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Designer gene circuits for basic science, engineering, and medicine

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

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2014
The dissertation of Arthur Prindle is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014
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LIST OF ABBREVIATIONS

A.U. ....................................................... arbitrary units
bp .......................................................... base pair
DNA ...................................................... deoxyribonucleic acid
E. coli .................................................... *Escherichia coli*
Eq. ........................................................... equation
fig. ........................................................... figure
FL. ........................................................... fluorescence
FP ........................................................... fluorescent protein
GFP ......................................................... green fluorescent protein
hrs. .......................................................... hours
kb ........................................................... kilobase
LB ........................................................... lysogeny broth
mRNA .................................................... messenger ribonucleic acid
OD ........................................................... optical density
ODE ....................................................... ordinary differential equation
PCR ........................................................ polymerase chain reaction
PDMS ..................................................... poly(dimethylsiloxane)
UV ........................................................... ultraviolet
YFP ........................................................ yellow fluorescent protein
\%w/v ..................................................... percent weight per volume (concentration)
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My academic path has been profoundly shaped by interactions with teachers. Chemistry was my favorite subject in school because of one exceptional teacher, Jason Rosé, who blended an incredible depth of knowledge with a willingness to do the hard work required to contextualize it for students. Combined with the engineering "bug", this experience led to my decision to pursue undergraduate training in chemical engineering. At Caltech I can clearly remember my first meeting with Richard Murray, who would be my senior thesis advisor. In that meeting he drew a diagram of an engineered genetic circuit (the “repressilator”) on the whiteboard and described how this network of genes resulted in a change in behavior at the organismal level. This was the day that my enthusiasm for experimental science was ignited, which lead directly to my decision to pursue graduate school with Jeff Hasty at UCSD. Over the last 5 years, Jeff and I have enjoyed an extremely productive relationship where he has imparted rare and valuable advice on adaptability in research, research planning, and grant writing. In particular, he expressed a confidence in my abilities even when I didn’t feel it, allowed me to enjoy opportunities to succeed and gave me safe ways to fail, and above all guided me to become the scientist I am today.

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ABSTRACT OF THE DISSERTATION

Designer gene circuits for basic science, engineering, and medicine

by

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Doctor of Philosophy in Bioengineering

University of California, San Diego, 2014

Professor Jeff Hasty, Chair

Gene regulatory networks lay at the foundation of biological function and are responsible for driving the diverse cellular decision making processes required to sustain life. Developing a comprehensive understanding of cellular function will require a quantitative description of the dynamics of these underlying interactions. The ability to design synthetic gene circuits offers the exciting prospect of prototyping new genetic subsystems inspired by the inherently complex networks of natural organisms. An increasingly vivid representation of the design principles that drive biological function is assembled through the systematic design and characterization of experimentally tractable synthetic modules of increasing complexity. The programming of living cells according to these principles will provide fundamental insights into the regulatory architecture, dynamics, and evolution of natural networks and open new avenues in biotechnology by revealing novel ways to program genetic modules.

In Chapter One, I give an introduction to the genetic circuits approach by relating...
a survey of recent research that has been influential for me. In Chapter Two, I discuss my work on integrated “bacto-electronic” sensors that communicate over supermicrobial spatial scales. In Chapter Three, I discuss engineered gene circuits in the clinically relevant microbe *S. typhimurium*. In Chapter Four, I discuss a rapid and tunable post-translational coupling method for genetic circuits. In Chapter Five, I discuss designer probiotics for non-invasive cancer diagnostics. The unifying goal of these efforts is a “bottom-up” approach to building gene circuits predicted to confer a particular behavior based on engineering principles.
Chapter 1

Introduction

As synthetic biologists, we must not forget about the biology. If there’s anything we’ve learned over the last decade, it’s that engineering biology requires a type of engineering that we’re not quite used to. And why should we be? Our knowledge of the parts, their function, and the way(s) to assemble them are changing almost daily. Biology continues to surprise us and we try our best to channel that surprise toward utility. But focusing first and foremost on the utility—the successful, eventual application of new biology—means that we overlook the majority of the process that gets us there. Instead, we would do well to keep an eye on fundamentals. Design good experiments, take good measurements, and make good models that inspire fresh thought. Then communicate those ideas honestly and clearly to capture the insights of others, especially biologists.

Indeed, the research process is all about people. Biological systems that make up living organisms are sufficiently complex that they will never be understood by any one individual. Only through pooling our efforts—by each individual communicating his own particular view—does understanding begin to emerge. A scientific field is the organized collection of many individual searches for understanding that have gone before in a particular area. There is another benefit to understanding the questions other
people ask and borrowing from their insights—it can help to refine one’s own identity. As such, for the introduction to my thesis, I am taking the somewhat unusual approach of describing the research of others that has been particularly important in a personal sense. I’ll take the next several pages to describe briefly some of the people who’s talks I’ve attended during my time at UCSD that, among many others that I’ve interacted with through reading their papers, have helped to form my identity as a scientist.

Uri Alon (Weizmann Institute) has made fundamental and pioneering contributions to the study of gene networks. The essence of this approach is that complex cellular networks are composed of smaller core subnetworks, or motifs, that can be understood quantitatively using mathematics. Because these small motifs often repeat throughout diverse networks, understanding a complex network is reduced to identifying the core motifs and separating out the superfluous details. In another talk, more personal and equally moving, he describes ‘the cloud’—the inevitable point in research along the path from A to B when everything stops making sense. What is most important to remember in the cloud is to keep on the lookout for C, the endpoint even better than B.

Bill Bialek (Princeton) described two striking examples of biological systems operating at the limits of the physical processes they interact with: chemical diffusion that coordinates gastrulation and pattern scaling in the early fly embryo and sound waves for ultra-precise echolocation hunting by the fruit bat. Bialek argues that studying examples of biological systems that operate at physical limits of their underlying processes (diffusion, sound) is our best hope for deciphering the underlying principles that describe them. In other words, it’s easy to make things noisier (by obscuring the guiding principal for the underlying process with confounding noise) but very hard to make things quieter. In the case of sensory systems, the ubiquitous and very expensive evolutionary drive towards physical limits indicates that we live in a world where the scarcity of information dominates the cost of getting it.
Jim Collins (Boston University) described the complementary approaches of systems and synthetic biology using a boombox illustration. Systems biology seeks to take apart the boombox to identify its components and their interactions, while synthetic biology attempts to put together individual components to assemble modules that approach boombox-like function.

Michael Elowitz (Caltech) described the single-cell gene expression dynamics of B. subtilis involved in sporulation. To precisely time a sporulation event several generations ahead, the cell must solve what Elowitz terms the water bucket problem, a task akin to filling a hole-filled bucket smoothly to the brim. The complication lies in the fact that the dilution rate depends on the concentration of the timer molecule, meaning that choosing a fixed production rate would require the cell to anticipate its growth conditions several generations in advance. His elegantly described solution involves a polyphasic positive feedback strategy analogous to infrequent compounding in finance: each cell cycle, add a pulse of timer molecule proportional to the current concentration.

James Ferrell (Stanford) explored questions of spatial and temporal synchronization during early cell division in frog embryos. How can this incredibly large egg divide synchronously in under 10 min when diffusive signal propagation would take 2 h? Using in vitro cell cycling by egg extracts, he devised an elegant experimental method to measure division signal propagation. We all watched as green foci formed and vanished along the axis of a long tube, undergoing what Ferrell called trigger waves. In essence, while diffusion slows as signal spreads, trigger waves remain fast since each new trigger point replenishes the signal as it switches.

Tim Gardner (Amyris Inc.) delivered a unique perspective on synthetic biology by recounting his journey from academic research to industrial biotechnology. Summarizing years of strain optimization in a plot of historical data, Gardner illustrated a dramatic hinge point at which research productivity nearly doubles. What key biolog-
ical insight could account for this? Not biology per se, but rather biological practice: reduce error bars! Gardner described how eliminating failures of quality allowed incremental improvements to rise above measurement noise. The message was well-received and concluded to applause with a quotation by Sydney Brenner, “Data should obey the CAP principle: it should be complete, it should be accurate, and it should be permanent. Otherwise there is no progress.”

Terrence Hwa (UCSD) described bacterial growth laws derived from empirical observation of the relationship between cellular growth rate and gene expression. He described the intrinsic constraints that govern the allocation of the proteome toward distinct protein types, where allocation to certain classes can be varied while other classes are fixed. This places boundaries on the maximum foreign protein that a cell can produce and quantitatively describes how foreign protein production affects cell growth. In analogy to the empirical discovery of Ohm’s Law, which allowed electrical circuits to be constructed in the absence of complete theoretical understanding, these phenomenological approaches can reveal the underlying design principles that shape cell behavior in the absence of complete molecular details about their regulation. This is a particularly encouraging message for those of us that study the complex biological systems that drive cellular behavior. It may be possible to gain predictive insights before full mechanistic detail is known.

Roy Kishony (Harvard Medical School) opened with a simple yet confounding question for antimicrobial medicine: how do we kill bugs with drugs without creating drug resistance? To tackle this problem, Kishony led us through what he calls “evolutionary medicine”. Devices such as his OMG (Observatory of Microbial Growth) produced beautiful spatial evolutionary lineages in response to increasing antibiotic concentrations. In clinical experiments, he tracked the path of a human outbreak by analyzing mutations over time, a form of molecular clock. Kishony proposed an unexpected solution to the resistance problem: suppressive (anti-synergy) drug combinations that
disfavor the development of resistance to any individual drug.

Galit Lahav (Harvard Medical School) focused on p53 dynamics in response to various forms of stresses. Sophisticated single cell studies examined the behavior of cells over time, after applying diverse forms of stresses, to uncover how cells transfer information through dynamics. She revealed that UV- and γ-irradiation prompted different dynamic behaviors and gave way to graded or digital-like p53 responses, respectively. Further work revealed that p53 dynamics control cell fate, whereby a sustained p53 response forced cells into senescence, and a pulsing p53 response rendered cells that are capable of dividing and growing normally. In the future, this work will be applied more broadly to understand the molecular mechanisms utilized at the cellular level to decode p53 dynamics.

Richard Murray (Caltech) described how biomolecular breadboards—in vitro transcription-translation (TX-TL) reactions using cell-free extracts—can accelerate the design-build-test cycle for genetic circuits. Murray’s group aims to debug prototype designs by rapid model-experiment iteration in less than a day, enabling construction of working circuits in no more than a week. Perhaps most appealing about his TX-TL platform is the possibility for precise control of circuit parameters that would normally be constrained by the cell, including copy number, resource limits, and component concentrations. This degree of control may facilitate progress toward spatiotemporal execution and improved modularity for genetic circuits.

Timothy Lu (MIT) described his engineering of analog and digital computation and memory in living systems. Why has the performance of our circuits not scaled with our ability to synthesize DNA? Under a purely digital paradigm, Lu explained, we are constrained by our lack of suitable parts. Instead, analog computing—few parts that do smart work, versus many parts doing individually stupid work—enables complex behavior by harnessing the built-in function of a comparatively small number of biological
Petra Schwille (Max Planck) aims to quantitatively understand living systems on the scale of individual molecules. What is particularly impressive about her group’s research is the goal of building complex systems from the bottom up—that is, reconstituting the molecular components of a particular biological subsystem in an extracellular and well-controlled environment. She presented work on the Min system in bacteria, a dynamically unstable set of proteins that collectively locate the midpoint of the cell by oscillating from side to side. To enable direct visualization of this dynamical system, Schwille constructed microfluidic “bathtubs” that confined the components to a particular region of space, enabling beautiful recapitulation of the Min oscillations in a completely abiotic environment.

Gürol Süel (UCSD) seeks to explain sophisticated cellular behaviors (decision making, differentiation) in terms of the underlying processes that drive them. Often, these apparently complex processes reduce to surprisingly simple dynamics. For example, differentiation to one of several phenotypes in the bacterium *Bacillus subtilis* is the result of multiple competing, yet independent, dynamics. There is no elaborate cross-regulation before the decision point because there doesn’t need to be. Süel’s work is building a theme of understanding the essence of complex biological behavior in the simplest and most fundamental terms.

Alexander van Oudenaarden (Hubrecht Institute) discussed the fundamental role of stochasticity in biological processes. In bacteria, random fluctuations can be advantageous to a population by ensuring that a number of different behavioral states are always present. This form of bet hedging ensures that, for a given environment, there are at least some individuals that are properly adjusted to survive. However, higher organisms require precise differentiation into specific roles to collaborate within a multicellular tissue. In an impressive career transition, van Oudenaarden’s current research utilizes
his own newly developed single cell measurement technologies to study how stem cells cope with and damp this molecular noise.

J. Craig Venter (J. Craig Venter Institute) compared life to a DNA software system, where DNA can be transformed into a cell to modify the species identity. For example, a chemically synthesized Mycoplasma mycoides genome was transplanted into Mycoplasma capricolum cells. After a few minutes the resulting cells looked identical to the donor strain. This work underscores the sufficiency of genomic DNA and demonstrates that phenotype is wholly specified by the genome.

And many more. From this collection of influences, and in large part due to Jeff’s persistently positive attitude, I have begun to assemble a scientific “self”. My overarching goal is to take a “bottom-up” approach to building circuits predicted to confer a particular behavior based on engineering principles. In contrast to human inventions, the design features of living systems display enormous intricacy and redundancy as a result of billions of years of trial-and-error evolution. In light of this complexity, the pursuit of biological understanding is best driven by the natural tension between applied and foundational research. To this end, I plan to pursue three distinct directions related to engineering, medicine, and basic science. The unifying goal of these efforts is the elucidation of the design principles that underlie biological function through a process of circuit design that iterates between learning-to-build and building-to-learn. I hope that I can illustrate some of the questions I find interesting in the following pages.

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Chapter 2

A sensing array of radically coupled genetic biopixels

Introduction

While there has been significant progress in the development of engineering principles for synthetic biology, a substantial challenge is the construction of robust circuits in a noisy cellular environment. Such an environment leads to considerable intercellular variability in circuit behavior, which can hinder functionality at the colony level. Here, we engineer the synchronization of thousands of oscillating colony “biopixels” over centimetre length scales through the use of synergistic intercellular coupling involving quorum sensing within a colony and gas-phase redox signaling between colonies. We use this platform to construct an LCD-like macroscopic clock that can be used to sense arsenic via modulation of the oscillatory period. Given the repertoire of sensing capabilities of bacteria such as *E. coli*, the ability to coordinate their behavior over large length scales sets the stage for the construction of low cost genetic biosensors that are capable of detecting heavy metals and pathogens in the field.

Synthetic biology can be broadly parsed into the “top-down” synthesis of genomes\(^44\) and the “bottom-up” engineering of relatively small genetic cir-
In the genetic circuits arena, toggle switches and oscillators have progressed into triggers, counters and synchronized clocks. Sensors have arisen as a major focus in the context of biotechnology, while oscillators have provided insights into the basic-science functionality of cyclic regulatory processes. A common theme is the concurrent development of mathematical modeling that can be used for experimental design and characterization, as in physics and the engineering disciplines.

The synchronization of genetic clocks provides a particularly attractive avenue for synthetic biology applications. Oscillations permeate science and technology in a number of disciplines, with familiar examples including AC power, GPS, and lasers. These technologies have demonstrated that operating in the frequency domain can offer significant advantages over steady-state designs in terms of information gathering and transmission. In particular, oscillatory sensors confer a number of advantages to traditional ones, since frequency is easily digitized and can be quickly updated with repeated measurements. For sensors that use optical reporters, measurements of frequency are less sensitive to experimental factors such as beam power and exposure time than intensity measurements which must be normalized and calibrated.

While the bottom-up approach to synthetic biology is increasingly benefiting from DNA synthesis technologies, the general design principles are still evolving. Within this context, a substantial challenge is the construction of robust circuits in a cellular environment that is governed by noisy processes such as random bursts of transcription and translation. Such an environment leads to considerable intercellular variability in circuit behavior, which can hinder their functionality at the colony level. An ideal design strategy for reducing variability across a cellular population would involve both strong and long-range coupling that would instantaneously synchronize the response of millions of cells. Quorum sensing typically involves strong intercellular coupling over tens of microns, yet the relatively slow diffusion time of molecular communication through cellular media leads to signaling delays over millimetre scales. Faster communication mechanisms, such as those mediated in the gas phase, may in-
crease the length scale for instantaneous communication, but are comparatively weak and short-lived since the vapor species more readily disperse.

**Synergistic Synchronization**

In order to develop a frequency modulated biosensor, we designed a gene network capable of synchronizing genetic oscillations across multiple scales (Fig. 2.1a). We constructed an LCD-like microfluidic array that allows many separate colonies of sensing bacteria to grow and communicate rapidly by gas exchange (Fig. 2.1b). Since previous work has demonstrated that coupling through quorum sensing leads to incoherent oscillations at the millimetre scale, this mode of cellular communication is too slow for the generation of synchronized oscillations at the macroscopic scale. However, the slower quorum sensing can be used to synchronize small local colonies, provided there is a second level of design that involves faster communication for coordination between the colonies. Therefore rather than attempting to engineer a sensor from a single large-colony oscillator, we wired together thousands of small oscillating colonies, or “biopixels”, in a microfluidic array. Coupling between biopixels involves redox signaling by hydrogen peroxide (H$_2$O$_2$) and the native redox sensing machineries of *E. coli*. The two coupling mechanisms act synergistically in the sense that the stronger, yet short-range, quorum sensing is necessary to coherently synchronize the weaker, yet long-range, redox signaling. Using this method we demonstrate synchronization of approximately 2.5 million cells across a distance of 5 mm, over 1,000 times the length of an individual cell (Fig. 2.1c-d). This degree of synchronization yields extremely consistent oscillations, with a temporal accuracy of about 2 minutes compared to 5 - 10 minutes for a single oscillator (Fig. 2.1d).

The global synchronization mechanism is comprised of two modes of communication that work on different scales. The quorum-sensing machinery (LuxI, AiiA) uses an acyl-homoserine lactone (AHL) to mediate intracolony synchronization. In our device, the degree to which neighboring colonies are able to influence each other via
Figure 2.1: Sensing array of radically coupled genetic biopixels. (a) Network diagram. The *luxI* promoter drives expression of *luxI*, *aiiA*, *ndh*, and *sfGFP* in four identical transcription modules. The quorum-sensing genes *luxI* and *aiiA* generate synchronized oscillations within a colony via AHL. The *ndh* gene codes for NDH-2, an enzyme that generates H$_2$O$_2$ vapor which is an additional activator of the *luxI* promoter. H$_2$O$_2$ is capable of migrating between colonies and synchronizing them. (b) Conceptual design of the sensing array. AHL diffuses within colonies while H$_2$O$_2$ migrates between adjacent colonies through the PDMS. Arsenite-containing media is passed in through the parallel feeding channels. (c) Fluorescent image of an array of 500 *E. coli* biopixels containing about 2.5 million cells. Inset: brightfield and fluorescent images display a biopixel of 5,000 cells. (d) Heatmap and trajectories depicting time-lapse output of 500 individual biopixels undergoing rapid synchronization. Sampling time is 2 minutes.
AHL diffusion is negligible owing to the high media channel flow rates. Instead, we engineered the cells to communicate via gas exchange by placing a copy of the gene coding for NADH dehydrogenase II (NDH-2) under the control of an additional lux promoter. NDH-2 is a membrane-bound respiratory enzyme that produces low levels of H$_2$O$_2$ and superoxide (O$_2^-$). Since H$_2$O$_2$ vapor is able to pass through the 25 µm oxygen-permeable PDMS walls that separate adjacent colonies, periodic production of NDH-2 yields periodic exchange of H$_2$O$_2$ between biopixels. When H$_2$O$_2$ enters the cell, it transiently changes its redox state, interacting with our synthetic circuit through the native aerobic response control systems, including arcAB which has a binding site in the lux promoter region. Under normal conditions, ArcAB is partially active so lux is partially repressed. In contrast, oxidizing conditions triggered by H$_2$O$_2$ inactivate ArcAB, relieving this repression. Each oscillatory burst promotes firing in neighboring colonies by relieving repression on the lux promoter. This constitutes an additional positive feedback that rapidly synchronizes the population (Fig. 2.1d).

We investigated the effects of catalase and superoxide dismutase (SOD) to probe the nature of H$_2$O$_2$ communication. When a population of synchronized colonies was exposed to a step increase of 200 U/ml catalase, an enzyme that rapidly degrades extracellular H$_2$O$_2$, synchronization was broken and colonies continued to oscillate individually. Since the cell membrane is impermeable to catalase, this confirms that communication between colonies depends on external H$_2$O$_2$ while oscillations within a colony do not. Conversely, when we enhanced the rate of superoxide conversion to H$_2$O$_2$ by expressing sodA from an additional lux promoter, colonies quickly fired in a spatial wave and failed to oscillate further despite no changes to growth rate or cell viability. Since H$_2$O$_2$ is produced internal to the cell, this confirms that H$_2$O$_2$ is capable of escaping the cell and activating lux-controlled genes in neighboring colonies via diffusion. The apparent higher output of H$_2$O$_2$ by SOD as compared to NDH-2 is likely due to its very high catalytic efficiency. Finally, we observed synchronization between arrays of traps even when they were fluidically isolated but held in close proximity. These devices share no common fluid sources or channels, making communication by
dissolved molecules like AHL impossible. Taken together, these results confirm that gaseous H$_2$O$_2$ is the primary mode of communication between oscillating colonies.

Based on our understanding of the mechanism for global synchronization, we expected that we could simplify the circuitry by eliminating *ndh* and achieve the same effect with intermittent bursts of high-intensity blue light. In this design, the GFP molecule acts as a photosensitizer, releasing free radicals upon exposure that produce oxygen species (ROS) including H$_2$O$_2$. At the peak of oscillation, significant vapor-phase H$_2$O$_2$ is produced by exposing GFP-containing cells to fluorescent light. Conversely, at the trough of oscillation, cells contain almost no GFP, and therefore produce very little H$_2$O$_2$ upon fluorescing. Bursts of light thus generate bursts of H$_2$O$_2$ vapor whose concentration depends on the oscillating GFP level, just as periodic production of NDH-2 did previously. Indeed, this strategy was similarly able to synchronize our sensor array (Fig. 2.1d). Numerous controls were performed to ensure that synchronized oscillations did not occur at low fluorescence intensities.

To probe this mode of synchronization, we investigated the effects of thiourea and the antibiotics ampicillin and kanamycin. When a synchronized population of colonies was exposed to 35 mM thiourea, a potent radical quencher, we observed sharply decaying synchronized oscillations while growth rate and cell viability were unaffected. This suggests that without O$_2^-$, oscillations cannot be produced. Next, we ran a series of experiments switching the antibiotic resistance genes on our plasmids. We noted that radical-producing antibiotics, particularly Ampicillin, significantly reduced the degree of synchronization, showing that an excess of radical species such as O$_2^-$ also hinders communication. Since our final constructs included a plasmid with kanamycin resistance, which was also found to produce some radicals, we used full (50 µg/ml) selection when growing up the cells but very low (5 µg/ml) selection during the experimental run. Persistence of oscillations, sequencing, and subsequent growth in full selection following the run confirmed the presence of all 3 plasmids despite this low experimental selection. Catalase and sodA results were identical to those with NDH-2 synchronization. These results show that fluorescence-mediated synchronization involves
the production of radical species following fluorescence exposure and communication via \( \text{H}_2\text{O}_2 \).

**Sensing Array of Biopixels**

With a platform for generating consistent and readily-detectable oscillations, we sought to use the circuit to engineer an arsenic-sensing macroscopic biosensor. We rewired the network to include an extra copy of the positive-feedback element, the AHL-synthase LuxI, under the control of a native arsenite-responsive promoter which is repressed by ArsR in the absence of arsenite (Fig. 2.2a). When arsenite is not present in the media, supplemental \( \text{luxI} \) is not transcribed and the circuit functions normally, generating baseline oscillations. However, the addition of trace amounts of arsenite relieves this repression and allows supplemental \( \text{luxI} \) to be produced, increasing the oscillatory amplitude and period. Tuning the level of LuxI by varying arsenite concentration results in clear changes to the oscillatory period b). To determine the range of detection, we swept arsenite concentrations from 0 to 1 \( \mu \text{M} \) and measured the oscillatory period (Fig. 2.2c, top). Using statistical methods, we generated a sensor calibration curve (Fig. 2.2c, bottom) that depicts the maximum possible arsenite concentration present (\( \alpha = 95\% \)) for a given measured period. This curve is an illustration of how data generated by our array would be used to measure arsenite concentrations in an unknown sample using our device. Our system was able to reliably quantify arsenite levels as low as 0.2 \( \mu \text{M} \), below the 0.5 \( \mu \text{M} \) WHO-recommended level for developing nations\(^97\).

As an alternative sensing strategy, we rewired the network to include a copy of the \( \text{luxR} \) gene controlled by an arsenic-responsive promoter while removing it from the rest of the circuit (Fig. 2.2a). Since the LuxR-AHL complex must be present to activate the \( \text{lux} \) promoter\(^{139} \), cells produce no LuxR when the media is free of arsenite, generating no fluorescence or oscillations. The addition of arsenite stimulates the production of LuxR, restoring circuit function and producing clear, synchronized oscillations (Fig. 2.2d). This ON/OFF detection system has a threshold of 0.25 \( \mu \text{M} \), a
Figure 2.2: Frequency modulated genetic biosensor. (a) Network diagrams depicting two constructed sensing modules. In thresholding (1), the luxR gene is removed from the oscillator network and supplemented by a new copy driven by an arsenic-responsive promoter. In period modulation (2), a supplemental luxI gene tagged for increased degradation is driven by the arsenic-responsive promoter which affects the period of oscillation. (b) A sample period modulation sensor output following a step increase of 0.8 μM arsenite. Oscillatory period increases from 69 minutes to 79 minutes. (c) (Top) Period versus arsenite concentration for the sensor array. Error bars indicate ± 1 standard deviation averaged over 500 biopixel trajectories. Dotted line represents model-predicted curve. (Bottom) Sensor calibration curve generated from experimental data. Points indicate the maximum arsenite level with 95% certainty for a given measured period as determined statistically from experimental data. (d) Thresholder output following a step increase of 0.25 μM arsenite. A dramatic shift from rest to oscillatory behavior is observed within 20 minutes following the addition of arsenite.
detection limit that can be adjusted by changing the copy number, ribosome binding site (RBS) strength, or promoter strength of the sensing plasmid.

The sensing array is also capable of producing complex behaviors arising from the dynamic interaction of cellular colonies. By making modifications to the size, number, and arrangement of biopixels in the device, we are able to dramatically alter the output waveforms. For example, when we constructed a device in which trap separation distance is increased (45 µm versus 25 µm), we observed local anti-phase synchronization between neighboring colonies (Fig. 2.3d, top right). To explore this phenomenon on a larger scale, we constructed a device that contains an array of 416 traps constructed according to the specifications above. In these experiments, we observe initial global synchronization that gradually falls into local anti-phase synchronization across the array (Fig. 2.3d, middle). Phase alignment is maintained over at least 48 hours, with patches of synchronization typically 3-6 colonies in size. Alternatively, by changing dimensions such that the array contains traps of two slightly different sizes, we observe a 1:2 resonance synchronization where larger traps pulse at double the frequency of smaller traps while maintaining synchronization (Fig. 2.3d, top). Finally, when LuxR is limited as in the thresholding scheme, we observe synchronized oscillations of alternating large and small peaks in both experiment and model. Our computational model (see Modeling Box) captures these effects (Fig. 2.3d, bottom) and indicates that further array manipulation will yield new, richer dynamics that could not be produced directly by changing circuit structure.

While our sensor array is capable of performing a variety of complex functions in the laboratory, adapting this technology to a real-world device will require the elimination of the expensive and bulky microscopy equipment. However, measuring genetic oscillations in the absence of any magnification or powerful illumination will require even further increased signal. Using this mechanism of global synchronization, we were able to scale up to a 24 mm x 12 mm array that houses over 12,000 communicating biopixels (Fig. 2.4a). Synchronization is maintained across the entire array, a distance over 5,000 times the length of an individual cell, using an inexpensive LED
Figure 2.3: Computational modeling of radical synchronization and biosensing. (a) Time series of a population of biopixels producing varying amounts of \( \text{H}_2\text{O}_2 \) vapor. Synchronization occurs only for moderate levels while high levels lock ON and low levels oscillate asynchronously. (b) A typical time series for our period modulation sensor undergoing a step increase of arsenite. Oscillations increase in both amplitude and period. (c) A typical time series output for the thresholding sensor. Oscillations arise following the addition of arsenite. (d) Experimental and computational output depicting complex dynamic behaviors between neighboring traps. (Top 2 panels) 1:2 resonance and anti phase synchronization observed when trap size (left, black/blue = 95 \( \mu \text{m} \) depth and red/magenta = 85 \( \mu \text{m} \) depth) and separation distance (right, same colors) are modified experimentally, (Middle) Scaled-up array experimental data for increased trap separation experiments demonstrating anti phase synchronization, (Bottom) Computational model trajectories depicting 1:2 resonance and anti phase synchronization when trap size (same colors as experimental data) and separation distance are changed.
(Fig. 2.4b,c). The signal strength generated by the large number of cells in the array (about 50 million) will allow us to adapt the device to function as a handheld sensor. In our conceptual design (Fig. 2.4d), the sensor will continuously read the oscillatory frequency using off-the-shelf electronic components costing less than $50.

There have been many examples of bacteria-based biosensors\textsuperscript{136:23:69}, usually involving an optical reporter driven by a single promoter. Since optical intensity readings are sensitive to imaging conditions like beam power and exposure time, measurements must typically be normalized and calibrated. Measuring period of oscillation allows us to avoid these issues since peak-to-peak time does not depend on individual peak intensity. Secondly, oscillations produced at the colony level effectively decouple the signal from the growth state of individual cells, which can also affect fluorescence intensity. By using a dynamic readout that depends on communication between biopixels, we scan and tune potential output signals by changing device parameters rather than redesigning the underlying circuit. For example, we might design a new sensing scheme in which oscillations synchronize with the addition of some toxin and shift to anti phase or resonant synchronization when critical toxin levels are present.

**Scaling Up Synthetic Biology**

By nesting two modes of communication we are able to expand the scale over which individual cells are coordinated and increase the complexity of their interaction. Indeed, there are many familiar examples of hierarchical systems. Airline routes are often designed such that small airports are connected locally to larger hubs that are connected internationally. It would neither be feasible nor desirable to connect every airport together. Similarly, individual cells communicate locally by one method, generating impulses large enough to enable colonies to communicate globally by another. Nesting communication mechanisms in this way may allow us to better scale up synthetic circuits of different types, such as switches and logic gates, paving the way for the next generation of synthetic biology pursuits.
Figure 2.4: Radical synchronization on a macroscopic scale. (a) The scaled-up array is 24 mm x 12 mm and houses over 12,000 biopixels that contain approximately 50 million total cells when filled. (b) Global synchronization is maintained across the array. Heatmap of individual trajectories of all 12,224 oscillating biopixels. (c) Image series depicting global synchronization and oscillation for the macroscopic array. Each image is produced by stitching 72 fields of view imaged at 4X magnification. (d) Schematic diagram illustrating our design for a handheld device utilizing the sensing array. An LED (A) excites the array (B) and emitted light is collected by a photodetector (C), analyzed by an onboard processor (D), and displayed graphically (E).
Computational Modeling

Our model of the frequency modulated (FM) biosensor is based on a published model for the quorum-sensing synchronized oscillator\(^20\). In addition to the reactions reflected in that model, we include the arsenite-induced production and degradation of LuxI and/or LuxR. From the biochemical reactions, we derived a set of delay-differential equations to be used as our model. These delayed reactions mimic the complex cascade of processes (transcription, translation, maturation, etc.) leading to formation of functional proteins. As expected, our model predicts oscillations that change frequency when changes in arsenite occur (Fig. 2.2c and 2.3b). The amplitude and period of the oscillations both depend on the concentrations of the toxin. We then modified the model to describe the LuxR-based detection system. Our model predicts a marked transition from rest to oscillations upon addition of arsenite, consistent with experimental observations (Fig. 2.3c).

The multi-scale nature of communication in our array allows us to treat colony and array-level dynamics separately, where arsenite affects the quorum-sensing machinery of a colony, producing changes to oscillatory period that propagate between biopixels in the array. To quantitatively describe the mechanisms driving synchronization at the array-level, we treat each colony as a single oscillator that acts according to degrade-and-fire kinetics\(^78\). We also include the production of H\(_2\)O\(_2\) and its interaction with neighboring colonies by two-dimensional diffusion. Using this model we identified three regimes that correlate well with experimental observations (Fig. 2.3a). When the effective production of H\(_2\)O\(_2\) is low, as with catalase, we observe unsynchronized oscillations owing to constant, mild repression of the lux promoter via ArcA (Fig. 2.3a, left). In contrast, when H\(_2\)O\(_2\) production is very high, neighboring colonies rapidly fire in succession and remain on due to the permanent activation of the lux promoter, consistent with the SOD experiment (Fig. 2.3a, right). Finally, at intermediate H\(_2\)O\(_2\), we observe globally synchronized oscillations (Fig. 2.3a, middle). As colonies are moved further apart, synchronicity breaks due to slowed migration of H\(_2\)O\(_2\) (Fig. 2.14).
Plasmid Construction

The oscillator plasmids were constructed by modifying the constructs used in a previous study. The antibiotic resistance genes of pTD103AiiA was switched to chloramphenicol. The reporter protein on pTD103LuxI/GFP was switched to a recently reported super folding green fluorescent protein, sfGFP. The ndh and sodA genes were amplified directly from the native E. coli genome by PCR. Promoter output was tuned by changing the RBS sequence and quantified using flow cytometry. We initially constructed the sensing plasmid with a published synthetic background reduced version that contains additional ArsR operator sites but failed to produce enough LuxR. To increase LuxR output, we reverted to the native promoter sequence, switched the RBS to that of pZ plasmids, and increased the copy number by a factor of 5 by switching to a mutated SC101 origin of replication. All circuit components except LuxR were tagged by PCR with a carboxy-terminal ssrA tag (AANDENYALAA) for fast degradation. Modular pieces (resistance genes, promoters, origins, and ORFs) were assembled using a PCR-based cloning scheme named CPEC.
Figure 2.5: Plasmids used in this study. Top row is the thresholding sensor: 2 oscillator plasmids with luxR genes removed and a plasmid containing pArs::luxR. Middle row is the period modulator: 2 oscillator plasmids and a plasmid containing pArs::luxI-laa. Bottom row contains 2 plasmids used to study H$_2$O$_2$ production and synchronization: pLux::ndh and pLux::sodA. NDH-2 synchronization strain is the oscillator plasmids with pZSm45 ndhII.
Additional Experimental Results

**Figure 2.6:** Biopixels with NDH-2 engineered synchronization observed at ultra-low fluorescence (4X, 20ms exposure, 3% power) using an EMCCD camera to ensure no fluorescence interaction. Synchronized oscillations are maintained across the array for the length of the experiment (14 hours).
Figure 2.7: Catalase degrades external H$_2$O$_2$ and prevents communication between colonies. When a synchronized population of biopixels was exposed to a step increase of 200 U/ml catalase, synchronization was broken and biopixels continued to oscillate individually. Since catalase can’t cross the cell membrane, this shows that synchronization between colonies depends on H$_2$O$_2$ but oscillations with a colony do not.
Figure 2.8: SodA produces \( \text{H}_2\text{O}_2 \) internal to the cell, permanently switching the cellular redox state (oxidizing) thereby activating lux-controlled genes. Biopixels rapidly fire and lock on in a spatial wave, far earlier than is typical for colonies of this size. The propagation of ON biopixels suggests that colonies are capable of activating those nearby via migrating \( \text{H}_2\text{O}_2 \) species.
**Figure 2.9:** Synchronized oscillations occur across 2 fluidically isolated devices held in close proximity. In this experiment, the devices were started at different times yet become synchronized. Since these devices share no common fluid sources or sinks, this confirms that synchronization is mediated by vapor species.
Figure 2.10: Heatmap of trajectories extracted from low fluorescence intensity control (Suppl. Movie 9) when NDH-2 plasmid is not present. Biopixels oscillate individually but fail to synchronize.
Figure 2.11: The introduction thiourea, a potent radical quencher, produces decaying synchronized oscillations across a population of biopixels. Because radical species are precursors for $\text{H}_2\text{O}_2$, eliminating them lowers the production of $\text{H}_2\text{O}_2$ and therefore dampens the oscillations. Colonies are still able to synchronize because, while thiourea eliminates radicals within cells, it does not prevent $\text{H}_2\text{O}_2$ from diffusing between cells.
Figure 2.12: Synchronization is prevented when 100 µg/ml Ampicillin is used in the media. The constructs, strains, and experimental conditions are otherwise identical.
Data Analysis

Fluorescence data was obtained by importing fluorescent images into ImageJ and subtracting cell signal from background signal. Oscillatory period was taken to be the average of peak-to-peak and trough-to-trough distance, calculated using a MATLAB script. The data represented in Fig. 1d and 2b-d were collected by stitching 4 images taken at 4X magnification. The mean trajectory in Fig. 1d was found by averaging 373 individual biopixel trajectories, of which 20 are shown. Biopixel trajectories were extracted from image series using a MATLAB script, where a bright field image of the corresponding array was used to generate a mask. The data shown in Fig. 2c was measured over 4 separate experiments using 10-30 oscillatory periods per data point.

Sensor calibration curve (Fig. 2c, bottom) was generated using a series of 2-population t tests comparing the experimental datasets to randomly generated new sample sets. The mean of generated sets was decremented until the test failed with $\alpha = 95\%$, indicating the lowest period that could be associated with that arsenite concentration. We repeated this process for each arsenite level and fit the points with a quadratic since we expected it to take the inverse shape of the period vs. arsenite measurements.

Microscopy and Microfluidics

We used a microscopy system similar to our recent studies, with the addition of a high-sensitivity Andor DU-897 EMCCD camera. Fluorescent images were taken at 4X every 30 seconds using the EMCCD camera (20ms exposure, 97% attenuation) or 2 minutes (2s exposure, 90% attenuation) using a standard CCD camera to prevent photobleaching or phototoxicity.

In each device, *E. coli* cells are loaded from the cell port while keeping the media port at sufficiently higher pressure than the waste port below to prevent contamination (Suppl. Fig 8). Cells were loaded into the cell traps by manually applying pressure pulses to the lines to induce a momentary flow change. The flow was then reversed and allowed for cells to receive fresh media with 0.075% Tween which prevented cells from adhering to the main channels and waste ports.
To measure fluid flow rate before each experiment, we measured the streak length of fluorescent beads (1.0 µm) upon 100 ms exposure to fluorescent light. We averaged at least 1,000 data points for each.

We constructed several microfluidic devices over the course of the study. The trap dimensions were always 100 µm x 85 µm x 1.65 µm high, which we previously found to be optimal for oscillator function, except when size was varied to study dynamic interactions. Spacing between traps was 25 µm, except in devices designed to study the effects of increasing separation distance between traps. For sensor array devices, we constructed 500 and 12,000 trap arrays as well as a tandem device which holds two 150 trap arrays in close proximity (25 µm) without sharing fluid sources or sinks.
Figure 2.13: Primary microfluidic device used for this study. Media containing variable arsenite concentration is fed through the cell port, flowing past the biopixel array into the cell and waste ports. During loading, pressure is increased at the cell port and decreased at the waste ports to reverse the flow, allowing cells to pass by the trapping regions. Other microfluidic devices used have the same layout with trap number, separation, and size varied.
Modeling for biosensor array

To model the dynamics of the quorum-sensing oscillator, we used our previously described model for intracellular concentrations of LuxI \((I)\), AiiA \((A)\), internal AHL \((H_i)\), and external AHL \((H_e)\):

\[
\frac{\partial A}{\partial t} = C_A [1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma A A}{1 + f(A + I)} \quad (2.1)
\]

\[
\frac{\partial I}{\partial t} = C_I [1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma I I}{1 + f(A + I)} \quad (2.2)
\]

\[
\frac{\partial H_i}{\partial t} = \frac{b I}{1 + k I} - \gamma H A H_i + D(H_e - H_i) \quad (2.3)
\]

\[
\frac{\partial H_e}{\partial t} = -\frac{d}{1 - d} D(H_e - H_i) - \mu H_e + D_1 \frac{\partial^2 H_e}{\partial x^2} \quad (2.4)
\]

In the original model, the concentration of the constitutively produced LuxR protein \(R\) was assumed constant. In the ON/OFF threshold arsenic biosensor circuit, LuxR production is induced by arsenic, which we model by the equation

\[
\dot{R} = \frac{\alpha_c A}{(A_0 + A)} - \gamma_R R \quad (2.5)
\]

in which the LuxR expression from the arsenic promoter follows a standard saturating function of the arsenic concentration \(A\). Accordingly, we modified the Hill function for Lux promoter to include the explicit dependence on \(R\):

\[
G(\alpha, \tau) = \frac{\delta + \alpha (R H_e)^2}{1 + k_i (R H_e)^2} \quad (2.6)
\]

For modeling the period-modulating sensor, we modified the equation for LuxI (3.2) to include additional production from the arsenic promoter,

\[
\dot{I} = C_I [1 - (d/d_0)^4] G(\alpha, \tau) + \frac{\alpha_c A}{(A_0 + A)} - \frac{\gamma_I I}{1 + f(A + I)} \quad (2.7)
\]

The following additional parameters were used for the biosensor simulations: \(\alpha_c = 50\), \(A_0 = 2\), \(\gamma_R = .1\).

Arsenic levels were swept across the dynamic range of the arsenic promoter to produce the curve in Fig. 2.2c. The period for each arsenic level was calculated from the peak-to-peak average of 15 oscillatory periods.
To model the spatial synchronization of oscillating colonies across a microfluidic array, we generalized a simplified “degrade-and-fire” model\textsuperscript{78}. The delay-differential equation
\begin{equation}
\dot{X}_{i,j} = \frac{\alpha(1 + \nu P_{i,j,\tau_2})}{\left(1 + \frac{X_{i,j,\tau_2}}{C_0}\right)^2} - \frac{\gamma X_{i,j}}{k + X_{i,j}}
\end{equation}
(2.8)
describes oscillations of individual biopixel \(\{i, j\}\) as a combined effect of production and delayed autorepression (first term in the r.h.s.) of the colony-averaged LuxI concentration \(X_{i,j}\) and its enzymatic degradation by ClpXP (second term). Unlike\textsuperscript{78}, the first (production) term in Eq. 1 describes both delayed auto-repression of LuxI and its delayed activation by H\textsubscript{2}O\textsubscript{2} proportional to its local concentration \(P_{i,j}\). Subscripts \(\tau_1\) and \(\tau_2\) indicate the delayed concentrations, \(X_{i,j,\tau_1}(t) = X_{i,j}(t - \tau_1)\) and \(P_{i,j,\tau_2}(t) = P_{i,j}(t - \tau_2)\). The dynamics of \(P_{i,j}\) is described by the equation
\begin{equation}
\dot{P}_{i,j} = \mu + \alpha_p X_{i,j} - \gamma_p P_{i,j} + \hat{S}\{P_{i,j}\}
\end{equation}
(2.9)
where the first three terms describe the basal and induced production and degradation of H\textsubscript{2}O\textsubscript{2}The last term models the spatial coupling of neighboring biopixels via the H\textsubscript{2}O\textsubscript{2} exchange. For a square \(N \times N\) array of traps, we used the following discrete diffusion form of the spatial operator,
\begin{equation}
\hat{S}\{P_{i,j}\} = D\Delta^{-2}[P_{i-1,j} + P_{i+1,j} + P_{i,j-1} + P_{i,j+1} - 4P_{i,j}]
\end{equation}
(2.10)
Each colony is affected by the H\textsubscript{2}O\textsubscript{2} produced in four neighboring colonies, two in each dimension of the array, separated by the equal distance \(\Delta\). We used the boundary condition \(P_{i,j} = 0\) for the edges of the array \(i, j = 0, N + 1\). This represents the infinite external sink of H\textsubscript{2}O\textsubscript{2} diffusing out of the microfluidic chip. The diffusion operator above can be generalized if the row spacing differs from the column spacing, or for other spatial arrangements of colonies within the biosensor.

We introduced variability among different traps by randomizing oscillator parameters for individual traps in each simulation. Specifically, LuxI (X) activation and degradation parameters \((p = \{\alpha, \gamma\})\) of each of the oscillators in the array were varied around their nominal values \((p_0)\) as \(p = p_0 + \delta\) where \(\delta\) is a random number uniformly distributed between \(-0.25\) and \(0.25\). We used the following dimensionless parameters for most of our simulations: \(\alpha_0 = 8.25\), \(\gamma_0 = 5.75\), \(\nu = 1\), \(\tau_1 = 10\), \(\tau_2 = 20\), \(C_0 = 6\), \(k = 10\), \(\mu = 20\), \(\alpha_p = 1\), \(\gamma_p = 10\), \(D = 7\), \(\Delta = 1\).
For the characterization of various regimes of array synchronization, 16 colonies were modeled in the $4 \times 4$ array. Scaling up the simulation with larger numbers of colonies produced equivalent results. Overproduction of H$_2$O$_2$ by expressing sodA was captured by increasing $\alpha_p$ 20-fold. This is consistent with expression from a pSC101m plasmid with a copy number of 20-30. Depletion of external H$_2$O$_2$ by catalase was modeled by increasing H$_2$O$_2$ degradation ($\gamma_p$) and decreasing H$_2$O$_2$ diffusion, D. In (Fig. 2.14) we show the variance of the concentrations $X_{i,j}$ within the array averaged over time and parameter variations. This plot demonstrates that the synchronicity among the biopixels decreases with increase of spacing among them, and for $\Delta > 5$ is completely lost.

![Average Simulation Variance](image)

**Figure 2.14:** Computational results depicting biopixel synchronicity as a function of trap separation distance. As biopixels are moved farther apart, the entropy increases due to decreased effective migration of H$_2$O$_2$ between colonies.

Increasing the trap spacing $\Delta$ 2-fold while simultaneously decreasing $k$ 4-fold allowed us to reproduce the more complex waveforms observed experimentally in our arrays. Note that changing $k$ models the change of the trap depth. As the size of the trap decreases, the flow of media is able to more rapidly sweep away AHL and increase the effective degradation for the colony. Simulating smaller and more sparse trap sizes recovered antiphase behavior for neighboring biopixels (Fig. 2.15). We also simulated the arrays with traps of two different sizes in different rows and recovered the experimental 2:1 biopixel resonance or 2:1 + antiphase behav-
ior depending on the trap spacing (Fig. 2.3d, bottom).

Figure 2.15: Antiphase behavior of 4 neighboring biopixels having equal trap sizes and spacing $\Delta = 3$.

The model was also able to capture the alternating large and small amplitude oscillations observed in the ON/OFF biosensor (Fig. 2.16). This behavior was seen when $C_0$ was increased 2-fold, capturing the decreased level of LuxR in ON/OFF experiments where it was the limiting factor for oscillations.

**Data Analysis**

Fluorescence data was obtained by importing fluorescent images into ImageJ and subtracting cell signal from background signal. Oscillatory period was taken to be the average of peak-to-peak and trough-to-trough distance, calculated using a MATLAB script. The data represented in Fig. 1d and 2b-d were collected by stitching 4 images taken at 4X magnification. The mean trajectory in Fig. 1d was found by averaging 373 individual biopixel trajectories, of which 20 are shown. Biopixel trajectories were extracted from image series using a MATLAB script,
Figure 2.16: Oscillations of alternating large and small amplitude when LuxR is limited in experiments and simulations. The alternating oscillations vanish when LuxR is restored to its normal level in the model. Experimentally, we were unable to build a system in which LuxR is tunable between big/small and normal amplitude regimes. This is probably due to the small dynamic range of arsenite promoter-driven output of LuxR compared to the level produced by 3 constitutively expressed copies in the original circuit.

where a bright field image of the corresponding array was used to generate a mask. The data shown in Fig. 2c was measured over 4 separate experiments using 10-30 oscillatory periods per data point.

Sensor calibration curve (Fig. 2c, bottom) was generated using a series of 2-population t-tests comparing the experimental datasets to randomly generated new sample sets. The mean of generated sets was decremented until the ttest failed with $\alpha = 95\%$, indicating the lowest period that could be associated with that arsenite concentration. We repeated this process for each arsenite level and fit the points with a quadratic since we expected it to take the inverse shape of the period vs. arsenite measurements.

Chapter 3

Genetic circuits in *Samonella typhimurium*

Introduction

Synthetic biology has rapidly progressed over the last decade and is now positioned to impact important problems in health and energy. In the clinical arena, the field has thus far focused primarily on the use of bacteria and bacteriophages to overexpress therapeutic gene products. The next generation of multi-gene circuits will control the triggering, amplitude, and duration of therapeutic activity *in vivo*. This will require a host organism that is easy to genetically modify, leverages existing successful circuit designs, and has the potential for use in humans. Here, we show that gene circuits that were originally constructed and tested in *E. coli* translate to *Salmonella typhimurium*, a therapeutically relevant microbe with attenuated strains that have exhibited safety in several human clinical trials. These strains are essentially non-virulent, easy to genetically program, and specifically grow in tumor environments. Developing gene circuits on this platform could enhance our ability to bring sophisticated genetic programming to cancer therapy, setting the stage for a new generation of synthetic biology in clinically relevant microbes.

An explosion of DNA sequencing\(^9\), synthesis\(^{80}\), and manipulation\(^{44}\) technologies
has driven the development of synthetic genetic programs of increasing complexity in living cells. Underlying this work is the hope that engineered biological systems will be used to solve important problems in energy and health over the coming years. Initially inspired by electronic circuits, researchers began by designing small transcriptional switches and oscillators. These early successes fostered a growing population of physicists, computer scientists, and engineers that aimed to apply an engineering-based methodology to the design of biological systems. In the past decade, substantial success has been achieved using this genetic circuits approach termed synthetic biology.

Multi-gene logic gates capable of integrating environmental signals have been constructed in bacteria, yeast, and mammalian cells. Electronics-inspired networks have included counters, pulse generators, filters, and communication modules. Sophisticated circuits can now be controlled by light, yielding genetic programs readily tunable both in vitro and in vivo in live animals. Dynamic genetic clocks have been constructed that function at the single-cell, colony, and multi-colony level in growing bacterial populations, and even in mammalian cells. In a recent study, redox signaling mediated by H$_2$O$_2$ vapor permitted the synchronization of millions of oscillating bacteria across an LCD-like sensor array.

Early efforts toward clinical applications have utilized bacteria and bacteriophages (viruses that infect bacteria) to perform therapeutic functions in vivo. Commensal bacteria have been engineered to fight diabetes, HIV, and cholera by producing and delivering therapeutic agents directly in the human microbiome. Because certain bacteria grow preferentially in hypoxic environments, a number of studies have engineered cancer-fighting bacteria to selectively attack tumors. Toward still another application, a pair of studies has engineered phages to produce foreign enzymes, making them far more potent than their unmodified counterparts at dispersing bacterial biofilms.

In most of these cases, the genetic programs involved were responsible for overexpressing target genes, similar to traditional genetic engineering where genes are added, removed, or modified one at a time in a stepwise fashion. To truly achieve its clinical potential, synthetic biology must continue to do what has made it successful: engineer progressively more complex, multi-input networks in which the triggering, amplitude, and duration of therapeutic activity is controllable. This will require using hosts that are easy to genetically modify and compatible...
with the clinical requirements regarding safety, immunogenicity, and drug resistance. While bacteriophage and adenovirus have their advantages, viruses have smaller genomes and therefore have a narrower range of genetic modifications, frequently induce host resistance, and are highly cell-type specific.\(^{85;105}\).

As one potential bridge between organisms such as *E. coli* and clinically relevant microbes, *Salmonella typhimurium* is a bacterial anti-cancer platform that is closely related to *E. coli*, has been extensively studied in vivo for therapeutic applications\(^{34;100;55;71;35;73}\) and has been shown to be safe in human clinical trials\(^{53;132;95}\). The development of attenuated strains has utilized auxotrophy and phoPQ deletions to suppress virulence cell invasion and virulence\(^{55}\). Lipid A mutations have been generated to reduce immunogenicity, stimulating a much weaker immune response than wild-type strains\(^{73}\). Despite this reduced potency, systemically injected *S. typhimurium* cells retain their ability to target and selectively replicate within tumors, displaying a thousand-fold growth preference relative to other organs\(^{34;100;35;73;55}\). Their motility allows them to follow chemical gradients and penetrate deep into the tumor vasculature\(^{61;62}\), much further than passively diffusing small molecules\(^{34}\). And many of these strains also display innate oncolytic activity, regressing tumors simply by growing in them\(^{100;93;73;60}\).

Perhaps the most important property of *S. typhimurium* for synthetic biology is the ease of genetic modification. It is a model organism whose genome is sequenced\(^ {81}\), has knockout collections, and the genetic tools are almost identical to *E. coli*. *S. typhimurium* is capable of stably expressing recombinant DNA from plasmid-based circuits *in vivo*. This approach has already been used to produce a number of therapeutic compounds directly within tumors, but most often via “always on” expression of well-established genes\(^ {55;51;96}\). This work has laid the foundation for more sophisticated functionality, such as programmed delivery profiles that take advantage of plasmid instability\(^ {18}\). Such a focus will merge the dynamic sensing, production, and delivery capabilities of genetic circuits with the native tumor seeking and penetration of *S. typhimurium*. 
Experimental Results

In order to test the degree to which existing synthetic circuits function in *S. typhimurium*, we transformed the attenuated strain ELH430 (SL1344 ΔphoPQ, gift of Elizabeth Hohmann, MGH) with several genetic oscillator constructs. First, we tested a single-plasmid variant of a published single-cell gene oscillator. Using our microfluidic platform, we observed robust oscillations for all *S. typhimurium* cells over many generations (Fig. 1A,B). While the qualitative period-inducer relationship was similar to *E. coli*, the curve was shifted toward faster periods as compared to *E. coli* strain JS006 (MG1655 ΔaraC,lacI) (Fig. 1C). In contrast, we initially expected *S. typhimurium* to oscillate slower since longer division times generally result in period lengthening. When we measured the dependence of oscillatory period on temperature in *S. typhimurium*, we found the trend qualitatively similar to *E. coli*, where lower temperatures (and therefore longer doubling times) resulted in longer oscillatory periods (Fig. 1D). We therefore hypothesized that the faster oscillations in *S. typhimurium* are not due to growth rate differences, but rather a strain-dependent factor such as mean promoter level, transcription rate, or enzymatic degradation rate.

To explore this quantitatively, we used automated single-cell tracking using a previously developed algorithm to compare a large number of single-cell time courses from *S. typhimurium* and *E. coli* (Supplementary Information). Oscillators are an ideal circuit to quantify strain-specific parameters such as transcription and degradation rates since they allow for hundreds of measurements in a single experiment. For each oscillatory period, the trough-to-peak and peak-to-trough slopes were measured. Since the ClpXP degradation machinery is likely saturated, the peak-to-trough slope yields an estimate for the zeroth-order enzymatic degradation rate in degrade-and-fire oscillators. Interestingly, we found that the apparent enzymatic degradation rate in *S. typhimurium* was roughly 1.5-fold that of *E. coli* (Fig. 4A). In our computational model of the oscillator, this increase reproduced the experimentally observed period-inducer relationship (Fig. 4B).

Next, we transformed *S. typhimurium* with a quorum-sensing oscillator that had been previously characterized in *E. coli*, and observed coherent, colony-level oscillations for more than 48 hours (Fig. 2A,B). Here, we found that the period-flow rate dependence was markedly different in *S. typhimurium* than in the original study, where oscillatory period was much longer...
and changed very little across a wide range of flow rates (Fig. 2C). Interestingly, while increased degradation rate resulted in faster oscillations for single-cells (Fig. 4B), our computational model correctly predicts the opposite trend for the quorum-sensing oscillator when degradation is increased (Fig. 4C).

Finally, we tested the original genetic toggle switch, plasmid pIKE107. In this circuit, a transient pulse of IPTG inducer turns the switch ON and reporter expression is maintained at a high level. A second pulse of ATC inducer turns the switch OFF, dropping reporter expression indefinitely. In periodically diluted batch culture experiments similar to the original study, we used flow cytometry to observe robust switching and bistability when inducing with either 2 mM IPTG or 500 ng/ul dox in cultures growing at 37 C (Fig. 3A-C). Interestingly, the fluorescence level at which *S. typhimurium* settled after we removed IPTG was lower than the same circuit in *E. coli* (Fig. 3A). We suspected that the differences in apparent degradation and expression rates (Fig. 4A) might explain this change, since the steady-state repressor balance would be adjusted.

To test this hypothesis, we used the original computational model of the toggle switch and quantified the steady-state expression level over time for strain parameters measured in *E. coli* and *S. typhimurium*. We found that the *S. typhimurium* parameters reproduced the experimentally observed curves, where expression rises to a higher level when switched ON then decays to a lower steady-state when IPTG is removed (Fig. 4D). While these parameters are particularly important for dynamic circuits, they can also impact the performance of stable switches since repressors are continuously being produced and degraded.

**Conclusions and outlook**

A central issue in the design of genetic circuits is the degree to which native and engineered networks should be integrated. Synthetic biology began by fully isolating itself from the strain background, using it solely to supply energy, enzymatic machinery, and a cellular volume in which to function. In contrast, industrial applications in medicine and energy have commonly utilized a variety of microbes for their native networks. As our biological knowledge of native networks and our ability to engineer new circuits has improved, it has become increasingly possible to blend these two strategies.
S. typhimurium is an ideal strain for clinical synthetic biology since it is closely related to E. coli, well studied in vivo, has safety precedence for clinical trials in humans, and displays a thousand-fold growth preference for tumor environments. Moving to other microbes for clinical and industrial purposes will require the determination of the critical strain parameters that define the space of bacteria capable of hosting genetic circuits. Next steps will involve measurement of these parameters and testing circuits in strains of interest that are further removed in the phylogenetic tree. One such roadmap would begin with more distantly related gamma proteobacteria like Pseudomonas aeruginosa before moving outside the phylum to alpha proteobacteria such as Calubacter crescentus. Additionally, individual components and modules can also receive a "portability" score that estimate the degree to which they translate to other hosts. For example, while lacI- and tetR-based circuits are nearly universal, more generally the function of other components are likely to be more sensitive to strain-specific parameters. This work will enable synthetic biology to move beyond E. coli into a diverse range of microbes for clinical and industrial applications.

Microscopy and Microfluidics

We used a microscopy system similar to our recent studies. Fluorescent images were taken at 4X every 30 seconds using the EMCCD camera (20ms exposure, 97% attenuation) or 2 minutes (2s exposure, 90% attenuation) using a standard CCD camera to prevent photobleaching or phototoxicity.

In each device, E. coli cells are loaded from the cell port while keeping the media port at sufficiently higher pressure than the waste port below to prevent contamination (Suppl. Fig 8). Cells were loaded into the cell traps by manually applying pressure pulses to the lines to induce a momentary flow change. The flow was then reversed and allowed for cells to receive fresh media with 0.075% Tween which prevented cells from adhering to the main channels and waste ports.

To measure fluid flow rate before each experiment, we measured the streak length of
fluorescent beads (1.0 \, \mu m) upon 100 \, ms exposure to fluorescent light. We averaged at least 1,000 data points for each.

We used several microfluidic devices over the course of the study. For single-cell oscillators (Fig. 1), we used a previously described device consisting of a trapping region and a dynamic switch\textsuperscript{88}. Traps have dimensions 40 \, \mu m wide \times 50 \, \mu m long \times 0.95 \, \mu m high, with the long sides open to media flow. Since \textit{E. coli} and \textit{S. typhimurium} cells have a 1 \, \mu m diameter, the trap maintains growing cells in a monolayer. For colony oscillators (Fig. 2), we used a previously described device consisting of arrays of square trapping regions\textsuperscript{20,102}. Trap dimensions were always 100 \, \mu m \times 85 \, \mu m \times 1.65 \, \mu m high and spacing between traps was 25 \, \mu m. This size allows cells to grow in a colony arrangement rather than a monolayer, while still allowing quantitative measurement of colony fluorescence.

**Degradation and Production Rate Quantification**

Single cell fluorescence trajectories were obtained from time-lapse movies using custom software previously developed in MATLAB\textsuperscript{88}. Each cell fluorescence trajectory represents the median GFP fluorescence signal inside that cell over time. Using built-in MATLAB functions we identified the peaks and troughs for each trajectory. The degradation rate was calculated by taking the amplitude change from peak to the successive trough and dividing by the time change between the peak and the trough. These peak-to-trough sections of the trajectory represent the time when the production of GFP is repressed and the observed dynamics are solely driven by degradation of GFP. Similarly we calculated the net production rate, by calculating the amplitude change from trough to successive peak and dividing by the time change between the trough and the peak. The measurement gives the net production rate, which includes the degradation of the protein.

<table>
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<tr>
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<th>Mean Degradation Rate (SE)</th>
<th>Mean Net Production Rate (SE)</th>
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<tbody>
<tr>
<td>\textit{E. coli}</td>
<td>0.024 (0.001)</td>
<td>0.035 (0.002)</td>
</tr>
<tr>
<td>\textit{S. typhimurium}</td>
<td>0.035 (0.002)</td>
<td>0.044 (0.002)</td>
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Modeling

To generate the plot in Figure 4D, we used previously described genetic toggle switch model\textsuperscript{40}. We included three additional parameters to model the effects of IPTG ($C_{\text{IPTG}}$), ATC ($C_{\text{ATC}}$), and dilution ($D$) on the synthesis and degradation of proteins:

$$
\frac{\partial u}{\partial t} = \frac{C_{\text{IPTG}}}{1 + \alpha_u} - (\gamma_u + D)u
$$

$$
\frac{\partial v}{\partial t} = \frac{C_{\text{ATC}}}{1 + \alpha_v} - (\gamma_v + D)v
$$

In this model, we set $n=2$ to allow for cooperativity of repression of both promoters. $C_{\text{IPTG}_0}$ and $C_{\text{ATC}_0}$ were set to 1 for the case of no inducers present. Next, we used metropolis algorithm to find the rest of the parameters to fit the qualitative nature of the curves from Figure 1A. The parameters found to generate the $E.\ coli$ curve were: $C_{\text{IPTG}_1} = 1.25, C_{\text{ATC}_1} = 1.68, \alpha_u = 4.28, \alpha_v = 5.80, \gamma_u = 1.76, \gamma_v = 2.37, D = 0.11$. The parameters found to generate the $S.\ typhimurium$ curve were: $C_{\text{IPTG}_1} = 1.25, C_{\text{ATC}_1} = 1.68, \alpha_u = 11.00, \alpha_v = 8.36, \gamma_u = 4.86, \gamma_v = 3.21, D = 0.08$. It is interesting to note that the optimized parameters show higher production and degradation as well as lower dilution for $S.\ typhimurium$ curve relative to $E.\ coli$ curve, which correlates well with our experimental measurements.

The dynamics of single cell oscillator were modeled using previously described model for activator ($a_2$) and repressor ($r_4$) proteins\textsuperscript{124}. The production and degradation of these proteins is described by the following set of reactions:
We updated the degradation function $F(X)$ to include dilution as follows:

$$f(X) = \frac{\gamma}{c_e + X} + DX$$

Here, $X$ is the total number of ssrA tags in the system (one for each monomeric version, two for dimers, and four for tetramers, including proteins bound to operator sites). We varied the parameter $\gamma$ from 1x to 2x to evaluate the effect of degradation difference between *E. coli* and *S. typhimurium* on the period of oscillation calculated from single cell model simulations. Dilution rate was calculated from experimentally measured cell half life as $\frac{\ln(2)}{T_{1/2}}$. 

---

\[ p_{a/r}^{0,0} \xrightarrow{b_{a/r}} p_{a/r}^{0,0} + m_{a/r} \]
\[ p_{a/r}^{1,0} \xrightarrow{a b_{a/r}} p_{a/r}^{1,0} + m_{a/r} \]
\[ m_a \xrightarrow{t_a} m_a + a_{uf} \]
\[ m_r \xrightarrow{t_r} m_r + r_{uf} \]
\[ a_{uf} \xrightarrow{k_{fa}} a \]
\[ r_{uf} \xrightarrow{k_{fr}} r \]
\[ a + a \xrightarrow{k_{da}} a_2 \]
\[ r + r \xrightarrow{k_{dr}} r_2 \]
\[ r_2 + r_2 \xrightarrow{k_{l}} r_4 \]
\[ a_{uf} \xrightarrow{\lambda_f(X)} \emptyset \]
\[ r_{uf} \xrightarrow{f(X)} \emptyset \]
\[ a \xrightarrow{\lambda_f(X)} \emptyset \]
\[ r \xrightarrow{f(X)} \emptyset \]
\[ a_2 \xrightarrow{\lambda_f(X)} \emptyset \]
\[ r_2 \xrightarrow{f(X)} \emptyset \]
\[ r_4 \xrightarrow{f(X)} \emptyset \]
To model the dynamics of the quorum-sensing oscillator, we used our previously described model for intracellular concentrations of LuxI ($I$), AiiA ($A$), internal AHL ($H_i$), and external AHL ($H_e$)\textsuperscript{20},

\[
\frac{\partial A}{\partial t} = C_A [1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma_A A}{1 + f(A + I)} - DA \tag{3.1}
\]

\[
\frac{\partial I}{\partial t} = C_I [1 - (d/d_0)^4] G(\alpha, \tau) - \frac{\gamma_I I}{1 + f(A + I)} - DI \tag{3.2}
\]

\[
\frac{\partial H_i}{\partial t} = \frac{b I}{1 + k I} - \frac{\gamma_H A H_i}{1 + g A} + D(H_e - H_i) - DH_i \tag{3.3}
\]

\[
\frac{\partial H_e}{\partial t} = -\frac{d}{1 - d} D(H_e - H_i) - \mu H_e + D_1 \frac{\partial^2 H_e}{\partial x^2} \tag{3.4}
\]

To model the difference in periods of oscillation between \textit{E. coli} and \textit{S. typhimurium} we varied the degradation parameters $\gamma_A$ and $\gamma_I$. We looked at the changes in the period over different values of the flow rate parameter $\mu$, while varying the degradation parameters from 1x to 2x of the original model value. To account for the difference in doubling time between the two strains, we introduce exponential decay terms into the model to account for dilution in addition to the enzymatic degradation terms. We add terms $-DI$, $-DH_i$, and $-DH$ to the first three equations respectively, with $D = \frac{\ln(2)}{T_1}$. We then looked at how the change in doubling time affected the period of both strains shown in Figure 2D.

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Figure 3.1: A fast, robust, and tunable genetic oscillator in *S. typhimurium*. (a) Timelapse fluorescence microscopy depicting asynchronous oscillations in a growing colony of *S. typhimurium*. (b) A single-cell trajectory extracted from image data. (c) Period vs. inducer concentration for *S. typhimurium* compared to original data taken in *E. coli*. The trends are qualitatively similar yet *S. typhimurium* is shifted toward shorter periods. Points are experimental measurements fit to a line generated by computational modeling. (d) Period vs. temperature for *S. typhimurium* compared to original data taken in *E. coli* with similar trends.
**Figure 3.2:** A synchronized quorum of genetic clocks in *S. typhimurium*. (a) Timelapse fluorescence microscopy depicting coherent oscillations at the colony-level for a growing colony of *S. typhimurium*. (b) A colony trajectory extracted from image data that illustrates the regularity of oscillations over time. (c) Period vs. flow rate for *S. typhimurium* compared to original data taken in *E. coli*. *S. typhimurium* displays much higher periods that appear to be independent of flow rate.
Figure 3.3: A genetic toggle switch in *S. typhimurium*. (a) A time course of fluorescence output that illustrates switching by both IPTG and ATC quantified by flow cytometry in periodically diluted batch culture experiments. (b) Raw flow cytometer data illustrating switching by 2mM IPTG and (c) 500 ng/µl ATC.
Figure 3.4: Computational modeling of *S. typhimurium* genetic circuits. (a) Comparison of enzymatic degradation rate between *S. typhimurium* and *E. coli* generated from automated single-cell tracking. Degradation rate is approximately 1.5X higher in *S. typhimurium*. (b) A higher degradation rate results in the shorter periods observed experimentally for the single-cell oscillator. (c) In contrast, increased degradation rate results in longer periods for the quorum-sensing oscillator that are comparatively unchanged with flow rate. (d) Increased degradation and expression rates produce the experimentally observed behavior for the *S. typhimurium* toggle switch.
Chapter 4

Rapid and tunable post-translational coupling of genetic circuits

Introduction

One promise of synthetic biology is the creation of genetic circuitry that enables the execution of logical programming in living cells. Such “wet programming” is positioned to transform a wide and diverse swath of biotechnology ranging from therapeutics and diagnostics to water treatment strategies. While progress in the development of a library of genetic modules continues apace\textsuperscript{89,119,131,152}, a major challenge for their integration into larger circuits is the generation of sufficiently fast and precise communication between modules\textsuperscript{72,24}. An attractive approach is to integrate engineered circuits with host processes that facilitate robust cellular signaling\textsuperscript{94}. In this context, recent studies have demonstrated that bacterial protein degradation can trigger a precise response to stress by overloading a limited supply of intracellular proteases\textsuperscript{37,84,15}. Here, we use protease competition to engineer rapid and tunable coupling of genetic circuits across multiple spatial and temporal scales. We characterize coupling delay times that are more than an order of magnitude faster than standard transcription-factor based coupling methods (less than one minute compared with \(\sim20-40\) minutes) and demonstrate tunability through manipulation of the linker between the protein and its degradation tag. We use this mechanism as a platform to couple genetic clocks at the intracellular and colony level, then
synchronize the multi-colony dynamics to reduce variability in both clocks. We show how the coupled clock network can be used to encode independent environmental inputs into a single time series output, thus enabling the possibility of frequency multiplexing in a genetic circuit context. Our results establish a general framework for the rapid and tunable coupling of genetic circuits through the use of native queueing processes such as protein degradation.

**A rapid and tunable post-translational coupling scheme**

In order to engineer rapid coupling between synthetic genetic modules, we developed a post-translational coupling platform that operates via shared degradation by the ClpXP protease (Fig. 1a). In this scheme, all LAA-tagged components are dynamically linked via competition for a limited number of proteases, such that tagged modules remain tightly aligned ($1 \pm 1$ min, GFP-CFP curve pairs in Fig. 1a) despite significant induction delay ($31 \pm 5$ min, inducer-GFP offset in Fig. 4.1a). This coupling method produces delays that are more than an order of magnitude faster than standard transcription-factor based coupling methods ($\sim 20-40$ min).

To illustrate directly the response time that can be achieved by coordinating module output via modulating ClpXP activity, we show that low levels ($90 \mu$M) of externally provided H$_2$O$_2$ “inducer” rapidly (< 2 min, our experimental timestep) and reversibly modulates the concentration of constitutively expressed GFP in a ClpXP-dependent manner (Fig. 4.1b). Here, H$_2$O$_2$ reduces the native substrate load on ClpXP by obstructing RssB, the adapter protein that targets the alternative sigma factor $\sigma^S$ for degradation by ClpXP. Since $\sigma^S$ is continuously produced and degraded by ClpXP, inactivating its rate-limiting adapter protein results in an instantaneous increase in the effective ClpXP degradation rate for LAA-tagged proteins.

We systematically explored the coupling mechanism by driving a constitutive module with a quorum-sensing (Fig. 4.1c). As the pacemaker, the quorum clock generates density-dependent synchronous oscillations at the colony level via acyl-homoserine lactone (AHL), a small molecule capable of synchronizing cellular behavior across distances up to 100 $\mu$m. Using microfluidic devices we observed the colony-level expression of the constitutive module, finding oscillating expression synchronized to the quorum clock (Fig. 4.1c, top right). We then constructed a library of degradation tags by adding a series of variable-length spacer regions...
Figure 4.1: A rapid post-translational coupling platform based on shared degradation. (a) We measured the delays associated with module-module coordination by ClpXP (1 ± 1 min) and input-output response via transcription/translation (31 ± 5 min) in a single experiment by inducing the lux promoter and tracking the response of sfGFP-LAA (lux promoter) and CFP-LAA (p_{lac/ara-1} promoter) in single cells (55 cell trajectories). (b) Rapid (< 2 min, our experimental timestep) induction of protein degradation by externally provided H\textsubscript{2}O\textsubscript{2} produces reversible changes in ClpXP load in response to obstruction of RssB\textsuperscript{87,37,84}. (c) To use post-translational coupling to drive downstream modules, we linked a quorum clock to a constitutively expressed fluorescent protein via the addition of identical LAA tags. With identical degradation tags, the constitutive module couples tightly to the quorum pacemaker. The addition of a variable-length linker (TS repeats) before the degradation tag phase-shifts the degradation dynamics, where longer linkers produced longer delays. The error bars indicate s.d. of offset time, centered at the mean (50-200 cells for each TS-linker length).
between the downstream protein and its degradation tag. Spacer regions contained between one and five copies of the amino acid sequence “TS” and their effects on offset time compared to that of a previously published alternate degradation tag. While all spacer sequences produced synchronous activation dynamics, the degradation dynamics of the downstream module were offset depending on the length of the linker sequence, where longer linkers produced greater GFP-CFP offset time (Fig. 4.1C, bottom). Thus, our ClpXP coupling platform rapidly links genetic modules via shared degradation, where the strength and timing of coupling can be tuned by changing the degradation kinetics of individual modules.

**Post-translationally coupled genetic clocks at multiple scales**

In order to engineer coupling between genetic modules capable of generating their own dynamics, we designed a circuit containing the quorum clock and a variant of a previously described intracellular clock (Fig. 4.2a)\textsuperscript{125}. This \textit{p}_{lac/ara-1} intracellular clock variant retains the fast dynamics and simple genetic architecture of the published \textit{P}_{LlacO-1} negative feedback oscillator, yet its period is tunable by both isopropyl β-D-1-thiogalactopyranoside (IPTG) and arabinose in the presence of chromosomal araC. We first used small microfluidic devices (100 cells) and observed fast and asynchronous intracellular clock oscillations without quorum clock contribution, since the quorum clock requires a critical colony size to function. In larger devices (5,000 cells), we observed a transition from asynchronous oscillations to identical intracellular/quorum clock oscillations as the population grew larger (Fig. 4.2b). In the case of the larger population, the substrate load on ClpXP during the quorum clock pulse is sufficient to shift the intracellular clock out of its oscillatory regime, enabling complete linkage between the two clocks despite their vastly different spatial and temporal scales. Thus, despite lacking a mode of cell-cell communication itself, the intracellular clock is effectively synchronized at the colony level via ClpXP-mediated coupling with the quorum clock.

We found that changing the intracellular clock period of individual cells indirectly tuned the quorum clock period, where IPTG values associated with longer intracellular clock periods inversely produced shorter quorum clock periods (Fig. 4.2c). We developed a computational
Figure 4.2: Post-translationally linked genetic clocks at multiple scales. (a) The network is composed of coupled intracellular and quorum clocks. The intracellular clock oscillates as a result of delayed negative feedback on its own promoter and its period is tunable by IPTG/arabinose. Quorum clock oscillations are tunable by media flow rate and are synchronized via AHL at the colony level. (b) The coupled intracellular-quorum clock system oscillates asynchronously in small populations and transitions to synchronized oscillations in larger populations once the quorum clock fires. Despite lacking a mode of cell-cell communication itself, the coefficient of variation of the intracellular clock drops markedly via host-linked coupling with the quorum clock (bottom, data from 28 single cell traces). (c) IPTG reduces the intracellular clock period in small cell populations without the quorum clock (blue) and increases the coupled period in larger populations with the quorum clock (red). Each data point taken from 10–30 oscillatory peaks. The error bars indicate s.e.m. of the period, centered at the mean. (d) In our computational model, load-mediated coupling allows the intracellular clock to modulate the quorum clock period via degradation coupling at ClpXP, where the intracellular clock continues oscillating between coupled pulses and accelerates the pulse onset. (e) This adaptive form of pulse frequency modulation ensures that the pulse dynamics remain unchanged while the inter-pulse duration is adjusted (left: model and right: experimental, 6–9 oscillatory peaks) The error bars indicate s.e.m. of relative quorum clock period. (f) This mechanism also makes the coupled system more robust by enabling oscillation at higher media flow rates.
model of the oscillator network involving a form of load-mediated pulse frequency modulation to explain this effect (Fig. 4.2d-f). Between coupled pulses, the intracellular clock accelerates the quorum pulse onset via load-mediated decreases in the degradation rate of LuxI, where larger intracellular clock load produces higher levels of the AHL-synthase (Fig. 4.2e, left). During the coupled pulse, contributions of the intracellular clock leave the duration of the pulse itself unchanged (Fig. 4.2e, left: model and right: experimental). Linking the intracellular and quorum clocks via degradation also yielded an expansion in the oscillatory regime for the coupled system with respect to flow rate compared to the quorum clock alone (Fig. 4.2f). In this way, the intracellular clock continually excites the quorum clock to fire, enabling more robust function at higher external flow rates.

**Genetic frequency multiplexing**

With a platform for rapidly coupling genetic clocks at multiple scales, we sought to engineer a system capable of frequency encoding information from both clocks into the multispectral time series of a single reporter (Fig. 4.3a). Here, the measured output of the intracellular clock reporter contains contributions from its own fast intracellular clock dynamics between slow quorum clock bursts. Since the range of natural periods for the faster $\text{p}_{\text{lac}}/\text{ara-1}$ intracellular clock is fully separated from the slower quorum clock, both IPTG/arabinose and flow rate inputs can be encoded into frequency-modulated oscillations in the time domain where they can be independently extracted by Fourier transform. Thus, the measurement of a single clock history reveals the activities both underlying clock networks.

We began by characterizing the frequency response curves for both the intracellular and quorum clocks in isolation, finding ranges of 7-25 min and 55-95 min, respectively, when sweeping IPTG/arabinose and flow rate inputs (Fig. 3b, top: intracellular clock in araC+ strain and bottom: quorum clock, original study data). We then measured trajectories taken from the coupled clock system and extracted the frequency components of both clocks by Fourier transform (Fig. 3c and Power spectra analysis). In sweeping IPTG/arabinose inducers, we found the frequency response of the intracellular clock contribution to the multispectral reporter to be unchanged by the inclusion of the quorum clock, where the intracellular frequency response to
Figure 4.3: Genetic multispectral encoding. (a) Separate IPTG/arabinose and flow rate inputs are encoded into frequency-modulation oscillations that can be measured from the time series of the reporter for the intracellular clock. This engineered system is capable of encoding information from two underlying networks into a single multispectral time series. (b) Frequency response curves generated from experimental data and computational models for the intracellular clock (top, data from 30 single cell traces each) and quorum clock (bottom, model applied to data from the original study) in isolation. The error bars indicate s.e.m. of the period, centered at the mean. (c) In the coupled system, frequency-modulated oscillations from both clocks can be observed in the output of the intracellular clock and extracted by inverse Fourier transform (inset). (d) Independent recovery of both IPTG/arabinose and flow rate inputs, where the frequency response of the intracellular clock to IPTG/arabinose is equivalent to the isolated clock (top) and the frequency response of the quorum clock is shifted by the intracellular clock (bottom). Periods calculated from 5-10 single cell traces for each condition. The error bars indicate s.e.m. of the period, centered at the mean.
IPTG/arabinose was equivalent to the isolated clock (Fig. 4.3d, top: coupled and Fig. 4.3b, top: isolated). We then swept flow rates at 3 fixed inducer levels, finding distinct response curves for the quorum clock contribution to the multispectral reporter shifted in accordance with our model for ClpXP-mediated frequency modulation by the intracellular clock (Fig. 4.3d, bottom). Thus, to decode a given pair of IPTG/arabinose and flow rate inputs, we first recover the intracellular clock frequency as a measure of IPTG/arabinose and then use the corresponding quorum clock response curve to measure flow rate.

Post-translational coupling at the array level

To extend rapid coupling to greater spatial scales, we added a genetic H$_2$O$_2$ signaling cassette to the network and observed synchronization at the multi-colony level (Fig. 4.4a). In conducting these experiments, we also observed H$_2$O$_2$-mediated interaction between the native stress response network and our synthetic circuit at ClpXP (Fig. 4.4b). In the original design, H$_2$O$_2$ synchronized quorum clock oscillations by transcriptional upregulation of the lux promoter via the aerobic response control system ArcAB$^{102}$. In addition to transcriptional increase (Fig. 4.4c, top), we found an increase in the apparent degradation rate with H$_2$O$_2$ (Fig. 4.4c, bottom), consistent with increased ClpXP activity in response to externally provided H$_2$O$_2$. The coupled increases in transcriptional output and effective ClpXP degradation rate in response to H$_2$O$_2$ also tightens the period distribution at the multi-colony level by mitigating the effects of period variation in an individual colony (Fig. 4.4c, top).

Engineering synthetic circuits composed of interacting modules is an ongoing effort$^{89,119,131,152}$ that has generally relied on transcription and translation, with less attention paid to post-translational coupling mechanisms$^{49}$. Protease competition offers the advantages of rapid response, modularity with distinct recognition sequences, and simultaneous control over multiple circuits with protease adapters$^{83,47}$. More generally, in natural biological networks, competition for cellular resources (e.g., metabolites, enzymes, transcription factors, binding sites) produces nonlinear coupling effects that serve to reduce noise, increase sensitivity to input concentrations, and discriminate between multiple inputs$^{45,13,90,12,127}$. We envision that coordinating engineered circuits via built-in cellular processes—what we term “host-linked”
**Figure 4.4:** Post-translational coupling at the multi-colony level. (a) At the multi-colony level, interaction of H$_2$O$_2$ generated by redox signaling with the cellular stress response network synchronizes quorum clock oscillations between colonies. Traces taken from 10 separate colonies across the array. (b) Host-linked oscillations change distinct aspects of the waveform in response to H$_2$O$_2$ produced by the enzymatic activity of NDH. With H$_2$O$_2$, oscillations have larger amplitudes and steeper downslopes, revealing increases in both transcription and degradation produced by the interaction of the synthetic clock network with the native stress response. (c) H$_2$O$_2$ increases the oscillatory amplitude while decreasing the required degradation time, revealing an increase in ClpXP activity. This increase in ClpXP capacity in response to H$_2$O$_2$ serves to mitigate the effects of transcriptional noise by minimizing the effects of amplitude variation on the period, resulting in a tightening of the period distribution with H$_2$O$_2$.

coupling—has the potential to produce more sophisticated circuits by facilitating robust signaling between synthetic modules.

**Additional Experimental Results**

**Degradation tag experiments**

In addition to exploring the effect of variable-length linker (TS repeats) on the phase-shift in module degradation (Supplementary Fig. 1c-f), we tested a well characterized AAV degradation tag$^2$. In Andersen *et al*, GFP-AAV was shown to have 50% higher half-life than GFP-LAA. In this study, downstream module (CFP-AAV) showed a delay in degradation relative to the driver module (GFP-AAV) that was similar to that of the 2 TS-linker sequence (Supplementary Fig. 1b bottom). Further characterization is required to determine the differences in the mechanism of action between variable-length TS linker sequence before the SspB binding
Figure 4.5: Increasing length of the TS linker sequence results in increasing downstream module degradation delay. (a) Detailed breakdown of single fluorescent trajectory analysis. Peaks are identified in red, troughs in green, upslope 10% points in purple and downslope 10% points in dark beige. The two period measurements are peak to peak and the time between two successive 10% upslope points. (b) Top: sfGFP does not show bleed-over into CFP fluorescence channel. Induction of sfGFP with 10nM AHL (dashed line) showed increase in fluorescence of sfGFP, which was not detected in CFP channel. Bottom: the use of the published AAV degradation tag\textsuperscript{2} shows delay in the downstream module degradation of 15min. (c) Without the TS linker sequence, there is very little delay in downstream module degradation. (d) Single TS linker sequence results in 10 min delay. (e) Double TS linker sequence results in 16 min delay, similar to that of AAV degradation sequence. (f) 5-TS linker sequence results in 25 min delay (data shown in panels c-f was used to generate Fig. 1C).

region and the AAV degradation tag. While CFP to GFP bleed-over is more significant than GFP to CFP bleed-over, the CFP to GFP bleed-over is not relevant to our experiment in Figure 1a, where the induced protein (GFP) drives the protein level of the coupled protein (CFP). Thus, we performed an experiment to test the potential for bleeding from sfGFP into CFP fluorescence
**Figure 4.6:** Cell-cell communication by AHL reduces variability in the quorum clock. (a) Individual "leader" cells show early activation of quorum clock proteins relative to the mean population response. (b) In a 2-cell simulation, cells 1 and 2 start out unlinked with slightly different constitutive production of AiiA and LuxI ($a_0$). At $t = 100$ min the two cells are linked through external AHL in the media, showing the cell with slower dynamics (2) linking up to cell 1 with shorter periods. (c) Cells 1 and 2 start out unlinked with cell 1 including intracellular clock dynamics (green) that result in higher frequency oscillations in cell 1. When the cells are linked ($t = 100$), the slower cell 2, without the intracellular clock, links on to the faster cell through external AHL communication between the cells. (d) Trajectories of 20 cells with noisy constitutive production at lux promoter synchronize when their external AHL pool is mixed at $t = 400$ min. Mean trajectory is shown in black. (e) Period variability after cell synching (red) is lower than in individual cells (blue).

channel by activation sfGFP with 10nM AHL in a strain that lacked CFP fluorophore. We saw no change in CFP fluorescence while sfGFP increased as expected (Supplementary Fig. 1b top).

**NFB helps $\text{H}_2\text{O}_2$ synchronize oscillations between colonies**

We defined the inter-pulse (wait) time as the time between the 10% downslope point of one peak and 10% upslope point of the following peak (Supplementary Fig. 1a).The mean QS inter-pulse time decreased with addition of IPTG (0.5 mM) to the coupled system, while the time of each pulse stayed constant. In addition, we find that QS trajectories from the coupled oscil-
Figure 4.7: The intracellular clock increases robustness in the coupled oscillator system by reducing the period of the quorum clock. (a) Removal of IPTG, which increases intracellular clock strength, leads to more regular oscillations (experimental). (b) The decrease in variability of the inter-pulse time of the coupled oscillator without IPTG suggests that the intracellular clock plays a significant role in the inter-pulse dynamics (experimental). (c) At very high flow rate, the quorum clock oscillates irregularly. Tuning up the intracellular clock reduces the quorum clock period, restoring regular oscillations and allowing for global level synchronization between colonies due to H$_2$O$_2$ biopixel coupling. Genetic addition of the intracellular clock (0.1mM IPTG) helps synchronize the quorum clock at high flows (430µm/s). Increasing the strength of the intracellular clock with removal of IPTG further enhances H$_2$O$_2$ inter-colony synchronization (experimental).

The intracellular clock showed significantly lower variability without IPTG as compared to 0.5mM IPTG (Supplementary Fig. 3a-b). These results suggest that stronger NFB (0mM IPTG) associated with higher NFB protein production$^{125}$ leads to shorter and more robust inter-pulse behavior in the coupled system. In large biopixel devices, less robust colony-level oscillations prevent H$_2$O$_2$ from effectively coupling neighboring pixels, resulting in unsynchronized QS oscillations (No NFB in Supplementary Fig. 3c). NFB reduces inter-pulse duration noise, which allows
Figure 4.8: \( \text{H}_2\text{O}_2 \) increases degradation rate by ClpXP that, in combination with transcriptional increase at the \textit{lux} promoter, decreases variability in the oscillator period. (a) There is a significant decrease in the degradation time due to \( \text{H}_2\text{O}_2 \) (experimental). (b) This is due to effective increase in ClpXP degradation rate (experimental). (c) \( \text{H}_2\text{O}_2 \) activation of \textit{lux} promoter alone would only increase the amplitude of quorum clock oscillations. Similarly, \( \text{H}_2\text{O}_2 \)-dependent increase in ClpXP activity results only in steeper degradation and longer inter-pulse duration. Combination of the two effects leads to increase in amplitude and decrease in inter-pulse duration, which matches experiments (model). (d) Individually the two \( \text{H}_2\text{O}_2 \) effects do little to lower the quorum clock period CV, which is reduced when both are present (model).

\( \text{H}_2\text{O}_2 \) to synchronize QS oscillations in neighboring colonies in biopixel devices (0.1mM IPTG in Supplementary Fig. 3c). Increasing NFB strength, further

**\( \text{H}_2\text{O}_2 \) increases protein degradation rate**

Our analysis of \( \text{H}_2\text{O}_2 \) synchronized quorum clock trajectories showed decrease in the period and increase in the amplitude of oscillations (Fig. 4b Top). \( \text{H}_2\text{O}_2 \) synchronization leads to clear reduction of the degradation time in these trajectories (Supplementary Fig. 4a). One of the significant contributors to the decrease in the period is the increase in the activity of ClpXP targeted proteins, which we quantified as the rate of CFP fluorescence decrease from the peak.
time to the 10% downslope time. Supplementary Figure 4b shows a significant increase in the ClpXP degradation rate (3X) due to H$_2$O$_2$ coupling.
Model Formulation

QS oscillator

To describe dynamic behavior of uncoupled QS oscillator, we expanded on the delay-differential equation model presented in \(^{21}\). In addition to the equations for LuxI (I), AiiA (A), internal AHL (\(H_i\)), external AHL (\(H_e\)), we included AHL substrate (S), consisting of acyl-ACPs and S-adenosylmethionine (SAM)\(^{99}\), to account for the slowing down of \(H_i\) production while the number of LuxI molecules is still on the rise. Transcription, translation, and maturation rate of proteins are combined into a single time-delay parameter \(\tau_H\). Transcriptional activation by the LuxR and AHL complex (2 of each LuxR and AHL molecules) give delayed production term \(P(\tau_H)\), which depends on the past concentration of internal AHL, \(H_i(t - \tau_H)\). We assumed a constant level of LuxR since it is not tagged for fast degradation and has a large amount of genetic copies on the plasmid (it is on colE1 twice and p15A once). We used hill coefficient of 4 in accordance with \(^{92}\) to account for high AHL cooperativity possibly due to AHL-LuxR polymerazation. Diffusion of AHL through cell membrane is described by terms proportional to \(D\), while dilution of external AHL is described by the term proportional to \(\mu\). Cell density parameter \(d\) was incorporated into the system to account for the difference in the total cell volume and media volume. Enzymatic degradation terms proportional to \(\gamma_I\) and \(\gamma_A\) describe enzymatic degradation of LuxI and AiiA respectively through Michaelis-Mentent kinetics. Different values of \(k_I\) and \(k_A\) represent different preferential binding dynamics of LuxI and AiiA to ClpXP.

\[
\begin{align*}
\frac{dA}{dt} &= C_A P(\tau_H) - \frac{\gamma_A(A/k_A)}{1 + A/k_A + I/k_I} \\
\frac{dI}{dt} &= C_I P(\tau_H) - \frac{\gamma_I(I/k_I)}{1 + A/k_A + I/k_I} \\
\frac{dH_i}{dt} &= \frac{bI(S/k_S)}{1 + S/k_S} - \frac{\gamma_H A(H_i/k_H)}{1 + H_i/k_H} + D(H_e - H_i) \\
\frac{dH_e}{dt} &= -\frac{d}{1 - d}D(H_e - H_i) - \mu H_e \\
\frac{dS}{dt} &= S_0 - S - \frac{bI(S/k_S)}{1 + (S/k_S)} \\

P(\tau_H) &= \alpha_0 + \frac{\alpha_H(H(t - \tau_H)/h_0)^4}{1 + (H(t - \tau_H)/h_0)^4}
\end{align*}
\]
Experimentally relevant scaled parameters used with this model are described in Extended Data Table 1.

**NFB oscillator**

To describe dynamic behavior of NFB oscillator, we used a single delay-differential equation for LacI ($L$) based on\(^7\). Transcription, translation, and maturation of proteins are lumped together into time-delay parameter $\tau_L$. Transcriptional inactivation of LacI gives the delayed production term $Q(\tau_L)$, which depends on the past concentration of LacI, $L(t - \tau_L)$. Enzymatic degradation of LacI is described by the term proportional to $\gamma_L$ through Michaelis-Mentent kinetics. Parameter $C$ in production expression $Q$ represents the effect of IPTG on the strength of LacI repression.

\[
\frac{dL}{dt} = Q(\tau_L) - \gamma_L \frac{L/k_L}{1 + L/k_L} \tag{4.6}
\]

\[
Q(\tau_L) = \frac{\alpha_L}{1 + (L(t - \tau_L)/C)^2}
\]

The dynamics of the above model accounted for most of the experimental results. To resolve the amplitude increase in the NFB oscillator when coupled to the QS oscillator during the QS pulse we had to include reporter dynamics with equations for YFP precursor ($Y_p$) and mature YFP ($Y_m$). These additional equations are not required to explain the QS dynamics in the coupled system. Experimentally relevant scaled parameters used with this model are shown in Extended Data Table 1.

\[
\frac{dL}{dt} = Q(\tau_L) - \gamma_L \frac{L/k_L}{1 + L/k_L + Y_p/k_L + Y_p/k_L} \tag{4.7}
\]

\[
\frac{dY_p}{dt} = Q(\tau_L) - \gamma_L \frac{Y_p/k_L}{1 + L/k_L + Y_p/k_L + Y_m/k_L} - Y_p \tag{4.8}
\]

\[
\frac{dY_m}{dt} = Y_p - \frac{\gamma_L (Y_m/k_L)}{1 + L/k_L + Y_p/k_L + Y_m/k_L} \tag{4.9}
\]

\[
Q(\tau_L) = \frac{\alpha_L}{1 + (L(t - \tau_L)/C)^2}
\]
Coupled NFB and QS oscillators

Coupling of the two oscillators was accomplished by increasing the effective "queueing" effect through ClpXP degradation\(^\text{15}\). In the uncoupled case, the degradation of the two oscillator components would be independent, \(\text{ClpXP}_{1+\text{QS}}^{+} + \text{ClpXP}_{1+\text{NFB}}^{+}\), while in the coupled scenario, \(\text{ClpXP}_{1+\text{QS}+\text{NFB}}^{+}\), the degraded components end up in the same degradation term. To couple NFB and QS oscillators through ClpXP degradation, we added LuxI and AiiA from QS system to the degradation expressions in NFB system and LacI \((L)\) from NFB system to the degradation expression in QS system.

\[
\frac{\partial A}{\partial t} = \frac{C_A P(\tau_H)}{1 + A/k_A + I/k_I + L} - \frac{\gamma_A (A/k_A)}{1 + A/k_A + I/k_I + L} \tag{4.10}
\]

\[
\frac{\partial I}{\partial t} = \frac{C_I P(\tau_H)}{1 + A/k_A + I/k_I + L} - \frac{\gamma_I (I/k_I)}{1 + A/k_A + I/k_I + L} \tag{4.11}
\]

\[
\frac{\partial H_i}{\partial t} = \frac{b I (S/k_S)}{1 + S/k_S} - \frac{\gamma_H A (H_i/k_H)}{1 + H_i/k_H} + D(H_e - H_i) \tag{4.12}
\]

\[
\frac{\partial H_e}{\partial t} = -\frac{d}{1 - d} D(H_e - H_i) - \mu H_e \tag{4.13}
\]

\[
\frac{\partial S}{\partial t} = S_0 - S - \frac{b I (S/k_S)}{1 + (S/k_S)} \tag{4.14}
\]

\[
\frac{\partial L}{\partial t} = \frac{Q(\tau_L)}{1 + L/k_L} - \frac{\gamma_L (L/k_L)}{1 + L/k_L + A + I} \tag{4.15}
\]

\[
P(\tau_H) = \alpha_0 + \frac{\alpha_H (H(t - \tau_H)/h_0)^4}{1 + (H(t - \tau_H)/h_0)^4}
\]

\[
Q(\tau_L) = \frac{\alpha_L}{1 + (L(t - \tau_L)/C)^2}
\]

Experimentally relevant scaled parameters used with this model are described in Extended Data Table 1. We varied the flow \(\mu\), IPTG concentration \(C\), and arabinose concentration \(\alpha_L\) to recapture many of the experimental findings.

Leader cell wait time shortening

To understand the multicellular dynamics of QS pulse activation we constructed a model with two identical cells that share external AHL \((H_e)\). We first considered a QS only system consisting of two cells with slightly different constitutive production of AiiA and LuxI. In this
system, the slower cell couples to the faster one, suggesting that cells whose QS pulse fires first cause QS pulse activation in the nearby cells through AHL cell-to-cell communication (Supplementary Fig. 2a). Next we added NFB to cell 1 in a two-cell system, resulting in period shortening of that cell. As the result, when the two cells were linked through external AHL, the slower cell 2 (without NFB), coupled to the faster cell 1 (Supplementary Fig. 2b). Consequently, even though NFB might be out of phase in different cells, the onset of QS pulse in the faster cells can initiate the propagation of the QS pulse through the rest of the cells in the nearby region. This effect further reduces cell-cell QS variability, which we see from period variability reduction in a 20-cell model (Supplementary Fig. 2d). We added noise to constitutive production of AiiA and LuxI proteins ($\alpha_0 = 0.6 \pm 0.1$) of each of the 20 cells and showed period variability reduction in synched vs unsynched cells (Supplementary Fig. 2e).

**QS and H$_2$O$_2$ coupled through queueing**

To describe dynamic behavior of QS oscillator in response to H$_2$O$_2$ produced during LuxI fluorescent reporter expression, we added a differential equation describing production and degradation of H$_2$O$_2$ ($V_i$ and $V_e$) to the QS oscillator delay-differential equation model. We assumed that the production of H$_2$O$_2$ is dependent on the concentration of LuxI, which is under the same promoter as the CFP fluorescent protein. Degradation of H$_2$O$_2$ by catalase is proportional to its concentration. H$_2$O$_2$ affects the QS oscillator in two characteristic ways. First, ArcA, which is under normal conditions partially represses Lux promoter, is inactivated under oxidizing conditions triggered by H$_2$O$_2$, relieving Lux repression and increasing LuxI and AiiA production. We model this phenomenon by adding a multiplier to the production term that is dependent on H$_2$O$_2$ concentration. Second, H$_2$O$_2$ has been shown to reduce ClpXP load, leading to increased rate of AiiA and LuxI degradation. Again, we model this behavior by adding a multiplier in front of the degradation term, dependent on H$_2$O$_2$ concentration. Finally, H$_2$O$_2$ can freely diffuse across cell membrane, which we describe a diffusion term characterized by diffusion parameter $D_V$. Extracellular H$_2$O$_2$ ($V_e$) can further leave the system with the rate proportional to its concentration.
\[
\frac{\partial A}{\partial t} = C_A P(\alpha_H, \tau) - (1 + V_i) \frac{\gamma_A(A/k_A)}{1 + A/k_A + I/k_I} 
\]

\[
\frac{\partial I}{\partial t} = C_I P(\alpha_H, \tau) - (1 + V_i) \frac{\gamma_I(I/k_I)}{1 + A/k_A + I/k_I} 
\]

\[
\frac{\partial H_i}{\partial t} = b I(S/k_S) - \frac{\gamma_H A(H_i/k_H)}{1 + H_i/k_H} + D(H_e - H_i) 
\]

\[
\frac{\partial H_e}{\partial t} = -\frac{d}{1 - d} D(H_e - H_i) - \mu H_e 
\]

\[
\frac{\partial S}{\partial t} = S_0 - S - \frac{b I(S/k_S)}{1 + (S/k_S)} 
\]

\[
\frac{\partial V}{\partial t} = \frac{\delta (1/C_I)}{1 + I/C_I} - V_i + D_V(V_e - V_i) 
\]

\[
\frac{\partial V_e}{\partial t} = \frac{d}{1 - d} D_V(V_e - V_i) - \mu_V * V_e 
\]

\[
P(\tau_H) = (1 + f_p V)(\alpha_0 + \frac{a_{\mu}(H(t-\tau_H)/\mu_0)^4}{1+(H(t-\tau_H)/\mu_0)^4})
\]

**H₂O₂ increases QS period robustness**

As we have mentioned before, reduction in inter-pulse duration leads to reduction in period variability arising from noise. Incorporating H₂O₂ effects on QS oscillator into our model (see above) results in several major changes in QS trajectory. First, as expected the amplitude of QS and the downslope time of QS decrease with addition of H₂O₂ (Supplementary Fig. 4c). The result of these two effects also results in shortening of inter-pulse duration, which leads to more robust QS oscillations (Supplementary Fig. 4d). We simulated the model to obtain at least 50 period measurement for period CV calculation. The noise was introduced into the model through addition of a noisy production term (\(\alpha_v = \pm 0.1\)) to the delayed production term

\[
P(\tau_H) = \alpha_v + (1 + f_p V)(\alpha_0 + \frac{a_{\mu}(H(t-\tau_H)/\mu_0)^4}{1+(H(t-\tau_H)/\mu_0)^4})
\]

Interestingly, our model shows that individual effects of H₂O₂ activation of lux promoter and increase in ClpXP activity result in the increase the CV of the QS period (Supplementary Fig. 4d). With respect to increased ClpXP activity, higher CV is mainly due to the resulting longer inter-pulse duration (Supplementary Fig. 4c green). Increased lux promoter activity, however, leads to more variable degradation due to higher pulse amplitude variability. The two counteracting H₂O₂ effects seem to cancel each other’s variability generating more robust QS oscillations.
Fitting model parameters to experimental results

To fit the NFB period data from experiments we used the following parameter scaling functions for the LacI production term \( Q(\tau_L) = \frac{\alpha_L}{1 + (L(\tau_L)/C)^2} \) to fit IPTG and arabinose (ARA) concentrations:

\[
\alpha_L \propto A_A + D_A \left( \frac{\text{ARA}}{\text{CA}} \right)^{H_A} \left( 1 + \frac{\text{ARA}}{\text{CA}} \right)^{H_A}
\]

\[
A_A = 0.2758, D_A = 1.6291, C_A = 0.5638, H_A = 0.9029
\]

\[
C \propto A_C + D_C \left( \frac{\text{IPTG}}{\text{CC}} \right)^{H_C} \left( 1 + \frac{\text{IPTG}}{\text{CC}} \right)^{H_C}
\]

\[
A_C = 0.0968, D_C = 60.8510, C_C = 8.2451, H_C = 0.4334
\]

Similarly we fit the model flow term \( \mu \) to the experimental flow values using the following function

\[
\mu = A_\mu \mu^2 + B_\mu \mu + C
\]

\[
A_\mu = 1.2e - 7, B_\mu = 0.0022, C_\mu = -0.11
\]

Model parameter values

\( C_A = 1 \) (AiiA copy number); \( C_I = 4 \) (LuxI copy number); \( \gamma_A = 8 \) (ClpXP degradation of AiiA); \( \gamma_I = 8 \) (ClpXP degradation of LuxI); \( K_A = 1 \) (AiiA binding affinity to ClpXP); \( K_I = 0.2 \) (LuxI binding affinity to ClpXP); \( \alpha_0 = 0.6 \) (Lux promoter basal production); \( \alpha_H = 3 \) (Lux promoter AHL induced production); \( h0 = 0.1 \) (AHL promoter binding affinity); \( \tau_H = 1 \) (delay in LuxI and AiiA production); \( b = 1 \) (AHL synthesis rate by LuxI); \( k_S = 25 \) (AHL substrate binding affinity to LuxI); \( S_0 = 50 \) (basal AHL substrate production); \( \gamma_H = 1 \) (AHL degradation rate by AiiA); \( k_H = 0.1 \) (AHL binding affinity to AiiA); \( D = 0.8 \) (AHL diffusion across the membrane); \( d = 0.1 \) (cell density); \( \mu = 0.5 \) (flow rate); \( \alpha_L = 1 \) (LacI/YFP production rate); \( C = 0.0025 \) (LacI promoter binding affinity); \( \tau_L = 0.7 \) (delay in LacI/YFP production); \( k_L = 0.001 \) (LacI/YFP binding affinity to ClpXP); \( \gamma_L = 0.05 \) (ClpXP degradation of LacI/YFP); \( \delta = 1 \) (H\(_2\)O\(_2\) production due to QS fluorophores); \( C_I = 2 \) (Michaelis constant); \( f_p = 1.3 \) (strength of H\(_2\)O\(_2\) activation of
LuxI promoter; $D_V = 8$ (H$_2$O$_2$ diffusion across membrane); $\mu_V = 0$ (extracellular H$_2$O$_2$ dilution)

Chapter 5

Programmable Probiotics: Gene circuit chaperones for non-invasive cancer detection

Introduction

Rapid advances in the forward engineering of genetic circuitry in living cells has positioned synthetic biology as a potential solution to numerous biomedical problems. As advances toward clinical applications of synthetic biology continue, one challenge involves the creation of technologies that integrate with existing clinical paradigms of diagnosis and therapy in the near-term. We develop a platform termed "programmable probiotics" (PROP) that involves the delivery of synthetic biological constructs into primary and metastatic tumor microenvironments following oral administration of engineered probiotics. As a proof-of-concept, we engineer a point-of-care diagnostic capable of noninvasive detection of disseminated hepatic tumors using simple and low-cost urinalysis methods. The diagnostic generates a high-contrast readout via three modes of amplification: selective expansion in the tumor (10E6-fold enrichment over control tissues), high reporter expression enabled by an engineered maintenance system, and enzymatic gain by the encoded reporter. Our results demonstrate how probiotic chaperones can be programmed to deliver synthetic gene circuits to disease microen-
environments where they can be exploited to integrate with existing clinical paradigms to develop next-generation diagnostics.

**Programmable Probiotics**

The ability of certain strains of bacteria to undergo tumor-specific growth has been explored for use as a potential cancer therapy and the growing ability to engineer genetic circuits with synthetic biology now enables the design of novel therapies and diagnostics with programmed behavior. We develop a simple and non-invasive method for delivering gene circuits to systemic tumors via oral delivery of the probiotic bacterium *E. coli* Nissle 1917 (EcN) resulting in execution of EcN’s genetic program within the tumor microenvironment (Figure 1). Our platform (PROP, for programmable probiotics) merges the safe and proven delivery characteristics of probiotics such as EcN with sophisticated programmed sensing and delivery capabilities drawn from synthetic biology. The PROP platform may then aid in developing next-generation diagnostics by integrating programmable tumor-specific gene expression with common modes of clinical diagnosis, such as urinalysis.

We reasoned that oral probiotic delivery might be a specific route to liver tumor colonization since the blood flow from the gut flows through the portal vein and onto the liver. To test this prediction, we chose an immunocompetent model of colorectal cancer liver metastases. To directly visualize probiotics within the liver metastases, we orally administered PROP-Z bacteria expressing a chromosomally-integrated luminescence cassette. The small doses of bacteria in the tumor were sufficient to detect luminescent signals in metastases as small as 1 mm, and we observed bacterial infiltration in virtually all tumor cores (Figure 2A). To determine the robustness of tumor colonization by orally delivered EcN, we measured robust colonization of a variety of tumor types including a human ovarian cancer cell line (TOV21G), a syngeneic cell line (MC26), and 2 cell lines obtained from a genetically engineered mouse models (GEMM, 393m lung metastasis and KBP22 pancreatic line) (Figure 2B,C). We then performed dosage experiments to characterize the minimum number of bacteria required to reliably colonize tumors, finding colonization for both nude and immunocomponent mice well below the observed toxicity level of approximately 1E7 CFU (Figure 2D).
Figure 5.1: PROP-Z probiotics for noninvasive cancer detection. a) The PROP-Z diagnostic platform is made up of probiotic E. coli Nissle 1917 (EcN) bacteria transformed with a dual-stabilized, high expression lacZ vector. (1) PROP-Z is delivered orally. (2) Bacteria rapidly (24 hours) translocate across the GI tract and (3) specifically amplify within metastatic tumors in the liver. (4) PROP-Z express high levels of the lacZ enzymatic marker, enabling urinary detection via injected cleavable substrates.

We then performed a series of experiments to measure the specificity of tumor colonization relative to off-target organs and sites of injury that might also attract bacteria, such as fibrotic tissue. To quantify the specificity of colonization, we developed a quantitative PCR-based assay to measure PROP-Z bacteria in various organs following oral administration. Over a period of 7 days, we measured the number of bacteria in liver metastases and found a striking colonization level of 10E6 bacteria (Figure 2E). In stark contrast, PROP-Z biodistribution in healthy spleen, kidneys, and livers was below the limits of detection for our q-PCR assay (< 500 bacteria/g). As confirmation of this negative result, we assayed for off-target colonization by performing colony counts of entire organs, and observed zero PROP-Z bacteria in any control organ tested. As a result of zero off-target colonization, PROP-Z treated mice survived without any noticeable adverse effects for at least 12 months. To further quantify the specificity for tumors, we performed colonization studies using DDC-induced liver damage, finding that orally delivered EcN does not colonize fibrotic tissue as measured by colony counts and IVIS (Figure 2F). Collectively,
Figure 5.2: Robustness and specificity of EcN colonization. a) We generated liver models of metastasis to test the performance of our diagnostic on a relevant clinical problem—the early detection of small tumors difficult to detect using traditional methods. Healthy and metastatic livers, showing small tumor nodules (top). 24 hours following oral administration, PROP-Z bacteria were detected in tumor nodules via IVIS measurement (middle). PROP-Z bacteria colonize the inner core of metastatic nodules are often not visible from the exterior but are revealed by cutting to the interior, dashed line indicates cut site (bottom). b) Robust colonization of a variety of different tumor models in immunocompetent and immunocompromised mice, with either human (LS174T, TOV21G) or mouse cell lines (MC26, KBP22, m393) via intravenous delivery. Colonization of liver mets observed post oral delivery of Prop-Z in immunocompetent MC26 and 393m cell lines. c) Quantification of tumor colonization for each of the subcutaneous cell lines (n=6 tumors per model). d) Colonization as a function of dosage (in CFU) for both immunocompromised as well as immunocompetent models, showing a sharp transition for immunocompetent models, whereas a gradual transition is shown for immunocompromised models (n=6 tumors per dosage). e) To determine safety and specificity, we colony counted PROP-Z in organs following oral administration. We observed 106 bacteria colonize tumors after 24 hours with zero colonization (0 CFU/g) in off-target organs and no growth over time (means ± s.e.m, n=4 organs each). f) A DDC-induced liver injury models shows no colonization of Prop-Z via IVIS as compared to a strong signal in the liver met case. Quantification of the amounts of bacteria in all three cases. No detectable levels of bacteria are observed in Control or DDC cases, whereas livers with metastases have a significantly higher bacterial colonization.

these findings constitute demonstration of specific colonization of liver metastases by oral delivery of the probiotic EcN, setting the stage for chaperoned delivery of gene circuits to systemic tumors via oral delivery.\textsuperscript{17}
Figure 5.3: Dual-stabilized vector efficiently maintains diagnostic activity in vivo. a) Constitutive and inducible plasmid constructs were tested in vivo using a genetically-encoded luciferase as a proxy for diagnostic activity. Inducible circuits were based on the lux promoter, where AHL was added to the drinking water at a concentration of 10 uM. b) Dual-stabilized maintenance system ensures total plasmid stability in the tumor environment. c) We found nearly 100% maintenance over 72 hours with the combination of hok/sok and alp7CAR (means ± s.e.m, n=4 each). d) Enzymatic activity was also maintained over 72 hours in vitro. e) To quantify the performance of our PROP-Z diagnostic in stringent tumor microenvironment conditions, we performed growth assays under low nutrient (varying glucose %), pH, and oxygen conditions (top). In addition to growth, enzymatic activity was maintained by the dually-stabilized vector (bottom). f) Further, to quantify in vivo maintenance, we injected PROP-Z bacteria intravenously in a subcutaneous model of cancer. Plasmids were quickly lost in the absence of maintenance, hok enabled stability for 24 hours, and the combination of hok and alp7 ensure total stability for 72 hours (means ± s.e.m, n=5 each).

Non-Invasive Cancer Detection

Since synthetic genetic circuits have generally been engineered using plasmids that enable high signal, low noise, and modularity, we used our PROP platform to test two different...
circuit architectures (constitutive and inducible) using a subcutaneous xenograft model of colorectal cancer (Figure 3A). Since plasmids are often unstable in nutrient-poor conditions like the tumor microenvironment\textsuperscript{146}, we engineered a dual-maintenance vector to ensure long-term stability for our PROP platform (Figure 3B). The first maintenance device is an R1-derived toxin-antitoxin system that simultaneously produces a toxin (hok) and a short-lived antitoxin (sok), killing the cell in the event of plasmid loss\textsuperscript{146;43}. The second device, alp7, comes from the B. subtilis plasmid pLS20 and produces filaments that dynamically push plasmids to the poles, ensuring equal segregation during cell division\textsuperscript{25}. To measure the performance of our maintenance systems, we performed colony counts (+/- S-Gal) after successive subcultures in vitro, observing nearly complete plasmid maintenance (Figure 3C) and lacZ activity for at least 48 hours (Figure 3D). We performed further in vitro experiments under conditions characteristic of the tumor environment, such as low nutrient levels, pH, and oxygen content\textsuperscript{54;137} (Figure 3E). We observed that PROP-Z function was generally maintained with respect to bacterial growth and lacZ function, in that both measures were mildly reduced, proportional to the extent of the condition tested.

Since the complex selective pressures of tumor microenvironment cannot be completely modeled in vitro, we performed further diagnostic testing in vivo using our subcutaneous cancer model. We found that less than 10\% of bacteria remained in the tumors after 24 hours if no stabilization circuits were utilized (Figure 3F). With the addition of the hok/sok system, 75\% of the bacteria were retained for the first 24 hours, followed by a decline to 45\% of the initial bacterial load after 72 hours. With the combination of hok and alp7, over 96\% PROP-Z bacteria remained after 72 hours in vivo. Although alp7 was the dominant factor, the addition of hok/sok led to a significant increase in maintenance at 72 hours (p<0.05, SI). This substantial improvement in stability likely results from the combination of pre- and post-division mechanisms that promote plasmid maintenance, mirroring natural strategies used to achieve this outcome\textsuperscript{43}. The fully stabilized, high-copy expression vector may also be relevant for additional applications of synthetic biology in vivo.

We next sought to apply PROP-Z probiotics to the clinical challenge of detecting cancer metastases, which are ultimately responsible for 90\% of all cancer-related deaths but
Figure 5.4: Rapid, specific, and minimally invasive detection of millimeter-scale metastatic tumors. a) To measure PROP-Z diagnostic amplification due to tumor-specific EcN growth, we analyzed tumor homogenates for lacZ activity, finding a total in vivo amplification of around 5 for the relative cleavage rate from Day 3 to Day 0 (means ± s.e.m, n=5 each). b) PROP-Z bacteria detect the presence of metastatic tumors via tumor-specific growth leading to high-level expression of lacZ. We quantified enzymatic activity by injecting Lu-gal, a lacZ substrate that when cleaved produces luciferin, and assayed the urine for luminescence (means ± s.e.m, n=6 for each condition, 2 tail students t-test, p<0.05). c) Paired day0/day1 urine values for the entire cohort indicate growing diagnostic power as EcN amplify. Inset shows a correlation plot for each of 10 mice in the cohort. d) Receiver operator characteristic (ROC) curve. We measured a high area (A=0.93) compared to the PSA blood biomarker test (A=0.6834), where area is a metric that characterizes the predictive power of the diagnostic.

remain difficult to detect because of their small size. In order to precisely quantify the capacity of our diagnostic to detect small metastatic tumors noninvasively from the urine, we performed luciferase assays using the systemically administered substrate LuGal. LuGal is a safe, commercially available luciferin-galactoside conjugate that, when cleaved by lacZ, acts as a substrate for luciferase to produce luminescence. To quantify the degree of amplification achieved by our diagnostic, we excised and homogenized colonized subcutaneous tumors over the course of 3 days, performing colorimetric assays for lacZ activity at each time point.
After subtracting background due to nonspecific cleavage, the combined signal amplification of bacterial exponential growth and enzymatic turnover produced signals nearly 5-fold greater than the initial injected dose (Figure 4A). To test the urine diagnostic in our liver metastasis model, we used only a small sample (1µl) of the collected urine and detected millimeter-scale metastatic tumors as early as 24 hours following oral administration of PROP-Z bacteria (Figure 4B). In quantifying diagnostic performance on multiple replicates including healthy mice, mock surgeries, and unmodified EcN bacteria, we measured a total in vivo signal-to-noise ratio (SNR) of approximately 4. This SNR was sufficient for the CPRG substrate to achieve readily visible urinary color changes, specifically in the presence of small tumors (Figure 4B, inset). As further evidence of robust diagnostic performance, we generated paired Day0/Day1 urine values taken for the entire cohort, demonstrating signal increase due to EcN growth for every animal (Figure 4C).

Since clinically relevant point-of-care diagnosis is dependent on a strong SNR to accurately classify a disease state based on patient samples, the most critical aspects of any diagnostic technology are its sensitivity and specificity\textsuperscript{154}. To quantify the performance of our diagnostic, we performed an ROC analysis and measured a high area in comparison to a current blood biomarker (A=0.93 for Prop-Z versus A=0.68 for PSA\textsuperscript{130}) indicative of low false negative and false positive rates (Figure 4D). A notable advantage of our PROP-Z platform is its capacity for enhancing sensitivity via three modes of signal amplification—bacterial growth, enzyme production, and enzymatic turnover. While traditional diagnostics are limited by the small fraction of the injected dose that reaches the site of interest\textsuperscript{116}, the small number of PROP-Z bacteria that initially reach the tumor quickly expand to achieve a maximized signal. In addition, enzymatic turnover further multiplies signal gain since each enzyme can cleave many substrates. The multiple modes of amplification help generate signals that rise above the in vivo background, a significant challenge in point-of-care diagnostics\textsuperscript{154}.

In translating any new medical technology, the central questions involve the degree to which the platform will function safely and effectively in human patients. While diagnostic performance in humans is certainly beyond the scope of this proof-of-principle study, we
understand the importance of justifying our approach in terms of its translational potential. There have been several lines of evidence that probiotics and other bacteria can translocate the GI tract in both mouse and humans. An excellent recent study measures the presence of bacteria within both healthy human breast tissue as well as cancerous breast tissue\textsuperscript{135}. The presence of the bacteria in both of these scenarios does not lead to any detectable infection, and since many of the species are present in the GI tract, we would speculate that some must originate from the gut microbiome. It seems then that persistent and harmless traversal of the GI tract by ingested bacteria is a normal part of human biology, and would therefore not preclude the possibility of our diagnostic working in humans. A remaining open question regarding the efficacy of our PROP-Z platform relates to the trafficking of microbes in the human gut. It has yet to be determined to what extent the specific microbiome of a patient may influence the rate of EcN translocation, nor can it be predicted how an individual’s immune response might affect the specificity of colonization.

The PROP-Z system features a probiotic chaperone that is currently prescribed in humans and can be cleared readily with antibiotics. In addition, safety in humans has also been previously demonstrated using attenuated S. typhimurium bacteria in human clinical trials. As a modular, evolving platform, subsequent iterations of the process may extend to support paper test modalities, incorporate substrates for colorimetric and/or MRI-based diagnosis, and be programmed to integrate with other synthetic biomarkers for cancer\textsuperscript{68}. In the present proof-of-concept study, we have already established a diagnostic strategy compatible with the existing clinical paradigm of urinalysis. Ultimately, we envision that complex synthetic gene circuits may reach beyond the broad diagnostic domains discussed here, and be extended to applications in therapeutic strategies that utilize self-triggered gene circuits, such as quorum sensing, to deliver clinical payloads to the densest tumor regions.

**Strains and Plasmids**

Plasmid pTKW106alp7A was constructed by adding the 3.5kb alp7AR cassette from the B. subtilis natto plasmid pLS20 to the pTKW106 lacZ expression vector containing the
hok/sok plasmid maintenance system\textsuperscript{146,149}. We amplified the alp7AR by PCR and added AvrII/NheI restriction sites using primers P1-2. For the purpose of cloning the alp7AR cassette into pTKW106, we amplified the entire 9kb pTKW106 backbone and added a single AvrII restriction site using primers P3-4. This PCR product was then digested with AvrII and ligated with the AvrII/NheI digested alp7AR insert, producing pTKW106alpA. The lux integrated strain was produced by transforming EcN bacteria with the p16Slux plasmid\textsuperscript{112}. Then this EcN-luxCDABE strain was transformed with our pTKW106alpA to create the Prop-Z diagnostic. The plasmid with no stabilizing element (pTKW106delhok) was created by PCR by removing the hok/sok region of pTKW106. The plasmids in Fig. 3A are the plux plasmid (), pTD103luxCDABE (inducible by 10uM AHL), and pTD104luxCDABE (inhibited by (Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone at 10uM). The latter pTD plasmids were constructed by replacing the GFP with lux genes in previous work\textsuperscript{22}.

**Plasmid Stability and Beta-Galactosidase activity in vitro**

Strains of E.coli lacZ deletion mutants (Mach One, Invitrogen) were transformed with either pTKW106, pTKW106alp7A, or pTKW106delhok. Cultures were diluted into LB media (Sigma-Aldrich) with antibiotics and no IPTG, and grown till an approximate OD=0.1. Cultures were then diluted 1/100x into deep 96 well-plates (Corning 3600) with 500uL LB without antibiotics and grown for 24 hours. Each 24 hours, cultures were diluted 1/10,000x and grown again in an additional well plate for 24 hours. Beta-galactosidase (lacZ) activity was measured using a Tecan i200 plate reader by diluting wells 1-10x as needed to obtain a cleavage rate during the 5-60min time when readings were obtained (Fig. 4A. First column is a PBS control cleavage showing stability of Lugal). Maximal activities were obtained from the linear portion of absorbance curve at 575 nm for each strain and each day. Each condition was replicated 4 times. Plasmid stability in Fig. 3C was calculated by plating on an LB + S-Gal and determining the number of white and black colonies\textsuperscript{146}, with the 0 hours time point being a pre-culture grown with antibiotics. In vitro activities in panel Fig. 2D are normalized for each strain at the 24 hour time point.
Tumor cell lines, animal models and in vivo tests

Plasmid stability and enzymatic tests (Fig 3F,4A) were performed in 6 week old nude mice (NcrNu, Female, Taconic) bearing tumors from a colorectal cancer cell line, LS174T (ATCC). Tumors were implanted with 5e6 cells in 100uL of PBS per flank and grown for 1-2 weeks till they reached a size of 5-10mm. Mice were randomized before the start of the experiment. To obtain measures of plasmid stability in vivo, EcN bacteria with pTKW plasmids were injected intravenously at a dosage of 1e6 bacteria, and tumors were steriley extracted and homogenized using a Tissue Dissociator (Miltenyi) and plated on both LB and LB with antibiotics to obtain the percent of plasmids with cells or measured for lacZ activity as mentioned above 5. Enzyme activities of homogenates in vivo were obtained by the same method as in vitro.

The liver metastasis model was generated by intraspenically injecting immunocompetent Balb/c mice (6 week old, Balb/c, Female, Taconic) with a luciferized, metastatic colorectal cell line (MC26 cell line, Tanabe Lab MGH, 5e4 cells in 100uL PBS per flank)\(^{111,145}\). Animals were monitored via IVIS (IVIS 200, Calipers) for approximately 20 days with intravenous injection of D-Luciferin (100uL, 30mg/mL, 15 min wait time). When the radiance values of tumors reach 5e9, mice were gavaged with 2-5e9 EcN bacteria resuspended in PBS. The drinking water was supplemented with IPTG at 10mM concentration to induce lacZ expression for 24 hours. Some mice were euthanized and livers extracted for quantification and detection of bacterial luminescence via IVIS 200 (Calipers) (Figures 2A).

To measure bacteria luciferase, a luminescence measurement was performed before injection of luciferin with an open filter, while mammalian luciferase measurements were made 15 mins post i.v. injection of 100uL of D-luciferin with an open filter. The Firefly values resulted in radiance units of about 1e7-1e10, while bacteria values were 1e5-1e7, thus the contribution of bacteria luciferase to mammalian luciferase values was negligible for most cases. Both bacteria luciferase (luxCDABE) and mammalian luciferase (Firefly) can be imaged in this way.
Urine Diagnostic assay

24 hours post gavage of bacteria, mice were injected subcutaneously with 1ml sterile PBS 1.5 hours before tail vein injection with 100ul Lugal [75ul PBS, 25ul Lugal (.01mg/ul)]. The Lugal injection was immediately followed by a second injection of 1ml sterile PBS subcutaneously to allow for sufficient volume of urine to be retrieved. Mice were placed in urine collection tubes for 1-2 hours. Typically 500-1000uL of urine was collected and 1 uL of urine was used to test for luciferin with a luciferase assay kit (Promega QuantiLum rLuciferase Kit) via luminescence in a Berthold Centro LB 960 reader (measurements done in triplicate). The final values plotted for the test were the total lacZ activity (product of luminescence and urine value) measured across the volume of urine obtained, with values typically in the range of [5e5-5e6]. ROC analysis was performed in Graphpad with a 95% confidence interval using the the PropZ+LM and PropZ-LM cases, representing mice with tumors receiving the PropZ diagnostic or mice without tumors receiving the PropZ diagnostic. The inset in Fig. 4B shows an example of the urine diagnostic assay where CPRG (100ug/100uL?) was injected intravenously and an observed color change in urine was present (+ case represents a high cleavage case , - case represents a negative control with no bacteria present).

Quantification of Tumor colonization

Colonization of the Prop-Z strain was determined by IVIS imaging 2-3 days post-injection and observing if a localized luminescent signal from the tumor was present (contains a luxCDABE chromosomal integration). Tumors were implanted in each of the models using a density of 5e6 cells per flank. 6 tumors were quantified from each case.

DDC-liver induced injury model

6 week old FVB/NJ mice were fed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 3 weeks. Control mice were the same type fed with regular food. After 3 weeks, urine diagnostic experiments were performed for n=4 mice for each group and compared using a 2 tailed students t-test. Colony counts were performed for n=5 mice from each group.
**Biodistribution/q-PCR experiments.**

Organs were harvested steriley by using a bead sterilizer for organs and washing in ethanol and then water before extraction. A small tissue sample between 10-100mg was cut from the organ and DNA was isolated using the UltraClean Tissue&Cells DNA Isolation Kit (MoBio, Carlsbad, CA). 1 uL of DNA sample was used in a subsequent q-PCR experiment run according to the manufacturerÕs parameters (Qiagen, Quantitech SYBR Kit) on a Bio-RAD iCycler machine. We used specific primers for EcN Nislux. Control curves were run for each qPCR experiment. Colony counts were obtained in separate experiments where whole organs were excised, homogenized in a Tissue Dissociator (Miltenyi) and plated on erythromycin to detect presence of EcN. Comparisons of colony counting from tumors and q-PCR experiments were equivalent.

**Additional controls and urine experiments.**

To further understand the background and signal during our urine assays we performed a variety of control animal studies. We determined that the majority of background cleavage comes from in vivo injection of Lugal, and not specifically tumor cleavage. Oral delivery of EcN tends to reduce the background, whether it contains lacZ or not.

![Figure 5.5: qPCR calculation of the number bacteria in a tumor sample. a) E.coli Nissle DNA samples were diluted in DNA-free water and DNA was purified as above. Automated Ct values generated from a Bio-RAD iCycler machine showing a linear correlation with Ct value across several orders of magnitude. B) The number of bacteria were calculated using a semi-log regression fit and the calculated number of bacteria is plotted in b, showing a sensitivity of detection of around 100 bacteria per sample. Samples were repeated in quadruplicate, standard error shown is smaller than the points.](image-url)
**Figure 5.6:** Colony forming units with hok and hok+alpA stability systems. a) We measured the absolute levels of bacteria in tumors in our athymic colorectal cancer model using colony-counting methods. The growth rates of both strains appear similar, suggesting that the stability of the combined system is not due to slower growth (in fact it grows slightly faster and thus stability results would likely be underestimated). b) Plasmid stability at 72 hours shown with an athymic colorectal cancer model. The difference between alp7A and hok+alp7A is significant (p<0.05), though the dominant contribution to stability observed is the alp system.

**Figure 5.7:** Growth and Activity of N-ColoniZe platform in different oxygen, inducer, and antibiotic conditions. a) OD600 values are shown in varying oxygen conditions after 48 hours of growth (subcultured at 24 hours) in 14mL Falcon tubes capped or uncapped with high level of media (14mL) or low level of media (3mL). b) Measured lacZ activity values in the same conditions. Norm indicates EZ-Rich media with 0.2% glucose, 3mL of media, and uncapped. Kanamycin growth conditions for the cap/high case were not tested in either panel.

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