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INTEGRATING AND AMPLIFYING SIGNAL FROM RIBOSWITCH BIOSENSORS

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14. ABSTRACT
Biosensors offer a built-in energy supply and inherent sensing machinery that when exploited correctly may surpass traditional sensors. However, biosensor systems have been hindered by a narrow range of ligand detection capabilities, a relatively low signal output, and their inability to integrate multiple signals. Integration of signals could increase the specificity of the sensor and enable detection of a combination of ligands that may indicate nefarious activities when detected together. Amplifying biosensor signal output will increase detector sensitivity and detection range. Riboswitches offer the potential to widen the diversity of ligands that may be detected, and advances in synthetic biology are illuminating myriad possibilities in signal processing using an orthogonal parts-based engineering approach. In this chapter we describe the design, building, and testing of a riboswitch-based Boolean logic AND gate in bacteria, where an output requires the activation of two riboswitches, and the biological circuitry required to amplify the output of the AND gate using natural extracellular bacterial communication signals to 'wire' cells together.

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Biological circuitry, Biosensor, Chemical wiring, Genetic logic, Synthetic Biology

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1.0 INTRODUCTION

Nature has developed myriad solutions to detecting and responding to external environmental ligands. By exploiting a cell's natural sensing and signal processing capabilities, modular, sensitive, and selective engineered sensors can be produced. The discovery and development of riboswitches has opened the door to their usage in ligand-dependent reportable devices, or biosensors. The ideal biosensor would be self-sustaining and generate a signal that could be detected from afar without the need for energy-hungry electronics, i.e., a passive sensor. Indeed, the major advantage of biosensors is that they can overcome the limitations of traditional sensors by having a built in energy generator and utilize sensing machinery already present in nature (Bhatia, 2006). The advantage of using riboswitches as sensors is that the output of the sensor can also be modular, i.e., it can be the expression of any gene. This modularity provides a wealth of options regarding the functionalization of the sensor and its integration into a wider sensing array. The simplest detectable biological sensor output is the expression of a fluorescent protein. This is advantageous since the fluorescent signal is additive, meaning that signal intensity is dependent upon the amount of protein produced; it generally requires illumination by a specific wavelength to emit a signal, meaning that knowledge of the correct excitation wavelength can be controlled; and the signal is often outside the visible spectrum, making it covert. However, a limitation of biological sensors that produce a fluorescent output is that the amount of fluorescence produced is usually sufficient only for detection by sensitive lab-based detection equipment. In addition, unlike electronic-based sensors, the output of biological sensors is generally not conducive to signal integration. Thus, signal integration and signal amplification in biological sensors is required for the development of fieldable, sensor platforms.

1.1 Biological Circuits

Synthetic biology has taken the lead from electrical engineering to construct circuits and logic gates that have been used in pharmaceutical and biotechnological applications (Guet, Elowitz, Hsing, and Leibler, 2002; Mayo, Setty, Shavit, Zaslaver, and Alon, 2006; Anderson, Voigt, and Arkin, 2007; Rinaudo, Bleris, Maddamsetti, Subramanian, Weiss, and Benenson, 2007; Weber, Schoenmakers, Keller, Gitzinger, Grau, Daoud-El Baba et al., 2008; Ellis, Wang, and Collins, 2009; Tamsir, Tabor, and Voigt, 2011). Indeed, multiple logic gates have been combined to perform more complex 'programs' within cells (Friedland, Lu, Wang, Shi, Church, and Collins, 2009; Tabor, Salis, Simpson, Chevalier, Levskaya, Marcotte et al., 2009; Lou, Lio, Ni, Huang, Huang, Jiang et al., 2010; Moon, Lou, Tamsir, Stanton, and Voigt, 2012), and even between cells using bacterial cell-cell communication pathways as 'wires' (Tamsir et al., 2011). These recent advances have opened the way to incorporate biological circuitry into biosensors to address integration of information and signal amplification. To attain biological sensor integration and signal amplification, we consulted biology. In nature, multiple inputs are integrated into one signal via a signaling cascade (e.g., the phosphorylation cascade network incorporating many inputs to activate a common transcriptional activator such as in the NF-kappaB pathway; Kawai and Akira, 2007) and signals are amplified by a positive feedback mechanism whereby activation of an output signal causes more signal to be produced (Afroz and Beisel, 2013). The latter example often leads to run-away production of the output which is generally detrimental to natural systems (e.g., cancerous growth), but is beneficial to signal amplification. Using inference from natural systems, we will describe methods to utilize the modularity of our sensor output and synthetic biology to integrate the output of riboswitches and amplify their output

signal. In this method, each compartment of the biological 'program' is a separate group of bacterial cells with the same genetic identity. This has the benefit of producing a more reliable computation by population averaging the response. We will use plasmids to act as the computational units of each compartment (Tamsir et al., 2011). The plasmids are constructed so that they contain all of the required information and are compatible in the same cell. However, between cells, components can be re-used because plasmid function is 'insulated' within the cell. This allows for re-use of transcription factors in multiple cells allowing a program to be built from a smaller number of orthogonal parts (Tamsir et al., 2011).

There are a number of considerations when designing the circuit and the plasmids that will convey the information. These include not only the genes to be activated, but also genetic controllers such as strength of promoters and riboswitch binding sites, degradation of the product, how tightly a riboswitch is regulated, and the level of output required. Many of these genetic circuit components are gathered in public accessible repositories, such as the 'Registry of Standard Biological Parts (http://parts.igem.org). Computational modeling is achieving huge strides in being able to predict which genetic components will form a reliable circuit, and many of the 'industrial' scale synthetic biology laboratories successfully utilize programs such as Clotho (Xia, Bhatia, Bubenheim, Dadgar, Densmore, and Anderson, 2011) to design complex circuitry. However, for many researchers that want to apply synthetic biology principles to enhance their research, there is currently no better way than to follow the engineering mantra of 'design-build-test.'

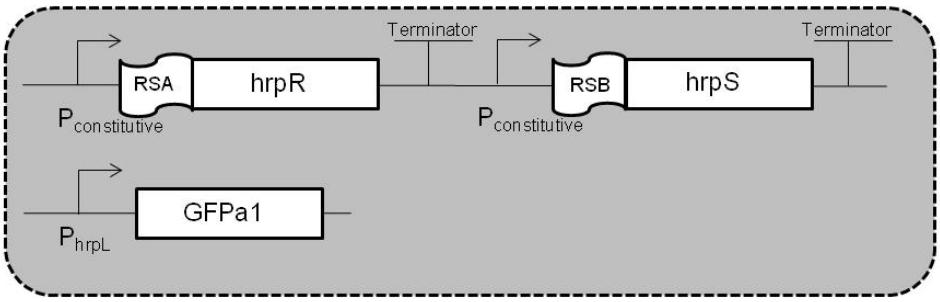
2.0 RIBOSWITCH SIGNAL INTEGRATION

2.1 Design

The specificity of biological sensors would be increased, and potentially dangerous chemical interactions could be detected if two (or more) signals from signature sensing elements could be integrated into a single report. For example, the likelihood of false positives could be reduced if two sensing elements that detected different parts of the same molecular signature were integrated such that a reporter was expressed only if both were activated. Similarly, such a circuit would also be advantageous in the detection of two chemicals that together produce hazardous consequences although they may be independently innocuous. Traditionally, biological sensors have been unable to be integrated. However, using recent advances in synthetic biology, we plan to achieve sensor integration by developing a genetic program that utilizes an orthogonal genetic Boolean logic gate in individual cells. In current genetic logic systems, inputs are promoters that activate expression of a protein (the output) that is either detected or becomes the input for another logic gate. In our system, we plan to use riboswitches to activate expression of transcription factors that serve as the input to the logic gate.

2.1.1. AND gate

Using data mining and directed evolution synthetic biologists have built transcriptional AND gates (Wang, Kitney, Joly, and Buck, 2011; Wang, Barahona, and Buck, 2013; Moon et al., 2012). The inputs to these gates induce expression of two proteins that are both required to activate a promoter. Activation of this promoter results in expression of the output of the gate. Thus both inputs must be present to produce an output. Orthogonal AND gates have been connected to form programs, the largest of which is a four-input AND gate that consists of three circuits that integrate four inducible systems, requiring 11 regulatory proteins, all within the same cell (Moon et al., 2012). We will describe integrating two riboswitches in *E. coli* using a hetero-regulation module from the hypersensitive response and pathogenicity (hrp) system for Type III secretion in the bacterium *Pseudomonas syringae* (Hutcheson, Bretz, Sussan, Jin, and Pak, 2001; Wang et al., 2011). This device comprises riboswitch control of two co-activating genes *hrpR* and *hrpS*, and a sigma^{54}-dependent *hrpL* promoter driving the output, a fluorescent reporter gene (Figures 1 and 2). The *hrpL* promoter is activated only when both *hrpR* and *hrpS* are expressed. Riboswitch integration using AND gate logic opens the path to increased layering and complexity of genetic programming in biological sensors. In addition, because of the modularity of biological circuits, the integration circuits described here and the amplification circuits described below may be linked simply by swapping the output of the integration circuit from a fluorescent protein to a bacterial communication molecule.



Input A	Input B	Output GFPa1
0	0	0
1	0	0
0	1	0
1	1	1

Figure 1: Genetic Integration Circuit -Including the Truth Table for the Circuit

Both riboswitches must be activated for the expression of hrpR and hrpS. The hrpL promoter is only activated in the presence of both hrpR and hrpS. RSA: Riboswitch to ligand A; RSB: Riboswitch to ligand B.

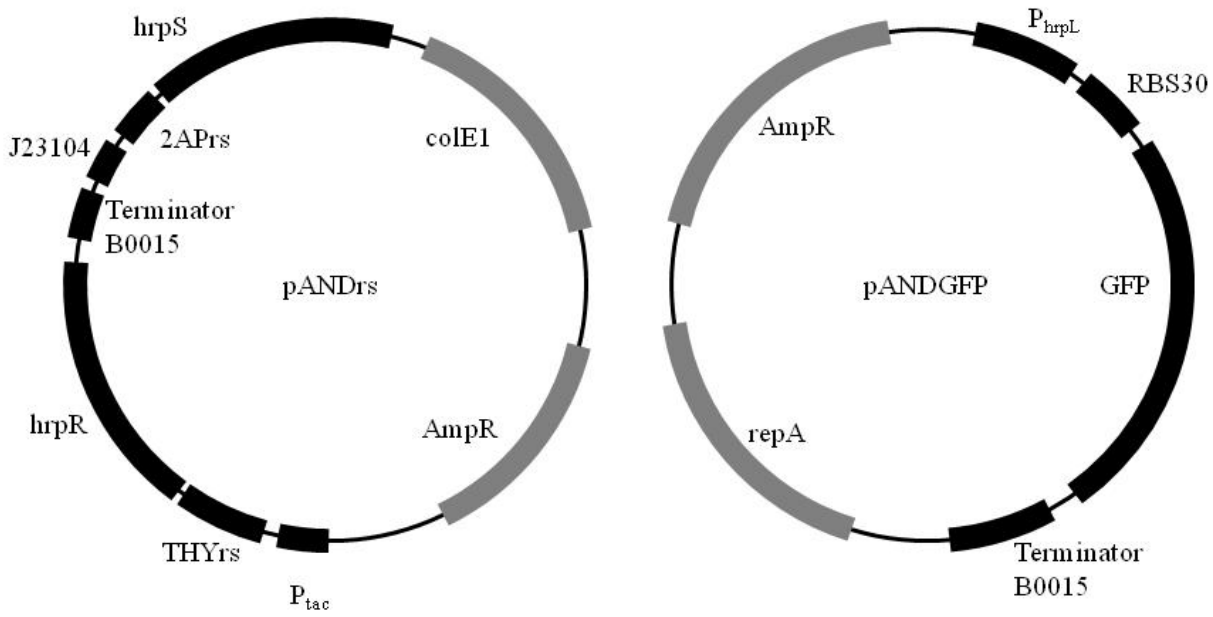


Figure 2: Plasmid Maps of Genetic Integration Circuit

AmpR: ampicillin resistance; P_{tac}: tac promoter; THYrs: theophylline riboswitch; J23104: strong constitutive promoter; 2APrs: 2-amonipruine riboswitch; colE1: origin of replication; P_{hrpL}: hrpL promoter; RBS30; strong ribosome binding site; repA: origin of replication.

2.1.2. Selection of Riboswitches

The riboswitches that will be used for the signal integration AND gate need to have a tight regulation as well as a well-defined activation ratio (the difference in output from 'off' to 'on'). For the purposes of this study, we will use the theophylline synthetic ribsowitch developed by Justin Gallivan's lab (Desai and Gallivan, 2004; Lynch and Gallivan, 2009), and the 2-aminopurine (2AP) riboswitch described by Dixon, Duncan, Geerlings, Dunstan, McCarthy, Leys, and Micklefield (2010). The theophylline riboswitch 12.1 has a large activation ratio of 96 and a half-maximal expression concentration of ~250 □M. The 2AP riboswitch is normally found in the 5' untranslated region of adenine deaminase in *Vibrio vulnificus* (Mandal and Breaker 2004) and has an activation ratio of ~12 and a half-maximal expression concentration of ~20 □M. As described above, we will be using these riboswitches to induce expression of *hrpR* and *hrpS* genes to form an AND gate. Since these genes have not, to our knowledge, been incorporated into the THY and 2AP riboswitches before, it is essential that some basic modeling using mfold is performed to make sure that the riboswitch will perform as expected. The strength of riboswitch and gene sequence binding can be predicted by inputting a sequence containing the riboswitch and the first 100-150 nucleotides of the downstream gene into mfold (Zuker, 2003) and forcing the aptamer region into configurations corresponding to before and after ligand binding. The energy required to switch the riboswitch on can then be compared to that imparted by the binding of the ligand to the aptamer. When the THY riboswitch is placed upstream of hrpR, the riboswitch basepairs well with the gene resulting in a conformation that is unlikely to result in exposure of the RBS on ligand binding (Table 1). To overcome this issue we inserted a 'linker' sequence between the riboswitch and hrpR to ensure the proper conformational change in the presence of the ligand. We used the first 61 amino acids of IS10 transposase, a gene that has been shown to produce a functional switch when placed downstream of THY riboswitch (Desai and Gallivan 2004). In contrast, when the 2AP riboswitch is placed upstream of hrpS, a very weak binding occurs that is likely to result in the riboswitch spontaneously turning 'ON' without the presence of the ligand. To rectify this, the first 35 amino acids of eGFP were inserted between the 2AP riboswitch and hrpS to ensure proper functioning of the riboswitch (Dixon, Robinson, Geerlings, Drummond, and Micklefield, 2012). The THY riboswitch will be promoted using the strong *tac* constitutive promoter as described in Desai and Gallivan (2004), while the 2AP riboswitch will be promoted using a strong constitutive promoter described in the parts registry, J23104 (website of constitutive promoter from iGem: parts.igem.org/Part:BB_J23104). The first 35 amino acids of eGFP were inserted between the 2AP riboswitch and hrpS to ensure proper functioning of the riboswitch (Dixon et al., 2012).

Table 1: Results of Modeling Riboswitch Activation Energy

Riboswitch Construct	eOFF (kcal/mol)	eON (kcal/mol)	□G (kcal/mol)	□Ligand (kcal/mol)
THYrs-hrpR	-37.0	-27.2	-9.8	-9.2[$]
THYrs-linker-hrpR	-47.0	-40.7	-6.3	-9.2[$]
2APrs-hrpS	-33.0	-32.3	-0.7	-9.4[*]
2APrs-linker-hrpS	-43.6	-36.1	-7.5	-9.4[*]

eOFF: energy required to linearize ribsowitch in OFF state
eON: energy required to linearize riboswitch in ON state
□G: energy difference between eOFF and eON, i.e. energy required to turn on riboswitch
□Ligand: energy provided by the ligand binding to the aptamers
$: Jenison et al., 1994
*: Gilbert et al., 2006

2.1.3. Using Plasmid Backbones Available from the Parts Registry

The components of the AND gate will be carried on plasmids within *E . coli* cells. There are many plasmid backbones described on the partsregistry website. The plasmids described herein were generous gifts from synthetic biologists. The plasmid backbone provides a vehicle to get the genetic components into the cell and, as such, isn't critical apart from ensuring that the plasmids are compatible if co-transforming multiple different plasmids into a cell.

2.2 Build

2.2.1. Materials for Construction of a Riboswitch-Based AND Logic Gate

- Plasmids containing the theophylline riboswitch upstream of LacZ (pSAL, a generous gift from Dr. Justin Gallivan, Emory University, Atlanta, GA), an aadA riboswitch with eGFP linker (Dixon et al., 2010) preceded by a strong constitutive promoter J23104, and proceeded with hrpS-NotI site (generated by DNA2.0, Menlo Park, CA), and plasmids conferring AND gate behavior (one plasmid containing hrpR and hrpS, and a plasmid containing GFP promoted by the hrpL promoter; generous gifts from Dr. Baojun Wang, Imperial College London, UK)
- Proof-reading high-fidelity DNA polymerase (e.g., Phusion DNA polymerase, New England Biolabs)
- Gibson assembly cloning kit (New England Biolabs)
- Primers designed to amplify genes of interest and including a 18-20 bp overlapping sequence required for Gibson assembly. There are many options available for the synthesis of primers. Ours were purchased from Integrated DNA Technologies (Coralville, IA) and designed using their free oligo design software.
- UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA)

- Ampicillin (Sigma, St. Louis, MO): 50 mg/mL stock solution in water, final concentration 100 μg/mL of media for all procedures
- Cloramphenicol (Sigma, St. Louis, MO): 34 mg/mL stock solution in 100% ethanol, final concentration 25 μg/mL of media for all procedures
- LB Agar: 35 g/L Difco LB Agar (Becton, Dickinson and Company, Franklin Lakes, NJ)
- Sterile Petri dishes, 100 mm (Fisher Scientific, Pittsburg, PA)
- Agarose (Ambion, Austin, TX)
- 1X Tris-Acetate-EDTA (TAE) buffer prepared from TAE 50X Solution (Fisher Scientific, Pittsburg, PA)
- 2-Log DNA Ladder (0.1-10.0 kb) and Gel Loading Dye, Blue (6X) (New England Biolabs, Ipswich, MA)
- SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA)
- Nucelospin II gel extraction kit (Clontech, Mountain View, CA)
- Restriction endonucleases KpnI and BlpI (New England Biolabs, Ipswich, MA)
- QIAprep plasmid miniprep kit (QIAgen, Valencia, CA)
- Costar 96-well assay plate 3631 (Corning, NY)
- Spectrophotometer (SpectroMax M5; Molecular Devices, Sunnyvale, CA)

Prepare all media and buffer solutions using ultrapure water (prepared by purifying deionized water to obtain a resistance of 18 MΩ cm at 25º C). For antibiotic solutions and enzymatic reactions, use nuclease-free water. Autoclave media solutions, and filter-sterilize antibiotic solutions. Store media and buffer solutions at room temperature. Store antibiotic solutions at -20º C.

2.2.2. Methods to Construct pANDrs

- Using the appropriate plasmid as template, perform PCR to produce three amplicons, ensuring 15-20 bp overlap of each fragment: theophylline riboswitch with IS10 linker; hrpR-terminator B0015-constitutive promoter J23104-aadA riboswitch with eGFP linker; hrpS
- Digest pSAL with KpnI and BlpI
- Run PCR products and pSAL digestion on a 1% agarose-TAE gel and gel extract appropriate bands
- Quantify extracted bands spectrophotometrically (we use a NanoDrop, Wilmington, DE)
- Assemble gel extracted products using Gibson Assembly method (Gibson, Young, Chuang, Venter, Hutchison, and Smith, 2009). Briefly, this method can join multiple overlapping DNA fragments by using a 5' exonuclease to create single-stranded 3' overhangs that anneal and are acted upon by a DNA polymerase to extend the 3' ends. A DNA ligase then seals the nicks.
- Transform into NEB 5-alpha competent *E. coli*

- Plate on LB-ampicillin plates
- Purify the plasmid from overnight cultures of individual colonies
- Confirm correct sequence
- 10) Co-transform pANDrs and plasmid containing PhrpL promoted GFP (pANDGFP) in *E. coli* BL21 competent cells. These cells were chosen since they are good for protein expression cells because they are deficient in *lon* and *ompT* proteases.

2.3 Test

- Use a single cfu of a) co-transformed cells, b) single transformed cells containing the riboswitch AND gate, and c) the reporter plasmid, to inoculate LB-ampicillin
- Incubate at 37^{o}C with 220 rpm shaking overnight
- Inoculate 100 □l of overnight cultures into 3 ml aliquots of LB-ampicillin with and without appropriate concentrations of theophylline ([final] = 2.5 mM) and 2AP ([final] = 500 □M)
- Inoculate ligand-doped cultures with 100 □l overnight culture
- Incubate at 37^{o}C with 220 rpm shaking overnight
- Record absorbance at 600 nm and fluorescence using an excitation wavelength of 480 nm and an emission wavelength of 510 nm (bandpass filter of 495 nm). Co-transformed cultures exposed to both theophylline *and* 2AP will fluoresce at a higher intensity than co-transformed cultures exposed to either theophylline *or* 2AP. No fluorescence is expected from single plasmid transformed bacteria.

3.0 RIBOSWITCH SIGNAL AMPLIFICATION USING BIOLOGICAL CIRCUITRY

3.1 Design

In biological circuits, the output of an upstream component serves as the input to the next component. Since each component in our system is contained within a cell, each cell needs to be connected for the circuit to perform. Bacterial cell-cell communication is abundant in nature in a process known as 'quorum sensing'. Quorum sensing allows appropriate genes to be activated in a high cell density environment. This is achieved by each cell expressing a quorum sensing protein which is exported out of the cell. When the concentration of this protein reaches a threshold level, the protein activates transcription of genes that have a quorum sensing promoter. This biological wiring allows a signal to be transmitted to surrounding cells, thus propagating any input signal across multiple cell types. Separations of up to 1 cm can be bridged between individual cell types containing different biological circuit components using natural cell-cell communication involved in quorum sensing (Tamsir et al., 2011).

In our design, the input of the amplification circuit will be the output of a sensing device. To form a positive feedback amplification circuit, receipt of the output of the riboswitch must activate transcription of the reporter protein and activate a cascade that provides an intra- and extracellular signal to produce more of the reporter protein. Thus a bicistronic arrangement will be constructed. Quorum sensing molecules are produced by acyl homoserine lactone (AHL) synthetases LasI and RhlI. Both of these are originally from the bacterium *Pseudomonas aeruginosa* PA01 and have no homologues in the *E. coli* chassis we are using. LasI synthetsizes the AHL *N*-3-oxo-dodecanoyl-omoserine lactone (3OC12-HSL), while RhlI synthetsizes the AHL *N*-butryl-homoserine lactone (C4-HSL) (Pesci, Pearson, Seed, and Iglewski, 1997). These AHLs bind to their cognate transcription factors (LasR and RhlR) and activate them. The promoter that is turned on by the transcription factor is used as the input to the next biological circuit component. Thus in our design, activation of the sensor will result in production of an AHL. This signal is received by cells containing different plasmids to the sensor resulting in expression of a fluorescent reporter and AHL (Figure 3). This signal will propagate throughout the sensor resulting in increased reporter protein production. It will also result in an increased sensitivity of the sensor, since only a few cell types are required to detect the molecular signature of interest for all of the cells in the biological sensor to react. The reporter protein we have chosen is GFPa1, a green fluorescent protein from the cephalochordate *Branchistoma floridae*. This protein has been shown to exhibit extremely strong fluorescence (Bomati, Manning, and Deheyn, 2009), and thus makes an ideal reporter.

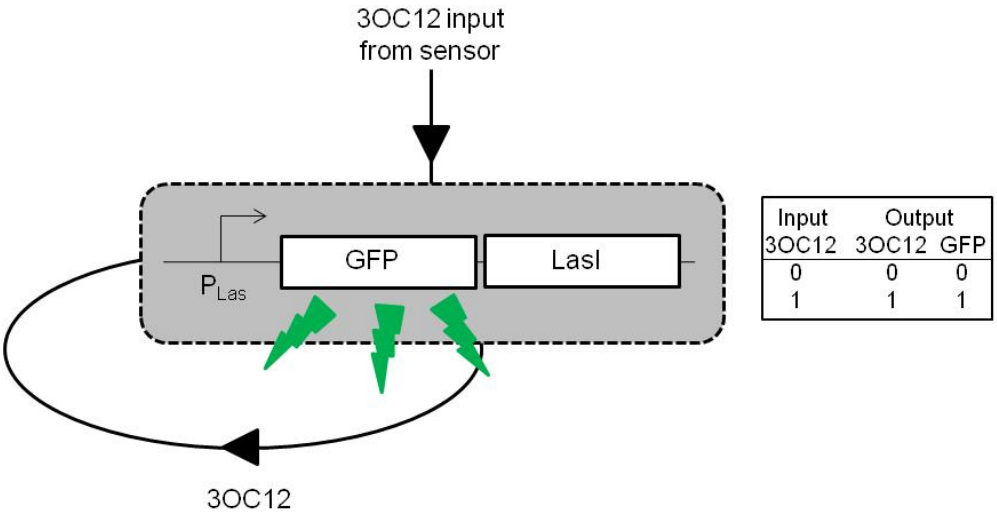


Figure 3: Genetic Amplification Circuit - Including the Truth Table for the Circuit

Detection of a molecular signature by a sensor such as a riboswitch results in expression of LasI and production of 3OC12-HSL. 3OC12-HSL activates the Las promoter resulting in expression of GFP and LasI. 3OC12-HSL activates the Las promoter of the same cell and also diffuses out of the cell resulting in activation of the Las promoter in surrounding reporter cells. Thus a progression of GFP and LasI expression is propagated.

In designing this circuit we need to tune the production of each protein such that the fluorescent protein is strongly expressed on receipt of the input signal, but HSL production is kept below its threshold until a positive upstream signal is received by the cell. Since the promoters used in this circuit are restricted to those responsive to HSL, the remaining synthetic biological parts that can tune expression are Ribosome Binding Sites (RBS) and protein degradation tags. We referenced constitutive RBS listed in the Parts Registry (parts.igem.org/Ribosome_Binding_Sites/Prokaryotic/Constitutive/Community_Collection) and also quantified by Wang et al. (2011), and used degradation tags also listed in the parts registry (parts.igem.org/Protein_domains/Degradation) (Figure 4).

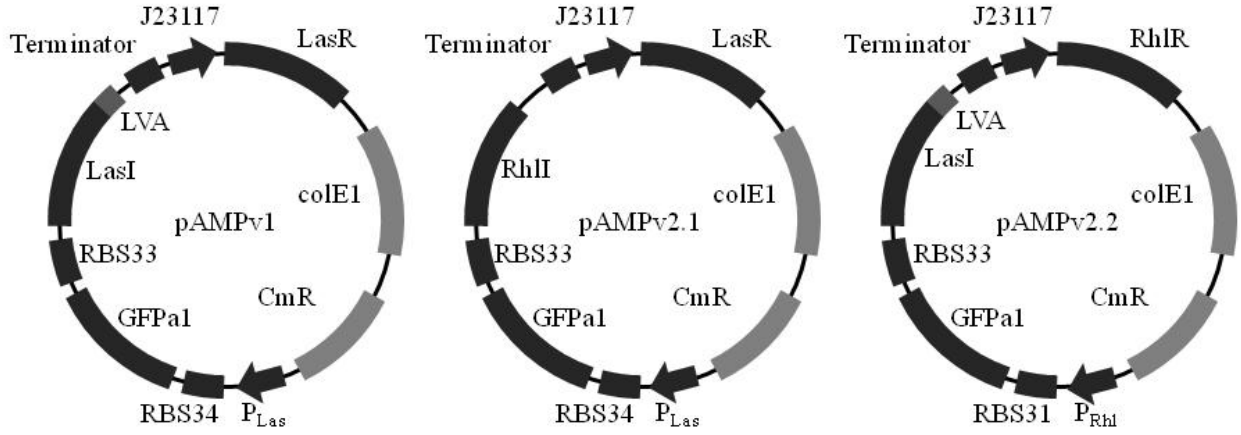


Figure 4: Plasmid Maps of Amplification Circuits

CmR: chloramphenicol resistance; P_{Las}: Las promoter; RBS34: strong ribosome binding site; RBS33: weak ribosome binding site; LVA: protein degradation tag; J23117: weak constitutive promoter; P_{Rhl}: Rhl promoter.

3.2 Build

3.2.1. Materials for Construction of an Amplification Circuit

- Plasmids containing LasR, PLas, RhlR , PRhl (pOR30 and pNOR40, generous gifts from Dr. Christopher Voigt, MIT, MA), and GFPa1 (a generous gift from Dr. Dimitri Deheyn, University of California, San Diego).
- Plasmid backbones pSB3K3 and pSB4A3 (generous gifts from Dr. Christopher Voigt, MIT, MA).
- Primers to amplify *LasI* and *RhlI* from *Pseudomonas aeruginosa* PA01 (ATCC 47085).
- Restriction endonucleases KpnI, BsrGI, BstBI, and BmrI (New England Biolabs, Ipswich, MA)
- PCR and cloning and fluorescent detection materials listed above
- Synthetic 3OC12-HSL and C4-HSL (Cayman Chemical Co., Ann Arbor, MI)
- DarkReader transilluminator (Clare Chemical Research, Dolores, CO)

3.2.2. Methods to Construct an Amplification Circuit - pAMPv1

PLas_ RBS34(strong)_GFPa1_Terminator_Weak RBS_LasI +/-degradation tag:

- Digest pOR30 with KpnI and BsrGI
- Amplify GFPa1 and LasI using primers that have a 18-20 bp overlapping sequence required for Gibson assembly. In this case our GFPa1 forward primer included a KpnI site immediately 5' of the GFPa1 sequence, and our LasI forward primers contained a GFPa1 overlap-BsrGI site-RBS at its 5' end and the LasI+LVA reverse

primers contained a LVA degradation tag (http://parts.igem.org). Two different RBS were tested: B0032 (weak) and B0033 (very weak).

- Run PCR products and plasmid digestion on a 1% agarose-TAE gel
- Gel extract appropriate amplicon and the large band corresponding to plasmid backbone
- Quantify extracted bands spectrophotometrically
- Assemble gel extracted products using Gibson assembly method
- Transform into NEB 5-alpha competent *E. coli*
- Plate on LB-chloramphenicol plates
- Purify the plasmid from overnight cultures of individual colonies
- Confirm correct sequence by sequencing

3.3 Test

- Grow sequence verified clones in LB-chloramphenicol at 37^{o}C with 220 rpm shaking overnight
- Record absorbance at 600 nm and fluorescence using an excitation wavelength of 480 nm and an emission wavelength of 510 nm (bandpass filter of 495 nm)
- From this experiment we saw that even when using a weak RBS before LasI with or without the addition of a degradation tag, fluorescence was recorded without the addition of an input signal (Figure 5). In other words, the positive feedback loop was too good and within a cell, any LasI expression resulted in a 3OC12-HSL concentration above threshold level, thus activating the pathway.

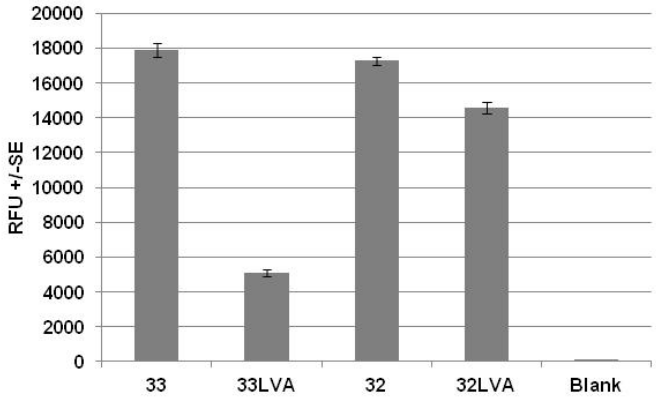


Figure 5: Auto-Induction of Fluorescence when the Amplification Circuit is Encoded on a Single Plasmid

The amplification cassette included a weak ribosome binding site upstream of a LasI gene with or without the LVA degradation tag. Mean values of 2 replicates. 33: P_{Las}_RBS34_GFPa1_RBS33_LasI; 33LVA: P_{Las}_RBS34_GFPa1_RBS33_LasILVA; 32: P_{Las}_RBS34_GFPa1_RBS32_LasI; 32LVA: P_{Las}_RBS34_GFPa1_RBS32_LasILVA; Blank: empty E. coli NEB 5-alpha.

3.4 Redesign and Build

To overcome amplification circuit self-activation, we made use of the flexibility afforded by utilizing biological wiring to devise a different amplification circuit (Figure 6). In this circuit, quorum sensing molecule production genes and the promoters responsible for quorum sensing molecule detection were insulated from each other by separating them in different cell types. Thus, 3OC12-HSL activates PLas_GFPa1_RhlI in a cell to produce fluorescence and C4-HSL. C4-HSL is exported from the cell and activates PRhl_GFPa1_LasI in a different cell type. This results in the production of fluorescence and 3OC12-HSL, and the cycle repeats. This design should reduce the likelihood of self-activation (Figure 4) since 3OC12-HSL does not bind to RhlR and C4-HSL does not bind to LasR (Pesci et al., 1997), and thus there is no positive feedback loop within a cell. A similar circuit was successfully used to create a microbial consensus consortium that expressed reporter proteins at >100-fold than the responses of the individual cell type sin isolation (Brenner, Karig, Weiss, and Arnold, 2007). The LasI promoter is more sensitive to 3OC12-HSL than the RhlI promoter is to C4-HSL (Figure 7). Thus to tune the circuit so that induction results in a comparable fluorescence in each cell type, a degradation tag was added to LasI to dampen the signal production in the PRhl_GFPa1_LasI cassette.

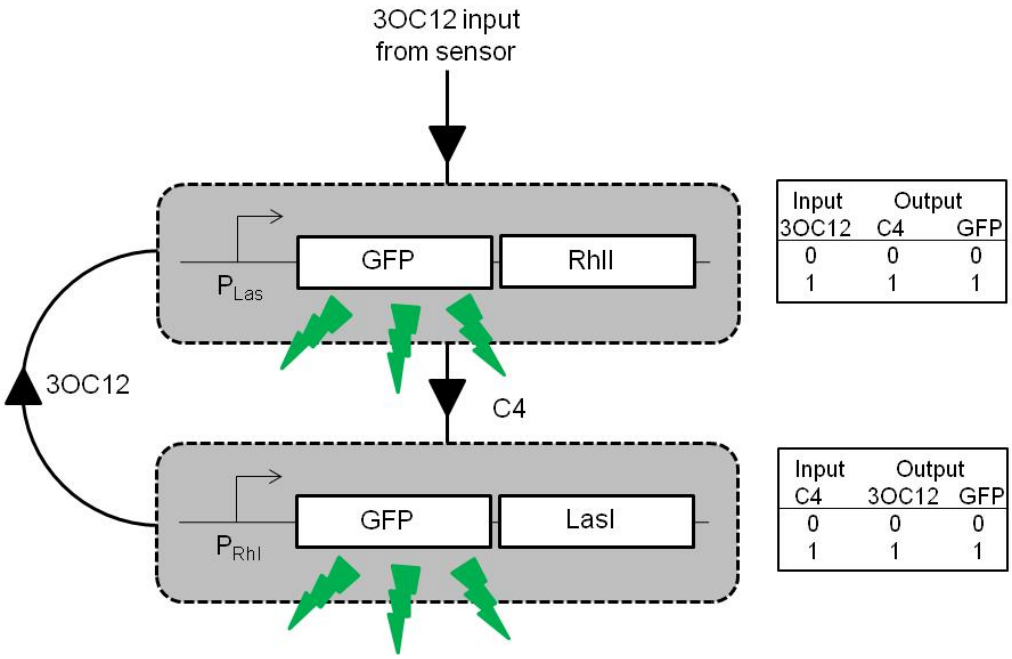


Figure 6: The Genetic Amplification Circuit - Including the Truth Table for the Circuit

Detection of a molecular signature by a sensor such as a riboswitch results in expression of LasI and production of 3OC12-HSL. 3OC12-HSL activates the Las promoter resulting in expression of GFP and RhlI. RhlI produces C4-HSL. C4-HSL diffuses out of the cell and activates the Rhl promoter of a different cell type resulting in expression of GFP and LasI. This initiates a signaling cascade that will spread to all reporter cell types in the sensing device.

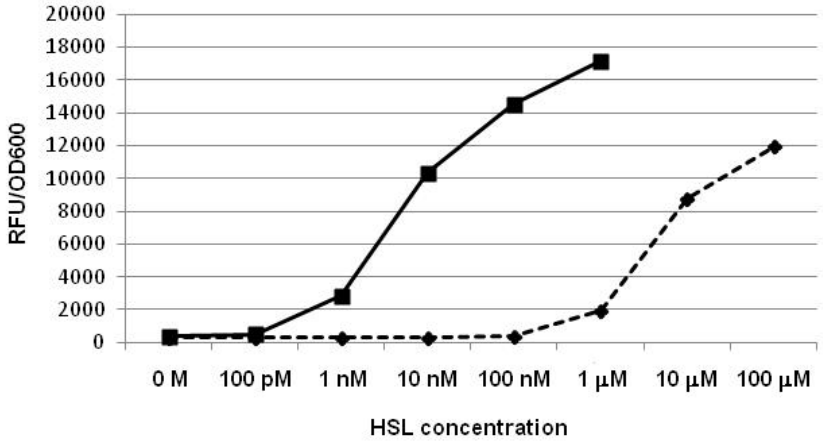


Figure7: The Individual Plasmids of Amplification Circuit 2.0 Produce a Dose-Dependent Fluorescence

Solid line, pAMPv2.1 exposed to 3OC12-HSL; dotted line, pAMPv2.2 exposed to C4-HSL.

3.4.1. Methods to construct amplification circuit version 2

3pAMPv2.1: P_{Las}_RBS 34 (strong)_GFPa1_Terminator_ RBS 33 (very weak)_RhlILVA:

- Digest the plasmid constructed above with BsrGI
- Amplify RhlI using primers that have a 18-20 bp overlapping sequence required for Gibson assembly. In this case our forward primer included a GFPa1 overlap-BsrGI site-RBS 33 at its 5' end and the RhlI+LVA reverse primer contained a LVA degradation tag.
- Follow protocol Steps (3)-(10) described above.

3.4.1.2 pAMPv2.2: P_{RhL}_RBS 34 (strong)_GFPa1_Terminator_ RBS 33 (very weak)_LasILVA

- Digest pNOR40 with BstBI and BmrI
- Amplify GFPa1 and LasI using primers that have a 18-20 bp overlapping sequence required for Gibson assembly. In this case our GFPa1 forward primer included a BstBI site 5' of the GFPa1 gene, and our LasI forward primers contained a GFPa1 overlap-BsrGI site-RBS 33 (very weak) at its 5' end and the LasI+LVA-Terminator-BmrI reverse primers contained a LVA degradation tag.
- Follow protocol Steps (3)-(10) described above.

3.5 Test

3.5.1. Fluorescence activation

- Grow sequence verified clones in LB-chloramphenicol with varying concentrations of synthetic HSL at 37^{o}C with 220 rpm shaking overnight

- Record absorbance at 600 nm and fluorescence using an excitation wavelength of 480 nm and an emission wavelength of 510 nm (bandpass filter of 495 nm)

Unlike the previous iteration, the two plasmids used in this amplification circuit produce a dose-dependent fluorescence (Fig. 7). Therefore these plasmids can be used in an experiment to show signal progression.

3.5.2. Signal progression

- Use a single cfu of each cell type to inoculate LB-chloramphenicol
- Incubate at 37^{o}C with 220 rpm shaking until OD600 reaches 0.5
- On a LB-chloramphenicol plate, alternately spot 1 □l of each culture in a straight line with a spacing of 1 cm between spots
- Add 1 □l of 10 □M synthetic 3OC12-HSL 1 cm from first PLas_GFPa1_RhlI spot
- For a diffusion of signal control, also add 1 □l of 10 □M synthetic 3OC12-HSL 4 cm from first PLas_GFPa1_RhlI spot (the limit of diffusion of this concentration of 3OC12-HSL in standard agar plates is ~2cm; Tamsir et al., 2011).
- Incubate at 37^{o}C for 24 h
- Visualize fluorescence. Signal will progress 'along' the line of spots by a cell type receiving a quorum sensing signal that activates expression of GFPa1 and production of the other quorum sensing signal, which then activates the next cell type, and so on. Controls that rely on passive diffusion of signal will not exhibit fluorescence.

Thus, using relatively straight-forward molecular biological techniques and 'off-the-shelf' biological parts, synthetic biology provides an engineering environment in which to integrate riboswitches into biological circuitry.

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