

# Towards Covalent Fluorescent Light-Up Aptamers (coFLAPs)

Martin Bitsche, Malou Hanisch, Alexandra Lusser, and Ronald Micura\*

**Abstract:** The fluorogen-activating aptamer, Spinach, was a milestone in cellular RNA imaging and conceptually similar to the green fluorescent protein (GFP). Since then, more than ten FLAP systems have been developed across a broad spectral range. All of these systems differ fundamentally from GFP in that FLAPs rely on noncovalent fluorogen–RNA interactions, whereas GFP integrates its fluorophore covalently. In this article, we discuss recently developed FLAPs, including the first covalent FLAP, coPepper, as a promising new strategy to overcome current limitations in FLAP-based RNA imaging. Moreover, the bioconjugation chemistry developed for coFLAPs has immediate impact on covalent RNA labeling, RNA drug targeting, and in general, on covalent drug design.

**Keywords:** Covalent RNA labeling · Fluorescent RNA (FR) · Fluorogen-activating aptamers · Fluorophore synthesis



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## 1. Introduction – Visualizing RNA *in Vivo*

Fluorescent proteins transformed cell biology by enabling the direct visualization of proteins in living systems. Green fluorescent protein (GFP) became the archetype thanks to its intrinsic brightness, rapid maturation, and compatibility with standard optics. However, extending this success to RNA has proven to be very challenging.<sup>[1–4]</sup>

Fluorogenic RNA aptamers are structured RNAs that bind weakly-fluorescent small molecules and thereby activate strong emission. They offer a genetically encodable solution for live-cell RNA imaging.<sup>[5–11]</sup> Selected *in vitro*, these ‘light-up’ aptamers restrict dye motions and thus reduce non-radiative decay, producing high signal upon binding. Early systems established the concept: for instance, the malachite green-binding aptamer turns on triphenylmethane dyes;<sup>[12]</sup> Spinach<sup>[13]</sup> and its successors (Broccoli,<sup>[14]</sup> Squash<sup>[15]</sup>) activate GFP-like chromophores such as DFHBI (Fig. 1); Mango binds thiazole orange derivatives;<sup>[16]</sup> and additional families such as Corn<sup>[17]</sup> and Pepper<sup>[18]</sup> broadened the spectral palette (Fig. 1). More recently, non-G-quadruplex designs, including dimethylindole red (DIR) binders,<sup>[19,20]</sup> and Pepper,<sup>[18,20]</sup> were introduced exhibiting improved folding robustness *in vivo*.

Yet several obstacles limit their widespread adoption in live cells. Unbound dyes can contribute significant background through residual fluorescence and nonspecific interactions, eroding contrast. Many aptamers rely on G-quadruplex elements that are prone to misfolding and sensitive to cations, reducing the fraction of functional RNA in physiological contexts. Photostability is often inadequate: DFHBI- and HBC-based complexes bleach quickly, curtailing long-term or single-molecule imaging. Cellular brightness can be compromised by

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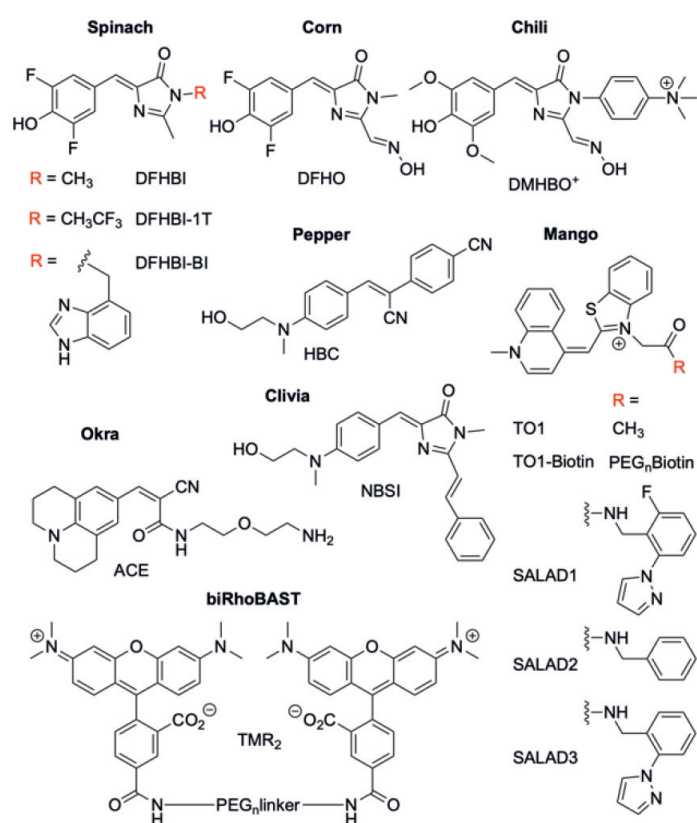


Fig. 1. Overview of some FLAPs and their corresponding fluorophores.

poor dye uptake, sequestration, or efflux, even when *in vitro* brightness is high. Biorthogonality poses another challenge. For instance, DFHBI ligands can be activated by Spinach, Broccoli, and Squash, while thiazole orange variants can light up with Mango or Spinach, which complicates multiplexed experiments.

Fluorophores with large Stokes shifts are particularly valuable because they simplify spectral separation, reduce bleed-through, and mitigate autofluorescence. The Chili aptamer exemplifies this advantage by producing strongly shifted emission with DMHBI<sup>+</sup> analogs (Fig. 1),<sup>[22,23]</sup> but the lower quantum yields limit *in vivo* utility. Conversely, Pepper paired with the GFP-like dye HBC (Fig. 1) achieves markedly higher intracellular brightness than earlier green systems, yet still suffers from photobleaching. These trade-offs underscore the need for coordinated optimization of both RNA scaffolds and dye chemistry.

The field is now converging on the design principles for next-generation tools, which include high on-off contrast in cells, robust folding without G-quadruplex dependence, elevated cellular brightness *via* strong absorption and a high quantum yield in the bound state, excellent photostability, large Stokes shifts, and strict orthogonality among aptamer-dye pairs. Meeting these criteria will enable the simultaneous tracking of multiple RNAs, longer time-lapse imaging, and integration with complementary modalities, such as bioluminescence. Continued advances in selection strategies, structural RNA engineering, and dye synthesis will deliver fluorogenic aptamers, enabling dynamic and minimally disruptive measurements of RNA localization, trafficking, and function in living systems. This will accelerate the transition from static transcript inventories to real-time views of the transcriptome in action.

## 2. The Most Recent FLAPs – Clivia, Okra, SALAD, biRhoBAST and biSiRA

### 2.1 Clivia

Clivia is an RNA aptamer that activates a red-emitting fluorophore, NBSI (Fig. 1), to produce bright, low-background signals with a large Stokes shift (up to 108 nm).<sup>[24]</sup> The crystal structure clarifies how the dye is accommodated and fluorescence is enhanced.<sup>[25]</sup> The major impact lies in the advantages it provides for cellular applications because Clivia's orange-to-red emission reduces cellular autofluorescence, light scattering, and phototoxicity, enabling deeper, gentler imaging in live cells and tissues.<sup>[24]</sup> Its dyes show good membrane permeability and low cytotoxicity, supporting routine labeling. Because Clivia recognizes ligands through a binding mode distinct from many G-quadruplex aptamers, it can be used orthogonally for multiplex experiments. Pairing Clivia with Pepper enables single-excitation, dual-emission imaging of RNAs and genomic loci, cutting acquisition time and instrumentation costs. A minimized 24-nt 'CM' module and multivalent assemblies further boost brightness without perturbing RNA localization.<sup>[24]</sup> Furthermore, Clivia supports bioluminescent readouts of RNA-protein interactions in live cells and *in vivo* (mice), broadening its utility for tracking, quantifying, and mechanistically probing RNAs across diverse biological contexts.

### 2.2 Okra

Okra (Okra505) is a green fluorogenic RNA aptamer that activates a small-molecule dye, ACE (Fig. 1), to deliver markedly brighter and far more photostable signals than existing green fluorescent RNAs.<sup>[26]</sup> With nanomolar affinity, robust folding with low ion dependence, and high thermal stability, Okra achieves about tenfold higher cellular brightness than Pepper and about thirtyfold higher cellular brightness than Broccoli-BI or Broccoli-TBI.<sup>[26]</sup> It withstands extended illumination – surpassing even EGFP (Enhanced Green Fluorescent Protein) in long-term imaging. These features enable reliable mRNA visualization in both bacteria and mammalian cells, such as time-resolved tracking of ACTB (beta actin) mRNA recruitment to stress granules. Furthermore, dual-color super-resolution imaging was achieved by pairing with Pepper620, which revealed distinct, nonuniform mRNA distributions within stress granules.<sup>[26]</sup> Mechanistically, Okra's performance can be attributed to the following factors: firstly, the compact donor- $\pi$ -acceptor design of ACE, and secondly, the fast binding kinetics that limit light-driven Z-E isomerization. In addition, the rapid unbinding of the nonemissive E form allows for efficient fluorescence recovery. Okra fills a critical gap for bright, stable green RNA imaging and sets the stage for next-generation fluorescent RNA development.

### 2.3 SALAD

SALAD is a bright, RNA-activated dye family engineered for Mango aptamers using a structure-guided, fragment-based discovery workflow.<sup>[27]</sup> Starting from a small molecule fragment that fits into a cavity next to the thiazole orange (TO) (Fig. 1) binding pocket of Mango, this fragment was covalently linked to the TO core without expanding the  $\pi$ -system, yielding ligands with subnanomolar affinity. The lead, SALAD1 (Fig. 1), delivers about 3.5-fold higher brightness than the widely used Mango II–TO1–biotin pair and markedly boosts quantum yield and turn-on. Crystal structures show why:<sup>[27]</sup> SALAD1 occupies the pocket more completely and positions a carbonyl to interact with the potassium that stabilizes the proximal G-quadruplex, constraining the fluorophore for enhanced emission. These stereo-electronic and recognition features translate into higher signal and low background in cells, enabling improved confocal imaging of RNA localization. Although Mango III–SALAD1 is even

brighter (with weaker binding), Mango II–SALAD1 combines tight affinity, high brightness, and large turn-on, illustrating how fragment linking and structural insight can systematically upgrade FLAP ligands and guide future probe design.<sup>[27]</sup>

## 2.4 biRhoBAST and biSiRA

biRhoBAST and biSiRA are engineered fluorescent light-up aptamers that overcome common FLAP limitations such as weak binding, modest turn-on, or poor photostability by pairing dimeric RNA aptamers with bivalent rhodamine dyes (Fig. 1).<sup>[28]</sup> biRhoBAST binds TMR2 to yield bright red emission, while biSiRA pairs with SiR2 for near-infrared signals; the two systems are orthogonal, enabling simultaneous tracking of distinct RNAs in live cells. The multivalent design drives exceptionally tight aptamer–dye binding (low picomolar), high fluorogenicity, and remarkable bleaching resistance, delivering far higher signal-to-background than earlier platforms.<sup>[28]</sup> A single biRhoBAST tag supports robust mRNA imaging without extensive washing, and arrays of eight repeats permit single-molecule detection and tracking with minimal impact on RNA localization, translation, or decay. Compared with prior TO-, cyanine-, or quencher-based systems, these probes combine brightness, stability, and permeability to meet single-particle demands. The strategy of dimerizing both ligand and aptamer generalizes to other rhodamines, opening paths to expanded multiplex palettes for real-time, dual-color RNA imaging and quantitative analysis of RNA lifecycles in living cells.

## 3. Covalent FLAPs

### 3.1 A New Concept

Fluorescent light-up aptamers (FLAPs) have undeniably advanced RNA imaging, yet they also carry well-recognized drawbacks, as outlined in the introduction. A universal feature of current FLAPs is that their fluorophores bind to the aptamer noncovalently. This noncovalent interaction is a major source of sensitivity issues in biological applications: ligands wash out, and fluorescence from the RNA target is lost. It can even preclude techniques widely used in protein imaging, such as fluorescence recovery after photobleaching (FRAP).<sup>[29]</sup> Because of rapid ligand exchange, a bleached dye can leave the pocket and be replaced by an unbleached molecule that immediately relights, obscuring RNA movement.

These limitations motivated us to introduce FLAP systems with covalently attached fluorophores (coFLAPs). First promising results with Pepper RNA were reported in 2025.<sup>[30]</sup> Conceptually, coFLAPs bring FLAPs closer to their original inspiration, the green fluorescent protein (GFP), in which the chromophore is covalently integrated into the protein scaffold (Fig. 2).

Importantly, rather than relying on *in vitro* selection, which is the prevailing route for developing classical FLAPs, we leverage our long-standing expertise in RNA structure analysis to pursue a structure-informed multidisciplinary approach for designing, synthesizing, and identifying reactive fluorophores to generate coFLAPs.

### 3.2 coPepper

Covalent Pepper (coPepper) is the first covalently tethered fluorescent light-up aptamer reported.<sup>[30]</sup> This system stabi-

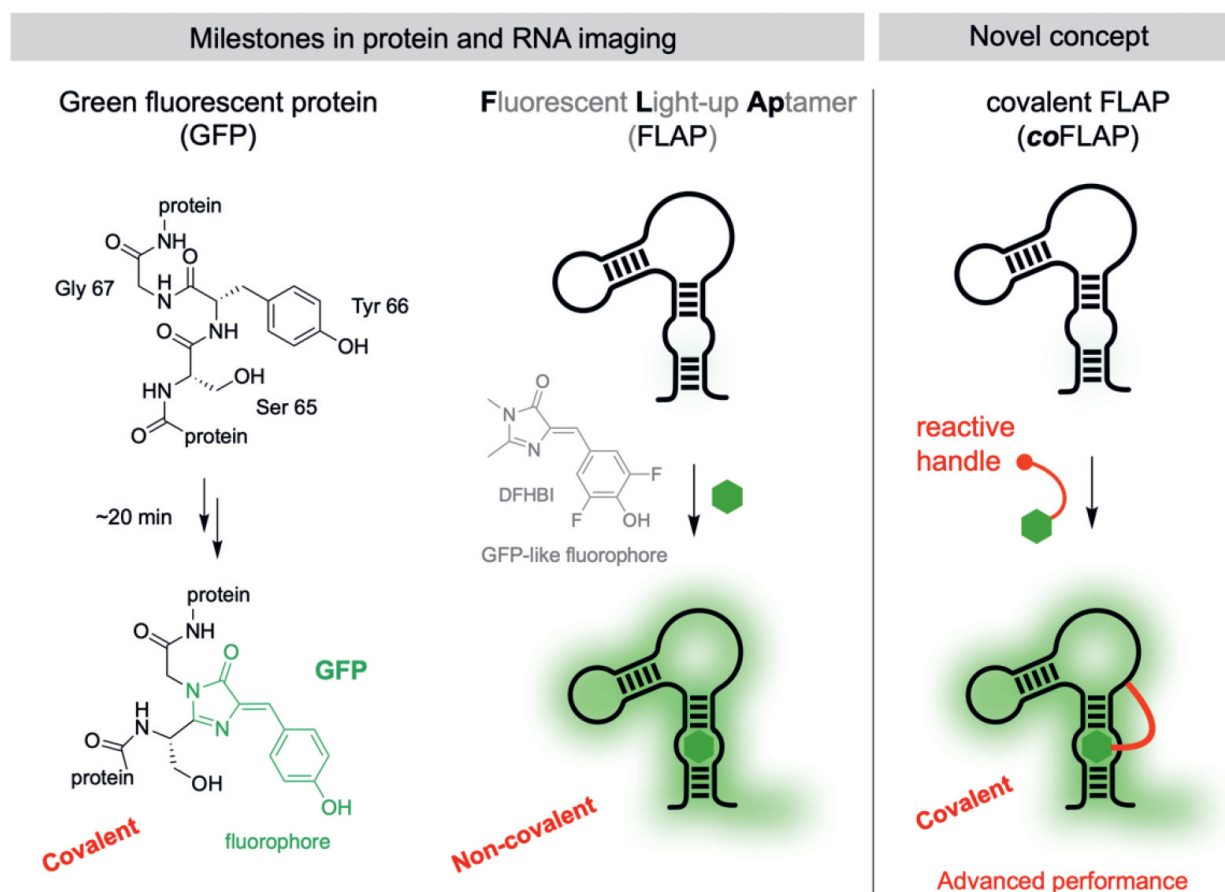


Fig. 2. coFLAPs bring FLAPs closer to their original inspiration: green fluorescent protein (GFP). In GFP, the chromophore is covalently integrated into the protein scaffold while in conventional FLAPs, it is not.

lizes Pepper–HBC complexes in cells and boosts signal retention and imaging performance. Our team synthesized a panel of HBC dyes bearing electrophilic handles and identified an N-(3-mesyloxypropyl) variant (MsOc<sub>3</sub>HBC) as the most effective for linking to Pepper, with favorable solubility for cellular use (Fig. 3).<sup>[30,31]</sup> Unlike the noncovalent ligand HBC, MsOc<sub>3</sub>HBC remained bound after washes, improving signal-to-background in live-cell microscopy. Mechanistically, short mesyloxy-alkyl substituents (or likewise bromo-alkyl substituents), placed away from the RNA recognition elements, positioned the electrophile for selective SN<sub>2</sub> reaction at an aptamer guanine (N7 atom of G41), yielding site-specific, high-yield attachment. In HEK293T cells, coPepper supported diffraction-limited confocal imaging and super-resolution methods (Airyscan, stimulated emission depletion (STED)). Furthermore, circular constructs of Pepper RNA were investigated. These constructs occasionally concentrated as nuclear speckles, where super-resolution microscopy revealed fluorescent RNA arranged at the speckle periphery and time-lapse imaging showed dynamic exchange.<sup>[30]</sup> Fluorescence correlation spectroscopy reported roughly twofold higher intensity in speckles versus cytoplasm (mean ~13 μM) and distinct mobility components: a slower fraction enriched in speckles (longer diffusion time, lower diffusion coefficient) alongside a faster, freely moving pool. Crucially, coPepper enabled FRAP readouts of RNA movement at the speckles: with noncovalent HBC, fluorescence recovered almost instantly due to ligand exchange, whereas covalently linked MsOc<sub>3</sub>HBC showed slow recovery consistent with RNA redistribution.

The covalent strategy thus broadens Pepper's utility for live-cell imaging across multiple microscopic platforms and techniques, such as confocal, STED, Airyscan, fluorescence corre-

lation spectroscopy (FCS), and FRAP. coPepper is therefore an optimal choice for use as a sensitive, wash-resistant RNA probe to study a broad range of research questions including RNA-containing bodies, phase-separation phenomena, or RNA localization dynamics.

#### 4. Implication for Selective Covalent RNA Labeling and RNA Drug Targeting

The work on covalent FLAPs directly informs selective covalent RNA labeling strategies that, in the long term, could also mature into designable modalities for covalent small-molecule RNA drugs.<sup>[32–35]</sup> While this may still seem aspirational, chemistry that tailors electrophiles to react selectively within RNA microenvironments is advancing rapidly. Our coPepper studies highlight primary alkyl halides and alkyl mesylates as underused yet practical handles for covalent tethering to aptamer targets, with the latter offering favorable solubility.<sup>[30,31]</sup>

Broader electrophile families previously applied to nucleic acids include α-halocarbonyls,<sup>[36–38]</sup> nitrogen (half-)mustards,<sup>[39,40]</sup> epoxides,<sup>[41,42]</sup> and photo-activated diazirines.<sup>[39]</sup> Recently, epoxide- and aziridine-2-carboxamides were defined that target guanines with tunable reactivity, linkage, and stereochemistry, enabling cellular modulation of expanded triplet-repeat RNA and flavin mononucleotide (FMN) riboswitches.<sup>[43]</sup> Complementarily, phenylglyoxals were shown to covalently address unpaired guanines in the FMN riboswitch pocket.<sup>[44]</sup> Further, the FMN-riboswitch inhibitor Ribocil, modified with N-acylimidazolides or photoactivated diaziridines, also reacted base- and site-selectively with the FMN RNA.<sup>[45]</sup> Finally, genetically encoded thioether macrocyclic peptide libraries generated by an mRNA display using a dibromoxylene linker and its fluorosulfonyl derivative were also shown to covalently engage RNA nucleophiles.<sup>[46]</sup>

Across these studies, specificity stems from RNA scaffold-guided pre-organization of the electrophile, the targeted nucleophile class (ribose 2'-OH vs N7 of guanine), and stereochemical control. The drug-design opportunity lies in converting modest-affinity recognition into durable occupancy.<sup>[47]</sup>

#### 5. Conclusions

In 2011, the Spinach FLAP<sup>[13]</sup> marked a milestone in cellular RNA imaging, conceptually echoing the green fluorescent protein (Nobel Prize, 2008).<sup>[48]</sup> Since then, more than ten FLAP systems have been developed across a broad spectral range.<sup>[4]</sup> While each offers distinct advantages, all share a fundamental difference compared with GFP: in FLAPs, fluorogen–RNA interactions are noncovalent, whereas GFP integrates its chromophore covalently.

One possibility to advance the performance of FLAPs is the creation of covalent FLAPs (coFLAPs). To do so, rather than relying on *in vitro* selection, a structure-informed approach is feasible, as demonstrated for the Pepper RNA system.<sup>[30]</sup> The broad applicability of the concept has yet to be shown for a broader range of FLAPs, and therefore we will put substantial efforts in engineering coFLAPs out of the most recent platforms, such as Clivia, and Okra, SALAD and biRhoBAST.

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#### Author Contributions

M. B., M. H., A. L. and R. M. cowrote this perspective.

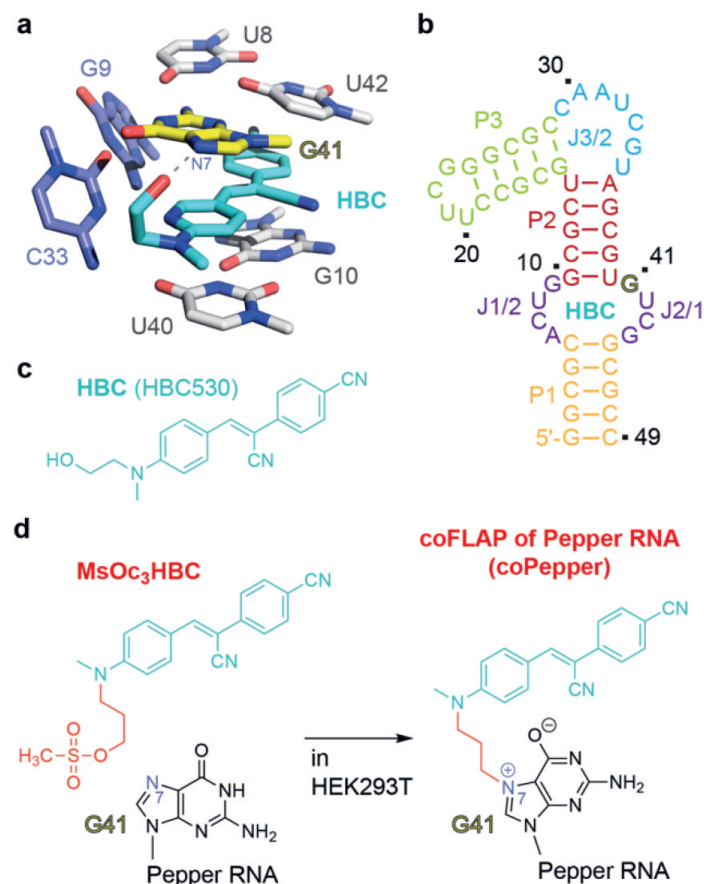


Fig. 3. The first coFLAP (coPepper). a) Architecture of Pepper with the non-covalent HBC fluorophore (PDB 7EOM).<sup>[20]</sup> b) Secondary structure of Pepper RNA. c) Chemical Structure of HBC (HBC530). d) 'Reactive' fluorophore MsOc<sub>3</sub>HBC and reaction *in vitro* and in cells.<sup>[30,31]</sup>

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