EXPANDING THE UTILITY OF RNA-BASED TRANSCRIPTION ACTIVATION MECHANISMS FOR DIAGNOSTIC APPLICATIONS

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Master of Science in Chemical & Biomolecular Engineering

by

Karl Brennan

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ABSTRACT

From its inception, one of the major aims of the field of synthetic biology was to reliably manipulate microorganisms to create novel solutions in medicine and biotechnology. This is possible due to the remarkable diversity of natural interactions they carry out to regulate gene expression. Bacterial cells for instance contain sophisticated regulatory networks that modulate expression based on environmental cues. This sense and response mechanism could have tremendous utility in resource-limited environments. In such places, lack of access to affordable, rapid diagnostic tools means that viral and bacterial contamination of public water sources present a serious threat to public health. Before applications can be addressed, cellular circuits must be synthetically repurposed. Traditionally, engineers have relied upon protein-based regulatory networks. In recent times however, the mechanistic flexibility of RNA has emerged. It is now recognized as a powerful tool for rewiring biological circuits. In the present paper, combining a toehold-based design with STAR transcriptional activators resulted in the development of a flexible RNA-based sensing device. A colorimetric LacZ assay, *in vivo* fluorescence assay and a suite of molecular biology techniques demonstrated the sensing capability of the modified RNAs with a fold activation of 1.5. This work provides direction for the development of high-performance flexible RNA sensors. The paper concludes with recommendations for future work in this area including RNA structural approaches and an exploration of *in vitro*, paper based circuitry.

BIOGRAPHICAL SKETCH

Karl Brennan completed a Bachelors degree in Chemical & Bioprocess Engineering in University College Dublin, Ireland in 2014. He was the recipient of the BMS Gold Medal in Chemical Engineering. During his undergraduate studies, he participated in the Renewable Energy Materials Science & Engineering Center (REMRSEC) Research Experience for Undergraduates (REU) at the Colorado School of Mines in the summer of 2012 thanks to the Pat McAdam Scholarship. In the summer of 2013, he completed an internship with Merck, Sharp & Dohme in Ballydine, Ireland. Having enjoyed the industrial experience, but feeling the need to supplement his knowledge of biological systems before entering industry, he worked in the Lucks group at Cornell between Fall 2014 and December 2015. Brennan will take up a graduate engineering role with MSD in the south of Ireland in January 2016.

ACKNOWLEDGMENTS

I would like to sincerely thank my advisor Julius Lucks for all his help and support during my time here at Cornell. Secondly, I would like express my gratitude to the Department of Chemical & Biomolecular Engineering – especially Prof. Matthew DeLisa - for their generous backing over my three semesters at Cornell. Thank you.

I have learnt an enormous amount during my time in Cornell in what has been a challenging and enriching experience. I am looking forward to leveraging this knowledge in an industrial setting in my next role.

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LIST OF ABBREVIATIONS

STAR: Small Transcription Activating RNAs

RNA: Ribonucleic acid

TX-TL: Cell-free Transcription-translation system

LacZ: β-galactosidase blue-white assay

PCR: Polymerase Chain Reaction

MFE: Minimum Free Energy

RBS: Ribosome Binding Site

ORI: Origin of Replication

pT181: Natural sequence occurring in *B. subtilis*

FL: Bulk fluorescence (a.u.)

CHAPTER 1

INTRODUCTION

The flexibility of RNA is leading synthetic biology from the laboratory to the field.

Lack of access to clean drinking water is an acute global health problem that affects 748 million people [1], particularly in the developing world [2,3]. Biologically contaminated, typically unmanaged, water sources are important carriers of diseases including typhoid, dysentery and cholera. Rapidly characterizing these sources on site could minimize the likelihood of human exposure. Access to an inexpensive, easy-to-use biological sensor would inform the user of contamination, allowing them to either treat the water sample or avoid it altogether. With this problem in mind, the aim of my project was to progress RNA-based circuitry towards diagnostic application for the identification of a contaminating RNA**.**

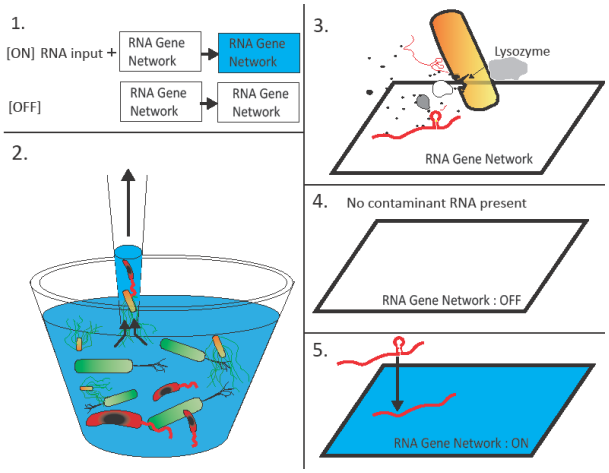


Figure 1. Schematic of the proposed diagnostic. **1.** After gene expression, the diagnostic is ON and returns a blue colour in the presence of an RNA input, OFF and white in its absence. **2.** Liquid is withdrawn from a container of contaminated water. **3.** When added to the paper strip, bacteria within the sample lyse and eject their cargo on contact with a lytic enzyme expressed by the genetic network on paper. **4.** If RNA from a contaminant is not present, a colorimetric assay is white. **5.** Contaminant RNA activates the network; the assay cleaves an expressed protein to yield a blue color.

In this paper, I will demonstrate the expanded utility of toehold-based RNA biosensors *in vivo*. My work will establish a future direction for development of flexible RNA diagnostics. This paper will conclude with a proposal for next steps in future work, aside from this project. This will involve exploring key engineering challenges associated with the diagnostic including the process for extracting detectable molecules from contaminant cells and the tool's sensitivity. In addition, the range of characterized contaminants should be expanded to include small molecules and heavy metals.

The use of paper for a diagnostic test is not a new concept, but combining it in tandem with engineered biological systems is. This novel approach is important because the use of biological systems in the wider world is heavily restricted due to concerns over toxicity and biosafety. Paper offers a potentially safe route for application outside the laboratory [4]. On paper, cells are not viable, so many of the toxicological fears associated with cell-free systems are redundant. The internal cellular machinery needed to produce a biological system is present, but the cells are not intact and are not capable of reproduction. Recently, a biological paper application of this kind was used to distinguish between two emerging strains of the Ebola virus in under 24 hours [5], during a severe outbreak. This application highlighted other strengths of paper detection. Firstly, the open, inexpensive nature of the platform means specific sensors can be rapidly built and applied. Next, the quick response of the device in delivering its output means that diagnostic decisions are made *in situ*, with no delay for off-site lab analysis. When the problem at hand is whether or not to drink from an unknown source, speed is essential. Overall, the use of biological networks on paper is a transformative idea that

is ready for application with novel biological designs and important problems.

Engineered biological systems make logical candidates for use in a biosensor diagnostic since can they harness the extraordinary natural abilities of micro-organisms such as bacteria [6,7]. Microbes possess inbuilt sensing behavior [8–12] that is driven by patterns of gene expression – the timing of when and how genes are switched on and off [13]. The recognition that this type of cellular response was controlled by circuits of biological components, was an important step for the synthetic biology field [14]. From there, a vision of programmable circuitry emerged [15]. To realize this idea in a synthetic context, biological parts or regulators are rationally combined [16,17] in genetic circuits to perform computational operations analogously to electrical engineering. Combinations of DNA, RNA and proteins are engineered to function like transistors, transducers and resistors. Of all the regulators available for engineering genetic networks, RNA is tailor made for high-performing, predictable circuitry [3,4,6]. Firstly, it can be manipulated with great freedom at the DNA level with an understanding of Watson-Crick base pairing to predict RNA-RNA interactions. Next, RNA-only circuits regulate gene expression at the transcription level, where DNA is written to messenger RNA (mRNA), and can transfer signals at a faster rate than protein-based circuits [13]. Functionally speaking, it is a remarkably diverse molecule. However, the flexibility of RNA has only recently come to light. For many years, it was considered a passive carrier of genetic information. The significant discovery of ribozymes, catalytic RNAs [20], reversed that perception. Since then, RNA's ubiquity across a multitude of biological interactions has been unraveled [21,22]. The diversity of natural and synthetic RNA structures [23–25] controlling these

interactions means that there is a significant and ever-expanding toolbox from which to construct genetic circuits. Recently, small transcription activating RNAs (STARs) were developed to address a functional gap that was absent in nature [26,27]. This family of synthetic switches [28] will be adapted to respond to a trigger input RNA, with unrestricted sequence, from a contaminant bacterium, at the heart of the proposed diagnostic. In summary, innovative RNA design in the current project and the solution of some important outstanding engineering challenges in future work will make *in vitro* RNA diagnostics a clinical reality.

CHAPTER 2

SIGNIFICANT BACKGROUND LITERATURE

Paper-based diagnostics signal a new era for affordable, point-of-care diagnostics.

While the synthetic circuitry is the sensing core of the planned diagnostic tool, the paper substrate will be crucial in developing it as an *in vitro* application. Recently, a protein-RNA hybrid network demonstrated the compatibility of a gene network with paper for the first time. As discussed previously, the deployment of synthetic gene networks in the wider world has been restricted because of fears over biosafety and the format of the cell host [5]. Generally speaking, to date, efforts to transition from *in vivo* work to an *in vitro* biosensor have involved reactions with cells in solution phase and in lipid vesicles called liposomes [5]. In some cases, they have relied exclusively upon interactions between a sensed small molecule and the promoter region of DNA that initiates transcription [9–11,29,30,39]. These approaches are either impractical, awkward to handle or lack generic utility. A much-needed new approach involves fixing a *cell-free* genetic network onto paper. The resulting material imitates the in-cell environment and can perform gene expression but is sterile. It can be stored for up to a year [5], perhaps even longer. When functional, a hydrophobic layer works to separate adjacent reaction spaces on the surface [31]. The diagnostic becomes active for sensing when a small quantity of water is added. As proof-of-concept, a diagnostic of this type was used to target distinct regions in the mRNA encoded by the Ebola virus (Zaire and Sudan strain) nucleoprotein, which is associated with viral replication [32]. This work highlighted other strengths of the medium. Firstly, in terms of cost, sensors could be built for in the Ebola

detection study for as little as 35-65¢ each, and significant savings are thought to be possible using an in-house cell-free system such as is currently used by the Lucks group [5,13,33,34], not a commercial system. Next, the output of the diagnostic - unambiguous, visible, color change - was illustrated. To achieve this, the sequence encoding the β-galactosidase (LacZ) enzyme was inserted on the expressed RNA. A layer of the liquid-phase X-galactosidase assay was added to the paper surface. When LacZ is expressed by the genetic network, it cleaves the white chlorophenol-β-D-galactopyranoside, contained on the paper strip to produce a blue product [5,35,36].

Cell-free transcription-translation, TX-TL, replicates the internal cellular environment, outside the cell.

Engineering gene expression on paper in the proposed diagnostic will require a platform that mimics the internal cellular environment, but lacks much of its toxicological potential. Thankfully, cell-free protein systems have been modified to carry out that exact role with genetic networks. In a TX-TL system, the internal cellular machinery is present, but the cell wall is not and DNA replication cannot occur. As a result, DNA can be added to the reaction at any time. The open nature of the system has resulted in testing cycles lasting 2-3 hours, compared to 2-3 days *in vivo*. TX-TL requires the mixing of three inputs in a test-tube: DNA encoding a genetic circuit, extracted cellular machinery and a buffer. Although still in its infancy as a tool for the analysis of genetic networks, the dynamic behavior of the platform has been extensively studied [13,37]. For instance, the response time was found to be approximately 5 minutes per step within an RNA-only transcriptional network [13].

pT181 attenuation establishes RNA-only circuitry, for the first time.

A crucial requirement of the proposed diagnostic is that it responds rapidly to the presence of a contaminant. To meet this need for fast dynamics, the circuitry must be exclusively composed of RNA components. Regulating gene expression in this way, with no protein intermediates, is based on binding interactions between RNA strands. The convention when discussing DNA and RNA, which are said to have 5' and 3' ends, is that gene expression proceeds in the 5' to 3' direction. Furthermore, two complimentary nucleic acid strands, referred to as the sense and antisense strands, bind such that the 5' end of the sense strand aligns with the 3' end of the antisense strand and vice versa. Antisense-controlled RNA interactions make RNA-only circuitry possible. One such antisense interaction, the pT181 attenuation mechanism was found to occur on a *Staphylococcus aureus* bacterial mRNA [38]. Although this interaction was discovered in an mRNA region that is not translated to protein, it plays an important role in regulating gene expression from its position upstream of the coding region. Novel work within the Lucks group exploited this natural interaction in *E. coli* [18]. The pT181 attenuator works like a switch, with ON and OFF states dictating whether a gene is expressed. At the heart of the switching process is an RNA secondary structure called an intrinsic terminator hairpin that forms when two sequences within the same sense strand can base-pair with themselves, forming the 5' and 3' stems of the hairpin. It is thought that as the enzyme RNA polymerase is transcribing DNA to mRNA, it is slowed by the hairpin-loop structure, then forced to dissociate completely from the mRNA transcript when it encounters a chain of repeating nucleotides after the hairpin. This stops transcription. In the pT181 mechanism, the antisense and sense RNAs interact to

expose the terminator, switching transcription OFF or repressing gene expression, while in the absence of the antisense RNA, the terminator is sequestered and transcription is ON. The level of repression was quantified through the expression of a reporter gene, Superfolder Green Fluorescent Protein (SFGFP) [39], that was fused downstream of an terminator on the sense RNA. Finally, specific pairs of orthogonal or independently acting sense-antisense RNA pairs were built. Interacting a pair of orthogonal strands resulted in high repression and low fluorescent output, while reacting a sense RNA with an off-target or non-cognate antisense delivered low repression and high fluorescence.

STARs address a gap in natural RNA regulation, creating transcriptional activators.

While the synthetic pT181 attenuation mechanism demonstrated that RNA-only circuitry could be built for the first time, it functioned to repress gene expression when

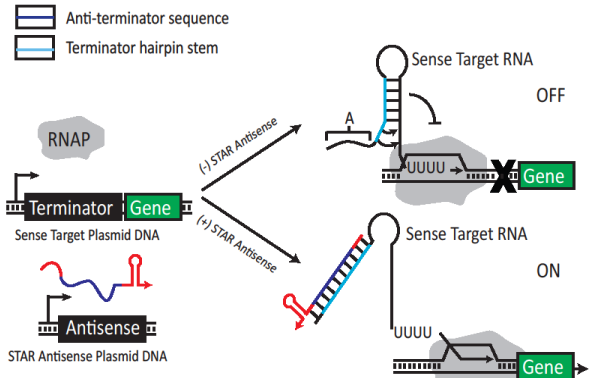


Figure 2. The STAR mechanism. Binding of an antisense RNA activates gene expression by unzipping a terminator hairpin. Linear recognition region, A and the 5' terminator hairpin stem are highlighted.

an antisense strand was present. The circuitry in the proposed diagnostic must transcriptionally activate with the addition of a trigger RNA. In the absence of a natural bacterial transcriptional activator, this transition requires a synthetic solution. To bridge this gap, STARs or small transcription activating RNAs were developed [26]. The STAR mechanism, illustrated in Figure 2, is an extension of the pT181 attenuator that relies on

the interaction between sense and antisense RNAs. The sense RNA contains the terminator hairpin and features two notable elements: a linear antisense recognition region, labelled A and the 5' terminator stem, colored in Figure 2. With no antisense present, the terminator is exposed and transcription is OFF. When the antisense is present, it binds, first to the recognition sequence, then along the 5' stem, which unzips the terminator and turns gene expression ON. The level of expression is characterized by the fold activation, the ratio of fluorescence/cell culture optical density for the ON state divided by the OFF state. Varying the linear recognition region, A, has been shown to be an effective design strategy for generating orthogonal variants.

Toehold switches demonstrate a novel approach for RNA sensor design.

While STARs provide a high-performing template mechanism for building transcriptional activating sensors in the planned diagnostic, it is possible to engineer the sense-antisense interaction to provide sharper fold activation. Recently, a novel toehold-

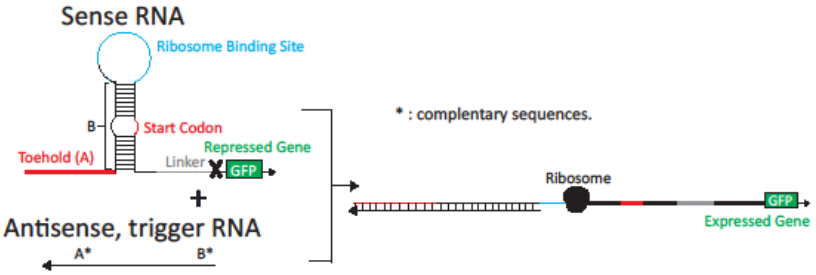


Figure 3. Toehold riboregulation. A trigger RNA unzips the sense hairpin, exposing translational regulatory features. The novel design element is the labelled toehold sequence, A, at the 5' end of the hairpin.

based design, illustrated in Figure 3, increased the fold activation of translational switches [40] by greater than an order of magnitude [5,41]. In this mechanism, regulatory elements required for translation are sequestered in a hairpin structure. An antisense RNA unzips the hairpin, exposing the regulatory features (RBS and start codon), allowing the ribosome to bind, and switching the state from OFF to ON. The main

difference between this mechanism and STARs, aside from the fact they function at the translational and transcriptional respectively, is the presence of a short toehold sequence at the 5' end of the hairpin, as illustrated. This is shorter (approximately 15 nucleotides) than the long (47 nucleotide) recognition sequence in the STAR mechanism [26]. Forcing the antisense to bind to a short linear region initially is a fast kinetic reaction [42,43] that seeds the overall interaction.

Computational tools facilitates scalable, varied switch design

In order to build sets of transcriptionally active RNA sensors, to integrate into circuitry in the proposed diagnostic, a computational tool would be very useful. When desired RNA structures are inserted into the NUPACK *test tube* algorithm [44], it specifies output nucleotide sequences according to equilibrium base pairing [45] to form those desired structures. The algorithm minimizes a physically relevant objective function [46], the ensemble defect, the fraction of incorrectly paired bases in the target structures to form the minimum free energy (MFE) complex [48,49] and the optimal RNA design.

CHAPTER 3

RESULTS

Demonstration of a qualitative colorimetric output in-vivo

The first step involved the downstream component of the proposed diagnostic device: the colorimetric readout [24]. The aim here was to demonstrate the use of the β-

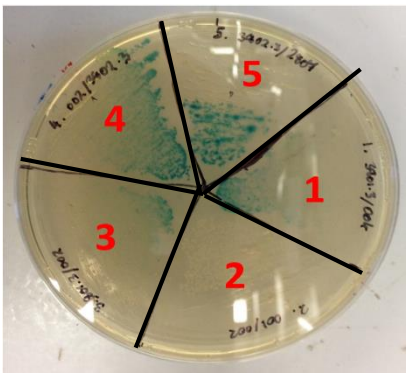
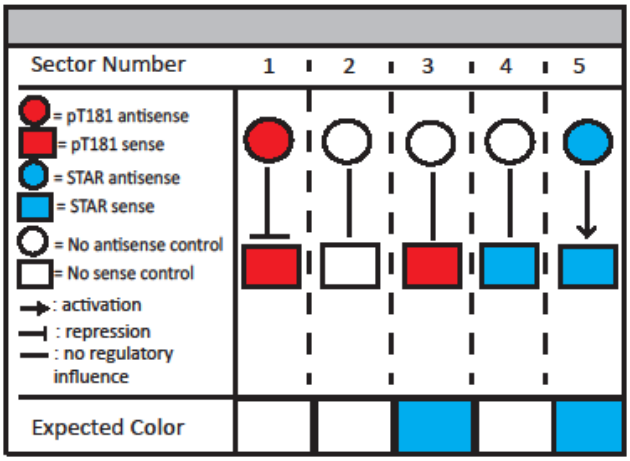


Figure 4. Using the β-galactosidase assay, colonies were blue or white based on whether the LacZ was expressed or not. Table 1 summarizes each plasmid set.

galactosidase assay with existing Lucks group circuit components: the pT181 attenuator [1,33] and STAR activator discussed previously [25]. The SFGFP sequence on each sense plasmid was replaced with the LacZ gene. The assay was spread on an agar plate containing the appropriate antibiotics. Pairs of sense and antisense plasmids and their controls were transformed, as detailed in Table 1, spread on the plate and incubated overnight at 37˚C. When viewed by eye, as in Figure 4, the colony color broadly reflected the expected state of the switch: ON or OFF. A small amount of leak, undesired activation, was observed in the OFF levels, but that was expected. Examining sectors 2, then 4 then 5 reveal a gradual increase in the number of blue colonies, reflecting the increase in activation. Similarly, there is a decrease in the number of blue colonies across sectors 1, 3 and 2, as the extent of repression increases.

Table 1. Summary of the plasmid combinations analyzed in each sector in the form of gene regulatory networks (GRNs). **Arrows-** pointed: activation, blunted: repression and uncapped: no regulatory influence, control plasmid.



Analysis of the impact of antisense to sense RNA ratios on GFP expression output in two plasmid systems.

At this point, the goal was to give the STAR pAD1 mechanism the flexibility to respond to any antisense RNA, not simply the specific naturally occurring antisense sRNA in the pAD1 mechanism, depicted in Figure 2. For the purpose of this project, three plasmid backbones were available, corresponding to three potential levels (L1, L2, L3) of a cascade of RNAs. Each backbone contained a different antibiotic resistance selection gene and a distinct origin of replication (ORI) DNA plasmid element [49]. The ORI dictates the number of DNA plasmids that are replicated and directly impacts on the concentration of transcribed RNA. To promote an interaction, the concentration ratio of binding RNAs is biased towards the antisense RNA [26]. The likelihood of a successful interaction, which hinges on the frequency and speed of biomolecular binding [26,49], is increased in this way. As a first step in modifying this interaction, the sense and antisense sequences were fused onto low and medium copy plasmids respectively. By examining three different two plasmid combinations, the ratio of antisense to sense

RNAs was varied *in vivo*. In combination 2, with the sense RNA expressed on L3, the

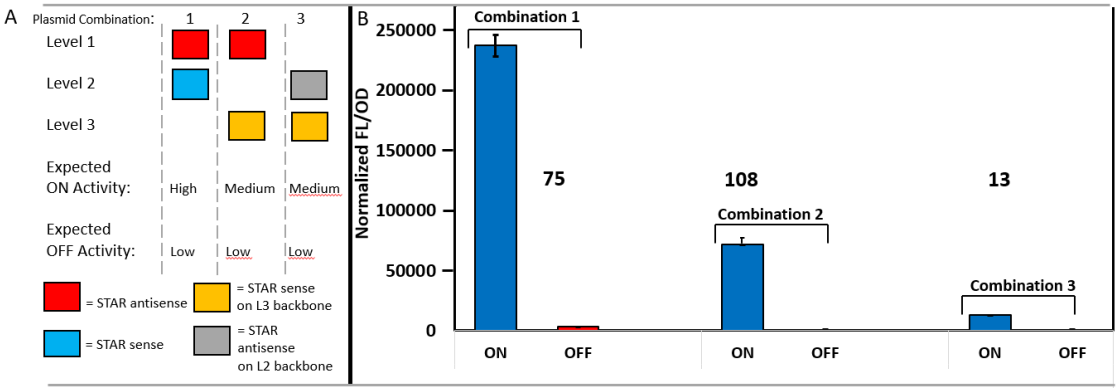


Figure 5. Examination of the influence of STAR antisense and sense concentration on GFP output. A: Gene Regulatory Network illustrating the two plasmid combinations used. B: Fluorescence/OD data for each of the plasmid combinations. Fold activation ratios are included.

fold activation was improved from 75 to 108. This was attributed to a higher ratio of antisense to sense strands and an increase in the proportion of activated sense. Likewise, in combination 3, the relative ratio decreased and the fold change dropped as expected, to 13. This established a benchmark for the order of fold activation to be expected with the sense RNA expressed on a low copy plasmid.

Design of three plasmid level flexible toehold STAR sensors

Next, the aim was to combine the STARs mechanism with the toehold design and yield a more flexible transcription activation mechanism. Where pAD1 involved one interaction between a sense and antisense RNA to activate, the proposed mechanism requires two, as illustrated in Figure 6A. Firstly, the trigger RNA binds to a toehold hairpin RNA, as in Figure 3. This interaction exposes a linear sense recognition sequence, for binding to the third, target RNA, the site of the intrinsic terminator and gene of interest. Figure 6A illustrates the three RNAs required for activation to occur. In more detail, Figure 6B shows the three level interaction, with complimentary strands

highlighted. The medium and low copy variants of the STAR antisense and sense RNAs respectively, were used from Figure 5. The STAR toehold RNA was expressed on a medium copy plasmid while a trigger RNA, was expressed on the high copy, L1 plasmid.

Characterization of three level STAR toehold sensors for response to Ebola sequence trigger RNAs.

To transition to three level sensor diagnostics, a trigger RNA from the Ebola strain detection work discussed previously [5], was used. Complimentary transcriptional STAR toehold and target RNAs were built, consistent with the mechanisms in Figures 6A and

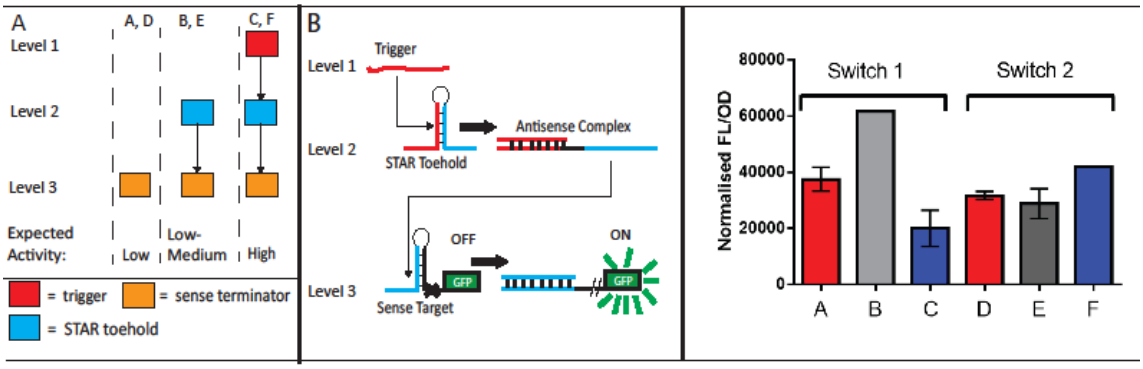


Figure 6. Toehold STAR sensors. A: Simplified gene network schematic that illustrates the levels in the diagnostic and the combination of levels (RNAs) required for activation. B: More detailed schematic illustrating the interaction between the trigger and the toehold STAR, then between the linear 3' end of the toehold STAR and the recognition region on the sense target, resulting in activation. C: The RNA subsets in A were characterized using bulk fluorescence and optical density measurements.

B. By truncating this trigger strand at distinct points, switches with different toehold lengths were made. Furthermore, in terms of experimental design, since this is a three level mechanism, and with complimentary sections of sequence between the trigger and target, there is an opportunity for the trigger in level 1 to interact directly with the target in level 3, bypassing the STAR toehold in between. To test for this, subsets involving

1, 2 and 3 plasmids were characterized. Figure 6A illustrates the possible subsets, the expected level of activity and the levels required for full activation. In Figure 6C, characterizing the activity of each subset revealed very different behavior across the two switches. In switch 1, instead of sequential activation as the levels are added, an initial increase in activation was observed between A and B, but curiously repression occurs between B and C. This is undesirable behavior. In contrast, for switch 2, within the standard deviation, there is parity between bars D and E and an activation increase when the trigger is added. This is the desired behavior. However, the level of fold activation for switch 2 was modest at 1.5. This is consistent with the fold activation observed in the early stages of STAR design [26]. Even so, it highlights the early potential of the transcriptional sensing diagnostics. Further design work will deliver sharper fold activation and superior switch performance.

CHAPTER 4

FUTURE DIRECTIONS

Having broadened the scope of the STAR pAD1 mechanism to sense any small RNA sequence at a modest level, future work will definitively refine this approach. Several next steps will be essential for the development of an effective sensing diagnostic.

Optimization of RNA transcriptional sensors to address OFF level leak

Although the desired sequential increase in fluorescence output was observed when the trigger level was added to switch 2 in Figure 6C in the current paper, the interaction between levels 2 and 3 was unacceptably high or transcriptionally leaky, in the absence of the trigger.

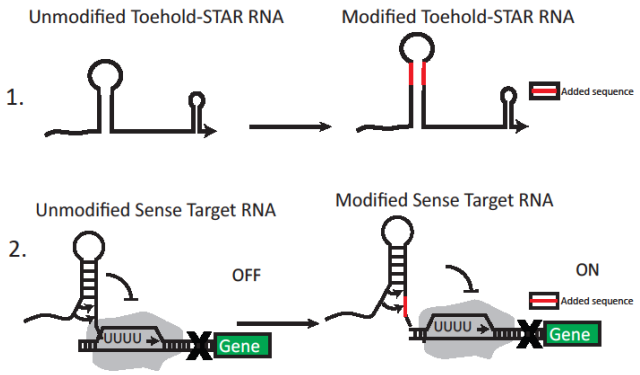


Figure 7. Two proposed design solutions to tighten the OFF level leak: (1) Sequence added to extend the hairpin on the toehold STAR, would strengthen it and sequester the linear target recognition sequence to a greater extent. (2) Sequence added at the base of the 3' terminator stem would bring more of the linear recognition region into the hairpin, making it more difficult for the trigger to directly activate the target.

To address this, I propose two solutions, as illustrated in Figure 7. By (1) strengthening the toehold hairpin on the toehold STAR RNA or (2) by adding extra complimentary sequence at the 3' end of the sense target terminator to further sequester the terminator stem, the issue should be remedied. These designs should use the functioning sensor

from Figure 6C as a template and build on it to rationally improve performance. Testing should be performed *in vivo* and *in vitro* in TX-TL.

The construction of an orthogonal library of transcriptional sensors

To transition towards a water-borne contaminant sensor, a library of three level combinations should be constructed to interact in a highly specific manner *in vivo* and *in vitro* (TX-TL) to target distinct domains on mRNA in three bacterial water contaminants: *Vibrio cholerae*, *Salmonella enterica* and *Shigella dysenteriae*, responsible for cholera, typhoid and dysentery respectively [3]. Combining variations in both the length and sequence of the toehold domain (A) in Figure 3 with the design proposals in Figure 7 would be an effective approach.

Anticipated results and alternative approaches

The two proposed design solutions are sensible approaches to tightening sensor leak. If successful, they would raise the observed fold activation for this set of RNA sensors, and switch the focus towards the construction of orthogonal variants for application *in vivo* and *in vitro*. In the event that neither of the proposed approaches was successful, many alternatives exist. Although, it has displayed lower fold activation than direct termination to date (11-fold maximum), the anti-anti termination STARs [26] mechanism offers more points of control for design, through the involvement of an additional strand that is complimentary to the anti-terminator sequence. Alternatively, the toehold translational switch sequences may serve as a useful template for building transcriptional sensors, once the Ribosome Binding Site and start codon are removed, and a poly uracil sequence is added after the terminator.

Key engineering challenges remain for paper-based sensing

To date, the paper-based diagnostic platform has been explored at a relatively superficial level [5]. To accelerate the platform further towards a clinical application [50], key engineering challenges must be solved. The first challenge involves the process of extracting trigger mRNA from an intact bacterial cell. The sensing diagnostic can only function when the cell has been lysed open to release its cargo. The Ebola paper-based detection study discussed previously [5] did not introduce a means of lysing cells that would be accessible to a non-specialist in a resource-limited area. I propose a solution: the lysing functionality should be built into the paper diagnostic. To demonstrate paper-based lysis, a BL21 *E. coli* strain could be engineered to express a T7 lysozyme, an enzyme responsible for cell lysis, on a chloramphenicol plasmid *in vivo* first, then *in vitro*. Secondly, the response time of paper–based transcriptional circuitry should be tested by monitoring the response to a small ubiquitous biomolecule, arabinose. Using an arabinose-sensitive promoter [51], which regulates gene expression in response to arabinose binding, the *in vitro* delay time in TX-TL and on paper should be measured. The faster response time of transcriptional circuits over translational circuits, would be expected to be observed [5]. Finally, the sensitivity of the detection diagnostic can be analyzed in response to water-based cell cultures over a range of concentrations. The correlation between pathogenic contaminant concentrations and the lower detectable limits of the diagnostic would be of particular interest.

CONCLUSIONS

In summary, this project has broadened the utility of transcriptional activator diagnostics. My work in toehold STAR regulators hints at real potential for sensing nucleic acids. In addition, the *in vivo* β-galactosidase work demonstrates the compatibility of colorimetric output with STAR componentry. In short, the outlook for the future is clear and strongly motivated. Pressing health concerns in resource-limited environments necessitate the development of an inexpensive, unambiguous diagnostic. This paper sets out a roadmap towards meeting this goal.

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