

# UC Irvine

## UC Irvine Previously Published Works

### Title

An Optimized Bioluminescent Lantern for RNA Imaging

### Permalink

<https://escholarship.org/uc/item/3gz2m40h>

### Journal

ACS Chemical Biology

### ISSN

1554-8929

### Authors

Fuller, Erin B

Chan, Christelle ET

Tran, Casey

et al.

### Publication Date

2026-09-14

### DOI

10.1021/acschembio.6c00479

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

# An Optimized Bioluminescent Lantern for RNA Imaging

Erin B. Fuller,<sup>‡</sup> Christelle E. T. Chan,<sup>‡</sup> Casey Tran, Katrina A. Green, Christoph Gohlke, Michelle A. Digman, Oswald Steward, Andrej Lupták,<sup>\*</sup> and Jennifer A. Prescher<sup>\*</sup>



Cite This: <https://doi.org/10.1021/acscchembio.6c00479>



Read Online

ACCESS |



Metrics & More

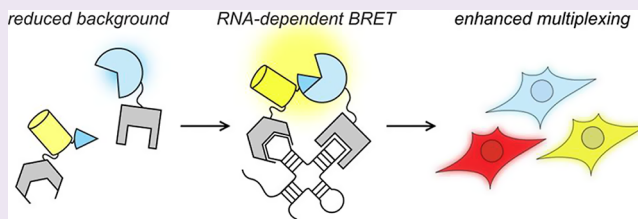


Article Recommendations



Supporting Information

**ABSTRACT:** A complete understanding of RNA dynamics requires methods to continuously image transcripts in living systems. We recently developed a panel of bioluminescent tools for multiplexed RNA detection, comprising luciferase fragments fused to RNA-binding proteins (RNA lanterns). The RNA lanterns assembled a light-emitting luciferase upon binding unique, structured RNA tags. Different colors of emission were produced via bioluminescence resonance energy transfer to fluorescent protein fusions. In this work, we systematically optimized the RNA lantern platform for enhanced performance in live cells. A luciferase-fluorescent protein linkage was identified that enabled improved energy transfer and enhanced photon outputs. The RNA lantern was further optimized for selective recognition of structured RNA tags. The new RNA lantern-tag combination is orthogonal to existing probes and enabled the first demonstration of three-color bioluminescent RNA visualization in live cells. Overall, this work expands the suite of probes for real-time, multiplexed imaging of RNA.



## INTRODUCTION

RNA imaging techniques can provide detailed views of transcript localization in live cells. For example, CRISPR-based technologies harness guide RNAs to target fluorescently labeled proteins to endogenous transcripts of interest.<sup>1,2</sup> With recently developed CRISPR methods, it is now possible to capture RNA movements on the seconds-to-minutes timescale, providing improved overall resolution.<sup>3–5</sup> Fluorogenic aptamers have also expanded the capabilities of live-cell RNA imaging. These tools typically comprise multiple copies of RNA tags that bind distinct fluorophores, resulting in turn-on signal.<sup>6</sup> Single-tag detection is also possible with recent advances in fluorescence lifetime imaging.<sup>7</sup> Additionally, several fluorogenic aptamers are available that span the visible spectrum, enabling numerous multiplexed applications.<sup>8–13</sup>

We recently reported a method for live-cell RNA tracking that builds on classic MS2 and PP7 aptamer tagging. The technique features single RNA aptamer tags that are incorporated into transcripts of interest and recruit discrete RNA binding proteins (RBPs).<sup>14–16</sup> The RBPs are fused to fragments of a split NanoLuc luciferase (NanoBiT).<sup>17–20</sup> The resulting fusions (termed RNA lanterns) are minimally emissive on their own, but form light-emitting enzymes upon binding to the aptamer tags. The method does not require excitation light, but rather a luciferin substrate (furimazine), and can be advantageous for certain applications in living systems.

We subsequently developed orthogonal RNA tags that pair MS2 with either PP7, boxB, or a CS2 aptamer.<sup>21–27</sup> All three tags feature a Ni-NTA aptamer that rigidifies the structure in

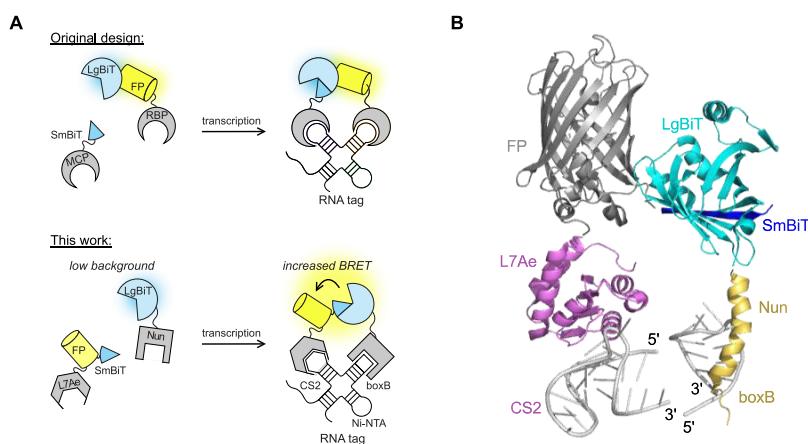
an optimal conformation for lantern assembly. The Ni-NTA aptamer further enables transcript retrieval for downstream biochemical analyses.<sup>28</sup> Multicolor detection with the orthogonal tags was possible using engineered RNA lanterns. We fused different fluorescent proteins to the larger (LgBiT) portion of NanoBiT.<sup>17,18</sup> Upon aptamer binding and luciferase assembly, photon emission from the fluorescent acceptors was observed via bioluminescence resonance energy transfer (BRET). However, the energy transfer efficiencies were suboptimal. Residual background signals were also detected in the absence of the RNA targets, likely due to the small amount of catalytic activity retained by LgBiT. These features complicated the detection of low-abundance targets.

We aimed to design new RNA lanterns with both improved dynamic range and spectral resolution (Figure 1A). Probes with lower background emission would enhance detection sensitivity. More spectrally distinct lanterns would also aid multiplexed imaging efforts. We hypothesized that improved optical outputs could be achieved by fusing the fluorescent protein to the SmBiT (instead of LgBiT) portion of NanoBiT. SmBiT does not catalyze the bioluminescent reaction, mitigating premature energy transfer to and emission from the fluorescent protein acceptor.

Received: May 16, 2026

Revised: July 30, 2026

Accepted: July 31, 2026



**Figure 1.** Bioluminescence imaging platform for improved RNA detection. (A) Schematic representation of the RNA lantern strategy. In this work, the two halves of NanoBiT (SmBiT and LgBiT) were fused to RNA binding proteins (L7Ae and Nun, respectively) that selectively bind to orthogonal RNA motifs (CS2 and boxB, respectively). A structured RNA tag comprising CS2, boxB, and Ni-NTA aptamers drives luciferase complementation and bioluminescence emission. A fluorescent protein inserted between the L7Ae and SmBiT shifts emission via bioluminescence resonance energy transfer (BRET) only upon RNA binding, resulting in brighter emission and improved BRET. (B) RNA lantern design was modeled by aligning existing structures using PyMOL, including L7Ae (purple, 1RLG), Nun (yellow, 1HJ1), a fluorescent protein (gray, SLK4), and NanoLuc (SIBO), highlighting LgBiT (cyan) and SmBiT (dark blue).

Only upon binding the target RNA would SmBiT-LgBiT complementation be achieved, providing a functional luciferase donor. BRET in this case would track with the transcript of interest, and any residual activity from uncomplemented LgBiT would be spectrally distinct from the SmBiT-LgBiT adduct. The SmBiT fusion could also potentially bring the fluorescent protein acceptor into closer proximity to the NanoBiT active site, improving the overall BRET efficiency and providing distinct colors.<sup>29,30</sup> Red-emitting lanterns are especially desirable, as they emit more tissue-penetrant light for imaging in heterogeneous biological samples.<sup>31</sup> Bright, red emission has been historically difficult to achieve, though, due to dim luciferase donors and the poor spectral overlap with red-shifted fluorescent proteins.

Here we report the development, characterization, and application of new RNA lanterns for improved RNA imaging. We prepared a panel of SmBiT-fluorescent protein fusions with varying linkers. These fusions, along with LgBiT, were appended to distinct, monomeric RNA binding proteins. NanoBiT complementation and BRET were measured upon incubation with target RNA tags. A new lantern was identified with improved BRET efficiency to multiple fluorescent proteins. Additional tuning of the probe and RNA tag provided the brightest and most sensitive bioluminescent tools in this class to date. We further optimized the expression and localization of the lantern in live mammalian cells. The new probe and tag combination were orthogonal to previously reported RNA lanterns and could be spectrally resolved. We used the new lantern, along with two existing ones, for three-color multiplexed RNA imaging, the first demonstration of its kind. Collectively, this work expands the RNA lantern toolkit and sets the stage for improved RNA imaging capabilities in living systems.

## RESULTS AND DISCUSSION

### Design of an Improved RNA Lantern

We first aimed to generate an RNA lantern with more efficient energy transfer and reduced background. We reasoned that

fusion of a fluorescent protein to the SmBiT peptide, which forms a strand of the barrel structure of NanoLuc, could enhance BRET efficiency. This arrangement could potentially increase the proximity of the luciferase active site to the fluorescent acceptor or otherwise improve dipole-dipole coupling.<sup>32,33</sup> Crude modeling of fluorescent protein fusions to both the SmBiT and LgBiT portions of NanoLuc supported the overall design (Figure S1). We further reasoned that the configuration would decrease background in the absence of RNA-dependent BRET. LgBiT itself can generate some photons in the presence of the furimazine substrate. The SmBiT piece, by contrast, emits essentially no luminescence on its own.<sup>19</sup> SmBiT-fluorescent protein fusions were thus expected to mitigate energy transfer in the absence of lantern assembly, but provide both a signal increase and color change upon RNA binding.

We also desired RNA binding proteins (RBPs) that would enable efficient luciferase complementation. Our previous work suggested that a one-to-one ratio of LgBiT and SmBiT bound to a target RNA results in optimal lantern assembly.<sup>17,18</sup> All probes generated to date, though, feature MS2 and/or PP7 coat proteins, RBPs that are known to form dimers and higher multimers with their RNA binding partners.<sup>21,22</sup> Aggregation of the lantern fragments can abrogate binding of the desired RNA tag and thus reduce bioluminescent outputs. Other RBPs, including L7Ae and Nun, bind their target RNAs as monomers.<sup>23,24,34,35</sup> L7Ae and Nun recognize the CS2 and boxB RNA motifs, respectively. The two RBPs were previously incorporated into RNA lanterns, but were used in combination with the aggregation-prone MS2 coat protein (MCP).<sup>18,23,24,34,35</sup> For this work, we hypothesized that use of both L7Ae and Nun in a single lantern would reduce background and enhance RNA-dependent bioluminescence. The desired lanterns thus comprised L7Ae and Nun RNA binding proteins, and a SmBiT-fluorescent protein fusion (Figure 1A). VenusΔC12 was initially selected as the fluorescent protein acceptor.<sup>17,18,29</sup> Simple modeling of the desired RNA lantern, generated by aligning the termini of existing protein structures in PyMOL,

indicated that the design could accommodate a structured RNA tag (Figure 1B).<sup>36</sup>

### Optimization of BRET Efficiency

Multiple Venus $\Delta$ C12-SmBiT linkages were examined to determine the optimal configuration for energy transfer. Variants were generated with a short, two amino acid linker, as well as flexible glycine-serine repeats (Figure 2A). For initial measurements, each half of the RNA lantern (L7Ae-Venus $\Delta$ C12-SmBiT or Nun-LgBiT) was expressed separately in HEK293T cell lysate. The lysates were then combined and analyzed. Bioluminescence emission spectra were recorded upon the addition of exogenous CS2-boxB structured RNA tags. Energy transfer from NanoBiT to Venus $\Delta$ C12 was observed across the panel of constructs (Figure 2B), with the two- and five-amino acid linker variants (AM and G<sub>4</sub>S) exhibiting the highest BRET efficiencies ( $\sim 0.84$  and  $\sim 0.83$ , respectively). These values were also higher than the BRET efficiency measured for the previously reported inverse design ( $\sim 0.67$ ), where the fluorescent protein was attached to LgBiT ("LgBiT fusion"). Fusion of mScarlet-I to SmBiT also resulted in efficient energy transfer in the context of the L7Ae-Nun based RNA lantern, though further optimization would likely improve performance (Figure S2). The SmBiT-fluorescent protein configuration also improved BRET for previously reported MCP-PCP and MCP-L7Ae based RNA lanterns, further validating the strategy (Figures S3–S6). Fluorescent protein linkages on both the SmBiT and LgBiT of the same MCP-Nun lantern generated even more unique spectral outputs, consistent with other recently developed bioluminescent reporters (Figure S7).<sup>37</sup>

The Venus $\Delta$ C12-SmBiT RNA lanterns were further evaluated for brightness and RNA sensitivity. We observed that the G<sub>4</sub>S-linked variant produced the brightest bioluminescence in the presence of the target RNA tag (Figure S8). While the two-amino acid linker demonstrated equally high BRET efficiency compared to the G<sub>4</sub>S linker, overall brightness was greatly reduced, potentially due to improper folding or abrogated SmBiT/LgBiT binding. In contrast, longer linker variants (GGs and G<sub>4</sub>S  $\times 3$ ) facilitated SmBiT/LgBiT complementation, but at the cost of BRET efficiency (Figure S8). We thus moved forward with the G<sub>4</sub>S-linked variant based on its overall brightness and high BRET efficiency.

### Optimization of the RNA Tag

We next aimed to tune the structure of the RNA tag to ensure efficient lantern assembly. A panel of structured tags was generated with varying numbers of nucleotides between boxB and CS2 (C-X-B, where X is the number of nucleotides between the aptamers). The RNA tags sampled approximately one half of a helical turn between boxB and CS2 (C-1-B through C-13-B) with C-11-B having a secondary structure-predicted phase angle of  $\sim 180^\circ$  between the aptamers. The varied spacers served to modulate the distance and angle between the RNA motifs, thereby sampling different configurations for lantern assembly. We used a similar method to enhance SmBiT/LgBiT complementation, and therefore bioluminescence intensity, with previous lantern designs.<sup>17,18</sup>

The structures were assayed using a mixture of HEK293T cell lysates expressing either protein component of the RNA lantern. The RNA tags were transcribed *in vitro* and added exogenously to lantern lysate before measuring photon outputs. The C-11-B tag produced the brightest bioluminescence, with a

nearly 1000-fold signal enhancement over a no-RNA control. The fold change observed was the highest recorded among the existing RNA lanterns (Figures 2C and S9). Additional biological replicates with differing levels of lantern expression resulted in varying intensity, underscoring the need for careful optimization of RNA lantern and tag expression to achieve maximal intensity. Despite the variance in signal outputs, overall RNA tag preference remained consistent across experiments (Figure S10). The high fold changes can be attributed, in part, to low background signals. The no-RNA samples produced very little bioluminescence in all cases (Figures 2C, S9 and S10). No differences were observed in luminescence emission spectra across the panel of RNA tags, suggesting that any background signal resulted from complementation in the absence of the RNA tag (Figure S9). The preference for the C-11-B tag remained constant across the panel of Venus $\Delta$ C12-SmBiT linker variants examined (Figure S11).

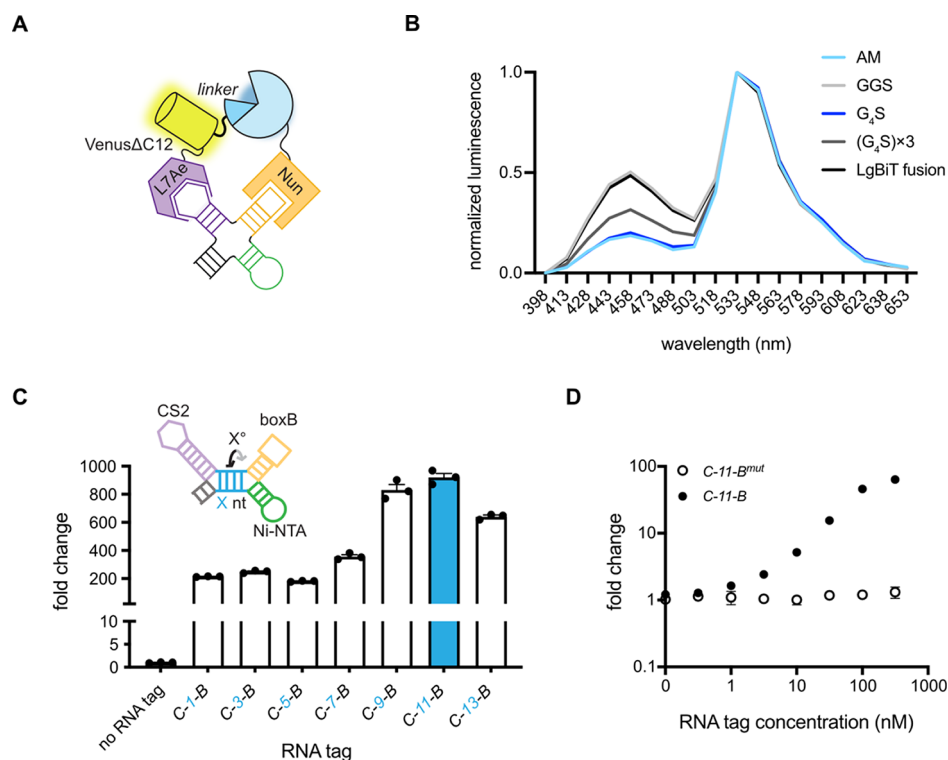
As the phase angle between CS2 and boxB increased, signal increased, peaking when the aptamers were predicted to be out of phase with one another ( $\sim 180^\circ$  phase angle). This result is in contrast to previous RNA lantern designs, where more compact tags were preferred on the whole.<sup>17,18</sup> The variance in tag preference may be explained by differences in binding position between the RBPs and the RNA motifs, potentially orienting the lantern fragments differently upon RNA target recognition. It is also likely that the large bulk of the fluorescent protein increased the preferred spacer length between the aptamers. In any case, C-11-B was chosen as the optimal RNA tag for further experiments.

The lanterns also demonstrated RNA-dependent emission. When increasing amounts of the C-11-B tag were added to cell lysates containing the lantern components, increased bioluminescence was detected (Figure 2D). We further observed that decreasing the expression of the RNA lantern, via serial dilution, resulted in peak luminescence at lower RNA tag concentrations (Figure S12). Lantern dilution further revealed an RNA-dependent hook effect, wherein peak luminescence was observed when the optimal ratio of lantern components to RNA tag was achieved. The hook effect is a feature of the three-part RNA lantern system. Large amounts of RNA result in reduced signal, as the lantern components are bound on separate transcripts and unable to form a light-emitting complex.

We further evaluated the potential for the RNA lanterns to assemble in the presence of non-target RNA tags. As a negative control, a single point mutation was introduced within the boxB motif to disrupt binding to the Nun peptide. In the presence of the mutant RNA (C-11-B<sup>mut</sup>), background levels of bioluminescence were observed at all concentrations, on par with the no-RNA controls (Figures 2D and S13). We additionally determined the L7Ae-Nun RNA lantern to be orthogonal to other existing lantern designs (MCP-PCP, MCP-Nun, and MCP-L7Ae).<sup>17,18</sup> Luminescence was measured in the presence of varying orthogonal RNA tag concentrations (0–100 nM) and no off-target binding was observed in any case (Figure S14). The L7Ae-Nun lantern is thus orthogonal to existing probe designs and can be used in tandem with any combination of the existing set of RNA lanterns.

### Optimization of the L7Ae-Nun Lantern for Live-Cell Imaging

To evaluate the RNA lantern for live cell imaging, we targeted a model fluorescent protein transcript, TagRFP657 (RFP). The



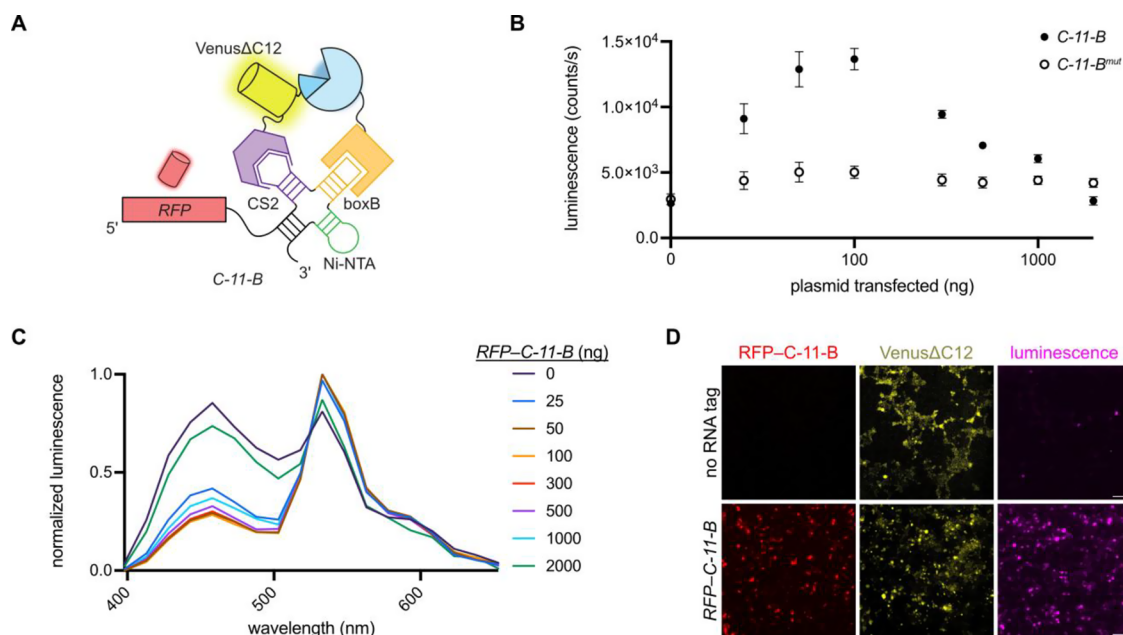
**Figure 2.** Optimization of the RNA lantern in vitro. (A) Schematic of the RNA lantern highlighting the fluorescent protein-SmBiT linker. VenusΔC12 was selected as a model fluorescent protein and varying linker lengths were assayed for fusion with SmBiT. (B) Normalized bioluminescence emission spectra of the various RNA lantern designs. The lantern halves (L7Ae-VenusΔC12-SmBiT and Nun-LgBiT) were expressed in HEK293T cell lysate and mixed together in an equal ratio. Luminescence intensity was measured in the presence of the RNA tag (1 nM) and normalized to directly compare emission spectra. Results represent the mean of  $n = 3$  technical replicates (AM, GGS, and LgBiT fusion) or  $n = 2$  biological replicates ( $G_4S$  and  $G_4S \times 3$ , each comprising 3 technical replicates). (C) RNA tag optimization. The fold change in bioluminescence intensity over a no RNA tag control was plotted for HEK293T cell lysate expressing the RNA lantern across a panel of RNA tag structures (at 1 nM). Varying spacer lengths, highlighted in blue below each bar, modulated the distance and phase angles between the CS2 and boxB aptamers. (D) Evaluation of the lantern at varying RNA tag concentrations. Each half of the lantern was expressed separately in HEK293T cell lysate and mixed together with either the target RNA tag or a mutant control (0–316 nM). Fold change was calculated over a no RNA tag control. Error bars represent the standard error of the mean for (C, D)  $n = 3$  technical replicates.

structured RNA tag, C-11-B, was fused to the 3' untranslated region (UTR) of the transcript where it would, in principle, facilitate RNA lantern assembly. Expression of the RNA tag could further be confirmed by RFP fluorescence resulting from protein translation. DNA expressing each portion of the RNA lantern was co-transfected with DNA expressing RFP-C-11-B into HEK293T cells. Samples lacking the target RNA or one portion of the lantern were included as negative controls, and bioluminescence and fluorescence imaging were performed using an ultra-cooled EMCCD camera. Bright bioluminescence was observed when both halves of the RNA lantern were expressed together with the RNA tag (Figure S15). Equally bright emission was also detected, though, in samples lacking the RNA tag, indicating that background complementation of the RNA lantern was prevalent in live cells. This result contrasted with the low background signal measured in cell lysate. We hypothesize that co-transfection of the RNA lantern fragments may have enabled more efficient complementation, because they were transcribed and translated together.

We continued to optimize the lantern expression system to decrease background and enhance dynamic range in live cell models. Different configurations of nuclear localization sequences (NLS) fused to either lantern fragment were

evaluated (Figure S16). We anticipated that spatial separation of the SmBiT and LgBiT fragments, in either the nucleus or cytoplasm, would prevent background complementation in the absence of the RNA tag. We, and others, have previously implemented this strategy to reduce background signal in split-reporter assays.<sup>16–18</sup> Transcription of a target RNA and subsequent transport from the nucleus to the cytoplasm would therefore be expected to result in lantern assembly. RNA lantern configurations tagged with either one or multiple NLS copies were co-transfected with or without DNA encoding RFP-C-11-B. Constructs were evaluated for luminescence turn on in bulk-cell populations and via bioluminescence imaging. Spatial separation of the lantern components greatly reduced background and enhanced the dynamic range (Figures S16 and S17). The largest signal enhancement was achieved with a single NLS fused to the SmBiT portion of the lantern, which was implemented in further experiments.

We also generated a plasmid for bicistronic expression of both RNA lantern fragments. The design featured the NLS-fused SmBiT fragment and the LgBiT fragment following an internal ribosomal entry sequence (IRES). When expressed in live HEK293T cells, the bicistronic plasmid resulted in slightly reduced overall signal, but a larger dynamic range, likely due



**Figure 3.** Detection of a model RNA transcript in live mammalian cells. (A) Schematic representation of mRNA encoding a TagRFP6S7 (RFP) fluorescent protein with a structured RNA tag inserted into the 3' untranslated region (*RFP-C-11-B*). Transcription of *RFP-C-11-B* is expected to drive lantern complementation and result in bioluminescence emission with high BRET efficiency. (B) HEK293T cells stably expressing the L7Ae-Nun RNA lantern were transfected with varying amounts of DNA encoding *RFP-C-11-B* or *RFP-C-11-B<sup>mut</sup>* (0–2000 ng). Total bioluminescence intensity was measured and plotted. Error bars represent the standard error of the mean for  $n = 9$  total replicates (3 biological replicates, each comprising 3 technical replicates). (C) Bioluminescence emission spectra were also measured and normalized to the maximum intensity to compare relative BRET efficiencies. Normalized values represent the mean of  $n = 9$  total replicates (3 biological replicates, each comprising 3 technical replicates). (D) HEK293T cells stably expressing the RNA lantern were transfected with DNA expressing *RFP-C-11-B* or not transfected (no RNA tag). Cells were imaged using an ultra-cooled EMCCD camera. Fluorescence from the RFP (red) indicated expression of the RNA tag and fluorescence from the VenusΔC12 (yellow) indicated lantern expression. Scale bars = 100  $\mu\text{m}$ .

to attenuated expression of the LgBiT portion of the lantern (Figure S18).<sup>38</sup> Variable light outputs were also observed across biological replicates due to differing RNA lantern expression levels. Nonetheless, an RNA-dependent increase in signal was observed. Based on this result, we also evaluated a PEST tag for degradation of excess LgBiT proteins and observed similar bioluminescence turn-on in the presence of the *C-11-B* tag compared to the bicistronic plasmid (Figure S18).<sup>39</sup> We therefore chose to move forward with the bicistronic plasmid as the optimized construct for RNA imaging in live cells for maximum dynamic range and ease of expression.

The lantern was further evaluated for live-cell RNA detection in bulk populations. HEK293T cell lines stably expressing the probe were generated and transfected with varying amounts of DNA encoding *RFP-C-11-B* (Figure 3A). Increased luminescence was observed with increasing amounts of transfected DNA, with maximum signal ( $\sim 4.8$  fold increase over background) achieved using 100 ng of vector (Figure 3B). The signal output detected in live cells, though consistent, was notably reduced compared to *in vitro* measurements. Further increases in the concentration of the RNA target resulted in reduced intensity, potentially explained by the RNA-dependent hook effect. It is also possible that transfection of high amounts of plasmid resulted in compromised cell viability and less efficient RNA expression.<sup>40</sup> Additionally, RNA tags with a point mutation in the boxB motif (*C-11-B<sup>mut</sup>*) produced abrogated signal in comparison to the target sequence (Figure 3B).

We also observed a color change that correlated with overall signal intensity (Figures 3C and S18). In the absence of the RNA tag, the bioluminescence emission spectrum indicated more blue photons. More yellow photons were observed with increasing RNA expression, indicating BRET from NanoLuc to the VenusΔC12 fluorescent protein (Figures 3C and S19). At the highest levels of RNA produced, though, a larger percentage of blue photons was observed again as lantern assembly was disrupted. The RNA-dependent shift in emission validated the hypothesis that a SmBiT-fused fluorescent protein would yield a color change upon tag binding. Although more blue emission was observed in the absence of RNA, some yellow signal was still evident, indicating background RNA lantern complementation. BRET was also observed in the presence of the mutant RNA tag (Figure S19). The spectral shift was only observed in live cells, though, and not detected when the lanterns were evaluated in cell lysate. This result can be explained by spatial separation of the lantern components in live cells. In lysate, by contrast, the lantern fragments are not spatially separated and therefore have a higher chance of complementation in the absence of the RNA tag.

The optimized lantern construct was next used for RNA imaging on a single-cell level. HEK293T cells stably expressing the lantern were transiently transfected with DNA encoding *RFP-C-11-B*. Cells were treated with furimazine and imaged using an ultra-cooled EMCCD camera. RNA tag expression was confirmed by RFP fluorescence and bright bioluminescence

was widely detected throughout the cells (Figure 3D). In comparison, cells that did not express the RNA tag produced minimal luminescence. Background signal that was detected corresponded to cells with high lantern expression (indicated by Venus $\Delta$ C12 fluorescence, Figure 3D). Reduced signal outputs on a single-cell basis were observed when using a point mutant RNA tag as well as a mutant with a scrambled boxB aptamer sequence (*C-11-B<sup>mut</sup>* and *C-11-B<sup>scramble</sup>*, respectively, Figure S20). The RNA lantern was also used to image a hybrid *mCherry- $\beta$ -actin 3'UTR* transcript. The signal observed matched the expected cytoplasmic localization for the mRNA of interest (Figure S21).<sup>41</sup> Overall, the optimized RNA lantern enabled imaging of multiple target transcripts in live mammalian cells.

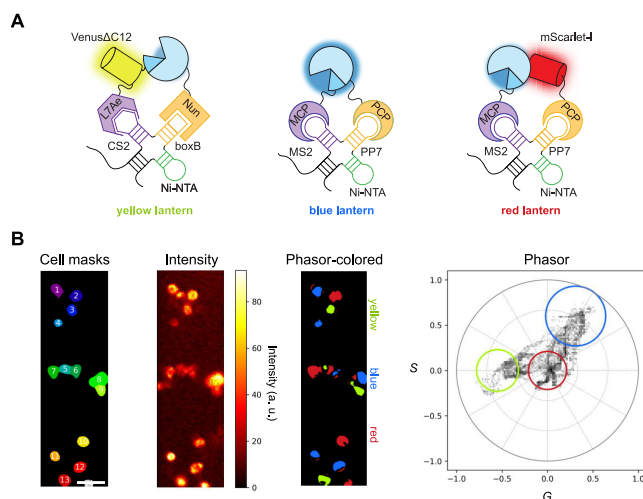
### Three-Colored RNA Imaging Using Bioluminescent Phasor Analysis

We next turned to multiplexed imaging of the L7Ae-Nun lantern with existing multicolored RNA lanterns. Multiplexed bioluminescence is challenging using traditional microscopy setups, due to broad and overlapping emission spectra that are characteristic of bioluminescent reporters. To address this limitation, we recently developed bioluminescent phasor analysis.<sup>42,43</sup> This technique involves simultaneously imaging through sine- and cosine-shaped filters referenced to an unfiltered channel. An optical Fourier transform is then performed to calculate spectral phasors at the level of individual pixels. Bioluminescent phasor analysis was previously employed for two-color imaging using the RNA lantern platform.<sup>18</sup>

We anticipated that the enhanced BRET efficiency of the newly optimized RNA lantern would provide improved spectral resolution and more facile multiplexing, enabling three-color imaging. Toward this end, three RNA lanterns were selected: the blue and red MCP-PCP RNA lanterns, and the yellow L7Ae-Nun lantern (Figure 4A). HEK293T cells stably expressing each RNA lantern were generated and co-cultured. The cells were transiently transfected with DNA encoding model mRNAs with the corresponding tags inserted into the 3' UTR (*RFP-M-3-P* and *RFP-C-11-B*). Bioluminescence imaging was performed using a custom optical setup, and the emission data were converted to spectral phasors (Figure 4B). Luminescence was false-colored at each pixel in the mixed cell images, using the phasor coordinates determined for the individual RNA lanterns (defined by the cursor regions shown in Figures 4B and S22–S25). As shown in Figure 4B, cells expressing each lantern were clearly resolved, demonstrating the utility of the platform for three-color imaging. Co-cultured cells expressing multiple RNA lanterns also demonstrated target-specific luminescence when transfected with orthogonally tagged RNAs, further indicating utility of the lanterns for live-cell multiplexing applications (Figures S26 and S27). We note that though three-color imaging was achieved, only two distinct RNA species were illuminated. Multiplexed detection was also only achieved by expressing the components in separate cells. Imaging three orthogonal RNAs in the same cell is possible, although technically challenging based on the larger number of components that must be introduced and the potential for more overlapping signals.

### CONCLUSIONS

Visualizing RNA dynamics has been complicated by a lack of tools for long-term tracking in physiological environments.



**Figure 4.** Three-colored RNA imaging using bioluminescent phasor analysis. (A) Schematic of the three RNA lanterns used for multiplexed imaging. The lanterns (yellow L7Ae-Nun lantern or blue/red MCP-PCP lanterns) were stably expressed in separate HEK293T cells. The cells were cocultured and transfected with DNA encoding a model transcript tagged in the 3'UTR (*RFP-C-11-B* and *RFP-M-3-P*). (B) Cells were imaged using a custom-built optical setup for bioluminescent phasor analysis. Cell masks (left) were assigned for single-cell analysis. Total intensity was measured in arbitrary units (a. u.), and cells were false-colored (based on their phasor coordinates) according to the multicolored cursors on the phasor plot. Scale bar = 100  $\mu$ m.

Conventional fluorescence-based methods require delivery of excitation light, which can limit applications in living systems. Bioluminescence does not require external illumination and is well-suited for continuous imaging in live cells and organisms. In previous work, we developed bioluminescent RNA imaging tools which harness split-luciferase fragments fused to RBPs (RNA lanterns). The lanterns bind corresponding RNA aptamer tags, resulting in luciferase complementation and bioluminescence emission. Previously developed tools also employed fluorescent protein fusions to generate multicolored emission using BRET. The dynamic range and efficiency of energy transfer, though, were limited with existing tools.

In this work, we developed an optimized lantern for enhanced RNA detection. The key change in design involved a fluorescent protein-SmBiT fusion strategy, which improved BRET efficiency and resulted in a color change upon RNA binding. More red-shifted emission resulted in greater spectral resolution from existing probes, and is anticipated to enhance utility for in vivo applications. The SmBiT fusion approach also improved BRET efficiency of existing RNA lanterns, further validating the strategy. The new RNA lantern features a unique combination of RBPs (L7Ae and Nun), resulting in a fourth orthogonal tool that can be used in tandem with any combination of existing lanterns. The L7Ae-Nun probe and corresponding tag structure were systematically optimized for sensitive RNA detection in mammalian cell lysate and in live cells, resulting in the brightest RNA-dependent signal achieved thus far.

The enhanced probe design was applied to three-color RNA imaging in tandem with existing lanterns. For the first time, blue, yellow, and red RNA lanterns were readily resolved using bioluminescent phasor analysis. We anticipate that

the improved lantern strategy can be applied to any number of fluorescent protein fusions and RBP/aptamer pairs, further enhancing multiplexing capacity and utility of the probes. Overall, this work expands the capacity of the RNA lanterns for multiplexed imaging and will facilitate RNA tracking at all scales.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.6c00479>.

Experimental details, materials and methods, additional probe characterization and optimization, raw imaging data, additional microscopy data, and sequencing information (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

**Andrej Lupták** – Department of Chemistry, University of California Irvine, Irvine, California 92697, United States; Department of Pharmaceutical Sciences, University of California Irvine, Irvine, California 92697, United States; Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, California 92697, United States; [orcid.org/0000-0002-0632-5442](https://orcid.org/0000-0002-0632-5442); Email: [aluptak@uci.edu](mailto:aluptak@uci.edu)

**Jennifer A. Prescher** – Department of Chemistry, University of California Irvine, Irvine, California 92697, United States; Department of Pharmaceutical Sciences, University of California Irvine, Irvine, California 92697, United States; Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, California 92697, United States; [orcid.org/0000-0002-9250-4702](https://orcid.org/0000-0002-9250-4702); Email: [jpresche@uci.edu](mailto:jpresche@uci.edu)

### Authors

**Erin B. Fuller** – Department of Chemistry, University of California Irvine, Irvine, California 92697, United States; [orcid.org/0009-0006-8711-5960](https://orcid.org/0009-0006-8711-5960)

**Christelle E. T. Chan** – Department of Pharmaceutical Sciences, University of California Irvine, Irvine, California 92697, United States

**Casey Tran** – Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, California 92697, United States

**Katrina A. Green** – Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, California 92697, United States

**Christoph Gohlke** – Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California Irvine, Irvine, California 92697, United States; [orcid.org/0000-0001-8108-7707](https://orcid.org/0000-0001-8108-7707)

**Michelle A. Digman** – Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California Irvine, Irvine, California 92697, United States; Beckman Laser Institute and Medical Clinic, University of California Irvine, Irvine, California 92697, United States; [orcid.org/0000-0003-4611-7100](https://orcid.org/0000-0003-4611-7100)

**Oswald Steward** – Department of Anatomy and Neurobiology, University of California Irvine, Irvine, California 92697, United States

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acscchembio.6c00479>

### Author Contributions

<sup>‡</sup>E.B.F. and C.E.T.C. contributed equally.

### Funding

This study was funded by the National Institutes of Health (R01GM161051 to J.A.P. and A.L.).

### Notes

The authors declare the following competing financial interest(s): J. Prescher is an Associate Editor for the journal.

## ■ ACKNOWLEDGMENTS

Some cartoon images were created with [BioRender.com](https://www.biorender.com). The authors also thank members of the Prescher, Lupták, Steward, and Digman laboratories for insightful discussions.

## ■ REFERENCES

- (1) Nelles, D. A.; Fang, M. Y.; O'Connell, M. R.; Xu, J. L.; Markmiller, S. J.; Doudna, J. A.; Yeo, G. W. Programmable RNA Tracking in Live Cells with CRISPR/Cas9. *Cell* **2016**, *165* (2), 488–496.
- (2) Abudayyeh, O. O.; Gootenberg, J. S.; Essletzbichler, P.; Han, S.; Joung, J.; Belanto, J. J.; Verdine, V.; Cox, D. B. T.; Kellner, M. J.; Regev, A.; Lander, E. S.; Voytas, D. F.; Ting, A. Y.; Zhang, F. RNA Targeting with CRISPR–Cas13. *Nature* **2017**, *550* (7675), 280–284.
- (3) Yang, L.-Z.; Wang, Y.; Li, S.-Q.; Yao, R.-W.; Luan, P.-F.; Wu, H.; Carmichael, G. G.; Chen, L.-L. Dynamic Imaging of RNA in Living Cells by CRISPR–Cas13 Systems. *Mol. Cell* **2019**, *76* (6), 981–997.e7.
- (4) Xia, C.; Colognori, D.; Jiang, X. S.; Xu, K.; Doudna, J. A. Single-Molecule Live-Cell RNA Imaging with CRISPR–Csm. *Nat. Biotechnol.* **2025**, *43* (12), 2023–2030.
- (5) Mitkas, A. A.; Valverde, M.; Chen, W. Dynamic Modulation of Enzyme Activity by Synthetic CRISPR–Cas6 Endonucleases. *Nat. Chem. Biol.* **2022**, *18* (5), 492–500.
- (6) Paige, J. S.; Wu, K. Y.; Jaffrey, S. R. RNA Mimics of Green Fluorescent Protein. *Science* **2011**, *333* (6042), 642–646.
- (7) Sarfraz, N.; Moscoso, E.; Oertel, T.; Lee, H. J.; Ranjit, S.; Braselmann, E. Visualizing Orthogonal RNAs Simultaneously in Live Mammalian Cells by Fluorescence Lifetime Imaging Microscopy (FLIM). *Nat. Commun.* **2023**, *14* (1), 867.
- (8) Kong, K. Y. S.; Jeng, S. C. Y.; Rayyan, B.; Unrau, P. J. RNA Peach and Mango: Orthogonal Two-Color Fluorogenic Aptamers Distinguish Nearly Identical Ligands. *RNA* **2021**, *27* (5), 604–615.
- (9) Cawte, A. D.; Unrau, P. J.; Rueda, D. S. Live Cell Imaging of Single RNA Molecules with Fluorogenic Mango II Arrays. *Nat. Commun.* **2020**, *11* (1), 1283.
- (10) Wu, J.; Zaccara, S.; Khuperkar, D.; Kim, H.; Tanenbaum, M. E.; Jaffrey, S. R. Live Imaging of mRNA Using RNA-Stabilized Fluorogenic Proteins. *Nat. Methods* **2019**, *16* (9), 862–865.
- (11) Jiang, L.; Xie, X.; Su, N.; Zhang, D.; Chen, X.; Xu, X.; Zhang, B.; Huang, K.; Yu, J.; Fang, M.; Bao, B.; Zuo, F.; Yang, L.; Zhang, R.; Li, H.; Huang, X.; Chen, Z.; Zeng, Q.; Liu, R.; Lin, Q.; Zhao, Y.; Ren, A.; Zhu, L.; Yang, Y. Large Stokes Shift Fluorescent RNAs for Dual-Emission Fluorescence and Bioluminescence Imaging in Live Cells. *Nat. Methods* **2023**, *20* (10), 1563–1572.
- (12) Zuo, F.; Jiang, L.; Su, N.; Zhang, Y.; Bao, B.; Wang, L.; Shi, Y.; Yang, H.; Huang, X.; Li, R.; Zeng, Q.; Chen, Z.; Lin, Q.; Zhuang, Y.; Zhao, Y.; Chen, X.; Zhu, L.; Yang, Y. Imaging the Dynamics of

Messenger RNA with a Bright and Stable Green Fluorescent RNA. *Nat. Chem. Biol.* **2024**, *20* (10), 1272–1281.

(13) Mi, L.; Yu, Q.; Karunanayake Mudiyanse, A. P. K. K.; Wu, R.; Sun, Z.; Zheng, R.; Ren, K.; You, M. Genetically Encoded RNA-Based Bioluminescence Resonance Energy Transfer (BRET) Sensors. *ACS Sens.* **2023**, *8* (1), 308–316.

(14) Bertrand, E.; Chartrand, P.; Schaefer, M.; Shenoy, S. M.; Singer, R. H.; Long, R. M. Localization of ASH1 mRNA Particles in Living Yeast. *Mol. Cell* **1998**, *2* (4), 437–445.

(15) Brodsky, A. S.; Silver, P. A. Pre-mRNA Processing Factors Are Required for Nuclear Export. *RNA* **2000**, *6* (12), 1737–1749.

(16) Wu, B.; Chen, J.; Singer, R. H. Background Free Imaging of Single mRNAs in Live Cells Using Split Fluorescent Proteins. *Sci. Rep.* **2015**, *4* (1), 3615.

(17) Halbers, L. P.; Cole, K. H.; Ng, K. K.; Fuller, E. B.; Chan, C. E. T.; Callicote, C.; Metcalfe, M.; Chen, C. C.; Barhoosh, A. A.; Reid-McLaughlin, E.; Kent, A. D.; Torrey, Z. R.; Steward, O.; Lupták, A.; Prescher, J. A. A Modular Platform for Bioluminescent RNA Tracking. *Nat. Commun.* **2024**, *15* (1), 9992.

(18) Fuller, E. B.; Cole, K. H.; Halbers, L. P.; Chan, C. E. T.; Ng, K. K.; Scipioni, L.; Gohlke, C.; Digman, M. A.; Steward, O.; Prescher, J. A.; Lupták, A. Bioluminescent Probes for Multiplexed RNA Imaging. *J. Am. Chem. Soc.* **2026**, *146* (11), 11521–11530.

(19) Dixon, A. S.; Schwinn, M. K.; Hall, M. P.; Zimmerman, K.; Otto, P.; Lubben, T. H.; Butler, B. L.; Binkowski, B. F.; Machleidt, T.; Kirkland, T. A.; Wood, M. G.; Eggers, C. T.; Encell, L. P.; Wood, K. V. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.* **2016**, *11* (2), 400–408.

(20) Eguchi, M.; Yoshimura, H.; Ueda, Y.; Ozawa, T. Split Luciferase-Fragment Reconstitution for Unveiling RNA Localization and Dynamics in Live Cells. *ACS Sens.* **2023**, *8* (11), 4055–4063.

(21) Bernardi, A.; Spahr, P.-F. Nucleotide Sequence at the Binding Site for Coat Protein on RNA of Bacteriophage R17. *Proc. Natl. Acad. Sci. U. S. A.* **1972**, *69* (10), 3033–3037.

(22) Lim, F.; Downey, T. P.; Peabody, D. S. Translational Repression and Specific RNA Binding by the Coat Protein of the *Pseudomonas* Phage PP7. *J. Biol. Chem.* **2001**, *276* (25), 22507–22513.

(23) Faber, C.; Schärpf, M.; Becker, T.; Sticht, H.; Rösch, P. The Structure of the Coliphage HK022 Nun Protein- $\lambda$ -Phage *boxB* RNA Complex. *J. Biol. Chem.* **2001**, *276* (34), 32064–32070.

(24) Chattopadhyay, S.; Hung, S. C.; Stuart, A. C.; Palmer, A. G.; Garcia-Mena, J.; Das, A.; Gottesman, M. E. Interaction between the Phage HK022 Nun Protein and the *Nut* RNA of Phage Lambda. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92* (26), 12131–12135.

(25) Kuhn, J. F. Archaeal Ribosomal Protein L7 Is a Functional Homolog of the Eukaryotic 15.SkD/Snu13p snoRNP Core Protein. *Nucleic Acids Res.* **2002**, *30* (4), 931–941.

(26) Omer, A. D.; Ziesche, S.; Ebhardt, H.; Dennis, P. P. *In Vitro* Reconstitution and Activity of a C/D Box Methylation Guide Ribonucleoprotein Complex. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (8), 5289–5294.

(27) Fukunaga, K.; Yokobayashi, Y. Directed Evolution of Orthogonal RNA–RBP Pairs through Library-vs-Library *In Vitro* Selection. *Nucleic Acids Res.* **2022**, *50* (2), 601–616.

(28) Chizzolini, F.; Passalacqua, L. F. M.; Oumais, M.; Dingilian, A. I.; Szostak, J. W.; Lupták, A. Large Phenotypic Enhancement of Structured Random RNA Pools. *J. Am. Chem. Soc.* **2020**, *142* (4), 1941–1951.

(29) Suzuki, K.; Kimura, T.; Shinoda, H.; Bai, G.; Daniels, M. J.; Arai, Y.; Nakano, M.; Nagai, T. Five Colour Variants of Bright Luminescent Protein for Real-Time Multicolour Bioimaging. *Nat. Commun.* **2016**, *7* (1), 13718.

(30) Yeh, H.-W.; Xiong, Y.; Wu, T.; Chen, M.; Ji, A.; Li, X.; Ai, H. ATP-Independent Bioluminescent Reporter Variants To Improve *In Vivo* Imaging. *ACS Chem. Biol.* **2019**, *14* (5), 959–965.

(31) Weissleder, R.; Ntziachristos, V. Shedding Light onto Live Molecular Targets. *Nat. Med.* **2003**, *9* (1), 123–128.

(32) Russo, F.; Civili, B.; Winssinger, N. Bright Red Bioluminescence from Semisynthetic NanoLuc (sNLuc). *ACS Chem. Biol.* **2024**, *19* (5), 1035–1039.

(33) Tran, S.; Usavage, K.; Halbers, L. P.; Choplick, C.; Helton, G.; Young, B.; Prescher, J. A.; Rathbun, C. M. Semisynthetic, Multicolor Probes for Bioluminescence Imaging. *ACS Omega* **2025**, *10* (49), 60416–60421.

(34) Moore, T.; Zhang, Y.; Fenley, M. O.; Li, H. Molecular Basis of Box C/D RNA-Protein Interactions. *Structure* **2004**, *12* (5), 807–818.

(35) Huang, L.; Lilley, D. M. J. The Molecular Recognition of Kink-Turn Structure by the L7Ae Class of Proteins. *RNA* **2013**, *19* (12), 1703–1710.

(36) The PyMOL Molecular Graphics System Version 3.0 Schrödinger, LLC.

(37) Hattori, M.; Wazawa, T.; Orioka, M.; Hiruta, Y.; Nagai, T. Creating Coveted Bioluminescence Colors for Simultaneous Multicolor Bioimaging. *Sci. Adv.* **2025**, *11* (4), eadp4750.

(38) Mizuguchi, H.; Xu, Z.; Ishii-Watabe, A.; Uchida, E.; Hayakawa, T. IRES-Dependent Second Gene Expression Is Significantly Lower Than Cap-Dependent First Gene Expression in a Bicistronic Vector. *Mol. Ther.* **2000**, *1* (4), 376–382.

(39) Rogers, S.; Wells, R.; Rechsteiner, M. Amino Acid Sequences Common to Rapidly Degraded Proteins: The PEST Hypothesis. *Science* **1986**, *234* (4774), 364–368.

(40) Gibson, T. J.; Seiler, M.; Veitia, R. A. The Transience of Transient Overexpression. *Nat. Methods* **2013**, *10* (8), 715–721.

(41) Li, X.; Kim, H.; Litke, J. L.; Wu, J.; Jaffrey, S. R. Fluorophore-Promoted RNA Folding and Photostability Enables Imaging of Single Broccoli-Tagged mRNAs in Live Mammalian Cells. *Angew. Chem., Int. Ed.* **2020**, *59* (11), 4511–4518.

(42) Yao, Z.; Brennan, C. K.; Scipioni, L.; Chen, H.; Ng, K. K.; Tedeschi, G.; Parag-Sharma, K.; Amelio, A. L.; Gratton, E.; Digman, M. A.; Prescher, J. A. Multiplexed Bioluminescence Microscopy via Phasor Analysis. *Nat. Methods* **2022**, *19* (7), 893–898.

(43) Scipioni, L.; Torrado, B.; Tedeschi, G.; Halbers, L.; Torrey, Z. R.; Fuller, E. B.; Fersini, F.; Gohlke, C.; Luptak, A.; Prescher, J. A.; Digman, M. A. An Open, Integrated Platform for Multiplexed Bioluminescence Microscopy. *Chem. Biomed. Imaging* **2025**.