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 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 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 fuzed 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 small 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 small, 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 superresolution imaging.
The molecules developed in *Aim 1* will be screened against libraries of aptamers to further improve probe properties. Libraries of RNA aptamers will be generated and screening will be carried out in mammalian cells via flow cytometry. Candidate probes will be verified through superresolution imaging of stress granules 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 X and Y as they interact in the cell.*

SIGNIFICANCE RNA lies at the center of cellular function. Its most appreciated function is to carry protein blueprints from the genetic code of the nucleus to be manufactured into the protein machines of the cell at the ribosome. Only recently have researchers begun to appreciate its myriad of other functions, many of which also lie at the center of important cellular processes. *X, Y, and Z (maybe the proposed test systems??)* have been recently found to have crucial RNA components. This importance for cellular function necessitates a toolset of probes to study these RNAs as they traverse the cell. Indeed, the location of these RNA transcripts has been implicated to be instrumental in their function.[2]

BACKGROUND Proteins 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[5], Broccoli[6], and Mango[7, 8]), RNA-binding protein fusions (MS2-FPs), and RNA hybridization probes (RNA FISH).

The most analogous to fluoresecent proteins, dye-binding aptamers utilize exogenously administered dyes that give fluorescence induction upon binding their aptamer.[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] Additionally, though fluorescence turn-on is excellent *in vitro*, signal induction in cells is low, *due to nonspecific binding of the dye [cite]*.

RNA-binding protein fusions are a much more robust technique for imaging RNA transcripts.[9] This technique often utilizes the MS2 bacteriophage coat protein that binds a 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, 10] the resulting protein-RNA complex is prohibitively large for many studies.

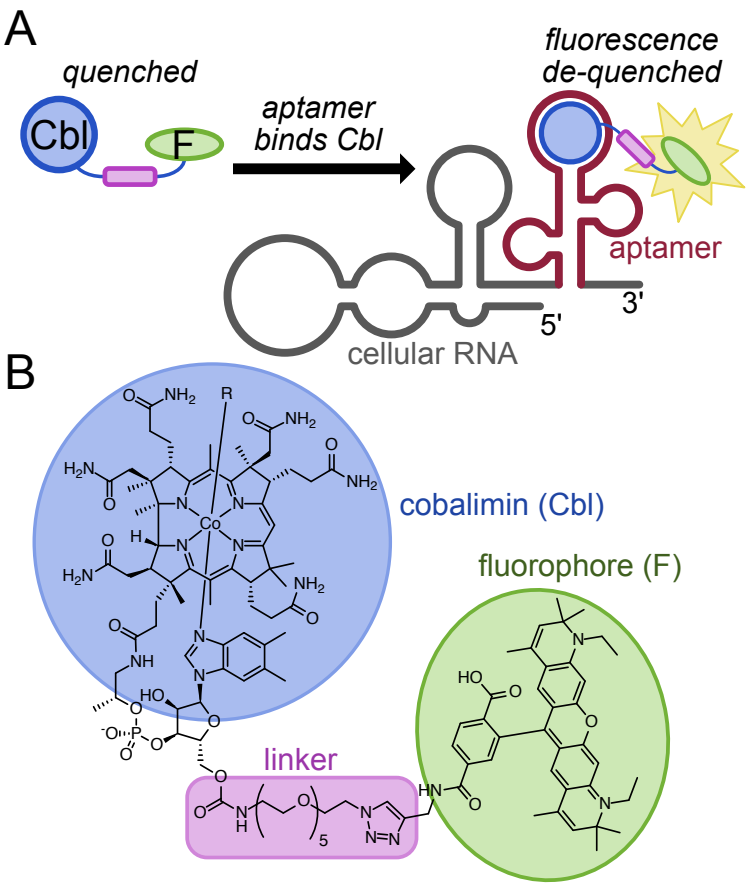


Figure 1. A) Cobalamin acts as a quenching and localization moiety to guide a fluorescent probe to an RNA transcript of interest. When unbound, fluorescence is quenched. In the presence of RNA tagged with the cobalamin aptamer, fluorescence is restored. B) Structure of the generation 1 Riboglow probe. 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.

Discuss RNA Fish here??

Riboglow is a recently developed platform in the Palmer lab that solves many of the drawbacks of the previously discussed techniques (Fig. 1). 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. Like the dye-binding aptamers, Riboglow utilizes a riboswitch receptor domain that natively binds vitamin B_{12} (cobalamin, Cbl, Fig. 1B).[4] Conveniently, cobalamin is known to act as a fluorescence quencher for a variety of fluorophores.*[cite]* 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).

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. Intuitively, the largest amount of quenching was observed in the probes with the shortest linkers, and the largest spectral overlap. *To optimize probe function, I will vary linker composition, linker attachment point, and pendant fluorophore.*

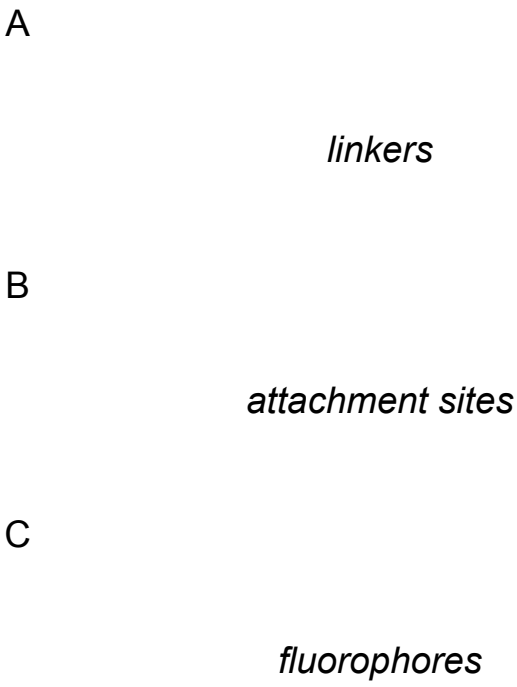


Figure 2: A) Linkers. B) Conjugation sites. C) Fluorophores.

Ideally, in the unbound state, the cobalamin and the fluorophore would be closely associated to maximize FRET and contact quenching.[11] 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. 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.[12] 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.

Discussion about changing linker position here.

The final variable in the molecular structure of the Riboglow probe is the fluorophore itself. Previous work showed that probes were quenched by the cobalamin center to varying degrees. Quenching correlated somewhat with the spectral overlap of the fluorophore and cobalamin absorption.

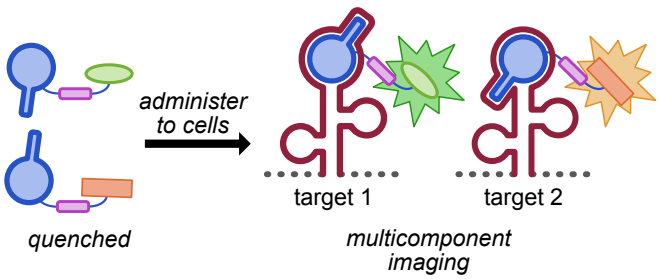
The new Riboglow probe constructs that I synthesize will be evaluated for brightness in the presence and absence of the cobalamin aptamer.

<u>Aim 2.</u> 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 should be possible via superresolution imaging and a probe with adequate photostability. Such requirements should be achievable with the Riboglow platform. With a variety of new probe constructs in hand, changes will be made to the cobalamin aptamer to increase fluorescence turn-on. The Palmer lab is a leader in technologies for tool development in mammalian cells.*[Cite]* This expertise will be leveraged for screening libraries of cobalamin aptamers in mammalian cells. Libraries of transcripts will be transduced into mammalian cells, the probe of interest will be administered, and cells will be sorted via flow cytometry. In this way, libraries of up to one million members will be screened. Bright variants will be collected, cultured, and resubjected to sorting until the library converges. Sequences will be evaluated through deep sequencing.

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

Don't forget Szostak precedent![13]



proposed structures here

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