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 B12) 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.*

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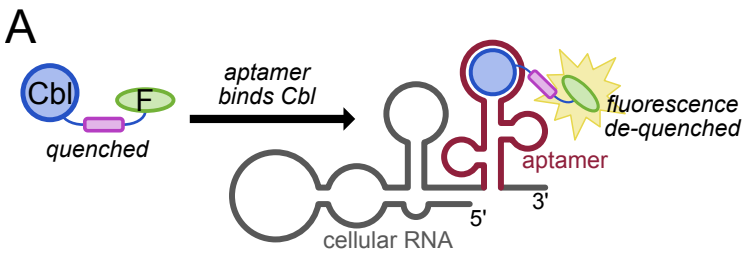

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Figure 1. A) Cobalamin acts as a quenching and localization moiety to guide a fluorescent probe to an RNA transcript of interest. When unbound, fluorescence is quenched. In the presence of RNA tagged with the cobalamin aptamer, fluorescence is restored.

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. Though some degree of quenching occurred in all of the constructs synthesized, the best probes always had the shortest linkers. Intuitively, the shortest linkers also gave the poorest fluorescence induction. *To increase quenching I will vary linker composition to maximize quenching in the unbound state, and minimize it in the bound state.* Ideally, in the unbound state, the cobalamin and the fluorophore would be closely associated to maximize FRET and contact quenching.[11] In the 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.

<u>Aim 2.</u> Adapt Riboglow for superresolution imaging.
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Aim 3. Develop mutually orthogonal Riboglow probes for multicomponent imaging.
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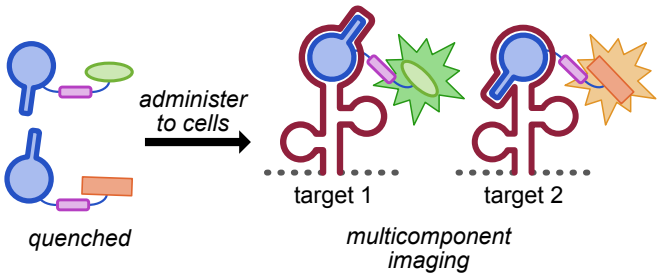

proposed structures here

Figure 2. A) Mutually orthogonal cobalamin analogs will enable multicomponent RNA imaging. Signal turn-on will only be observed in the presence of the matched pair. B) *Propose some structures.* C) SELEX will be used to screen for aptamers that bind each cobalamin in a mutually-exclusive manner.

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