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REVIEW

Control of synthetic gene networks and its applications

David J Menn, Ri-Qi Su and Xiao Wang*

School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ 85287, USA
* Correspondence: xiaowang@asu.edu

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***Background*: One of the underlying assumptions of synthetic biology is that biological processes can be engineered in a controllable way.**
***Results*: Here we discuss this assumption as it relates to synthetic gene regulatory networks (GRNs). We first cover the theoretical basis of GRN control, then address three major areas in which control has been leveraged: engineering and analysis of network stability, temporal dynamics, and spatial aspects.**
***Conclusion*: These areas lay a strong foundation for further expansion of control in synthetic GRNs and pave the way for future work synthesizing these disparate concepts.**

Keywords: synthetic biology; gene regulatory networks; modeling; GRN control; stochasticity

INTRODUCTION

Synthetic biology, since its flourishing in the early 2000s, has undergone rapid advancement. The is due in a large part to the promise that the field holds for such diverse applications as bioenergy [1–3], personalized medicine and therapeutics [4], bioremediation [5,6], and biopharmaceuticals [7]. Paired with advancements in DNA synthesis [8–10] and sequencing [11], the field has grown exponentially in the last 15 years. This growth has seen the development of new tools, such as the implementation of the Cas9 protein for gene editing and transcriptional regulation [12–14], to further plumb the depths of our biological understanding and the applications thereof. In particular, advancements in synthetic biology have allowed the study of gene regulatory networks (GRNs) in a simplified setting amenable for precise experimental controls [15,16].

Using a build-to-understand, bottom-up approach [17–19], synthetic biologists can strip away much of the complexity of highly interconnected natural biological systems while studying gene regulation within an *in vivo* [17,18] or *in vitro* system [20,21]. Synthetic networks which function orthogonally to natural networks give researchers more control over their behavior and avoid the confounding effects of the many unknown genetic interactions endemic to natural systems [22,23]. These functional synthetic networks have been used to demonstrate many fundamental biological processes such as multiple stabilities [18,24,25], complex temporal behavior [16,17,26,27], and rich spatial patterning [28–30]. Many of these small functional networks, often referred to as motifs, utilize positive or negative feedback topologies. For example, bistable GRNs can be constructed either through the use of two mutually inhibitory components [18] or with self-activating components [25]. From these small functional networks, great efforts have been made to build up, combining multiple motifs into larger and more complex networks [31–34]. In addition to realizing immediate applications for complex synthetic networks, engineered circuits also shed additional light on the underlying mechanisms of biological regulation and control [22,24]. For example, by constructing a symmetrical circuit expressing two different fluorescent proteins, Elowitz *et al.* demonstrated the existence of intrinsic and extrinsic stochasticity within a cell [22]. While Wu *et al.* illustrated impacts of such stochasticity on cell fate determination using a synthetic toggle switch in yeast [24].

This article is dedicated to the Special Collection of Synthetic Biology, Aiming for Quantitative Control of Cellular Systems (Eds. Cheemeng Tan and Haiyan Liu).

Control of GRNs has been a constant research focus and is of paramount importance to continued advancement in synthetic biology. In its most abstract sense, GRN control refers to methods by which researchers can engineer, modulate, and predict robust network behavior. In a physical sense, this entails proper selection of internal and external factors which influence network behavior. Internal factors function as a closed feedback control loop within the cell and include the selection of cell type, GRN topology and motifs, and specific components comprising the GRN: promoters driving individual gene's expression [8,27], transcription factors modulating expression of downstream genes [16,18], localization signals or synthetic protein domains affecting protein interactions [23,28,29], etc. Once put into the cell, many of these components do not leave researchers with a direct means of interaction. External factors form an open control loop and are therefore easier to control throughout the course of an experiment. These include factors such as growth media composition [8,24,35], ambient temperature [16,36,37], light exposure and wavelength [38,39], magnetic fields [36,40], or small molecule inducers which either bind surface receptors or permeate the cell to cause changes in protein behavior [8,16,18,23]. A crucial third component in GRN control is the theoretical framework that describes the predictability of the system and allows synthetic biologists to compose networks toward a desired outcome, rationally select components to achieve that outcome, and predict the parameters under which that desired outcome is attainable [41–43].

In the following sections, we first examine in close detail the theoretical basis of GRN control. We then use this framework to inform discussion on three aspects of GRN behavior: multi-stability, temporal dynamics, and spatial relations. Each of these areas is further explored with discussion of how the intrinsic and extrinsic biological control factors relate to the theoretical framework, problems faced in realizing these behaviors, and examples and applications of the behavior to broader aims.

THEORIES AND COMPUTATION OF CONTROL

The mathematical foundation of control theory has been well developed for both linear and nonlinear dynamical systems [44,45]. Its application to many biological fields, especially systems biology, has produced progress in both designing experiments and understanding results [46,47]. In synthetic biology, with its bottom-up design mindset, the functional motifs are well isolated from the cellular environment and thus provide better test platforms for control theories in biology. However, there are several challenges remaining in mathematically modeling and predicting gene network functions: genetic networks are often highly nonlinear, cellular environments and internal kinetics are stochastic, and natural genetic networks can have high dimensionality with unknown interconnectivity between genes.

Several approaches have been taken in addressing GRN nonlinearity. Ordinary differential equations (ODEs) are frequently used to model deterministic systems with the aim of obtaining a sketch of the underlying interactions and the effect of varying parameters within these systems [18,24]. The regulation of gene expression has often been described in the form of nonlinear Hill equations [48]. Many theoretical approaches for analyzing nonlinear GRNs [42,49,50] have borrowed from the large amount of work that has been developed around linear control theory [44,45,51]. These approaches have been adapted into several network simplification methodologies. By linearizing nonlinear Hill functions around an equibriumn point, Shin *et al.* studied the transfer function for simple GRNs and reproduced experimental results in continuous models [42]. Liu *et al.* proposed a linear control theory for large networked systems [49] and used it to analyze minimum control inputs in metabolic networks [50].

However, most of these theoretical approaches still cannot be directly applied to model and predict complex behaviors of GRNs, and an *ad hoc* model based on ODEs is still required for each specific system. After developing a system of ODEs to describe a GRN, bifurcation analysis is often employed to investigate how the network's deterministic behaviors change with system parameters. This can reveal parameter regions of multistability and phase transitions [23,24,51,52], as it is shown in Figure 1A. This is important for investigating networks which can have multiple states, such as toggle switches which can switch states in response to environmental stimuli [18,26], and it can also be applied to oscillatory systems [16]. This *in silico* method can be paired with hysteresis analysis, an experimental design which is used to probe dynamical systems without knowing their detailed dynamical form or parameters [18,53,54]. Hysteresis analysis involves performing experiments to investigate the parameter space and its effect on system stability. It is often paired iteratively with bifurcation analysis to further develop the model to more accurately describe the system in question [18]. Figure 1A demonstrates how the bistable region can be identified by hysteresis analysis without knowing exact system parameters.

ODEs and other deterministic methods have been invaluable in many GRN analysis applications; however, the strict determinism of these equations limits their application to cellular behaviors influenced by stochasticity [55]. Stochastic simulation tools, including the stochastic differential equation (SDE), cellular automata (CA), potential landscape [56], and Gillespie algorithm

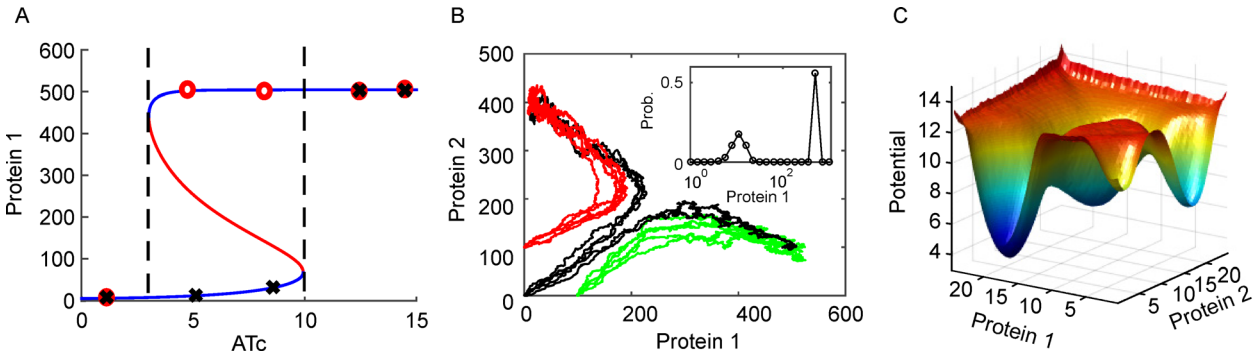

Figure 1. Mathematical frameworks for GRN Control. (A) Bifurcation diagram of the toggle switch controlled by the concentration of ATc [26] and illustration of hysteresis analysis. The blue lines represent the SSSs under a range of ATc concentrations, while the red line represents the unstable steady states. The black crosses are the predicted SSSs for the cell which is first grown in media lacking ATc then transferred to media with variable ATc concentrations, while the red circles represent SSSs for cells initially grown in high ATc concentrations (250 ng/mL). By performing hysteresis analysis, the bistable region can be identified without knowing the system parameters, denoted as the area between the two dash lines. (B) Temporal trajectories simulated using Gillespie algorithm for experiments in [24]. The black trajectories were initiated directly on the separatrix, while the red and green ones were not. The inserted panel shows the resultant histogram for trajectories which began on the separatrix and subsequently differentiated into two populations. (C) A pseudo potential landscape for systems with four SSSs, as shown in [41]. SSSs are represented by local minima within the parameter space, while the stability of any given state is represented by the depth of the energy well relative to its neighbors.

(GA) [57] are used to simulate and study inherently noisy processes within the cell [22,41,56]. Deterministic models fail in situations in which there are several potential outcomes from a common set of parameters and initial conditions, such as the stochastic differentiation from an undetermined state to one of two stable steady states (SSSs) shown in Figure 1B. When the trajectories are initiated from the separatrix dividing the two states' energy wells [24], they differentiate into one or the other population as a result of gene expression noise. The resulting distribution can be predicted using Gillespie algorithm. When the system's gene expression is perturbed by external stimuli, the overall expression distribution amongst various attractor basins can be quantified using SDEs or GA. Furthermore, the pseudo potential landscape can be portrayed from the stationary distribution, which provides better characterization of system stabilities and state regulation behaviors for the GRN [24,41], as shown in Figure 1C. The positions together with the stabilities of all SSSs of a system can be illustrated by the pseudo landscape. PDEs and GA can describe the stochastic interactions between different cells and simulate how the cell population is distributed in space [30,56,58]. In their study of synthetic ecosystems, Song *et al.* modeled the spatio-temporal dynamics of two synthetic *Escherichia coli* populations using PDEs [59,60]. On an intracellular level, GA (or the related Monte Carlo simulation) is often used to simulate stochastic fluctuations in transcriptional regulator numbers [24,61]. These types of simulations can be used to determine likelihood of state transitions under varying amounts of noise [52] or to thoroughly analyze the GRNs potential landscape under a single noise condition [56].

Though synthetic networks are, thus far, limited in size, understanding the regulation of cell differentiation and state transitions within a natural system requires computational tools capable of dealing with large dimensionality [62]. On one hand, researchers have tried to abstract the large scale GRNs into different motifs with varying functions [63]. They then tried to understand the relationship between the motifs' structure and cellular behavior [17,18,64]. On the other hand, PDEs and CA have also been applied to model gene expression distribution over time of high dimensional systems. For example, Wang *et al.* proposed a pseudo potential landscape based on the equilibrium distribution in state space of gene expression levels, and solved the equilibrium distribution using a PDE model [56]. CA models have been utilized to simulate complex stochastic interactions between cells. These are used frequently in tumor modeling, where individual cell behavior within a group is highly dependent on its immediate neighbors and environment rather than relatively simple chemical gradients [65]. The primary drawback of CA models is that they tend to consume large computational resources [30].

Studying control problems in GRNs will generally require the application of multiple theoretical tools at the same time. One prominent example which has important ramifications in many areas is how to control transitions between different SSSs in gene expression space. This is important in an area like cancer research, since cancer is

frequently characterized as cells which have fallen into an unhealthy but stable gene expression state [66]. With better control strategies, it may be possible to transition these genes' expression back to a healthy state via a novel route: a different tactic from what current therapies provide. To this end, Wang *et al.* used bifurcation analysis to identify possible transitions paths between different SSSs in multistable GRNs, and then suggested to model the GRNs as a network of attractors to reduce its dimensionality. Based on hysteresis analysis, they proposed transient and sequential control signals to navigate the state transition from an arbitrary cancer attractor to a health attractor [41]. Separately, work has been done on minimizing the effects of failed nodes within a larger network and on determining the best methods to limit large-scale cascading effects if single nodes display anomalous behavior [67]. Finally, ongoing progress has been made in developing frameworks for understanding and controlling genetic regulation and metabolic flux in complex biological networks [50].

MULTISTABILITY OF GRNs

Multistable systems can hold two or more stable gene expression profiles, SSS, with the same set of parameters. This ubiquitous property of natural systems allows isogenic populations to express a range of behaviors in response to their needs and environment [68]. In single-celled organisms, this division of labor can lead to increased population fitness. In bacteria, for example, often a sub-population can enter a competent state in which the uptake of foreign DNA is increased, allowing the bacteria to increase genetic diversity [69]. Similarly, some bacteria within a population may enter a state called persistence, in which the cell becomes dormant [70]. If a catastrophic event, such as contact with an antibiotic, wipes out the colony, these persistent cells can remain unaffected, thereby ensuring the colony's survival [71]. In multi-cellular organisms, the role of multistability is primarily to allow the development of a multitude of tissues from a single stem state [72].

Topologically, there are two general ways for a system to achieve multiple SSSs: mutual inhibition and auto-activation. In a mutually inhibitive GRN, the gene or genes associated with one state actively repress the expression of those associated with one or more competing genes and vice versa [18,73], as seen in Figure 2A. Synthetic mutual inhibition circuits have been

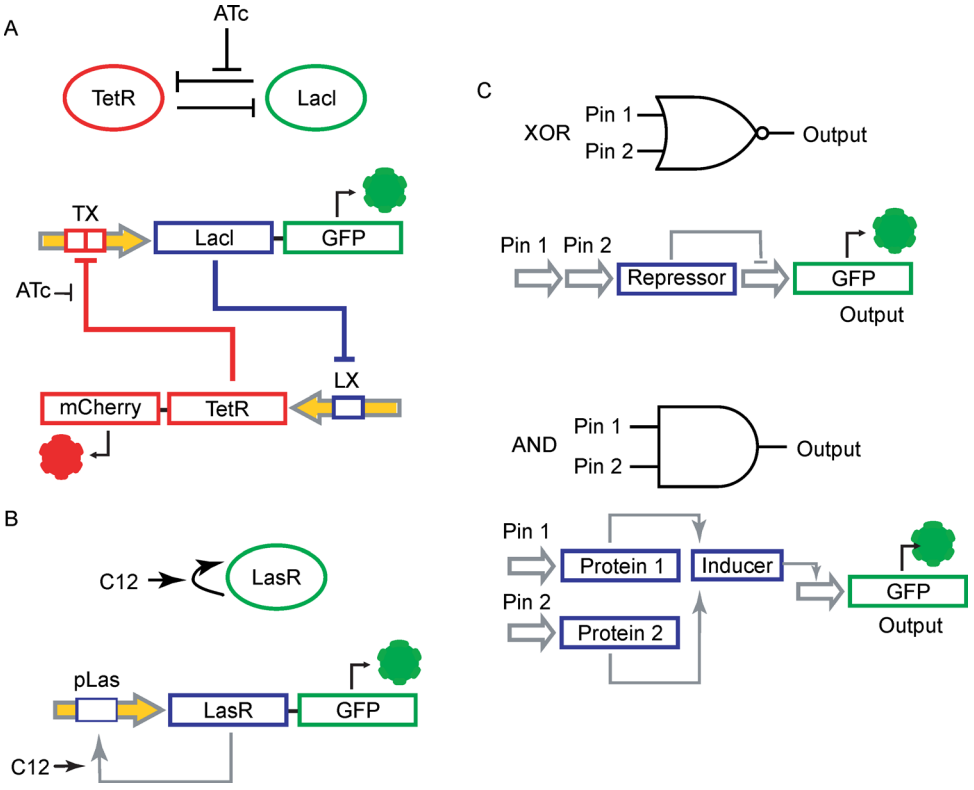

Figure 2. GRN multistable behavior. (A) Schematic diagram and simplified schematic for the mutual inhibition toggle [18]. (B) Another bistable circuit of autoactivation [74]. (C) Schematic diagram for two representative logic gates, the XOR and AND gates reviewed by Singh *et al*. [75].

demonstrated in multiple organisms [18,24,76], and examples of similar topologies are rife in nature [68]. A system can also express multistable behavior through autoactivation [74]. As illustrated in Figure 2B, a single gene can keep itself activated if its expression has passed a certain threshold; below this threshold, however, the gene remains inactive [77]. Self-activating motifs like this tend to be noisy on their own, but they can also play a stabilizing role to the expression of mutual inhibition GRNs [35]. In addition to these two primary topologies, multistability has also been shown to emerge from linear networks as a result of circuit load and other interactions affecting the growth rate of the host cell [78]. These host-circuit interactions may play an increasingly important role in future synthetic network design. From these basic underpinnings, several control problems have come to define the study of multistability.

First, researchers have sought to better understand and control the proportionality of differentiation into various SSSs. Wu *et al.*, studied the effect of both internal and external factors on differentiation into one of two states in a synthetic yeast network [24]. Using a novel design strategy, researchers positioned the cells' expression near the separatrix dividing the energy wells of mutually inhibitive red-expressing or green-expressing states. The stochastic process of gene expression then caused the cells to gravitate towards either the red or the green state. By changing the promoters driving the antagonizing repressors, and by changing inducer concentrations to alter the efficiency of those repressors, the percentage of cells falling into each state can be tuned. Both methods of controlling cell fate determination show how changing the underlying energy landscape of a multistable system can affect the behavior of the system itself. Ishimatsu *et al.* built on this foundation, using gene overexpression to force a bistable network into temporary monostability [35]. By tuning overexpression, the single steady state could be adjusted in state-space, and this adjusted location became the new initial point from which the cells would differentiate upon cessation of overexpression. By placing the cell expression near the system's separatrix, a tuning of the population fraction in each state was observed, similar to that demonstrated by Wu *et al.* [24].

This leads to a second area of study: how to control the transitions between states in multistable systems. Bifurcation analysis is a commonly employed method for investigating the parameter space in which a system can maintain multistability. On either side of the multistable region there is a bifurcation point: a parameter value at which one of the SSSs disappears or emerges. Using transient chemical or thermal induction, Gardner *et al.* showed that bistable networks could be switched between states by temporarily forcing them out of the bistable region [18]. Ellis *et al.* further demonstrated that the transition time between states could be both predicted mathematically and tuned through selection of different promoters from a synthetic library in order to temporally control the flocculation of yeast [26]. Unlike purely stochastic cell fate determination process used to tune population percentages, these experiments showed that full populations' expression could be controlled essentially deterministically with a high degree of accuracy, accounting for both expression levels and transition times.

Related to both of these areas of study is the control of a system's multistable region itself. Multistability generally occurs only within a small range of parameter and induction values. To engineer robust networks, expansion of multistable regions is crucial. This is partially determined by the network topology and relies on proper selection of network components. Using a library of synthetic promoters, it has been shown that the same topology can yield bistable regions responding to low, mid, or high levels of induction [24]. Additionally, the regulatory proteins used have a profound effect on hysteresis behaviors. In an autoactivation network, Wu *et al.* demonstrated that different pairs of activator and chemical inducers produced different bistable regions [23]. Interestingly, it was also demonstrated that pairing poorly interacting inducer/activator pairs — due to quorum sensing (QS) crosstalk in this instance — yielded an expanded toolbox of parts with a range of bistable regions to choose from.

As the physical construction of multistable networks has expanded, so too has the investigation of the theoretical underpinnings of stability. A large amount of *in silico* work has been dedicated to understand the topological basis of multistability. For example, Yao *et al.* identified a minimal circuit to generate bistability from a simplified Rb-E2F network which regulates the initiation of DNA replication [79]. Faucon *et al.* looked for instances of possible ways in which a three-gene network could exhibit multistability [52]. Additional work has been done to demonstrate the role that small motifs play in enhancing network stability [80].

Studies such as this focus on both how to attain multistability as well as on quantifying how stable the discovered multistable states are. Quantification of the stability of an energy well is still an evolving field, and stochastic simulations are often applied to determine how likely a cell is to jump out of a given SSS due to inherent noise.

Finally, there are also stationary synthetic circuits that are not multistable but have multiple outputs and can be used to control and integrate environmental and cellular signals. By layering multiple feedback systems, researchers have created digital logic gates, as reviewed in [32,33,75]. Researchers have demonstrated the ability to engineer AND, OR, NOR, XOR, NOT, and NAND gates

in multiple organisms [34,75,81–83]. Two examples of such logic gates are shown in Figure 2C. However, with increasing complexity comes increasing design constraints. Synthesis of two or more inputs can require engineering of new synthetic promoters capable of interacting with multiple proteins, and layered circuits require that upstream gene expression be clear enough that the signal translates into downstream expression. Regulator crosstalk can become a problem, as more parts are added to a circuit. Wu *et al.* designed an orthogonal AND gate in *E. coli* and studied the effects of regulators' crosstalk in autoactivated quorum sensing circuits [23]. The effect of integrating multiple module layers can be even more unpredictable, so additional design principles from engineering of digital control systems, such as timescale separation, have been introduced to overcome these obstacles. Mishra *et al.* designed a genetic device called the "load driver" to mitigate the interference between different genetic modules in *Saccharomyces cerevisiae* [84,85].

TEMPORAL DYNAMICS OF GRNs

While gene network stability is important for developmental processes, cell differentiation, and population fitness, dynamic temporal behavior is equally relevant to sustained biological processes. For instance, many cellular processes are informed by the oscillatory dynamics of the cell cycle [86] or by the daily circadian rhythm [87]. Additionally, certain sensory inputs are subject to the phenomenon of adaptation, in which a stimulatory signal has a reduced effect if introduced repeatedly within a short period [88]. Relatedly, a large amount of intercellular signaling is due to temporal bursts of activity, as seen in neuronal spiking [89] and the subsequent release of regulatory neurotransmitters [90]. To be able to engineer biological processes effectively, researchers need to be able to control the time scales together with the stabilities of these types of behaviors by utilizing internal and external control methods.

Oscillators were some of the earliest dynamic synthetic GRNs [17]. Most instances of oscillation have been shown to arise from two primary topologies: a three-node negative feedback loop known as the represillator [17,27] and a two-node network comprised of one autoactivator and one repressor [16] (see Figure 3A and 3B). In both topologies, the oscillator relies on two kinetic elements: negative feedback and a delay which grants enough time for one gene to turn on before being turned off by an antagonizing gene. To this end, control of oscillatory GRNs relies on tuning the negative feedback loop and/or the regulation duration, thus selection of GRN components and external regulators which alter degradation, production, and regulation kinetics play an important role in producing the desired oscillatory behavior. Much research has been done to modulate the frequency, stability, and synchronization of oscillations within a large population [16,17,27,91].

The negative feedback is the most critical component for generating oscillations. Since the kinetics associated with feedback loops can be described by nonlinear ODE functions, possible approaches to control the oscillatory behavior can be analyzed and predicted using bifurcation analysis across a range of parameters. Changes in

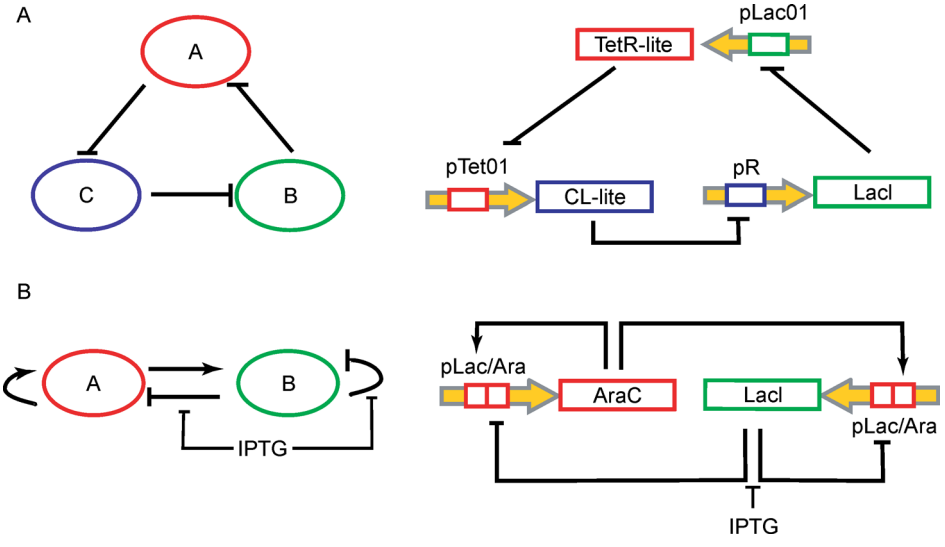

Figure 3. GRN temporal behavior. (A) Simplified illustration and schematic diagram of the represillator from [17] and [27]. The three nodes A, B and C jointly form a negative feedback loop. (B) Simplified illustration and schematic diagram of the two-node oscillatory network [16] with autoactivation on node A. The negative feedback strength can be controlled by the concentration of IPTG which regulates the repression strength from node B to A.

parameters can be caused by altering production efficiency of network genes, regulation strength between genes, concentration of inducers, and environmental conditions. As with multistability, bifurcation analysis can be utilized in conjunction with oscillatory network models to determine parameter ranges at which one is likely to observe oscillation, and it can give insight into the range and frequency of the expected fluctuations [92,93]. For examples, Stricker *et al.* introduced auto-activation into the negetive feedback circuit which enhanced the production activities in node A (See Figure 3B). Also, by changing the concentration of IPTG, which inhibited the repression of node A by node B, the regulation of negative feedback was be modulated, allowing further control of the oscillatory periods. It was also shown that the oscillatory period decreased when the temperature was increased due to a decrease in cell doubling time [16]. Additionally, together with the frequency, the ampitude of oscillations can also be controlled with relative plasmid dosage changes in mammalian cells [91]. The negative feedback loop can be extended to intercellular processes with the help of QS genes, which produce diffusible signaling molecules, and further achieve synchronization within a population. This sort of synchronization behavior has been modeled synthetically in bacteria [16], and it is a first step toward engineering large scale oscillation synchronization, an important aspect of multicellular life.

Time delay within the feedback loop is another critical element that generates oscillatory behavior in GRNs. A sufficiently long delay has been numerically demonstrated to be one of the required conditions to generate oscillations from a single autoinhibitory gene [94]. The time delay in the feedback loop arises from finite interactions and production time in stochastic gene expression and can be highly noisy, so the effect of delays needs to be analyzed via stochastic simulation tools. There are many ways to extend or shorten the delay to further control the period and robustness of oscillation. In their pioneering work of synthetic oscillatory GRNs, Elowitz *et al.* synthesized three cascading repressors into a represillator [17] and extended the delay duration by introduction of additional cascading processes. Genomic structure can also affect the delay; Swinburne *et al.* engineered oscillatory GRNs in animal cells and found that longer introns, which require longer production time, can increase the transcriptional delays, thus generating longer expression pulses [95]. The delay duration will also affect the stability of resulting oscillations. Potvin *et al.* reduced the delay in the original represillator by choosing low copy plasmids and thereby generated more stable oscillations while maintaining a minimal topology [27].

Another biologically relevant temporal behavior is that of spiking. This can be seen in neural signaling or in the response to certain sensory inputs, in which an external stimulus causes a short burst of activity before the system returns to a resting state. Adaptation is a dynamic behavior in which an extracellular signal causes the temporary excitation of a GRN which eventually returns to its basal level despite the signal's continuation [64]. Ma *et al.* exhaustively identified all possible three node GRNs topologies that can generate adaptation signals, and they found that the precision and sensitivity of adaptation can be independently modulated by tuning the system's parameters. Unlike oscillations, adaptation does not rely on a bifurcation for the desired behavior to appear; rather, it is a result of temporary perturbation of an otherwise stable network. Alternatively, spiking signals of neural systems exist in networks which operate close to a bifurcation point, so small environmental cues can push the cell into a region of either random or periodic spiking, depending on the network topology [96].

SPATIAL ASPECTS OF GRNs

In addition to the multistability and temporal aspects of GRNs, another active area of synthetic biology research is the exploration of the spatial properties of complex networks. Organization of individual cells into population wide patterns is a common behavior found throughout nature [28,97] and biomedical applications [29,98]. There are many open questions in controlling GRN regulated spatial patterns. In lower organisms, there have been two primary foci in exploring GRN spatial properties: pattern formation and population density control [97]. Besides their biological significance, synthetic GRNs in bacteria also serve as platforms to study how the cells communicate with each other and respond to the environmental signals. In higher organisms, the primary thrust in studying spatial patterning has been to better understand tissue and organ development [98]. Understanding how this emergent behavior can be engineered and controlled can lead to a better understanding of developmental processes, cellular signaling and signal processing paradigms, and construction of complex behavior from simple components.

Typically, synthetic GRN directed pattern formation requires three basic functional modules: a mechanism to send signals, a way to receive extracellular signals, and cellular actions responding to signals. Since spatial distribution and patterning is a population-wide phenomenon, intercellular signaling is required. It has been demonstrated by using QS genetic components [97] that bacterial populations can be engineered to form patterns in response to extracellular concentrations of acyl-homoserine lactones (AHLs), a class of signaling molecules used by QS [28]. Once received, the signal is

then processed into a stable output phenotype or behavior, typically through a negative feedback topology. When the signal is being processed by the synthetic GRNs the cells can respond by utilizing one or more mechanisms of cell movement [29], proliferation, and/or death [99,100] in order to form the intended patterns.

The control schemes used in cell-cell communication involve how the specific intercellular regulator molecules are added to the system (external addition or internally produced by the GRN) [28]. By controlling the local concentrations AHL, Basu *et al.* used programmed synthetic GRNs to form different pattern shapes. They employed co-cultures of engineered sender and receiver cells. The sender cells were designed to synthesize AHL under user defined gradients, while the receiver cells were engineered to operate like a bandpass filter. By placing sender cells in different configurations, through their fluorescent outputs the engineered cells jointly expressed different shapes such as a bullseye, ellipse, heart, and clover. Important control variables within the network topology and genetic components used are also widely studied. There are currently two types of underlying GRNs that can sense environmental signals. One type of GRN, such as that in Figure 4A, senses morphogen gradients and expresses weaker with increasing distance from the region of highest concentration [28]. Conversely, Payne *et al.* developed a novel pattern formation schedule in *E. coli* equipped with intracellular autoactivation and intercellular negative feedback motifs, shown in Figure 4B [30]. By employing this mechanism, the pattern scale could self-organize into intended patterns without reliance on a morphogen gradient. The pattern could also be controlled by biological processes. Payne *et al.* discussed that the metabolic burden caused by the activated synthetic circuit could actually enhance the pattern robustness [30]. Liu *et al.* demonstrated control of the mobility of cells and further achieved periodic striped patterns, using the circuit shown in Figure 4C [29]. They synthesized a LuxR/LuxI module to synthesize and excrete AHL when the cell density was high, which in turn further activated expression of LuxR. Additionally, the LuxR-AHL complex drove the expression of lambda repressor and further regulated *CheZ* expression, so as to reduce the mobility of the *E. coli*. Integrating many of these concepts — feedback and feedforward motifs, intercellular communication, and nutrient consumption — Cao *et al.* showed that scale-invariant patterns could be produced [101]. This is of crucial importance for understanding development in higher organisms and a clear frontier in GRN construction.

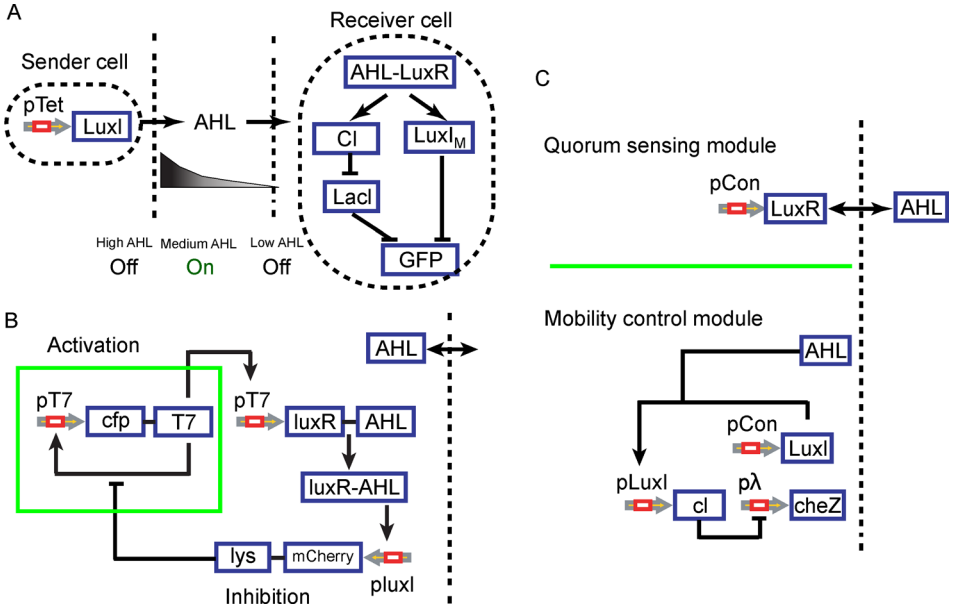

Figure 4. GRN spatial behavior. (A) Schematic diagram of the sender-and-receiver GRNs from [28]. The sender cells (left) were placed in the middle of the environment and contain a different GRN than the receivers , which surround the sender cells (right). AHL secreted by the sender cells has a concentration gradient depending on the distance from the sender cells, and it controls the response of the feedforward loop in the receiver cells (bottom). (B) Schematic diagram of circuit in [30]. The activation subunit forms an auto-activation circuit, as shown in the green box. The other portion of the GRN secretes AHL into the medium, which can diffuse back into the cell and induce inhibition of the sub-circuit of activation (outside the green box). (C) Schematic diagram for the mobility control GRNs in [29]. The upper layer is the quorum-sensing module which can secrete AHL into the environment, while the lower layer is the mobility control module which senses the AHL concentration and regulates the mobility of cells. High mobility will reduce the cell population density.

A similar application of pattern formation is to control population density. When bacteria are used for bioproduction applications, it may be beneficial to halt cellular growth to force the population to focus on producing the molecule of interest [102]. While pattern formation work has primarily taken place in bacteria plated in a dish, density control work seeks to understand and control the requirements for adjusting growth behavior in liquid culture. Similar topologies have been employed: cells were modified with LuxR/LuxI QS together with CcdA/CcdB toxin/antitoxin systems to study population density and individual fitness [102]. QS can also be controlled by changing the specific QS regulators employed, considering any crosstalk which might occur between QS components. Wu *et al.* demonstrated the important role that crosstalk between signaling molecules and transcriptional regulators (signal crosstalk) or between regulators and promoters (promoter crosstalk), as well as the overall expression intensity of QS components as determined by each component's promoter, can play in a synthetic system [23]. They also found that such regulation and crosstalk may induce novel host-circuit interaction in the QS system of LuxR/LuxI and LasR/LasI, and can be engineered to generate varying population dynamics.

CONCLUSION

There is much room for expansion in each of these areas, as synthetic biology is still very much a field in its infancy. Looking forward, there are several directions in which this work could be expanded. Topologically, GRN construction has primarily focused on using proteins to perform major functions. As we come to understand a wide variety of cellular regulatory mechanisms, we will likely see use of a more diverse set of cellular components. This is already the case with the rise of CRISPR technology, which uses RNA to guide circuit interactions; however, there are still major limitations in interfacing protein and RNA components in a single network. Beyond CRISPR, the toolbox may also be expanded to include other regulatory such as epigenetic marks like phosphorylation, methylation, and acetylation [103,104]. This could conceivably prepare the field to integrate chromatin interactions to stabilize desired behaviors, improving robustness in increasingly complex GRNs.

In addition to expanding the component toolbox, moving from proof-of-concept GRNs to development of networks for applications is likely in the near future for this field. Whether used for biosynthesis applications, understanding aberrant developmental processes, clinical diagnostics, or targeted genetic interventions, improved knowledge surrounding GRN control is sure to make an impact.

Control of GRNs in synthetic biology is a quickly expanding field covering all types of network behavior. Here we have provided examples of pioneering work on four key aspects of GRN control: its theoretical basis, stability analysis, temporal dynamics, and spatial distributions. Each aspect holds promise on its own and can be expanded into more complex, robust, and diverse applications. Beyond this, however, synthesis of these aspects of GRN control also promises powerful new tools for understanding and interacting with developmental processes, which innately possess multistable, temporal, and spatial properties.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors David J Menn, Ri-Qi Su and Xiao Wang declare they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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