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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Engineered synchrony of bacterial lysis and its applications

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

in

Bioengineering

by

Muhammad Omar Zanolabe Din

Committee in Charge:

Jeff Hasty, Chair
Terence Hwa
Justin Meyer
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Bernhard Palsson
Lev Tsimring

2017

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Chair

University of California, San Diego

2017

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LIST OF ABBREVIATIONS

A.U.	arbitrary units
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. typhimurium</i>	<i>Salmonella enterica subspecies enterica serovar typhimurium</i>
Eq.	equation
fig.	figure
FL.	fluorescence
GFP	green fluorescent protein
h.	hours
kb	kilobase
LB	lysogeny broth
OD	optical density
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
SLC	Synchronized Lysis Circuit
sfGFP	superfolder GFP
E-GFP	Lysis protein E fused to GFP

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Chapter 2 contains material originally published as Din MO*, Danino T*, Prindle A, Skalak M, Selimkhanov J, Allen K, Julio E, Atolia E, Tsimring LS, Bhatia SN, Hasty J. Synchronized cycles of bacterial lysis for in vivo delivery. *Nature*. 536.7614 (2016). (*equal contribution). The dissertation author was one of the primary researchers and is the first author of this paper.

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FIELDS OF STUDY

Major Field: Bioengineering (Synthetic Biology)

Studies in Biological Dynamics

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ABSTRACT OF THE DISSERTATION

Engineered synchrony of bacterial lysis and its applications

by

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Doctor of Philosophy in Bioengineering

University of California, San Diego, 2017

Jeff Hasty, Chair

Programmed cell death is a common theme in biology. Apoptosis in eukaryotic cells is typically mediated by external signals and is important for development and tissue homeostasis. There is also a growing body of evidence showing that programmed cell death also occurs in bacteria, and is important for biofilm formation. With the growing amount of tools for the genetic engineering of bacteria, researchers have made genetic constructs where bacterial cell death could be controlled or induced to further our understanding of bacterial cell death and its prospective use in applications. Concurrently, researchers in the field of synthetic biology have succeeded in developing dynamic gene circuits, such as oscillators and toggle switches, which operate at the intracellular (protein) level. In this dissertation, I discuss the engineering of bacteria via a gene circuit which can produce dynamic behaviors of the population by synchronizing bacterial cell lysis.

In Chapter Two, I discuss the construction and characterization of this circuit, which is called the Synchronized Lysis Circuit (SLC). The engineered bacteria are then

used as a vehicle to deliver therapeutic proteins to tumors via synchronized lysis. In Chapter Three, I discuss the application of the SLC in stabilizing a competitive ecosystem of bacteria. In Chapter Four, I discuss the effects of lysis protein production at the single-cell level on growth and survival. In Chapter Five, I discuss the behavior of the SLC at different scales (micro to macro) and its evolution over time. All of these sections combine to describe the behavior of synchronized lysis from single-cells to populations, and the applications of such a system.

Chapter 1

Introduction

The idea that a set of genes can be organized into a network controlled by regulatory elements to produce different dynamic behaviors has been proposed several decades ago[1]. This idea was followed up by the discovery and characterization of natural gene regulatory networks, such as the lysis-lysogeny switch in phage lambda, and mathematical descriptions of different network topologies via nonlinear models[2, 3]. The first experimental construction of non-natural networks was later accomplished by bringing together genes and regulatory elements originating from different contexts (natural or synthetic)[4, 5]. In the engineering sense, these non-natural gene networks are typically (although not exclusively) referred to as genetic circuits, synthetic gene circuits, or engineered gene circuits. Ideas borrowed from electrical circuits are often used describe different motifs and behaviors of genetic circuits[6, 7].

The development of genetic circuits has become a central goal for many researchers in the field of synthetic biology. The discussion of what synthetic biology as a field entails overall is more difficult to explain at the present moment. Succinctly, it may be said that synthetic biology is generally viewed as a field which seeks to engineer biology with our increasingly advanced molecular biology tools (it should be noted also that there are scientists who perform research of a very similar flavor to synthetic biologists, yet they do not ascribe to a field called synthetic biology). One of the results of this

engineering effort is the construction of small, synthetic gene circuits. Among the first examples of synthetic gene circuits were the genetic toggle switch, a memory switch where bacterial cells switch between two expression states based on external chemical input, and the oscillatory ‘repressilator’ circuit. Both of these circuits, and circuits that were developed thereafter, typically made use of a small repertoire of characterized genes and regulatory elements from previous literature (such as promoters, ribosome binding sites, and terminator sites). Among some groups, these elements are referred to as ‘genetic parts’ and there is ongoing research in way of characterizing new parts for synthetic biology[8]. Concurrently, there has been rising interest in the development of synthetic ecologies with engineered microbial members. The construction of these ecologies may be for different purposes. Researchers may want to study a natural ecological relationship with a small and controlled environment of engineered microbes (for example, a predator-prey or mutualistic ecology), or to use multiple members of a microbial ecosystem to produce valuable chemicals which would otherwise be difficult to produce with a single strain[9, 10]. Interestingly, while recent evidence has hinted at the idea that competition is the dominant interaction in microbial communities, ecologies made in the lab have thus far relied on engineering circuits with beneficial interactions or interdependencies to stabilize populations of multiple microbes (since competitive cultures in the lab will almost always be dominated by the faster growing strain)[11].

There has been increasing focus on developing genetic circuits in living cells (while this distinction may sound outdated, it is more relevant now due to the recent interest in *in vitro* circuits) for ‘real-world’ applications[12]. One of these general applications is the use of engineered bacteria for therapeutic purposes[13, 14]. The human body has a thriving community of microbes which can contribute to disease and various aspects of health (such as host metabolism and immune system function)[15]. As such, bacteria make for an interesting prospect as genetically engineered therapies.

Of particular interest to the work presented in this dissertation is the long historical precedent for using bacteria to treat cancer. William Coley was arguably the first scientist to systematically test the efficacy of this approach (albeit with pathogenic

bacteria) over a hundred years ago[16, 17]. While the use bacteria to treat cancer was not accepted as a mainstream treatment, many years later there was a resurgence in this idea. An attenuated strain of *M. bovis* was found to be a good candidate for the treatment of bladder cancer (the only bacterial strain currently used in the clinic for cancer therapy)[18]. Other strains of bacteria were also investigated for their prospective efficacy in treating cancer[19, 20]. More recently, facultative anaerobes such as *E. coli* and *S. typhimurium* were found to naturally target tumors in animal models[21]. This was significant due to the fact that these organisms were the main microbial models for molecular biology in the last century, and as such they were much easier to genetically manipulate over other bacteria given the larger repertoire of molecular biology tools. While initial clinical trials did not find good efficacy against cancer with attenuated strains (using *S. typhimurium*), researchers concluded that the bacteria were safe to use in humans[22]. Many studies since have focused on engineering these bacteria with different therapeutic strategies to help increase their efficacy[23].

One useful behavior that may be engineered into a genetic circuit for a variety of applications is population control via self-activated cell death. In nature, programmed cell death is a commonly observed phenomena, typically in eukaryotes (for example, apoptosis)[24]. This behavior is important for the development and maintenance of multi-cellular organisms. There is also ample evidence for programmed cell death occurring in bacteria[25]. Examples include cell death via toxin-antitoxin modules in response to starvation and cell death in a subpopulation of bacteria during sporulation events.

In this dissertation I describe the development of a genetic circuit that controls the bacterial population via synchronized lysis, and examples of it's applications. While previous examples of gene circuits controlling cell lysis in literature have focused mainly on the induction of cell death upon the addition of external inducers, here I describe work on constructing a circuit which auto-activates cell lysis via quorum sensing[26, 27]. In the context of bacterial therapies, this serves the dual purpose of pruning the bacterial population *in vivo* to reduce the possibility of deleterious immune responses, and

to release genetically encoded therapies. In the context of synthetic ecologies, self-attenuation of multiple bacterial populations serves to potentiate coexistence in a competitive state.

This circuit also exhibits different dynamic behaviors, such as oscillatory lysis and constant lysis, which I then further investigate by observing the expression of the lysis protein inside the bacteria. These behaviors would not be expected based on previous literature describing the action of the lysis protein (E from the phage ϕ X174). Investigation into the intracellular behavior of the lysis gene in the context of the synchronized lysis circuit (SLC) may inform us of behaviors possible in natural settings that have not been previously observed. This may have wider implications given that some antibiotics have the same intracellular target as E.

I also investigate the behavior of the circuit across different scales and its evolution over time. Currently, much literature on the development of genetic circuits has a focus on circuit design (for example, by arranging known or developed genetic parts to produce novel circuits) and then measuring their behavior. Circuit measurement techniques may be performed across physical scales: for example, a milliliter scale culture may be subject to fluorescence measurement on a plate reader, or a small number of cells may be tracked under a microscope. One rarely studied aspect of genetic circuits is how their behavior changes between physical scales. Another rarely studied aspect of genetic circuits are their evolution over time where mutations deleterious to circuit function may appear (except in the cases when directed evolution of the circuit is being performed). These two properties of genetic circuits are important to understand and eventually optimize for applications.

The work presented in the following chapters is not entirely chronological in the order in which it was done. While, Chapters 2 and 3 are from published material describing the application of the SLC to therapeutic delivery and the stabilization of ecosystems, the rest of the dissertation describes the work that has been ongoing in the background of these two projects. Altogether, the following chapters describe the results from investigating this genetic circuit, the SLC, from its micro-scale and macro-scale

properties to it's evolution over time and implementation for different applications.

Chapter 2

Synchronized cycles of bacterial lysis for *in vivo* delivery

Introduction

The pervasive view of bacteria as strictly pathogenic has given way to an appreciation of the widespread prevalence of beneficial microbes within the human body[15, 28, 29]. Given this milieu, it is perhaps inevitable that some bacteria would evolve to preferentially grow in environments that harbor disease and thus provide a natural platform for the development of engineered therapies[21, 30, 13]. Such therapies could benefit from bacteria that are programmed to limit bacterial growth while continually producing and releasing cytotoxic agents *in situ*[31, 17, 32, 33]. Here, we engineer a clinically relevant bacterium to lyse synchronously at a threshold population density and to release genetically encoded cargo. Following quorum lysis, a small number of surviving bacteria reseed the growing population, thus leading to pulsatile delivery cycles. We use microfluidic devices to characterize the engineered lysis strain and we demonstrate its potential as a drug delivery platform via co-culture with human cancer cells *in vitro*. As a proof of principle, we track the bacterial population dynamics in ectopic syngeneic colorectal tumors in mice. The lysis strain exhibits pulsatile popula-

tion dynamics *in vivo*, with mean bacterial luminescence that remained two orders of magnitude lower than an unmodified strain. Finally, guided by previous findings that certain bacteria can enhance the efficacy of standard therapies[34], we orally administer the lysis strain, alone or in combination with a clinical chemotherapeutic, to a syngeneic transplantation model of hepatic colorectal metastases. We find that the combination of both circuit-engineered bacteria and chemotherapy leads to a notable reduction of tumor activity along with a marked survival benefit over either therapy alone. Our approach establishes a methodology for leveraging the tools of synthetic biology to exploit the natural propensity for certain bacteria to colonize disease sites.

Circuit construction, characterization, and quantitative modeling

In order to control population levels and facilitate drug delivery using bacteria, we engineered a synchronized lysis circuit (SLC) using coupled positive and negative feedback loops that have previously been used to generate robust oscillatory dynamics[35, 36]. The circuit (Fig. 1a) consists of a common promoter that drives expression of both its own activator (positive feedback) and a lysis gene (negative feedback). Specifically, the *luxI* promoter regulates production of autoinducer (AHL), which binds LuxR and enables it to transcriptionally activate the promoter. Negative feedback arises from cell death that is triggered by a bacteriophage lysis gene (ϕ X174 E) which is also under control of the *luxI* promoter[36, 37, 38]. Importantly, AHL can diffuse to neighboring cells and thus provides an intercellular synchronization mechanism.

The bacterial population dynamics arising from the synchronized lysis circuit can be conceptualized as a slow buildup of the signaling molecule (AHL) to a threshold level, followed by a lysis event that rapidly prunes the population and enables the release of bacterial contents (Fig. 1b). After lysis, a small number of remaining bacteria begin to produce AHL anew, allowing the “integrate and fire” process to be repeated in a cycli-

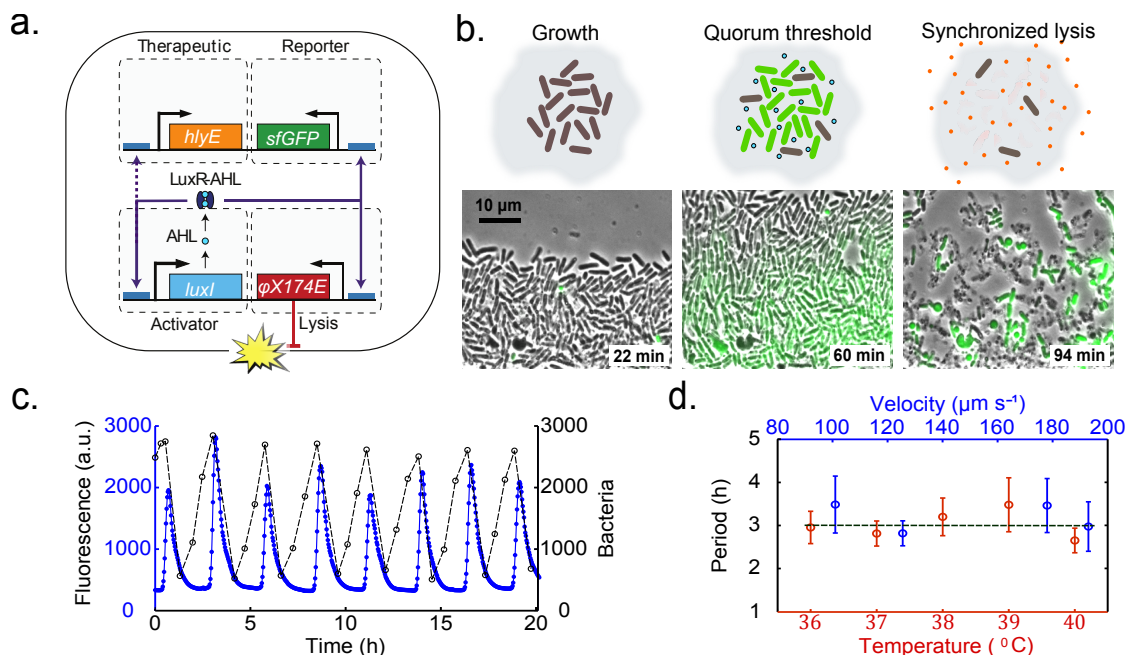


Figure 2.1: Construction and characterization of the SLC. (a) The circuit contains an activator[36] and lysis plasmid. When the population reaches the quorum threshold at a critical AHL concentration, the *luxI* promoter drives the transcription of gene *E* for lysis, LuxI, and *sfGFP* or *luxCDABE* as the reporter module. The *luxI* or the *ptac* promoter also drives the transcription of the therapeutic gene for the stabilized circuit used *in vivo*. LuxR in this system is driven by the native pLuxR promoter. (b) A schematic that illustrates the main stages of each lysis cycle from seeding to quorum ‘firing’. Shown below are typical time series images of the circuit-harboring cells undergoing the three main stages of quorum firing in a microfluidic growth chamber[35]. (c) Fluorescence profile of a typical microfluidic experiment. The estimated cell population trajectory reveals that lysis events correspond to peaks of sfGFP fluorescence. (d) Period as a function of estimated flow velocity in the media channel of the microfluidic device and environmental temperature. Error bars indicate ± 1 standard deviation for 13 - 50 peaks. The above experiments were performed with Strain 1, see Supplementary Information for complete strain information.

cal fashion. We used microfluidic devices to observe growth and lysis with a fluorescent protein (sfGFP) as a proxy for circuit dynamics in attenuated *S. typhimurium* (Supplementary Videos 1 and 2). We observed periodic lysis events characterized by peaks in the fluorescent reporter expression that correspond to population lysis (Fig. 1c). The fraction of lysed cells remains consistent across subsequent cycles, suggesting that lysis and survival occur in a stochastic manner (Extended Data Fig. 1a-b). Given the ultimate goal of implementation in an *in vivo* microenvironment characterized by variable growth conditions, we tested a range of incubation temperatures (36°C to 40°C) and perfusion rates (100 $\mu\text{m/s}$ to 200 $\mu\text{m/s}$), measuring an average period of 3 hours across all conditions (Fig. 1d). These findings demonstrate that the SLC has the capacity to generate robust cycles of bacterial lysis in our microfluidic devices across a spectrum of environmental fluctuations that is likely to exist in an *in vivo* context.

The emergence of bacterial therapies in synthetic biology has accentuated the need for predictive modeling. This need stems from a bottleneck created by a difference in the timescales for bacterial cloning versus animal experiments; the circuits required for candidate therapies can be created much faster than they can be tested *in vivo*. Therefore, in order to quantitatively characterize the SLC concept before testing in animal models, we developed a computational model (Fig. 2a and Supplementary Information) in order to define an optimal strategy for subsequent testing in a lower-throughput animal model setting. We found that high production and degradation rates of the feedback-controlling proteins resulted in a wider domain of oscillatory dynamics in the parameter space (Fig. 2b). This model is consistent with our observations that oscillations in *S. typhimurium* were more robust than in *E. coli*, where rates of protein production and degradation were previously found to be lower[39] (Extended Data Fig. 1c and Supplementary Video 3). Since the ability to manipulate circuit behavior enhances the versatility of the system, we explored the tunability of the lysis period by adding an *ssrA* degradation tagging sequence on the LuxI protein. Consistent with model predictions, we observed an increased period and colony firing amplitude when tracking bacterial population dynamics (Fig. 2c-d and Extended Data Fig. 1d). The SLC

thus enables tuning of the period and magnitude of delivery, which will be necessary for eventual application of this platform in the complex and fluctuating conditions present *in vivo*.

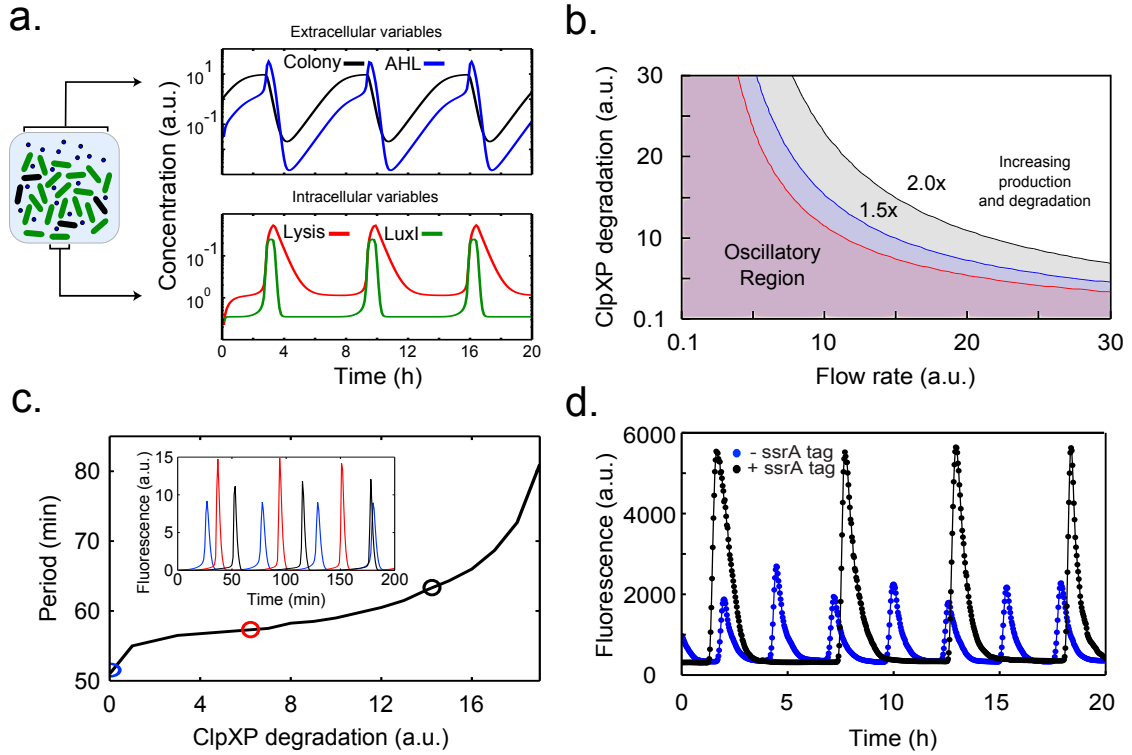


Figure 2.2: Computational modeling and tunability. (a) The model consists of intracellular variables (lysis gene E and LuxI concentrations) and extracellular variables (colony size and AHL concentrations). A time series of colony size (black line), colony AHL (blue line), intracellular LuxI (green line) and lysis protein concentrations (red line) are shown on the right. (b) The region in the model parameter space for clpXP mediated degradation (see Supplementary information) and flow where the model output is oscillatory increases with higher production and degradation terms. (c) Results from the computational model showing the ability to tune the oscillatory period by varying ClpXP mediated degradation of LuxI. (d) Fluorescence profiles showing lysis oscillations for LuxI *ssrA* (black line, Strain 2) and non-*ssrA* (blue line, Strain 1) tagged versions of the circuit. See Supplementary Information for complete model information.

***In vitro* characterization with cancer cells**

To incorporate a cytotoxic payload into the SLC strain, we added expression of Hemolysin E, encoded by *hlyE* of *E. coli*, which has been tested as a pore-forming anti-tumor toxin[40]. We initially confirmed the capability of the circuit to release intracellular contents by visualizing released sfGFP with a small microfluidic sink located beneath the growth chamber (Extended Data Fig. 2a-c). Then, in order to visualize bacterial lysis and killing of cancer cells *in vitro* via HlyE, we engineered a microfluidic device so that cancer cells adhere inside a growth channel that is flanked by smaller bacterial growth chambers, which permits simultaneous single-cell visualization of bacterial lysis and cancer cell death (Extended Data Fig. 2d). After co-culturing human cervical cancer HeLa cells with *S. typhimurium* harboring the SLC circuit, we observed HeLa cell death upon the onset of bacterial lysis, indicating efficient toxin release (Fig. 3a-b, Supplementary Videos 4 and 5). Complete cell death occurred in the growth channel within ~ 111 min of initial sfGFP fluorescence (Fig. 3c). Thus, the SLC bacteria were capable of releasing HlyE at levels necessary to kill tumor-derived cells.

We assessed the toxicity of released SLC or control bacterial contents in batch culture. As anticipated, we found that HeLa cells exposed to supernatant from a culture of the SLC bacteria bearing the *hlyE* module exhibited almost complete loss of viability (Fig. 3d), while the viability of HeLa cells exposed to supernatants of and equivalent dose of non-payload bearing SLC bacteria were only slightly impacted ($\sim 15\%$). We concluded that bacterial lysis allowed for efficient HlyE release *in vitro* and that natural intracellular bacterial contents do not significantly affect HeLa cell viability. We further investigated the delivery characteristics of the SLC bacteria with *hlyE* by seeding variable amounts of circuit-harboring bacteria with HeLa cultures in well plates. We observed that the time to HeLa cell death following initial seeding increased with lower bacterial seeding volumes, presumably resulting from the extended time needed for bacteria to reach the quorum threshold (Fig. 3e and Supplementary Video 6). Initial seeding with a larger volume of bacteria resulted in increased firing rates which corresponded

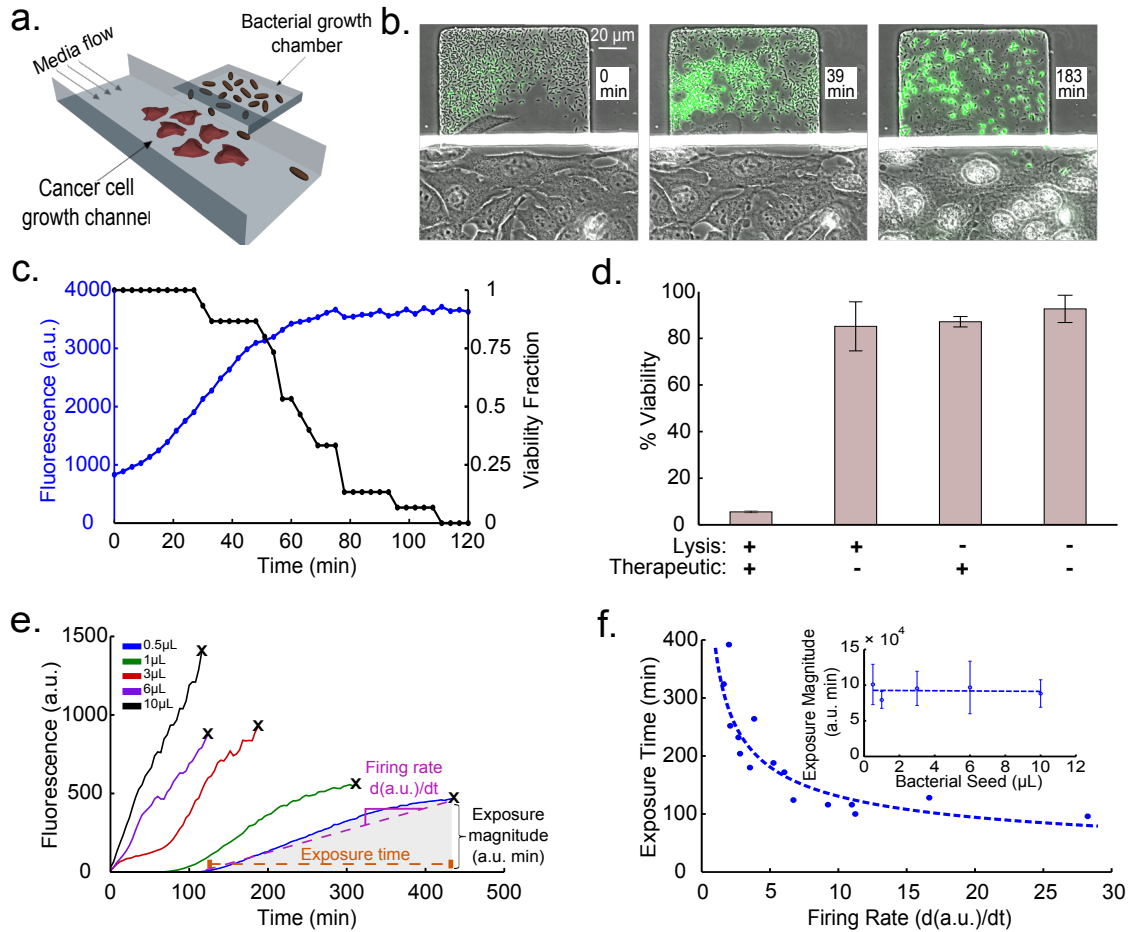


Figure 2.3: *In vitro* co-culture. (a) Schematic of the microfluidic co-culture with cancer cells and bacteria. Fluidic resistance was modified in this chip to achieve stable near-stagnant flow reduction to allow for cancer cell adherence and for diffusion of released therapeutic from the trap to the channel (methods in Supplementary Information). (b) Frames from the co-culture time series sequentially visualizing *S. typhimurium* (Strain 3) ‘firing’, lysis, and HeLa cell death. (c) Fluorescent profile of the bacteria and HeLa cell viability fraction (# live cells / # dead cells in image frames) from (b) with time. (d) % viability of HeLa cells co-cultured with supernatant from *S. typhimurium* culture harboring the SLC + HlyE (Strain 4), the SLC only (Strain 5), constitutive *hlyE* only (Strain 6), or no plasmid (Strain 7). Error bars indicate ± 1 standard error for $n=3$ wells. (e) Fluorescence profile of the SLC + HlyE (Strain 4) co-cultured with HeLa cells at various initial seeding densities. The black ‘x’ marks the point of complete HeLa cell death. (f) The toxin exposure time, measured from the initial presence of fluorescence to HeLa cell death, as a function of the sfGFP production rate (see example in (e)). Although the time to death depends on seeding, the total magnitude of exposure remains conserved (inset). Error bars indicate ± 1 standard error for $n=3$ wells. See Supplementary Information for ELH1301 host strain information.

to shorter HlyE exposure times until cell death, consistent with a greater magnitude of lysis and payload release, although the cumulative toxicity threshold appears to be similar in all cases (Fig. 3f). Based on these observations, one can adjust the seeding size of the bacterial population to determine the initial timing and release characteristics of the circuit.

***In vivo* characterization in hind-flank tumor grafts**

We next used a luciferase reporter to monitor bacterial population dynamics in grafted syngeneic colorectal tumors in mice. To minimize the extent of plasmid loss in the absence of antibiotic selection *in vivo*, we incorporated previously described stabilizing elements for plasmid retention and segregation into the SLC strain[41, 42, 43, 44, 45]. Additionally, we placed both the payload and *luxCDABE* genes (the *in vivo* reporter module) under the *luxI* promoter as an indicator of *hlyE* production and quorum firing via bacterial luminescence (Fig. 1a). Using a subcutaneous model of colorectal cancer (MC26 cell line) in immunocompetent mice, we intratumorally injected a strain of SLC bacteria (SLC-hly). We observed pulsatile bacterial population dynamics within the tumor (Fig. 4a-c, Extended Data Fig. 3a-b) using In-Vivo Imaging (IVIS) technology[46], consistent with the design and *in vitro* characterization (Fig. 2). Importantly, the end luminescence intensity was on average ~ 300 -fold lower than the constitutive control strain, indicating a significant decrease in bacterial population levels within the tumor (Extended Data Fig. 3c).

Given the ability to engineer bacterial population dynamics in tumor grafts, we next leveraged the versatility of the SLC bacteria as a delivery system to compare different classes of previously developed payloads. In addition to the hemolysin strain that was characterized in microfluidic devices, we created two additional SLC strains expressing genes to activate a host immune response (via T-cell and dendritic cell recruitment, using mCCL21) or trigger tumor cell apoptosis (using CDD-iRGD)[47, 48]. Upon intratumoral injection, the immune recruitment strain elicited the strongest ef-

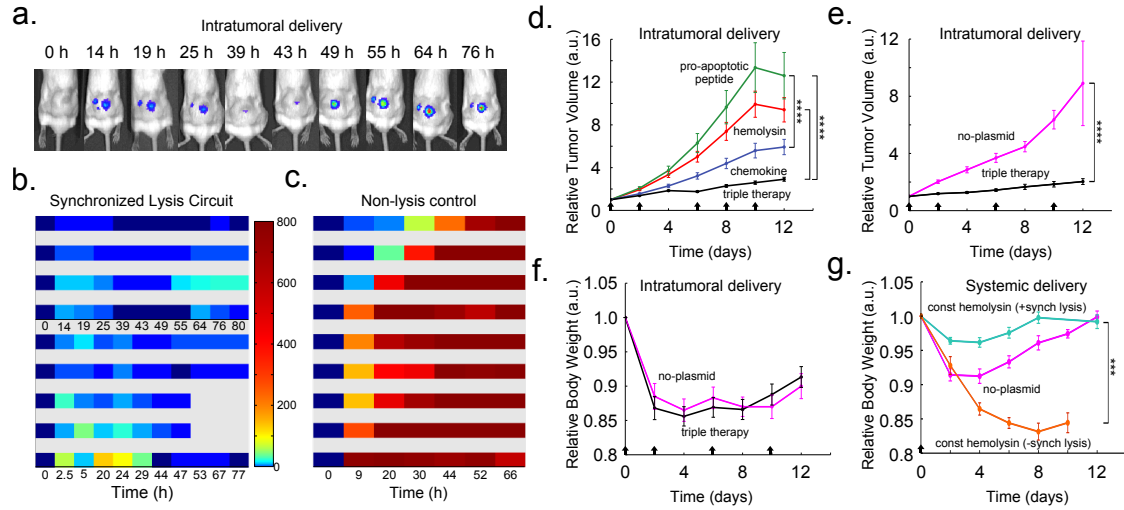


Figure 2.4: *In vivo* bacterial dynamics, tumor impact, and tolerability in a subcutaneous tumor model. (a) IVIS imaging over time of a mouse bearing two hind flank tumors injected once with the stabilized SLC-hly strain (Strain 8). (b) Single tumor density map trajectories of bacterial luminescence (relative to luminescence at 0h) for the SLC-hly strain (Strain 8). Data for each axis represents separate experiments. (c) Single tumor density map trajectories of bacterial luminescence for the genomically integrated constitutively luminescent strain (Strain 9). Intratumoral injection resulted in over 35-fold higher post-injection luminescence compared to intravenous injection (Extended Data Fig. 3d). (d) Average relative tumor volume over time for subcutaneous tumor bearing mice injected with SLC-hly (red line, Strain 10), SLC-cdd (green line, Strain 14), SLC-ccl21 (blue line, Strain 15), and all together (SLC-3) (black line). Bacteria were injected intratumorally on days 0, 2, 6, 8, and 10 (black arrows) (**** $P < 0.0001$, two-way ANOVA with Bonferroni post test, $n = 14 - 17$ tumors, s.e.). (e) Average relative tumor volume over time for mice with subcutaneous tumors injected with the SLC-3 strains (black line, Strain 10, 14, and 15) and the no-plasmid control (magenta line, Strain 7). Bacteria were injected intratumorally on days 0, 2, 6, and 10 (black arrows) (**** $P < 0.0001$, two-way ANOVA with Bonferroni post test, $n = 18 - 19$ tumors, s.e.). (f) Average relative body weight over time for mice with subcutaneous tumors injected with the SLC-3 strains (black line, Strain 10, 14, and 15) and the no-plasmid control (magenta line, Strain 7). Bacteria were injected intratumorally on days 0, 2, 6, and 10 (black arrows) ($n=10$ mice for both cases, s.e.). (g) Average relative body weight over time for subcutaneous tumor-bearing mice with a single intravenous injection of the SLC + constitutive hlyE (turquoise line, $n=9$ mice, Strain 11), a non-SLC strain with constitutive hlyE (orange line, $n=5$ mice, Strain 12), or the no-plasmid control strain (magenta line, $n=9$ mice, Strain 7) (*** $P < 0.001$, two-way ANOVA with Bonferroni post test, s.e.).

fect on tumor growth when compared to the hemolysis or apoptotic strains (Fig. 4d). We observed that an equal mixture of the three strains generated a stronger response than any single strain (Fig. 4d and Extended Data Fig.3 e-g), and on this basis we elected to pursue the “triple-strain” dose for further testing in order to minimize animal usage. In a side by side comparison, we observed that the tumor response to SLC triple-strain (SLC-3) injections was significantly larger than the response to unmodified bacteria (Fig. 4e). Additionally, upon necropsy, histopathological analysis of remnant tumors was performed for mice treated with the SLC-3 strains, chemotherapy, or unmodified bacteria. In mice treated with SLC-3 and non-circuit bacterial strains, robust staining of bacteria was observed by anti-Salmonella antibodies, showing localization of Salmonella within tumors. TUNEL staining indicated higher levels of apoptosis and cell death in SLC-3 treated tumors (Extended Data Fig. 4).

As a first step towards monitoring the effect of bacterial injections on the host, we compared how the triple-strain system affected body weight when administered intratumorally and intravenously, since the administration route affects bacterial localization (Extended Data Fig. 3d). We found that treatment with the SLC strains generated the same weight change as unmodified bacteria when administered intratumorally (Fig. 4f). However, intravenous administration of the SLC conferred a greater health benefit based on observations that SLC strains producing constitutive therapy were better tolerated than unmodified bacteria or non-SLC strains producing constitutive therapy (Fig. 4g). While further targeted studies are required to systematically explore the impact of these bacteria on host health, these preliminary experiments suggest that the SLC design can reduce the burden of bacterial injections.

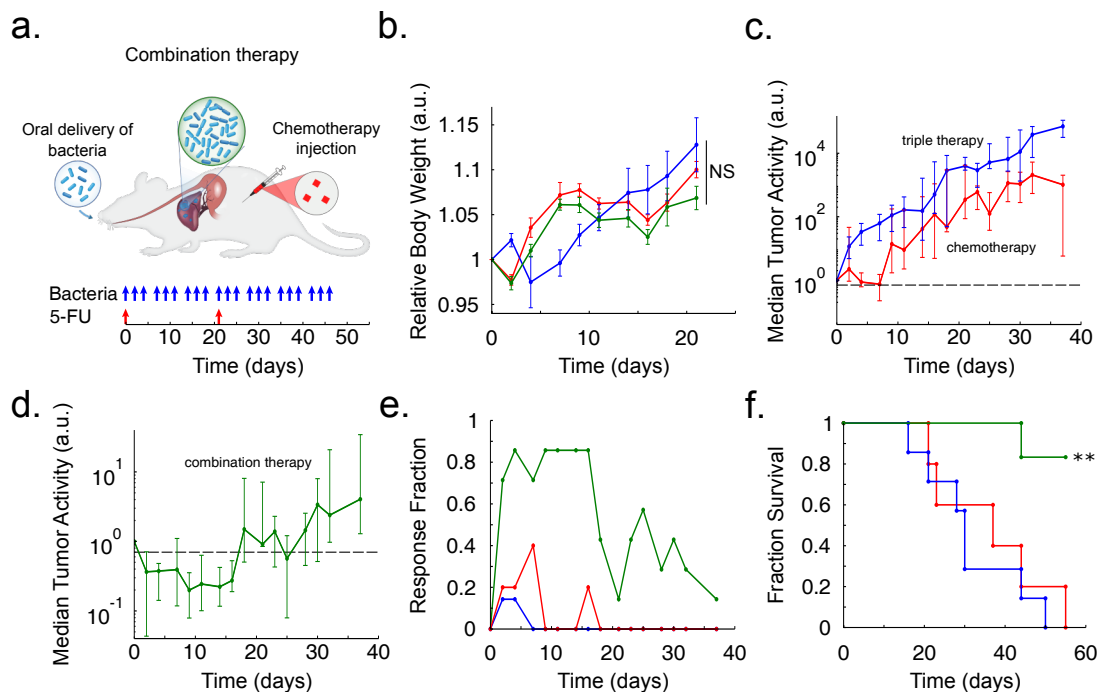


Figure 2.5: *In vivo* testing in an experimental model of colorectal metastases in the liver via oral delivery of bacteria. (a) Schematic of the experimental syngeneic transplantation model of hepatic colorectal metastases in a mouse, with the dosing schedule of either engineered bacteria (SLC-3) or a common cytotoxic chemotherapeutic, the antimetabolite 5-FU. The SLC-3 strains were delivered orally while 5-FU was delivered via intraperitoneal injection. (b) Relative body weight over time for the mice with hepatic colorectal metastases fed with the SLC-3 strains (blue line), injected with 5-FU chemotherapy (red line), or a combination of the two (green line). Error bars indicate ± 1 standard error for 5 - 7 mice. (c) Median relative tumor activity, measured via tumor cell luminescence using IVIS imaging, for the chemotherapy and SLC-3 cases from (b). (d) Median relative tumor activity for the combination therapy case from (b). Error bars for (c) and (d) indicate the interquartile ranges for 5 - 7 mice. The dashed line marks relative tumor activity of 0.70. (e) Fraction of mice from the cases in (b) which respond with 30% reduction of tumor activity over time. (f) Fraction survival over time for the mice in (b) (** $P < 0.01$, log rank test; $n = 5 - 7$ mice).

***In vivo* characterization in a transplantation model of colorectal metastases**

To explore a proof-of-principle for the application of our circuit in the context of *in vivo* tumors, we examined the efficacy of our system in an experimental syngeneic transplantation model of colorectal metastases within the liver. We had previously established that oral delivery of these bacterial strains led to safe and efficient colonization of hepatic colorectal metastases (see Methods), and that mice tolerated repeated dosing without overt adverse effects (Fig. 5a-b)[45]. In the context of bacteria-based therapeutic candidates, previous studies have shown that anaerobic bacteria can occupy avascular tumor compartments where chemotherapy is thought to be ineffective due to poor drug delivery[34]. Thus a synergistic effect may arise when bacteria are used to deliver drugs to the necrotic core of a tumor, while standard chemotherapy is used for the vascularized regions[34, 49]. Inspired by this paradigm, we tested the combination of SLC-3 bacteria with a common clinical chemotherapy (5-fluorouracil, 5-FU). Tumors exhibited similar growth trajectories in response to repeated oral delivery of either the bacterial therapy alone, or two i.v. doses of 5-FU on Days 0 and 21 (Fig. 5c). In contrast, combination of these two applications led to a marked decrease in tumor activity over a period of 18 days, followed by a return to growth (Fig. 5d). During the initial 18-day period, a large fraction of the tumors were scored as eliciting at least a 30% reduction in tumor activity (Fig. 5e). The overall response led to roughly a 50% increase in the mean survival time for animals harboring incurable colorectal metastases (Fig. 5f). Improvements may arise from strategies for long term circuit stability or the utilization of additional therapeutic cargo.

Conclusion

The synchronized lysis circuit exemplifies a methodology for leveraging the tools of synthetic biology to exploit the ability of certain bacteria to colonize disease

sites. In contrast to most drug delivery strategies, the synchronized lysis paradigm does not require pre-loading of a drug or the engineering of additional secretion machinery. In addition, it has the potential to decrease the likelihood of a systemic inflammatory response through population control; since the bacterial colony is pruned after each oscillatory lysis event, the design could mitigate an undesirable host response. The circuit may enable new bacterial drug delivery strategies through modulation of the frequency and amplitude of the population cycles over time.

Along these lines, cyclic drug release may have broader implications, given recent insights into the effects of circadian rhythms on host-microbial interactions and metabolic disorders[50, 51]. Such engineering strategies may allow for the development of therapeutic communities within *in vivo* environments, where population dynamics are driven by interacting viruses, bacteria, and host immune cells[52].

Extended data figures

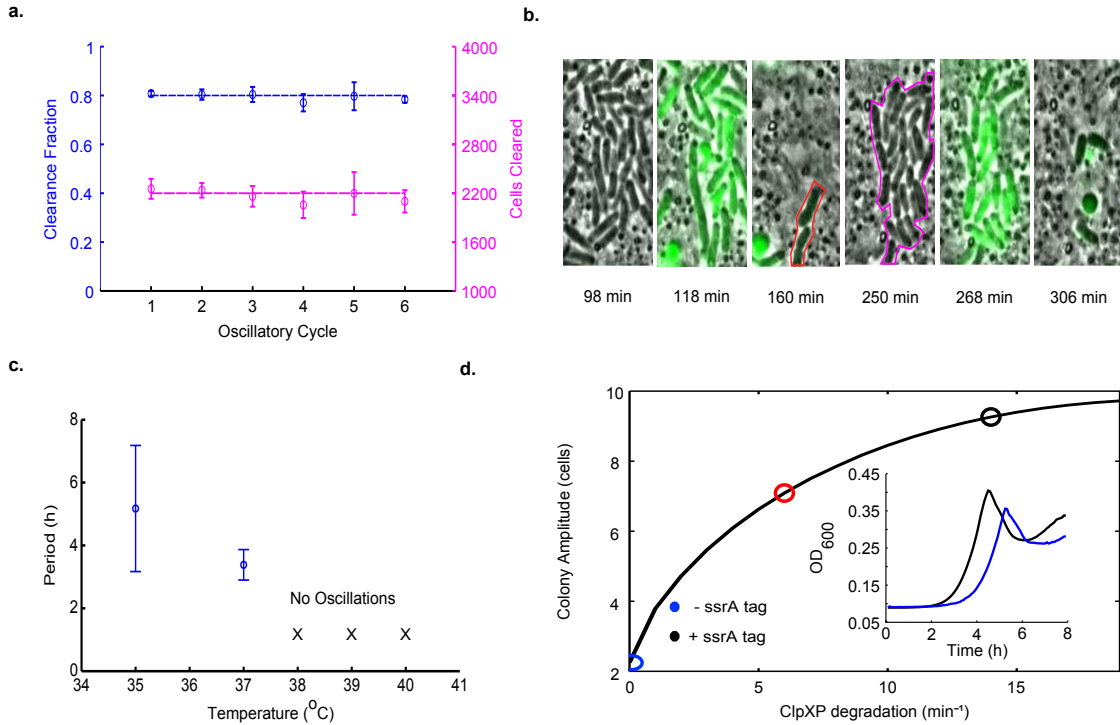


Figure 2.6: Various properties of the SLC. (a) The fraction and number of bacterial cells cleared per consecutive oscillatory cycle in the growth chamber for a typical microfluidic experiment for *S. typhimurium*, including the effects of lysis and flow of cells outside of the trap (Strain 1). (b) Subset of time series images from the experiment in (a) showing a portion of the growth chamber where survivors of the initial lysis event (160 min frame, red outline) produce progeny (250 min frame, magenta outline) which are lysis sensitive. (c) Period as a function of the environmental temperature for *E. coli* (Strain 13). The circuit does not oscillate for temperatures above 37°C in *E. coli*. Error bars indicate ± 1 standard deviation for 12 - 19 peaks. (d) Colony amplitude at quorum firing for increasing degradation on the LuxI activator protein in the computational model. These simulation results are supported by batch well-plate experiments of the LuxI ssrA (black line, Strain 2) and non-ssrA (blue line, Strain 1) tagged versions of the circuit in *S. typhimurium* (inset).

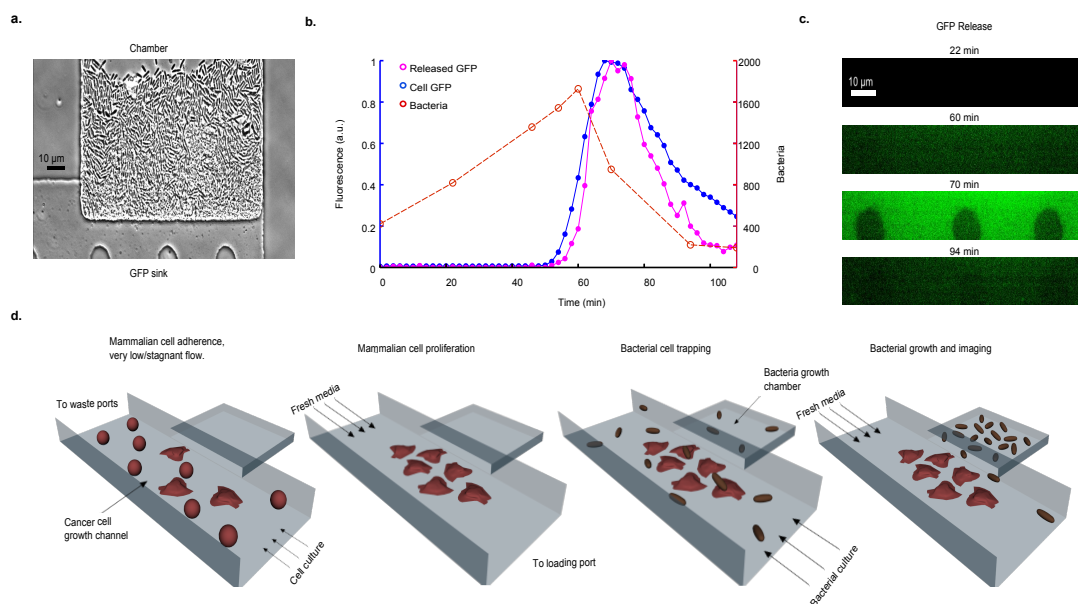


Figure 2.7: Investigating lysis mediated intracellular release. (a) A bacterial growth chamber with a $0.4\mu\text{m}$ sink for sfGFP visualization after release. (b) Number of bacteria (red line), bacterial fluorescence (blue line), sink fluorescence (pink line) for a typical oscillatory cycle (Strain 1). (c) Fluorescence time series images of the microfluidic sink from (b). (d) General procedure for performing bacterial and cancer cell co-culture experiments in a microfluidic device (also see Supplementary Information)

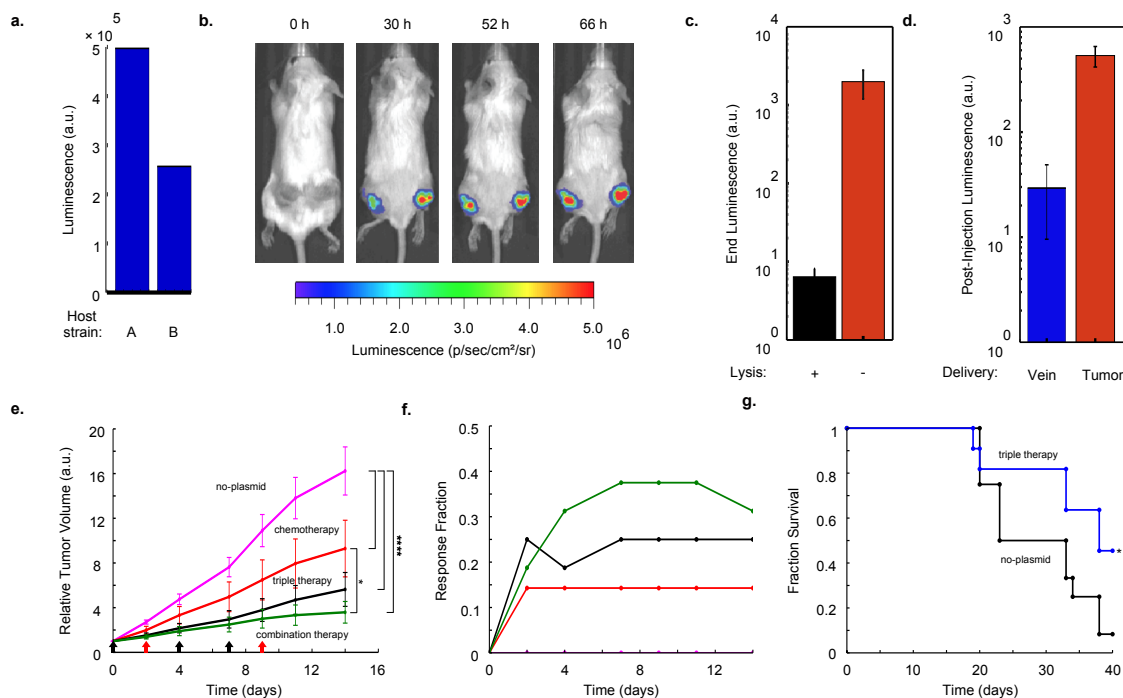


Figure 2.8: *In vivo* expression and therapy testing. (a) End-point *in vitro* luminescence intensity for SLC strains after ~ 20 h of growth. Host strains A and B are the host bacteria for Strains 8 and 10. They are ELH1301 and ELH 430, respectively. Host A exhibits ~ 2 -fold higher luminescence with the same circuit than Host B. (b) IVIS imaging over time of a mouse bearing subcutaneous tumors injected with a genomically integrated constitutively luminescent strain (Strain 9). (c) End-point *in vivo* bacterial luminescence of the SLC-hly strain and the constitutively luminescent strain from the experiments presented in Fig. 4. Error bars represent the standard error of the mean bacterial luminescence from 9 tumors. (d) Post-injection *in vivo* bacterial luminescence for the constitutively luminescent strain administered intravenously (vein) or intratumorally (tumor). Luminescence was measured ~ 20 h post-injection. Error bars represent the standard error of the mean bacterial luminescence from 6 and 9 tumors for the intravenous and intratumoral cases, respectively. (e) Average relative tumor volume over time for subcutaneous tumor bearing mice injected with the no-plasmid bacterium (Strain 7), 5-FU chemotherapy, the SLC-3 strains, and the combination of SLC-3 with chemotherapy. Bacteria were injected intratumorally on days 0, 4, and 7 (black arrows), and chemotherapy was administered on days 2 and 9 (red arrows) (*P < 0.05, ****P < 0.0001, two-way ANOVA with Bonferroni post test, n = 12 - 16 tumors, s.e.). (f) Fraction of mice from the cases in (e) which respond with 30% reduction of tumor volume over time. (g) Fraction survival over time for mice with hepatic colorectal metastases fed with either the SLC-3 strains (blue line) or the no-plasmid control (black line) (*P < 0.05, log rank test; n = 11 - 12 mice).

a.

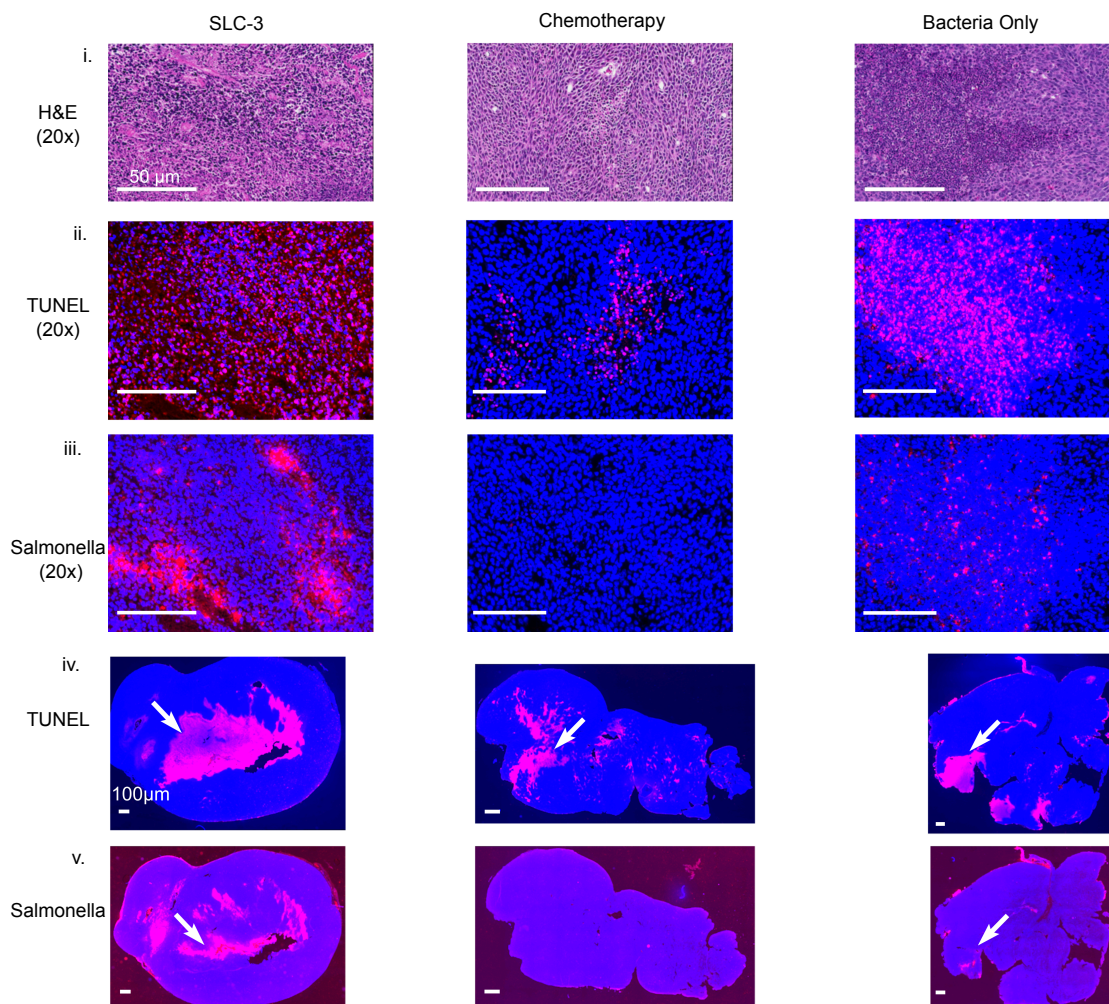


Figure 2.9: (a) Histology of tumor sections taken from mice with different treatments 3 days post administration: (i) H and E staining for tissue sections intravenously injected with a combination of therapeutic bacteria (SLC-3), chemotherapy (5-FU), or a bacteria control with no therapeutic (Strain 7). (ii) TUNEL staining (red) in the same sections indicating cell apoptosis. (iii) Salmonella immunohistochemistry (red) in the same sections confirming presence of bacteria in tumors. Scale bars for (i), (ii), and (iii) denote $50\mu\text{m}$. (iv) and (v) TUNEL and Salmonella staining (red) in the entire tumor sections (examples indicated by arrows). Scale bars for (iv) and (v) denote $100\mu\text{m}$. DAPI staining (blue) was used to obtain a measure of live and dead cells in (ii) - (iv). Histology slices ($n=6$) from 20x images were compared across the groups and mean intensity of TUNEL staining, normalized by sample area, was demonstrated to be significantly higher for SLC-3 compared to the other two groups ($P<0.0001$, one-way ANOVA), and not significantly different between the chemotherapy and bacteria only cases.

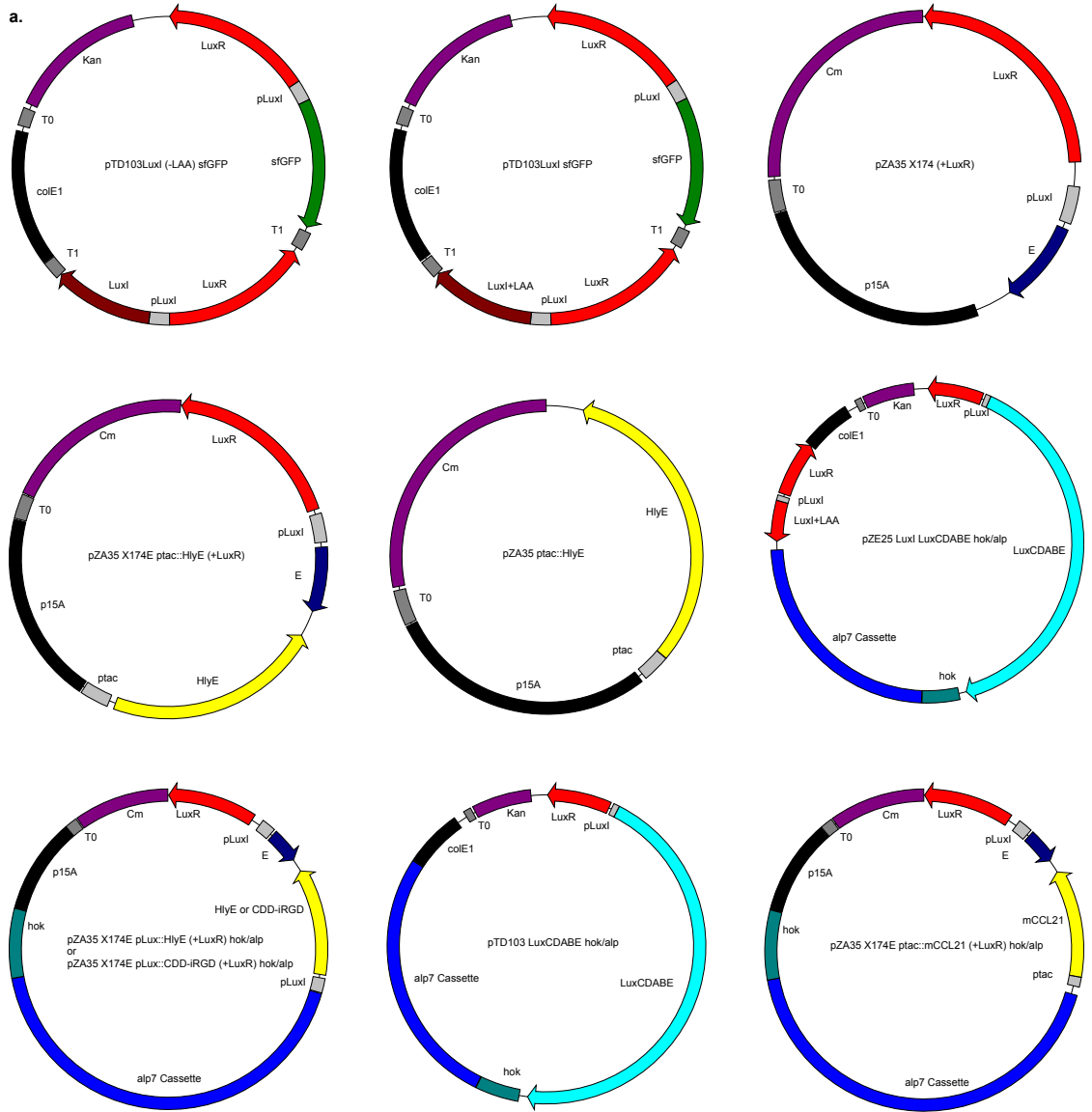


Figure 2.10: Shown are the main plasmids used in this study.

Table 2.1: A list of strains and respective plasmids used in this study.

Strain #	Strain Name	Host Bacterium	Plasmid(s)
1	MOD47	SL1344, M913	pTD103 luxI (-LAA) sfGFP + pZA35 X714E (+LuxR)
2	MOD46a	SL1344, M913	pTD103 luxI sfGFP + pZA35 X714E (+LuxR)
3	MOD67	SL1344, M913	pTD103 luxI (-LAA) sfGFP + pZA35 X714E (+LuxR) ptac::HlyE
4	MOD61	SL1344, ELH1301	pTD103 luxI sfGFP + pZA35 X714E (+LuxR) ptac::HlyE
5	MOD64	SL1344, ELH1301	pTD103 luxI sfGFP + pZA35 X714E (+LuxR)
6	MOD65	SL1344, ELH1301	pZA35 ptac::HlyE
7	ELH1301	SL1344, ELH1301	N/A
8	MOD105	SL1344, ELH430	pZE25 luxI luxCDABE hok/alp + pZA35 X714E (+LuxR) pLux::HlyE hok/alp
9	EcN-luxCDABE	Nissle 1917	N/A
10	MOD101	SL1344, ELH1301	pZE25 luxI luxCDABE hok/alp + pZA35 X714E (+LuxR) pLux::HlyE hok/alp
11	MOD102	SL1344, ELH1301	pZE25 luxI luxCDABE hok/alp + pZA35 X714E (+LuxR) ptac::HlyE hok/alp
12	MOD69	SL1344, ELH1301	pTD103 LuxCDABE hok/alp + pZA35 X714E (+LuxR) ptac::HlyE hok/alp
13	MOD29	JS006, BW25113	pTD103 luxI sfGFP + pZA35 X714E (+LuxR)
14	MOD110	SL1344, ELH1301	pZE25 luxI luxCDABE hok/alp + pZA35 X714E (+LuxR) pLux::CDD-iRGD hok/alp
15	MOD112	SL1344, ELH1301	pZE25 luxI luxCDABE hok/alp + pZA35 X714E (+LuxR) ptac::mCCL21 hok/alp

Methods

Strains and Plasmids

Our circuit strains were cultured in LB media with $50 \mu\text{g ml}^{-1}$ and $34 \mu\text{g ml}^{-1}$ of Kanamycin and Chloramphenicol respectively, along with 0.2% glucose, in a 37°C incubator. Mammalian cells (HeLa CCL-2 from ATCC, verified by third party cell line authentication services using an STR multiplex system) were cultured in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (CellGro 30-002-CI), placed inside a tissue culture incubator at 37°C maintained at 5% CO_2 . Plasmids were constructed using the CPEC method of cloning or using standard restriction digest/ligation cloning. The activator plasmid (Kan, ColE1) was used in previous work from our group, while the lysis plasmid was constructed by taking the lysis gene, *E*, from the ePop plasmid via PCR and cloning it into a vector (Chlor, p15A) under the control of the LuxI promoter[36, 38]. The *hlyE* gene was taken via PCR from the

genomic DNA of MG1655, while mCCL21 (mouse CCL21) and CDD-iRGD were synthesized. These genes were cloned into the lysis plasmid, under the control of either the ptac or pLuxI promoters (Extended Data Fig. 5). Co-culturing was performed with HeLa cells and either motile or non-motile *S. typhimurium*, SL1344 (Extended Data Table 1). For full strain and plasmid information, please refer to the Supplementary Information.

Microfluidics, Microscopy, and Well Plate experiments

The microfluidic devices and experiment preparation protocols used in this study are similar to those previously reported from our group[36]. The bacteria growth chambers were 100x100 μm in area and approximately 1.4 μm in height. For co-culture experiments on the chip, we first loaded a suspended culture of HeLa cells in the device media channels at very low flow rates, to allow for adherence, and then incubated the device in a tissue culture incubator for 0.5 - 2 days to allow for proliferation. On the day of the experiment, the device was transferred to the microscope and circuit-containing bacteria were loaded in the growth chambers before imaging. Acquisition of images was performed with a Nikon TI2 using a Photometrics CoolSnap cooled CCD camera. The scope and accessories were programmed using the Nikon Elements software.

Co-cultures for well plate experiments were performed in Falcon 96 well tissue culture plates. HeLa cells were allowed to adhere to the wells before the addition of bacteria and subsequent imaging under the microscope or measurement in a TECAN Infinite M200 Pro plate reader. For viability measurements using an MTT assay, there were two technical replicates per well. For fluorescence measurements of co-cultures with variable seeding densities of bacteria, there were three technical replicates per case.

Additional details on microfluidics, microscopy, and well plate experiments can be found in the Supplementary Information.

In vivo Experiments

All animal work was approved by the committee on animal care (MIT, protocol 0414-022-17). The cell line (MC26-LucF, Tanabe lab, Massachusetts General Hospital) was obtained from, and authenticated by, the Tanabe lab, MGH. The cell line was tested several times to be mycoplasma free before implantation in mice. Sample sizes for mice were determined by expected effect size to produce a power of 0.8-0.9. Mice were blindly randomized into various groups using a random number generator.

Subcutaneous Tumor Model: Animal experiments were performed on 6 week old female BALB/c mice (Taconic Biosciences, Inc.) with bilateral subcutaneous hind flank tumors from an implanted mouse colon cancer cell line. The concentration for implantation of the tumor cells was $1e8 \text{ cells ml}^{-1}$ in DMEM (no phenol red). Cells were then implanted subcutaneously at a volume of $100 \mu\text{L}$ per flank, with each implant consisting of $1e7$ cells. Tumors were typically grown to an average of 300 mm^3 before experiments.

Experimental Liver Metastasis Model: The experimental metastasis model was generated by injecting luciferase-producing mouse cancer cells into surgically externalized spleens of immunocompetent mice. Tumor cells seeded the liver during 90 seconds after which the spleen was removed to prevent ectopic tumor growth[53]. The MC26-LucF cell line was used (Tanabe Lab, MGH) and injected at $5e4 \text{ cells}/100 \text{ uL}$ PBS into the spleens of female Balb/c mice 6 weeks of age (Taconic Biosciences, Inc.). For the liver metastasis model, tumors were grown for 5-7 days to an average total tumor burden of 143 mm^3 before experiments.

Bacterial Growth and Administration: Bacterial strains were grown overnight in LB media containing appropriate antibiotics and 0.2% glucose as for the *in vitro* experiments. A 1/100x dilution in fresh media with antibiotics was started the day of injection and grown until an $\text{OD} < 0.1$ to prevent bacteria from reaching the quorum threshold (for SLC specifically). Bacteria were spun down and washed 2-3x with sterile PBS before injection into mice. Intratumoral injections of bacteria were performed at

a concentration of 5×10^7 cells ml^{-1} in PBS with a total volume of 10-20 μL injected per tumor, while intravenous injections were given at a total volume of 100 μL . For the SLC-3 strains injection, this final volume was equally divided between the three strains at the indicated density. For liver metastasis experiments, bacteria were grown in LB media containing appropriate antibiotics and 0.2% glucose until they reached an OD of 0.05, after which they were concentrated to $1-5 \times 10^9$ bacteria/mL and delivered via oral gavage.

Post-Administration Monitoring for Subcutaneous Liver Metastasis Models: Luminescent signal was measured with the IVIS Spectrum *in vivo* imaging system following bacterial injection. Measurements were compared relative to pre-injection values to follow dynamics, with two biological replicates for the SLC experiment. Subcutaneous tumor volume was quantified using calipers to measure the length, width, and height of each tumor throughout the imaging course ($V=L \times W \times H$). Volumes were compared to pre-injection values to follow physical tumor growth. Survival of mice was measured as the time from the beginning of the experiment up to the day when mice were moribund and euthenized. Survival for the experiment in Fig. 4f was measured with two biological replicates.

Statistical Analysis: Statistical tests were calculated either in Microsoft Excel (Student's t-test) or GraphPad Prism 5.0 (ANOVA with Bonferroni post test, Log-rank test). The details of the statistical tests carried out are indicated in respective figure legends. Where data were approximately normally distributed, values were compared using either a Student's t-test or one-way ANOVA for single variable, or a two-way ANOVA for two variables. Mice were randomized in different groups before experiments.

Supplementary Information

Host Strains and Culturing

Strains with our lysis circuit were grown in LB media with $50 \mu\text{g ml}^{-1}$ and $34 \mu\text{g ml}^{-1}$ of the respective antibiotics (kanamycin and chloramphenicol) along with 0.2% glucose in a 37°C shaking incubator. The glucose was added in order to decrease expression from the Lux promoters. There is a binding site for the CAP-cAMP activating complex located between the *luxI* and *luxR* promoters, which is believed to activate transcription of the *luxR* promoter[54]. When glucose levels in the cell are high, levels of cAMP are low, decreasing the transcriptional activation from CAP-cAMP.

For the microfluidics experiments we selected a non-motile *S. typhimurium* host for our circuit, SL1344 (M913: *fliGHI* mutant)[55]. For well-plate co-culture experiments and *in vivo* experiments, we utilized an attenuated *S. typhimurium* host, SL1344 (ELH1301: $\Delta\text{phoPQ } \Delta\text{aroA}$)[56], that was shown in previous work to have good efficiency in plasmid retention[44]. For *in vivo* experiments where the luminescence expression of the circuit was measured, we utilized another attenuated strain of *S. typhimurium* SL1344 as the circuit host (ELH430: ΔphoPQ) where luminescence expression was higher (see Extended Data Fig. 3a). For the constitutive luminescence case we used a well characterized bacterium with a constitutive luminescence cassette, *E. coli* Nissle 1917 integrated with p16Slux[57]. A list of strains and plasmids are shown in Extended Data Table 1.

For co-culture experiments, we used the HeLa cell line in DMEM (complete medium) supplemented with 10% fetal bovine serum and appropriate antibiotics for the circuit. HeLa cells were initially grown with penicillin/streptomycin (CellGro 30-002-CI) in the growth medium and placed inside a tissue culture incubator at 37°C with 5% CO_2 before the experiment. For loading on a microfluidic chip or well-plate, HeLa cells were initially washed with dPBS and dis-adhered with 0.05% or 0.25% trypsin EDTA. Cells were then pelleted and resuspended in DMEM + FBS and the appropriate

antibiotics.

Plasmids

Our circuit is composed of two plasmids, an activator plasmid and a lysis/therapeutic plasmid. The main activator plasmid is pTD103LuxI sfGFP which was used in previous work from our group[36]. This plasmid contains the *ssrA*-LAA degradation tag (amino-acid sequence of AANDENYALAA) on LuxI and sfGFP, a superfolding green fluorescent protein variant[58]. pTD103LuxI (-LAA) was constructed by removing the *ssrA*-LAA tag from LuxI. Most of the construction was done using the CPEC method of cloning[59].

The lysis plasmids were constructed using the modular pZ plasmid set-up with a p15a origin of replication and a chloramphenicol resistance marker[60]. The lysis gene, E from the bacteriophage ϕ X174, was kindly provided by Lingchong You and was taken from the previously reported ePop plasmid via PCR[38]. The E gene was placed under the expression of the LuxR-AHL activatable *luxI* promoter. In order to construct the therapeutic versions of the lysis plasmids, we extracted the *hlyE* gene from the genomic DNA of *E. coli* strain MG1655 via PCR and inserted it into the lysis plasmid. The promoter used to drive expression of HlyE was the *tac* promoter for *in vitro* characterization, and the *luxI* promoter for *in vivo* testing. The CDD-iRGD and mCCL21 genes were synthesized as fragments before inserting into the appropriate plasmids.

For the plasmids used in the circuit strains tested *in vivo*, we inserted two stabilizing elements, the *hok/sok* system and *alp7* partitioning system, into the activator and lysis/therapeutic plasmids. Recent work has shown that addition of the *hok/sok* toxin-antitoxin system and the *alp7AR* cassette from the *B. subtilis* plasmid pLS20 enables plasmid retention *in vivo*[41, 42]. See Extended Data Fig. 5 for maps of the the plasmids used in this study.

Microscopy and Microfluidics

The microscopy and microfluidics techniques described here are similar to those reported previously from our group[35]. Briefly, our microfluidic devices were constructed from PDMS (polydimethylsiloxane) which was molded and baked on a silicon wafer with micron-scale features formed by cross-linked photoresist. Individual devices (formed by a set of features transferred from the wafer to the PDMS) were then cut out of the baked PDMS and holes were punctured in the devices to allow for the connection of fluid lines. The devices were then bonded onto coverslips and placed on a microscope stage for cell loading and imaging. Fluid lines were connected to the devices from various syringes supplying media, cells, or acting as waste reservoirs. The flow direction in the device was controlled by changing the relative heights between the relevant syringes resulting in hydrostatic pressure driven flow.

We cultured cells to an optical density (A_{600nm}) of approximately 0.1 (using 1.5 ml cuvettes from Plastibrand) before loading a device. Before loading, devices were ‘wetted’ with the media syringes to remove bubbles in the channels[61]. Devices were loaded from the cell port by lowering the designated waste port such that the relative changes in height of the cell loading syringe and waste syringe resulted in flow of cells from the cell port to a waste port. Once cells were loaded in the traps, the flow direction was reversed allowing media to flow into all ports, thus supplying the trapped cells with a continuous perfusion of nutrients. In these microfluidic experiments we added 0.075% Tween20 to the media and cell suspension fluid to prevent cells from adhering to channels and ports within the device. Experiments for characterizing circuit behavior in Fig. 1-2 were done in a side-trap array device, as described previously[35]. The device used with the GFP sink is also arranged as a side-trap array. See Extended Data Fig. 2a for a schematic of the trap and sink.

For the co-culture experiments on the chip, we utilized the side-trap array device and added fluidic resistance on all of the inlet/outlet ports with sinuous channels to

increase the dynamic range of achievable flow rates. For mammalian cell loading, cells were trypsinized, pelleted, and resuspended in 0.5 - 1.0 mL DMEM+FBS+antibiotics (Kanamycin and Chloramphenicol) before loading. Cells were loaded under a light microscope such that un-adhered cells were localized in the media channels under near-stagnant flow conditions. The microfluidic chip and syringe apparatus was then carefully placed in the CO₂ incubator while avoiding any changes in the relative heights of the syringes to maintain near-stagnant flow. The cells were allowed to adhere within 2 - 4 hours before slightly raising the media syringe to supply fresh media to the channels, and then left to proliferate overnight. On the next day, the chip and syringe apparatus was transferred to the microscope under a temperature and CO₂ environmental chamber as described previously[62]. The bacterial culture was then prepared and loaded as described above before imaging. If flow rate was too high, it was lowered when the bacterial population reached the quorum threshold (indicated by the appearance of sfGFP) to allow for better diffusion of the released therapeutic into the mammalian growth channel. For a schematic of the main steps involved, refer to Extended Data Fig. 2d.

For microscopy we used the same system as described in our previous work[36]. Briefly, we used a Nikon Eclipse TI epifluorescent microscope with phase-contrast based imaging. For the acquisition of images, we used a CoolSNAP HQ2 CCD camera from Photometrics. The microscope and acquisition was controlled by the Nikon Elements software. A plexiglass incubation chamber connected to a heating unit, which encompassed a wide area around the stage, was used in order to maintain the temperature of the microfluidic device. Phase-contrast images were taken at 60x magnification at 50-200ms exposure times. Fluorescent imaging at 60x was performed at 150ms for GFP, 30% setting on the Lumencor SOLA light source. Images were taken every 2 - 3 minutes for the course of a typical experiment. In order to estimate the flow rate in the device channel, we measured the length of traces of fluorescent beads (1.0 μm) upon 200ms exposure of fluorescent light to estimate the average fluid velocity. Further information on the analysis of these images is presented in the Data Analysis section below.

Well-plate Experiments

For the viability experiments, we seeded a monolayer of HeLa cells on standard tissue culture 96-well flat bottom plates (Fisher Scientific) with penicillin and streptomycin antibiotics. We grew the four bacterial strains from Fig. 3d in 50 mL cultures to an optical density of 0.08 before pelleting and re-suspending in 1.2 mL media with the appropriate antibiotics. The bacterial cultures were then grown for one hour and then pelleted in a 1.5 mL micro-centrifuge tube. 100 μ L of each resulting supernatant was then added to three HeLa culture wells. Thereafter we implemented the protocol for the Vybrant MTT Cell Proliferation Assay Kit (V-13154, Molecular Probes) to measure HeLa cell viability.

For the variable seeding experiments (Fig. 3e-f), we also utilized the same well-plates. We grew the SLC + HlyE strain to an optical density of ~ 0.07 before pelleting the cells and re-suspending in 1 mL of fresh media and antibiotics. Variable volumes of this dense culture were then seeded to three wells for each respective case, and imaging was performed at 20X magnification.

Data Analysis

Fluorescence intensity profiles were obtained by analyzing frames from the fluorescent channel and plotting the mean pixel intensity over time. The period measured is the peak-to-peak period of the fluorescence profile. The number of cells in the trap was found by analyzing the phase-contrast images in ImageJ. Since bacteria formed a monolayer in the growth chamber, we first estimated the average area of an individual bacterial cell and the average void fraction (open space between bacteria in the trap). Taking into account the $\mu\text{m}/\text{pixels}$ of the image, we measured the area of the trap taken up by cells using ImageJ and divided by the average area of a bacterial cell. This value was then multiplied by (1 - void fraction) to yield the total estimated number of cells in the trap. Bacteria that were not close to the main group of cells were counted individually and added to the final number. The fraction of cells lysed per period was estimated

by dividing the number of lysed bacteria by the maximum number of bacteria before lysis. Plots were generated by using MATLAB.

In order to estimate the flowrate in the media channel, we imaged fluorescent 1 μm beads at 20X and analyzed the images using MATLAB. The length of the fluorescent bead traces was measured in pixels and converted to microns. The length of the trace was then divided by the exposure time (200 ms) to yield the flow velocity. In this study we report the median flow velocity because it is less sensitive to outliers in the bead traces.

Modeling

To describe the dynamic behavior of the SLC we developed an ordinary differential equation model where the populations dynamics track the intracellular concentrations of the variables LuxI (I) and the lysis protein E (L). The population variables are cell population number (N) and extra-cellular AHL (H) (we assume LuxR-AHL binding is fast). This model can be thought of as a system where we follow a surviving lineage (via a single cell) throughout the experiment which responds to extracellular AHL, which in turn increases with cell number. Once the extracellular AHL threshold is reached, the intracellular production of the Lux driven genes, LuxI and E, are brought to the ON state (second stable state), due to the positive feedback provided by the activation term P_{lux} . Increase in the lysis protein leads to a rapid reduction of cell number via the killing term, γ_N , modeled as a hill function to switch killing ON/OFF based on the concentration of lysis protein. Once AHL and lysis protein levels decay, P_{lux} and γ_N turn back to their OFF stable states, allowing cell number to rise and repeat the process. Since firing is dependent on a threshold value of AHL, the SLC can be thought of as a circuit displaying integrate-and-fire behavior.

Maximum cell population (N_0) is defined by the maximum number of cells that could fit inside a single cell trap. Cells leave the trap as the consequence of cell growth (μ_G). The rate of cell degradation through lysis is described by hill function γ_N . We assumed AHL diffusion through the cell membrane to be fast, allowing for dynamic description of total AHL (H) in the trap. Production of AHL (H) is proportional to the product of cell population (N) and per-cell concentration of LuxI (I). AHL dilution is taken to be inversely proportional to N due to the increased blockage of AHL clearance from the trap as the result of cell accumulation. Internal production of LuxI and lysis proteins is described by P_{lux} . Degradation of both proteins is due to cell growth (μ_G) as well as some basal degradation (γ and γ_L). In addition, LuxI is further degraded by ClpXP machinery (γ_C). ClpXP-mediated degradation is used as a proxy for temperature

since it is affected by changes in temperature[63].

$$\frac{dN}{dt} = \mu_G N(N_0 - N) - \gamma_N N \quad (2.1)$$

$$\frac{dH}{dt} = bNI - \frac{\mu H}{1 + N/N_0} \quad (2.2)$$

$$\frac{dL}{dt} = C_L P_{lux} - \gamma_L L - \mu_G L \quad (2.3)$$

$$\frac{dI}{dt} = C_I P_{lux} - \gamma_I I - \mu_G I - \gamma_C I \quad (2.4)$$

$$P_{lux} = \alpha_0 + \frac{\alpha_H (H/H_0)^4}{1 + (H/H_0)^4}$$

$$\gamma_N = \frac{kL^n}{L_0^n + L^n}$$

Parameters:

We chose model parameters to qualitatively fit the experimental population and GFP (proxy for LuxI) trajectories (Fig 2a). Increased protein production and degradation in *S. typhimurium* as compared to *E. coli* was reported in previous work[39]. We find that the production and degradation terms in our model (μ , LuxI production and basal degradation rates: C_L , C_I , γ_L , γ_I) account for the qualitative differences in behavior between *E. coli* and *S. typhimurium* (Fig. 2b).

Model parameter values:

μ_G (Dilution due to cell growth) 0.2; N_0 (Maximum cell population size) 10; k (Maximum rate of cell lysis) 10; L_0 (Conc. of lysis gene resulting in half maximum lysis) 2; n (Hill coefficient of lysis function) 2; b (AHL production rate) 25; μ (Maximum AHL clearance rate due to flow) 12; C_L (Lysis gene copy number) 0.5; C_I (LuxI copy number) 1; α_0 (Lux promoter basal production) 0.5; α_H (Lux promoter AHL induced production) 35; H_0 (AHL binding affinity to Lux promoter) 5; γ_L (Basal degradation of lysis protein) 2; γ_I (Basal degradation of LuxI) 2; γ_C (ClpXP degradation of LuxI) 12.

Supplementary Videos

Supplementary Video 1. This video shows timelapse fluorescence microscopy of the Synchronized Lysis Circuit (SLC) in Strain 1 (*S. typhimurium*, no *ssrA* tag on *LuxI*) at 60X magnification. Images were taken every 2 min at the bottom portion of a 100x100 μ m chamber.

Supplementary Video 2. This video shows timelapse fluorescence microscopy of the SLC in Strain 2 (*S. typhimurium*, *ssrA* tag on *LuxI*) at 60X magnification. We observe a longer lysis period with a higher degradation efficiency on *LuxI*. The chamber size is 100x100 μ m and images were taken every 2 min.

Supplementary Video 3. This video shows timelapse fluorescence microscopy of the SLC in Strain 13 (*E. coli*) at 60X magnification. The chamber size is 100x100 μ m and images were taken every 2 min.

Supplementary Video 4. Video of bacteria and cancer cell co-culture on a microfluidic device at 60X magnification. Strain 3 (non-motile *S. typhimurium*, SLC with HlyE) was loaded in the growth chambers while HeLa cells grow in the main channel of the device. Timelapse fluorescence microscopy images were taken every 3 min.

Supplementary Video 5. Second video of bacteria and cancer cell co-culture on a microfluidic device at 60X magnification. Strain 3 (non-motile *S. typhimurium*, SLC with HlyE) was loaded in the growth chambers while HeLa cells grow in the main channel of the device. Timelapse fluorescence microscopy images were taken every 3 min.

Supplementary Video 6. Video of bacteria and cancer cell co-culture in a tissue culture well-plate at 20X magnification. Strain 4 (motile *S. typhimurium*, SLC with HlyE) was loaded in the well with a monolayer HeLa cells. Timelapse fluorescence microscopy images were taken every 1 min.

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Chapter 3

A stabilized microbial ecosystem of self-limiting bacteria using synthetic quorum-regulated lysis

Introduction

Microbial ecologists are increasingly turning to small, synthesized ecosystems[64, 65, 66, 67, 68] as a reductionist tool to probe the complexity of native microbiomes[69, 70]. Concurrently, synthetic biologists have gone from single-cell gene circuits[5, 4, 71, 72] to controlling whole populations using intercellular signaling[27, 73, 35, 74, 75]. The intersection of these fields is giving rise to new approaches in waste recycling,[76] industrial fermentation[77], bioremediation[78], and human health[79, 75]. These applications share a common challenge[70] well known in classical ecology[80, 81]; stability of an ecosystem cannot arise without mechanisms that prohibit the faster growing species from eliminating the slower. Here, we combine orthogonal quorum sensing systems and a population control circuit with diverse self-limiting growth dynamics in order to engineer two ‘ortholysis’ circuits capable of maintaining a stable co-culture of metabolically competitive strains in microfluidic devices. While no successful co-

cultures are observed in a two-strain ecology without synthetic population control, the 'ortholysis' design dramatically increases the co-culture rate from 0% to approximately 80%. Agent-based and deterministic modeling reveal that our system can be adjusted to yield different dynamics, including phase-shifted, anti-phase or synchronized oscillations as well as stable steady-state population densities. The 'ortholysis' approach establishes a paradigm for constructing synthetic ecologies by developing stable communities of competitive microbes without the need for engineered codependency.

SLC dynamics and identification of orthogonal signaling systems

In order to engineer a stable co-culture of two competitive bacterial strains, we first characterized the dynamics of a small library of quorum sensing (QS) components (Supplementary Fig. 1a-c). This was achieved by evaluating different components of natural quorum sensing systems to identify receptor-promoter pairs and signals (AHL) that yield the desired characteristic upon combination (Supplementary Fig. 1d)[82]. From a range of possible configurations (Supplementary Fig. 2b), we identified that the Lux and Las systems were suitable for one-way orthogonal signaling, and the Lux and Rpa systems were suitable for two-way orthogonal signaling (Supplementary Fig. 1e-g). We used these components to design synchronized lysis circuits (SLCs)[75] in two bacterial strains, whereby each strain is programmed to lyse upon reaching a critical population density.

To understand how an ecosystem harboring the synchronized lysis circuit (SLC) can be altered, we established the range of possible self-limiting dynamics of the circuit (Fig. 1a-b). The circuit exhibits oscillations, characterized by periodic lysis events, which are driven by the activation of the Lux-controlled positive feedback loop upon reaching a quorum threshold of AHL, as was seen in earlier work[75]. A lysis event reduces the population dramatically, and a few survivors resume the process starting again

below the quorum threshold. In microfluidic devices, the fluorescent protein sfGFP reports the activation state of the circuit in this oscillatory state (Fig. 1c, Supplementary Video 1). We also discovered a constant lysis state that is characterized by a steady-state in which growth and lysis are approximately balanced, and the stable ON state of the circuit is evidenced by the constant production of sfGFP (Fig. 1d, Supplementary Video 2). Tuning the degradation efficiency of the activator LuxI by changing its *ssrA* degradation tag, we demonstrated a bifurcation in lysis dynamics of the population between these two states. In a deterministic model of the circuit (Fig. 1b), lower α_q corresponds to stronger enzymatic degradation of LuxI (see Methods section for details). Consistently, the oscillatory lysis behavior was observed for the highest level of activator degradation (Fig. 1e-f), dampened oscillations were observed at a lower level of degradation (Fig. 1g-h), and constant lysis behavior was observed for the lowest levels of degradation (Fig. 1i-j). The SLC therefore exhibited two main modes of dynamics lysis with respect to changes in circuit parameters.

Construction and characterization of a dual strain ecosystem with orthogonal SLC signaling

To build a synthetic ecosystem of two orthogonal SLC strains, we used the previously built circuit based on the Lux quorum sensing system and constructed a new circuit with the Rpa system. The Rpa system had RpaR in place of LuxR and an *ssrA* tagged RpaI in place of LuxI (Fig. 2a). These strains are called Lux-CFP and Rpa-GFP, respectively, for convenience. Both strains' gene expression is controlled by the *PluxI* promoter for consistency, considering pC-bound RpaR can activate *PluxI* at about 90% the efficiency of AHL-bound LuxR (Supplementary Fig. 2b).[82] Although these strains are in the same bacterial host, when started from equal densities in batch culture, Rpa-GFP shows a significant growth advantage over Lux-CFP (Fig. 2b). Because of this growth advantage, a 1:1 mixture of these strains in a batch culture (with or without the

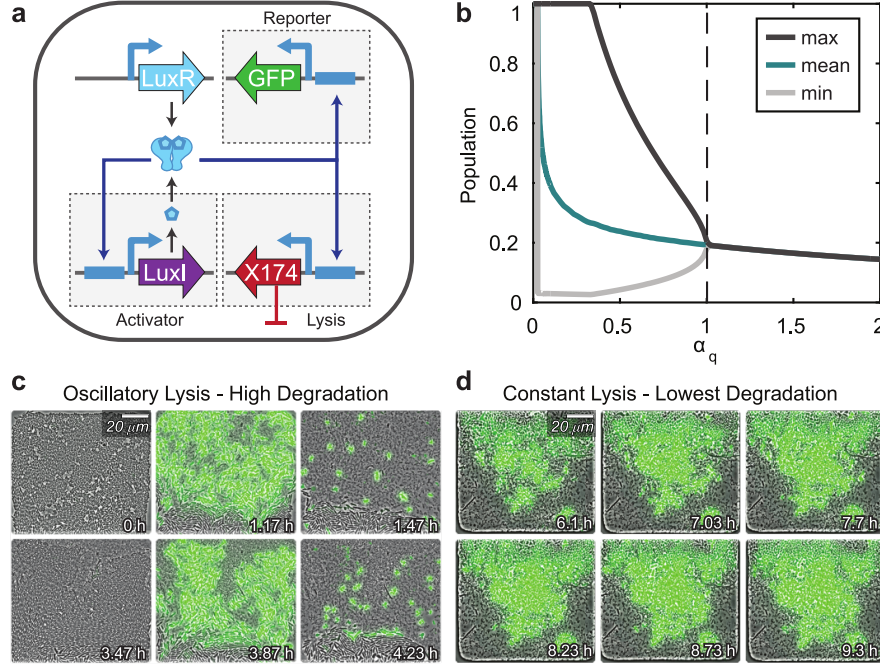


Figure 3.1: Experimental investigation into the space of population dynamics of a self-communicating synchronized lysis circuit. **a**, Genetic diagram of the synchronized lysis circuit (SLC). The circuit contains a lysis plasmid and an activator/reporter plasmid. Transient production of LuxI eventually leads to an accumulation of AHL above the quorum threshold needed to activate LuxR which begins a positive feedback loop by driving transcription off the PluxI promoters that control production of LuxI, GFP, and the lysis gene Φ X174E. LuxR in this system is driven by the native luxR promoter. **b**, Bifurcation to oscillations in deterministic model of the lysis circuit. Ignoring initial transient behavior, minimum, maximum, and mean population density over time were determined for each parameter value. Lower α_q corresponds to stronger degradation (see Methods section for details). **c**, Video stills showing bacteria harboring the SLC with strong degradation of the LuxI activator (LuxI-LAA) exhibiting oscillations in a microfluidic growth chamber. Oscillations result from repeated cycles of growth, quorum threshold reached, and self-limitation by lysis activation. **d**, Video stills depicting bacteria harboring the SLC with weaker degradation of LuxI (LuxI with no degradation tag) exhibiting constant lysis. Constant activation of the lysis circuit results in the continual activation of GFP as well as continuous growth and lysis events within the microfluidic chamber. **e-j**, Changing certain properties, such as the degradation tag of the SLC will result in different population dynamics. **e-f**, Fluorescence (green) and population (black) over time for cells harboring the SLC with $\alpha_q=0.4$ for the deterministic model (**e**) and LAA-tagged LuxI for a typical experimental run (**f**), as seen in **c**. **g-h**, Fluorescence (green) and population (black) over time for cells harboring the SLC with $\alpha_q=1.1$ for the deterministic model (**g**) and TS-LAA-tagged LuxI for a typical experimental run (**h**). **i-j**, Fluorescence (green) and population (black) over time for cells harboring the SLC with $\alpha_q=2$ for the deterministic model (**i**) and untagged LuxI for a typical experimental run (**j**), as seen in **d**. The video stills **c-d**, as well as the fluorescence and population profiles in **f**, **h**, and **j** are all representative of typical microfluidic experiments with these strains.

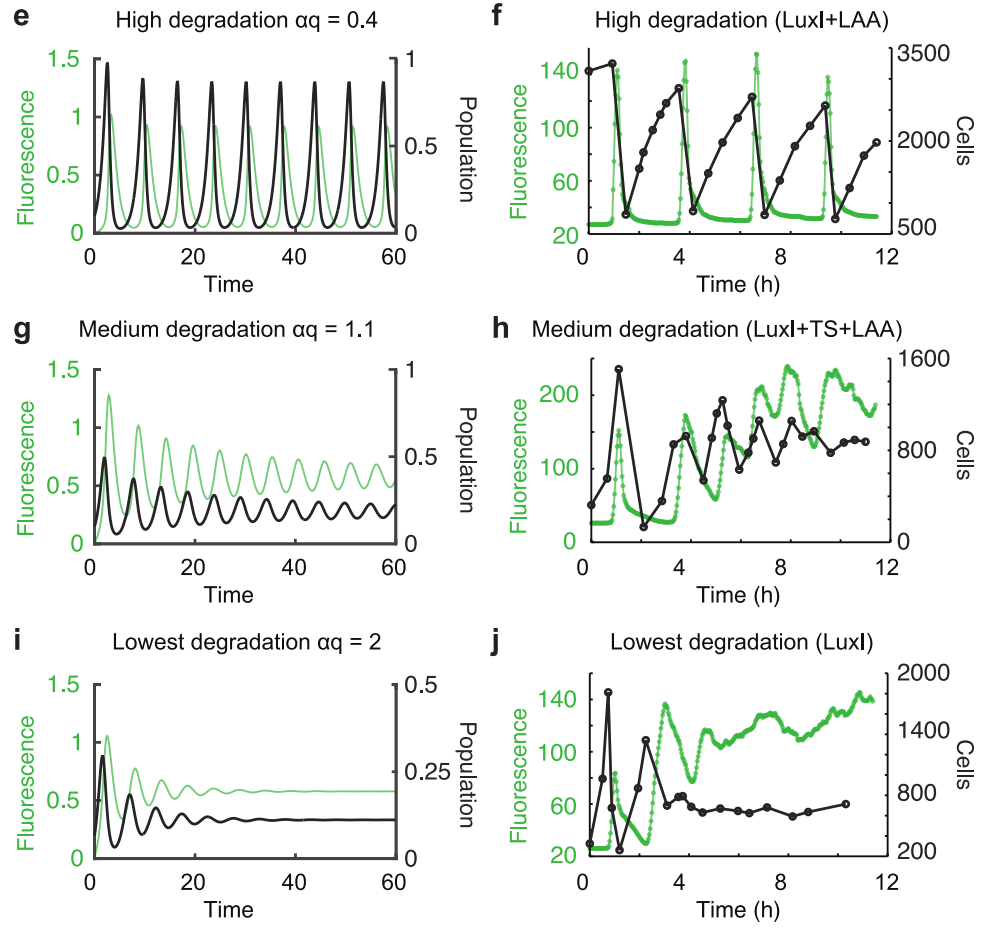


Figure 3.1: Experimental investigation into the space of population dynamics of a self-communicating synchronized lysis circuit (Continued).

lysis gene), is primarily taken over by the faster growing Rpa-GFP strain by the time the strains reach stationary phase (Fig. 2c). However, if the slower growing Lux-CFP strain is enriched 100x more than the green strain, the population stabilizing effects of the lysis circuit becomes evident. Without the lysis gene, the mixture is taken over by the Lux-CFP strain, however with the lysis gene, the population ratio over the initial 10 hours keeps close to a 1:1 ratio. The ‘ortholysis’ strategy thus showed promise in batch co-culture.

We then grew the strains in microfluidic devices, with a seeding ratio of 1:10 (Rpa-GFP to Lux-CFP) optimized for the new system, in order to examine the long-term dynamics of the co-culture. The microfluidic trap (growth chamber) harboring the two strains without the lysis gene quickly lost its co-culture and was taken over by the Rpa-GFP strain alone (Fig. 2d and Supplementary Video 3). This process was observed for 60 traps, and the time duration of the co-culture was measured over two days. All traps eventually lost their co-culture completely, with an average co-residence time of 6.5 hours (Fig. 2h). However, when the two orthogonal lysis strains were grown together, most of the 60 traps maintained a co-culture for the duration of the two-day experiment (Fig. 2e and Supplementary Video 4); all traps that lost co-culture were completely taken over by the Rpa-GFP strain. Due to differences in the inherent parameters of the two quorum sensing systems, the Rpa-GFP circuit remains in the constant lysis regime and is therefore perpetually producing sfGFP. However, the Lux-CFP strain is in the oscillatory regime and remains dark until it reaches quorum threshold and its lysis events are characterized by a punctuated burst of CFP production (Fig. 2g and Supplementary Fig. 3a-d). The bimodality of the co-residence time (either lost in the first couple hours, or maintained through the end of the experiment) suggests the small volume of these reactors, and the non-deterministic loading conditions, predisposes some wells with very few Lux-CFP cells to stochastic loss of co-culture. Seemingly, depending on the environmental context, oscillatory strains are more susceptible to environmental perturbations than a strain in the constant lysis regime. However, the benefit of using a strain in the oscillatory lysis regime is that it leaves the possibility of engi-

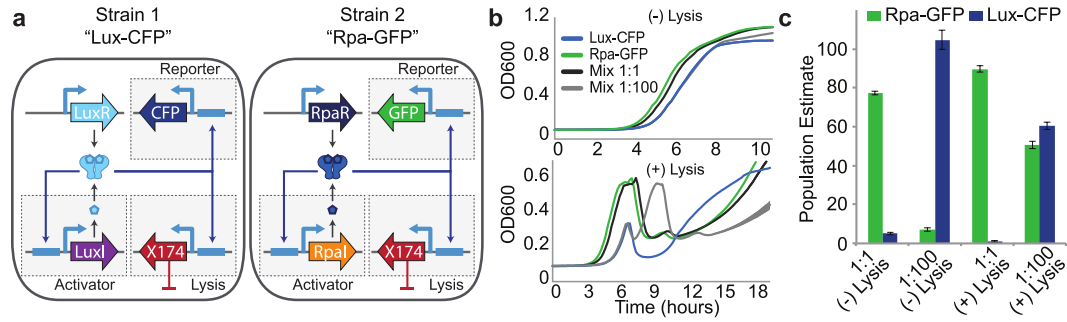


Figure 3.2: Experimental demonstration of long-term co-culture of competitive species with unequal growth rates using signal orthogonal self-lysis. **a**, Genetic diagram of a two-strain ecosystem of self-lysing *Salmonella* constructed with two signal orthogonal quorum sensing systems, *rpa* and *lux*. **b**, Batch culture growth curves of the Lux-CFP strain alone (blue), Rpa-GFP strain alone (green), a 1:1 mixture (black), and a 1:100 (Rpa-GFP:Lux-CFP) mixture (gray), both without the lysis gene (top) and with the lysis gene (bottom). All strains were started from the same diluted density and under the same growth conditions. Width of lines represent s.d. ($n=3$) **c**, Batch culture population estimates of Lux-CFP and Rpa-GFP co-cultures. Rpa-GFP population estimated as GFP fluorescence (integrated over the full length of the experiment) of the mixture normalized by the time-integrated GFP fluorescence of Rpa-GFP cells alone. Lux-CFP population estimated as CFP fluorescence (integrated over the full length of the experiment) of the mixture normalized by the time-integrated CFP fluorescence Lux-CFP cells alone. Error bars represent s.d. ($n=3$) **d**, Video stills of a representative co-culture of non-lysing Lux-CFP and Rpa-GFP strains showing the eventual takeover by the green strain. **e**, Video stills of a representative co-culture of the Lux-CFP and Rpa-GFP strains with the lysis plasmid. The addition of the lysis plasmid prevents either strain from taking over for the duration of the experiment. **f**, Time trace of the GFP and CFP Fluorescence of the trap in the video shown in **d**. **g**, Time trace of the GFP and CFP Fluorescence of the trap in the video shown in **e**. **h**, Graph showing the length of co-culture for each of the sixty traps containing the non-lysing strains. **i**, Graph showing the length of co-culture for each of the sixty traps containing the strains with the lysis plasmids.

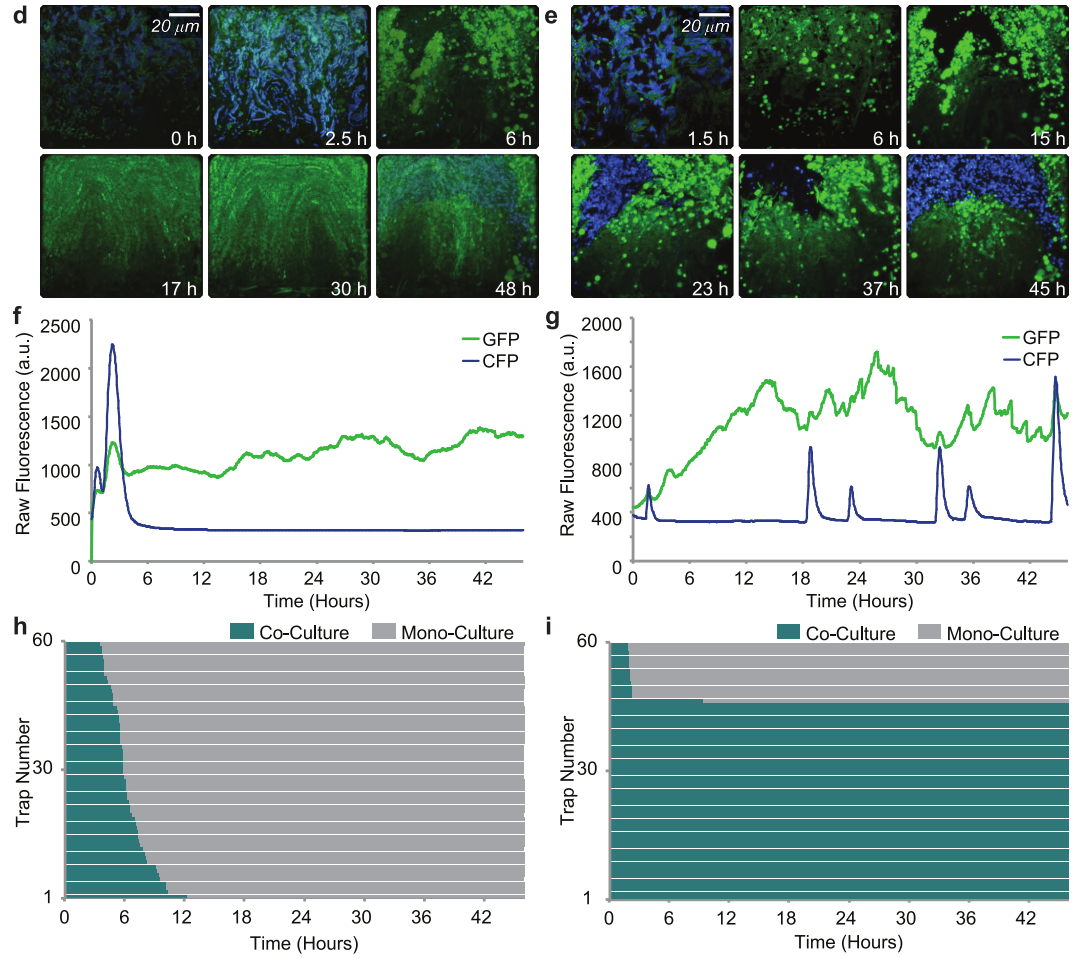


Figure 3.2: Experimental demonstration of long-term co-culture of competitive species with unequal growth rates using signal orthogonal self-lysis (Continued).

neering dynamic population profiles which may be useful for certain applications like timed delivery of two different payloads. Nevertheless, within our microfluidic device, the 'ortholysis' method is rather robust at co-culturing even competitive strains for long periods of time (Fig. 2i).

Agent based modeling of the dual strain SLC system

We used agent-based modeling to visually show how the 'ortholysis' strains might behave with different quorum sensing parameters. We first modeled a system where the quorum sensing parameters of the Rpa system were the same as the Lux system parameters used in previous studies[75]. However, we used the experimental difference in growth whereby the Rpa-GFP strain grows at 110% the rate of the Lux-CFP strain. With the Lux-CFP strain seeded in a 10:1 ratio to the Rpa-GFP strain in the model simulation, the resulting dynamics show anti-phase oscillations (Fig. 3a and Supplementary Video 5). Seemingly due to volume exclusion, as shown by their fluorescence time series, the populations enter an anti-phase pattern where the strains switch off growing and lysing (Fig. 3c).

We then took into consideration the innate differences between the two quorum sensing systems[82] by changing several of the Rpa-GFP strain's quorum sensing parameters in relation to the Lux parameters used. Furthermore, based on observed phenotypic phenomenon the probability of lysing was reduced by 10-fold which allows more AHL to build up and a constant lysis dynamic to develop (Fig. 3b and Supplementary Video 6). The resulting dynamics were similar to the experimental observations, with a constantly lysing Rpa-GFP strain maintaining the majority of the population share, and the Lux-CFP strain intermittently firing and lysing (Fig. 3d). In order to understand how these dynamics and the size of the growth container affect stability, the agent-based model was run many times under different conditions. For conditions where Lux-CFP is oscillating and Rpa-GFP is in constant lysis (lys/osc) or where both are oscillating (osc/osc), ten simulations were done in volumes of 20, 40 and 60 each. As the size of

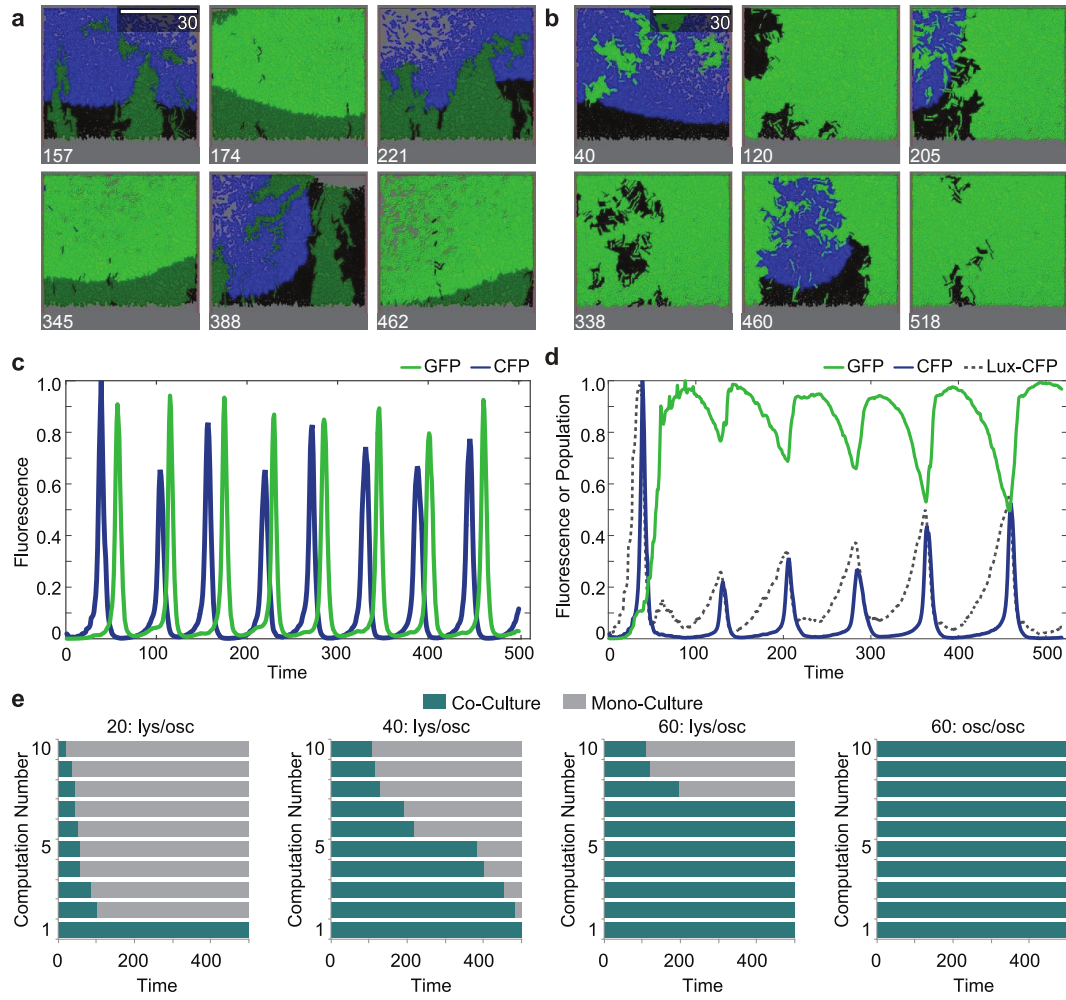


Figure 3.3: Agent-Based model elucidating experimental dynamics. **a**, Video stills of a representative, virtual co-culture of two self-lysing strains both in the oscillatory regime of the lysis circuit in a simulated trap of size 60. Scale bar at top, right of micrograph indicates half of the size of the trap. Number at the bottom of the micrographs indicate iteration "Time". **b**, Video Stills of representative, model-generated video recreating experimental dynamics. Number at the bottom of the micrographs indicate iteration "Time". **c**, Time trace of the GFP (green) and CFP (blue) "Fluorescence" of the trap in **a** over time. **d**, Time trace of the GFP (green) and CFP (blue) "Fluorescence" of the trap in **b**, as well as population of the "Lux-CFP" strain (black, dashed line). **e**, From left to right: (1) green in constant lysis phase, blue in the oscillatory phase in a trap with size 20. (2) green in constant lysis phase, blue in the oscillatory phase in a trap with size 40. (3) green in constant lysis phase, blue in the oscillatory phase in a trap with size 60. Video in **B** is in this size trap with these lysing conditions. (4) Both strains in oscillatory phase with trap size 60. Video in **a** is in this size trap with these lysing conditions.

the space increases, so does the average residence time of the co-culture (Fig. 3e), suggesting that, as we expected, larger traps will have fewer issues with losing co-culture to stochastic events.

Deterministic modeling of the dual strain SLC system

As evidenced by the agent-based model, our strains demonstrate only one particular dynamic of a wide-range of possibilities facilitated by quorum sensing controlled self-lysing microbes with varying levels of orthogonality. We developed a reduced deterministic model to explore a wider space of possible dynamics achieved through differences in growth rates, QS systems, and lysis circuit regimes. For each case, communication motifs are distinguished and suitable experimental candidate QS systems are chosen to achieve the displayed dynamic. For the two individual lysis circuits, we consider either Non-lysing (no SLC), Lysing (SLC), or Weak Lysing (less effective SLC). With two non-lysing strains, the faster growing strain will eventually dominate the population (Fig. 4a). However, even a single strain equipped with the SLC can stabilize the co-culture, provided the non-lysing strain has the lower growth rate (Fig. 4b). In cases where both strains harbor an SLC, but there is one-way cross-talk, the strain that responds to both signals becomes entrained to the strain that only responds to its own (Fig. 4c-d). An example would be the Lux and Las systems, the Lux can respond to the Las signal, but Las is orthogonal to the Lux signal. The strength of the cross-talk determines the strength and delay of the entrainment, with strong cross-talk (Fig. 4c) exhibiting strong entrainment, and weak cross-talk (Fig. 4d) showing time-delayed entrainment. In cases where each SLC operates independently, by using signal orthogonal QS systems, the most robust co-culturing is achieved where for large ranges of growth rates, the time-averaged population ratio remains around 50/50 (Fig. 4e). If one of the strains exhibits weaker lysing dynamics, in that it has a lower probability of lysing given a quorum threshold, we get dynamics similar to those observed in our experimental system (Fig. 2g and Fig. 4f). As seen in the experiment, the Rpa-GFP strain inhabits most

of the space, with blue periodically displacing it until it reaches quorum and self-limits its population. This dynamic, as with the dynamics of each set-up, offers a distinct advantage for certain purposes. For example, a system requiring a constant production of a particular chemical and periodic bursts of a second chemical could appropriate the set-up in Fig. 4f to its advantage.

Conclusion

Synthetic biologists have used lysis to control populations before[27], but not until recently have populations been engineered to dynamically control their own population without exogenous input[75]. Since our system relies on DNA parts carried on plasmids, undesired mutations may arise which can hinder the function of the circuit. Bacteria may mutate toxic or burdensome genes, and any possible mutants may gain a selective advantage over non-mutated members of the population. In this regard, strategies to enhance stability of the circuit components inside the host cells would be necessary to ensure long term robustness of the synthetic ecosystem[83]. Additionally, in the absence of antibiotics, bacteria would encounter a selective pressure to lose the circuit plasmids. This would be problematic when introducing the synthetic ecosystem to an environment without any selective agents. Possible ways this could be addressed are by either integrating circuit components within the genome or using plasmid-stabilizing elements in the circuit. Elements such as toxin/antitoxin systems and actin-like protein partitioning systems have previously been shown to stabilize plasmids in environments without antibiotics[45]. The emergence of escapees is a direct consequence of strong selection imposed by periodic lysis, and recent evidence also suggests that repeated pruning of a population suppresses beneficial mutations that confer growth advantages unrelated to the lysis circuit[84]. Therefore, the ortholysis strategy might be an attractive methodology to impose certain population dynamics or types of selection in evolution experiments.

The challenge in maintaining a population of metabolically competitive micro-

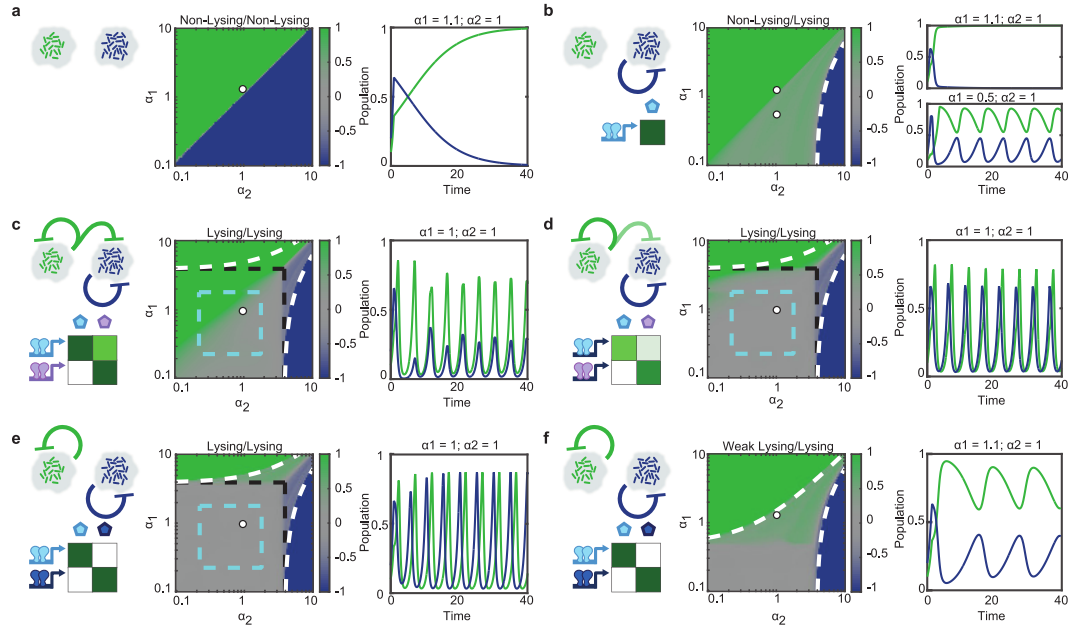


Figure 3.4: Prediction of synchronized lysis circuit dynamics in a dual strain population using various communication motifs. Model-generated heat maps depicting time-averaged population ratio of green and blue strains in a well-mixed, constant flow co-culture, as function of green's growth rate α_1 against blue's growth rate α_2 . Each panel has a particular combination of lysis regimes for each of the strains, either Non-Lysing, Lysing, or Weak Lysing (see Methods Summary for details). On the left of each heat-map is the communication motif it exhibits and experimental candidate QS systems to achieve the desired signaling characteristic. These traits determine the behavior and composition of the co-culture. The white dot on the heat map indicates the growth rate parameters selected for the time-series plots. Time series plots show population of the green and blue strains as a function of time. **a**, Two non-lysing strains. **b**, One non-lysing strain and one lysing strain. White, dashed lines indicate the growth rate at which one strain's growth rate exceeds that of the other one even for maximum lysis activation. **c**, Two lysing strains with one strain having a strong response to the other's QS signal. Cyan dashed lines indicate the region where both strains are in the oscillatory regime, black dashed lines mark the area in which strains are self-limiting. **d**, Two lysing strains with one strain having a weak response to the other's QS signal. **e**, Two completely orthogonal lysing strains. The *rpa* and *lux* systems could be used for this dynamic as they are signal orthogonal. **f**, Two completely orthogonal strains with the green strain in the weak lysis regime (leading to constant lysis), and the blue strain in the lysis regime. This is the regime corresponding to our experimental system. Oscillations in the green strain's population are imposed by the oscillatory blue strain through volume exclusion.

bial organisms has long been recognized [80]. Strategies to maintain the long-term stability of engineered microbial ecosystems that have thus far been investigated mainly consist of mutualistic interactions, such as metabolic interdependencies, or predator-prey type interactions[9, 10]. Recent evidence suggests, however, that competition is likely the dominant interaction in microbial communities[11]. In this vein, the ‘ortholysis’ system can be viewed as a strategy to stabilize competitive strains without engineering positive and negative interactions between members of the population. Moreover, recent evidence has identified quorum-sensing controlled self-lysis as a naturally occurring phenomenon in *Pseudomonas aeruginosa*[85], which is a relevant example of how the interests of synthetic biologists and microbial ecologists are merging in the field of engineered microbial ecosystems.

With the additional modeling of our circuit it becomes clear that the transition from monoculture synthetic biology to synthetic engineered ecosystems will be marked by an explosion of possibilities. A circuit designed for monocultures, such as the SLC, can have drastically broadened use-cases when expanded into the setting of a community. The ‘ortholysis’ system is immediately applicable for further expansion on the periodic *in situ* drug delivery system[75]. However, this phenomenon of stably co-culturing two metabolically competitive strains through orthogonal self-lysing offers the possibility of many unique applications beyond drug delivery where the use of synthetic microbial ecosystems is advantageous.

Supplementary figures

Table 3.1: A list of strains used in this study and their respective chassis and plasmid(s). JS006, BW25113 is a lab strain *E. coli* chassis. SL1344 is an attenuated *Salmonella enterica* subsp. *enterica* serovar Typhimurium host. The components of the plasmids listed in the table are shown in Supplementary Figure 4.

Strain #	Strain Name	Host Bacterium	Plasmid(s)
1	MOD41	JS006, BW25113	pTD103luxI(-LAA)sfGFP + pZA35 X174E (+LuxR)
2	MOD29	JS006, BW25113	pTD103luxI sfGFP + pZA35 X174E (+LuxR)
3	MOD42	JS006, BW25113	pTD103luxI(TS) sfGFP + pZA35 X174E (+LuxR)
4	SRS732	SL1344	pTD103-LuxI-CFP
5	SRS800	SL1344	pTD103-LuxI-CFP + pZA35-X174E (+LuxR)
6	SRS840	SL1344	pTD103-RpaR-RPal-LAA-sfGFP
7	SRS841	SL1344	pTD103-RpaR-RPal-LAA-sfGFP + pZA35-X174E (+RpaR)

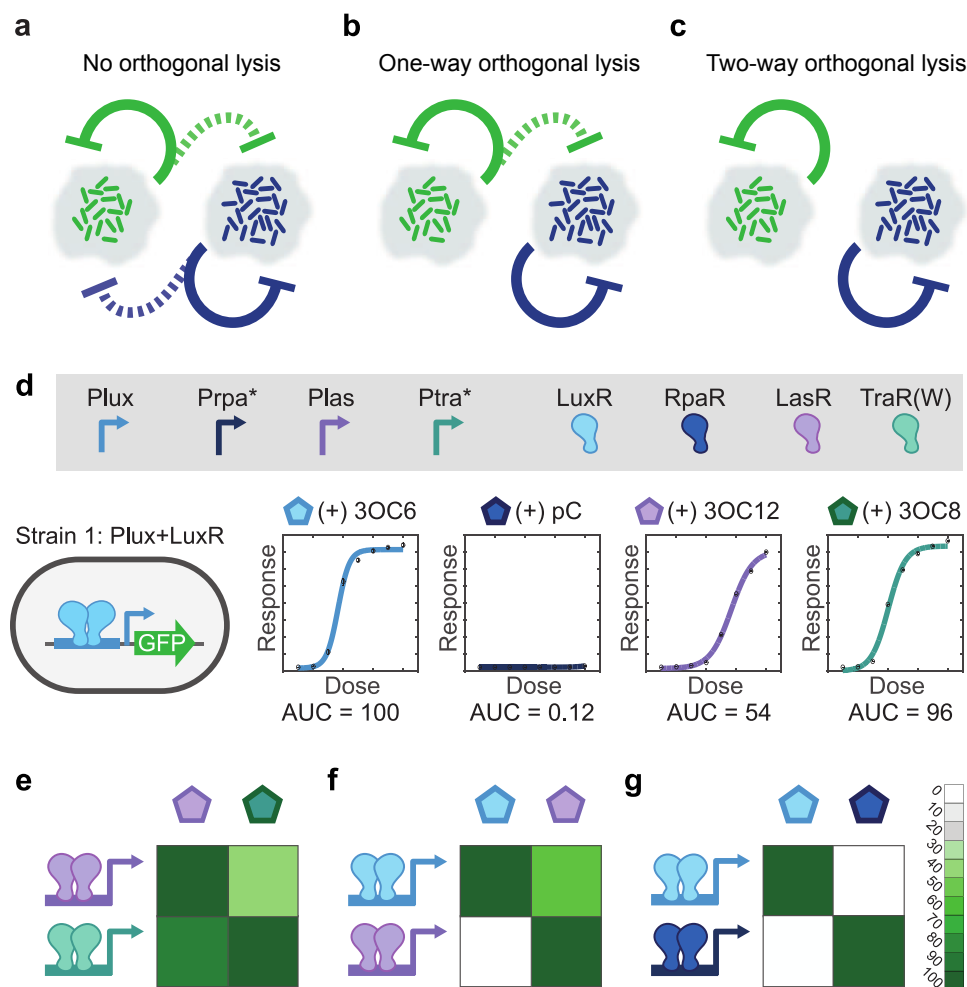


Figure 3.5: Communication motifs and quorum sensing signaling for synthetic microbial consortia. a-c. Schematic showing some of the QS communication possibilities between two members of a microbial consortia. a, With each strain capable of either sending, receiving, both or neither, there are generally 16 possible communication motifs (Supplementary Figure 2a). With the dual-strain consortia interfaced with the synchronized lysis circuit, we can achieve either one-way orthogonal signaling (b) or two-way orthogonal signaling (c). d, Schematic showing how QS systems can be tested and characterized for easy categorization. A strain containing one QS promoter and one QS receptor is subjected to a range of signals and its dose response curve is quantified by the area under its curve (AUC), which becomes the heat map parameter in e-g. Signaling homoserine lactones (HSL), 3-oxo-C6 HSL (3OC6), p-Coumaroyl HSL (pC), 3-oxo-C12 HSL (3OC12), and 3-oxo-C8 HSL (3OC8), are represented as pentagons color-coded to their native QS system. Error bars indicate the standard error of the mean (n=3). e-g, Heat maps of aggregated QS systems and their AUC responses to different signals; data is representative of 3 technical replicates. This methodology allows for quick identification of signal orthogonal strains classified by their diagonal matrices (g). Square matrices with significant induction in all squares indicates two-way signal cross-talk (e), while one-way signal cross-talk will only have one square not on the diagonal with a significant value (f). Additional combinations of promoters, receptors and signals are shown in Supplementary Figure 2b.

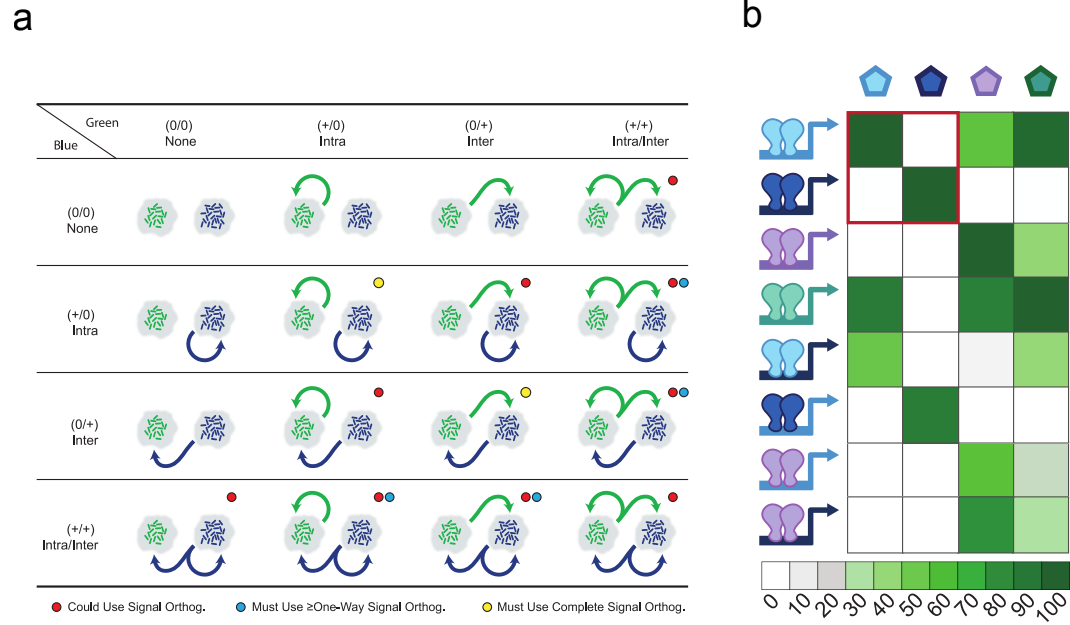


Figure 3.6: Possible Two-Strain Quorum Sensing Communication Motifs. **a**, Each strain is capable of no, intra, inter or both intra and inter-communication. This gives 16 general QS dynamics between two strains. Certain communication motifs require different level of signal orthogonality. Yellow dot means that motif requires complete signal orthogonality. Blue dot means that motif requires at least one-way signal orthogonality. Red dot means the motif doesn't necessarily need signal orthogonality, but it could utilize that dynamic. **b**, Example heat map of aggregated QS systems and their AUC responses to different signals. The meaning of the column and row label pictograms is the same as in Supplementary Figure 1.

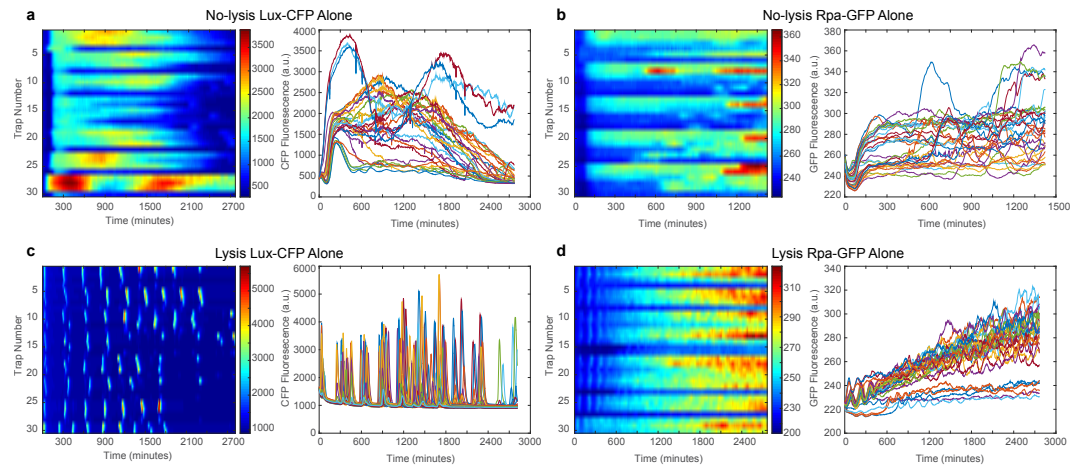


Figure 3.7: Phenotypes of Lysis and Non-Lysis Monocultures. a, Fluorescence intensity heat map of individual traps plotted against time and raw CFP fluorescence time-series of non-lysis Lux-CFP cells grown alone. b, Fluorescence intensity heat map of individual traps plotted against time and raw GFP fluorescence time-series of non-lysis Rpa-GFP cells grown alone. c, Fluorescence intensity heat map of individual traps plotted against time and raw CFP fluorescence time-series of oscillatory lysing Lux-CFP cells grown alone. d, Fluorescence intensity heat map of individual traps plotted against time and raw GFP fluorescence time-series of constantly lysing Rpa-GFP cells grown alone.

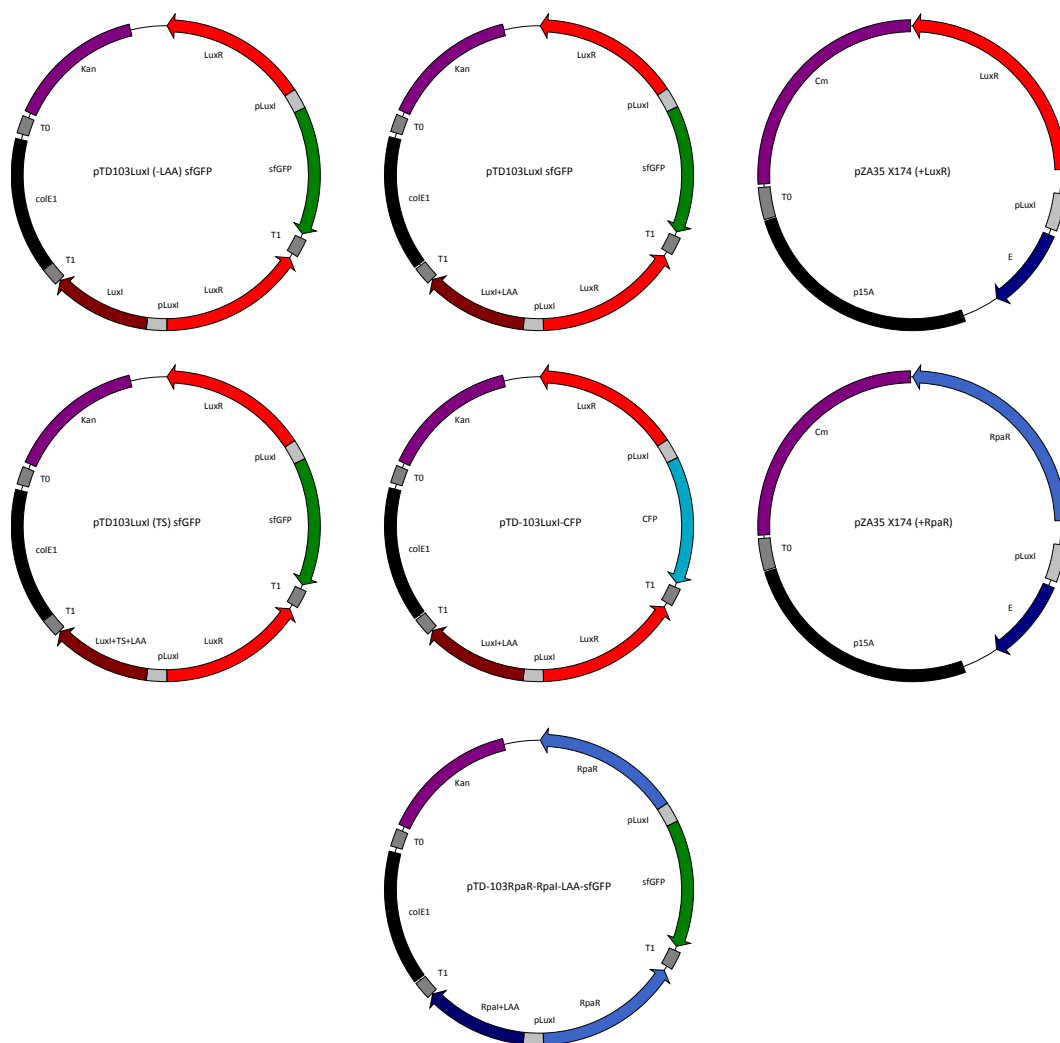


Figure 3.8: Plasmid maps of the main DNA constructs used in this study. Arrows in Red represent LuxR. Dark red arrows represent LuxI. Orange elements represent the pLuxI promoter. Yellow arrows represent RpaR. Green arrows represent sfGFP. Light blue arrows represent CFP. Dark blue arrows represent RpaI. Pink arrows represent the lysis gene E. Purple elements represent antibiotic resistance markers. Black elements represent origins of replication. Gray elements represent transcription terminators. See Methods section for more details.

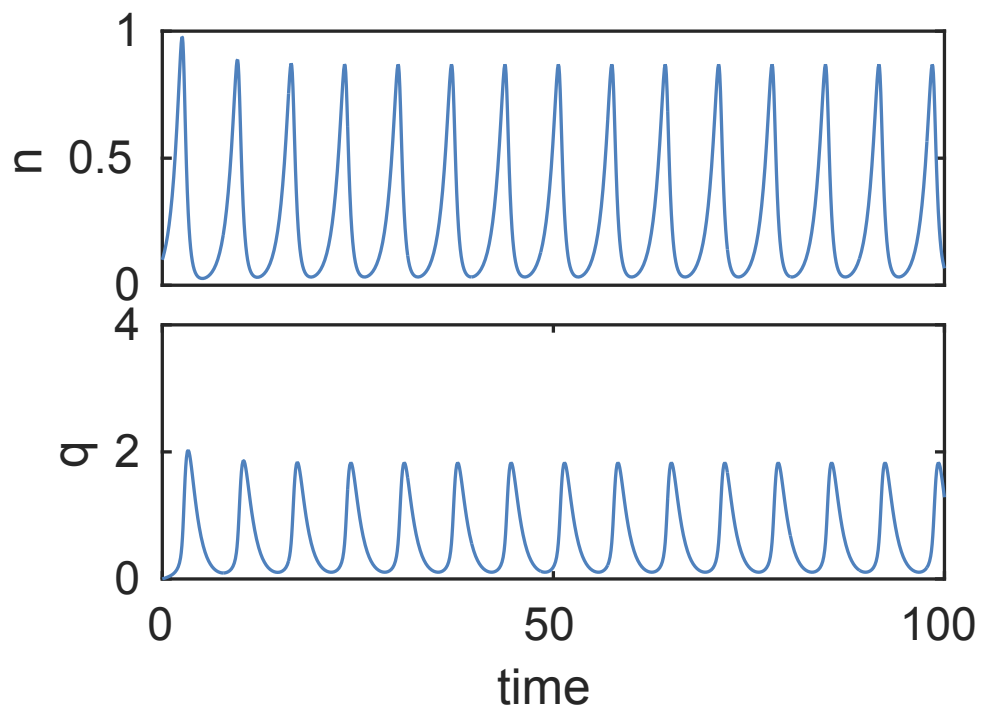


Figure 3.9: Dynamics of the model equation 1 in the Methods section. n represents the population of cells, and q represents the amount of fluorescent protein in the system.

Methods

Plasmids and Strains

Our circuit strains without the lysis plasmid were cultured in LB media with 50 $\mu\text{g ml}^{-1}$ kanamycin, in a 37°C incubator. Our circuit strains with the lysis plasmid were cultured in the same way but with 34 $\mu\text{g ml}^{-1}$ of chloramphenicol as well along with 0.2% glucose. For microscopy and plate reader experiments 1nM of 3-oxo-C6-HSL was added to all media. Plasmids were constructed using the CPEC method of cloning or using standard restriction digest/ligation cloning. The lux activator plasmid (Kan, ColE1) and lux-lysis plasmid (Chlor, p15A) were used in previous work from our group[36, 75]. The RpaR and RpaI genes were obtained via PCR off the *Rhodopseudomonas palustris* genome obtained through ATCC to create the Rpa-activator and Rpa-lysis plasmids. The lux-sfGFP lysis circuit alone was characterized in *E. coli*. Co-culturing was performed with non-motile *S. typhimurium*, SL1344.

The SLC, in both the Lux and Rpa case, is composed of an activator plasmid and a lysis plasmid. For the circuit characterization experiments, there were three variations of the activator plasmid. The first is pTD103LuxI-sfGFP which was used in previous work from our group[36]. This plasmid contains a LuxI with the ssrA-LAA degradation tag (amino-acid sequence of AANDENYALAA) and sfGFP, a superfolding green fluorescent protein variant[86]. pTD103LuxI (TS) sfGFP was constructed by adding the TS-linker (amino acid sequence of TS) between the ssrA-LAA tag and LuxI. pTD103LuxI (-LAA) sfGFP was constructed by removing the ssrA-LAA tag from LuxI. For the dual lysis experiments, the Lux-CFP strain used the activator plasmid with the ssrA-LAA tagged LuxI instead with a CFP in place of the sfGFP. The Rpa-GFP strain's activator plasmid was created by replacing LuxR with RpaR, and the LuxI with an ssrA-LAA tagged RpaI.

The lysis plasmids have a p15a origin of replication and a chloramphenicol resistance marker[60] and have been previously described by our group[75]. The lysis

gene, E from the bacteriophage ϕ X174, was kindly provided by Lingchong You and was taken from the previously reported ePop plasmid via PCR[38]. The E gene was placed under the expression of the LuxR-AHL activatable luxI promoter for both the Lux-CFP and Rpa-GFP strains. Most of the construction was done using the CPEC method of cloning[59]. See Supplementary Fig. 4 and Supplementary Table 1 for maps of the the plasmids used in this study.

Microfluidics and Microscopy

Our group has previously described in depth the microscopy and microfluidics techniques used in this study[35]. In short, micron-scale features are baked onto silicon wafers using cross-linked photoresist. The microfluidic device resin, PDMS (polydimethylsiloxane), is then poured over the wafers and solidified by baking. The PDMS, which contains numerous devices, is peeled off and individual devices are cut out from the whole. Holes are then punched into the device at their input and output where the fluid lines will eventually plug in. After puncturing, the devices are bonded onto glass coverslips via plasma-activation. The devices were then put in a vacuum and the outlet was loaded with cells and the inlet with media. Vacuum pressure loads cells into the traps and media lines are plugged in before the cells can contaminate the upstream section of the device. The flow was then adjusted by changing the relative heights of the syringes, which for all experiments the meniscus of the media was set to one inch above the meniscus of the waste, resulting in a low, constant hydrostatic pressure driven flow.

All microfluidic experiments were done in a side-trap array device as previously described[35], and in all cases 0.075% Tween20 was added to the media to deter cells from sticking to the channels and the ports of the device. The bacteria growth chambers were 100 μ m wide 85 μ m deep and approximately 1.6 μ m in height.

For lysis characterization (Figure 1): Cells were cultured until they reached an optical density of approximately 0.1 (Plastibrand 1.5mL cuvettes were used) at which point they were spun-down and loaded via vacuum pressure the chip. Media was LB

with Kanamycin and Chloramphenicol.

For dual lysis and co-culturing experiments (Figure 2): Cells were cultured until they reached an optical density of approximately 0.1 (Plastibrand 1.5mL cuvettes were used to test OD) and 1.5mL was spun down and resuspended in 50ul of media. This concentrate was used to vacuum load the cells for single strain experiments, or it was mixed at a 10:1 ratio (Lux-CFP:Rpa:GFP) in the co-culturing experiments before loading via vacuum pressure. Media was LB with Kanamycin (and Chloramphenicol for lysis experiments) with 1nM 3OC6 HSL added.

The microscope system used has also been previously described by our group[36]. In short, a Nikon Eclipse TI epifluorescent microscope with phase-contrast based imaging was used. Our camera is a Photometrics CoolSNAP HQ2 CCD. The acquisition software used is Nikon Elements. Our microfluidic devices are housed in a plexiglass incubation chamber that is maintained at 37C by a heating unit. For dual lysis and co-culturing experiments: Phase-contrast images were taken at 20x magnification with 50-200ms exposure times. Fluorescent imaging at 20x was performed at 300ms for GFP, 30% setting on the Lumencor SOLA light source, and 300ms and 35% for CFP. Images were taken every 3 minutes for the course of the experiment (~2 days). Co-culture was determined to be lost if the fluorescence of either CFP or GFP went below background fluorescence, and then was checked manually in cases of the oscillatory lysing CFP strain which can go below threshold between lysis events.

For the lysis characterization (Figure 1), we counted cells using the following strategies: for experiments where the cell population was mostly aggregated together (non-sparse population), we first estimated the average area of an individual bacterial cell and the average void fraction (open space between bacteria in the trap). Taking into account the pixel density of the image, we measured the area of the trap taken up by cells using ImageJ and divided by the average area of a bacterial cell. This value was then multiplied by (1 - void fraction) to yield the total estimated number of cells in the trap. Bacteria that were not close to the main group of cells were counted individually and added to the final number. For experiments where the growing population was sparse

(due to the constant lysis regime), we utilized the Trainable Weka Segmentation plug-in for ImageJ to count cells. Plots were generated by using MATLAB.

For co-culture experiments: Co-culture was determined to be lost if the fluorescence of either CFP or GFP went below background fluorescence, and then images were checked manually in cases of the oscillatory lysing CFP strain which can go below threshold between lysis events.

Plate Reader Fluorescence and Population Estimates

For the well-plate experiments the strains were grown in a standard Falcon tissue culture 96-well flat bottom plate with appropriate antibiotics (kanamycin only for non-lysis and kanamycin and chloramphenicol for lysis strains). For consistency with microfluidic experiments, 1nM of 3OC6-HSL was added to all media. We grew the bacterial strains used in Fig. 2b in 4mL cultures to an optical density of 0.15 before adding 10uL of this culture to 10mL of fresh LB with appropriate antibiotics and added HSL. For single strain tests, 200ul of the dilution was distributed into the well-plate. For the 1:1 mixtures, 100ul of each dilution was added to the same well. For the 1:100 mixtures 200ul of the Lux-CFP dilution was added with 2ul of the Rpa-GFP dilution. For all cases there were four technical replicates.

These dilutions were then grown for 10 hours (non-lysing), or 19 hours (with lysis) and their OD600nm, GFP, and CFP levels were measured every 10 minutes in a Tecan Infinite M200 Pro. GFP readings had an excitation of 485nm and emission of 520nm. CFP readings had a an excitation of 433nm and emission of 475nm. The resulting fluorescence curves were used to calculate estimated populations of the co-cultures.

Population estimates in the co-culture mixtures was estimated in the following way. The GFP fluorescence time-series trace of Rpa-GFP alone was integrated and used as a standard for accumulated fluorescence of a culture with 100% of the Rpa-GFP strain. In the same way, the CFP fluorescence time-series trace of Lux-CFP alone

was integrated and used as a standard for accumulated fluorescence of a culture with 100% of the Lux-CFP strain. The integrated GFP and CFP fluorescence curves of the mixtures was then divided by the standards to give a population estimate of Rpa-GFP and Lux-CFP, respectively. For all cases, the area of the background fluorescence was subtracted. Additionally, the GFP fluorescence required extra signal normalization because the Tecan's GFP sensor reads into the CFP emission profile (but not the other way around).

Here are the equations used to calculate the population estimates with appropriate filtering and normalization:

$$\begin{aligned}
 Population_{Lux} &= \frac{Area(CFP_{mix}) - Area(BG_{CFP})}{Area(CFP_{Lux}) - Area(BG_{CFP})} \\
 \eta &= \frac{Area(GFP_{Lux}) - Area(BG_{GFP})}{Area(CFP_{Lux}) - Area(BG_{CFP})} \\
 GFP_{Crosstalk} &= [Area(GFP_{mix}) - Area(BG_{GFP})] \\
 GFP_{Real} &= GFP_{Crosstalk} - [Area(CFP_{mix}) - Area(BG_{CFP})]\eta \\
 Population_{Rpa} &= \frac{GFP_{Real}}{Area(GFP_{Rpa}) - Area(BG_{GFP})}
 \end{aligned}$$

$Population_{Lux}$ is the population estimate of the Lux-CFP strain in a co-culture. $Area(CFP_{mix})$ is the area of the CFP fluorescence time-series curve of a given co-culture. $Area(BG_{CFP})$ is the area of the background CFP fluorescence time-series line. $Area(CFP_{Lux})$ is the average area of the CFP fluorescence time-series curve in the wells with only the Lux-CFP strain. $Area(GFP_{Lux})$ is the average area of the GFP fluorescence time-series curve in the wells with only the Lux-CFP strain (For this strain the GFP fluorescence should technically be at background, further normalization is done because the Tecan's GFP sensor reads into the CFP emission profile). $Area(BG_{GFP})$ is the area of the background GFP fluorescence time-series line. η is the calculated fluorescence emission cross-talk scalar, and is only needed to scale GFP values as the CFP sensor does not read any GFP. The normalized, filtered, GFP value is thus given

by GFP_{Real} . $Area(GFP_{mix})$ is the area of the GFP fluorescence time-series curve of a given co-culture. $Area(GFP_{Rpa})$ is the average area of the GFP fluorescence time-series curve in the wells with only the Rpa-GFP strain. Finally, $Population_{Rpa}$ is the population estimate of the Lux-CFP strain in a co-culture.

Agent-Based Modeling

For the agent-based model, to simulate bacterial motion, we adapted the mechanical agent-based model developed in our earlier work[87, 88]. Each cell is modeled as a spherocylinder of unit diameter that grows linearly along its axis and divides equally after reaching a critical length $l_d = 4$. It can also move along the plane due to forces and torques produced by interactions with other cells. The slightly inelastic cell-cell normal contact forces are computed via the standard spring-dashpot model, and the tangential forces are computed as velocity-dependent friction.

To describe the intracellular dynamics of each cell, we adapted the ordinary differential equation model from[75]. Specifically, the intracellular dynamics are

$$\begin{aligned}
 P_{lux} &= \alpha_0 + \alpha_H \frac{(\frac{H_i}{H_0})^m}{1 + (\frac{H_i}{H_0})^m} \\
 \frac{dH_i}{dt} &= b \frac{I_i}{K_I + I_i} + D_m(H_e(\mathbf{x}_i, t) - H_i) \\
 \frac{dI_i}{dt} &= C_I P_{lux} - \gamma_I I_i \\
 \frac{dL_i}{dt} &= C_L P_{lux} - \gamma_L L_i
 \end{aligned}$$

Here the variables P_{lux}, H_i, I_i and L_i are the activity of luxI promoter, intracellular AHL, LuxI and lysis protein of the i -th cell. $H_e(\mathbf{x}_i, t)$ is the extracellular concentration of AHL at the location of the i -th cell. luxI promoter is induced by AHL. $b \frac{I_i}{K_I + I_i}$ is the production term for AHL. $D_m(H_e(\mathbf{x}_i, t) - H_i)$ describes the exchange of intra- and extra-cellular AHL across the cell membrane. $C_I P_{lux}$ and $\gamma_I I_i$ are the production and

degradation terms for LuxI. $C_L P_{lux}$ and $\gamma_L L_i$ are the production and degradation terms for lysis protein.

The extracellular AHL concentration $H_e(\mathbf{x}, t)$ is governed by linear diffusion equation

$$\frac{\partial H_e(\mathbf{x}, t)}{\partial t} = D_m (\sum H_i \delta(\mathbf{x} - \mathbf{x}_i) - H_e(\mathbf{x}, t)) - \delta_H H_e(\mathbf{x}, t) + D_H \nabla^2 H_e(\mathbf{x}, t)$$

In the simulation, we use 2D finite difference methods to describe the diffusion of AHL.

We implement the model in traps with different side lengths (20, 40 and 60). To simulate the lysis of each cell, we assume that when the concentration of lysis protein L_i is above a threshold L_{th} , the cell has a probability of $P_r = p_L(L_i - L_{th})$ per unit of time to lyse and once a cell lyses, it is removed from the trap.

We chose model parameters to qualitatively fit the experimental results and the parameters H_0, m, b, p_L were chosen to account for the differences of experimental measurements and dynamic behaviors between Lux-CFP and Rpa-GFP strains. The parameter values for the Lux-CFP strain are $\alpha_0 = 0.1$ (Lux promoter basal production); $\alpha_H = 2$ (Lux promoter AHL induced production); $H_0 = 1$ (AHL binding affinity to Lux promoter); $m = 4$ (Hill coefficient of AHL induced production of Lux promoter); $b = 1.5$ (AHL production rate); $K_I = 1$ (Conc. of LuxI resulting half maximum production of AHL); $D_m = 10$ (Diffusion constant of AHL across cell membrane); $C_I = 1$ (LuxI copy number); $\gamma_I = 1$ (Degradation rate of LuxI); $C_L = 1$ (Lysis gene copy number); $\gamma_L = 0.5$ (Degradation rate of lysis protein); $\delta_H = 0.1$ (Dilution rate of extracellular AHL); $D_H = 65$ (Diffusion constant of extracellular AHL); $p_L = 0.3$ (Probability of lysing); $L_{th} = 1.6$ (Threshold of lysis protein for lysis).

To simulate the constant-lysis Rpa-GFP strain, these parameters have different values: $H_0 = 0.2, m = 1, b = 0.8, p_L = 0.03$. Besides, Rpa-GFP strain's growth rate is 10% larger than Lux-CFP strain.

Deterministic Modeling

Single lysis oscillator strain: We describe the population level mechanisms that lead to oscillations in population size as observed with the synchronized lysis circuit. To gain an intuitive understanding, we use a reduced model that aims to reproduce the observed population level behavior using only the fundamental ingredients of the circuit: Autocatalytic production of quorum sensing agent and quorum sensing agent-induced lysis of cells. The basic equations for a single strain equipped with the lysis circuit are as follows (see Supplementary Fig. 5 for model traces):

$$\frac{dn}{dt} = \alpha n - f(q)\gamma n \quad (3.1a)$$

$$\frac{dq}{dt} = [\alpha_q + \alpha_q^* f(q)]n - \gamma_q q \quad (3.1b)$$

The cell density is denoted by n . Cells divide with a rate α and die with a maximal rate γ due to lysis. $0 \leq f(q) \leq 1$ characterizes the promoter under which the QS and lysis proteins are expressed, so it determines the dependence of the death rate on q and the auto-catalyzed production of the QS agent q . α_q is the basal production rate of QS agent, which can be increased by the presence of q to a maximum production rate of $\alpha_q + \alpha_q^*$. q is diluted in the environment with a rate γ_q . We use a standard Hill function for $f(q)$:

$$f(q) = \frac{q^m}{q_c^m + q^m}, \quad (3.2)$$

where q_c is the concentration of q that results in the half-maximum death rate (and auto-catalyzed production of q) and m is the Hill coefficient.

A linear stability analysis shows that the system (3.1) has a stable fixed point when

$$m \left(1 - \frac{\alpha}{\gamma} \right) < 1 + \frac{\alpha_q \gamma}{\alpha_q^* \alpha}. \quad (3.3)$$

The border of this stability region corresponds to the onset of oscillations. Basal parameters are, unless otherwise mentioned: $\alpha = 1$, $\gamma = 4$, $\alpha_q = 0.4$, $\alpha_q^* = 8$, $\gamma_q = 1$, $q_c = 1$, $m = 2$. These parameters lead to oscillations according to (3.3). All simulations are

carried out using the Runge-Kutta-Fehlberg (RKF45) method. An example trajectory is depicted in Supplementary Figure 5.

While we do not explicitly model individual proteins or enzymes, we can gain an understanding for the influence of LuxI degradation by ClpXP with the model (3.1) using the following logic: When there is very little LuxI (i.e. the positive feedback loop has not been activated), fast degradation by ClpXP will have a strong influence on the steady-state level of LuxI. LuxI with a strong degradation tag will experience fast degradation by ClpXP leading to a low basal production rate of QS agent (α_q), whereas LuxI with a weak degradation tag will have a higher steady-state level and therefore a higher basal production rate α_q . In contrast, once the positive feedback has been activated, the concentration of LuxI (and consequently the parameter α_q^* of the model) have a much weaker dependence on its degradation tag since an abundance of LuxI produced from a fully activated promoter saturates the limited enzymatic processing capacity of ClpXP and therefore the level of LuxI will be determined mainly by dilution due to cell growth. As seen from (3.3), decreasing α_q by a larger factor than α_q^* generally brings the system closer to oscillations, which is consistent with the requirement of a strong degradation tag for sustained oscillations demonstrated in Figure 2 of the main text. In summary, we model stronger (weaker) enzymatic degradation of LuxI by a lower (higher) value of α_q .

Microfluidic traps and multiple strains: A microfluidic trap is clearly a finite environment, but because nutrients are constantly replenished by diffusion from fresh media in the channel, logistic growth (as is often assumed in other scenarios with finite carrying capacities) would be an unrealistic description of the population dynamics. Instead, we assume that growth is unaffected as long as the population density is below the carrying capacity c of the trap. We then cap the cell density at c , corresponding to any extra cells being washed away by the flow in the main channel (“spillover”). Numerically, we reset the cell density to c after every time step of the simulation if it exceeds c . In all our simulations $c = 1$. Supplementary Figure 5 shows that the system with standard parameters lyses just before it reaches the carrying capacity of the trap, so

it is truly self-limiting.

For simulations of multiple strains, we simulate two copies of the system (3.1) with variables $\{n_1, q_1\}$ and $\{n_2, q_2\}$. Again, we let the system evolve freely as long as $n_1 + n_2 < c$. If $n_1 + n_2$ exceeds c after any time step, we set n_1 and n_2 according to

$$n_1 = \frac{n'_1}{n'_1 + n'_2}c \quad n_2 = \frac{n'_2}{n'_1 + n'_2}c \quad \text{if } n'_1 + n'_2 > c, \quad (3.4)$$

where n'_1 and n'_2 correspond to the population densities before the reset. More specifically, this way of limiting the total population density to the carrying capacity c corresponds to assuming a well-mixed environment, such that the relative population densities of the two strains remain unchanged upon spillover.

Consequently, two oscillating strains in one trap that use completely orthogonal quorum sensing systems only interact if the total population density hits the carrying capacity c . As shown in the main text, the strains will eventually lock into an anti-phase pattern where they avoid reaching their peak density at the same time. In order to model cross-talk, we modify the equation of the “receiver” strain (strain 2 in this case) to read

$$\frac{dn_2}{dt} = \alpha_2 n_2 - f(q_2 + \xi q_1) \gamma_2 n_2 \quad (3.5a)$$

$$\frac{dq_2}{dt} = [\alpha_{q,2} + \alpha_{q,2}^* f(q_2 + \xi q_1)] n_2 - \gamma_{q,2} q_2 \quad (3.5b)$$

where ξ determines how much strain 2 responds to the QS agent of strain 1, i.e. the strength of the cross-talk.

Additional parameters used in the main text: For the parameter scan of a single strain in Figure 1, the model equations were simulated for 2000 time units for different values of the model parameter α_q . The last 400 time units were used to determine the minimum, mean and maximum population density. For all parameter scans of two strains, the model equations were simulated for 500 time units and the last 100 time units were analyzed to determine the average cell densities $\bar{n}_1$ and $\bar{n}_2$ of the two strains. The “steady-state population ratio” shown in Figure 4 was then calculated as $(\bar{n}_1 - \bar{n}_2)/(\bar{n}_1 + \bar{n}_2)$, ranging from -1 (strain 2 dominates) to 1 (strain 1 dominates). For non-lysing strains, the model parameter q_c was set to infinity. Cross-talk parameters in

Figure 4c and d, are $\xi = 0.6$ and $\xi = 0.12$, respectively. Weak lysis (strain 1, Figure 4f) was achieved by reducing the lysis rate of the strain to $\gamma = 0.5$.

Supplementary Videos

Supplementary Video 1. This video shows timelapse fluorescence microscopy of the Synchronized Lysis Circuit (SLC) in Strain 2 (ssrA tag on LuxI) at 20X magnification. Images were taken every 2 min of a 100x100 μ m chamber. Timestamp is in minutes.

Supplementary Video 2. This video shows timelapse fluorescence microscopy of the Synchronized Lysis Circuit (SLC) in Strain 1 (no ssrA tag on LuxI) at 20X magnification. Images were taken every 2 min of a 100x100 μ m chamber. Timestamp is in minutes.

Supplementary Video 3. Video of non-lysis co-culture on a microfluidic device at 20X magnification. Strain 4 (non-lysis Lux-CFP) and Strain 6 (non-lysis Rpa-GFP). Timelapse fluorescence microscopy images were taken every 3 min. Timestamp is in minutes.

Supplementary Video 4. Video of dual lysis co-culture on a microfluidic device at 20X magnification. Strain 5 (lysis Lux-CFP) and Strain 7 (lysis Rpa-GFP). Timelapse fluorescence microscopy images were taken every 3 min. Timestamp is in minutes.

Supplementary Video 5. Timelapse agent-based simulation of two lysis strains both in the oscillatory regime.

Supplementary Video 6. Timelapse agent-based simulation of two lysis strains one in the oscillatory regime and the other in the constant lysis regime.

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Chapter 4

Single-cell behavior of the SLC across different dynamic regimes

Introduction

In the previous chapters I have described the results of investigating the behavior of the SLC at the population level. In this chapter I will discuss the work I have done investigating the behavior of the circuit at the level of single-cells. While the circuit is used to control the population, there are some striking aspects of the circuit that immediately stand out from the time-lapse experiments. For example, why do some bacteria survive the lysis event and re-populate after activating the quorum machinery? Also, why are some cells entering the constant lysis mode upon constant activation of the circuit with external inducer (AHL) instead of exhibiting immediate lysis? Both of these phenomena are unexpected and have no immediate explanation available from literature. As a first step toward understanding these behaviors, it is important to know what the expression of the lysis gene looks like in single-cells.

In this chapter I first describe results from investigating the survivors in the lysis oscillations using a fusion of the lysis protein E to GFP. I then show how bacterial growth at the single-cell level changes in different dynamic regimes. Lastly, I present data

on how these results may describe similar phenomena observed with another bacterial killing gene, *ccdB*.

Survivors produce lysis protein and transiently tolerate its expression

In order to investigate the expression of lysis protein E at the single cell level, I modified the circuit to include a fusion protein of E with GFP (E35-GFP, or E-GFP), which was originally made by the Young Lab, to be used as a reporter for the lysis protein[26]. The sfGFP was removed from this version of the SLC so that the only fluorescent signal provided by the circuit would be via E-GFP. In a microfluidic device with rectangular growth chambers (or ‘traps’), the cells exhibited oscillations as expected. The oscillations with this modified circuit appeared less consistent than the original strains used in Chapter 2, as evidenced by the atypical peaks. This may be due to changing circuit parameters by switching the original lysis protein E for the modified E-GFP. The circuit exhibited oscillations nonetheless, providing a model system to investigate survivors in these oscillations. It should be noted here that the cells of interest in these experiments are cells near the middle or deep-end of the microfluidic growth chambers. There are cells near the opening of the growth chambers that most likely survive due to diffusion of the AHL (these cells are also less activated than the cells near the middle or deep-end of the growth chambers as evidenced by lower activation of sfGFP).

As shown in Fig. 1a, cells harboring the circuit express E-GFP during the lysis event, meaning that survival is not due to non-expression of the lysis protein. Compared to the E-GFP trajectory of the entire population in the chamber, surviving cells appear to stay in the activated ON state of the circuit for some time before the expression of E-GFP lowers (Fig. 1b-c). It appears that the survivors are oscillating between two states as time progresses, the ON and OFF states of the circuit, yielding trajectories that resemble relaxation oscillations. This behavior can be made sense of by thinking of the positive

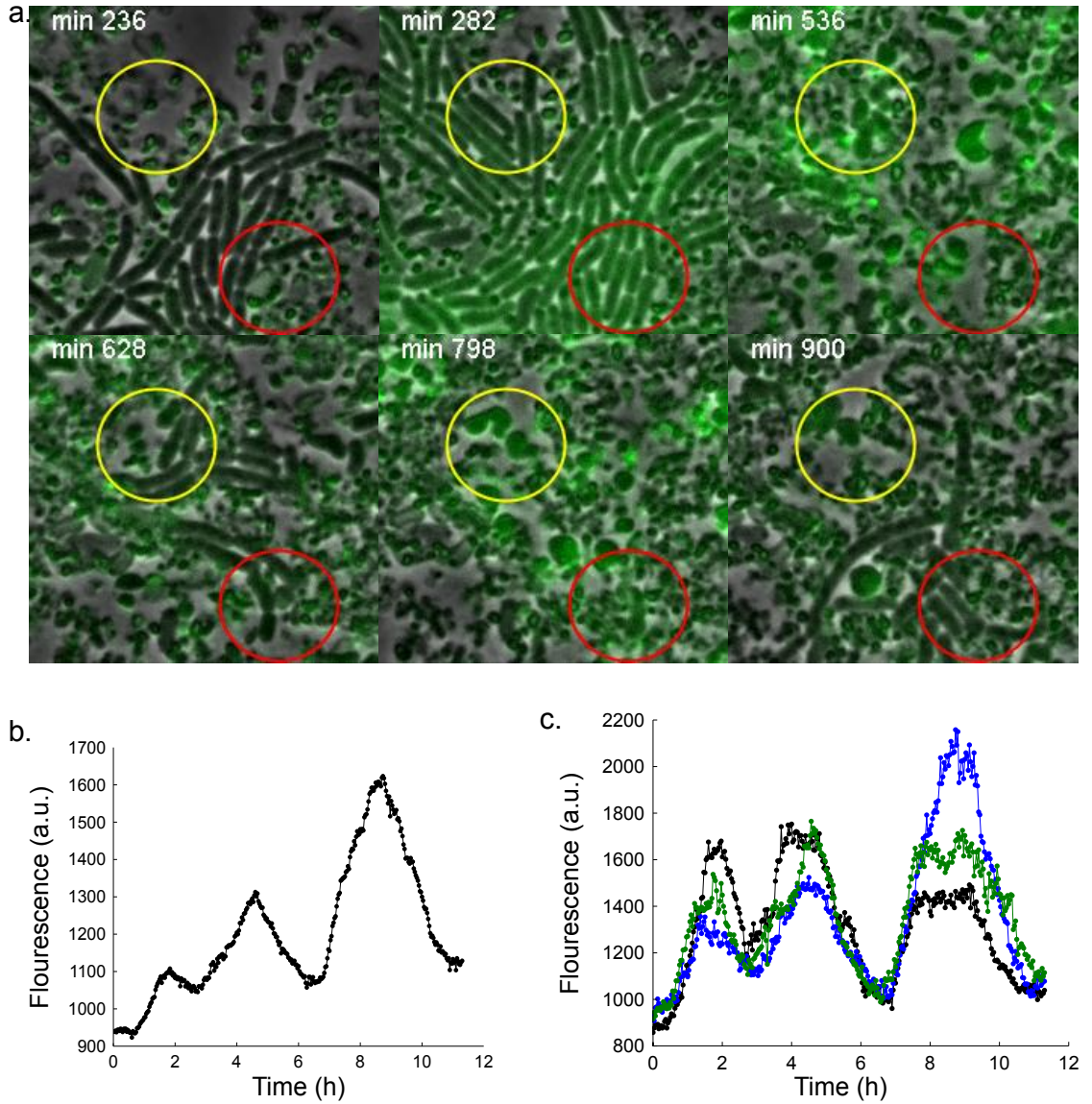


Figure 4.1: Single-cell behavior E-GFP in the oscillatory regime of SLC dynamics. (a) Typical time series showing part of a growth chamber with bacteria harboring the SLC circuit with E-GFP. The yellow and red circles indicate areas where a survivor cell appears during the lysis events. (b) Mean E-GFP fluorescence profile of the entire growth chamber from the experiment in (a). (c) Individual single-cell E-GFP trajectories from three surviving lineages in this experiment. This experiment was done with *E. coli*, strain JS006[89].

feedback quorum sensing system of the circuit pushing the cells to the ON stable state when enough AHL is available, and then relaxing to the OFF state once neighboring cells die and AHL is sufficiently diffused from the local area.

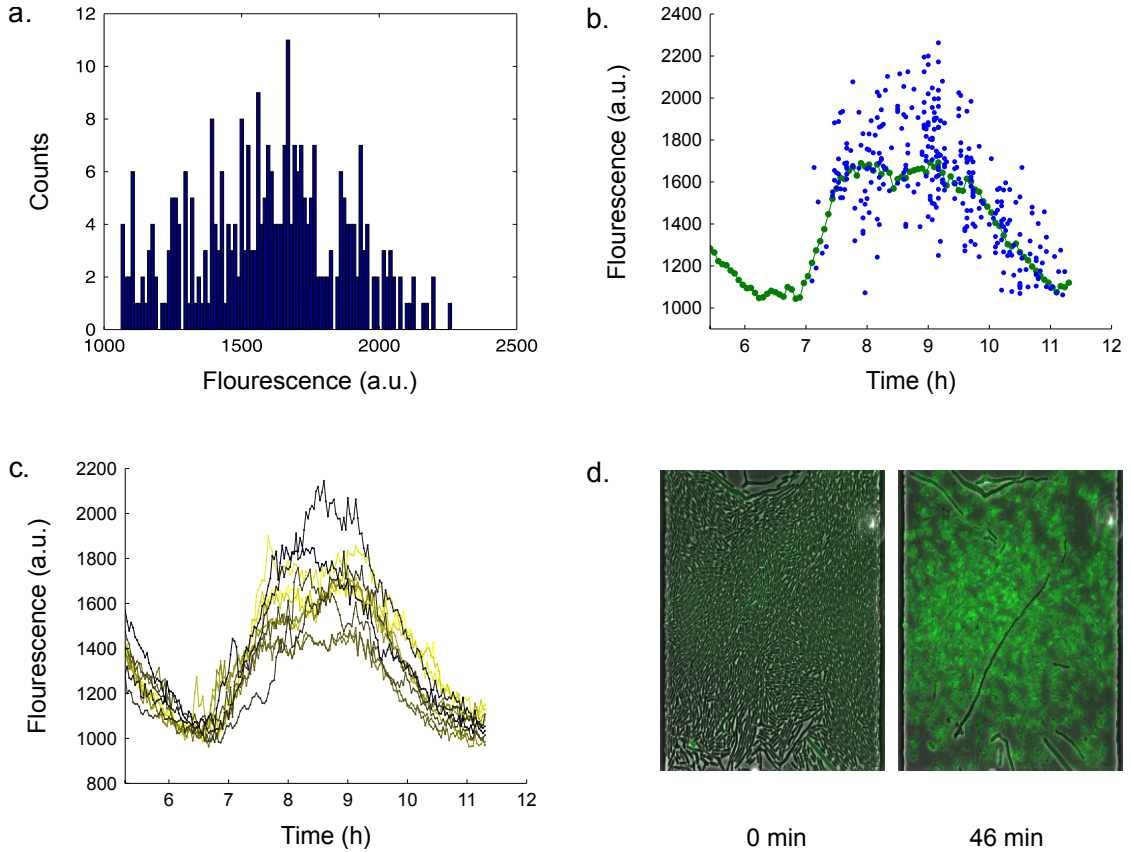


Figure 4.2: Distribution of E-GFP levels across lysed cells and surviving lineages. (a) Histogram showing the distribution of E-GFP fluorescence levels of cells that lyse during the last lysis cycle from the experiment shown in Fig. 1. (b) Distribution of E-GFP fluorescence levels of lysed cells across time (blue dots). Also shown is the E-GFP fluorescence trajectory of a surviving lineage (green). (c) Individual single-cell E-GFP trajectories from 12 surviving lineages during the last lysis cycle showing the distribution of E-GFP levels. (d) Time series frames showing *E. coli* with a plasmid containing pLlacO-1 driving E-GFP in a microfluidic growth chamber. The cells were induced with IPTG at time 0 min, and all cells were lysed by 46 min after induction. This strain was kindly provided by the Young Lab.

In order to show whether lysis occurs at a threshold level of the lysis protein, I tracked the E-GFP levels via fluorescence for single cells that lysed during the last lysis cycle of the experiment shown in Fig. 1. Considering the distribution in E-GFP levels at

lysis, it does not appear that lysis occurred in this system at a strict threshold concentration of lysis protein (Fig. 2a). Additionally, single-cell lysis appeared distributed across the cycle, with many deaths occurring at the tail end of the cycle, suggesting that cell death due to the action of the lysis protein has a stochastic quality that may be dependent on intracellular parameters (Fig. 2b). Trajectories of surviving cells also exhibited some variability as the value of the ON state E-GFP was variable (Fig. 2c). This may in part be due to the location of the cell in the growth chamber, as cells closer to the entrance of the chamber will exhibit a higher degree of local diffusion and thus lower AHL levels. Interestingly, when using a construct with the pLlacO-1 promoter driving E-GFP, all cells lyse in microfluidic growth chambers upon induction by IPTG (Fig. 2d). This might indicate that the level of expression possible under pLlacO-1 is higher than the pLux in the SLC, that after some threshold not achievable by pLux the bacterial population will encounter complete lysis. Inadvertently, these results also show that the lysis protein E by and large appears to be stuck to its intracellular target on the membrane after lysis.

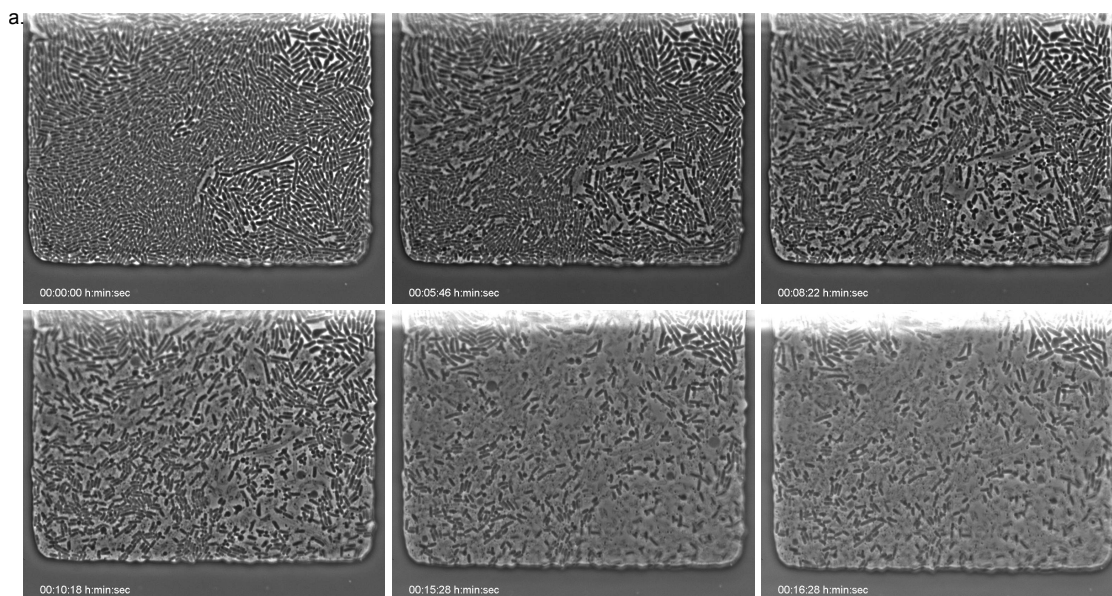


Figure 4.3: High time resolution time-lapse of an SLC mediated lysis event. (a) Time-series frames from a microfluidic experiment where a lysis event was imaged in real-time every 2 seconds.

To investigate how lysis propagates during the quorum lysis event with the SLC, I imaged a lysis event at high time resolution (Fig. 3a). Although the images are difficult to analyze with image processing software due to the proximity of the cells to each other and the appearance of 'ghost' cells after death, lysis events appeared to be spatially disconnected and not following a specific spatial pattern across the chamber. The only exception to this observation are two groups of cells near the opening of the trap that do not exhibit lysis, likely due to higher local diffusion of AHL. Images such as these may benefit from advanced image processing techniques using machine learning to correctly identify cell edges over time. Until such a system is implemented, it remains difficult to determine spatial patterns over time by eye.

Transient or constant expression of the lysis protein E results in different growth states

To elucidate how expression of E affects the growth of survivors, or cells that are in constant lysis mode via external AHL, I tracked cell division of the bacteria in these experiments by plotting cell perimeter trajectories. Surprisingly, during a lysis event survivors are still growing and dividing while they are expressing the lysis protein (E-GFP), albeit at a slower rate as evidenced by a sample trajectory in Fig. 4a. The size of the cell also decreased during this time. The division times before E-GFP expression ranged from 12 - 24 min, and during expression of E-GFP the division time ranged from 44 - 76 min. After E-GFP expression decreased, the division times were 20 and 28 min.

Interestingly, when external AHL is applied to the cells and they enter the constant lysis mode, they grow slower than non E-GFP expressing cells but faster than E-GFP expressing cells in the oscillatory mode (survivors as in Fig. 4a). Out of the 10 division times measured, most fall between 16 - 36 min and two above this range that are 48 and 60 min, respectively (Fig. 4b). These data show that the constant lysis mode most likely represents a physiological state of the cells that is different from when they

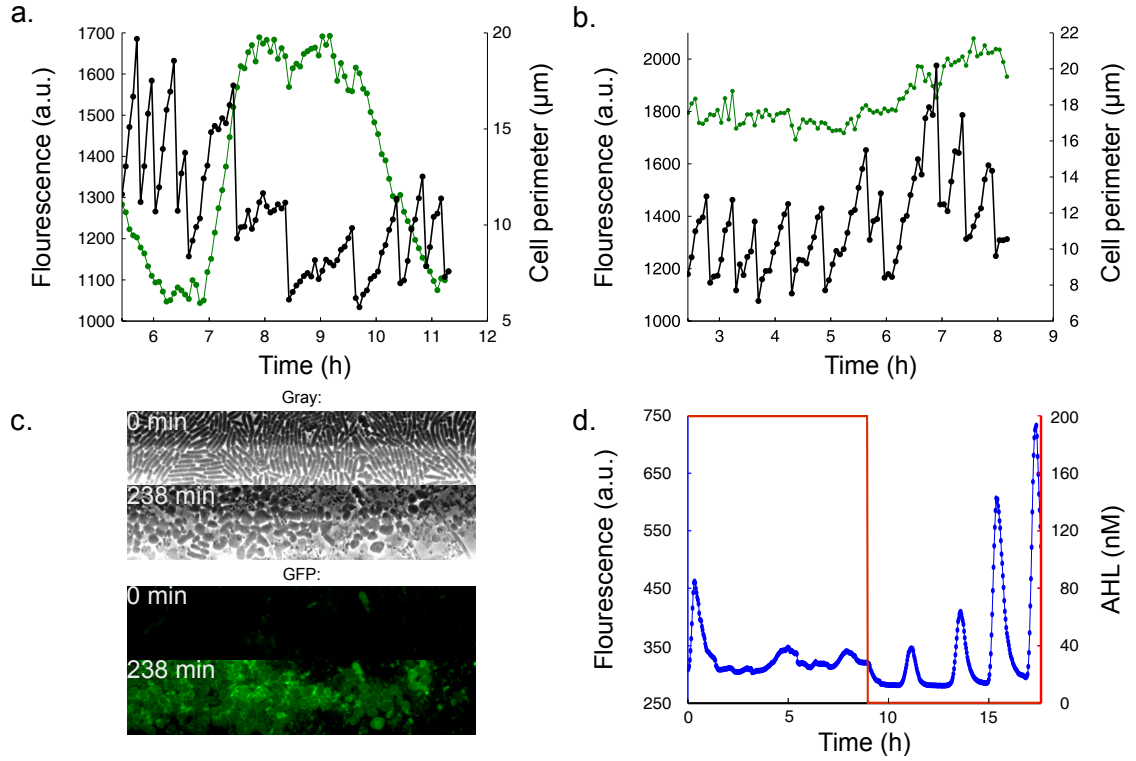


Figure 4.4: Different growth states possible with SLC mediated E-GFP expression. (a) E-GFP fluorescence and cell perimeter trajectories of a single-cell from the last cycle in the experiment from Fig. 1. (b) E-GFP fluorescence and cell perimeter trajectories of a single-cell after external AHL was added to the experiment from (a). (c) Phase-contrast and GFP images from the experiment in (b) taken at time 0 min and 238 min after induction with external AHL. (d) sfGFP trajectories of a *S. typhimurium* strain with the SLC (no E-GFP) during induction with external AHL and after it is removed. The strain used in this experiment is MOD 47 (refer to Chapter 2).

are in the oscillatory mode. It appears that the bacteria are somehow able to acclimate to the constant levels of lysis protein present without affecting growth as much as it would had the lysis protein only been expressed in a transient episode. This is supported by the fact that, at least in *E. coli*, the shape of the cell is altered upon entering the constant lysis mode to become more rounded (Fig. 4c). Also, the hypothesis that this state is a physiological state is supported by the results in Fig. 4d which show that a population of SLC bacteria can be switched from constant lysis behavior to oscillatory behavior upon the removal of external AHL.

Although the data from Fig. 4a-b are from a single cell, the conclusions are consistent with visual observations made from the time-lapse videos of the two experiments. Further quantitative data on the division times from the images is necessary before making a definitive claim, however, this is currently difficult to have automated with image processing software for these experiments.

Dynamic behavior in a quorum based circuit with ccdB

In the previous sections I described results with E-GFP which provide a better understanding of the dynamics of the lysis protein in the SLC behavior, namely that the bacteria expressing the lysis protein can transiently tolerate it's expression or possibly acclimate to it's presence by entering a different physiological state. One interesting question is whether similar dynamics as encountered with the SLC (such as oscillations) are possible with other kill genes. In this section, I describe some of my early work on a quorum based circuit which drives the gyrase inhibitor ccdB, instead of the lysis protein E.

The design of the circuit is very similar to the SLC, except that ccdB is present where E would have been in the SLC. Also, present is the ccdA gene (which encodes the anti-toxin to ccdB) which is expressed during propagation of the cells to exert tight control over the possible leaky expression of ccdB (Fig. 5a). This circuit exhibits behavior similar to the oscillations with the SLC where the bacteria reach quorum and express

ccdB, and survivors repopulate to perpetuate the process (Fig. 5b). Cell death here is not lysis, but filamentation and subsequent death of the cells (as evidenced by the diffusion of sulforhodamine 101 dye into the cells). The filamentation causes dead cells to take up space in the growth chamber and slow down growth and subsequent take-over by the growing survivors (Fig. 5c). This results in trajectories that are not typically regular in time.

The results described above with E-GFP may explain the phenomena observed here with ccdB, a killing gene with a different mode of action. Survivors may transiently tolerate ccdB during the extent of the quorum firing until AHL levels decrease afterwards. More generally, these results may hint at a behavior possible with any killing gene that bacteria can stochastically tolerate for a short period of time combined with quorum sensing machinery. That is, punctuated episodes of killing once the population reaches quorum and subsequent re-growth by survivors.

Conclusion

The results described in this section appear to support the idea that bacterial cells can encounter a physiological change in growth behavior depending on whether E-GFP is expressed transiently (via quorum sensing) or constantly via external AHL. It is interesting to consider whether these phenomena are encountered in nature, for example, when the phage ϕ X174 naturally infects populations of *E. coli*. Do some *E. coli* cells naturally persist or grow in the face of lysis protein E expression? Is ‘persistence’ to lysis genes a common occurrence? ϕ X174 is a lytic phage, so it is conceptualized as a phage which infects, replicates, then subsequently lyses its host. It may be also that the expression levels encountered with the quorum machinery is different than that of the natural system, where enough protein may be made to ensure lysis.

These results may have wider implications given that there are some antibiotics which can target the intracellular target of the lysis protein E, the translocase MraY. It may be possible that different levels and dynamics of induction with these antibiotics

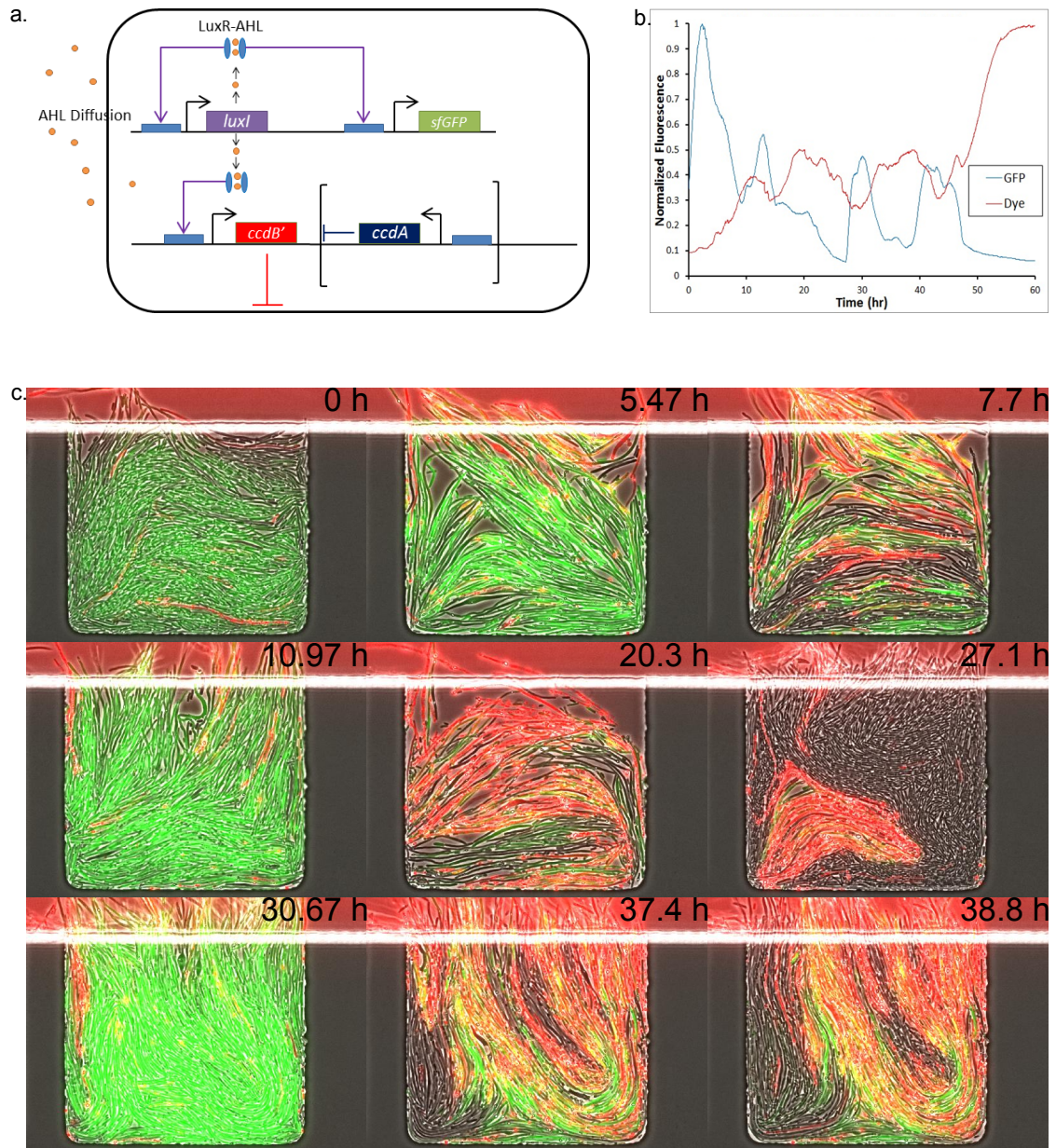


Figure 4.5: A quorum based killing circuit with *ccdB*. (a) Diagram of the quorum based circuit with *ccdB* and a *lac* sensitive promoter driving *ccdA*. (b) Typical sfGFP trajectory of cell with this circuit growing in a microfluidic growth chamber (blue). Also shown is the trajectory of fluorescence from the sulforhodamine 101 dye (red) which gets internalized into dead cells. (c) Time-series image frames from the experiment in (b). This experiment was done with *E. coli* MG1655 Z1, which provided that *lac* repressor necessary to turn off expression of *ccdA*.

can also result in bacteria adjusting to take on different growth states. It is also unknown whether this phenomenon has ever been encountered with any known antibiotic or killing gene; the results presented in this section may represent the first example of bacteria ‘adjusting’ their growth state to different dynamic levels of lysis protein (transient or constant).

Chapter 5

Dynamics and evolution of the SLC at different scales

Introduction

In this chapter, I discuss investigations into two important aspects of the SLC; circuit behavior and evolution across different scales. Up until this point, characterization of the SLC has been primarily presented inside microfluidic devices with $100\mu\text{m} \times 100\mu\text{m}$ growth chambers. It is important to know what the behavior of the circuit looks like in larger geometries as well as in macro-culture (for example, in a culture tube). While there has been some data presented for the latter in the form of batch well-plate experiments (see Chapters 2 and 3), here I will investigate the behavior of the circuit in large millimeter scale microfluidic devices and at the macro-scale with continuous flow. Additionally, another critical aspect of the circuit that needs to be discussed is its evolutionary instability (either from losing circuit components or mutating them over time). Here I will discuss the results of experiments showing evolution over time in microfluidics and in macro-scale experiments.

The SLC exhibits both constant lysis and oscillatory dynamics in a millimeter length scale microfluidic growth chamber

From the experiments shown thus far from the previous chapters, a conceptual picture of the space of behaviors possible by the SLC can be obtained. For some space of parameters the circuit oscillates (Ch. 2). For some space of parameters the circuit enters a constant lysis mode (Ch. 3-4), notably when the AHL threshold is raised (either by introduction of external AHL, or the removal of the *ssrA* tag from the LuxI protein). Additionally, it is possible to lyse all of the cells with a different promoter in *E. coli*, pLlacO-1 driving E-GFP (albeit with a different strain of *E. coli* as this construct was not tested in JS006).

Using the model presented in Chapter 2, a bifurcation diagram using XPP-AUT can be constructed to visualize what such a space of parameters might look like (Fig. 1a). The the two sample parameters chosen for this plot, flow rate and AHL induced lysis protein production, are reasonable given their relevance to the existing data. Oscillations occur in this diagram for some intermediate levels of flow, where a higher flow rate results in no quorum firing. If the parameter for AHL induced protein production increases in value, the system encounters the constant lysis mode (or the ‘lysis persis-tece’ region as noted on the plot), and above some threshold all cells lyse. The results of this model generated plot are very reasonable given that at some high flow rate, AHL will diffuse too quickly for the system to reach the quorum threshold. Whereas, for very low flow rates, the AHL will not diffuse quickly enough after quorum lysis to allow survivors to regrow and quorum lyse again (thus possibly leading to constant lysis). For higher levels of AHL mediated lysis protein production, it is known from the data that cells exhibit constant lysis behavior; and the theoretical upper limit on lysis protein concentration before all cells lyse is supported by the pLlacO-1::E-GFP results presented in Chapter 4.

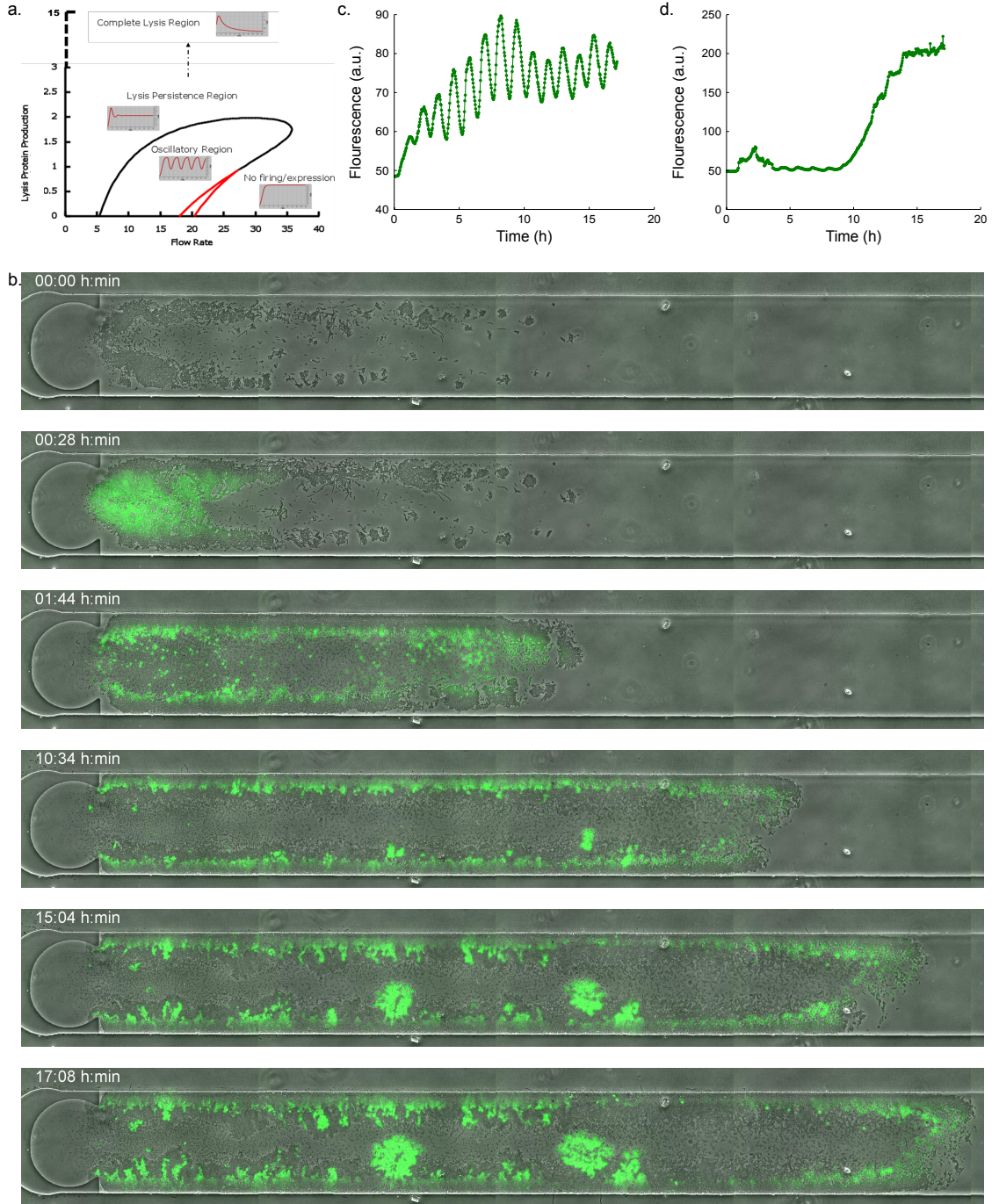


Figure 5.1: Oscillations and constant lysis in a single growth chamber. (a) Bifurcation diagram of the SLC model from Chapter 2 for the flow rate and AHL induced lysis protein production parameters. Sample trajectories and what they most likely represent from experimental observations are shown on the appropriate regions in the plot. (b) Time-series image frames of JS006 *E. coli* with the SLC growing in a long microfluidic growth chamber (200 μ m wide and 5 mm long). (c) sfGFP trajectory of SLC cells growing at the edges and (d) in the center of the long microfluidic growth chamber shown in (b).

Interestingly, both the oscillatory and constant lysis behaviors can be shown to co-exist in a single microfluidic growth chamber via regions of varying diffusion of AHL. I built a microfluidic device with a growth chamber that is $200\mu\text{m}$ wide and 5 mm long. The device was designed such that cells were only loaded at a single end of the growth chamber, allowing the cells to grow from one end to the other over time. After the initial quorum lysis event, all of the cells in the center of the chamber lysed and growth continued at the sides of the chamber and the leading edge of the center of the chamber (Fig. 1b). While the cells at the edges exhibited oscillations, cells that eventually started growing into the less diffuse center of the chamber switched to constant lysis, as exhibited by the constant activation of the quorum machinery (Fig. 1c-d). These results have implications for the behavior of the circuit in the context of an application, as it is most likely to be the case that local diffusion of AHL is not as homogenous in a field application as in microfluidic devices.

Dynamics of quorum based circuit with *ccdB* in a millimeter length scale microfluidic growth chamber

As mentioned in the previous chapter, a quorum based killing circuit with *ccdB* exhibits dynamics similar to those seen with the SLC (i.e. punctuated killing events followed by re-growth). In this section, I take a slight detour from the SLC to present data on the *ccdB* based circuit in the same microfluidic device described in the previous section. This data is relevant as it may show whether the results from the SLC in this device are generalizable to quorum based circuits with different kill genes.

The device used is shown in Fig. 2a, with the cell loading scheme highlighted on the diagram. Growth of the *ccdB* circuit cells in this device gives rise a few different cell fates: growing cells, filamentous dead cells, and seemingly non-growing cells (Fig. 2b). The cells grow until the first quorum killing event, after which many cells turn filamentous and die and others within the column survive and push out dead cells in their local

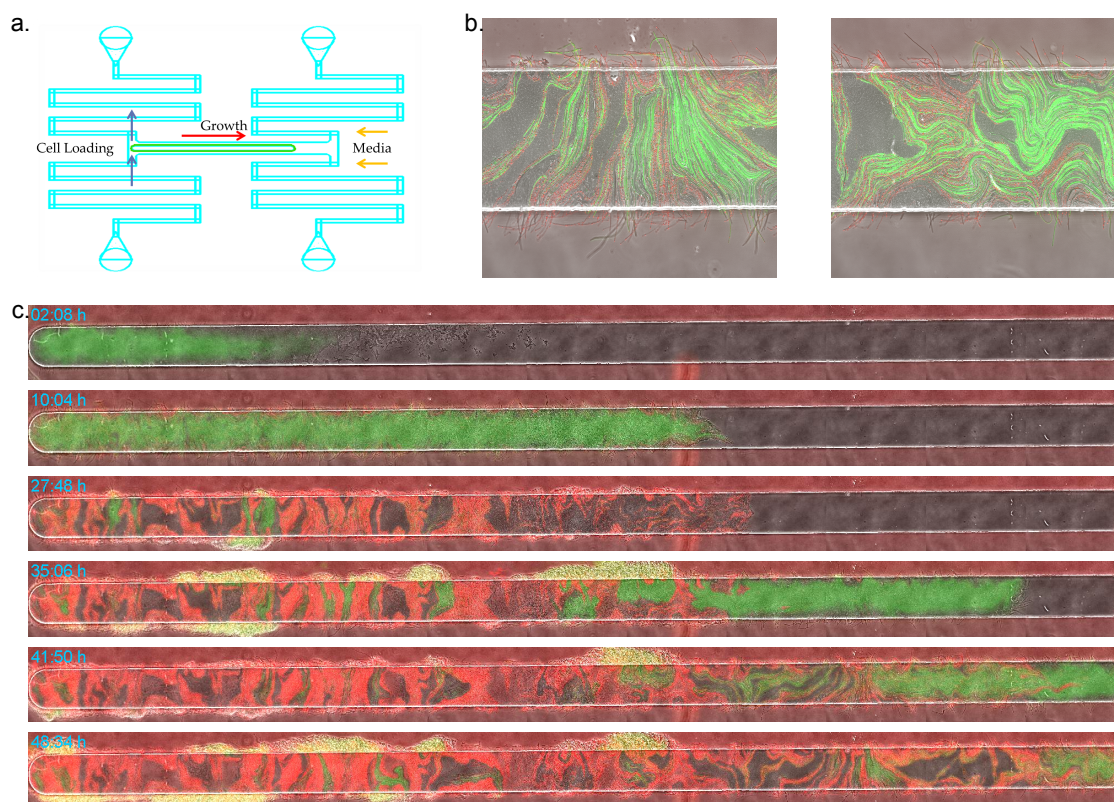


Figure 5.2: Formation of patterns with a quorum based ccdB circuit. (a) Schematic of the device used for the experiment. The cell loading protocol is used to seed cells at one end of the growth chamber, while media flows in the opposite direction of cell growth. (b-c) Time-series image frames of MG1655 Z1 *E. coli* with the the quorum based ccdB circuit growing in a long microfluidic growth chamber (200 μ m wide and 5 mm long).

area. The leading edge remains stagnant for some time (presumably because surviving cells are blocked by the physical presence of filamentous dead cells) before cells start growing there to the open area of the chamber. Two more growth and quorum killing events occur before the entire chamber is full of cells (Fig. 2c). The final colony of cells in the chamber is very homogeneous, with many regions throughout the chamber retaining the a somewhat static pattern of dead and live cells through time.

Unlike the results from the SLC cells, quorum killing with *ccdB* creates a physical barrier within the colony in the form of dead filamentous cells which then are difficult to move around. The end result is then a colony which appears more static over time than quorum killing with lysis. These results may be of interest if a quorum based killing circuit were to be used to create semi-static patterns via cell death. Are the patterns observed in this experiment repeatable or are the results very sensitive to initial conditions of the experiment? The latter appears likely. Additionally, there is the inherent stochasticity of cell death during quorum-killing to consider as well.

Dynamics of the SLC at the macro-scale

Most studies of dynamic circuits involves some form of microscopy and may include agar pads or microfluidic devices. In this section I present data on the dynamics of the SLC at the macro-scale, or in this case, milliliter size cultures. Since the SLC is an inherently dynamic circuit, it requires a constant flow of nutrients and diffusion to bring about the dynamic behaviors. For this, I put together a very simple chemostat to run experiments at constant flow.

The set-up of the chemostat is shown in Fig. 3a. Running an experiment with SLC cells (Strain MOD 101, refer to the Extended Data in Chapter 2 for further strian information) at a dilution ration of approximately 0.8 (2mL culture), oscillations in the culture turbidity are apparent over time (Fig. 3b). These results were surprising, given that there is (according to the author's knowledge) no known example of a synchronized population oscillator at this scale.

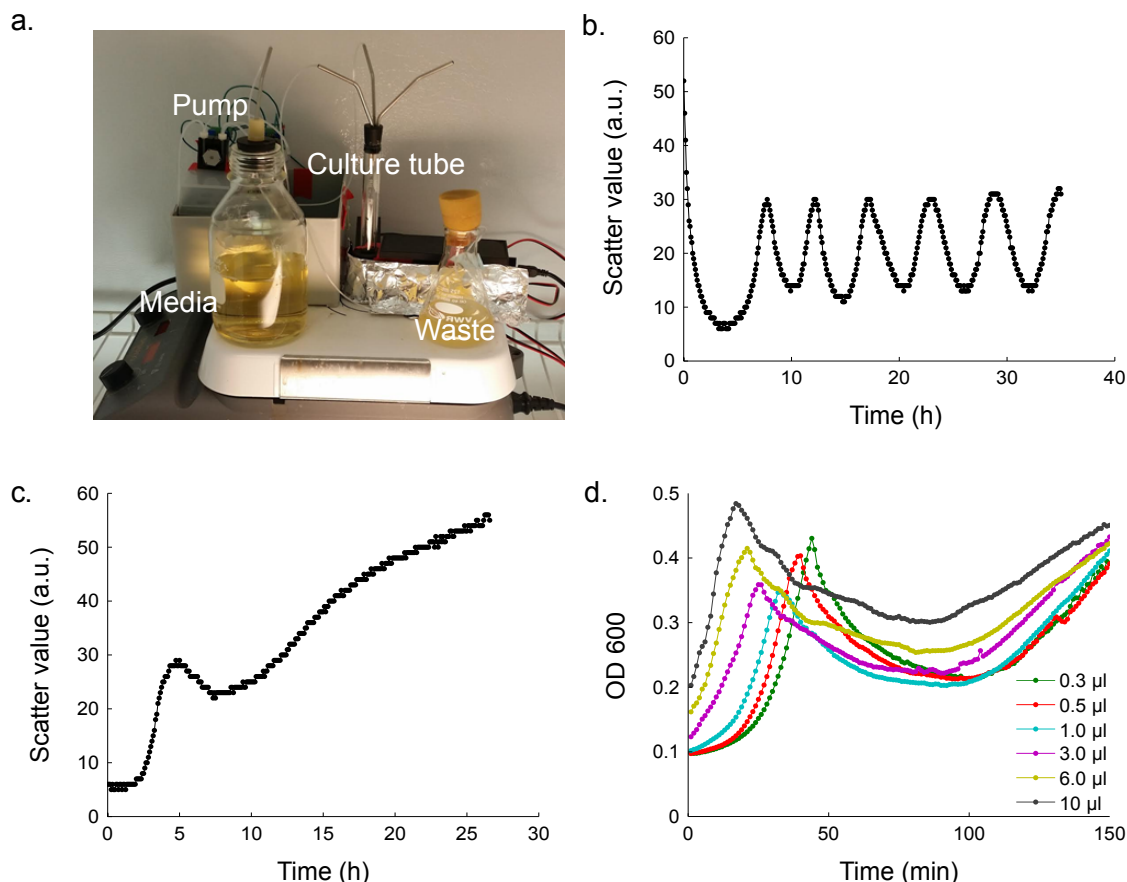


Figure 5.3: Behavior of the SLC at the macro-scale. (a) Set-up of the chemostat used for the macro-scale experiments. It includes a pump for constant flow (from the media to the waste) and a turbidity sensor fabricated in-house to measure cell density (the sensor was built by a previous graduate student, Ivan Razinkov). The entire set-up was put in an incubator to maintain the temperature at 37°C, and the culture tube was placed on a stir plate to allow a spinner to keep the culture well mixed. A 3-D printed assembly held the sensor and kept the culture tube in place. (b) Culture turbidity trajectories as measured by the turbidity sensor for an experiment with constant flow on the SLC culture. (c) Culture turbidity trajectories of an SLC culture without constant flow (batch culture). The strain used in (b) and (c) is MOD101 (refer to extended data in Chapter 2). (d) Varying seeding densities with SLC bacteria in batch culture (the strain is MOD61, refer to extended data in Chapter 2). This experiment was done on a well-plate and optical densities were measured on a TECAN plate reader.

When the circuit was run without constant flow (batch case), a single lysis event was seen, similar to the batch experiments done in well-plates presented in Chapters 2 and 3 (Fig. 3c). Interestingly, when varying the initial seeding density in the batch case (0.3 mL culture) a non-linear relationship between seeding density and culture density at lysis is revealed. Gradually increasing the seeding density from the lowest value tested results in decreasing threshold lysis density, until it reverses for very high seeding densities. Precise initial seeding of bacteria is difficult in the microfluidic devices used in the SLC studies, highlighting the utility of using macro-scale approaches to investigate non-linearities in these types of phenomena.

Evolution of the SLC in microfluidics

Evolution of circuit components is an important consideration for the implementation of synthetic gene circuits in ‘real-world’ applications. Given the selective pressure against expressing genes not essential to the survival of the bacterium, especially a lysis gene which kills a large fraction of the population in the case of the SLC, plasmid loss (in environments without antibiotic selection) and mutations which deactivate the circuit are expected occur at some point during growth. In this section, I present the work I have done investigating the evolutionary changes which occur in the bacteria over time in microfluidic cultures which either change or diminish the effects of the SLC circuit.

In order to assess how long the SLC can remain active in bacteria, I grew *S. typhimurium* harboring the SLC for over 7 days in a microfluidic device with square $100\mu\text{m} \times 100\mu\text{m}$ growth chambers (similar to the devices used in Chapters 2 and 3). Imaging the dynamics of the bacteria on the first day (Day 1), I observed oscillations consistent with those obtained in the data presented in Chapter 2 (Fig. 4a-b). Imaging after one week (Day 7), I observed oscillations that were less regular than typically seen with this strain (Fig. 4d). Looking at individual growth chambers, it was clear that the build-up of bacterial ghosts over time took up a substantial portion of the chambers, thereby affecting subsequent growth and lysis dynamics of the survivors (Fig. 4c).

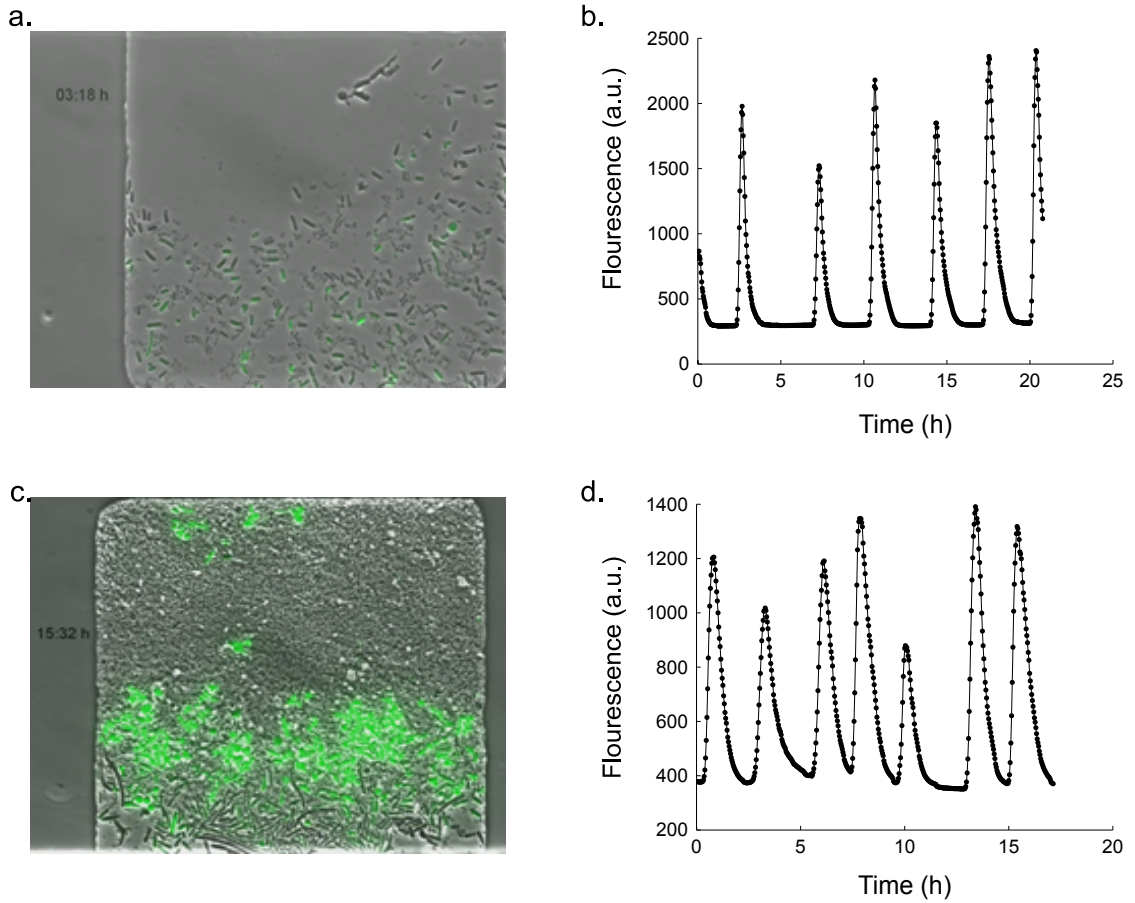


Figure 5.4: Long-term culturing of the SLC in microfluidics. (a) Image frame of the SLC bacteria growing in one of the growth chambers in the device on Day 1 of the experiment. (b) Fluorescence trajectory of sfGFP from the chamber in (a) on Day 1. (c) Image frame of the SLC bacteria growing in growth chamber from (a) in the device on Day 7 of the experiment. (d) Fluorescence trajectory of sfGFP from the chamber in (a) on Day 7. The strain used in this experiment was a *S. typhimurium* strain with the SLC (MOD47). Refer to the extended data in Chapter 2 for further information on this strain.

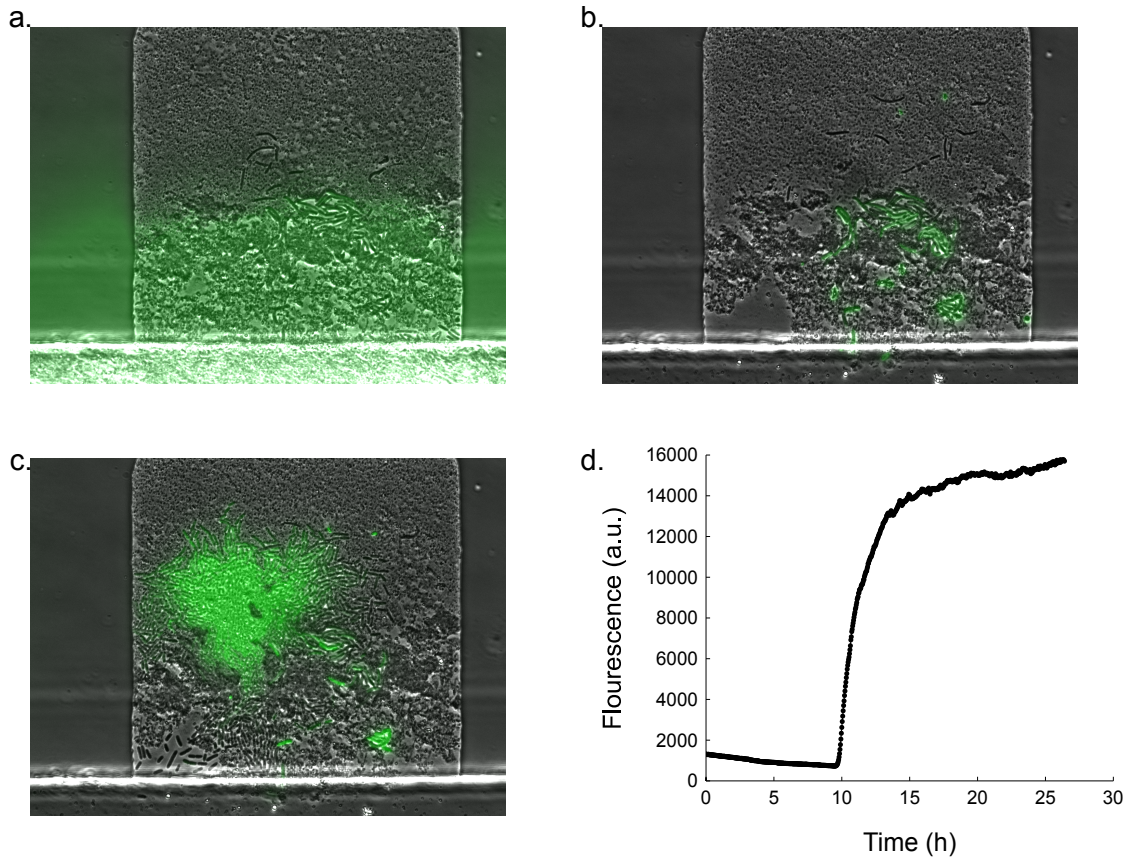


Figure 5.5: Re-growth of SLC bacteria after starvation. (a) Image frame of the SLC bacteria in one of the growth chambers in the clogged channel on Day 7 of the experiment. The bacteria in the chamber are not growing and are fluorescent due to high levels of sfGFP (the signal is saturating in the fluorescence imaging channel). Notice the cells residing in the channel directly outside of the chamber, due to the absence of media flow in the channel. (b) Image frame of the SLC bacteria growing in the growth chamber from (a) after the channel was manually cleared on Day 8. (c) Image frame of the SLC bacteria growing in the growth chamber during re-growth under typical flow conditions on Day 8. (d) Fluorescence trajectory of sfGFP from the chamber in (c) on Day 8.

Clogging was also observed in one of the channels in this device during the experiment, leading to a row of colonies in growth chambers becoming starved and ceasing to replicate. In this device there are several main channels that feed a row of growth chambers. Occasional clogging of the channels is a not so uncommon occurrence, potentiated typically by large particulates introduced during the initial set-up which may eventually end up blocking the inlet or outlet of the channel. During clogging, flow of fresh media is blocked and cells typically starve without nutrients. In this particular instance, clogging occurred on Day 7, as seen in Fig. 5a. Notice the cells stuck in the channel on the bottom of the image, and the constant expression of sfGFP (indicating the local AHL levels are very high). Once the clog was cleared via manual intervention (Day 8), most of the cells in the channel and in the chambers were ejected (Fig. 5b). During re-growth under typical flow conditions, the colony failed to lyse, instead growing until the colony filled up the chamber fully expressing sfGFP (Fig. 5c-d).

This indicated that the cells had their quorum machinery intact; and since the cells were still growing in the presence of chloramphenicol (the antibiotic selection for the lysis plasmid), it is most likely the case that the bacteria mutated the lysis gene under the starving conditions. It is possible that the starving conditions may have potentiated the appearance of the mutation that disrupted the ability of the cells to lyse. This may be likely, as it has been shown in the literature that error-prone DNA polymerases contribute to the stationary-phase mutagenesis in *E. coli*[90].

In non-starving conditions, evolutionary changes other than mutations affecting the lysis machinery were observed with a version of the SLC in *E. coli* where the LuxI gene was appended with a TS (threonine-serine) linker immediately prior to the *ssrA* tagging sequence (MOD42, see Chapter 3 for further strain details). Microfluidic experiments done with this strain yielded growth chambers where non-circuit expressing bacteria were observed to appear at some times during growth (from the time-lapse movies, these non-expressing cells did not appear to be initially seeded during cell loading of the device). The cells in these growth chambers would exhibit some quorum lysis events before eventually exhibiting constant lysis (from the data in Chapter 3, this strain

exhibits dampened oscillations until it reaches the constant lysis state) (Fig. 6a). During this time, the non-expressing cells would appear and proliferate for a limited amount of time before they halt growth. This may happen more than once in the same chamber over time, where one event is followed by another. Once these cells halt growth, they mainly appear not to divide for the rest of the experiment and eventually they die off (Fig. 6b).

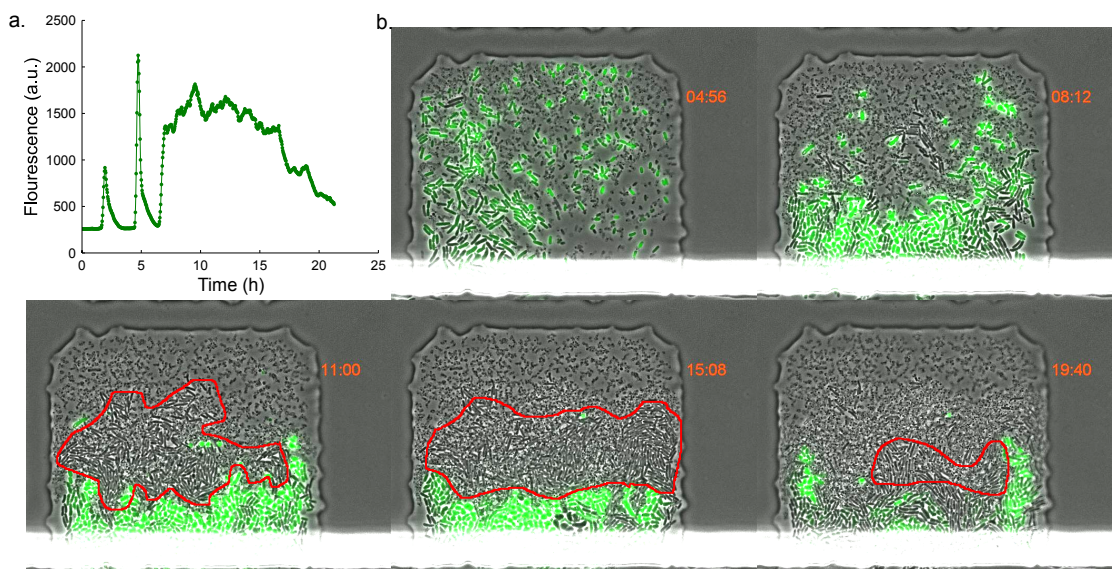


Figure 5.6: Appearance of antibiotic sensitive, non-expressing bacteria with the SLC. (a) Typical fluorescence trajectory of sfGFP from SLC cells with LuxI-TS-ssrA in *E. coli* growing inside a microfluidic growth chamber (the strain is MOD42, refer to Chapter 3 for further strain information). (b) Time-series image frames from the experiment in (a) showing the appearance of non-circuit expressing cells which are antibiotic sensitive, as evidenced by eventual stasis and cell death.

This phenomenon is most likely plasmid loss as these cells eventually die off after their short spurt of growth (i.e. they are sensitive to the antibiotics in the media). It is unclear which plasmid they would have lost given that they neither lyse with the circuit cells nor express sfGFP. As for why they exhibit a short growth spurt after appearing, it may be that enough antibiotic resistance protein is released by lysing cells to allow the non-expressing cells to grow. A more difficult question to answer is why these cells appear in the first place. It may be that they normally appear during growth

in microfluidic growth chambers (or in any culture more generally) after having lost externally introduced plasmid(s), but without the lysis mechanism to release antibiotic resistance proteins to the local area they are quickly killed off by the action of the selecting antibiotic. This idea would be consistent with the observation that these cells may become transiently protected by the action of released antibiotic resistance proteins with the SLC, and exhibit a short spurt of growth before being killed off by the antibiotics in the medium after the concentrations of said proteins decreases due to flow and diffusion.

A similar phenomenon was observed in larger microfluidic growth chambers with an SLC strain used for *in vitro* circuit characterization in Chapter 2 (MOD47, see Chapter 2 for further strain information). Using a circular chamber $400\mu\text{m}$ in diameter, I observed the appearance of non-expressing, antibiotic resistant cells appear in the center of the chamber with this strain (Fig. 7a). The strain exhibited typical oscillatory behavior until the non-expressing cells appeared and spread across most of the inner chamber through punctuated bursts of growth and stasis, diminishing the regularity of the lysis events (Fig. 7b).

It is important to note that this strain was used extensively in the $100\mu\text{m} \times 100\mu\text{m}$ square growth chambers, where the appearance of these non-expressing cells was rarely observed. It is possible that due the large area of this growth chamber, the rise of non-expressing antibiotic resistant cells was partly potentiated by the appearance of antibiotic diffusion gradients made once the cells and lysed ghosts filled up most of the trap area. This would then allow the cells that lost the plasmid to flourish in the center of the chamber for a short time. This idea is strengthened by the fact that once diffusion was increased by raising media flow, most of the non-expressing cells were killed and the colony returned to exhibiting regular oscillations (Fig. 7c-d).

Evolution of the SLC in macro-scale experiments

In this section I describe the the evolution of the SLC at the macro-scale using the experimental set-up describe in Fig. 3a. While the individual mutants can't be visualized

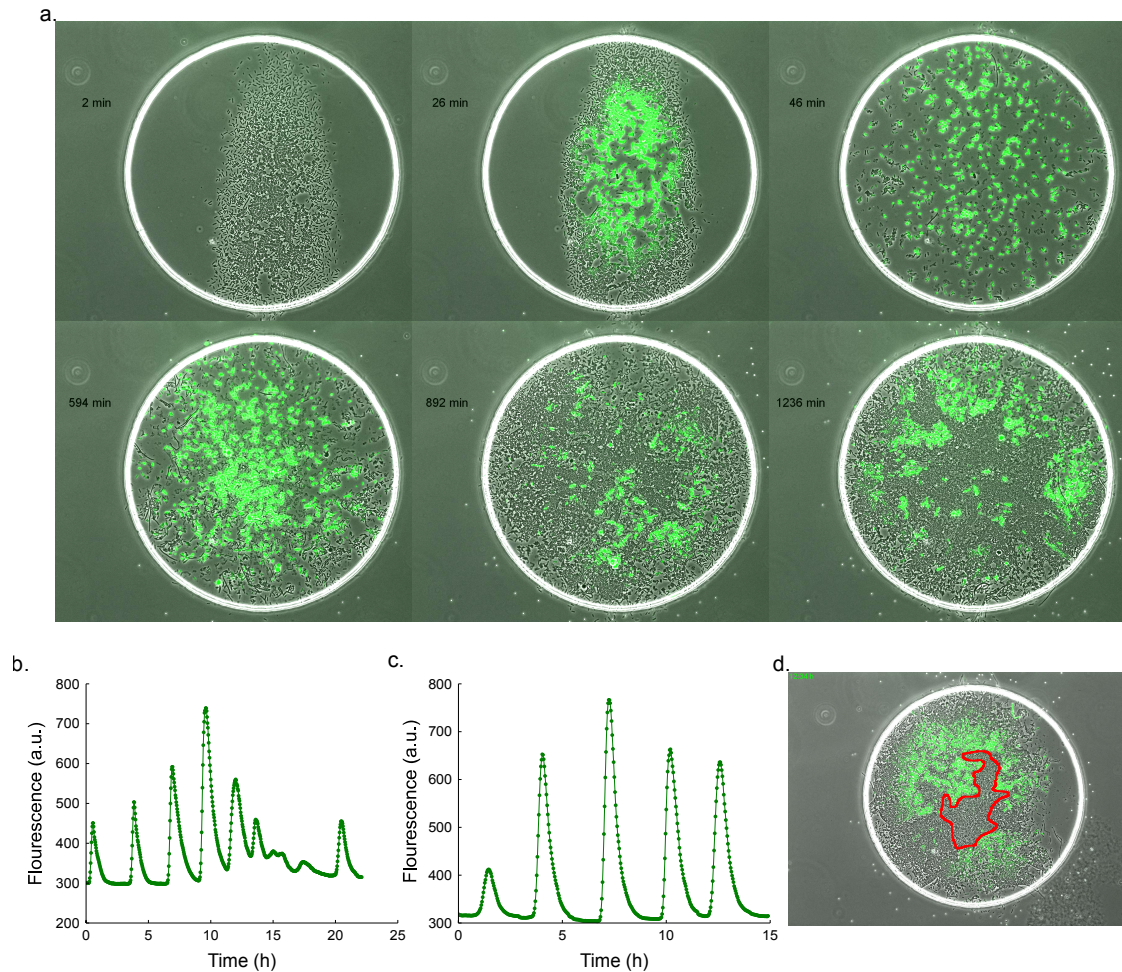


Figure 5.7: Appearance of antibiotic sensitive, non-expressing bacteria in a large growth chamber. (a) Time-series image frames of SLC cells in *S. typhimurium* (MOD47) growing in a large circular microfluidic growth chamber ($400\mu\text{m}$ in diameter, and $1.3\mu\text{m}$ high)). (b) sfGFP fluorescence trajectory from the experiment in (a). (c) sfGFP trajectory from the time after the media flow was raised at the end of the experiment from (b). (d) A single image frame from near the end of the experiment in (c) with the remaining non-expressing, antibiotic sensitive bacteria which have mostly been killed of by antibiotics in the media.

over time in this set-up, one advantage here is that cells can be sampled from the culture tube for sequencing. This is not easy with the microfluidic devices presented thus far, as isolating the cells from a single growth chamber and sequencing them would be very difficult.

When SLC cells (MOD101) are run for several days in a chemostat set-up, oscillations are observed for close to two days before a mutation appears to take over the population, diminishing the ability of the circuit to lyse (Fig. 8a). A sample of this population was taken after approximately three days (after the growth appears to stabilize), and the lysis plasmid was sequenced for mutations. Interestingly, a stretch of DNA on the lysis plasmid, including a part of LuxR, the pLuxR and pLuxI promoters, and part of the lysis gene, was missing from the plasmid (Fig. 8b). It appears surprising that the oscillations last for so long at this scale, given that the genetic diversity present in the culture tube is much higher than in microfluidic growth chambers (the approximate number of cells present in both cases is on the order of 10^8 and 10^3 , respectively).

Plasmid loss in microfluidics was discussed in the previous section, and was observed under a couple circumstances. In the chemostat set-up, the culture is well-mixed, making it unlikely that any cells would lose the plasmid (as antibiotic concentrations should be homogeneous in the culture tube). Additionally, the strain tested in the chemostat (MOD101) is an SLC construct with the stabilizing elements that were presented in Chapter 2 (the hok/sok toxin/anti-toxin system, and the alp7a plasmid partitioning system), which were borrowed from previous literature using plasmid-based circuits *in vivo*. I tested the effectiveness of this system for stabilizing the SLC plasmids in culture in the absence of antibiotics with the chemostat. This set-up is different from the *in vivo* environment, since it is unlikely that the bacteria *in vivo* will proliferate to the scale of a 2mL *in vitro* culture. The cells oscillate with an increasing basal density and dampened peaks until it appears to reach a stable density (Fig. 8c). After plating the cells taken from the end of this experiment, it was clear that many cells retained either plasmid, but not both (Fig. 8d). The population, then, appeared to be bifurcating into two populations over time, each containing one of the circuit plasmids. This is most

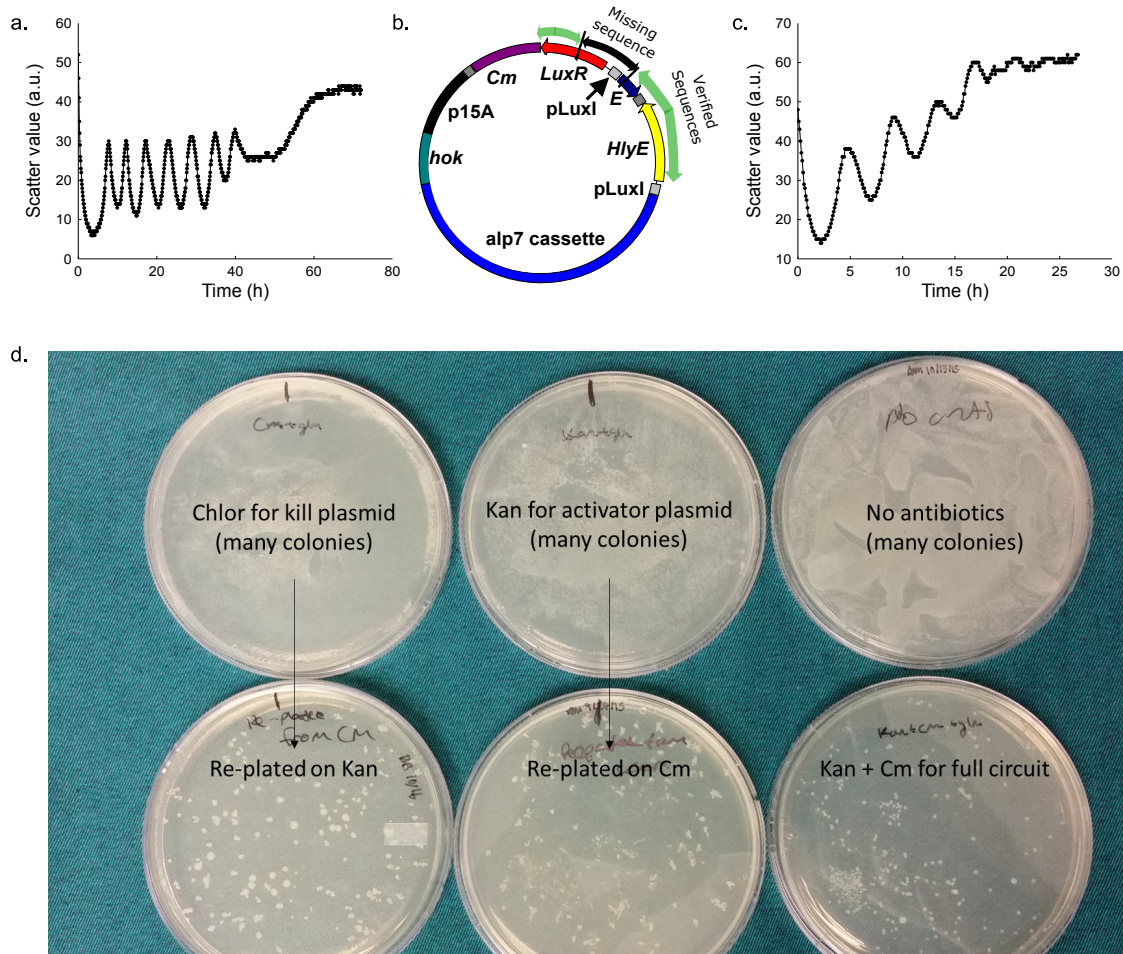


Figure 5.8: Evolution of the SLC at the macro-scale. (a) Culture turbidity trajectory of MOD101 cells in the chemostat set-up described in Fig. 3a and continued from Fig. 3b. (b) Sequencing results of cells taken from the end of the experiment in (a). The black arrows represent missing sequences and the green arrows represent verified sequences on the plasmid. (c) Culture turbidity trajectory of MOD101 cells grown in the chemostat without antibiotics. (d) Plating results from cells taken at the end of the experiment in (c). Cells were spread on plates with chloramphenicol, kanamycin, both, or no antibiotics. The cells initially plated on chloramphenicol and kanamycin were individually re-plated on plates with the other antibiotics to confirm the amount of colonies with both plasmids.

likely due to the fact that the same plasmid stabilization mechanisms are being used for both the activator and lysis plasmid, therefore the cells cannot ‘discriminate’ between the two plasmids. It is possible that putting all of the circuit components on a single plasmid with the stabilization genes could greatly enhance the stability of the circuit in non-selective environments.

Conclusion

In a long microfluidic growth chamber, the SLC can exhibit different growth behaviors (oscillations and constant lysis). This is possible due to the fact that diffusion of the quorum component (AHL) drives the expression of the circuit, and longer chambers result in larger diffusion gradients compared to the smaller square chambers used in experiments from the previous chapters. Using the quorum based circuit with the *ccdB* killing gene in place of the lysis gene with the long growth chamber, a different phenomenon is observed. The colony grows in punctuated events followed by cell death upon reaching the quorum threshold. Within the column of cells that fire and express *ccdB*, survivors grow up and repeat the process in localized areas within the chamber. This is because they are blocked by static regions of long, filamentous dead cells left behind in the chamber. The end result in the growth chamber is a population of cells that have left behind a pattern of dead cells interspersed with pockets of living cells.

The SLC can evolve over time in microfluidics to a state where the cells no longer express the lysis protein, but retain both plasmids and highly express the quorum machinery. This state appears after starvation of the SLC cells for an extended period of time. From previous literature, it is possible that this may be due to error-prone DNA polymerases becoming activated during stationary-phase conditions. In growth chambers where starvation did not occur, the circuit remained functional for at least 7 days. Additionally, there are instances where the SLC cells do not express any circuit machinery and become sensitive to the selecting antibiotics in the medium, indicating that these cells have likely lost the plasmids. This occurs with a circuit with LuxI-TS-

ssrA that exhibits dampened oscillations and in a circular microfluidic growth chamber with a large diffusion gradient.

Surprisingly, the SLC can oscillate in milliliter-scale chemostat cultures. To my knowledge, this is the first example of a synchronized oscillator in bacteria working at this scale. It also appears surprising given that the genetic diversity present at this scale is orders of magnitude higher than in microfluidic cultures, resulting in increased chances for a mutant to arise and take over the population. Mutants do eventually arise in the chemostat after approximately two days after inoculation of the culture. From sequencing results, these cells mutate the lysis gene but retain most of the other components on the lysis plasmid. Additionally, in the absence of antibiotics, the SLC cells with the stabilization machinery (hok/sok and alp7a systems) eventually bifurcate into two populations with either the activator or lysis plasmids. These results indicate that a further improvement on the stabilization of the SLC *in vivo* included finding unique mechanisms to stabilize each plasmid.

Chapter 6

Summary and outlook

In the preceding chapters I have described the construction of a novel genetic circuit which controls the bacterial population, the synchronized lysis circuit (SLC), and two possible applications of this circuit. While the two applications described here are specific (delivering therapeutics to tumors and stabilizing competitive ecosystems), they represent two general classes of applications that may be addressed using synchronized bacterial lysis. Firstly, using the SLC as a strategy to deliver therapeutics *in vivo* where the bacteria can colonize the site of disease. And secondly, using the SLC as a circuit motif to create different synthetic ecosystems, which may include self-attenuation, killing off other members of the population, or helping other members of the population by releasing beneficial proteins/molecules.

Synchronized expression of the lysis gene, E from ϕ X174, revealed two possible dynamic states with this protein. The first one is oscillatory lysis, where upon quorum threshold and lysis gene expression a portion of the population that expresses the lysis protein survives and re-populates the colony until the next quorum firing event. The second state is constant lysis, where the population is constantly expressing the lysis protein, and where growth and death appear to be close to a balanced point. These results are intriguing because it shows that bacteria can tolerate transient or prolonged expression of this lysis protein. Even more surprising is that transient or constant expression

of the lysis protein results in different physiological (for example, irregular shape of the bacteria) and growth characteristics of the bacteria. Upon transient expression of the lysis protein, bacterial growth is on average much lower than under constant expression. It appears as if bacteria are somehow able to acclimate to the prolonged expression of the lysis protein via physiological changes, while still allowing them to grow and divide. To my knowledge this is the first example of such a behavior in bacteria ever observed with a lysis protein. It is possible that this behavior may be observed with other killing genes, or perhaps with antibiotics which have the same intracellular target as E.

Lastly, I described the results of investigating the behavior of the SLC across different scales and its evolution over time. These two properties of synthetic circuits are important because of their relevance to potential applications. For example, is the response of a genetic sensor strain to external inducer the same when looking at an isolated population in a microfluidic device as opposed to testing the same strain in a milliliter scale chemostat? Does a genetic oscillator strain behave the same in a small microfluidic growth chamber as opposed to a culture tube (or even a much larger microfluidic chamber)? There are environmental differences between these physical scales, like mixing and oxygen diffusion. Also, bacteria may encounter different physiological states in well mixed milliliter scale cultures as opposed to geometrically constrained microfluidic growth chambers (and even between different types of chambers, depending on their height and diffusion properties). With respect to evolution, the effects may be even more difficult to address. Circuit components introduced into bacteria present an added burden on the bacteria, and there is a fitness advantage to mutating the expression of the circuit (especially if it encodes for lysis, as is the case with the SLC). These questions regarding scale and evolution will become more relevant as researchers continue exploring applications of gene circuits.

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