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Programming Cells to Work for Us

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Abstract

The past decade has witnessed the rise of an exciting new field of engineering: synthetic biology. Synthetic biology is the application of engineering principles to the fundamental components of biology with the aim of programming cells with novel functionalities for utilization in the health, environment, and energy industries. Since its beginnings in the early 2000s, control design principles have been used in synthetic biology to design dynamics, mitigate the effects of uncertainty, and aid modular and layered design. In this review, we provide a basic introduction to synthetic biology, its applications, and its foundations and then describe in more detail how control design approaches have permeated the field since its inception. We conclude with a discussion of pressing challenges in this field that will require new control theory, with the hope of attracting researchers in the control theory community to this exciting engineering area.

1. INTRODUCTION TO SYNTHETIC BIOLOGY

Synthetic biology is a nascent, interdisciplinary research field at the intersection of many areas, including biotechnology, genetic engineering, molecular biology, biophysics, electrical engineering, control engineering, and evolutionary biology (1). One of its aims is to program cells—ranging from single-celled organisms (e.g., bacteria) to cell populations, tissues, and organs—for a variety of applications, including health (e.g., developing revolutionary cures for cancer and diabetes and reprogramming cell identity in regenerative medicine), the environment (e.g., biosensing and bioremediation), and energy (e.g., biofuels) (2, 3). In this section, we provide some brief background on synthetic biology and then describe some of its many applications that can potentially revolutionize the areas of health, the environment, and energy.

1.1. Background on Synthetic Biology

In this section, we provide a brief introduction to the history of synthetic biology and to the advancements that enabled the birth of the field. We then introduce the central paradigm used for modeling biomolecular processes.

1.1.1. Historical perspective. Synthetic biology is based largely on scientific advances in biotechnology that have occurred over the past 50 years, chiefly DNA cloning, amplification, and sequencing techniques, as well as the ability to insert extraneous DNA within a cell (transformation or transfection) (1) (**Figure 1*a***). Specifically, the discovery of DNA restriction enzymes in the late 1960s enabled DNA to be cut and pasted at targeted sites (4). In the 1970s, new technologies allowed the insertion of synthetic DNA into host cells (5). These scientific advances enabled one of the first applications of engineering biology—the production of synthetic insulin (6). The discovery of the polymerase chain reaction (PCR) (7) and DNA sequencing technology (8) in the 1980s made modification of DNA for insertion into cells quicker and easier. The construction of the first two synthetic genetic circuits—a ring oscillator (9) and a toggle switch (10)—in 2000 was based on these technologies. At this time, much work was focused on the combination of a few DNA parts to form simple circuits, with the aim of understanding the purpose of similar naturally occurring motifs (11) (**Figure 1*a***, subpanel *ii*). More recently, the field has progressed to a systems view of biological processes (12), focusing on creating larger systems composed of well-characterized parts and subsystems. To this end, intense research has gone into strategies for enabling modular and layered design (13) (**Figure 1*a***, subpanel *iii*). This research direction is important to set the basis for the rational design of systems that are sophisticated enough to solve real-world problems. Therefore, the community has made substantial efforts to create novel parts [e.g., CRISPR-based regulators (14)], characterize parts (15), provide insulation between modules (16), and enforce functional circuit modularity [e.g., against the effects of loads through the design of load drivers (17)].

1.1.2. Encoding programs on DNA through core biomolecular processes. A genetic circuit realizes its functionalities by encoding the production and subsequent interactions of biomolecules (such as proteins) on DNA sequences. Historically, early genetic circuits operated via transcriptional regulation, by which a protein x alters the rate at which another gene expresses its protein y (see **Figure 1*a***). Specifically, protein x can repress or activate the rate at which protein y is produced by binding to the promoter region upstream of the y gene and by recruiting or inhibiting gene expression machinery. In this sense, we can view a genetic circuit as a network of input/output (I/O) dynamical systems. Inputs and outputs represent the amounts of proteins (here, x and y),

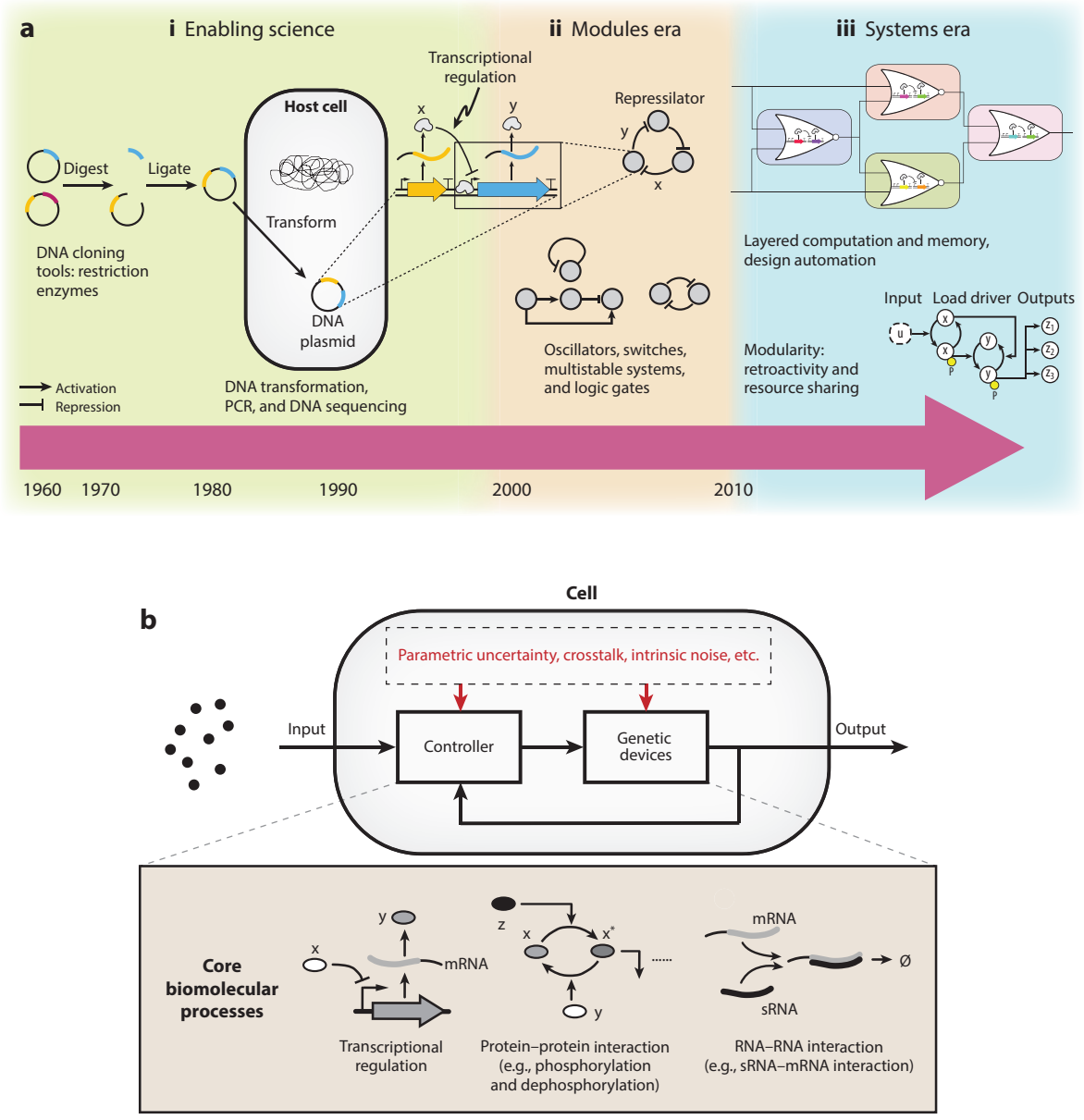


Figure 1

Overview of synthetic biology. (*a*) Synthetic biology has developed from key technological advancements in the 1960s to the systems approach used today. (*i*) Cloning allows for cutting (digesting) and pasting (ligating) pieces of DNA together, enabling one to encode a circuit on a DNA plasmid. (*ii*) The first synthetic systems were simple modules performing tasks such as oscillations and switching. (*iii*) The construction of more complex circuits is based on a modular or layered design approach. (*b*) Core biomolecular processes, including transcriptional regulation, protein–protein interaction, and RNA–RNA interaction, can be exploited to build genetic circuits in vivo. In transcriptional regulation (*left*), protein x alters the rate at which protein y is expressed. In phosphorylation (*center*), kinase z transfers a phosphate group to substrate x, resulting in a conformational change of the substrate to become active (x*); in the complementary process of dephosphorylation, phosphatase y removes a phosphate group from the active substrate. In small RNA (sRNA)–messenger RNA (mRNA) interaction (*right*), sRNAs can bind with their target mRNAs to expedite their degradation and/or inhibit translation. Additional abbreviation: PCR, polymerase chain reaction.

and each subsystem (node) in the network represents the dynamical process of protein production from DNA (**Figure 1*a***, subpanel *ii*). Any gene, including a synthetic one, utilizes the cell's built-in machinery to create proteins. First, RNA polymerases read the gene sequence and create a mirrored messenger RNA (mRNA) through a process called gene transcription. The mRNA is then read by another cellular enzyme known as the ribosome to create the amino acid chain that forms the protein, a process called mRNA translation. This dynamic process of protein production from DNA is known as the central dogma of molecular biology (18).

The process of transcriptional regulation has been studied at length, and well-characterized mathematical models are available (19). When molecular counts are sufficiently high, the simplest mathematical model uses ordinary differential equations to describe the protein and mRNA concentrations. Referring to protein x repressing protein y in **Figure 1*b*** and using m and y (in italic) to represent the concentrations of protein y's mRNA and protein y, respectively, we can write the dynamics as

$$\frac{\mathrm{d}}{\mathrm{d}t}m = \alpha \frac{1}{1+(x/k)^n} - \delta m \qquad \frac{\mathrm{d}}{\mathrm{d}t}y = \beta m - \gamma y, \qquad 1.$$

where α is the maximum rate of transcription and k is the dissociation constant between protein y's DNA and protein x. Stronger binding affinity between the two molecules can be represented by a smaller k value. Parameter n describes the number of x molecules that must bind together before they can act to regulate expression of protein y, also known as cooperativity. Parameters δ and γ represent the mRNA and protein decay rate constants, respectively, that result from dilution (arising from cell volume increase as the cells grow) and/or degradation (by degradation enzymes in cells), and β represents the translation rate constant. This model can be derived from chemical reactions under suitable quasi-steady-state assumptions (19).

In addition to transcriptional regulation, a variety of other biomolecular mechanisms regulate protein activities in nature and have recently been engineered for synthetic biology applications. A large portion of such regulations are carried out through protein–protein interactions, including allosteric modification and covalent modification (19). One of the most common types of covalent modification is the process of phosphorylation, illustrated in **Figure 1*b***. Phosphorylation and dephosphorylation dynamics are much faster than gene expression and can be used in genetic circuits where rapid responses are required. This property has been exploited, for example, to design biomolecular insulation devices (see Section 4.1). Other common types of protein–protein interactions that have been successfully engineered include allosteric regulation, phosphotransfer, and regulation of protein degradation. Reference 19 provides detailed descriptions of these core processes.

An increasing amount of experimental evidence since the 1990s has suggested that RNAs are functional not only as messengers between DNA and proteins, but also as important regulators for gene expression (for a review, see 20). For example, many regulatory small RNAs (sRNAs) have been identified in bacteria, where they are involved in a variety of adaptive responses (20, 21). As shown in **Figure 1*b***, most commonly, sRNAs can bind with their target mRNAs to expedite their degradation and/or inhibit translation. Quantitative modeling of sRNA-mediated regulation has revealed distinctive features compared with transcriptional regulations, such as faster responses and switch-like behaviors (22). RNA-mediated regulations are also prevalent in eukaryotes, where single-stranded microRNAs (miRNAs) inhibit mRNA translation and double-stranded short interfering RNAs (siRNAs) can cleave mRNAs (20). Finally, the advent of CRISPR/Cas9 technology has provided another class of highly efficient tools to perform gene regulation through guide RNAs (14). However, although initial experimental results have achieved remarkable success, mathematical characterizations of these processes are still largely lagging behind.

1.2. Applications of Synthetic Biology

In the past two decades, researchers have built genetic circuits to address a variety of societal problems, including in the health, environment, and energy industries. Here, we describe a few success stories that may be particularly interesting to systems and control engineers.

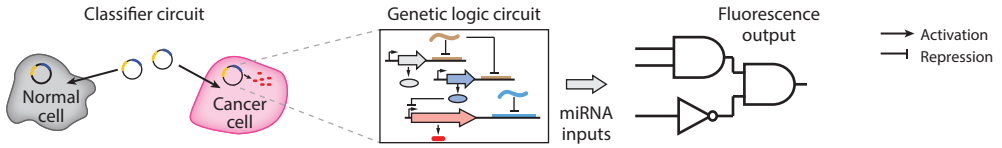
1.2.1. Health. Synthetic biology can revolutionize disease diagnosis and treatment. Synthetic genetic circuits can sense the intracellular concentrations of multiple molecular species, carry out logic computations through biomolecular reactions, and output a visible signal (e.g., a fluorescent reporter protein) when a set of logical conditions are met. For example, these logical conditions can be specified to recognize the chemical signature of cancerous cells to trigger a number of actions (23) (**Figure 2*a***). Similar circuits provide a promising approach to reduce invasive tests for diagnosis and health monitoring (29, 30). Programmed bacteria can also serve as smart vehicles for drug delivery by lysing at the tumor site and periodically releasing therapeutic proteins to reduce tumor activity (24) (**Figure 2*b***).

Synthetic biology also provides powerful tools to program T cells, a type of body immune cells, to specifically attack cancer cells. This type of treatment, known as immunotherapy, has recently been demonstrated to be successful in clinical trials (25). As shown in **Figure 2*c***, synthetic receptors engineered on T cells, possibly combined with biomolecular logic gates, can identify cancer cells with high specificity. Synthetic genetic controllers may then interact with the cellular chemotaxis pathway to migrate T cells to tumor sites and regulate the duration and strength of T cell activity to protect noncancerous cells (26).

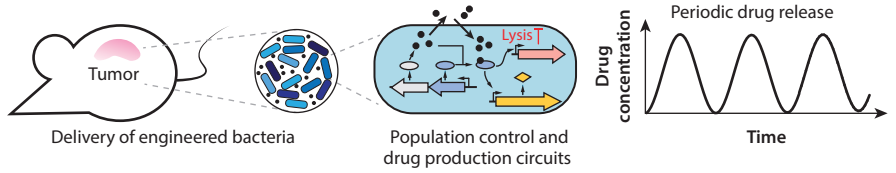
Both theoretical and experimental studies in synthetic biology enhance our understanding of natural systems, including cell differentiation and cancer biology (31–33). For instance, such understanding can provide unprecedented tools to reprogram cell fate for regenerative medicine (27, 34). Saxena et al. (27) designed a reprogramming circuit that converts pancreatic progenitor cells derived from human induced pluripotent stem cells into insulin-secreting beta-like cells by strictly regulating the timing and expression of three key transcription factors in vivo (**Figure 2*d***). Consequently, it has become possible to implant functional beta cells in diabetes patients that are derived from the patient's own tissue cells.

1.2.2. Environment and energy. Programming microbes to detect and report toxicants in water, air, soil, and food was one of the earliest applications of synthetic biology. To create an environmental biosensor, genes encoding the reporter proteins and proteins that carry out logic computation are artificially brought under the control of the sensory-regulatory system of the host cell (35). This design technique has been utilized to detect TNT, heavy metals, and antibiotics (for a comprehensive review, see 35). More recently, sensors that produce a dynamic output have been developed. **Figure 2*e*** illustrates a bacterial biosensor that produces oscillatory fluorescence output, the magnitude and frequency of which reflect the concentration of arsenic in the environment (28). In addition, microbes can be programmed to remove contaminants, including heavy metals and organic pollutants for bioremediation (36, 37). Microbes may also be programmed to convert biomass feedstock into biofuels (38), and synthetic controllers have been implemented to improve productivity (39, 40) (see Section 3.2). Finally, biosafety is also a concern for mass application of microbial biosensors, as they may escape and proliferate. To ease concerns about this safety issue, genetic toggle switches (see Section 2.1) have been engineered so that the host microbes survive only under specific conditions (41).

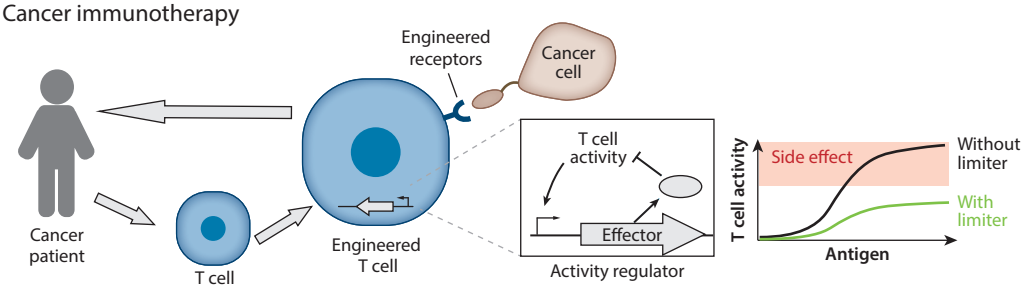
a Ex vivo cancer diagnostic



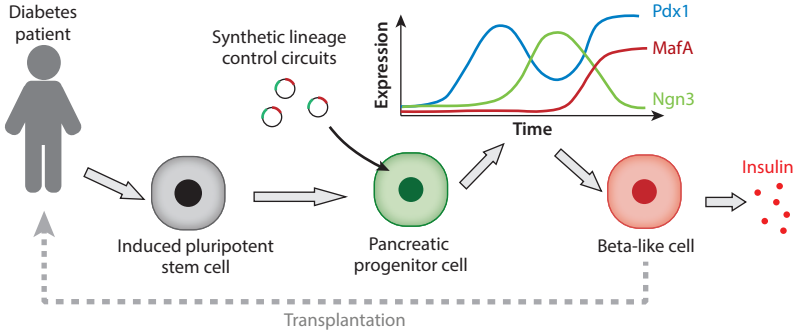
b Periodic drug release



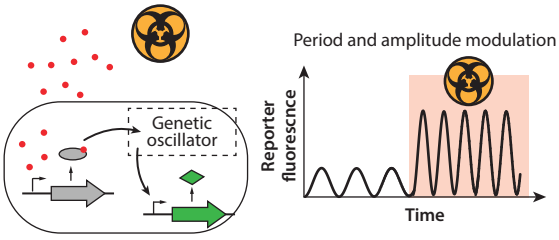
c Cancer immunotherapy



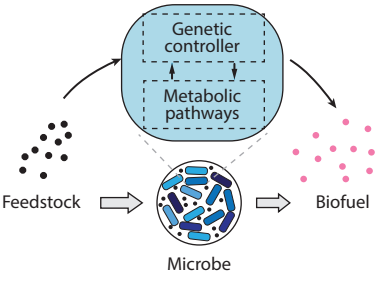
d Diabetes treatment/cell fate decision



e Hazard detection



f Biofuel production



1.3. Control Design for Synthetic Biology

Feedback control has permeated synthetic biology since its inception. In fact, the first two circuits built, which marked the beginning of the field in the year 2000, used feedback to design dynamics. The ability to design dynamics is one of the several celebrated applications of feedback control in traditional engineered systems. Feedback makes an unstable system stable at a desired attractor by virtue of interconnections that result in closed-loop dynamics that modify natural behavior [e.g., highly agile, open-loop unstable aircraft (42)]. One example is the repressilator, which used negative feedback along with a sufficiently large phase lag to create an oscillating system (9). By contrast, the toggle switch used positive feedback along with the nonlinearity of the steady-state I/O characteristic to obtain a bistable system that can hold two different states in memory (see Section 2).

Synthetic genetic circuits are subject to several perturbations (e.g., noise and changes in temperature and cellular chassis) and uncertainty (e.g., 10–100× uncertainty on the parameter values). Managing uncertainty is a crucial ability in any engineered system. Feedback allows for high performance in the presence of uncertainty by comparing actual and desired output values through accurate sensing [e.g., repeatable performance of amplifiers with 5× component variation (43)]. In synthetic biology, negative feedback and feedforward control implementations, shown in **Figure 1*b***, have been used throughout to mitigate the effects of unknowns (see Section 3).

In the transition from the modules era to the systems era (**Figure 1*a***), the ability to perform modular design has become critical. Maintaining modularity is a remarkable achievement of feedback. Feedback can enable a system to maintain its I/O properties when connected and thus provides simplified abstractions for higher design layers. Feedback enables layered design abstractions by hiding the details of complex dynamics and uncertainty [e.g., Black's amplifier design (43)], so that a designer may ignore a system's internal structure and only reason about its I/O properties. Insulation devices in synthetic biology provide one example of the use of high-gain negative feedback to aid modular composition by buffering interconnected systems from impedance-like effects (17, 44) (see Section 4.1). We describe these applications of control-theoretic concepts to synthetic biology in detail in the next several sections.

2. FEEDBACK CONTROL TO DESIGN DYNAMICS

In this section, we describe several synthetic genetic circuits whose design and analysis are enabled by theoretic tools from control and dynamical systems, including genetic multistable systems and oscillators.

Figure 2

Applications of synthetic biology to health, the environment, and energy. (*a*) A cell type classifier circuit used for cancer diagnosis ex vivo (23). A reference profile of microRNAs (miRNAs) that are expressed in cancer cells is used to construct a genetic logic circuit realized through RNA interactions. When transfected into a cancer cell, the output of the logic circuit triggers the expression of a fluorescence protein. (*b*) Bacteria as smart drug delivery vehicles (24). A consortium of engineered bacteria is delivered to the target tumor site. Each cell contains a genetic clock, a cell lysis gene, a therapeutic protein production gene, and a cell–cell communication module. The synchronized clocks control cell lysis to release the therapeutic proteins periodically. (*c*) A synthetic genetic circuit for cancer immunotherapy (25, 26). Receptors can be engineered to trigger T cell activity when cancer cells are detected. Feedback loops can be used to regulate T cell activity to avoid side effects. (*d*) A synthetic lineage control circuit. Regulating the expression of three transcription factors according to a temporal pattern enables human induced pluripotent stem cells to be reprogrammed into insulin-secreting beta-like cells for diabetes treatment (27). (*e*) A biosensor that detects the presence of arsenic and indicates the amount by modulating the output period and amplitude of a genetic circuit (28). (*f*) A synthetic genetic controller that interacts with engineered microbial metabolic pathways to increase biofuel productivity.

2.1. Multistable Systems

Multistable systems are generally useful in enabling a system to maintain a particular state after the input is removed. One notable example is the toggle switch (10, 45), a circuit in which two proteins, x_1 and x_2, mutually repress each other (**Figure 3*a***, subpanel *i*). Under appropriate conditions, this circuit exhibits three steady states—two stable and one unstable. A simplified model governing the toggle switch's dynamics is

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \frac{\alpha_1}{1+x_2^n} - x_1 \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \frac{\alpha_2}{1+x_1^m} - x_2, \qquad 2.$$

where x_1 and x_2 represent the concentrations of proteins x_1 and x_2, respectively; α_1 and α_2 represent their maximal production rates; and n and m represent the cooperativity of proteins x_1 and x_2, respectively. Analytical conditions under which the system displays multistability can be given using this model. For example, the production rates α_1 and α_2 must be approximately balanced (10). More recently, the toggle switch has been used as a critical element in more complex circuits for applications such as biocontainment removal (41) and biosensors (28). Toggle switches may also be used in "digital" logic systems to maintain memory (49). These applications lead to the requirement to construct toggle switches with a faster switching time and lower metabolic burden, which are still among the current design challenges (50).

Multistable systems are frequently found in natural gene regulatory networks pertaining to cell fate determination (31, 51, 52), which is typically thought of as a potential landscape in which different potential wells correspond to different cell types (52, 53). **Figure 3*a***, subpanel *ii*, shows a popular motif in cell fate decision, which has three stable steady states (54). Major challenges in the control of natural multistable systems arise as complexity grows, including the need for methods that can trigger transitions to desired steady states to artificially reprogram cell identity (34).

2.2. Oscillators

Oscillators are prevalent in natural systems and are critical for multiple functions, such as the circadian pacemaker (55) and the timing of metabolism (56). Several synthetic genetic oscillators were constructed in the early–mid-2000s, with the initial goal of understanding nature's "design principles" of timekeeping (9, 45, 47). Lately, these circuits have found application in proposed novel cancer therapies based on synthetic biology to enable periodic drug release and in environmental sensing to determine the concentration of pollutants (Section 1.2).

The first synthetic oscillatory circuit built was the repressilator (**Figure 3*b***, subpanel *i*) (9). This circuit consists of three genes arranged in a ring configuration, with the protein produced by each gene repressing production of the protein produced by a downstream gene. Using x_1, x_2, and x_3 to represent the concentrations of the three proteins and, for simplicity of presentation, assuming the circuit is symmetric (i.e., identical parameters for all three genes), we can model the repressilator by

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \frac{\alpha}{1+x_3^n} - x_1, \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \frac{\alpha}{1+x_1^n} - x_2, \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_3 = \frac{\alpha}{1+x_2^n} - x_3, \qquad 3.$$

where α represents the maximal protein production rate constant and n is the cooperativity of the protein. In the original paper, mathematical analysis indicated that the unique equilibrium point of this system can become unstable provided α and n are sufficiently large, leading to a stable limit cycle (9).

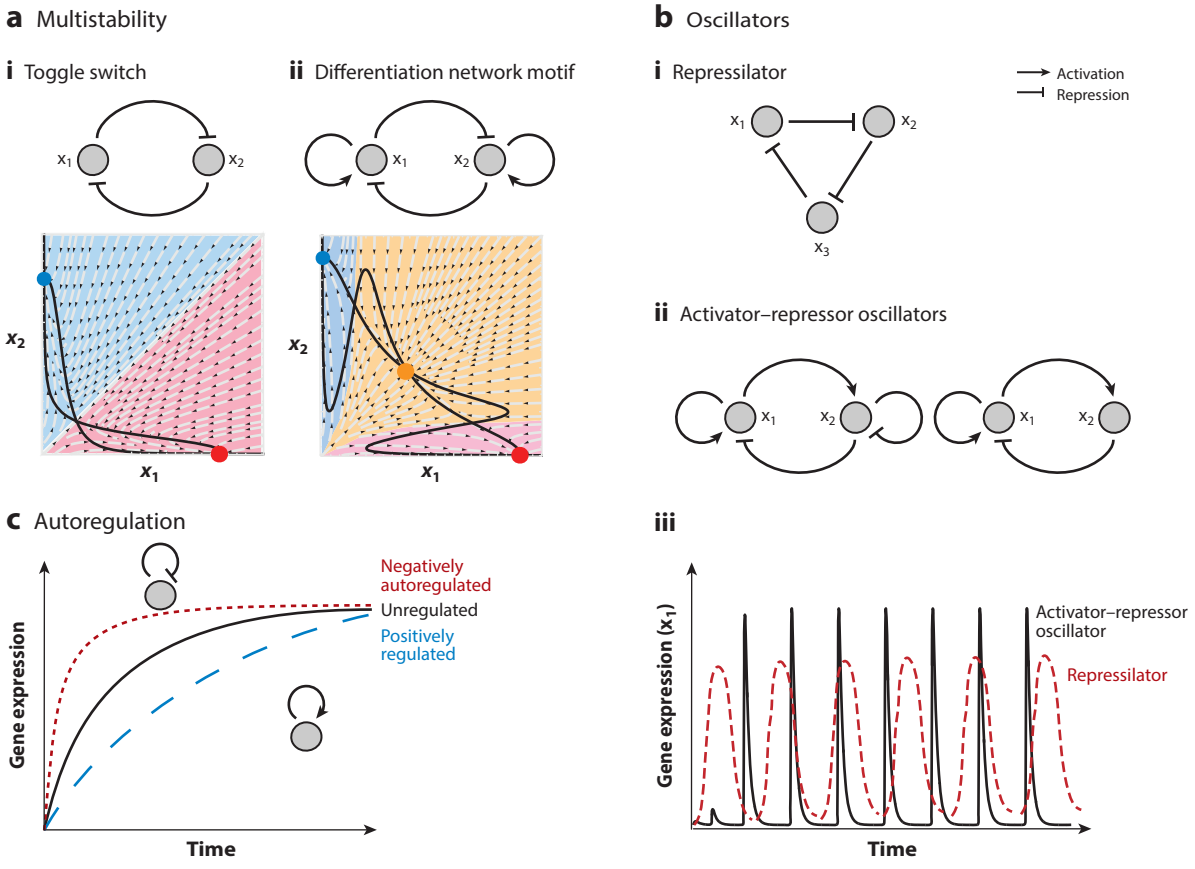


Figure 3

Feedback for designing dynamics. (*a*) Multistability in genetic circuits. Circles on the phase plot represent stable steady states, solid black lines are nullclines, and colored regions represent the regions of attraction of the respective stable steady states. (*i*) Circuit diagram and phase plot of the genetic toggle switch built in 2000 (10). (*ii*) Circuit diagram and phase plot of a tristable differentiation network motif built in 2017 (46). (*b*) Synthetic genetic oscillators. (*i*) The repressilator circuit built in 2000 (9). (*ii*) The activator–repressor oscillators built in 2008 (47) (*left*) and 2003 (45) (*right*). (*iii*) Sample trajectories of the oscillators. (*c*) Autoregulation shaping the temporal response of gene expression. The negatively autoregulated gene (48) (*dotted line*) has a shorter rise time than the unregulated gene (*solid line*) and positively regulated gene (*dashed line*), the latter of which has the slowest rise time.

Another class of synthetic oscillators are constructed based on a combination of activation and repression between two genes. As shown in **Figure 3*b***, subpanel *ii*, these circuits consist of protein x_1 activating both protein x_2 and itself (45) and protein x_2 repressing either only itself or both protein x_1 and itself (47, 57). A model for these activator–repressor oscillators is given by

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \frac{\alpha_1 x_1^n + \beta_1}{1 + x_1^n + x_2^m} - x_1 \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \frac{\alpha_2 x_1^n + \beta_2}{1 + x_1^n + c\,x_2^m} - x_2, \qquad 4.$$

where x_i represents the concentration of protein x_i for $i = 1, 2$; α_i represents the maximal production rate of protein x_i; and β_i represents the basal production rate of protein x_i. Here, $c = 0$ for the motif of Reference 45. One can derive parametric conditions under which this system displays a unique unstable equilibrium that is not a saddle, which guarantees oscillations

(58, 59). **Figure 3*b***, subpanel *iii*, shows sample temporal traces of the repressilator and of an activator–repressor oscillator.

While oscillators found in biological systems are remarkably robust (56), many synthetic oscillators are sensitive to parametric uncertainty and stochasticity, leading to poor predictability of design (45, 60). Therefore, the community is still actively seeking design principles for robust oscillators. Such efforts have been facilitated by (*a*) theoretical advancements that provide refined conditions for oscillations [e.g., the secant condition for cyclic systems (61)] and (*b*) novel biotechnological tools to robustify circuits [e.g., synchronized oscillators through cell–cell communication (62); see Section 6.3].

2.3. Speed of Response

Feedback may also be used to change the temporal response of a circuit. A simple instance of this is the use of negative autoregulation to speed up the response time of a genetic circuit (48). This is useful especially for biosensing applications, where faster response is desirable. By contrast, positive autoregulation slows down the response time compared with that of an unregulated gene (63) (**Figure 3*c***).

3. FEEDBACK CONTROL FOR ROBUSTNESS

Gene expression is inherently a noisy process (64). Theoretical and experimental studies have demonstrated that negative feedback can effectively increase the signal-to-noise ratio in genetic circuits (Section 3.1). While many biotechnological studies have attempted to standardize genetic parts (15, 65), their performance is often uncertain in practice. To solve this problem from an engineering perspective, negative feedback controllers can be implemented in vivo to increase circuits' robustness to model uncertainties (see Section 3.2). Finally, in Section 3.3, we review a synthetic feedback system recently constructed in *Escherichia coli* that enables gene expression to robustly track a dynamic input.

3.1. Feedback Control to Attenuate Noise

In practice, a population of genetically identical cells always leads to a distribution of protein molecular counts. Such heterogeneity (i.e., cell–cell variability) reflects the stochastic nature of gene expression. Both intrinsic and extrinsic noise contribute to stochasticity. Intrinsic noise arises from the randomness associated with biomolecular processes. For instance, binding and unbinding between molecules are innately probabilistic events. Extrinsic noise reflects the fluctuations in cellular components, such as enzyme quantity and gene copy numbers (64). These noise sources can substantially limit the precision to which genes are expressed. Furthermore, in large-scale circuits, noise propagation can significantly deteriorate circuit performance or even lead to complete circuit failure, as has been observed experimentally for a genetic cascade (66) and an oscillator (45). In multistable genetic circuits, noise can lead to random transitions among phenotypes (i.e., stable steady states) or to the creation of unexpected new states (31, 67, 68). While noise may be utilized by natural systems for differentiation and evolution (69), most of the research in synthetic biology has focused on reducing heterogeneity in engineered circuits. Many experimental studies of single-gene expression have demonstrated that negative feedback through transcriptional negative autoregulation is an effective approach to reduce noise in gene expression (70–72). These results are consistent with negative feedback's leveraged property of noise suppression in engineering systems and with negative autoregulation's repeated occurrence in natural gene networks (63).

3.1.1. Negative autoregulation suppresses intrinsic noise. To theoretically study gene expression in the presence of intrinsic noise, biomolecular reactions are often treated as discrete-state, continuous-time Markovian processes and modeled by the chemical master equations rather than ordinary differential equations (19). A simplified model of negative autoregulation consists of four chemical reactions (R_1–R_4 in **Figure 4*a***) that model the mRNA and protein production and decay. The probability that reaction R_i occurs during the interval $(t, t + dt]$ is quantified by $a_i(t)$. The fact that protein x represses its own transcription is described by the decreasing Hill-type function $k_m(x) := c/[1 + (bx)^n]$, where c is the basal transcription rate constant, b increases with the binding affinity between protein x and its own promoter, and n describes their binding cooperativity. Parameters δ and γ are the mRNA and protein decay constants, respectively, and k_p is the translation rate constant. Assuming that stochastic fluctuations are small, so that $k_m(x)$ can be linearized around steady-state average protein count $\mathbb{E}[\bar{x}]$, the steady-state coefficient of variation (CV_{in}) of x due to intrinsic noise can be computed (75) as

$$CV_{in}^2 = \frac{\mathrm{Var}[\bar{x}]}{\mathbb{E}^2[\bar{x}]} = \frac{k_p}{(\delta+\gamma)(1+\kappa)\mathbb{E}[\bar{x}]}, \quad \text{where } \kappa := -\frac{\mathbb{E}[\bar{x}]}{k_m(\mathbb{E}[\bar{x}])}\left.\frac{\mathrm{d}k_m(x)}{\mathrm{d}x}\right|_{x=\mathbb{E}[\bar{x}]} > 0 \qquad 5.$$

is the sensitivity of transcription rate $k_m(x)$ to the protein count x and can be effectively regarded as the feedback strength. As illustrated in **Figure 4*a***, it is immediate from Equation 5 that if expressions of two genes, gene 1 and gene 2, result in identical steady-state average protein counts ($\mathbb{E}[\bar{x}_1] = \mathbb{E}[\bar{x}_2]$), then the gene with stronger negative transcriptional autoregulation must have less cell–cell variability ($CV_{in,1} < CV_{in,2}$ if $\kappa_1 > \kappa_2$).

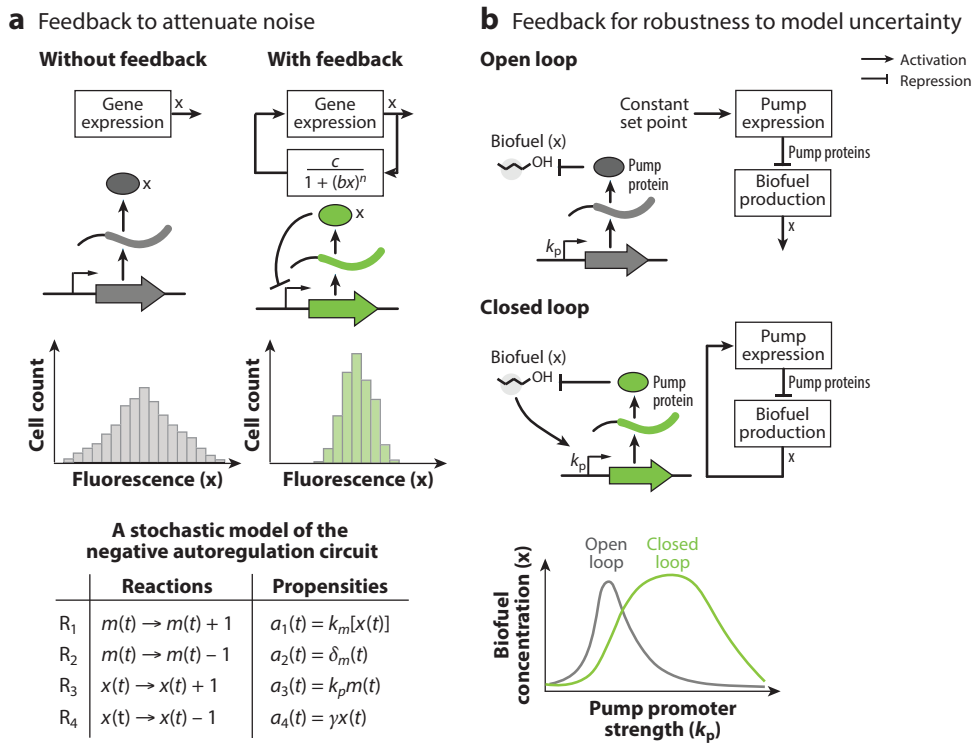
3.1.2. Negative autoregulation attenuates extrinsic noise. Experiments suggest that extrinsic noise often affects gene expression more significantly than intrinsic noise (76). The role of negative feedback in extrinsic noise attenuation is less subtle. This is because extrinsic fluctuations can be regarded as external inputs, and the ability of a negatively autoregulated gene to reject these noisy inputs can be inferred from its linear, deterministic approximation (70). In fact, using the mathematical tool in Reference 64, Shimoga et al. (72) were able to explicitly extract CV_{in} and CV_{ex} from experimental covariance data and found that negative autoregulation is much more efficient in reducing the effects of extrinsic noise than in reducing those of intrinsic noise.

Our current understanding of the relation between genetic circuit design and noise characteristics is largely limited to the benchmark problem of negative transcriptional autoregulation on a single gene. The repertoire of synthetic genetic circuits has rapidly expanded, but only a limited number of investigations of noise characteristics have been carried out at the system level (67, 75, 77). Developments in this direction are largely hindered by the lack of analytical tools to characterize circuits' stochastic properties, especially in the low-molecule count regime. There is a pressing need for analytical understanding of stochastic properties, especially as these unfold into the interconnection of I/O biomolecular processes (see, for example, 78, 79).

3.2. Feedback Control for Robustness to Uncertainty

Robustness to parametric uncertainty is a defining feature of negative feedback systems (80). Since most biological parameters either are difficult to measure or estimate or are highly sensitive to context, negative feedback can be applied to effectively improve circuit performance despite unknowns. In this section, we review a few biomolecular controllers designed toward this goal.

3.2.1. Transcriptional negative feedback reduces the sensitivity of biofuel production to parameter uncertainty. As illustrated in **Figure 2*f***, Dunlop et al. (40) proposed to apply negative feedback to improve the output from a synthetic biofuel production circuit. A major design



	Reactions	Propensities
R_1	$m(t) \to m(t)+1$	$a_1(t) = k_m[x(t)]$
R_2	$m(t) \to m(t)-1$	$a_2(t) = \delta_m(t)$
R_3	$x(t) \to x(t)+1$	$a_3(t) = k_p m(t)$
R_4	$x(t) \to x(t)-1$	$a_4(t) = \gamma x(t)$

Figure 4

Robust genetic circuit construction through in vivo feedback. (*a*) Transcriptional negative autoregulation decreases variability in genetically identical cells. (*b*) Negative feedback enables the output from a synthetic biofuel production circuit to be insensitive to parameter uncertainties, such as the promoter strength k_p, allowing productivity to be nearly optimal for a wide range of conditions. (*c*) A biomolecular integral controller (73) guarantees that the output of a circuit is robust to constant disturbances and parameter uncertainties. The controller uses two species, z_1 and z_2, to regulate the concentration of protein x_o to reference level u. (*d*) A biomolecular circuit was designed by Hsiao et al. (74) to track a temporal concentration profile. In the presence of a scaffold protein (input r), a histidine kinase (HK) can phosphorylate a response regulator (RR) to become RR*, which activates the production of output species y. The output contains an antiscaffold protein that can sequester the scaffold to reduce the input concentration.

trade-off in this circuit is that, although an increased number of efflux pumps (i.e., biofuel transporters) improves microbial tolerance to biofuel toxicity, overexpression of the pumps can lead to a reduction in cell growth, reducing population-wide biofuel output. As a consequence, in order to maximize biofuel production, the expression of efflux pumps must be regulated to an optimal level (40). Although this theoretical optimal pump expression level can be computed numerically, owing to uncertainty in system parameters and implementation, reaching it through fine-tuning of parameters is impractical. The authors thus numerically investigated whether a closed-loop circuit, where pump gene transcription is activated by the intracellular biofuel level, can outperform an open-loop circuit, where pumps are constitutively expressed, in the face of parametric uncertainty. When cellular biofuel concentration becomes too high, which hinders cell growth, pump gene production is activated to export biofuel, thus reducing toxicity to the host cell. As illustrated in **Figure 4*b***, the authors found that the closed-loop circuit can tolerate a much wider range of parametric uncertainty and still produce a near-optimal amount of biofuel. Since dealing with parametric uncertainty is a universal challenge for most biological systems, we expect that this advantage of negative feedback can be further exploited in other application scenarios.

3.2.2. Realizing integral controllers in living cells. In control design, parametric uncertainty is most effectively addressed using integral controllers (80). Assuming that the closed-loop system is stable, integral controllers can drive an unknown plant to reach a constant set point without steady-state error. These properties are particularly appealing to synthetic biology applications, where disturbances and uncertainties are often prevalent. In fact, integral control motifs have been identified in many natural biomolecular systems, including bacterial chemotaxis (81), calcium homeostasis (82), and yeast osmoregulation (83).

Recently, there has been increasing interest in synthesizing integral controllers in vivo to increase a genetic circuit's robustness to uncertainty and disturbances (73, 84, 85). In a theoretical study by Briat et al. (73), the authors proposed a type of integral controllers realizable through simple biomolecular mechanisms (see **Figure 4*c***). The integral controller consists of two controller species, z_1 and z_2, whose production rates are proportional to the concentration of input transcription factor u and the regulated output x_o, respectively. The controller species can bind with each other and degrade together according to the chemical reaction $z_1 + z_2 \xrightarrow{\theta} \emptyset$, where θ is the degradation rate constant. Biomolecular controllers of this type are named antithetic integral controllers, and their dynamics are

$$\frac{d}{dt}z_1 = u - \theta z_1 z_2, \qquad \frac{d}{dt}z_2 = x_o - \theta z_1 z_2. \qquad 6.$$

A linear transformation leads to the memory variable $z := z_1 - z_2$, the dynamics of which are the integral of the tracking error: $dz/dt = u - x_o$. Under suitable stability and reachability conditions,

the output of the regulated biomolecular process (x_o) can reach reference input u independent of parameters and constant disturbances (**Figure 4*c***). Physically, the dynamics in Equation 6 can be realized through, for example, RNA interactions (86) and σ/anti-σ factor interactions (73). Briat et al. (73) further showed that, even when the system operates with a small number of molecules (i.e., large intrinsic noise), the expectation of x_o is guaranteed to converge to the desired set point.

Another type of theoretically proposed biomolecular controller approximates integral action through the saturation of certain Michaelis–Menten-type kinetics (84–86). One such circuit, proposed in Reference 84, consists of activation of protein z (a memory variable) by transcriptional activator x_o (output protein) and a saturating amount of protease that degrades protein z, resulting in the following dynamics:

$$\frac{\mathrm{d}}{\mathrm{d}t}z = \alpha\frac{x_o}{x_o + k} - \gamma_{\max}\frac{z}{z + k_{\deg}} \approx \alpha\frac{x_o}{x_o + k} - \gamma_{\max}, \qquad 7.$$

where α is the maximum production rate of protein z, k is the dissociation constant between protein x_o and the promoter of protein z, $k_{\deg}$ is the dissociation constant between the protease and protein z, and $\gamma_{\max}$ is the maximum degradation rate constant with a saturating amount of protease. The approximation in Equation 7 is valid if $z \gg k_{\deg}$. Under this assumption, steady-state output $\bar{x}_o$ can be computed from $\alpha\bar{x}_o/(\bar{x}_o + k) = \gamma_{\max}$, whose solution is independent of any parametric uncertainty/disturbance in x_o dynamics. Satisfying this assumption, however, requires additional design considerations, such as engineering $k_{\deg}$ to be small (84).

Implementing integral controllers in vivo has tremendous potential to increase the robustness of genetic circuits and modularize their steady-state responses (87). Experimental characterizations of these integral controllers are in progress (88), but further theoretical studies to explore the fundamental limitations and design constraints of these biomolecular controllers are still needed (e.g., 86).

3.3. Robust Tracking

Less work has been devoted to designing biomolecular controllers that can achieve robust reference tracking, which is another important design objective in classical control theory. This is partly because the fidelity and resolution of time-course data in biomolecular systems have been limited, and, as a consequence, most of the current research has focused primarily on using feedback to achieve robust set-point regulation at the steady state (19). Nevertheless, we envision that feedback systems that track dynamic biomolecular signals will benefit genetic circuits with more versatile application-oriented functionalities in the near future.

Hsiao et al. (74) presented the design and implementation of a biomolecular concentration tracker. As shown in **Figure 4*d***, the reference input r to the circuit is the concentration of a scaffold protein, and the output y of the circuit is the concentration of an antiscaffold protein. A synthetic two-component system is utilized to actuate expression of the output. The two-component system consists of a histidine kinase donating a phosphate to the response regulator to become active in the presence of scaffold r. The active response regulator can then activate the expression of the antiscaffold (output y), which binds with the scaffold (input r). The antiscaffold (output) thus reduces the ability of the scaffold (input) to sequester the histidine kinase and response regulator to activate gene expression, closing the negative feedback loop. Hsiao et al. (74) demonstrated both numerically and experimentally in *E. coli* that the output can track a range of dynamic input, and the I/O gain of the tracker can be tuned efficiently in practice. Further analysis of this circuit has revealed that this sequestration-based negative feedback mechanism contains an approximate signal subtractor (89).

4. FEEDBACK CONTROL TO MAINTAIN MODULARITY

Modular and layered design is a convenient way to systematically create larger and more sophisticated systems (12, 90). A critical assumption in any modular design approach is that the salient I/O properties of a system do not change upon composition with other systems. Modularity allows for the design of complex systems by composing the I/O characteristics of elemental subsystems, without considering their internal details. Unfortunately, modularity is not a natural property of biomolecular systems, as their I/O properties depend on context, which includes both connections to and the presence of other systems. Direct connections create loading effects captured by the concept of retroactivity. The pure presence of a system can also affect the I/O properties of a different system because they compete for a limited pool of resources. Here, we review these system-level problems along with control-theoretic solutions proposed to address them.

4.1. Attenuation of Retroactivity

As in in electrical systems, impedance-like problems arise in biomolecular systems. In this section, we describe the problem of mitigating loading effects (retroactivity) at the interconnection of biomolecular modules.

4.1.1. Retroactivity. As shown in **Figure 5*a***, when an upstream system is connected to a downstream one, a signaling molecule generated in the upstream system becomes involved in chemical reactions in the downstream system. Because of this, the molecule becomes temporarily unavailable to the reactions that constitute the upstream circuit, resulting in a back effect on the upstream system that changes its dynamics. This loading effect on the upstream system is termed retroactivity and can be viewed as a disturbance signal s applied to the upstream system (91).

As an example, consider the interconnection of an upstream genetic clock (45) to a downstream genetic circuit (**Figure 5*a***). Letting A and R represent the concentrations of the activator and repressor proteins of the clock, the isolated clock dynamics are

$$\frac{\mathrm{d}}{\mathrm{d}t}A = f_A(A, R) - \gamma A, \qquad \frac{\mathrm{d}}{\mathrm{d}t}R = f_R(A) - \gamma R, \tag{8.}$$

where f_A and f_R are Hill functions describing transcriptional regulations between proteins A and R. When protein A becomes an input to the downstream system, it transcriptionally regulates the expression of a gene producing protein D by binding promoter sites $\mathrm{P_D}$. As a consequence, it is no longer available to the reactions constituting the clock's dynamics. Assuming that protein A binds with sites $\mathrm{P_D}$ according to $\mathrm{A} + \mathrm{P_D} \underset{\mathrm{k^-}}{\overset{\mathrm{k^+}}{\rightleftharpoons}} \mathrm{c}$, the dynamics of the connected clock become

$$\frac{\mathrm{d}}{\mathrm{d}t}A = f_A(A, R) - \gamma A + \overbrace{k^- c - k^+ A P_D}^{s}, \qquad \frac{\mathrm{d}}{\mathrm{d}t}R = f_R(A) - \gamma R, \tag{9.}$$

where $s := k^- c - k^+ A p_D$ is the retroactivity to the output, which, comparing to Equation 8, represents the effect of binding between protein A and sites $\mathrm{P_D}$ on the clock dynamics. As illustrated in **Figure 5*c***, while the isolated clock ($s = 0$) displays sustained oscillations, the connected clock no longer oscillates, and hence we fail to transmit the clock's signal to the downstream system (i.e., D does not oscillate) (92). Retroactivity therefore breaks modularity and renders layered design difficult. Effects of retroactivity have been experimentally demonstrated in both genetic circuits (17, 44, 93) and biomolecular signaling systems (94, 95). In these experiments, retroactivity can appreciably slow down upstream dynamics and/or change the steady-state I/O response.

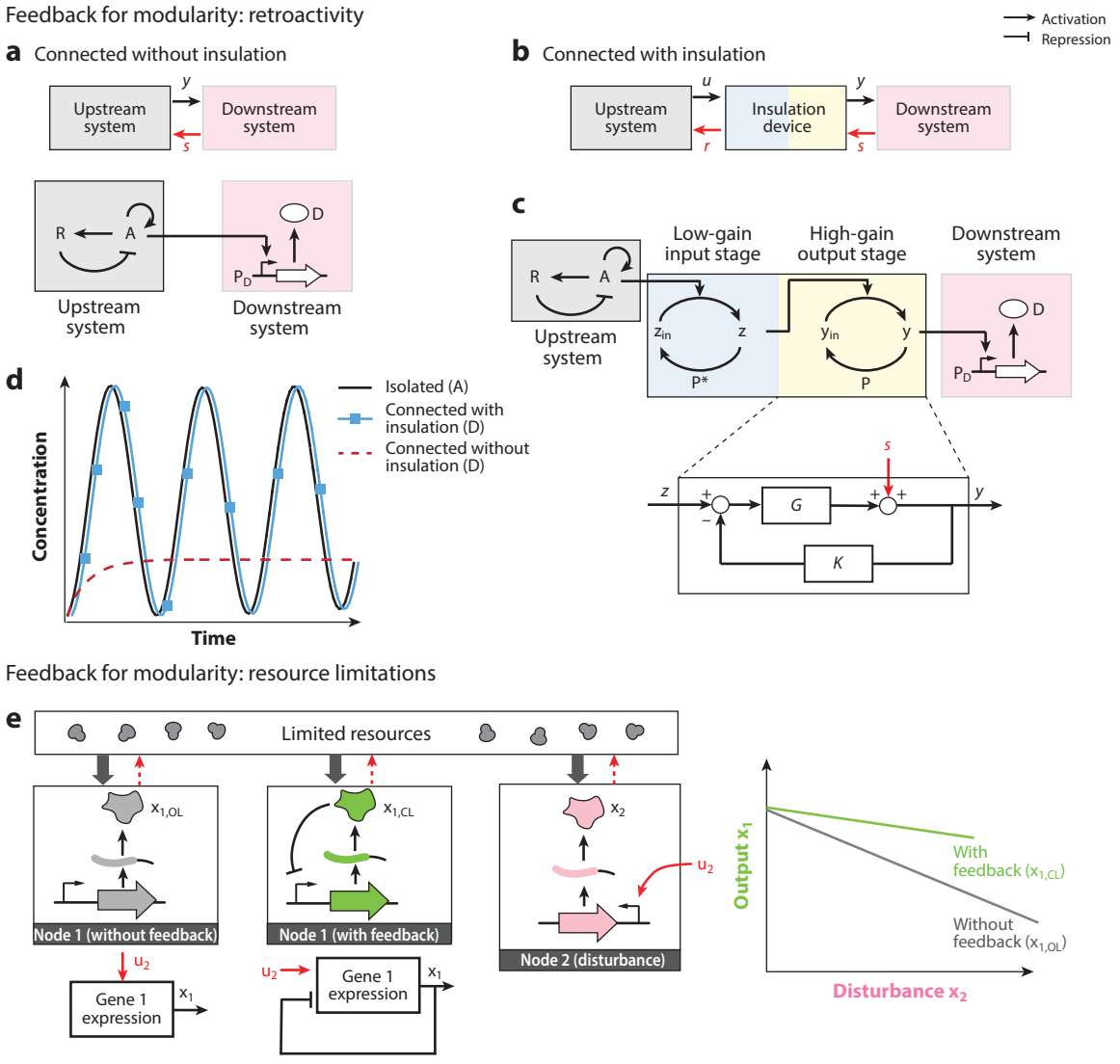


Figure 5

Modularization of genetic circuits using feedback control. (*a*) When an upstream system (e.g., a genetic clock) is connected to the downstream system (e.g., a reporter gene), a signaling molecule generated in the upstream system, y, binds to sites in the downstream system. The fact that some y molecules are sequestered by the downstream promoter introduces a loading effect on the upstream system, which can be viewed as a disturbance signal called retroactivity, s. (*b*) An insulation device can be placed between the upstream and downstream systems to allow faithful transmission of signals. Such a device attenuates the effect of s on y to allow y to track u and has small retroactivity to the input r so that u is not changed by loading. (*c*) A two-stage insulation device can be constructed from a cascade of two phosphorylation cycles. With large amounts of phosphatase P and substrate y_{in}, the output stage realizes high-gain negative feedback to attenuate disturbance s. The low-gain input stage uses timescale separation to mitigate potential loading effects imparted by the high-gain stage while ensuring low retroactivity to the input. (*d*) In the absence of the insulation device (panel *a*), the clock (upstream) dynamics are disrupted by loading. With the insulation device, the clock output signal is successfully transmitted to the downstream system. (*e*) When gene 2 is induced (by u_2), a disturbance is imparted to the expression of gene 1 since the production of protein x_2 uses RNA polymerases and ribosomes, reducing their availability to the expression of gene 1. The production of protein x_1 is thus affected by u_2. A gene with negative autoregulation is less affected by such nonregulatory interactions arising from resource competition.

4.1.2. Design of insulation devices to mitigate retroactivity. In order for the clock to transmit its signal to a downstream system despite potentially significant loading, we can place a special device between the clock and the downstream system, called an insulation device (**Figure 5*b***). An insulation device should be designed such that loading effects from the downstream system (i.e., retroactivity to the output, s) minimally affects y (i.e., y should track u independent of s), and it should have small retroactivity to the input, r, so that it does not affect the signal, u, that it receives from the upstream system. The result is that the signal of the upstream system, u, is faithfully transmitted to the downstream system, despite the possibility of imparting a large load.

If one regards s as a disturbance input to the insulation device, the requirement that y track u independent of s can be formulated as a disturbance attenuation problem, which can be solved using high-gain negative feedback (96). To illustrate this idea, we consider a negative feedback system subject to a disturbance input s, reference input z, and output y (block diagram in **Figure 5*c***). This diagram leads to

$$y = \frac{G}{1+KG}z + \frac{s}{1+KG}, \qquad 10.$$

from which $\lim_{G\to\infty} y = z/K$, which is independent of s. The high-gain negative feedback system in the block diagram of **Figure 5*c*** can be realized through a phosphorylation cycle (91). As shown in **Figure 5*c***, the cycle takes kinase z as an input to convert the inactive substrate y_{in} into active substrate y that regulates the downstream system. Phosphatase P converts substrate y back into y_{in}. In this system, the negative feedback is realized by phosphatase P, and the gain G is proportional to the total concentrations of the phosphatase and substrate (P and y_{in}, respectively). This design has been experimentally validated in Reference 44.

Implementing high-gain negative feedback through the above-mentioned phosphorylation cycle requires the substrate y_{in} to be present in large amounts. This design requirement creates a major trade-off because the presence of large amounts of y_{in} imparts a significant load to the input kinase, creating a large retroactivity to the input, r (97). To overcome this limitation, one can design a cascade of two phosphorylation cycles (**Figure 5*c***). The output stage is designed as before and is a high-gain stage. The input stage, by contrast, is designed to have a lower concentration of substrate z_{in} and phosphatase P* (low-gain output stage). Despite low substrate and phosphatase amounts, the input stage can still effectively attenuate retroactivity to its output arising from protein z binding to a large amount of protein y_{in}. This is because the dynamics of the phosphorylation cycle are much faster than protein expression, which determines the timescale of the input to the insulation device (e.g., A in **Figure 5*c***). In fact, a general theoretical result in Reference 98 states that the temporal effects of retroactivity can be attenuated by any biomolecular system with sufficiently fast dynamics compared with those of the input, which is consistent with fundamental studies of the relationship between high-gain feedback and timescale separation (96). Mishra et al. (17) constructed such a device in yeast, resulting in complete retroactivity attenuation (**Figure 5*d***).

4.2. Mitigation of Resource Competition Effects

Competition for a common pool of limited resources by multiple genes in a circuit results in unintended interactions. Here, we describe mathematical models that capture such interactions and discuss recent efforts to mitigate their effects.

4.2.1. Resource competition introduces nonregulatory interactions among genes. An important source of context dependence that has received much attention recently is the competition

for transcriptional and translational resources and machinery, chiefly for RNA polymerases and ribosomes. These resources are produced by the host cell, and their total concentrations can be regarded as conserved under constant growth conditions (99). To exemplify the effect of resource competition, Gyorgy et al. (100) and Shopera et al. (101) experimentally tested a simple genetic circuit composed of two nodes: node 1, producing protein x_1 constitutively, and node 2, producing protein x_2 under the control of transcription factor u_2. Contrary to expectations, they found that the steady-state levels of x_1 and x_2 were coupled and followed a linear relationship (see **Figure 4*b***), called an isocost line. This phenomenon can be explained by nonregulatory interactions among nodes due to resource competition: As the amount of u_2 increases, production of x_2 demands more resources, reducing the availability of x_2 to node 1, which consequently decreases the expression of x_1.

More generally, as demonstrated in Reference 102, in an n-node genetic circuit with the resource conservation constraint, the dynamics of node i can be written as

$$\frac{\mathrm{d}}{\mathrm{d}t}x_i = \frac{T_i F_i(u_i)}{1 + \sum_{k=1}^{n} J_k F_k(u_k)} - \gamma x_i, \qquad 11.$$

where $F_i(u_i)$ is the Hill function representing the intended regulatory interactions on node i by its own transcription factor input u_i and J_i is a resource demand coefficient, which is related to physical attributes of node i, such as its DNA copy number and ribosome binding site strength. As a consequence of Equation 11, the expression of each node is coupled to the expression of the other nodes, which may largely demolish a circuit's modularity. This model has been experimentally validated (102) to illustrate how the effective interactions in a genetic circuit can be determined by the superposition of intended regulatory interactions and nonregulatory interactions owing to resource competition.

4.2.2. Mitigation of resource competition effects through negative feedback. According to the model represented by Equation 11, for node i, we can regard resource demand by other nodes in the circuit as disturbances $d_i := \sum_{k \neq i} J_k F_k(u_k)$ that affect the I/O response from reference input u_i to output x_i. The idea of using negative feedback to modularize the I/O response of node i to resource competition has been theoretically explored in Reference 103 and experimentally investigated in Reference 101 for the simple circuit shown in **Figure 5*e***. In particular, because the product of gene 1 (x_1) has been engineered to repress itself, at steady state, the extent to which the steady state x_1 is coupled to x_2 decreases. It is unclear whether other biomolecular feedback controllers, such as the integral controllers discussed in Section 3.2, can mitigate the effects of resource competition more efficiently (86, 103) and whether the feedback strategy applies to other forms of competition, such as competition for degradation machinery (104). More importantly, since feedback controllers do not increase a host cell's ability to produce proteins but instead increase demand by the regulated genes in the face of resource depletion, scaling up this strategy to include multiple nodes with feedback may be limited by fundamental design trade-offs that are yet to be explored (87).

4.2.3. Circuit–host interaction. When resource demand by a synthetic circuit becomes too large, the physiology of the host cell may be affected (105–107), resulting in another form of context dependence known as host–circuit interaction, which is not accounted for in Equation 11. Host–circuit interaction arises from growth-modulated feedback, where synthetic circuit expression retards host cell growth, which in turn affects synthetic circuit expression, leading to unexpected behaviors (105, 108). While preliminary experiments using negative feedback to robustify circuits' responses to changes in cell physiology have been promising (106), the mechanistic link between

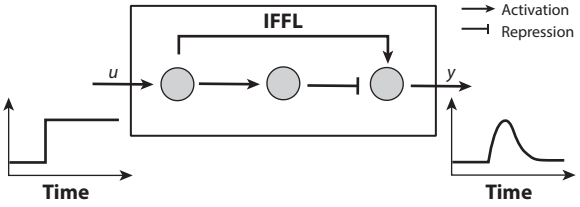


Figure 6

The incoherent feedforward loop (IFFL). If the two branches are well balanced, the system rejects a step disturbance input u.

host cell growth and synthetic circuit expression remains largely unexplored. An understanding of this link may allow the implementation of a central controller that interacts with the host cell to optimize resource production, distribution, and utilization (109, 110), a strategy often used to solve similar problems in engineering (111).

5. FEEDFORWARD CONTROL FOR COMPENSATION AND TEMPORAL RESPONSE SHAPING

In a classical control design setup, feedforward compensators are commonly designed to complement feedback controllers, especially when a model of the plant to be controlled is known (80). In genetic circuits, this is often accomplished by the incoherent feedforward loop (IFFL) motif (**Figure 6**). The IFFL is used by natural biological systems in a variety of settings, including miRNA degradation of mRNA (112), insulin release in beta cells (113), and robustness to temperature disturbances (114).

The standard topology of an IFFL motif consists of three nodes and two forward paths from the input to the output in which the gains on the paths have opposite signs. Owing to this incoherent nature, under constant input disturbances, one path compensates for the input transmitted by the other, allowing the output of the motif to approximately reject constant disturbances (**Figure 6**). This IFFL motif is found much more frequently in natural systems than would be expected in a random network (11). This discovery prompted further research into special properties of IFFLs that help explain their prevalence (115–117). For example, an IFFL acts as a pulse generator in response to a step input (117). The output initially increases in response to the step input, then decreases to approximately the original steady state as the two paths oppose each other, generating a pulse from the step input. Additionally, under appropriate conditions, the response of an IFFL may be sensitive only to the multiplicative factor (fold) by which the input is increased and not to the absolute value of the input. This property has been termed fold change detection (115, 116). Finally, IFFLs that perfectly adapt to constant or step inputs contain a hidden integrator, which can be made explicit through a change of coordinates (118). While IFFLs are known for their ability to provide compensation, for compensation to occur, the two branches of the feedforward motif need to be well balanced. This translates into specific choices of parameters that are difficult to set in practice. Novel designs that combine feedback with feedforward may be particularly useful for enhancing the robustness of incoherent feedforward architectures to parameter variation.

Because of the investigation of feedforward motifs in natural systems, researchers have used these motifs to engineer synthetic systems with improved disturbance compensation. For example, the copy number of the plasmids can be highly variable from cell to cell, leading to high variability in the concentration of expressed proteins, and IFFLs have been engineered to mitigate such variability (119).

6. COORDINATION OF MULTICELLULAR BEHAVIOR

In recent years, multicellular coordination has become a new frontier in synthetic biology. Multicellular coordination can be realized through cell–cell communication, in which small molecules synthesized in sender cells diffuse through the cell membrane to regulate the expression of genetic circuits in receiver cells, a mechanism well known in bacterial quorum sensing (120). While the biomolecular reactions that carry out computation and actuation still take place in individual cells, cell–cell communication enables each cell to have access to some states of its neighbors and then adjust its own activity accordingly to affect the collective population behavior. The system-level architecture of multicellular coordination resembles that of cooperative control (121).

The ability to program cellular behaviors collectively leads to genetic circuits with novel spatiotemporal functionalities, including population controllers (122, 123), synchronized oscillators (62), and spatial pattern generators (124, 125). In addition, multicellular coordination, combined with intracellular feedback control, can reduce the heterogeneity of gene expression in the population (126). Finally, multicellular coordination among different cell strains allows the engineering of distributed genetic circuits, where the burdens of sensing, computing, and actuation are distributed to multiple cell strains (127–129). The cooperation of multiple cell strains can increase productivity in biosynthesis applications (127). Additionally, a distributed genetic circuit can, in principle, circumvent the lack of modularity often found in circuits that operate at the single-cell level. In the following sections, we review these aspects of multicellular coordination in more detail.

6.1. Population Control

You et al. (122) constructed one of the earliest genetic circuits that uses multicellular coordination to maintain the density of *E. coli* at a desired level (**Figure 7*a***). The circuit realizes cell–cell communication through the well-characterized quorum-sensing system in the marine bacterium *Vibrio fischeri*, which consists of the proteins LuxI and LuxR (120). The LuxI protein is constitutively produced to catalyze the synthesis of the diffusible small molecule acyl-homoserine lactone (AHL), which can bind with a constitutively produced LuxR protein to activate a killer gene, leading to cell lysis. Since AHL diffuses freely across the membrane of the cell, its intracellular concentration reflects its intercellular level and can therefore be regarded as a proxy for population size. An increase in cell population increases AHL synthesis and, as a result, increases the intracellular AHL concentration, activating the killer gene to decrease population size and closing the feedback loop. In a more recent study, Scott et al. (123) constructed a similar population control circuit in the *Salmonella typhimurium* bacterium and demonstrated, both numerically and experimentally, that the degradation rate of LuxI is a key bifurcation parameter that controls bacteria population dynamics. As shown in **Figure 7*b***, when LuxI degrades rapidly, the amount of AHL is small, leading to weak cell–cell communication strength and oscillatory population dynamics. Conversely, communication strength is strong when LuxI degradation is slow, enabling the population to reach a consensus (i.e., population size reaches a steady state).

In contrast to the above-mentioned circuit, which regulates the population of a single cell strain, several studies have attempted to control the population dynamics of multiple cell strains or types (e.g., microbial consortia) (123, 130, 131). These studies increase our understanding of natural ecosystems (123, 130) and are critical to the implementation of distributed genetic circuits (131), which we discuss in Section 6.4. Maintaining a population of metabolically competing species remains challenging, as species with growth deficiencies are often taken over by those with growth advantages, and population dynamics are often oscillatory and sensitive to parameters and initial conditions (132, 133). Although experiments with preliminary multiple-strain population control circuits have shown promising results (e.g., 123), deeper control-theoretic studies are still critical to improve the robustness and predictability of these population control circuits.

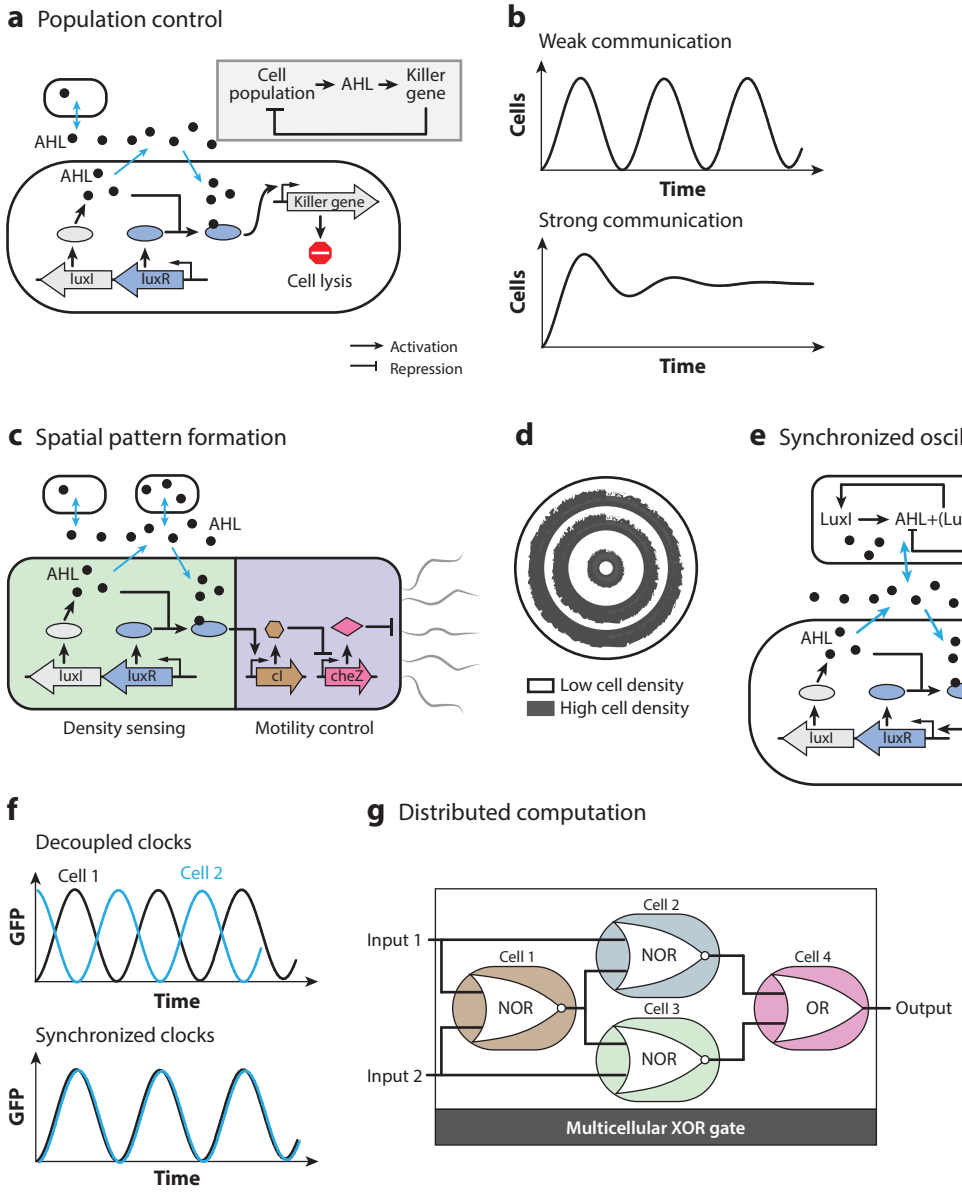


Figure 7

Multicellular coordination circuits. (*a*) Cell–cell communication enables the control of cell population density (122). Since each cell synthesizes the diffusible small molecule acyl-homoserine lactone (AHL), an increase in cell density results in an increase in intercellular AHL concentration, which triggers the expression of a killer gene to limit population growth. (*b*) Population dynamics are tunable through the degradation rate of LuxI protein. Strong degradation leads to weak cell–cell communication, resulting in oscillatory population dynamics. (*c*,*d*) Coupling the population-sensing circuit with bacteria motility control enables a population of engineered bacteria to form spatial patterns autonomously (125). (*e*,*f*) Cell–cell communication synchronizes a population of genetic clocks (62). (*g*) Multicellular coordination enables distributed computation in genetic circuits (129).

6.2. Pattern Formation

Synthetic pattern formation systems could lay the foundation for future biomaterials that self-organize into patterns of biological entities (134). They could also enhance our understanding of patterning in nature for developmental biology research (32). One of the earliest pattern formation circuits was developed by Basu et al. (124). The pattern forms on a plate containing a spatially homogeneous population of receiver cells that surround sender cells placed at the center of the plate. The sender cells produce diffusible AHL constitutively, resulting in a spatial AHL concentration profile on the plate that reduces radially from the center. The receiver cells contain an IFFL that takes AHL as input and produces a fluorescent reporter as output. The IFFL is tuned to produce a biphasic I/O dose–response curve, and fluorescent output is therefore produced at intermediate AHL concentrations, forming a fluorescent ring on the plate.

The circuit described by Basu et al. (124), however, is unable to produce a pattern autonomously, in that a predefined spatial concentration profile of AHL produced by the sender cells is required. More recently, Liu et al. (125) constructed an autonomous pattern formation circuit by coupling a LuxR/LuxI population-density-sensing module with a motility-control module, which includes the *CheZ* gene in the *E. coli* chemotaxis pathway so that cells aggregate into stripe patterns (see **Figure 7*c*,*d***).

6.3. Reduction of Cell–Cell Variability

Through population averaging, multicellular coordination can serve as an effective tool to reduce population-level heterogeneity in gene expression. Vignoni et al. (126) theoretically studied a circuit in which the intercellular concentration of diffusible AHL determines the strength of repression on the regulated gene in each cell. The regulated protein further catalyzes AHL synthesis, forming an effective negative feedback loop. The authors demonstrated that this control scheme can effectively reduce steady-state gene expression heterogeneity. This approach may find applications in biosensing, where increasing the signal-to-noise ratio is highly desirable (35).

Reducing population heterogeneity is especially crucial for multistable and oscillatory circuits. Cell–cell variation may lead to noise-induced transition among phenotypes (i.e., stable steady states) in a multistable circuit (31, 68), jeopardizing its desired functionality. A numerical study by Koseska et al. (135) found that coupling of genetic toggle switches through small molecules enhances the precision of cell decision. Similarly, Danino et al. (62) used cell–cell communication to reduce heterogeneity in a population of genetic clocks. As shown in **Figure 7*e***, the diffusible molecule AHL has two functions: enabling intracellular transcriptional activation that gives rise to oscillatory dynamics on a single-cell level, and mediating cell–cell communication to synchronize the genetic clocks. Experimentally, the synchronized genetic clocks can produce sustained oscillation at the population level (**Figure 7*f***). This contrasts with earlier experiments using decoupled genetic clocks, where population-level oscillation is damped out as cells become progressively out of phase because of noise (45).

6.4. Distributed Genetic Circuits

Cell–cell communication provides a promising tool to realize distributed genetic computation. The idea is to split functional modules in a genetic circuit into multiple cell strains and coordinate their behavior through diffusible small molecules. This is an appealing design concept in that it exploits the cell membrane to add another layer of compartmentalization and therefore increases circuits' modularity. In fact, distributed genetic circuits can circumvent several context-dependent

problems found in single-cell circuits, including retroactivity and resource competition. Preliminary experimental results have demonstrated the potential of this distributed approach (128, 129). For example, Tamsir et al. (129) built a genetic XOR gate using a composition of four NOR and OR gates distributed into four different *E. coli* strains (**Figure 7*g***). These distributed designs are particularly appealing for biosynthesis applications, in which the employment of multiple microbial strain can help divide labor and work cooperatively to increase productivity (127, 136).

Nevertheless, several technical challenges remain before this technology matures (137). A major system-level hurdle lies in the fact that communication strength (i.e., the concentration of communicating small molecules) depends on population size. As cells grow, robust population control for each cell strain needs to be devised to guarantee reliable signal transmission (see Section 6.1). In addition, an appreciable amount of delay may occur during signal transmission (i.e., diffusion), which may deteriorate circuits' temporal response or even cause instability (80). The solution to both problems may benefit significantly from a control-theoretic approach since closely related problems, such as multiagent coordination in the presence of communication delay, have been addressed in other engineering contexts (121). Meanwhile, the exploration and characterization of orthogonal cell–cell communication modules in bacteria (138) and eukaryotic cells (139, 140) remain preliminary, and more input from the biological engineering community is needed to expand the toolbox.

We envision that at least two control layers are required in future genetic circuits. Low-level intracellular controllers modularize the behavior of functional modules, distributed to distinctive cell strains, allowing their I/O behaviors to be robust to external disturbances and noise (see Section 4). Higher-level intercellular controllers can then be implemented to regulate the population size of various strains and coordinate strains' collective behaviors. This layered control architecture may enable synthetic biology to obtain a higher degree of modularity, facilitating the design and implementation of more sophisticated circuits.

7. SUMMARY AND OUTLOOK

In this review, we have discussed how control design principles have permeated synthetic biology to tackle fundamental problems encountered when programming cells to work for us: designing circuits' dynamics (Section 2), improving circuits' robustness to unknowns (Sections 3 and 5), aiding modular and layered design (Section 4), and programming the emergent behavior of cell populations (Section 6). While the field of synthetic biology has made rapid progress and has clearly demonstrated its remarkable potential in groundbreaking applications (Section 1), significant challenges remain. Many of these challenges are in essence system-level problems and, as such, can most likely be addressed by a control-theoretic approach.

The conceptually appealing, yet perhaps overused, analogy between a programmed cell and a robot breaks down as soon as the physical properties of biomolecular systems in living organisms are considered. Although we can clearly design the qualitative dynamics of simple functional modules (e.g., oscillators and multistable systems), the spectrum of functions that can be realized is still unclear, especially the extent of achievable precision for more quantitative design. Imposing strict analogies with engineering, chiefly with electrical engineering, may be misleading because of several factors, including the intrinsic (and most likely useful) nonlinearity and stochasticity of biomolecular systems. Furthermore, while basic components are well characterized in electrical engineering, the core I/O biomolecular processes (e.g., transcriptional regulation, protein–protein interactions, and RNA–RNA interactions) that are used in synthetic biology are plagued by 10–100× uncertainty in key parameters. These processes may also dramatically change with temperature, pressure, cell metabolism, and the specific circuit's context. Yet nature's design

strategy is remarkably robust to these sources of variability and may actually exploit them in its favor.

An interesting aspect of the field is the rapid development of new biological tools, which continually expands the set of core processes that can be used for design [e.g., CRISPR-based regulators (14)]. Compared with this rapid pace, theory is lagging behind, and new processes are used before they are systematically characterized. Systematic characterization presents significant challenges, including the need for system identification techniques that can handle nonlinear parameterizations typical of biomolecular processes and the lack of fast and precise sensors. At the same time, circuit design techniques that can produce reliable and repeatable outcomes despite all the unknowns that plague single components are largely lacking. Feedback design has been instrumental in engineering to obtain, for example, repeatable performance of amplifiers despite 5× variations in their components (43). The key is to compare the actual output of the system with the desired output, under the assumption that we have an accurate and precise sensor for the output. In synthetic biology, sensors are inaccurate, imprecise, and slow, and the uncertainty in components is much larger than that found in engineering systems.

At the system level, a modular and layered design approach is appealing to an engineering mind, yet it presents significant challenges. As described in Section 4, even with components that are well characterized in isolation, a system's behavior becomes unpredictable because of context dependence (16, 90). Context dependence leads to I/O characteristics of core processes that change greatly when the context (i.e., circuits around them and cell growth) changes. This results in a lengthy, ad hoc, and combinatorial design process, significantly limiting our ability to scale up circuits' size and sophistication. In addition to the remarkable advances in the biological engineering community toward minimizing interference among basic parts (for example, DNA promoters and terminators) (16), engineering in vivo biomolecular controllers provides a promising path toward making the I/O behavior of genetic circuits independent of context (Section 4).

At the multicellular level, programming the emergent behavior of a bacterial population remains a grand challenge (Section 6.4). Although the apparent analogy with cooperative and decentralized control problems is appealing, the large number of cells (e.g., on the order of trillions in human guts), communication delay resulting from diffusion, nonlinearity in agent dynamics, and spatial heterogeneity make traditional control-theoretic formulations inapplicable. Interestingly, as illustrated in Section 6, experimentalists are already implementing multicellular computation and using feedback control for coordination. However, these designs often miss theoretical guarantees and/or have poor robustness properties. More generally, the key question in any multicellular computation of how to robustly maintain desired cell populations in multistrain consortia remains largely open.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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