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Optimization of the split-Spinach aptamer for monitoring contiguous RNA nanoparticle assembly

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by

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Abstract:

The emerging field of RNA nanotechnology takes advantage of the RNA's ability to self-assemble into exquisite structures. As nanoparticle design continues to advance and move into increasingly complex biological systems, tools to monitor their assembly and location will be of great importance. Here, a split-aptamer system is used to monitor assembly of a six-membered nanoring based on fluorescence feedback of a fluorophore. First, the split-aptamer is designed into two of the six pieces of the ring. Through mutation and deletion, we optimize the fluorescence feedback established when a six membered nanoparticle assembles, compared to partial assembly. We demonstrate that with these new versions of the aptamer, the full assembly can be monitored and distinguished form partial assembly. Finally, the nanoring and aptamer are transcribed from DNA and assembled, to demonstrate the potential for *in vivo* application.

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Introduction

RNA nanotechnology exploits the formation of programmable base pairs and folding patterns of RNA to construct materials with precise, predefined shapes.[1–6] Using RNA as a building material includes benefits associated with biocompatibility, the introduction of biological functions, and the potential to isothermally fold nanoparticles directly from DNA transcripts. RNA nanoparticles have a variety of perceived uses including the delivery of therapeutics, as stable scaffolds for the addition of functional moieties, and as molecular signaling devices.[7] While much progress has been made in the manufacturing of rationally designed RNA structures, few tools exist to permit the monitoring of their assembly and/or the subsequent tracking of wholly formed nanoparticles.

As the design and utilization of nanostructures with increased complexity progresses, new methods and systems intended to monitor and verify the assembly of nanoparticles will be required to push the field of RNA nanotechnology forward. A current strategy in development to visualize RNA involves the use of RNA aptamer and fluorophore pairs. Such aptamers possess an affinity for a specific small molecule that fluoresces when bound by the aptamer.[8] The Broccoli aptamer has been previously split and utilized to monitor the assembly of two RNA strands.[8,9] The Spinach aptamer, as well, has been split into two, where the combination of the halves, in the presence of a small molecular fluorophore known as DFHBI, produces a fluorescent signal.[10,11]

Fluorescent-based label-free RNA tracking methods offer much promise. But, current techniques are limited in their ability to report on the assembly of more than two RNA strands. Because RNA nanoparticles are typically composed of many unique strands of RNA, the ability to monitor multiple RNA strands (i.e. two or more) is a primary requirement for the maturation of complex nanoparticles seeking broader applications in our view. Furthermore, while RNA light-up aptamers provide attractive, non-invasive means to monitor RNA nanostructures, they have not been used to monitor more than direct strand-strand interactions.

To expand and prove their effectiveness for tracking and monitoring RNA nanostructure assembly, we set out to integrate the split-Spinach aptamer into the previously reported RNA nanoring. Herein, we demonstrate that the split-Spinach aptamer can monitor the assembly of six strands of RNA. Furthermore, we demonstrate that the integrated light up aptamer has the ability to distinguish between full and partial assembly of a the six-stranded nanoparticle.[11] In doing so, we believe this to be the first system developed with the ability to detect adjacent, long-range tertiary interactions not directly linked to the aptamer itself.

Results & Discussion

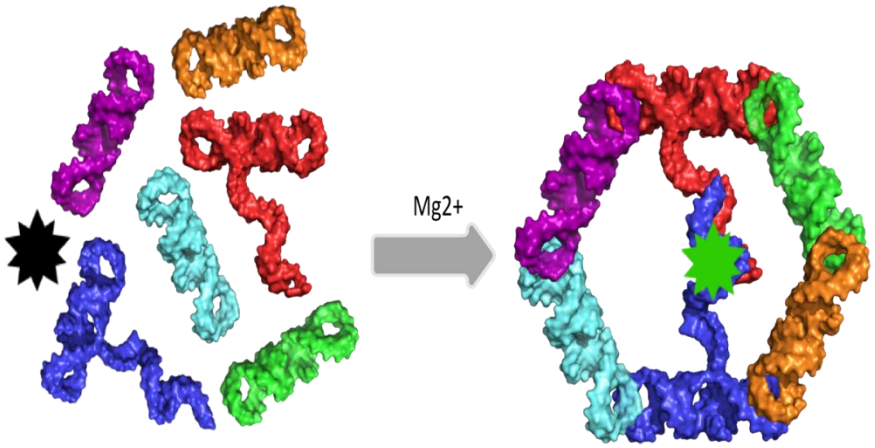
Initial Design

The goal of this research was to develop a system to detect RNA nanoring assembly by incorporating two halves of the split-Spinach aptamer into two of the six nanoring strands. To begin, the crystal structure of the full-length aptamer was artificially designed in two of the six-membered ring, and evaluated *in silico*.[12,13] For the ring to assemble with the aptamer in the middle, each ring strand must fold appropriately to include their respective kissing loops.[14] As well, the aptamer strands must retain free 3' ends for formation of the G-quadruplex necessary for the binding of DFHBI and fluorescence feedback.[15] We cut away portions of the two stems surrounding the fluorophore binding pocket until the aptamer fit inside the interior of the nanoring (**Figure 1A**). Initially, we wanted to make the stems long enough to ensure that they would properly connect to appropriate aptamer function. The aptamer was tethered to the helical struts of the nanoring via flexible single-stranded linkers. In this manner, the linker constituted a second variable to affect aptamer formation within the nanoring. Our original model indicated that the appropriate length of the linker was either five or six nucleotides because these lengths retained

the kissing loops. Modeling of the aptamer also showed that the stem of the aptamer could be six or seven nucleotides (**Figure 1B**).

Using the model as our guide, we tested a series of RNA sequences with variable stem and linker lengths. Testing of the aptamer i*n vitro* established that stem lengths of five base pairs on one side of the aptamer and six base pairs on the other—in conjunction with linkers of five nucleotides—produced the highest level of fluorescence. This data suggests that the longer stem lengths may have not fit properly within the interior of the ring. Additionally, the data suggests that the five-nucleotides-long aptamer was not sufficient to allow the aptamer to span the width of the ring's interior and adequately form. (**Figure 1B**).

A



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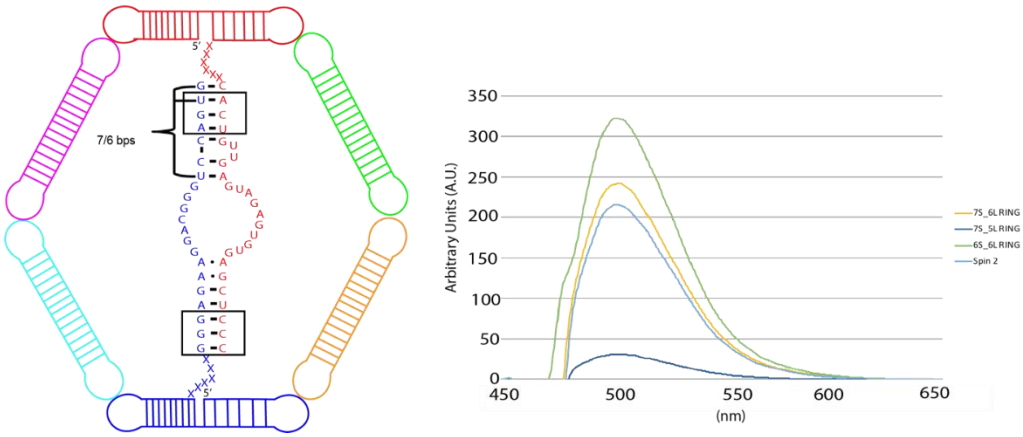


Figure 1 A demonstration of the bifurcated split-Spinach aptamer, grafted into two strands of the ring, forming the G-quadruplex necessary for binding of DFHBI and fluorescence. **B** *Left* The coded aptamer. The linker which connects the body of the aptamer to the ring is shown as nucleotides X as the identity of each nucleotide is varied later. The "stem" describes the base pairs formed at the top and bottom of the aptamer, before the linkers. The blue strand henceforth called the "A-strand" and the red the "B-strand." The boxed nucleotides become the only nucleotides

varied in this experiment as explained later. *Right* Fluorescence data recovered from testing differing aptamer stem and linker lengths. Because the green graph (6S_6L) gave the highest fluorescence peak, a linker and stem length of six nucleotides is optimal.

Further Optimization

Split aptamer assembly has been demonstrated for two separate RNA stands with the Broccoli[8] and the Spinach aptamer.[11] The current utilization of split-aptamer systems, however, are limited to the monitoring of two RNA strands. In our case, the split-aptamer system required further optimization to monitor the assembly of six RNA strands. Thus, an important aspect of our system involved engineering the split-aptamer to distinguish between partially and fully assembled nanorings. The best split-aptamer design is one that would fluoresce when the full ring forms, and not when part of the ring forms. To achieve this goal, we set out to engineer a split-aptamer system that abided by a Goldilocks-like principle: it would require just the right balance between being stable, but not too stable. It needed just the right amount of stabilization/destabilization.

Therefore, point mutation and deletion editing of the split-Spinach aptamer nucleotides was used to disrupt aptamer formation for partial ring formation events. Initial experiments demonstrated that mutation of nucleotides in most of the aptamer completely hindered aptamer formation (data not shown here). Yet, six base pair locations, the three base pairs formed by the 3' end nucleotides of the respective aptamer halves, were identified as mutable. Therefore, base pairs were systematically mutated and deleted at these locations with the goal of destabilizing partial ring assemblies so that the aptamer would not form. (**Figure 2**). It was thought that adding mismatched base pairs at any of the six locations would destabilize the aptamer. And, contrarily, that adding GCs to the aptamer would increase stabilization.

The various aptamers were evaluated by fluorescence feedback. Mixtures of all ring strands (6/6) were compared to mixtures of the two (2/6) aptamer strands (**Figure 2**). Deletion of a C nucleotide clearly gave favorable disruption for GGG-AGU/BACU-CC_.1 (**Figure 2**). A greater than tenfold fluorescence gap between 2/6 and 6/6 was found in the substitution of a G nucleotide the A nucleotide in the B-strand of GGG-AGU/BGCU-CC_.1. Again, this meant that destabilization was working to find the goldilocks middle.

This was not always successful, of course. In the case of CCC-AGU/BGCU-GGG.7, a simple switch of the location of the three Gs with the three Cs from A-strand to B-strand, paired with a G to U mismatch, left the split-aptamer completely unable to assemble because it was destabilized (**Figure 2)**. That is why the fluorescence for both 2/6 and 6/6 is so poor. In fact, for all aptamer attempts where the G nucleotides were swapped with the C nucleotides, fluorescence feedback for the 6/6 was low (**Figure 2**). In any case, all of this data was used as an initial screen for all aptamers. It was empirically determined that a successful aptamer gives ~10-fold fluorescence feedback for the 6/6 mixture compared to the 2/6 mixture.

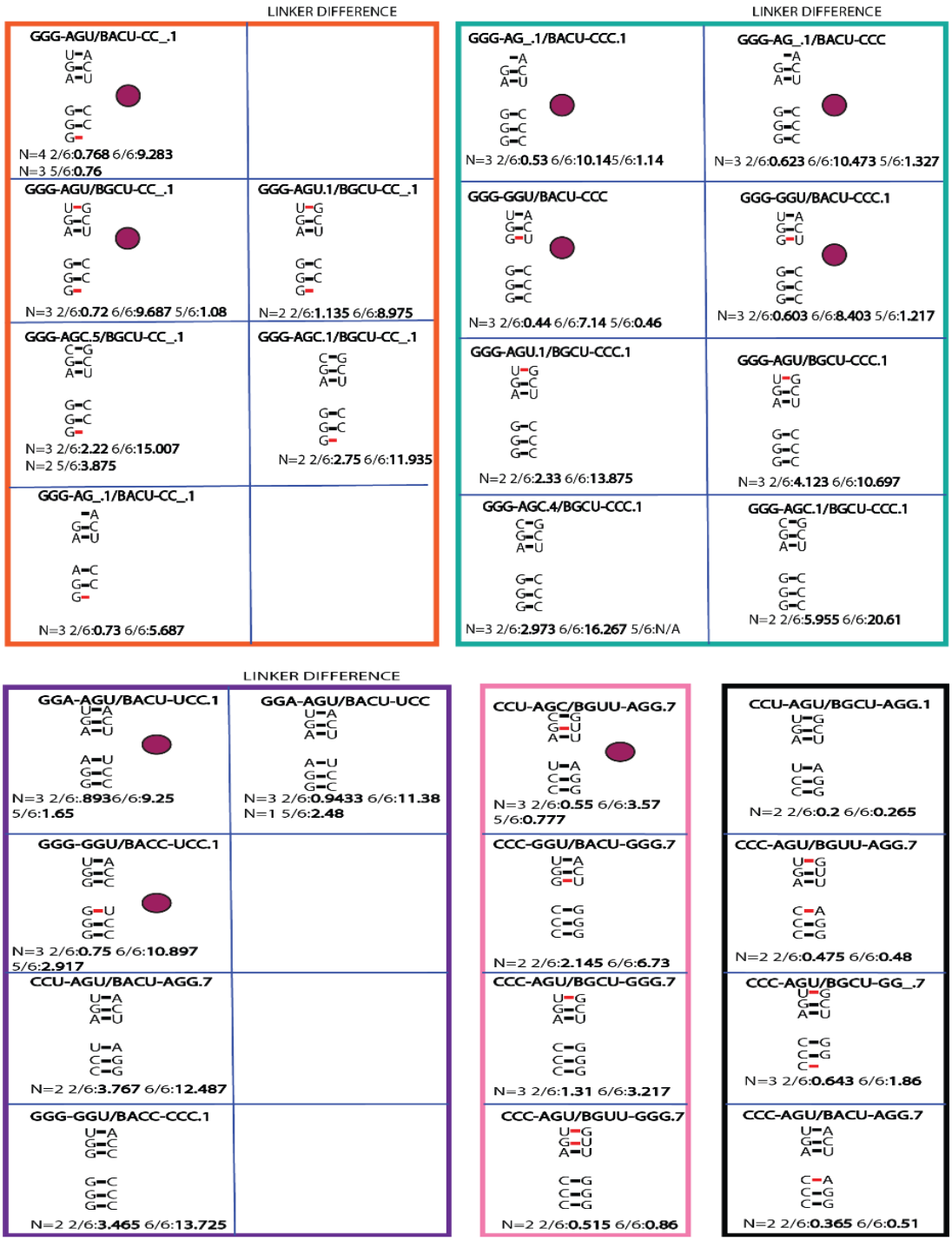


Figure 2 Twenty-seven versions of the split-Spinach aptamer. Left of the slash (XXX-XXX/) represent the A-strand nucleotides attached to the linker at the 3' end of the strand. Right of the slash (BXXX-XXX) represent the nucleotides attached to the linker and the 3' end of the B-strand. Linker identity is denoted by the value following the period after each strand. The standard linker nucleotides are UUAACA. Deviations from this are denoted by the value following the period after a strand. Linker identities are as follows, 1 = AAUUAU, 4 =UUAACU, 5 = UUAAUC, 7 = AAUAUU (These are numbered by chronological creation. Therefore, not shown here are many linkers which completely failed). The maroon dot indicates that the aptamer was tested in the co-transcription experiment (see below). 2/6 indicates data for a mixture of strands A and B, 5/6 a mixture of five of the six ring pieces, 6/6 the presence of all the ring strands in a mixture.

As successful aptamers appeared, they were further augmented by linker mutation. First, *in silico* testing on mfold[15] was used to evaluate the likely patters of mutant linker sequences. Again, the point of this stage of the experiment was to identify linkers that would aid the assembly of the nanoring/split-aptamer system. The main feature sought in the results was the formation of the two kissing loops, which allow the aptamer to assemble within the nanoring (**Figure 3)**. Seven linker sequences were identified that folded with the most free-energy to include the free half of the aptamer and the kissing loops of the nanoring (**Table 1**). Yet, it was postulated that linkers with multiple, favorable folding patterns would add an advantageous destabilizing factor to the aptamer. These could not assemble when only part of the ring pieces was present. So, linker sequence was mutated following the same goldilocks principle as before.

Figure 3 Split-Spinach aptamer GGG-AGU sporting the 0 aptamer (**Table 1).** Boxed in red are the loops which assemble the aptamer within the ring based on the kissing interaction. These loops were found among the top four predicted folding patterns for all seven linkers used in later experiments (**Table 1**).

Linker	Sequence
0	UUAACA
1	AAUUAU
2	AAUACU
3	AAUUCU
4	UUAACU
5	UUAAUC
6	UUCUCA
7	AAUAUU

Table 1 See in-text explanation.

The most stable partially assembled ring is composed of five strands (5/6). Therefore, many of the best candidates from the 2/6 screen were evaluated in a comparison of 5/6 to 6/6 (**Figure 4**). This is necessary because 5/6 data could be contaminating the feedback of presumed 6/6 assembly. Essentially, 5/6 strands could assemble and form the aptamer in the mixtures with 6/6 strands present.

Indeed, not all the aptamers demonstrated that the fluorescence feedback from mixtures of six ring pieces isn't corrupted by false positive partial ring assemblies. For 5/6, GGG-AGC.5/BGCU-CC_.1 gave 3.875 +/- 0.078 A.U. of fluorescence feedback. This means that almost four arbitrary units of fluorescence in the 6/6 data is indistinguishable from 5/6 data. In

other words, full and partial formation of the ring is not as discernable as initially determined by the 2/6 vs. 6/6 experiment. However, for the rest of the aptamer candidates, the 5/6 fluorescence was not impressively more than the 2/6 (**Figure 4**). This signifies that eleven of the aptamers identified by the initial 2/6 vs 6/6 screen also passed the more critical 5/6 vs 6/6 experiment.

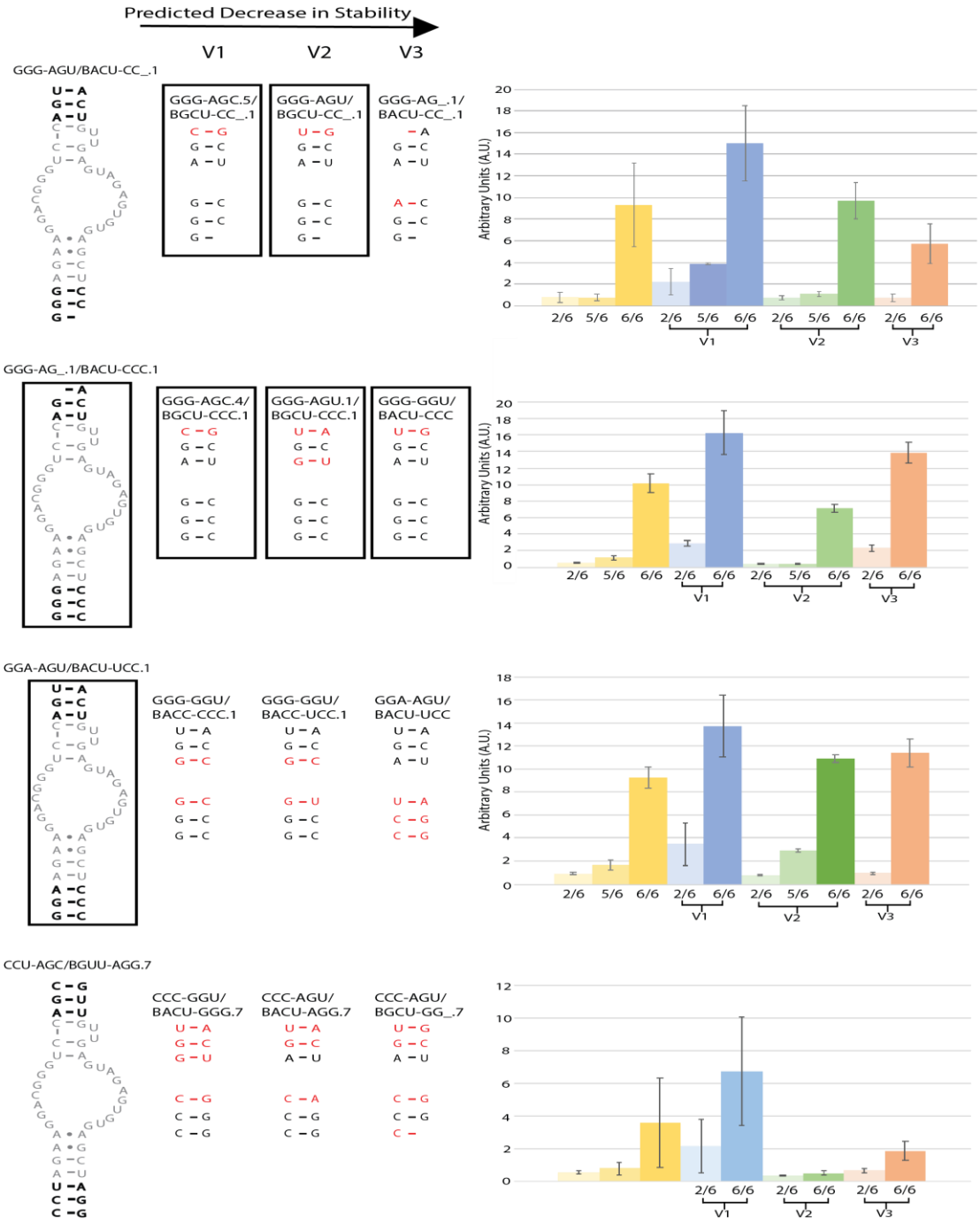
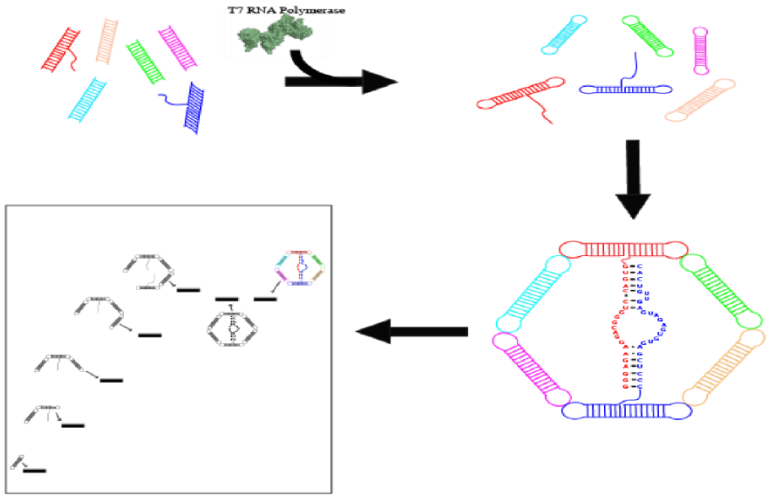


Figure 4 Sixteen versions of the split-Spinach aptamer. The first twelve demonstrated a significant level of separation in the fluorescence feedback for 2/6 and 6/6. The last four are examples of aptamers which failed the 2/6 vs 6/6 experiment.

Co-transcriptional Assembly

Split-Aptamer evaluation of nanoring assembly could be a valuable tool for use *in vivo*.[7] RNA will degrade in the cell over time and need to be replenished. However, DNA in the cell can always be used as a code to translate into corresponding RNA. Therefore, transcribing the strands of the nanoring together is meant to simulate an *in vivo* application of the split-Spinach aptamer. Six of the successful aptamers (**Figure 4**) underwent co-transcription. The results of the experiment were measured by fluorescence feedback and by gel electrophoresis (**Figure 5**). Of all the aptamers, three showed significant variation by standard deviation between the 5/6 and 6/6 fluorescence feedback (**Figure 5**).

A



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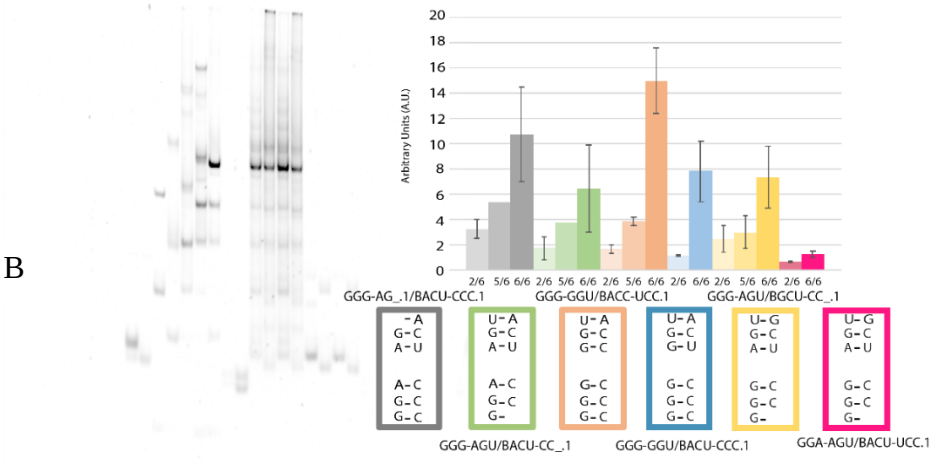

Figure 5 A Co-transcription involves the transcription of a mixture of unique RNA strands. Then, the assembly of the ring is examined by gel electrophoresis. **B** *Left* Representative experimental gel for the GGG-AG_.1/BACU-CCC.1 aptamer. *Right* The fluorescence data for the six aptamers which were most optimized as RNA. Three of the six show a significant difference based on their standard deviations: GGG-AG_.1/BACU-CCC.1; GGG-GGU/BACCC-UCC.1; GGG-AGU/BGCU-CC_.1.

Conclusion:

Here is an RNA based system which can identify perihperal tertiary interaction between RNA strands as shown for the six membered RNA nanoring. Six total aptamers fluoresceed prominently in the six strand mixtures, and not in the two or five strand mixtures, indicating fluorescence based on assembly. Co-transcriptional experimentation of the six aptamers was not as succesful as the RNA experiments. However, we do show as a proof-of-concept the potential for *in vivo* testing of the six aptamer canidates. This is signifigant because, as mentioned, tracking nanoparticles will become neccesarry as they become vehicles for insertion of therapies.[7]

Materials and Methods:

Design and Synthesis of Split-Spinach Aptamer and Fluorophore

The previously published Split- Spinach aptamer (PBD ID: 4TS2)[11,16]was modeled into the RNA nanoring[14] using the Swiss PDB-Viewer.[17] Modeling of the aptamer inside the nanoring provided a proof-of-concept and initial estimate for strand lengths. Individual, rationally-designed RNA strands were evaluated for unintended folding patterns prior to experimentation.[18,19] DNA sequences, corresponding to the RNA sequences of interest, were designed by adding a T7 polymerase promoter site sequence (TTCTAATACGACTCACTATA) to the 5' end of each RNA. DNA templates and primers were purchased from Integrated DNA technologies (IDT), amplified by PCR, and transcribed using T7 RNA polymerase *in vitro*. The RNA was purified by 8 M urea-10% polyacrylamide gel electrophoresis (PAGE). The fluorophore, DFHBI, was synthesized as previously reported according the protocol of the Paige research group.[10] A complete list of RNA sequences used in the study can be found in the Supporting Information.

Evaluation of Assembly

Assembly of the split-aptamer integrated nanoring was evaluated by native PAGE and fluorimetry. RNAs were assembled by combining equimolar concentrations of RNA strands (at a concentration of 500 mM unless noted otherwise) and the snap cool process (2 minutes at 95°C and 3 minutes on ice). After snap cooling, an association buffer was added to achieve a final concentration of 40 mM HEPES (pH 8.2), 1 mM $Mg(OAc)_2$, and 50 mM KCl. This mixture was incubated at 37°C for 20 minutes and evaluated by fluorescence spectroscopy with an LS 55 luminescence spectrometer (PerkinElmer). DFHBI was added (either before or after incubation) to final concentration of 1mM. Samples were loaded into a 40 uL quartz cuvette (Starna Cells, Inc.) and excited at 469 nm. Emission was recorded at 509 nm. Assembly products were also analyzed by a gel shift assay. Products were loaded into a 7% polyacrylamide gel of 1× HEPES (40 mM HEPES) buffer and 1 mM $Mg(OAc)_2$. Gels were run at 6 W for 3−4 h at 4 °C. Gels were stained with Sybr Gold (Invitrogen) and imaged using a FluoroChemQ gel imager (Protein Simple).

Co-Transcription Assembly

The DNA counterparts for the RNA ring pieces were combined in a concentration of 0.35 μM with a 5X co-transcription buffer (DTT (100 mM), NTPs (25 mM each), IPP (0.1 u/μL), RNasin (40 u/μL), and T7 RNA polymerase (120U)) and incubated at 37°C for 45 minutes. The amount of T7 RNA polymerase was normalized to the total amount of DNA in each reaction mixture. After incubation, 0.4 uL of DNase was added to each reaction mixture and then incubated for an additional 15 minutes at 37°C. Aliquots of each reaction mixture were evaluated by fluorescence and by gel electrophoresis as described above.

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Appendix I: Faith and Learning:

The Worship in Research

My grandmother on my mother's side, Grandma Karen, was one of my greatest role models growing up. I know some people say that casually, but I am serious. I actually made a poster about her for a project about mentors in the sixth grade. She was kind, tender, and understanding. Grandma Karen had a calming presence, which she used to help me through the terrible panic attacks I suffered as a child. Before I was born, she had battled and beaten cancer. When I was younger, I did not really understand what that meant, but I knew she was lucky to be cancer free. When I entered high school, Grandma Karen's cancer returned. This time, the tumors metastasized and spread throughout her body. My family and I watched on as she slowly became weaker and weaker. Grandma went from smiling every day, to wincing from the deep-set pain that comes with months of chemotherapy, from one unsuccessful surgery, and from cancer growing in the bones. She died when I was a junior in high school. I was devastated.

When I began to ask around the biology and chemistry department here at SPU, I wanted to avoid any computational chemistry research because it is primarily focused on making programs to simulate reactions. I am a hands-on individual and wanted to work in a lab with active chemicals. I talked with different professors and nothing seemed to fit my interests and needs. Dr. Bartlett's Organic Chemistry research sounded interesting, but it was unpaid. Dr. Pratt needed more of an administrator than a researcher. When I first heard about the work in Dr. Grabow's lab, I was disinterested because I knew little about RNA. However, I knew I wanted to work in the biochemical field, so I went to his office and interviewed him about his research. When I realized that part of his lab was involved in this split-aptamer project, which seemed to have a thread of connection to cancer therapy, I was sold. My Grandma Karen fought for breath, seated in her favorite living room chair, moments before she died. This memory compelled me to take up this research opportunity. It is true that this research primarily focused on tracking the formation of RNA nanoparticles and may never be specifically used to treat cancer patients. Yet, I fully believe all the science directed towards treating cancer is helping to narrow the focus of our research, bringing us ever closer to a cure.

> "The requirements of a work to be done can be understood as the will of God. If I am supposed to hoe a garden or make a table, then I will be obeying God if I am true to the task I am performing. To do the work carefully and well, with love and respect for the nature of my task and with due attention to its purpose, is to unite myself to God's will in my work. In this way I become His instrument. He works through me. When I act as His instrument my labor cannot become an obstacle to contemplation, even though it may temporarily so occupy my mind that I cannot engage in it while I am actually doing my job. Yet my work itself will purify and pacify my mind and dispose me for contemplation"[1]

As Christians, I believe God us to use our created minds to study and work with God's Creation. In this way, any act of contemplation and critical thought becomes a form of worship. I truly believe that "the will of God" for my time here, in undergraduate research, has included this project. I have done my best to "be true to the task I am performing" because I fully see it as a form of worship. When I perform research, and perform it well, I have the privilege of being caught up in God's plan the world. In every hour spent at the bench, I see the God of the Bible allowing me to be a part of God's action against pain, sickness, death.

Reference (Appendix I)

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