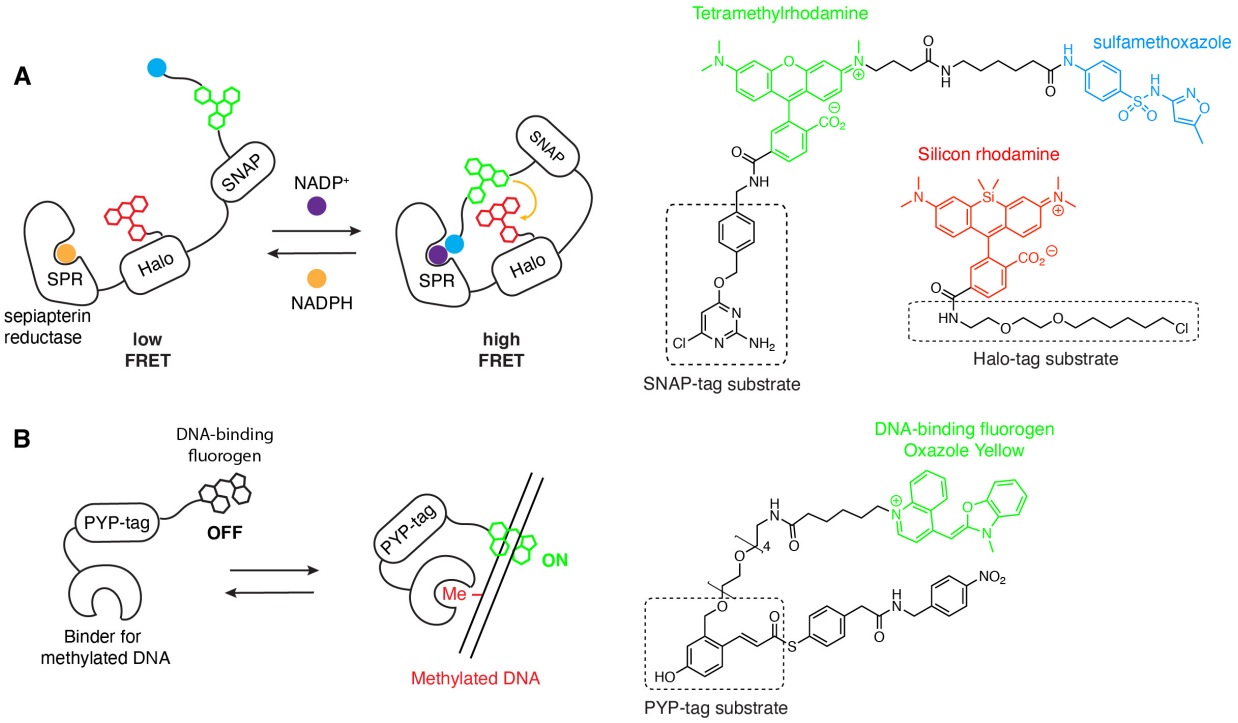

Figure 1. A) Next generation Snifits rely on co-recruitment of NADP and sulfamethoxazole to sepiapterin reductase to detect the NADPH:NADP ratio as a change in FRET efficiency between silicon rhodamine and tetramethylrhodamine. B) Live cell detection of DNA methylation functions via targeting with a protein binding domain that recognizes methylated DNA labeled (via the PYP-tag) with oxazole yellow, a fluorogenic dye that lights up upon interaction with DNA.

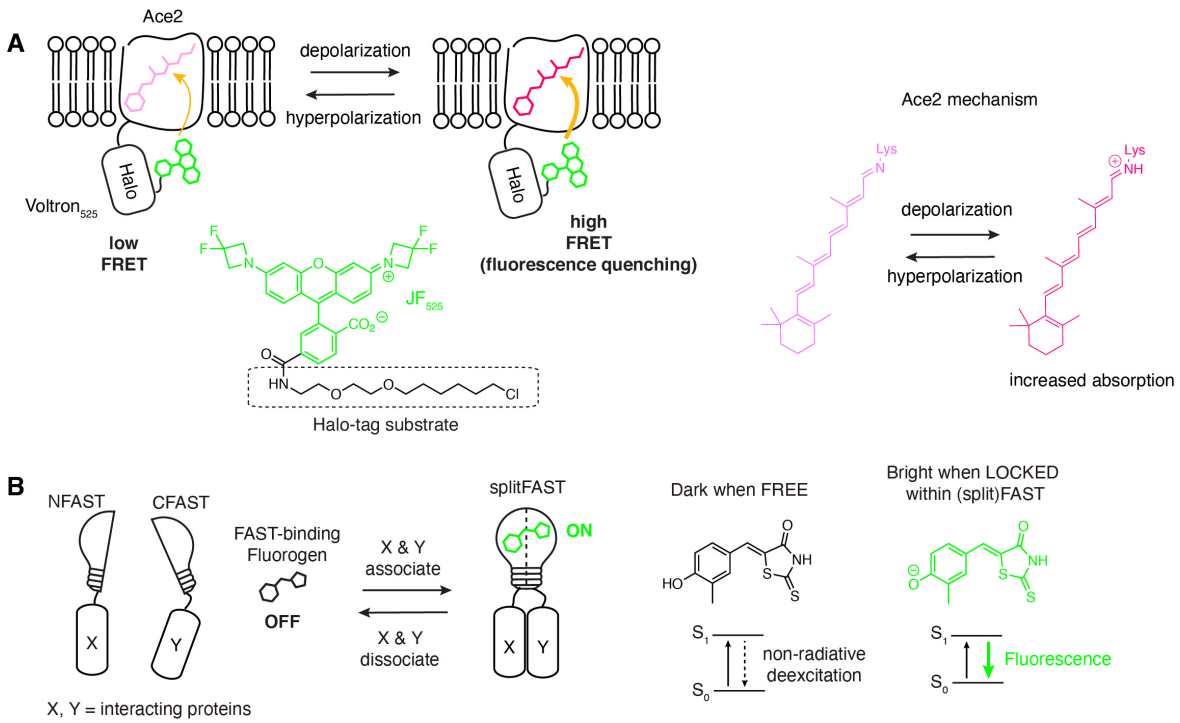

Figure 2. A) Voltron is a next generation genetically encoded voltage indicator comprised of the microbial rhodopsin, Ace2N, and a Halo tag conjugated with a Janelia Fluor. Upon membrane depolarization, the protonation of the retinal Schiff base within Ace2 changes. This protonation change leads to an increase in absorption that causes the quenching of JF fluorescence. B) splitFAST, derived from the fluorogenic tagging system FAST, is the first reversible bimolecular fluorescence complementation assay for the detection of protein-protein interactions. splitFAST complementation promotes the specific activation of hydroxybenzylidene rhodanine fluorogens through conformational locking.

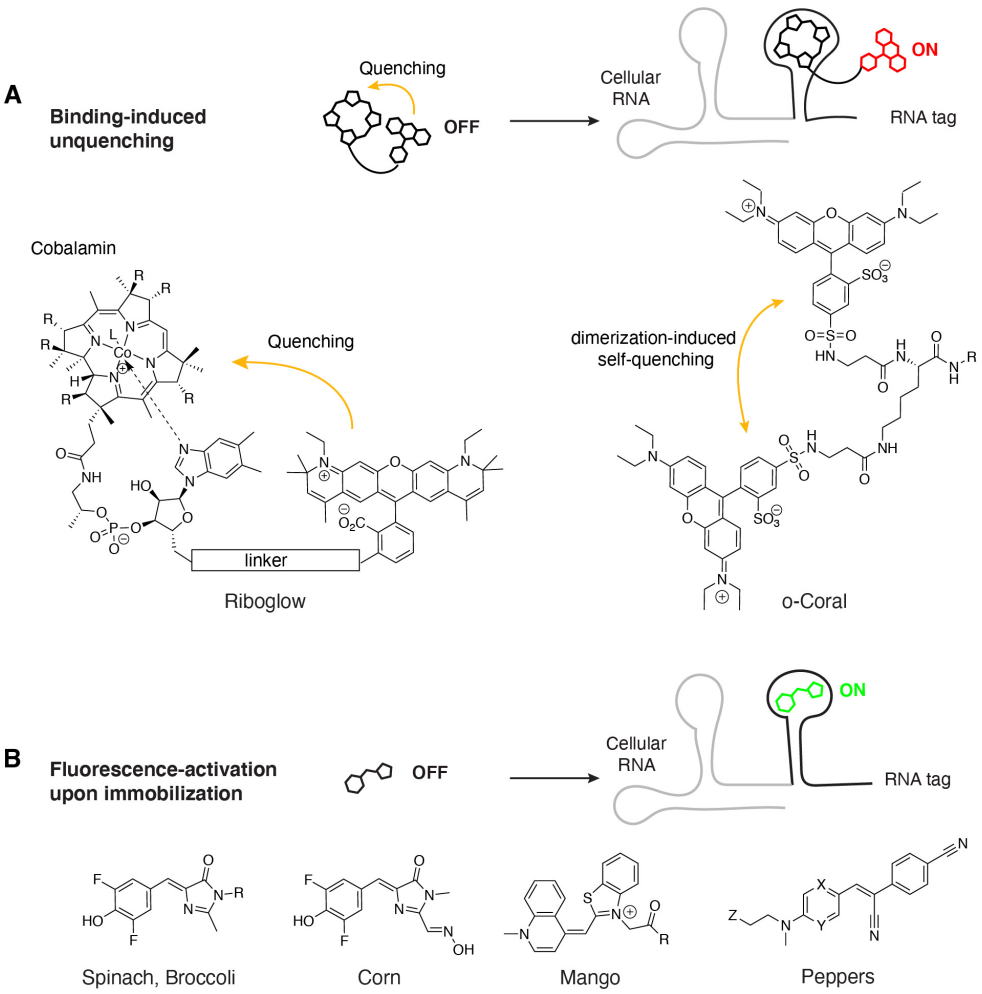

Figure 3. A) High contrast RNA detection can be achieved via de-quenching of probes mediated by aptamer recognition. B) RNA detection can also be achieved through the activation of fluorescence via aptamer-based immobilization of the probe, resulting in an increased fluorescence quantum yield.