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Split-Spinach Monitoring of RNA Aptamer Assembly

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SPLIT-SPINACH MONITORING OF RNA APTAMER ASSEMBLY

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ABSTRACT:

As insights into RNA's many diverse cellular roles continue to be gained, interest and applications in RNA self-assembly and dynamics remain at the forefront of structural biology. The bifurcation of functional molecules into nonfunctional fragments provides a useful strategy for controlling and monitoring cellular RNA processes and functionalities. Herein we present the bifurcation of the preexisting Spinach aptamer and demonstrate its utility as a novel split aptamer system for monitoring RNA self-assembly as well as the processing of pre-short interfering substrates. We show for the first time that the Spinach aptamer can be divided into two nonfunctional halves that, once assembled, restore the original fluorescent signal characteristic of the unabridged aptamer. In this regard, the split-Spinach aptamer is represented as a potential tool for monitoring the self-assembly of artificial and/or natural RNAs.

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Transformative knowledge can only exist in community. Indeed, this seems fundamental to the nature of creation: even God exists within a community of three persons, from whom His self-disclosure emanates. As I will later discuss in my statement on Faith and Learning, the multivalent nature of God's people ensures that we encounter God's revelation through multiple avenues as we interact with the multitude of His Church. Here, I hope to express in tangible terms the way that my community has revealed God, and in so doing made this project possible.

My project came about through those interactions among my professors, family and friends, who have channeled the revelatory self-disclosure of the Trinity. For this, I owe eternal gratitude to each and every one of them. Thank you to Dr. Stamatis Vokos, for drinking coffee with me over discussions of Greek Orthodoxy. Thank you Dr. Rod Stiling, for playing catch on the campus green with me over discussions of history and science. Thank you to Drs. Jeff Keuss, Diana Keuss, and Christine Chaney, for displaying capital-R Reconciliation through the streets of Dublin, Edinburgh, and Glasgow. Thank you Dr. Frank Spina for displaying, through both your ministry and your academic work, that true theology occurs hand-in-hand with pastoral care and the life of the Church. Thank you to Drs. Wade Grabow and Ben McFarland, for mentoring me as a scientist, a teacher, a believer, and a friend, and for believing in the capabilities of Grant and I. Thank you Ernie, Marit, Jeff, Dave Kragen, Jan Kragen, Dave Sellers, Rick, Trish, Uncle Greg, Theron, Robin, Rich, and Judi, for how you've raised me in the faith that I might "do justice, love kindness, and walk humbly with [our] God (Micah 6:8)." Thank you to Maddie, Nate, Mom, and Dad, for how you've taught me to take the world with the utmost seriousness by acknowledging my own absurdity; for how you've taught me to take joy in my work; for how you've taught me to love others as effectively as possible; and for putting up with all my bull crap, and calling it out when necessary. In all of these encounters I've learned something about creation, and the One that set that creation on its foundation. With that in mind, thank you to everyone who has taught me to love learning, and to treat life as an experience of wonder and doxology to the Creator.

On a more technical note, this research was supported (in part) by the Montana Family Endowment and the Seattle Pacific University Faculty Research Grant (FRG), which made it possible to be paid for my research work during the summer of 2013. Special thanks also to Dr. Bruce Congdon for providing funding for our Gel Imaging equipment; to Dr. Ben McFarland for the use of his Nanodrop; and to Dr. Kevin Bartlett for use of his synthesis glassware.

I dedicate this work to the memory of my Mother, Lynnette Rogers, who would have peed her pants knowing that her "Baby Boy!" was going to be published.

FLUORESCENT MONITORING OF RNA ASSEMBLY AND PROCESSING

USING THE SPLIT-SPINACH APTAMER

Introduction:

RNA is an exceedingly important molecule in an array of cellular processes (i.e., catalysis, gene regulation, and metabolite recognition) beyond its traditionally recognized roles involving protein expression. This increased awareness of RNA's utility calls for new tools that can be used to study and monitor RNA self-assembly, structure, and cellular dynamics. Split-protein and nucleic acid systems, which rely on the reassociation of independent nonfunctional fragments to conditionally restore the desired whole and operative moiety, [1,2] represent useful tool for identifying and ascertaining important molecular interactions [3] and have the potential to provide new devices for synthetic biology and/or biomedical applications. [4] The benefits of these split systems hinge on at least two important factors: the involved fragments' collective ability to readily and stably reassociate into the functional complex and their ability to elicit the desired functional response or signal. In the first regard, nucleic acids provide an accommodating platform for choreographing self-assembling, predefined molecular inter-actions because of their ability to form predictable and precise hydrogen bonds between complementary nucleobases. [5−7] With respect to this second point, molecules that provide fluorescent outputs are thought to offer highly sensitive signals with desirable signal-to-noise ratios.

RNA's versatility has been made all the more useful in the developing fields of RNA synthetic biology and nanotechnology with the advent of directed evolution techniques that may be used to select for novel synthetic RNAs possessing virtually any preconceived functionality.8 Using this approach, the previously reported Spinach aptamer was evolved and selected for its ability to produce a fluorescent signal when it is complexed with the small molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a mimic of the chromophore found in the green fluorescent protein.9 The scope and utility of the Spinach aptamer have been expanded further to include the ability to report the presence of various metabolites and, more recently, as a means of quantitatively monitoring protein production. [10,11]

In our view, the bifurcation of the full-length RNA aptamer into two segments provides an additional set of future applications that include the ability to monitor and/or visualize dynamic self-assembly. Herein, we describe the design of the split-Spinach system for monitoring the formation of synthetic RNAs for use in the RNA interference (RNAi) pathway as well as their subsequent processing by the human recombinant dicer enzyme.

Results and Discussion

Previous reports about the Spinach aptamer [10] demonstrated that the third stem (from the 5′ end of the full-length molecule) tolerates a range of different sequences, including the appendage of various aptamer moieties, without too much loss of function (70–80% signal strength of the original Spinach aptamer). Thus, we hypothesized this stem to be a suitable point for bifurcation of the full-length Spinach aptamer. As a way to selectively control the formation of the bipartite complex and demonstrate its functionality, we placed the split-Spinach aptamer in the context of a self-assembling RNA–DNA hybrid system, similar to that of Afonin et al. [12,13] The bifurcated stem of the aptamer complex was extended using different short interfering RNA (siRNA) sequences (see Figure SI1 of the Supporting Information). Three siRNA sequences were tested on the basis of previously reported work [4,14] (see the Supporting Information). Using the RNA–DNA hybrid system, complementary DNA "blocker" strands were annealed to individual RNA strands to prevent the formation of a functional split-Spinach aptamer (Figure 1). The "blocking" DNA strands could be selectively displaced by the addition of fully complementary "unblocking" DNA strands that could associate with the blocker strands through the 12-nucleotide toehold sequences, leaving the split-Spinach blocked stem of the aptamer free to assemble binding pocket.

Analysis of split-Spinach aptamer assembly was conducted using polyacrylamide gel electrophoresis (PAGE) and fluorescence spectroscopy. Conformational studies via PAGE show that the two aptamer segments, when annealed, produce a single band that migrates through the gel at a rate comparable to that of the full-length Spinach aptamer in native salt concentrations (Figure 2). In terms of functionality, the DFHBI fluorescence was observed only in the presence of both aptamer strands (i.e., DFHBI did not fluoresce in the presence of either split-Spinach aptamer alone). Furthermore, the split-Spinach aptamer showed fluorescence comparable to that of the full-length Spinach aptamer in the presence of DFHBI, indicating that bifurcation of the aptamer does not substantially reduce the binding affinity for DFHBI and that the bifurcated aptamer conforms to a tertiary structure analogous to that of the full-length Spinach aptamer.

Complementary blocker and unblocker DNA strands were introduced to demonstrate selective immobilization and assembly of the split-Spinach aptamer (Figure 2). PAGE and fluorescence spectroscopy show that the addition of complementary blocking DNA strands prior to the split-Spinach aptamer's assembly induces the bifurcated aptamer to form a nonfunctional DNA–RNA hybrid moiety that prevents the formation of the third stem that is critical for aptamer formation. Thus, the split-Spinach aptamer does not fully assemble or fluoresce in the presence of the blocker strands but assembles and exhibits fluorescence with the removal of the blocker strands via strand displacement when complementary unblocker DNA strands are introduced (Figure 2). Of the three siRNA sequences tested (validated sequences targeting GFP; two targeting HIV, protease (Pro) and Ldr3), only two proved to be suitable for study. The Ldr3 sequence contained a series

of four GA repeats that were prone to mispair. In the case of the other two sequences, the percent recovery of the functional aptamer upon unblocking at 37 °C was between 38 and 50%, which was confirmed by analysis of the assembly on native PAGE gels and fluorescence spectroscopy. We postulate that the split-Spinach system can be improved by tailoring the length and specific sequence of the toehold with the length of the desired siRNA sequence. We also saw that we could increase the overall recovery to >60% by assembling at 45 °C (see Figure SI2 of the Supporting Information).

In addition to its ability to monitor RNA self-assembly processes, the split-Spinach system can also be used to monitor RNA processing. The aptamer was subjected to selected degradation by the recombinant human dicer enzyme, targeting the 3□ overhang on the third stem, generating a siRNA (Figure 3). Independent analysis of the predicted product of the split-Spinach aptamer after dicing demonstrated no ability to fluoresce (data not shown). In the same way that blocking and unblocking DNA strands selectively induce fluorescence, dicing of the split-Spinach complex can selectively disrupt the split-Spinach aptamer's functionality, which was corroborated by the absence of fluorescence. Moreover, we show a direct correlation among the dicer concentration, siRNA formation, and the reduction of the fluorescence signal, further suggesting that the aptamer was selectively degraded by dicer processing of the bifurcated stem.

The split-Spinach aptamer complex, in the context of the DNA–RNA hybrid system, provides a successful demonstration of the bifurcated Spinach aptamer in vitro. We show that the modification of a previously reported RNA aptamer and fluorophore offers a promising tool for monitoring RNA assembly and RNA processing. Given that the split system reduces the magnitude of the signal by only □20% compared to that of the full-length sequence in vitro, it is anticipated that the split-Spinach aptamer could be used for in vivo applications similar to those previously reported for the original aggregate Spinach aptamer. [9–11] Alternatively, we hypothesize that the recently reported Spinach2 aptamer, which offers >3 times the fluorescence intensity of the first-generation Spinach aptamer, could be modified in the same fashion to create a programmable bifurcated aptamer. [15] The improved sensitivity of the Spinach2 aptamer is attributed to the elimination of mismatches in the first and third stems, which thereby improved the overall thermostability and folding efficiency of the Spinach aptamer. [15] Using the improved stem sequences associated with the Spinach2 aptamer to facilitate programmable assembly in the split-Spinach system (as the core of the aptamer sequence remains unchanged) would likely provide a better testing ground for future in vivo applications. Regardless of the precise stem sequences used, we posit that the split-Spinach concept offers a new tool for investigating a variety of RNA assembly and RNA–RNA interactions. [16,17] For example, the split construct has potential as a fluorescent signaling device in RNA-based sensors and/or programmable circuitry as well as the assembly of RNA nanostructures

similar to how the binary malachite green aptamer has been previously reported [12,18,19] but without the reported toxicity. [20]

Materials and Methods:

Synthesis of the Aptamer and Fluorophore:

DNA sequences and primers of interest were purchased from Integrated Data Technologies (IDT). RNA constructs were synthesized by in vitro transcription with T7 RNA polymerase from polymerase chain reaction-generated double-stranded DNA templates ordered from Integrated DNA Technologies (IDT), followed by 8 M urea−10% polyacrylamide gel electrophoresis (PAGE) purification. DFHBI was synthesized according to previously published protocols. [9] RNA and DNA sequences used here can be found in the Supporting Information.

Nucleic Acid Assembly Experiments:

To control the assembly of the split-Spinach complex, individual RNA sequences were annealed to complementary blocker DNA strands by being slowly cooled from 95 °C. Blocked RNA strands were mixed in a 1:1 ratio and incubated together for 20 min at 37 °C. Mixtures containing unblocker DNA strands and their controls were left to incubate at 37 °C for 25 min before being loaded into a 7% polyacrylamide gel of 1× HEPES (40 mM HEPES) buffer and 1 mM MgCl2. Gels were run at 8 W for 2−3 h at 4 °C. Aliquots of samples run on gel were also characterized by fluorescence spectroscopy. Identical assembly experiments were conducted using 10 to 12% polyacrylamide gels containing 8 M urea. Denaturing gels were run at 40 W for 1−1.5 h at room temperature. Gels were stained with Sybr Green II (Invitrogen) and imaged using ChemiDoc MP (Bio- Rad) or FluoroChemQ (Protein Simple).

Fluorimeter Studies:

The fluorescence of the fully assembled and unblocked Spinach aptamer was confirmed with an LS 55 luminescence spectrometer (PerkinElmer). Samples from reaction mixtures in assembly and dicer experiments were incubated with 1 mM DFHBI at 37 °C for 20 min and then loaded into a 40 μL quartz cuvette (Starna Cells, Inc.). Samples were excited at 469 nm and emission spectra recorded at 509 nm.

Dicer Experiments:

The recombinant human dicer enzyme kit was purchased from Genlantis. Samples were incubated overnight at 37 °C in the supplied dicer reaction buffer (Genlantis) or HEPES according to the manufacturer's suggested protocol. Dicing reactions were analyzed by PAGE on a 1 mM MgCl2 native 7% PAGE or 8 M urea−10% PAGE gel and by fluorescence spectroscopy as described above.

Figures

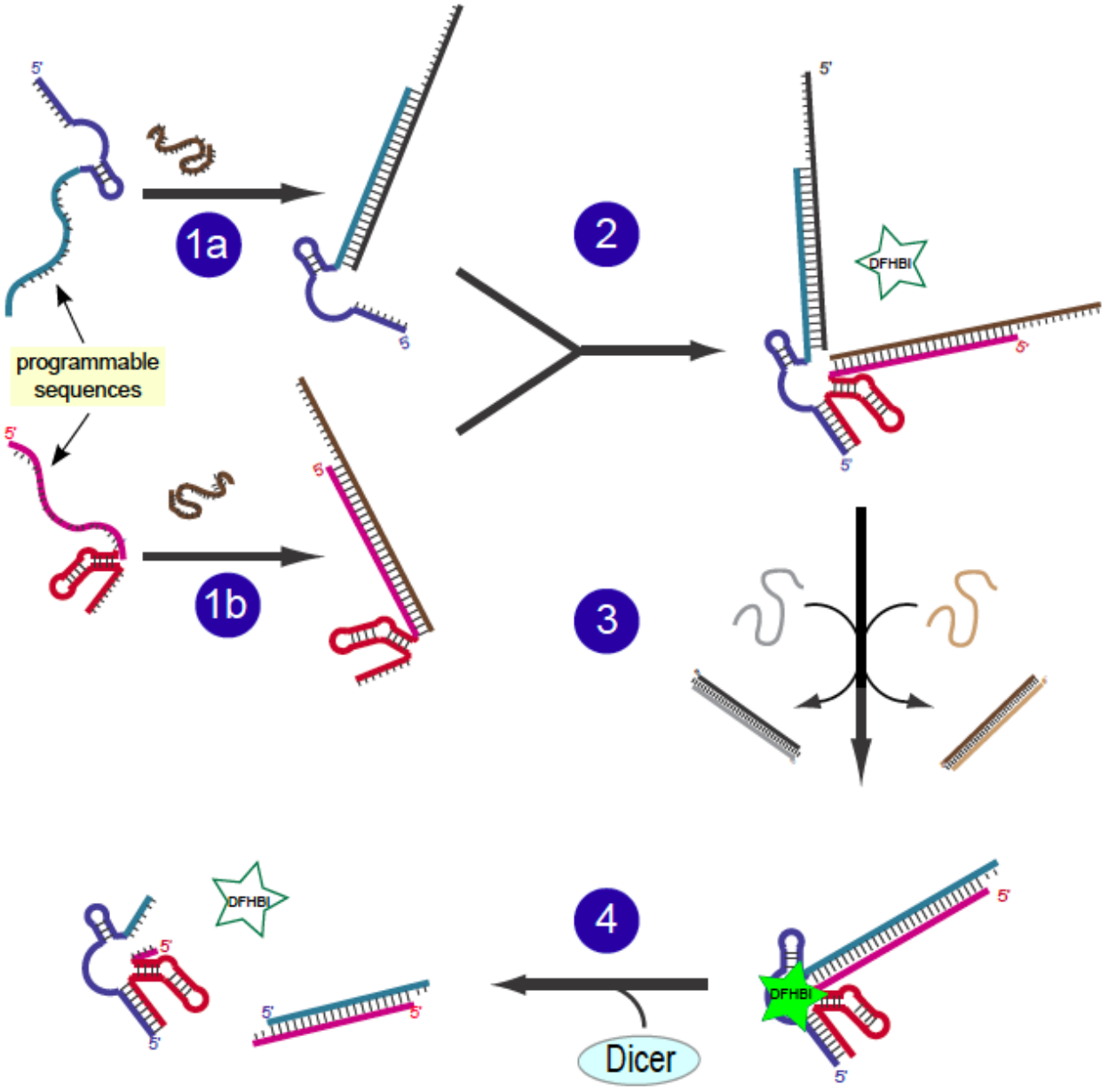
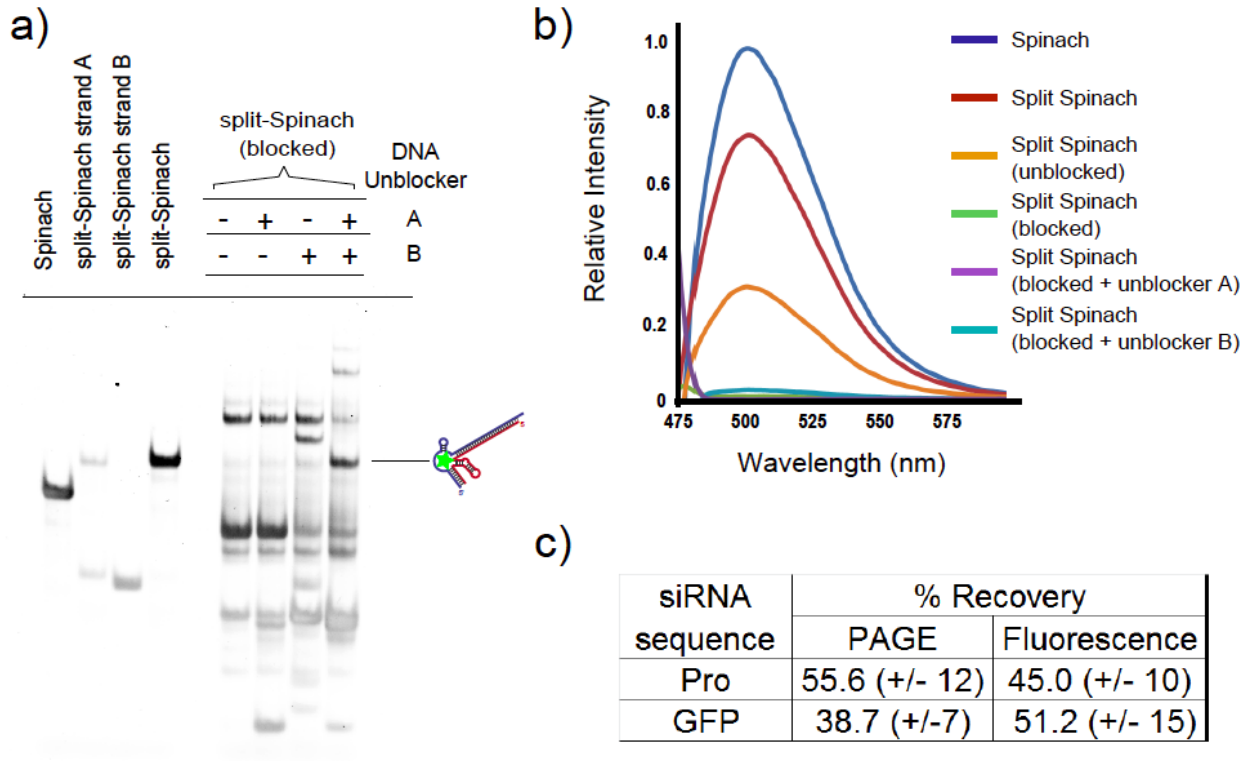


Figure 1

Schematic showing the methods used to control the assembly and processing of the split-Spinach system. In steps 1a and 1b, single strands of the aptamer were annealed to a complementary DNA blocker sequence on the elongated stem. In step 2, the products of steps 1a and 1b are combined to form the full Spinach aptamer with DNA blocker strands in place. In step 3, DNA toehold strands complementary to their respective DNA blocker strands "unzip" the DNA from the aptamer by maximizing Watson–Crick base pairs, allowing the elongated stem to bind with itself and form the DFHBI binding site and DFHBI to bind and fluoresce. In step 4, dicer cuts the dsRNA 21 nucleotides from the 3′ two-nucleotide overhang, destabilizing the binding site and ending fluorescence.



c)

siRNA sequence	% Recovery	
	PAGE	Fluorescence
Pro	55.6 (+/- 12)	45.0 (+/- 10)
GFP	38.7 (+/-7)	51.2 (+/- 15)

Figure 2

Monitoring the assembly and fluorescence of the controlled split-Spinach system. (a) A representative native PAGE gel (40 mM HEPES buffer and 1 mM Mg2+) confirms that the split-Spinach aptamer forms from the blocked complex only when both unblocker strands are added. Data represent the Pro-siRNA sequences. See the Supporting Information for sequence details. (b) Corresponding fluorescence data confirm that the unblocked split-Spinach system fluoresces. The split-Spinach system fails to fluoresce when blocker strands are added, but fluorescence occurs when both unblockers are added. (c) The percent recoveries (following the addition of the DNA unblocking strands) of the two programmable sequences tested were analyzed in triplicate by native PAGE and fluorescence microscopy.

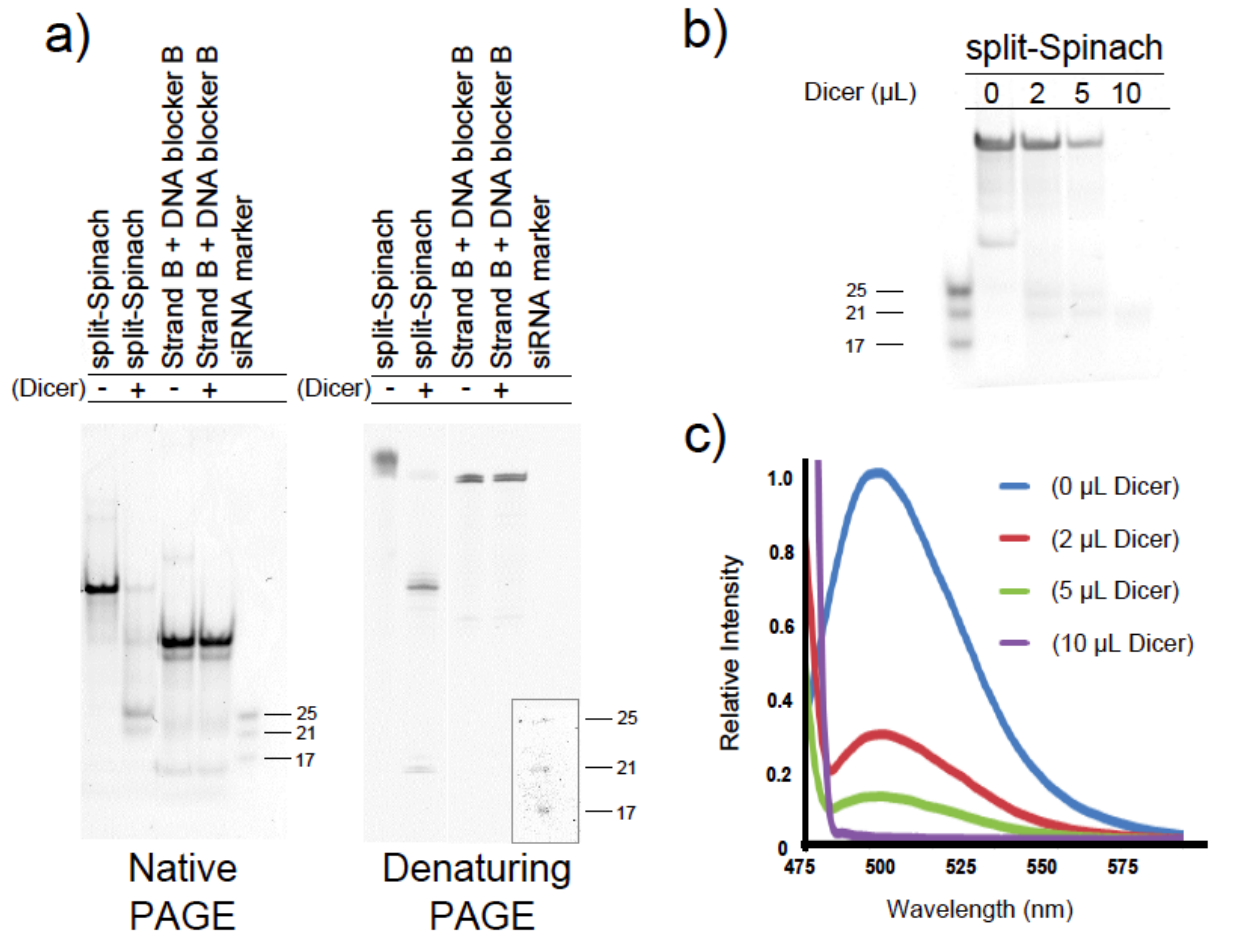


Figure 3:

Monitoring dicer processing. (a) PAGE gels demonstrate that dicer selectively processes double-stranded RNA in the third stem of the split-Spinach aptamer. The denaturing gel indicates that the dicer cuts the programmable stem of the assembled the split-Spinach system into ~21-nucleotide segments. No dicing was observed when RNA is annealed to the blocker strand. Note that the contrast was increased for the ladder portion of the denaturing PAGE gel to increase visibility. (b, c) As the concentration of the dicer increases, the concentration of the split-Spinach system decreases and fluorescence decreases.

BIBLIOGRAPHY

(1) Kolpashchikov, D. M. (2005) Binary malachite green aptamer for fluorescent detection of nucleic acids. J. Am. Chem. Soc. 127, 12442– 12443.

(2) Ozawa, T., Nogami, S., Sato, M., Ohya, Y., and Umezawa, Y. (2000) A fluorescent indicator for detecting protein-protein interactions in vivo based on protein splicing. Anal. Chem. 72, 5151–5157.

(3) Shekhawat, S. S., and Ghosh, I. (2011) Split-protein systems: Beyond binary protein-protein interactions. Curr. Opin. Chem. Biol. 15, 789–797.

(4) Afonin, K. A., Kireeva, M., Grabow, W. W., Kashlev, M., Jaeger, L., and Shapiro, B. A. (2012) Co-transcriptional assembly of chemically modified RNA nanoparticles functionalized with siRNAs. Nano Lett. 12, 5192–5195.

(5) Chworos, A., Severcan, I., Koyfman, A. Y., Weinkam, P., Oroudjev, E., Hansma, H. G., and Jaeger, L. (2004) Building programmable jigsaw puzzles with RNA. Science 306, 2068–2072.

(6) Grabow, W. W., Zhuang, Z., Swank, Z. N., Shea, J. E., and Jaeger, L. (2012) The right angle (RA) motif: A prevalent ribosomal RNA structural pattern found in group I introns. J. Mol. Biol. 424, 54–67.

(7) Severcan, I., Geary, C., Chworos, A., Voss, N., Jacovetty, E., and Jaeger, L. (2010) A polyhedron made of tRNAs. Nat. Chem. 2, 772–

(8) Cobb, R. E., Si, T., and Zhao, H. (2012) Directed evolution: An evolving and enabling synthetic biology tool. Curr. Opin. Chem. Biol.16, 285–291.

(9) Paige, J. S., Wu, K. Y., and Jaffrey, S. R. (2011) RNA mimics of green fluorescent protein. Science 333, 642–646.

(10) Paige, J. S., Nguyen-Duc, T., Song, W., and Jaffrey, S. R. (2012) Fluorescence imaging of cellular metabolites with RNA. Science 335,

(11) Pothoulakis, G., Ceroni, F., Reeve, B., and Ellis, T. (2014) The Spinach RNA Aptamer as a Characterization Tool for Synthetic Biology. ACS Synth. Biol. 3, 182–187.

(12) Afonin, K. A., Viard, M., Martins, A. N., Lockett, S. J., Maciag, A. E., Freed, E. O., Heldman, E., Jaeger, L., Blumenthal, R., and Shapiro, B. A. (2013) Activation of different split functionalities on re-association of RNA-DNA hybrids. Nat. Nanotechnol. 8, 296–304.

(13) Afonin, K. A., Desai, R., Viard, M., Kireeva, M. L., Bindewald, E., Case, C. L., Maciag, A. E., Kasprzak, W. K., Kim, T., Sappe, A., Stepler, M., Kewalramani, V. N., Kashlev, M., Blumenthal,

R., and Shapiro, B.A. (2014) Co-transcriptional production of RNA-DNA hybrids for simultaneous release of multiple split functionalities. Nucleic Acids Res. 42, 2085–2097.

(14) Grabow, W. W., Zakrevsky, P., Afonin, K. A., Chworos, A., Shapiro, B. A., and Jaeger, L. (2011) Self-assembling RNA nanorings based on RNAI/II inverse kissing complexes. Nano Lett. 11, 878–887.

(15) Strack, R. L., Disney, M. D., and Jaffrey, S. R. (2013) A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat-containing RNA. Nat. Methods 10, 1219–1224.

(16) Jaeger, L., and Chworos, A. (2006) The architectonics of programmable RNA and DNA nanostructures. Curr. Opin. Struct. Biol.16, 531–543.

(17) Grabow, W., and Jaeger, L. (2013) RNA modularity for synthetic biology. F1000 Prime Reports 5, 46.

(18) Afonin, K. A., Bindewald, E., Yaghoubian, A. J., Voss, N., Jacovetty, E., Shapiro, B. A., and Jaeger, L. (2010) In vitro assembly of cubic RNA-based scaffolds designed in silico. Nat. Nanotechnol. 5, 676–682.

(19) Kolpashchikov, D. M. (2010) Binary probes for nucleic acid analysis. Chem. Rev. 110, 4709–4723.

(20) Srivastava, S., Sinha, R., and Roy, D. (2004) Toxicological effects of malachite green. Aquat. Toxicol. 66, 319–329.

INTEGRATION OF FAITH AND LEARNING

"For by one Spirit we were all baptized into one body -- Jews or Greeks, slaves or free -- and all were made to drink of one Spirit. For the body does not consist of one member but of many. If the foot should say, "Because I am not a hand, I do not belong to the body," that would not make it any less a part of the body. And if the ear should say, "Because I am not an eye, I do not belong to the body," that would not make it any less a part of the body. If the whole body were an eye, where would be the hearing? If the whole body were an ear, where would be the sense of smell? But as it is, God arranged the organs in the body, each one of them, as he chose. If all were a single organ, where would the body be? As it is, there are many parts, yet one body. The eye cannot say to the hand, 'I have no need of you,' nor again the head to the feet, 'I have no need of you.'"

- 1 Corinthians 12: 13-21 [RSV]

When I think of a community I often have in mind the articles around which a community gathers: a common place, activity, experience. These articles of the community become inseparable from the people of the community's routine, behaviors, and personhood: these articles are formational to the community members. We are "Foot people" say these communities, or we are "hand people. We're dedicated to doing well whatever it is we do." What are we to make, then, of a diverse community? What are we to make of a community that gathers around its diversity, rather than something specific and shared? What do you do with a community of hands *and* feet, eyes *and* ears?

While I associate the Church with the common faith summarized in the Apostle's Creed, I also consider the Church to reflect God's diversity within the Trinity, as well as His creative agency. God built us for community as a reflection of His communal nature within the trinity. But our need for community is further reflected in our giftings. We have been created to feed the community in a manner anointed by God, without which the community of God is incomplete.

I think the same is equally true of our ways of knowing. In my experience throughout the UScholars program, we have not been united around a common discipline the way that a community might develop within the Biochemistry major, or the theology program, or amongst education majors. Rather, we have been united by our mutual, yet diverse, participation in the community of Faith through the act of learning. With the loss of any individual from this community, we would lose a unique testament to God's provision, truth and revelation, because we would lose a unique way of knowing God. We have a body that is can no longer function at its best.

In this sense, our diverse ways of knowing are all directed toward God as a *doxological* ways of knowing that are as multivalent as our community members. Dylan worships God with the study of Greek, as Lisa worships when she teaches Physics to undergrads, as Abby worships by taking fecal samples, as Gabe worships through the studying privilege, as Scott and Lauren worship through researching development, as Caroline worships through operating on patients in South Africa. Worship is an embodied experience, and every part of the body must participate.

This is what it means to be the people of God: not to gather around an article or a belief, but to see, glorify and pleasure in God's revelation through His people, and to never neglect His unique work in your sisters and brothers, because it is in this way that we *learn* about God. Nay, "learning" is far too mechanical of a term: we *learn* how fly chromosomes segregate; we *learn* how to operate a shotgun; we *learn* how to write a good sentence. We are *renewed*, *reconciled*, and *transformed* by a God who reveals Himself to His people through the Created order and through His scriptures. This is what it means to have faith. This is what it means to learn. This is what it means to be the body of Christ.