SIGNIFICANCE

RNA lies at the center of cellular function. Its most appreciated function 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.[1] *X, Y, and Z (maybe the proposed test systems??)* have been recently found to have crucial RNA components. *Need more on these systems here. Talk about how localization is important.* This importance for cellular function necessitates a toolset of probes to study these RNAs as they traverse the cell.

BACKGROUND

Protein studies benefit from a large imaging toolkit that has been developed over the last *XX* 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 remain limited. The most popular systems to date include dye-binding aptamers (Spinach[2], Broccoli[3], and Mango[4, 5]), and RNA-binding protein fusions (MS2-FPs).[6]

The most analogous to fluorescent proteins, dye-binding aptamers utilize exogenously administered dyes that give fluorescence induction upon binding their RNA partner.[2–5] 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 non-native structure.[7] Additionally, though fluorescence turn-on is excellent *in vitro*, signal induction in cells is low, most likely due to nonspecific binding of the dye.

RNA-binding protein fusions are a much more robust technique for imaging RNA transcripts.[6] 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,[6, 8] the resulting protein-RNA complex is prohibitively large for many studies.

Discuss RNA Fish here??

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 aptamer, 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 (Fig. 1).[9] The two-component system utilizes a synthetic fluorophore-quencher pair, and a genetically-encoded aptamer. When the construct binds the transcribed aptamer, 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, Fig. 1B).[10] Conveniently, cobalamin is known to act as a fluorescence quencher for a variety of fluorophores.[11–13] 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 (Fig. 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 other aptamer-based techniques. 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 stress granule (SG) detection in mammalian cells.

Though promising, the initial iteration of Riboglow has significant room for improvement. With initial constructs, signal induction upon aptamer binding never exceeded seven fold. Additionally, to effectively detect stress gran-
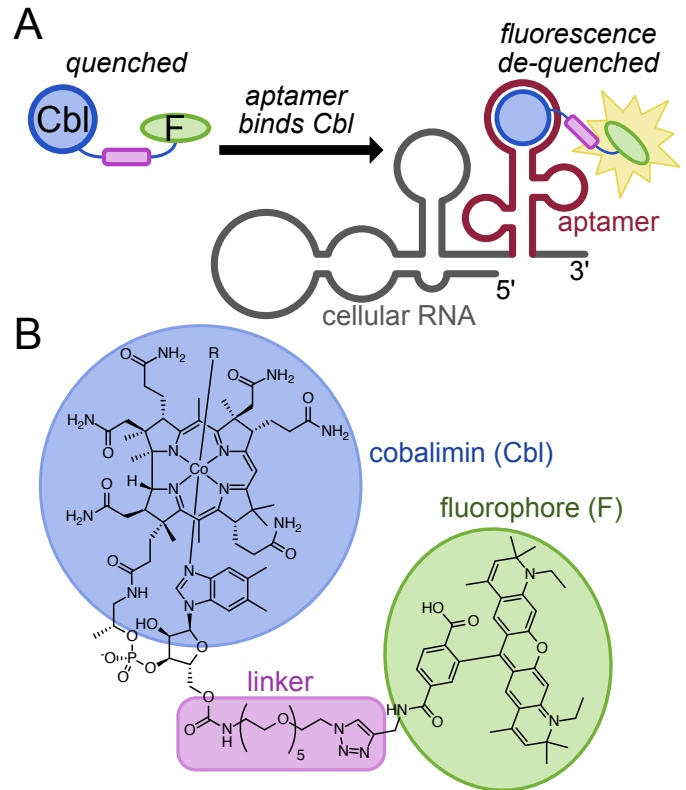
ules, four aptamers had to be placed in series to concentrate fluorescence signal. This high background precluded single-molecule imaging. Finally, the strategy cannot currently be used to monitor the location of multiple transcripts in tandem. Herein, I will propose strategies to overcome these limitations through simultaneous modulation of the small molecule construct and aptamer 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 sequence of the aptamer itself. I'll design a screen for brightness that will select the best aptamer sequence to match the optimized probe structure (Aim 2). This new, brighter pair will be tested for single-molecule detection. Finally, I will use SELEX to identify mutually orthogonal probe-aptamer pairs for multicomponent RNA imaging (Aim 3).

APPROACH <u>Aim 1</u>. 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. *To optimize probe function, I will vary linker composition, attachment point, and pendant fluorophore.*
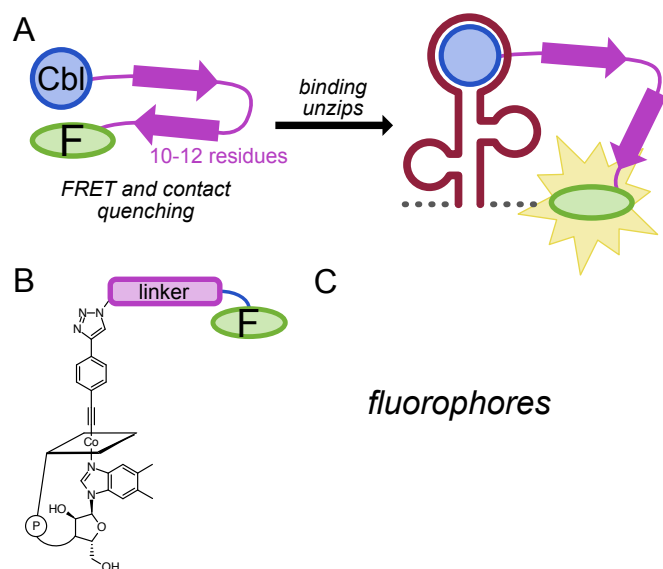
Figure 2: A) Linkers. B) Conjugation sites. C) Fluorophores.

Ideally, in the unbound state, the cobalamin and fluorophore would be closely associated to maximize FRET and contact quenching.[12] In the RNA-bound state, the molecules would reside at their maximal distance to promote fluorescence. To strike this balance, I propose the use of a synthetic beta turn as the linker between cobalamin and the fluorophore (Fig. 2A). Such a linker would hold the molecules close in solution, but would be linearized upon binding to the aptamer. A number of such beta turns have been developed. These motifs are as small as twelve amino acids and many are stable to denaturation up to 85 C.[14] 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). When the cobalamin is bound by an aptamer, steric occlusion would force the beta turn to unfold to place the fluorophore-quencher pair at a larger distance. The amino acids of the peptide linker will be varied to adjust the stability of the fold. *More or less specific here? Discuss other linker possibilities?* The new constructs will be tested for their ability to quench fluorescence, and their fluorescence signal induction in the presence of the Riboglow aptamer.

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,[15] others have found alkynyl modifications to be stable to air and light.[16, 17] Following this precedent, I will synthesize a cobalamin with an alkyne handle attached to the cobalt metal center (Fig. 2B). Such a molecule has already been synthesized in the lab of Dorota Gryko (see Gryko letter of support), and shown to be amenable to functionalization via dipolar azide-alkyne cycloaddition (AAC).[18] The alkyne handle will be readily conjugated to a variety of linkers with terminal azides. *More detail here?* These constructs will be evaluated for brightness in the presence and absence of the cobalamin aptamer.

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. I believe that the Janelia Fluor series of dyes is perfectly suited for our application (see Lavis support letter).[19] *Not sure how much else is needed here.*

Summary and alternate approaches:

Aim 2. Adapt Riboglow for superresolution imaging.

Visualization of the lifecycle of single RNA transcripts as they move throughout the cell remains a holy grail of RNA imaging.*[Cite]* Such a goal is possible via superresolution imaging and a probe with adequate photostability, and should be achievable with the Riboglow platform. With the wide variety of new probe constructs developed in Aim 1, changes will be made to the sequence of the cobalamin aptamer to increase fluorescence turn-on. *A directed evolution screen for fluorescence brightness will identify optimal aptamer sequences that promote fluorescence signal induction.*

First, a library will be designed that varies the environment surrounding the binding pocket of the cobalamin aptamer (Fig. 3A). Though the guidance of Robert Batey (see Batey support letter), sites will be chosen to retain binding of the cobalamin, yet maximize the distance between the quencher and the fluorophore upon binding.[10] The Palmer lab is a leader in technologies for tool development in mammalian cells.[20, 21] This expertise will be leveraged for screening libraries of cobalamin aptamers (Fig. 3B). Libraries of transcripts will be transduced into mammalian cells, the probe of interest will be administered, and cells will be sorted via flow cytometry. Cells that show elevated brightness relative to a fluorescent protein expression control will be sequestered. In this way, libraries of up to one million members will be screened. Bright variants will be collected, cultured, and resubjected to sorting until only bright variants remain. Sequences will be evaluated through deep sequencing.

Candidate probes found through cell sorting will be verified in single molecule imaging experiments under the guidance of Professor John Rinn... *Not sure what I should propose to image here, especially with respect to proving that single molecules can be visualized.*

Figure 3. A) Riboswitch sites for mutation will be targeted based on potential contact with the linker and fluorophore. PDB: 4FRN[10] B) RNA sequences will be screened for brightness relative to a fluorescent protein control.

Summary and alternate approaches:

Aim 3. 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 through the cell. *Independent RNA transcripts are implicated to interact in X and Y ways.* The modularity of the Riboglow platform is well suited to enable such an experiment. Probes will be distinguished through the use of different fluorophores, and different aptamer sequences will be used to bind each structurally different probe in a mutually exclusive manner (Fig. 4B).

Fortunately, screening for substrate selectivity is well-precedented in RNA engineering through systematic evolution of ligands by exponential enrichment (SELEX).[22, 23] In fact, *de novo* aptamers have already been developed for cobalamin and a few of its analogs via SELEX.[24] This important precedent shows that even small changes to the structure of the cobalamin can be distinguished by engineered RNA, and implies that no additional changes will need to be made to the cobalamin structure apart from those constructs already proposed above.

Importantly, for our selection experiments, we will not be starting 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. 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.[25] The selection will be carried out as shown in Figure 4A. Briefly, 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 aptamers will be incubated with the beads, and unbound sequences will be rinsed away. In a second rinsing step, the probe (or probes) intended to be orthogonal to the construct under selection will be incubated with the beads to wash away any aptamers that do not bind selectively. *Is this kind of negative screen known?* Aptamers that bind selectively will be collected off the beads and amplified (via reverse transcription, PCR amplification, and transcription) to undergo another round of selection. Aptamers will be deep sequenced to identify library convergence and optimal clones. This process will be repeated for each probe of interest, with negative screens against each other probe that is intended for multiplexed use.
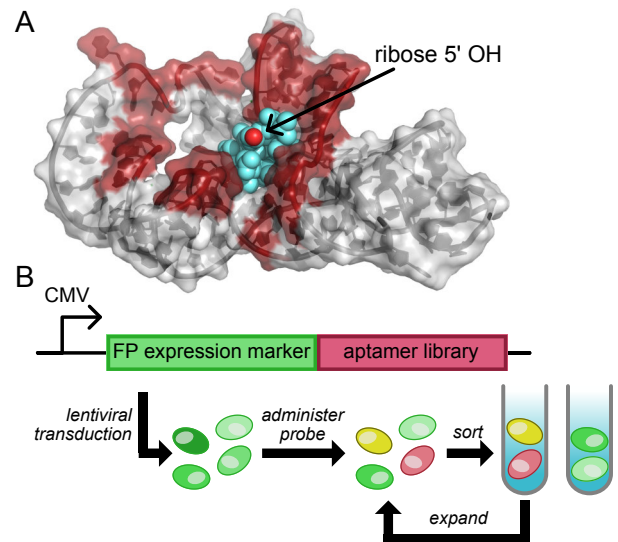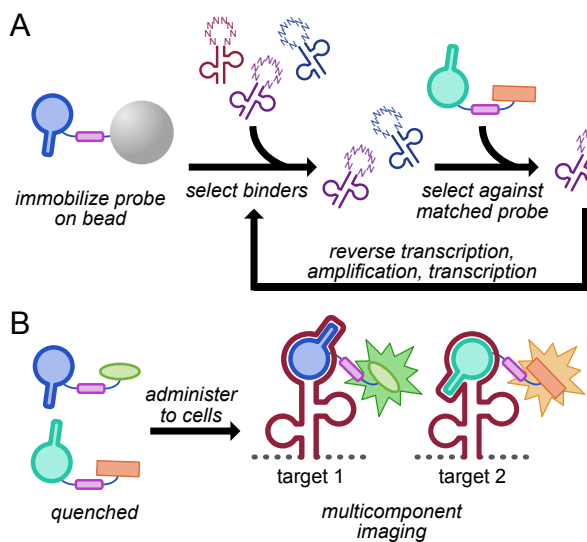
Once mutually-selective Riboglow probes are identified, they will be tested in mammalian cells... *Maybe look at two differently localized RNAs that both migrate to SGs upon arsenite addition?* **Summary and alternate approaches:**

A

immobilize probe
on bead

select binders

select against
matched probe

reverse transcription,
amplification, transcription

B

administer
to cells

quenched

target 1

target 2

multicomponent
imaging

Figure 4. A) SELEX will be used to screen for aptamers 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.

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