**PROJECT NARRATIVE**

Long noncoding RNA lie at the center of many cellular processes, yet our ability to visualize this biomolecule as it moves throughout the cell is limited. Our group is developing a new imaging tool to track individual RNA molecules within living cells. This technique will enable visualization of molecules that have never been imaged before, yet are implicated in a wide variety of disease states.

## SPECIFIC AIMS

Genetically-encoded probes have revolutionized our understanding of cellular processes. Fluorescent proteins are a powerful technology that enable tracking of protein concentration and localization over large timescales and with single-molecule resolution. Via simple genetic manipulation, virtually any protein of interest can be labeled and tracked in the cell. However, analogous techniques for study of other biomolecules through genetic encoding (e.g. RNA) are underdeveloped. There is currently no approach suitable for tracking the diverse types of RNAs found in mammalian cells.

The importance of RNA in controlling a wide range of cellular functions has only recently been realized [1]. Signaling by messenger and noncoding RNAs in the cell is highly dependent on the location of the transcripts. It is understood that cellular machinery modulates the location of these molecules, but these processes remain difficult to elucidate [2]. This lack of understanding is due in part to the lack of tools available to image this biomolecule. Localization of RNA on a single-molecule level, and multicomponent imaging of RNA transcripts remains difficult. Existing tools utilize aptamers that are unstable in mammalian cells [3], or constructs that are too large for imaging most transcripts. Multicomponent RNA imaging is also difficult due to the design of current tools.

To address this need, *I aim to develop a platform for RNA imaging that will enable facile tracking of multiple transcripts at high resolution*. Riboglow is an RNA imaging platform recently developed in the Palmer lab. It utilizes a fluorescence-quenched pair formed by cobalamin (vitamin $B_{12}$) and a pendant fluorophore. In solution, this construct shows low fluorescence. When bound to the cobalamin riboswitch aptamer domain [4], there is an increase in fluorescence. This tool shows promise for RNA imaging because it solves many of the problems faced by traditional RNA probes. The platform is similar to Spinach [5], Broccoli [6], and Mango [7, 8] in that it utilizes a small molecule-binding aptamer for localization, but improves on these tools because it utilizes a natural aptamer that is more stable in the cellular environment (because of its native fold). The current gold-standard for imaging is the MS2-FP system [9], which utilizes an stem-loop-binding bacteriophage coat protein fused to a fluorescent protein. Though this technique benefits from the modularity of fluorescent proteins, it requires large constructs to concentrate the fluorescent signal (24 stem-loops are often placed in series), thus precluding its use with most RNAs.

Riboglow is already a useful tool for RNA imaging, but several drawbacks are keeping it from widespread utility. The proposed work addresses these drawbacks, and seeks to utilize improved Riboglow tools to study outstanding questions in the field of noncoding RNA. The *objective* of the work is to image noncoding RNAs as they function within living cells. My *central hypothesis* is that the Riboglow platform can be improved through chemical modification and RNA selection to provide brighter, multicomponent probes.

**Aim 1. Synthesize improved Riboglow probes.**
Previously developed Riboglow constructs suffered from poor signal induction and low brightness. I aim to derivatize the native cobalamin structure to produce new probe scaffolds. The linker and fluorophore will also be varied with the goal to maximize fluorescence turn-on. These new molecules will be evaluated for quenching efficiency and will be tested with native cobalamin aptamers.

**Aim 2. Adapt Riboglow for single-molecule imaging.**
The molecules developed in *Aim 1* will be screened against libraries of aptamers to further improve probe properties. Screening will be carried out in mammalian cells via flow cytometry. Candidate probes will be verified through imaging of mRNA transcripts in living cells.

**Aim 3. Develop mutually orthogonal Riboglow probes for multicomponent imaging.**
In tandem with brightness optimization, I will develop mutually orthogonal probes to enable labeling of different RNA transcripts in the same cell. The power of SELEX to find selective and tight binders will be used to screen for mutually exclusive aptamer-cobalamin pairs. These pairs will be conjugated to spectrally-resolved fluorophores to enable tracking of multiple RNA simultaneously. These orthogonal probes will be used to image long noncoding RNA and mRNA as they interact in the cell.

## SIGNIFICANCE AND INNOVATION

RNA lies at the center of cellular function. Its most appreciated role is to carry protein blueprints to the ribosome for manufacture. Only recently have researchers begun to appreciate its myriad of other functions, many of which also lie at the center of important cellular processes and are implicated in a variety of disease states [1, 10, 11]. Long noncoding RNA (lncRNA) comprise one such important class of RNA that does not participate in the central dogma [12]. Alarmingly, the human genome encodes for as many lncRNA as proteins [12, 13]. These transcripts are typically greater than 200 bases, and participate in binding both proteins and nucleic acids, often both at the same time [11, 12]. In a striking example, *SNGH5* is a long noncoding RNA (lncRNA) of largely unknown function, that that has been linked to cancer cell survival and metastasis in a diversity of different cancers from leukemias to melanoma [14–21]. This 2 kilobase transcript localizes to the cytoplasm and is upregulated in these disease states. *SNGH5* has been implicated to interact with a variety of mRNAs to control expression, though little else is known about its role in normal cell function. Where and when does *SNGH5*, and other similar lncRNA, interact with their mRNA targets? How long do these interactions occur, and what other cellular machinery is present? Despite such burning questions in the field of lncRNA, a tool to directly image these species and their interacting partners in living cells has yet to be reported. *Herein, I propose a robust imaging tool for multicomponent, single-molecule visualization of RNA dynamics in living cells.*

## BACKGROUND

Protein studies benefit from a large imaging toolkit that has been developed over the last 25 years. Genetically encodable fluorescent proteins are now ubiquitous for the study of the localization of any translated target in the cell. While fluorescent proteins are a mature, well-understood technology, tools for imaging the localization of individual RNA transcripts in living cells remain limited. The most popular systems to date include dye-binding aptamers (Spinach [5], Broccoli [6], and Mango [7, 8]), and RNA-binding protein fusions (MS2-FPs) [9, 22].

The most analogous to fluoresecent proteins, dye-binding aptamers utilize exogenously administered dyes that give fluorescence induction upon binding their RNA partner [5–8]. This sequence is encoded downstream of an RNA of interest to track its location in the cell. Though excellent binders for their dyes, the aptamers utilized by this technology are unstable in mammalian cells due to their nonnative structure [3, 23–25]. Additionally, though fluorescence turn-on is excellent *in vitro*, often reaching 1,000 fold, signal induction in cells is typically two to four fold, most likely due to nonspecific binding of the dye, making them poor probes for single-molecule tracking [7].

RNA-binding protein fusions are a much more robust technique for imaging RNA transcripts [9]. This method often utilizes the MS2 bacteriophage coat protein that binds an encoded stem loop of RNA. When MS2 is fused to a fluorescent protein, transcripts can be visualized in the cell. In order to concentrate the fluorescence signal above background, multiple stem loops are placed in series (up to 24 in a row). Though this technique has enabled imaging of single transcripts in the cell [9, 26], the resulting protein-RNA complex is prohibitively large for many studies [22].

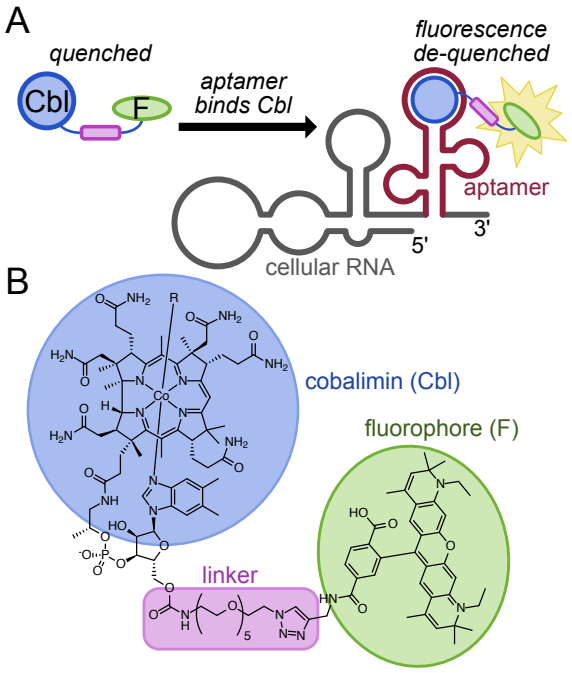


**Figure** 1. A) Cobalamin acts as a quenching and localization moiety to guide a fluorescent probe to an RNA transcript of interest. When unbound, fluorescence is quenched. In the presence of RNA tagged with the cobalamin riboswitch, fluorescence is restored. B) Structure of the generation 1 Riboglow probe. A polyethylene glycol linker of five units (5xPEG) connected to the 5' hydroxly of the cobalamin ribose was used to tether an ATTO 590 fluorophore to the construct.

Riboglow is a recently developed platform in the Palmer lab that solves many of the drawbacks of current RNA imaging techniques (Figure 1) [27]. The two-component system utilizes a synthetic fluorophore-quencher pair, and a genetically-encoded riboswitch. A transcript of interest is first tagged within the cell, and the probe construct is administered via bead loading [26, 28, 29]. When the construct binds the transcribed riboswitch, the fluorophore is dequenched, giving fluorescent signal. Like the dye-binding aptamers, Riboglow utilizes a riboswitch receptor domain that natively binds vitamin $B_{12}$ (cobalamin, Cbl, Figure 1B) [4]. Conveniently, cobalamin is known to act as a fluorescence quencher for a variety of fluorophores [30–32]. With Riboglow, the cobalamin center is conjugated to the fluorophore through a flexible linker that promotes quenching in the unbound state, but enables the fluorophore to reside at a distance in the bound state (Figure 1B).

The Palmer lab found that this initial generation of Riboglow has the potential to outperform the existing tools in the field. Its use of a naturally-occurring riboswitch imparts stability to the construct that is not present in aptamer-based techniques [33]. Additionally, the use of a donor-quencher pair reduces nonspecific fluorescence in the unbound state. Such signal induction is low in the aptamer-binding dyes, and cannot be obtained at all with the MS2-fluorescent protein fusions. Due to these advantages, Riboglow outperformed Broccoli and the MS2 system in initial studies of mRNA stress granule (SG) localization in mammalian cells.

Though promising, the initial iteration of Riboglow has significant room for improvement. With initial constructs, signal induction upon riboswitch binding never exceeded seven fold *in vitro*, and the pendant dye was never fully quenched (with 10-20% residual fluorescence) [27]. Additionally, to effectively detect stress granules, four riboswitches had to be placed in series to concentrate fluorescence signal [27]. This high background precluded single-molecule imaging. Finally, the strategy cannot currently be used to monitor the location of multiple transcripts in tandem. Herein, I will propose strategies to overcome these limitations through simultaneous modulation of the small molecule construct and riboswitch sequence. This is an undertaking for which I am uniquely suited, due to my experience of tandem modification of small molecule-protein interactions during my graduate studies. First, I will synthesize a variety of new Riboglow probes to optimize fluorescence quenching (Aim 1). Next, I will turn to the structure of the riboswitch itself. A screen for brightness will select the best riboswitch sequence to match the optimized cobalamin probe (Aim 2). This new, brighter pair will be tested for single-molecule detection. Finally, I will use SELEX to identify mutually orthogonal probe-riboswitch pairs for multicomponent RNA imaging, and will test these new tools by tracking lncRNA-mRNA interactions in living cells (Aim 3).

**APPROACH Aim 1. Synthesize improved Riboglow probes.**

The main drawback of Riboglow is the poor turn-on that is observed upon probe binding. In this aim, I intend to leverage my background in synthetic chemistry to produce a panel of diverse probe structures that improve fluorescence quenching (and thus signal induction). In previous studies in collaboration with Professor Dorota Gryko (see Gryko letter of support) a small number of linkers and fluorophores were evaluated for quenching and fluorescence turn-on. Linker length and fluorophore wavelength were varied to gauge the quenching ability of cobalamin. Remarkably, some degree of quenching occurred in all of the constructs synthesized, regardless of the spectral overlap of the fluorophore and the cobalamin. However, most retained 10-20% residual fluorescence, which increased background signal significantly. *To optimize probe function, I will vary linker composition, attachment point, and pendant fluorophore with the goal of obtaining 98% quenching in vitro.*

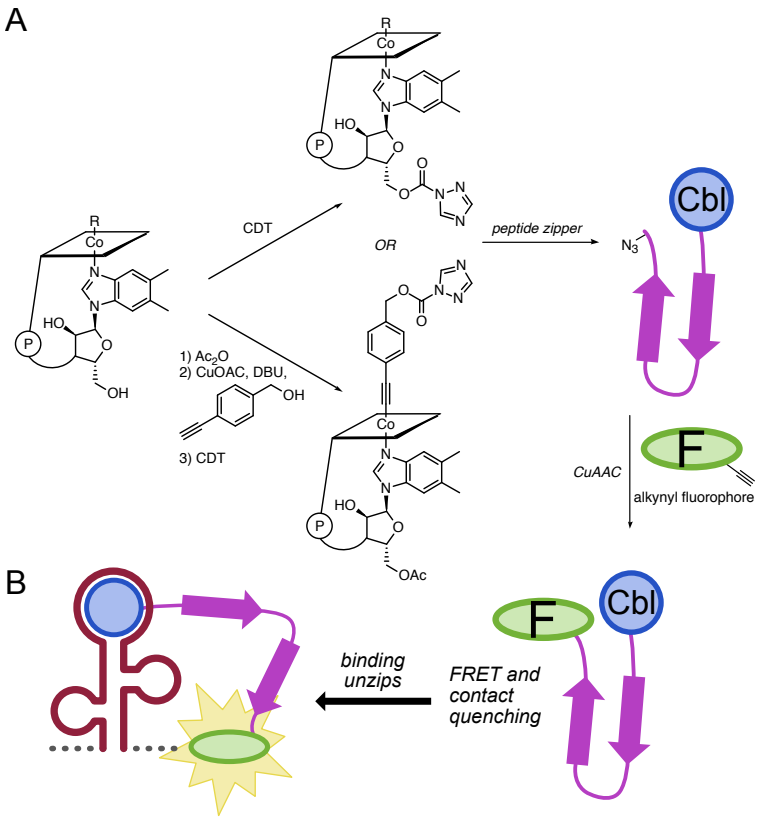


**Figure** 2. A) Synthesis of two structurally distinct attachment points for Riboglow probes. Both modes of conjugation have already been described in Ref. [34] (see Gryko support letter). Top: Conjugation with CDT at the ribose 5' OH was used in the first-generation probes [27]. Bottom: Alkynylation at the metal center with 4-ethynylbenzyl alcohol will enable subsequent coupling with CDT. $Ac_2O$: acetic anhydride, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, CDT: 1,1-carbonyl-di-(1,2,4-triazole), CuAAC: copper catalyzed azide-alkyne cycloaddition. B) Tryptophan zipper peptides will be used to maximize FRET and contact quenching in the unbound state.

Ideally, in the unbound state, the cobalamin and fluorophore would be closely associated to maximize FRET and contact quenching [30, 36, 37]. In the RNA-bound state, the molecules would reside at their maximal distance to promote fluorescence [31]. To strike this balance, I will use a synthetic β-hairpin as the linker between cobalamin and the fluorophore (Figure 2B, Table 1). A number of such β-hairpins have been developed, and are referred to as tryptophan zippers [38]. These motifs are as small as twelve amino acids and some are stable to denaturation up to 85 °C (Table 1). In the unbound state, such a linker would hold the quencher and fluorophore in close proximity (due to the short distance between the N and C-termini of the peptide, Figure 2B). When the cobalamin is bound by the riboswitch,

steric occlusion would force the β-hairpin to unfold and place the fluorophore-quencher pair at a larger distance. A wide range of tryptophan zippers have been developed with a variety of structures and stabilities, enabling easy construction of a range of different linkers via solid phase peptide synthesis [35, 38–40] (see Equipment document). To enable modularity, an azidoalanine amino acid will be appended to the C-termini of all linkers during peptide synthesis. Cobalamin will then be coupled to the linker via amide coupling and alkynyl fluorphores will be appended via copper catalyzed azide-alkyne cycloaddition (CuAAC, Figure 2A) [41, 42]. This general synthetic sequence is very similar to that used to make the original Riboglow probes [27]. A starting set if linkers are listed in Table 1. The polyglycine linker will be used as a negative control for a sequence with little secondary structure, but identical length. If the zipper folds prove to be too stable (dequenching is not observed), the amino acids will be varied to tune the melting temperature. Or, if adequate quenching is not observed, the relative positions of the N and C-termini will be adjusted based upon structural data available in the Protein Data Bank (PDB) [40]. All newly synthesized Riboglow probes will be purified via high-performance liquid chromatography (HPLC), and structurally verified via nuclear magnetic resonance (NMR) spectroscopy and high resolution mass spectrometry (HRMS). Each new construct will be tested for its ability to quench fluorescence in vitro by comparing the fluorescence of the construct with that of the free fluorophore in solution. Fluorescence signal in the presence of the Riboglow riboswitch will also be measured to calculate fluorescence turn-on.

| Sequence | Tm (°C) | % folded (25 °C) |
|---|---|---|
| KKWTW-NPATGK-WTWQE(Az) | 85 | >96 |
| KKYTW-NPATGK-WTVQE(Az) | 66 | 92 |
| GEWTY-NPATGK-FTVTE(Az) | 47 | 74 |
| GGGGG-GGGGGG-GGGGG(Az) | N/A | N/A |

**Table** 1. Tryptophan zippers of varying stability will be tested to maximize fluorescence quenching. Azidoalanine (Az) will be added to the C-terminus to enable easy fluorophore conjugation. Values were obtained from ref. [35] and reflect measurements for the peptide without the C-terminal azidoalanine.

Another underexplored variable of the original Riboglow probes is the linker attachment point. Though the 5' hydroxyl of the cobalamin ribose is the most accessible nucleophile on the structure, there exist several other possible sites of conjugation. Perhaps the second most common site is the axial ligand of the cobalt metal itself. Though many studies have taken advantage of the labile nature of certain alkyl modifications at this position [36], others have found alkynyl modifications to be stable to air and light [43, 44]. Following this precedent, I will synthesize a cobalamin with an alkyne handle attached to the cobalt metal center (Figure 2A, Bottom). Such a molecule has already been synthesized in the lab of Professor Dorota Gryko (see Gryko letter of support), and shown to be amenable to functionalization [34].

These constructs will also be evaluated for degree of fluorescence quenching, and brightness in the presence and absence of the cobalamin riboswitch. It is possible that incorporation of a ligand at this site on the metal center will abolish binding to the native riboswitch due to its positioning in the binding pocket [4]. If this is the case, these probes could serve as excellent starting points for directed evolution of orthogonal aptamer-probe pairs (see Aim 3). Additionally, perturbations to the native binding mode of cobalamin could reduce off-target association with native $B_{12}$ machinery [45], further reducing fluorescence background.

The final variable in the molecular structure of the Riboglow probe is the fluorophore itself. The ideal probe would minimize cellular autofluorescence through the use of a red fluorophore with a high extinction coefficient. The Janelia Fluor series of dyes is well suited for this application [46, 47]. With minor modifications to the rhodamine scaffold, Janelia Fluors have varying excitation and emission spectra, and excellent photophysical properties. Table 2 includes a subset of the probes that will be explored. Emphasis will be placed on fluorophores with high extinction coefficients ($\epsilon$) and quantum yields ($\phi$), with the intent for these probes to be used in single-molecule imaging (Aim 2). Additionally, $JF_{585}$ and $JF_{635}$ were shown to be highly fluorogenic upon conjugation to a protein tag, with absorbance increases of 80 and 113-fold respectively [46]. The Lavis lab makes these probes freely available, and has offered (personal communication) to provide us with alkynylated versions for easy conjugation via CuAAC (Figure 2A). Probes containing these new fluorophores will be evaluated as previously described, with the addition of a test for fluorophore photostability. Stability under extended illumination will be important for single-molecule localization (Aim 2), thus these experiments will give

| Dye | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | $\epsilon$ ($M^{-1}cm^{-1}$) | $\phi$ |
|---|---|---|---|---|
| Cy5 | 646 | 662 | 271,000 | 0.2 |
| ATTO 488 | 501 | 523 | 90,000 | 0.8 |
| ATTO 590 | 594 | 624 | 120,000 | 0.8 |
| ATTO 633 | 629 | 657 | 130,000 | 0.6 |
| **$JF_{549}$** | **549** | **571** | **101,000** | **0.88** |
| **$JF_{585}$** | **585** | **609** | **156,000** | **0.78** |
| **$JF_{635}$** | **635** | **652** | **167,000** | **0.56** |

**Table** 2. A range of fluorophores will be explored for optimal quenching and signal induction. Cy5 and the ATTO series were utilized in the first generation Riboglow system, and will also be used here with new linkers and Cbl attachment points. Photophysical values were obtained from Atto tec and refs. [27] and [46]. $\lambda$: wavelength, $\epsilon$: extinction coefficient, $\phi$: quantum yield.

an additional point of comparison for probe quality.

**Expected Outcomes and alternate approaches:**
To identify probes with improved quenching and fluorescence turn-on, linker, attachment point, and fluorophore will be varied. To start, I will target 98% fluorescence quenching *in vitro*. With four linkers, two attachment points, and seven fluorophores, 56 constructs are possible. Though not all of these will be evaluated, simple chemistries involving amide bond couplings and click chemistry will enable us to sample a large amount of this structure space in a rapid fashion. If β-hairpin zippers do not yield improvements in quenching, other linkers with varying rigidity and lengths will be explored [31], specifically those that promote direct contact between the fluorophore and the cobalamin. Also, it should be noted that new cobalamin constructs are not necessary for the success of aims 2 or 3. It is likely that all three aims will be pursued in parallel, with new discoveries in each informing project direction.

**Aim 2. Adapt Riboglow for single-molecule imaging.**

Visualization of the lifecycle of single RNA transcripts as they move throughout the cell remains a holy grail of RNA imaging [48]. This is an entirely feasible goal due to the fact that so few copies of an individual RNA transcript exist in a cell at any given time (the samples are by definition sparsely populated) [48–50]. Though several recent studies have demonstrated visualization of individual mRNA transcripts, all have required the use of at least 24 repeats of the MS2-binding stem loop [9, 51–53]. When these loops (which each bind two MS2 proteins) are fully populated, a complex of more than 2,000 kilodaltons is produced. Such a structure is several times larger than most mRNA, giving the potential to perturb normal mRNA behavior. Riboglow reduces the size of the fluorescent probe by 10–100-fold (relative to the MS2 system), however, high fluorescence background and poor signal induction precluded single-molecule studies [27].

With the wide variety of new probe constructs developed to optimize quenching (Aim 1), changes will be made to the sequence of the cobalamin riboswitch with the goal of increasing fluorescence turn-on by 50-fold. *A directed evolution screen for fluorescence brightness will identify optimal riboswitch sequences that promote fluorescence signal induction*.

First, a library will be designed that varies the environment surrounding the cobalamin binding pocket (Figure 3A). Through the guidance of Robert Batey (see Batey support letter), sites will be chosen to retain affinity for the cobalamin, yet maximize the distance between quencher and fluorophore upon binding [4]. Next, a screen for fluorescence intensity in mammalian cells will be used to select bright riboswitch sequences. A cellular screen for brightness is crucial because it ensures that the riboswitch aptamer maintains robust folding in a complex environment. The Palmer lab is a leader in technologies for tool development in mammalian cells [54, 55]. This expertise will be leveraged for screening libraries of cobalamin riboswitches (Figure 3B). Libraries of transcripts will first be transduced into mammalian cells using lentivirus at a low multiplicity of infection to ensure incorporation of a single library member per cell (a technique widely used in the Palmer lab). The probe of interest will be administered, and cells will be sorted via fluorescence activated cell sorting (FACS, see Equipment document). Cells that show elevated brightness relative to a fluorescent protein expression control will be binned. In this way, libraries of up to one million members will be screened. I will collect, culture, and resubject bright variants to sorting until only highly fluorescent cells remain. Sequences will be evaluated through high-throughput sequencing.

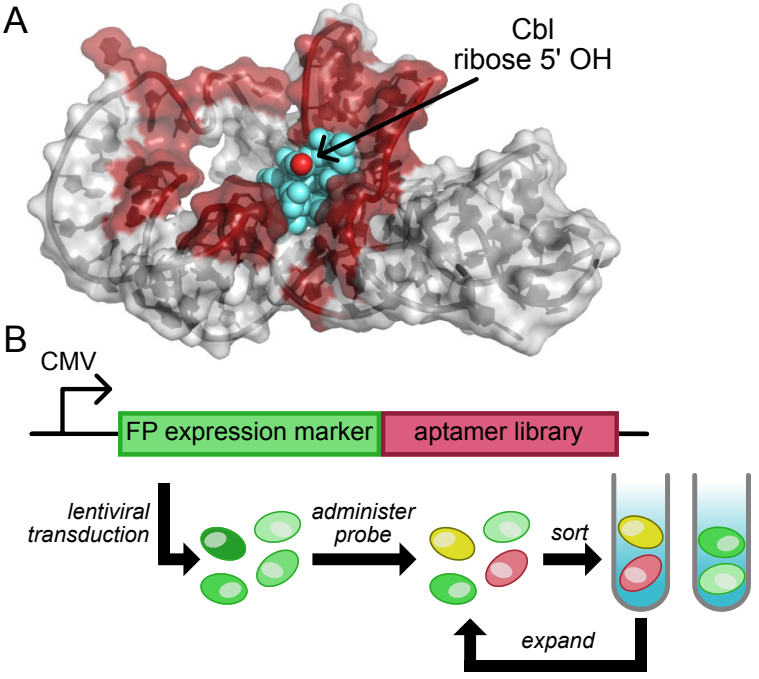


**Figure** 3. A) Riboswitch sites for mutation (colored red) will be targeted based on potential contact with the linker and fluorophore, while the cobalamin (blue) binding site will be retained. (PDB: 4FRN, ref. [4] B) RNA sequences will be screened in mammalian cells for brightness relative to a fluorescent protein control.

New sequences identified through this screen will undergo rigorous characterization of their biophysical and photophysical properties *in vitro*. It will be important to characterize the extinction coefficient of each complex upon binding the RNA tag, as well as their quantum yields and photostability. The binding affinity for each probe-

RNA combination will also be measured via isothermal titration calorimetry. I will aim to retain $K_D$ values in the nanomolar range (the native riboswitch binds cobalamin at $K_D$ = 37 nM). Such a value is important because it informs the probe dosage that will be necessary for imaging (and lower probe dosages will contribute less fluorescence background). Experiments to obtain these values are readily carried out in the Palmer Lab [27, 56].

Candidate probes that exhibit low background fluorescence, high signal induction, and high RNA affinity will be tested to determine the absolute level of turn-on fluorescence, and the minimum number of fluorophores that can be localized. To do this, β-actin mRNA will be tagged with one, two, or four repeats of the mutant riboswitch of interest in U2-OS cells through transient transfection. The cells will be imaged under conditions analogous to previous studies [51] using a TIRF microscope (see Equipment document). If puncta that appear to be single molecules are observed under these conditions, probe localization will be verified via fluorescence in situ hybridization (FISH) following cell fixation. If puncta are not visible in the initial TIRF images, cells will be treated with arsenite to induce the formation of stress granules (SGs) that also contain the protein G3BP1 [57–59]. G3BP1 can be tagged with the Halo-tag, and subsequently labeled with an orthogonal fluorophore to evaluate co-localization. A check of direct mRNA location can then be carried out with FISH. This is a technique that the Palmer lab has previously used successfully to test Riboglow probes [27].

To quantify our ability to resolve single mRNA molecules, we will utilize single-molecule FISH (smFISH) imaging to correlate RNA count with Riboglow signal [60]. This same method was recently used to quantify the sensitivity of an improved MS2 system [22].

**Expected Outcomes and alternate approaches:**
To attain single-molecule resolution with Riboglow, the probes synthesized in Aim 1 will be screened with libraries of riboswitch mutants *in cellulo*, targeting a 50-fold fluorescence turn-on. Cells containing bright mutants will be sorted via FACS and analyzed. Library hits will be characterized *in vitro*, and tested via established assays for single-molecule localization *in cellulo*. While screening for high fluorescence in cells via FACS is the most efficient way to ensure robust turn-on, an alternative approach could involve alternating a screen for binding via *in vitro* selection (see aim 3 for experimental detail) and in cellulo selection for fluorescence via FACS. If single-molecule resolution is not observed in the library hits, additional riboswitches (over the four repeats already planned) can also be added to concentrate fluorescent signal. Even if 24 repeats are required, the construct will still be less than half the size of the MS2 system.

**<u>Aim 3.</u> Develop mutually orthogonal Riboglow probes for multicomponent imaging.**

The ability to track multiple RNA transcripts simultaneously would enable study of the interactions of RNA as they move throughout the cell. Upregulation of *SNHG5* in colorectal cancer has been implicated in stabilization of a variety of mRNA in the cytoplasm that enable tumor survival [15]. Direct hybridization of this lncRNA with mRNA protects transcipts from degradation, and enables translation, promoting disease states. Despite these implications, the spatiotemporal dynamics of lncRNA and their interacting partners have never been visualized in living cells, and thus their functions remain unknown. Localization of these lncRNA and their mRNA targets would shed light on these “dark” processes.

The modularity of the Riboglow platform is well suited to enable such an experiment, however mutually orthogonal riboswitch-probe pairs must first be identified. *In vitro selection will enable identification of mutually-selective riboswitch-probe pairs for multicomponent RNA imaging* (Figure 4B). *To start, I will identify two orthogonal riboglow probes.*

Fortunately, screening for substrate selectivity is well-precedented in RNA engineering through systematic evolution of ligands by exponential enrichment (SELEX) [61, 62]. In fact, *de novo* aptamers have already been developed for cobalamin and a few of its analogs via SELEX [63]. This important precedent shows that even small changes to the structure of the cobalamin can be distinguished by engineered RNA, indicating that no additional changes will need to be made to the cobalamin structure apart from those constructs already proposed above. However, to improve our chances at identifying mutually-orthogonal probes, we will begin by screening probes that contain each of the two linker attachment points (Aim 1) to maximize structural differences at the cobalamin center.

Importantly, our selection experiments will not start from a completely randomized sequence of nucleic acids. It will be crucial to retain the original fold (and thus *in cellulo* stability) of the native riboswitch. RNA biosensors developed in this way are known to have increased stability relative to aptamers selected *de novo* [33]. Additionally, the brightest probe sequences identified in Aim 2 will be retained to preserve the bound conformation of the linker and fluorophore as much as possible. The design of the starting RNA libraries, and subsequent selection experiments will be carried out under the supervision of Professor Robert Batey (see Batey letter of support),

an expert in SELEX and RNA engineering [33, 64]. Libraries will target bases known to participate in contacts with the cobalamin small molecule [4, 33]. The selection will be carried out as shown in Figure 4A. Briefly, the cobalamin probe of interest will be immobilized on a bead via the same linker that will be used in the final construct. The synthetic library of riboswitches will be incubated with the beads, and unbound sequences will be rinsed away. Riboswitches that bind selectively will be collected and amplified (via reverse transcription, PCR amplification, and transcription) to undergo another round of selection (for a total of 7-10 rounds). In subsequent rounds, the stringency of the screen will be increased via additional washes and reduction of RNA concentration. In the final 2-3 rounds of selection, a counterselection will be conducted to ensure orthogonality with our other synthetic probes (Figure 4A). In this way, the probe (or probes) intended to be orthogonal to the construct under selection will wash away any riboswitches that do not bind selectively. Riboswitches will be deep sequenced to identify library convergence and optimal clones [33]. This process will be repeated for each probe of interest, with counterselections against each other probe that is intended for multiplexed use.

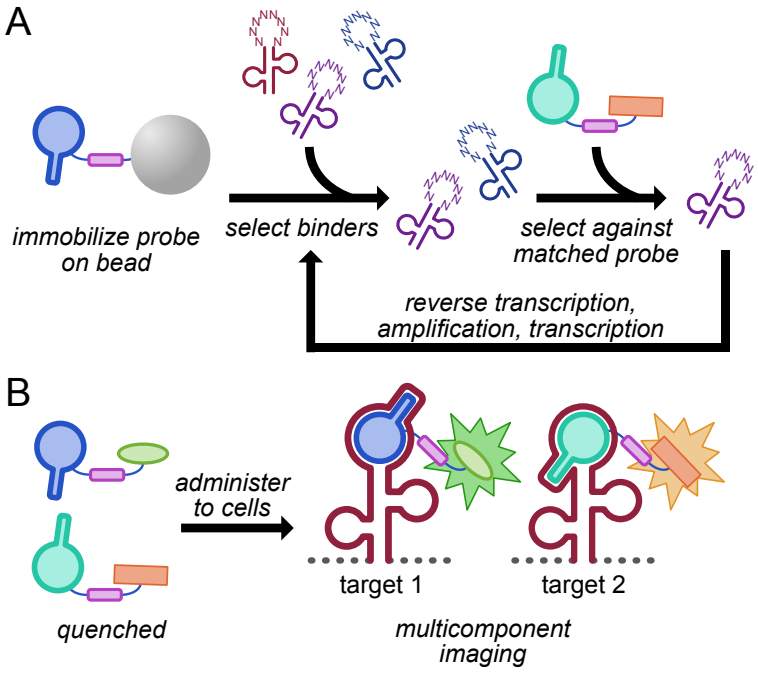


**Figure** 4. A) SELEX will be used to screen for riboswitches that bind each cobalamin in a mutually-exclusive manner. B) Mutually orthogonal cobalamin analogs will enable multicomponent RNA imaging. Signal turn-on will only be observed in the presence of the matched pair.

Mutually-orthogonal probes identified through the *in vitro* selection will be verified in a similar fashion as described in Aim 2. Probe extinction coefficient, quantum yield, photostability, and binding affinity will be characterized *in vitro*. Probes will also be independently verified *in cellulo* via the β-actin fusion assay described in Aim 2 to ensure signal induction was retained.

Once a robust pair of Riboglow probes is identified, a lncRNA will be imaged interacting with a putative binding partner mRNA as a proof-of-concept. Studies involving imaging of lncRNA will be carried out under the supervision of Professor John Rinn, an expert in lncRNA (see Rinn support letter). First, *SNHG5* will be tagged with one of our riboswitches and imaged in the cytosol of colorectal carcinoma (HCT116) cells, coupled with smFISH for verification. Known phenotypic checks will be used to ensure that *SNGH5* behaves normally upon tagging [15].

Next, our multicomponent Riboglow platform will be used to image *SNGH5* interacting with mRNA. *SNGH5* has been shown to promote colorectal cancer survival through base pairing in the 3'-UTR of mRNA that promote tumor growth [15]. *SPATS2*, *PTRM1*, and *GLE1* have been identified as interacting partners. To test whether these interactions can be detected, we will tag these three transcripts (one at a time) with an orthogonal riboswitch, and image them in living HCT116 cells alongside the tagged *SNGH5*. To further study these interactions, *SNGH5* will be both overexpressed (via transient expression) and knocked down (via RNA interference), which has been shown to increase and decrease, respectively, the levels of interacting transcripts [15]. An additional check for localization will be made through fluorescent tagging of *STAU1*, a protein that binds to the 3'-UTR of mRNA and mediates their degradation [65]. Single molecule FISH will provide a final check of probe selectivity.

**Expected Outcomes and alternate approaches:** To develop multicomponent Riboglow, *in vitro* selection will identify orthogonal riboswitch-probe pairs through a series of selections and counterselections. Following characterization *in vitro*, probes will be tested for multiplexed imaging of lncRNA-mRNA interactions *in cellulo*. It is possible that the *in vitro* selection will result in a loss of fluorescence turn-on. If this is the case, riboswitch sequences will be resubjected to the *in cellulo* fluorescence intensity screen described in Aim 2. Additionally, it may be difficult for the riboswitch to differentiate probes with similar cobalamin-linker structures. If the SELEX screen does not yield well-resolved pairs, additional steric bulk can be added to to the cobalamin (at either the 5' OH of the ribose, or the axial position of the cobalt), to aid in differentiation [34].

These studies will provide the first glimpse into the real-time behavior of lncRNA *SNGH5* and its interacting partners. With new tools in the RNA imaging toolkit, we will be able to ask new questions about the little understood biomolecule that accounts for half of all cellular transcription.

## RESPONSIBLE CONDUCT OF RESEARCH

My graduate studies at UC Irvine included significant early training in the conduct of research that has laid a foundation for the rest of my career. During my first year of study at UCI, I took a "Conduct of Research" class from Professor Dave Van Vranken. The class met three times a week for a quarter (30 hours), and discussed a range of issues relevant to research ethics at an academic institution. Additionally, on a quarterly basis throughout my graduate career, I met with my research advisor, Professor Jenn Prescher, to discuss my conduct in the lab, the clarity of my lab notebook, mentoring, and authorship. I remain committed to the highest standard of research conduct, and look forward to receiving additional training in this area as a postdoctoral researcher. During my time at the University of Colorado, I will be taking "Scientific Ethics and the Responsible Conduct of Research" from Professor Mary Allen. I have outlined the instruction I will be receiving in this class below.

1. **Format**

   On a weekly basis the class will meet to hear a lecture from a member of the CU Boulder research faculty on a relevant topic on conduct of research. Immediately following each lecture, the class will break into small groups for discussion. Between classes, there will be assigned readings with accompanying questions to facilitate class discussion. Over the course of the semester, two case study essays will be written, reporting on real issues that researchers faced regarding one of the topics in the course.

2. **Subject Matter**

   The class reading will be from "Responsible Conduct of Research" by Adil Shamoo and David Resnik. (3rd ed. 2015, Oxford University Press)

   *Topics will include:*

   (a) Why does RCR matter?
   (b) The Scientist in Society
   (c) Mentor/Trainee Issues
   (d) Data Acquisition, Management, and Reproducibility
   (e) Lab Safety
   (f) Authorship, Publication, and Peer Review
   (g) Conflict of Interest
   (h) Research Misconduct
   (i) Protection of Human Subjects
   (j) Collaborative Research
   (k) Intellectual Property

3. **Faculty Participation**

   In addition to interactions with faculty speakers during class, I will also meet with Professor Palmer on a biweekly basis to discuss responsible conduct of research during my first semester in the lab (Fall 2018). These meetings will cover data acquisition and storage, mentoring of graduate and undergraduate students, authorship, and collaboration in the Palmer lab. The lab also conducts monthly meetings on special research topics including data storage and management, conduct of research, responsible image analysis, and manuscript writing.

4. **Duration of Instruction**

   The class will meet on a weekly basis during the spring semester of 2019 for 15 weeks. Each class is two hours, giving a total of 30 contact hours.

5. **Frequency of Instruction**

   I will begin my postdoctoral studies in the fall of 2018, and enroll in the Responsible Conduct of Research class to be offered in the spring of 2019. This will fulfill the requirement for training during my postdoctoral career.

## SELECTION OF SPONSOR AND INSTITUTION

The proposed research will be carried out in the lab of Professor Amy Palmer in the Biofrontiers Institute at the University of Colorado Boulder. The Biofrontiers Institute is a world leader in both imaging and RNA research. In choosing this community to carry out my research, I am in an excellent position to be successful. RNA is a large focus of the institute. With Nobel Prize winner Tom Cech and its director, and the Rinn and Batey labs (see letters of mentoring support) in the same building, I will have easy access to the top researchers in the field of RNA. Collaboration with these labs will provide me with additional techniques and input that may not be readily available in the Palmer lab itself. The institute also provides superior research facilities and instrumentation that are maintained by full-time staff. Within the same wing as the Palmer lab is located both the cell culture facilities, and the microscopy core, and the building houses the deep sequencing and mass spectrometry cores.

I will also have a high chance of success due to my choice in mentor. Professor Palmer was educated by some of the best in the field, with her graduate degree in the lab of Edward Solomon at Stanford, and her postdoctoral work with Roger Tsien at the University of California, San Diego. She is an expert in the field of fluorescent tools development, and the study of fundamental cellular processes. When she was a postdoc in the Tsien lab, she was also awarded the Ruth L. Kirschstein National Research Service Award Postdoctoral Fellowship, thus she is aware of the level of training and mentorship that is necessary for awardees. Because my independent research goals lie in tool development at the interface of molecular organic chemistry and chemical biology, she is an excellent choice for training in this area. Additionally, professor Palmer has already mentored four group members that have gone on to acquire tenure-track professorships at research institutions.

## FACILITIES AND OTHER RESOURCES

More than adequate facilities for conducting the proposed research are available in the sponsor's laboratory, and in other core facilities across the Colorado University Boulder campus. These facilities, in addition to the scientific environment at CU Boulder, are more than sufficient to ensure the success of the proposed research. *Major equipment in the sponsor's laboratory is described in the Equipment form.*

**The Jennie Smoly Caruthers Biotechnology Building (JSCBB):** Dr. Palmers laboratory is located in the new state-of-the-art Jennie Smoly Caruthers Biotechnology Building (JSCBB). JSCBB is a four-story, 330,000 square foot research building that houses more than 60 faculty members from the BioFrontiers Institute, the Department of Chemical and Biological Engineering, and the Division of Biochemistry. The building contains most of the core facilities that are crucial to the proposed research, in addition to the labs of professors Rinn and Batey, who will be offering mentoring support (see support letters).

**The Palmer Laboratory:** The Palmer lab is located on the third floor of JSCBB and covers approximately 3,000 feet of lab space shared with the Liu Lab. The lab occupies 5 research bays, each with 3–4 benches and 3 desk spaces. Located directly across the hall is *(i)* the cell culture core facility, with all necessary equipment for mammalian cell culture, and *(ii)* the imaging core facility that houses a variety of state-of-the-art microscopes. A cold room, autoclave and dishwashing room, dark rooms, and shared instrument rooms are located near the lab in JSCBB as well.

**BSL-2 Certified Cell Culture Core Facility:** Located directly across the hall from the Palmer lab is a fully-equipped mammalian cell culture facility with 12 biosafety cabinets, 24 double door incubators, automated liquid nitrogen storage units, and appropriate equipment (centrifuges, light microscopes, etc). The facility is BL-2 plus certified, enabling work with viruses and pathogens. The facility is staffed by Theresa Nahreini, who provides training and support.

**BioFrontiers Advanced Light Microscopy Core:** Also located across the hall from the Palmer lab, the Advanced Light Microscopy Core houses a number of advanced microscopes: a Nikon A1R resonant laser scanning confocal microscope with perfect focus, full environment chamber for long term imaging, and equipped with TIRF; a Nikon spinning disc confocal microscope with seven laser lines, equipped with a fully enclosed environmental chamber; an Image Express high throughput high content microscope; and a Nikon N-STORM system for super-resolution (more details on specific microscope capabilities in Equipment statement). An image analysis station is available with software including MATLAB, Imaris, Nikon Elements, ImageJ/Fiji, ICY, Cell Profiler. The facility is staffed by core director Dr. Joe Dragavon, who offers training and assisted imaging sessions. Professor Palmer is the faculty advisor.

**BioFrontiers Next-Generation Genomics Facility:** Located in JSCBB, this facility houses an Illumina HiSeq 2000 sequencer, two MiSeq sequencers and associated Illumina iCompute infrastructure for analysis of sequencing data. Staff members aid in library construction and assessment of sequencing quality.

**Biomolecular Mass Spectrometry and Proteomics:** Also located in JSCBB, this facility specializes in both macromolecular and small-molecule mass spectrometry. The facility houses a LTQ-Orbitrap, and Orbitrap Velos mass spectrometer with CID/HCD/ETD fragmentation capabilities, interfaced with a Waters NanoAcquity 2D-UPLC system. Other instruments include a LTQ-Orbitrap mass spectrometer with CID fragmentation capabilities (used for other proteomics), interfaced with a Waters NanoAcquity 2D-UPLC system, a Waters Synapt G2 QqTOF mass spectrometer with Waters Acquity UPLC system (mainly used for small molecule and hydrogen-deuterium exchange studies), a PerSeptive Voyager DE-STR MALDI-TOF mass spectrometer, and a AB 4000 QTrap Linear Ion Trap mass spectrometer with Eksigent 2D-LC nanoflow HPLC. For small molecule analysis, the facility offers accurate mass determinations and complex mixture analysis.

**NMR Spectroscopy Facility:** In JSCBB, a Bruker Avance-III NMR spectrometer operating at 400MHz for proton NMR is available for walk-up use. It is capable of one and two-dimensional NMR experiments on most NMR-active nucleii, and is equipped with a 60-slot autosampler. This instrument is complemented by a larger facility nearby on campus containing a similar 300MHz instrument, a 400MHz Varian INOVA 400, and a 500 MHz Varian INOVA 500.

**Cell Sorting Facility:** Located directly across the hall from the Palmer Lab, the cell sorting facility contains a FACSAria III (BD Biosciences), and a MoFlo cell sorter from Cytomation. The facility, and training for its use is available to all labs in the building.

**Computers:** Several computers and data storage servers are available in the Palmer lab. The Biochemistry division of the Chemistry department also offers DEC and SUN workstations, and Silicon Graphics terminals for

molecular modeling.

**The JSCBB Shared Instruments Pool:** JSCBB also houses a shared instruments pool that provides access to a variety of analytical instruments necessary for biochemical studies. The facility contains EPR, CD, and fluorescence spectrometers, an isothermal titration calorimeter, and other instruments.

**Institutional Postdoctoral Support and Resources:** *See document "Institutional Environment and Commitment to Training" document.*

## EQUIPMENT

The Palmer lab has access to all of the equipment needed to perform the work proposed, most of which is present in the lab, the BioFrontiers Advanced Light Microscopy Core, and the Cell Culture Facility (both of which are across the hall from the lab).

- Synthesis
    1. 2 chemical fume hoods, 1 equipped with a vacuum line for airfree synthesis
    2. Mettler electronic analytical balances
    3. Rotary Evaporators
    4. LabAlliance high-performance liquid chromatography (HPLC) solvent delivery system equipped with a Rainin UV-1 detector (located in the lab of Zhongping Tan)
    5. Applied Biosystems Pioneer continuous flow peptide synthesizer (located in the lab of Zhongping Tan)
- Imaging
    1. Nikon Ti-Eclipse Inverted Fluorescence Microscope with a iXon3 CCD camera (Andor) and a Dual view camera (DV2, Andor) for FRET imaging
    2. Nikon A1R Laser Scanning Confocal and TIRF Inverted Microscope with iXon X3 EMCCD
    3. Molecular Devices ImageXpress Micro XL System for high throughput-high content fluorescence microscopy
    4. Olympus IX-81 Inverted Microscope
    5. Nikon N-STORM and TIRF inverted microscopes for super-resolution microscopy with three cameras (2x Andor Ixon Ultra 897 EMCCD, 1x Hamamatsu ORCA Flash4.0 sCMOS)
    6. Nikon Spinning Disc Confocal Microscope
    7. An image analysis workstation with software: MatLab, Imaris, Nikon Elements, ImageJ/Fiji, ICY, and Cell Profiler
- Cell Culture
    1. Biosafety cabinets for mammalian cell work
    2. BSL-2-certified biosafety cabinet for lentiviral work
    3. Centrifuge and ultracentrifuge
    4. $CO_2$ cell culture incubator
    5. Refrigerators and freezers (4, -20, and -80 $^\circ$C)
    6. Table top fluorescence microscope
    7. FACSAria III cell sorter (BD Biosciences)
- Molecular Cloning
    1. Molecular biology-grade water system
    2. Cold room (4 $^\circ$C)
    3. Temperature-controlled incubating shakers
    4. Protein and DNA gel electrophoresis and Western blotting equipment
    5. heating blocks
    6. 37 $^\circ$C incubator
    7. PCR thermocyclers

See document “Facilities & Other Resources” for shared equipment within the BioFrontiers Institute.

## INSTITUTIONAL ENVIRONMENT AND COMMITMENT TO TRAINING

1. **The University of Colorado Boulder:** University of Colorado Boulder is home to the Postdoctoral Association of Colorado. This group provides a regular mailing list of announcements for a wide variety of professional development and funding opportunities. They also organize regular events with a focus on career development, networking, social activities and advocacy. The Office of Postdoctoral Affairs is another avenue of support on campus for postdoctoral training and career development. They offer a number of services to CU postdocs such as networking events, seminars, and tutorials. Postdoctoral researchers are also welcome to attend events hosted by the graduate school at CU Boulder, including workshops, lectures, and networking events. Postdoctoral researchers are also invited to participate in an array of scientific communication forums, such as serving as speakers for Supergroup meetings and at the Biochemistry annual retreat.

2. **The BioFrontiers Institute:** The scientific environment within CU Boulder and the BioFrontiers Institute is excellent. Professor Palmer is a part of both the Department of Chemistry and Biochemistry, and the BioFrontiers Institute, directed by Nobel Laureate Tom Cech. The institute has become a leading organization for RNA research, with both highly established faculty such as Robert Batey (see support letter), Roy Parker (HHMI Investigator), and Tom Cech, and newcomers like John Rinn who has recently moved from Harvard (see support letter). Not only are all these labs within the same building (JSCBB), they also meet regularly as supergroups to talk about current research, and to find opportunities to collaborate. The Palmer lab participates in the Biophysics, Signaling and Cellular Regulation, Chemical Biology, RNA, and Bioinformatics supergroups which meet bi-weekly.

3. **The Palmer Lab:** Professor Palmer implements a lab structure that fosters an environment of collaboration and mentorship. She meets one-on-one with each student and postdoc every two weeks to discuss data, their research project, and career goals. Professor Palmer and I will also use these meeting times to *(a)* discuss my future research program, *(b)* grant writing and proposal construction, and *(c)* mentoring opportunities and strategies. The Palmer lab also holds weekly group meetings in which members of the lab present their recent findings. Once a month the lab holds a special topics meeting to discuss important practical matters surrounding research. Topics include data storage and management, conduct of research, responsible image analysis, and manuscript writing. Within the lab itself, benches and desks are arranged together, with an abundance of shared workspace that promotes conversation and collaboration amongst labmembers.

4. **Additional Individual Mentorship:** In addition to mentorship under Professor Palmer, I will meet regularly with professors Rinn and Batey (see letters of support) and attend both Rinn lab and Batey lab group meetings. Full Professor Batey has been a professor at CU Boulder for 15 years, and is an expert in riboswitch biology and engineering. His advise and mentorship will be instrumental for Aims 2 and 3 of my proposal. Full Professor Rinn only recently moved to CU Boulder from Harvard. He is an authority on long noncoding RNA, and will be instrumental in the experimental design regarding the use of our new tools for imaging these understudied RNAs. Taken together, professors Palmer, Batey, and Rinn form the ideal mentoring team for a postdoc that is seeking to advance to a tenure-track research professorship. Each will bring unique viewpoints and resources that will ensure my success if funded.

## RESOURCE SHARING PLAN

This work will generate new reagents and data that will be made freely available. Discoveries made as a result of this proposal will be published in peer-reviewed journals, presented at scientific conferences, and shared with other scientists and the community through open discussions. Information will only be withheld if it endangers chances of publication or communication to the community.

### Chemical Characterization and Resources

All original synthetic compounds and characterization obtained, including Riboglow probes and their associated NMR, UV/Vis, mass spectra, etc. will be stored in the Palmer lab indefinitely and will be distributed by the following plan:

- Original and unprocessed spectra and chromatographs will be sent directly to requesting laboratories via email within 2 weeks of the original request.
- All processed spectra will be provided in the supporting information of published journal articles for continuous long term access.
- If available, small samples of material will be sent directly to requesting laboratories using standard express mailing services within 2 weeks of the original request.

### DNA

All original DNA reagents (including primers, plasmids, and libraries) will also be stored in the Palmer lab indefinitely and distributed by the following plan:

- Genetic data, including high-throughput sequence reads will be submitted to the appropriate NIH-funded repositories including the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
- Genetic constructs in the form of plasmids will be submitted to Addgene (www.addgene.org).
- DNA reagents will be will be sent directly to requesting laboratories using standard express mailing services within 2 weeks of the original request, so long as sufficient stocks remain available

I agree to deposit these resources into the appropriate repository as soon as possible but no later than within one year of the completion of the funded project period for the parent award or upon acceptance of the data for publication.

## AUTHENTICATION OF CHEMICAL AND BIOLOGICAL RESOURCES

This application makes use of a variety of biological and chemical reagents: established cultured cell lines, specialty chemicals and peptides, and plasmid DNA. The authenticity of these resources will be established according to NIH guidelines. Our efforts in each area are briefly described below.

1. **Cell lines:** Model mammalian cell lines will be used for the proposed studies. All cell lines will be obtained from ATCC and will be used for less than 12 passages. Cell lines will be tested for mycoplasma every 6 months.
2. **Specialty chemicals and peptides:** Chemical reagents will be obtained from common suppliers (Sigma Aldrich, etc.) that provide QC authentication. The integrity of purchased chemicals will also be verified using common analytical techniques (NMR, mass spec, etc.) All new small molecules produced during the proposed work will also be rigorously characterized (via NMR, mass spec, HPLC, etc.). It should be noted that the PI has over 8 years of experience in small molecule synthesis and characterization. Peptides produced via solid-phase peptide synthesis will be confirmed using LCMS.
3. **Plasmids:** Plasmids used to produce protein conjugates will be obtained from commercial sources with sequencing validation (Addgene). Plasmids produced in-house will be sequenced to confirm the open reading frame.

## RESPECTIVE CONTRIBUTIONS

The original Riboglow probes were developed primarily in the Palmer lab with the aid of several collaborators including Roy Parker, Ralph Jimenez, Dorota Gryko, and Robert Batey. A majority of the lab work was conducted by postdoc Esther Braselmann.

In my initial interviews with Professor Palmer, she mentioned that I could have the opportunity to work in RNA imaging tool development in her lab (among other projects), and suggested areas in which the initial Riboglow methodology could be improved. The aims stated herein were conceived of by me, the applicant, after review of the literature and identification of the limitations present in the field of RNA imaging. Professor Palmer provided input with respect to the feasibility of the aims, and suggested additional assays for verification of the tool.

I wrote the research proposal and designed all figures. I am submitting the proposal with the approval of Professors Palmer, Gryko, Batey, and Rinn (see letters of support).

National Institutes of Health
Center for Scientific Review, Div. of Receipt and Referral
6701 Rockledge Drive MSC 7768
Bethesda, MD 20892-7768
(301) 435-1115

April 7, 2018

Greetings:
Thank you for receiving my F32 application. See below for information describing my submission:

**Title:** Multicomponent, superresolution imaging of RNA in mammalian cells

**FOA:** PA-16-307

**Please assign this application to:** Synthetic and Biological Chemistry [F04A]

**Referees:**

- Jennifer A. Prescher, Ph.D, U.C. Irvine
- Scott Rychnovsky, Ph.D, U.C. Irvine
- Chris Vanderwal, Ph.D, U.C. Irvine

Thank you for your time and consideration.

Sincerely,

Colin Rathbun

## PROJECT SUMMARY

RNA lies at the center of cellular function. Its most appreciated role is to carry protein blueprints to the ribosome for manufacture. Only recently have researchers begun to appreciate its myriad of other functions, many of which are implicated in a variety of disease states. Long noncoding RNA (lncRNA) comprise one such important class of RNA that does not participate in the central dogma. Alarmingly, the human genome encodes for as many lncRNA as proteins. These transcripts are typically greater than 200 bases, and are known to participate in binding both proteins and nucleic acids, often both at the same time. However, little else is understood regarding their function. Where and when do they interact with their targets? How long do these interactions occur, and what other cellular machinery is present? This lack of understanding is due in part to the lack of tools available to image this biomolecule. Localization of RNA on a single-molecule level, and multicomponent imaging of RNA transcripts remains difficult. Existing tools utilize aptamers that are unstable in mammalian cells, or constructs that are too large for imaging small transcripts. Multicomponent RNA imaging is also difficult due to the design of current tools.

To address this need, I aim to develop a platform for RNA imaging that will enable facile tracking of multiple transcripts at single cell resolution. Riboglow is an RNA imaging platform recently developed in the Palmer lab. It utilizes a fluorescence-quenched pair formed by cobalamin (vitamin $B_{12}$) and a pendant fluorophore. In solution, this construct shows low fluorescence. When bound to the cobalamin riboswitch aptamer domain, there is an increase in fluorescence. This tool shows promise for RNA imaging because it solves many of the problems faced by traditional RNA probes, however several drawbacks are keeping it from widespread utility. The proposed work addresses these drawbacks, and seeks to utilize improved Riboglow tools to study outstanding questions in the field of noncoding RNA.

Previously developed Riboglow constructs suffered from poor signal induction and low brightness. First, I aim to derivatize the native cobalamin structure, linker and fluorophore with the goal of maximizing fluorescence turn-on. These new molecules will be evaluated for quenching efficiency and signal induction. Next, the molecules I develop will be screened against libraries of riboswitch aptamers to further improve probe properties. Screening will be carried out in mammalian cells via flow cytometry, a specialty of the Palmer lab. Candidate probes will be verified through single-molecule imaging of mRNA in living cells. In tandem with brightness optimization, I will develop mutually orthogonal probes to enable labeling of different RNA transcripts in the same cell. The power of SELEX to find selective and tight binders will be used to screen for mutually exclusive aptamer-cobalamin pairs. These pairs will be conjugated to spectrally-resolved fluorophores to enable tracking of multiple RNA simultaneously. These orthogonal probes will be used to image lncRNA and mRNA as they interact in the cytosol.

## Bibliography and References Cited


[1] Cech, T. R. and Steitz, J. A.: The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. Cell. 157(1): 77–94, March 2014.

[2] Müller-McNicoll, M. and Neugebauer, K. M.: How cells get the message: Dynamic assembly and function of mRNA–protein complexes. Nature Reviews Genetics. 14(4): 275–287, April 2013.

[3] Etzel, M. and Mörl, M.: Synthetic Riboswitches: From Plug and Pray toward Plug and Play. Biochemistry. 56(9): 1181–1198, March 2017.

[4] Johnson Jr, J. E., Reyes, F. E., Polaski, J. T., and Batey, R. T.: $B_{12}$ cofactors directly stabilize an mRNA regulatory switch. Nature. 492(7427): 133–137, December 2012.

[5] Paige, J. S., Wu, K. Y., and Jaffrey, S. R.: RNA Mimics of Green Fluorescent Protein. Science. 333(6042): 642–646, July 2011.

[6] Filonov, G. S., Moon, J. D., Svensen, N., and Jaffrey, S. R.: Broccoli: Rapid Selection of an RNA Mimic of Green Fluorescent Protein by Fluorescence-Based Selection and Directed Evolution. J. Am. Chem. Soc. 136(46): 16299–16308, November 2014.

[7] Autour, A., Jeng, S., Cawte, A., Abdolahzadeh, A., Galli, A., Panchapakesan, S. S. S., Rueda, D., Ryckelynck, M., and Unrau, P. J.: Fluorogenic RNA Mango aptamers for imaging small non-coding RNAs in mammalian cells. Nature Communications. 9(1): 656, February 2018.

[8] Dolgosheina, E. V., Jeng, S. C. Y., Panchapakesan, S. S. S., Cojocaru, R., Chen, P. S. K., Wilson, P. D., Hawkins, N., Wiggins, P. A., and Unrau, P. J.: RNA Mango Aptamer-Fluorophore: A Bright, High-Affinity Complex for RNA Labeling and Tracking. ACS Chem. Biol. 9(10): 2412–2420, October 2014.

[9] Fusco, D., Accornero, N., Lavoie, B., Shenoy, S. M., Blanchard, J.-M., Singer, R. H., and Bertrand, E.: Single mRNA Molecules Demonstrate Probabilistic Movement in Living Mammalian Cells. Current Biology. 13(2): 161–167, January 2003.

[10] Della Ragione, F., Gagliardi, M., D'Esposito, M., and Matarazzo, M. R.: Non-coding RNAs in chromatin disease involving neurological defects. Front. Cell. Neurosci. 8, 2014.

[11] Anastasiadou, E., Jacob, L. S., and Slack, F. J.: Non-coding RNA networks in cancer. Nature Reviews Cancer. 18(1): 5–18, January 2018.

[12] Rinn, J. L. and Chang, H. Y.: Genome Regulation by Long Noncoding RNAs. Annual Review of Biochemistry. 81(1): 145–166, 2012.

[13] Rinn, J. L., Euskirchen, G., Bertone, P., Martone, R., Luscombe, N. M., Hartman, S., Harrison, P. M., Nelson, F. K., Miller, P., Gerstein, M., Weissman, S., and Snyder, M.: The transcriptional activity of human Chromosome 22. Genes Dev. 17(4): 529–540, February 2003.

[14] Tanaka Ritsuko, , Satoh Hitoshi, , Moriyama Masatsugu, , Satoh Kasumi, , Morishita Yasuyuki, , Yoshida Syouko, , Watanabe Toshiki, , Nakamura Yoshikazu, , and Mori Shigeo, : Intronic U50 small-nucleolar-RNA (snoRNA) host gene of no protein-coding potential is mapped at the chromosome breakpoint t(3;6)(q27;q15) of human B-cell lymphoma. Genes to Cells. 5(4): 277–287, December 2001.

[15] Damas, N. D., Marcatti, M., Côme, C., Christensen, L. L., Nielsen, M. M., Baumgartner, R., Gylling, H. M., Maglieri, G., Rundsten, C. F., Seemann, S. E., Rapin, N., Thézenas, S., Vang, S., Ørntoft, T., Andersen, C. L., Pedersen, J. S., and Lund, A. H.: *SNHG5* promotes colorectal cancer cell survival by counteracting STAU1-mediated mRNA destabilization. Nature Communications. 7: 13875, December 2016.

[16] Zhao, L., Guo, H., Zhou, B., Feng, J., Li, Y., Han, T., Liu, L., Li, L., Zhang, S., Liu, Y., Shi, J., and Zheng, D.: Long non-coding RNA SNHG5 suppresses gastric cancer progression by trapping MTA2 in the cytosol. Oncogene. 35(44): 5770–5780, November 2016.

[17] He, B., Bai, Y., Kang, W., Zhang, X., and Jiang, X.: LncRNA SNHG5 regulates imatinib resistance in chronic myeloid leukemia via acting as a CeRNA against MiR-205-5p. Am J Cancer Res. 7(8): 1704–1713, PMC5574942, August 2017.

[18] Yan, L., Wang, S., Li, Y., Tognetti, L., Tan, R., Zeng, K., Pianigiani, E., Mi, X., Li, H., Fimiani, M., and Rubegni, P.: SNHG5 promotes proliferation and induces apoptosis in melanoma by sponging miR-155. RSC Advances. 8(11): 6160–6168, 2018.

[19] Ma, Z., Xue, S., Zeng, B., and Qiu, D.: lncRNA SNHG5 is associated with poor prognosis of bladder cancer and promotes bladder cancer cell proliferation through targeting p27. Oncology Letters. 15(2): 1924–1930, February 2018.

[20] Shen, H., Wang, Y., Shi, W., Sun, G., Hong, L., and Zhang, Y.: LncRNA SNHG5/miR-26a/SOX2 signal axis enhances proliferation of chondrocyte in osteoarthritis. Acta Biochim Biophys Sin (Shanghai). 50(2): 191–198, February 2018.

[21] Zheng, L., Hu, N., Guan, G., Chen, J., Zhou, X., and Li, M.: Long noncoding RNA SNHG6 promotes osteosarcoma cell proliferation through regulating p21 and KLF2. Archives of Biochemistry and Biophysics. Forthcoming 2018.

[22] Tutucci, E., Vera, M., Biswas, J., Garcia, J., Parker, R., and Singer, R. H.: An improved MS2 system for accurate reporting of the mRNA life cycle. Nature Methods. 15(1): 81–89, January 2018.

[23] Iii, R. J. T., Demeshkina, N. A., Lau, M. W. L., Panchapakesan, S. S. S., Jeng, S. C. Y., Unrau, P. J., and Ferré-D'Amaré, A. R.: Structural basis for high-affinity fluorophore binding and activation by RNA Mango. Nature Chemical Biology. 13(7): 807–813, July 2017.

[24] Warner, K. D., Chen, M. C., Song, W., Strack, R. L., Thorn, A., Jaffrey, S. R., and Ferré-D'Amaré, A. R.: Structural basis for activity of highly efficient RNA mimics of green fluorescent protein. Nature Structural & Molecular Biology. 21(8): 658–663, August 2014.

[25] Jeng, S. C. Y., Chan, H. H. Y., Booy, E. P., McKenna, S. A., and Unrau, P. J.: Fluorophore ligand binding and complex stabilization of the RNA Mango and RNA Spinach aptamers. RNA. 22(12): 1884–1892, January 2016.

[26] Morisaki, T., Lyon, K., DeLuca, K. F., DeLuca, J. G., English, B. P., Zhang, Z., Lavis, L. D., Grimm, J. B., Viswanathan, S., Looger, L. L., Lionnet, T., and Stasevich, T. J.: Real-time quantification of single RNA translation dynamics in living cells. Science. 352(6292): 1425–1429, June 2016.

[27] Braselmann, E., Wierzba, A. J., Polaski, J. T., Chromiński, M., Batan, D., Gryko, D., Batey, R. T., and Palmer, A. E.: Development of a riboswitch-based platform for live cell imaging of RNAs in mammalian cells. bioRxiv. 199240 pp, October 2017.

[28] McNeil, P. L. and Warder, E.: Glass beads load macromolecules into living cells. Journal of Cell Science. 88(5): 669–678, December 1987.

[29] Hayashi-Takanaka, Y., Yamagata, K., Wakayama, T., Stasevich, T. J., Kainuma, T., Tsurimoto, T., Tachibana, M., Shinkai, Y., Kurumizaka, H., Nozaki, N., and Kimura, H.: Tracking epigenetic histone modifications in single cells using Fab-based live endogenous modification labeling. Nucleic Acids Res. 39(15): 6475–6488, August 2011.

[30] Rosendahl, M. S., Omann, G. M., and Leonard, N. J.: Synthesis and biological activity of a profluorescent analogue of coenzyme B12. PNAS. 79(11): 3480–3484, June 1982.

[31] Lee, M. and Grissom, C. B.: Design, Synthesis, and Characterization of Fluorescent Cobalamin Analogues with High Quantum Efficiencies. Org. Lett. 11(12): 2499–2502, June 2009.

[32] Smeltzer, C. C., Cannon, M. J., Pinson, P. R., Munger, J. D., West, F. G., and Grissom, C. B.: Synthesis and Characterization of Fluorescent Cobalamin (CobalaFluor) Derivatives for Imaging. Org. Lett. 3(6): 799–801, March 2001.

[33] Porter, E. B., Polaski, J. T., Morck, M. M., and Batey, R. T.: Recurrent RNA motifs as scaffolds for genetically encodable small-molecule biosensors. Nature Chemical Biology. 13(3): 295–301, March 2017.

[34] Chromiński, M., Lewalska, A., Karczewski, M., and Gryko, D.: Vitamin B12 Derivatives for Orthogonal Functionalization. J. Org. Chem. 79(16): 7532–7542, August 2014.

[35] Fesinmeyer, R. M., Hudson, F. M., and Andersen, N. H.: Enhanced Hairpin Stability through Loop Design: The Case of the Protein G B1 Domain Hairpin. J. Am. Chem. Soc. 126(23): 7238–7243, June 2004.

[36] Shell, T. A. and Lawrence, D. S.: Vitamin B12: A Tunable, Long Wavelength, Light-Responsive Platform for Launching Therapeutic Agents. Acc. Chem. Res. 48(11): 2866–2874, November 2015.

[37] Shell, J. R., Rodgers, Z. L., and Lawrence, D. S.: Tunable Visible and Near-IR Photoactivation of Light-Responsive Compounds by Using Fluorophores as Light-Capturing Antennas. Angew. Chem. Int. Ed. 53(3): 875–878, January 2014.

[38] Cochran, A. G., Skelton, N. J., and Starovasnik, M. A.: Tryptophan zippers: Stable, monomeric $\beta$-hairpins. PNAS. 98(10): 5578–5583, May 2001.

[39] Kier, B. L. and Andersen, N. H.: Probing the Lower Size Limit for Protein-Like Fold Stability: Ten-Residue Microproteins With Specific, Rigid Structures in Water. J. Am. Chem. Soc. 130(44): 14675–14683, November 2008.

[40] Andersen, N. H., Olsen, K. A., Fesinmeyer, R. M., Tan, X., Hudson, F. M., Eidenschink, L. A., and Farazi, S. R.: Minimization and Optimization of Designed $\beta$-Hairpin Folds. J. Am. Chem. Soc. 128(18): 6101–6110, May 2006.

[41] Kolb Hartmuth C., , Finn M. G., , and Sharpless K. Barry, : Click Chemistry: Diverse Chemical Function from a Few Good Reactions. Angewandte Chemie International Edition. 40(11): 2004–2021, May 2001.

[42] Patterson, D. M., Nazarova, L. A., and Prescher, J. A.: Finding the Right (Bioorthogonal) Chemistry. ACS Chem. Biol. 9(3): 592–605, March 2014.

[43] Chromiński, M., Lewalska, A., and Gryko, D.: Reduction-free synthesis of stable acetylide cobalamins. Chemical Communications. 49(97): 11406–11408, 2013.

[44] Ruetz Markus, , Salchner Robert, , Wurst Klaus, , Fedosov Sergey, , and Kräutler Bernhard, : Phenylethynylcobalamin: A Light-Stable and Thermolysis-Resistant Organometallic Vitamin B12 Derivative Prepared by Radical Synthesis. Angewandte Chemie International Edition. 52(43): 11406–11409, September 2013.

[45] Pathare, P. M., Wilbur, D. S., Heusser, S., Quadros, E. V., McLoughlin, P., and Morgan, A. C.: Synthesis of Cobalamin-Biotin Conjugates That Vary in the Position of Cobalamin Coupling. Evaluation of Cobalamin Derivative Binding to Transcobalamin II. Bioconjugate Chem. 7(2): 217–232, January 1996.

[46] Grimm, J. B., Muthusamy, A. K., Liang, Y., Brown, T. A., Lemon, W. C., Patel, R., Lu, R., Macklin, J. J., Keller, P. J., Ji, N., and Lavis, L. D.: A general method to fine-tune fluorophores for live-cell and *in vivo* imaging. Nature Methods. 14(10): 987–994, October 2017.

[47] Grimm, J. B., English, B. P., Chen, J., Slaughter, J. P., Zhang, Z., Revyakin, A., Patel, R., Macklin, J. J., Normanno, D., Singer, R. H., Lionnet, T., and Lavis, L. D.: A general method to improve fluorophores for live-cell and single-molecule microscopy. Nature Methods. 12(3): 244–250, March 2015.

[48] Li, G.-W. and Xie, X. S.: Central dogma at the single-molecule level in living cells. Nature. 475(7356): 308–315, July 2011.

[49] Cabili, M. N., Dunagin, M. C., McClanahan, P. D., Biaesch, A., Padovan-Merhar, O., Regev, A., Rinn, J. L., and Raj, A.: Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. Genome Biology. 16: 20, January 2015.

[50] Andrecka, J., Lewis, R., Brückner, F., Lehmann, E., Cramer, P., and Michaelis, J.: Single-molecule tracking of mRNA exiting from RNA polymerase II. PNAS. 105(1): 135–140, January 2008.

[51] Katz, Z. B., English, B. P., Lionnet, T., Yoon, Y. J., Monnier, N., Ovryn, B., Bathe, M., and Singer, R. H.: Mapping translation 'hot-spots' in live cells by tracking single molecules of mRNA and ribosomes. eLife Sciences. 5: e10415, January 2016.

[52] Yamagishi, M., Ishihama, Y., Shirasaki, Y., Kurama, H., and Funatsu, T.: Single-molecule imaging of $\beta$-actin mRNAs in the cytoplasm of a living cell. Experimental Cell Research. 315(7): 1142–1147, April 2009.

[53] Halstead, J. M., Lionnet, T., Wilbertz, J. H., Wippich, F., Ephrussi, A., Singer, R. H., and Chao, J. A.: An RNA biosensor for imaging the first round of translation from single cells to living animals. Science. 347(6228): 1367–1671, March 2015.

[54] Fiedler, B. L., Van Buskirk, S., Carter, K. P., Qin, Y., Carpenter, M. C., Palmer, A. E., and Jimenez, R.: Droplet Microfluidic Flow Cytometer For Sorting On Transient Cellular Responses Of Genetically-Encoded Sensors. Anal. Chem. 89(1): 711–719, January 2017.

[55] Dean, K. M., Davis, L. M., Lubbeck, J. L., Manna, P., Friis, P., Palmer, A. E., and Jimenez, R.: High-Speed Multiparameter Photophysical Analyses of Fluorophore Libraries. Anal. Chem. 87(10): 5026–5030, May 2015.

[56] Park, J. G. and Palmer, A. E. Quantitative Measurement of $Ca^{2+}$ and $Zn^{2+}$ in Mammalian Cells Using Genetically Encoded Fluorescent Biosensors. In Fluorescent Protein-Based Biosensors, Methods in Molecular Biology, pp 29–47. Humana Press, Totowa, NJ, 2014.

[57] Zurla, C., Lifland, A. W., and Santangelo, P. J.: Characterizing mRNA Interactions with RNA Granules during Translation Initiation Inhibition. PLOS ONE. 6(5): e19727, May 2011.

[58] Jain, S., Wheeler, J. R., Walters, R. W., Agrawal, A., Barsic, A., and Parker, R.: ATPase-Modulated Stress Granules Contain a Diverse Proteome and Substructure. Cell. 164(3): 487–498, January 2016.

[59] Nelles, D. A., Fang, M. Y., O'Connell, M. R., Xu, J. L., Markmiller, S. J., Doudna, J. A., and Yeo, G. W.: Programmable RNA Tracking in Live Cells with CRISPR/Cas9. Cell. 165(2): 488–496, April 2016.

[60] Mueller, F., Senecal, A., Tantale, K., Marie-Nelly, H., Ly, N., Collin, O., Basyuk, E., Bertrand, E., Darzacq, X., and Zimmer, C.: FISH-quant: Automatic counting of transcripts in 3D FISH images. Nature Methods. 10(4): 277–278, April 2013.

[61] Mairal, T., Özalp, V. C., Sánchez, P. L., Mir, M., Katakis, I., and O'Sullivan, C. K.: Aptamers: Molecular tools for analytical applications. Anal Bioanal Chem. 390(4): 989–1007, February 2008.

[62] Cho, E. J., Lee, J.-W., and Ellington, A. D.: Applications of Aptamers as Sensors. Annual Review of Analytical Chemistry. 2(1): 241–264, 2009.

[63] Lorsch, J. R. and Szostak, J. W.: In vitro selection of RNA aptamers specific for cyanocobalamin. Biochemistry. 33(4): 973–982, February 1994.

[64] Trausch, J. J. and Batey, R. T. Chapter Three - Design of Modular "Plug-and-Play" Expression Platforms Derived from Natural Riboswitches for Engineering Novel Genetically Encodable RNA Regulatory Devices. In Burke-Aguero, D. H. (Ed.): Methods in Enzymology, volume 550 of *Riboswitches as Targets and Tools*, pp 41–71. Academic Press, January 2015.

[65] Park Eonyoung, and Maquat Lynne E., : Staufen-mediated mRNA decay. Wiley Interdisciplinary Reviews: RNA. 4(4): 423–435, June 2013.