Programming cells to work for us

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Abstract

The past decade has witnessed the rise of a new exciting field of engineering: synthetic biology. Synthetic biology is the application of engineering principles to the fundamental components of biology with an aim of programming cells with novel functionalities for energy, environment, and health applications. Control design principles have been used in synthetic biology since its on-set in the early 2000's, for designing dynamics, mitigating the effects of uncertainty, and aiding modular/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 this review with a discussion of pressing challenges that the field is facing for which new control theory is required, with the hope of attracting researchers in the control theory community to this new exciting engineering application.

Contents

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 the aims of the field is to program cells, from single-cell organisms (e.g., bacteria) to cell populations, tissues, and organs for a variety of applications ranging from health (e.g., developing new revolutionary cures to cancer and diabetes), to energy (e.g., biofuels), to environment (e.g., biosensing and bioremediation), to regenerative medicine (e.g., reprogramming cell identity) (2, 3). In this section, we provide a short background on synthetic biology and then describe some of its many applications that can potentially revolutionize health and energy.

1.1. Background on synthetic biology

Historical perspective. Synthetic biology is largely based on scientific advances in biotechnology that have occurred over the past 50 years, chiefly DNA cloning techniques, DNA amplification techniques, the ability to insert extraneous DNA within a cell (transformation or transfection), and DNA sequencing (1) (Figure 1a). Specifically, the discovery of DNA restriction enzymes in the late 1960's allowed for the cutting and pasting of DNA at targeted sites (4). In the 1970's, new technologies allowed for the insertion of synthetic DNA into host cells (5). These scientific advances enabled one of the first applications of engineering biology with the production of synthetic insulin (6). The discovery of polymerase chain reaction (PCR) (7) and DNA sequencing technology (8) in the 1980's,

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 the year 2000 was based on these technologies. At this point in time, much work was focused on the combination of a few DNA parts to form simple circuit modules with the aim of understanding the purpose of similar naturally occurring motifs (11) (Figure 1a, Modules era). 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 1a, Systems era). 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 placed substantial efforts toward creating novel parts (for example, CRISPR-based regulators (14)), characterizing parts (15), providing insulation between modules (16), and enforcing functional circuit modularity against the effects of loads through the design of load drivers (17).

Encoding "programs" on DNA through core biomolecular processes. A genetic circuit realizes its functionalities by encoding the production and subsequent interactions of biomolecules (for example, proteins) on DNA sequences. Historically, early genetic circuits operate by transcriptional regulation, by which a protein, x, alters the rate at which another gene expresses its protein, y (see Figure 1a). Specifically, protein x can repress ($\dashv$) or activate ($\rightarrow$) 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) and each subsystem (node) in the network represents the dynamical process of protein production from DNA (Figure 1a, Modules era). Any gene, including synthetic ones, utilizes the cell's built-in machinery to create proteins. First, RNA polymerases (RNAPs) read the gene sequence and create a mirroring messenger RNA (mRNA), through a process called gene *transcription*. Then, the mRNA is translated by another cellular enzyme known as the ribosome to create the amino acid chain which forms the protein, a process called mRNA *translation*. This dynamic process of protein production from DNA is called 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 (ODEs) to describe the protein and mRNA concentrations. Referring to x$\dashv$ y in Figure 1b and using m and y (*italic*) to represent the concentrations of protein y's mRNA and protein y, respectively, the dynamics can be written as

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

where α is the maximum rate of transcription, and k is the dissociation constant between the y's DNA and x. Stronger binding affinity between the two molecules can be represented by smaller k value. Parameter n describes the number of x molecules required to bind together before they can act to regulate expression of y, also known as *cooperativity*. Parameters δ and γ represent the mRNA and protein decay rate constants, respectively, due to dilution (arising from cell volume increase as they 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).

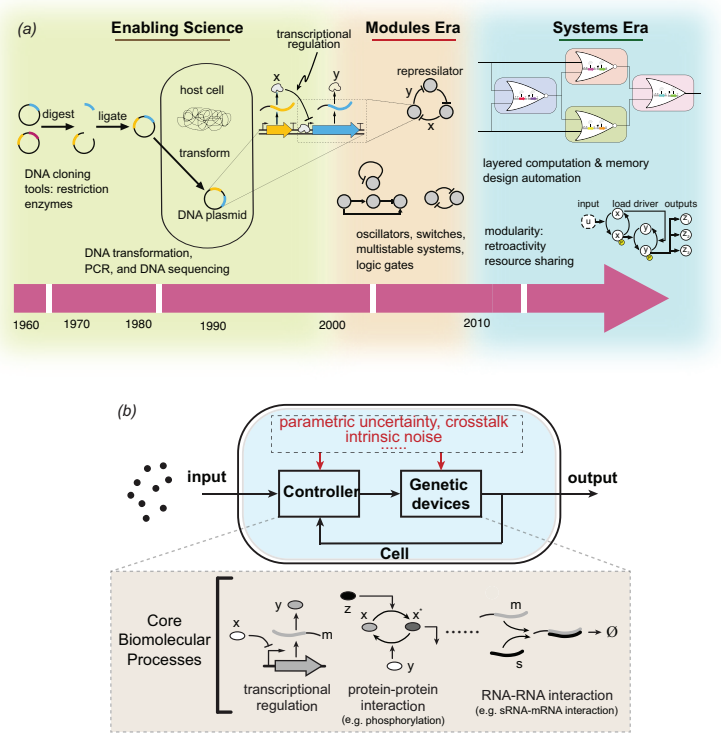

Figure 1

Overview of synthetic biology. (a) Synthetic biology on the temporal axis. (Enabling science) Cloning allows for cutting (digest) and pasting (ligate) pieces of DNA together, thus enabling one to encode a circuit on DNA plasmid. (Modules era) The first synthetic systems created were simple modules performing tasks such as oscillations and switching. (Systems era) Construction of more complex circuits is based on a modular/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 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, for instance, allosteric modification and covalent modification (19). One of the most common types of covalent modification is the process of *phosphorylation*, illustrated in Figure 1b. In this process, a *kinase* z transfers a phosphate group to the *substrate* x, resulting in a conformational change of the substrate to become active (x^*). *Dephosphorylation*, on the other hand, is a complementary process where a *phosphatase* (y) removes a phosphate group from the active substrate (x^*). 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 include allosteric regulation, phosphotransfer, and regulation of protein degradation. They have been successfully engineered in synthetic genetic circuit (see an example in Section 3.3). We refer the readers to (19) for detailed descriptions of these core processes.

Increasing experimental evidence since the 1990s have suggested that RNAs are not only functional as messengers between DNA and proteins, but also as important regulators for gene expression (see (20) for a review). For example, many regulatory small RNAs (sRNAs) have been identified in bacteria, where they are involved in a variety of adaptive responses (20, 21). With reference to Figure 1b, most commonly, sRNAs (s) can bind with their target mRNAs (m) to expedite their degradation and/or inhibit translation. Quantitative modeling of sRNA-mediated regulation has revealed distinctive features compared to transcriptional regulations, such as faster response 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 in recent years has provided another class of highly efficient tools to perform gene regulation through guide RNAs (14). However, while initial experimental results have achieved remarkable success, mathematical characterization of these processes are still largely lagging behind.

1.2. Applications of synthetic biology

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 reporter protein) when a set of logic conditions are met. For example, these logic conditions can be designed to classify the chemical signature typical of cancer cells, so that the circuit can recognize cancer and trigger a number of actions (23) (Figure 2a). As a result, synthetic genetic circuits can program bacteria to colonize target sites where cancer is detected, providing a promising approach to reduce invasive tests for diagnosis and health monitoring (29, 30). Programmed bacteria can further serve as smart vehicles for drug delivery by lysing at the tumor site and releasing therapeutic proteins to reduce tumor activity (24) (see Figure 2b).

Synthetic biology also provides powerful tools to program T cells, a type of body immune cells, to specifically attack cancer cells (25). This type of treatment, known as immunotherapy, has recently demonstrated successful preliminary clinical trials (25). As shown in Figure 2c, synthetic receptors engineered on T cells, possibly combined with biomolecular logic gates, can identify cancer cells with high specificity. Synthetic controllers can then interact with the cellular chemotaxis pathway to migrate T cells to tumor cites. After T cells reach the target site, *in vivo* genetic controllers can actuate negative feedback actions to regulate the duration and strength of T cell activity to protect non-cancerous cells (31). Synthetic biology may also be used to enhance understanding of natural systems, including understanding of cell fate decisions (32) and can provide unprecedented tools to reprogram cell fate for regenerative medicine (26, 33). For instance, Saxena et al. designed a synthetic reprogramming circuit that converts pancreatic progenitor cells derived from human induced pluripotent stem cells (hIPSCs) into insulin-secreting beta-like cells by strictly regulating the timing and expression of three key transcription factors *in vivo* (26) (see Figure 2d). Consequently, it has become possible to implant

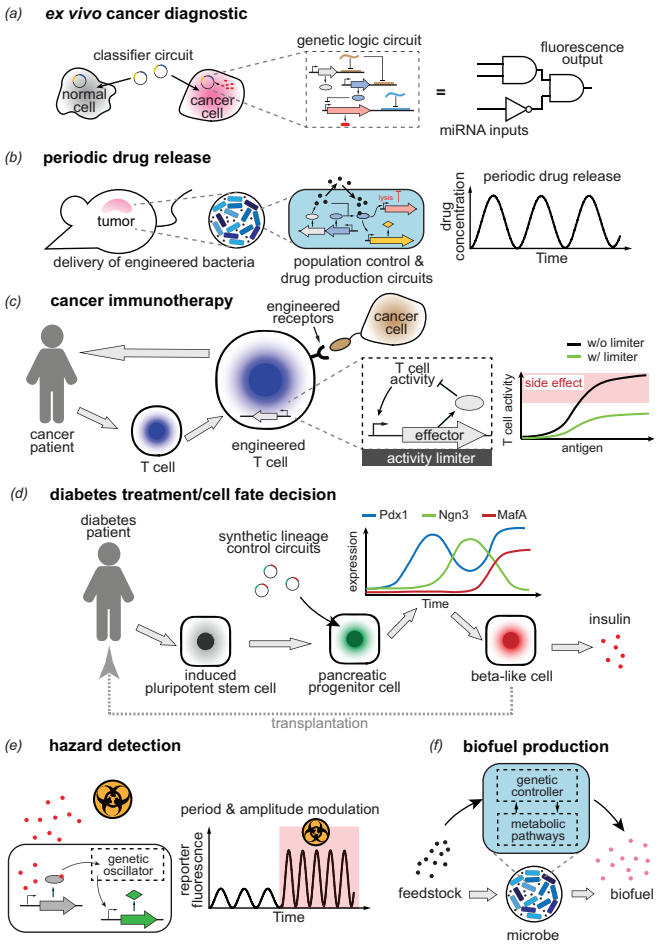

Figure 2

Applications of synthetic biology to health, environment and energy. (a) A multi-input cell type classifier circuit used for cancer diagnostic *ex vivo* (23). A reference profile of 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 expression of a fluorescence protein. (b) Bacteria can be engineered to periodically release drug *in vivo* (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 in a periodic manner to release the therapeutic proteins, resulting in periodic drug delivery to the patient. (c) Synthetic genetic circuits increase the specificity and safety of cancer immunotherapy (25). Receptors can be engineered to trigger T cell activity when cancer cells are detected. Feedback loops can be used to limit the response of T cell activity to avoid side effects. (d) A synthetic lineage control circuit, activating the expression of three transcription factors according to a temporal pattern, hIPSCs can be reprogrammed into insulin-secreting beta-like cell for treating diabetes (26). (e) A biosensor that detects arsenic presence and indicates its amount by modulating the period and amplitude of a oscillatory reporter gene expression (27). (f) Genetic controllers can be used to interact with engineered metabolic pathways in microbes to improve their biofuel productivity (28).

functional beta-cells in diabetes patients that are derived from the patient's own tissue cells.

Environment and energy. Programming microbes to detect and report toxicants in water, air, soil and food is 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 (34). This design technique has been utilized to detect TNT, heavy metals and antibiotics (see (34) for a comprehensive review). More recently, sensors that produce a dynamic output have been developed due to their advantages in signal transmission and processing. Figure 2e illustrates a bacterial biosensor that produces oscillatory fluorescence output, whose magnitude and frequency reflects the concentration of arsenic in the environment (27). In addition to sensing hazards, microbes can be programmed to remove contaminants, including heavy metals and organic pollutants for bioremediation (35, 36). Microbes may also be programmed to convert biomass feedstock into biofuels (37), and synthetic controllers have been implemented to improve productivity (28, 38) (Figure 2e and 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 not found in nature (39, 40).

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, both used feedback to design dynamics. The ability of *designing 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 resulting in "closed loop" dynamics that modify the natural behavior (e.g., highly agile, open-loop unstable aircrafts (41)). Examples of this include the repressilator, which used negative feedback along with a sufficiently large phase lag to create an oscillating system (9). In 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 (10).

In the transition from the modules era to the systems era (Figure 1a), the ability of performing modular design has arisen as critical to the field. *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 (42)), so that a designer may ignore a system's internal structure, enabling him or her to only have to reason about the system's 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 (e.g., retroactivity) (17, 43).

Synthetic genetic circuits are subject to a number of perturbations (e.g., noise, temperature changes, changes in the cellular chassis) and uncertainty (e.g., 10x-100x uncertainty on the parameter values). *Managing uncertainty* is a crucial ability in any engineered systems. 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 5x component variation (42)). In synthetic biology, negative feedback and feedforward control implementations, shown in Figure 1b, have been throughout used to mitigate the effects of unknowns (see (44, 45), for example). 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 a number of synthetic genetic circuits whose design and analysis are enabled by theoretic tools from control and dynamical systems, including the genetic multistable systems and oscillators.

2.1. Multistable systems

Multistable systems are generally useful in endowing a system with the ability to maintain a particular state after the input is removed. One notable example of this is the toggle switch (10, 46). This is a circuit in which two proteins, x_1 and x_2, mutually repress each other (Figure 3a.1). Under appropriate conditions, this circuit exhibits three steady states–two stable and one unstable. A simplified model governing the toggle switch's dynamics is given by

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

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 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 (40) and biosensors (27). Toggle switches may also be used in "digital" logic systems to maintain memory (47). These applications lead to requirements of constructing toggle switches with faster switching time and lower metabolic burden, which are still among some of the current design challenges (48).

Multistable systems are frequently found in natural gene regulatory networks pertaining to cellular fate determination (32, 49, 50). Cellular fate determination is typically thought of as a potential landscape in which different potential wells correspond to different cell types (50, 51). Figure 3a.2 shows a popular motif in cell fate decision, which has three stable steady states (52). Major challenges in the control of natural multistable systems arise as complexity grows, including methods to trigger transitions to desired steady states to artificially reprogram cell identity (33).

2.2. Oscillators

Oscillators are prevalent in natural systems and are critical for a number of functionalities, such as the circadian pacemaker (56) and the timing of metabolism (57). A number of synthetic genetic oscillators have been constructed in early-mid 2000s with the initial goal to understand nature's "design principles" at the basis of time-keeping (9, 46, 54). Lately, these circuits have found application in proposed novel cancer therapies based on synthetic

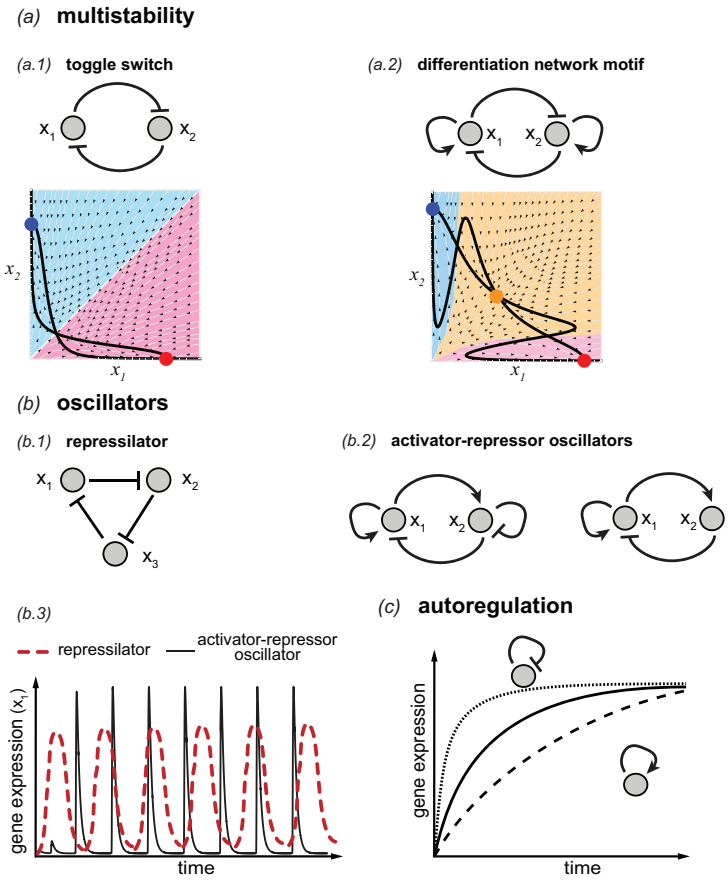

Figure 3

Feedback for designing dynamics. (a) *Multistability in genetic circuits.* Circles on the phase plot represent stable steady states, black solid lines are nullclines, and colored regions represent the region of attractions of the respective stable steady state. (a.1) Circuit diagram and phase plot of the genetic toggle switch built in 2000 (10). (a.2) Circuit diagram and phase plot of a tristable differentiation network motif built in 2017 (53). (b) *Synthetic genetic oscillators.* (b.1) The repressilator circuit built in 2000 (9). (b.2). The activator-repressor oscillators built in 2008 (54) (left) and 2003 (46) (right). (b.3). Sample trajectories of the oscillators. (c) *Autoregulation shapes temporal response of gene expression.* The negatively autoregulated gene (55) (dotted line) has a shorter rise time than that of the unregulated gene (solid line) and of the positively regulated gene, which has the slowest rise time (dashed line).

biology for enabling periodic drug release and in environmental sensing to determine the concentration of pollutants (Section 1.2).

The first synthetic oscillatory circuit built is the *repressilator* (see Figure 3b.1) (9). The 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. If we use x_1, x_2 and x_3 to represent the concentrations of the three proteins, and for simplicity of presentation, we assume the circuit is symmetric (i.e., identical parameters for all three

genes), then the repressilator can be modeled by:

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

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).

Another class of synthetic oscillators are constructed based on a combination of activation and repression between two genes. As shown in Figure 3b.2, these circuits consists of protein x_1 activating both protein x_2 and itself, and protein x_2 either repressing only itself or both x_1 and itself ((46) and(54, 58), respectively). A model for these activator/repressor oscillators is given by

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \frac{\alpha_1 x_1^n + \beta_1}{1+x_1^n+x_2^m} - x_1 \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \frac{\alpha_2 x_1^n + \beta_2}{1+x_1^n+cx_2^m} - x_2, \tag{4}$$

where x_i represents the concentration of protein i for $i = 1, 2$, α_i represents maximal production rate of protein x_i, and β_i represents its basal production rate. Here, $c = 0$ for the motif of (46). One can derive parametric conditions under which this system displays a unique unstable (not a saddle) equilibrium, which guarantees oscillations (59, 60). Figure 3b.3 shows sample temporal traces of the repressilator and of an activator-repressor oscillator.

While oscillators found in biological systems are remarkably robust (57), many synthetic oscillators are sensitive to parametric uncertainty and stochasticity, leading to poor design predictions (46, 61). Therefore, the community is still actively seeking design principles for robust oscillators. Such efforts have been facilitated by (i) theoretical advancements that provide refined conditions for oscillations (e.g., the "secant condition" for cyclic systems (62)), and by (ii) novel biotechnological tools to robustify circuits (e.g., synchronized oscillators through cell-cell communication (63), 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 (55). This is useful especially because the speed of response obtained with gene expression is typically very slow and one of the many design challenges is to obtain faster response speed, especially for biosensing applications. By contrast, positive autoregulation slows down the response time compared to that of an unregulated gene (64) (Figure 3c).

3. Feedback control for robustness

Gene expression is inherently a noisy process (67). 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, 68), their performance are 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 *E. coli* that enables gene expression to robustly track a dynamic input.

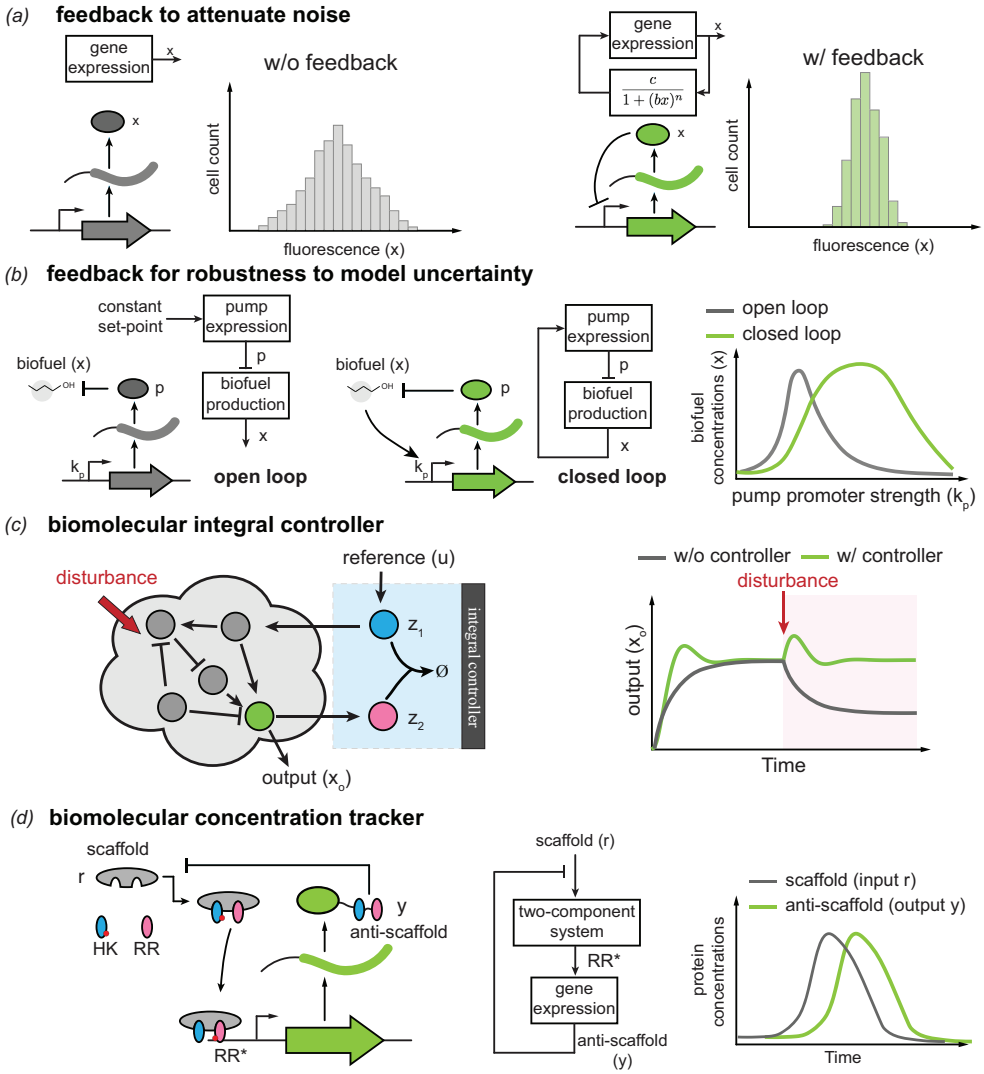

Figure 4

***In vivo* feedback control increases robustness of synthetic genetic circuits.** (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. Compared to constitutive pump production, a biofuel-responsive pump production circuit is less sensitive to system parameters such as the promoter strength, k_p, allowing productivity to be near-optimal for a wide range of conditions. (c) Biomolecular integral controller design proposed in (65). The controller uses two species z_1 and z_2 to regulate concentration of x_o to reference level u. This controller guarantees that the output of a circuit is robust to constant disturbances and parameter uncertainties. (d) Design of a biomolecular concentration tracker (66). In the presence of a scaffold protein (input r), HK can phosphorylate RR to become RR*, which activates production of the output y. The output contains an anti-scaffold protein that can sequester the scaffold to reduce the input concentration.

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 (67). 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 (69) and an oscillator (46). In multi-stable genetic circuits, such as the genetic toggle switch and the cell-fate decision network, noise can lead to random transitions among phenotypes (i.e. "stable steady states") or to the creation of unexpected ones (32, 70, 71). While noise may be utilized by natural systems for differentiation or evolution (72), most of the research in synthetic biology has been focused on reducing heterogeneity in engineered genetic circuits. In addition to optimizing the design and arrangement of basic genetic parts (see, for example, (73)), many experimental studies on single gene expression have demonstrated that negative feedback through transcriptional negative autoregulation is an effective approach to reduce noise in gene expression (44, 74, 75). These results are consistent with negative feedback's leveraged property of noise suppression in engineered systems and with negative autoregulation's repeated occurrence in natural gene networks (64).

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 (CME) rather than ODEs (19). A simplified model of negative autoregulation (Figure 4a) consists of the following 4 reactions ($\mathtt{R}_1$-$\mathtt{R}_4$) that model the mRNA and protein production and decay (76, 77):

$$\begin{aligned} &\mathrm{R}_1 : m(t) \to m(t)+1; \quad a_1(t) = k_m(x(t)), \quad \mathrm{R}_2 : m(t) \to m(t)-1; \quad a_2(t) = \delta m(t), \\ &\mathrm{R}_3 : x(t) \to x(t)+1; \quad a_3(t) = k_p m(t), \quad \mathrm{R}_4 : x(t) \to x(t)-1; \quad a_4(t) = \gamma x(t), \end{aligned} \tag{5}$$

where $a_i(t)$ is the probability that reaction $\mathtt{R}_i$ occurs during the interval $(t, t+dt]$, and m and x are the mRNA and protein counts, respectively. The fact that protein x represses its own transcription is described by 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 the cooperativity of their binding. The constant parameters δ, k_p and γ represent the transcription rate constant, mRNA decay rate constant, translation rate constant, and protein decay rate constant, respectively. Assuming that stochastic fluctuations are small so that $k_m(x)$ can be linearized about steady state average protein count $\mathbb{E}[\bar{x}]$, the steady state coefficient of variation (CV_{in}) of x due to intrinsic noise (i.e., noise generated by stochastic firing of reactions $\mathtt{R}_1$-$\mathtt{R}_4$) can be explicitly computed (76). In particular, we have

$$CV_{\mathrm{in}}^2 = \frac{\mathrm{Var}[\bar{x}]}{\mathbb{E}^2[\bar{x}]} = \frac{k_p}{(\delta+\gamma)(1+\kappa)\mathbb{E}[\bar{x}]}, \quad \text{where } \kappa := -\frac{\mathbb{E}[\bar{x}]}{k_m(\mathbb{E}[\bar{x}])} \left.\frac{\mathrm{d}k_m(x)}{\mathrm{d}x}\right|_{x=\mathbb{E}[\bar{x}]} > 0 \tag{6}$$

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 4a, it is immediate from (6) that if expression of two genes, gene 1 and gene 2, results in identical steady state average molecular count on the population level ($\mathbb{E}[\bar{x}_1] = \mathbb{E}[\bar{x}_2]$), then the gene with "stronger" negative transcriptional autoregulation must have less intrinsic noise ($CV_{\text{in},1} < CV_{\text{in},2}$ if $\kappa_1 > \kappa_2$).

It is worth noting that (i) the definition of "feedback strength" (κ) in equation (6) is different from the biological notion of feedback strength, which is often captured by parameter b in the Hill-type repression function, $k_m(x)$, and (ii) the comparison is valid only if two proteins have identical steady state average molecular count ($\mathbb{E}[\bar{x}_1] = \mathbb{E}[\bar{x}_2]$). Therefore, the claim that increasing the binding affinity of protein x with its own promoter (i.e parameter b) reduces intrinsic noise (i.e. reduce CV_{in}) is not always true, as noted theoretically in (78), demonstrated numerically in (79, 80), and verified experimentally in (75). Detailed parametric studies in (77, 78) demonstrate, however, that increasing binding cooperativity n can effectively reduce CV_{in}.

Negative autoregulation attenuates extrinsic noise. Experimental studies have suggested that extrinsic noise often affects gene expression more significantly than intrinsic noise (81). The role of negative feedback on 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 (44). While more rigorous analytical performance evaluation depends, in principle, on the source of extrinsic noise, it has been shown analytically that CV_{ex} decreases significantly in the presence of negative autoregulation when the extrinsic noise source is the stochasticity in gene copy number. Using the mathematical tool introduced in (67), the authors of (75) were able to explicitly extract CV_{in} and CV_{ex} from experimental covariance data. They found that, as expected, negative autoregulation is much more efficient in reducing the effects of extrinsic noise than those of intrinsic one.

The 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. As our repertoire of synthetic genetic circuits expands rapidly, only a very limited number of investigations on noise characteristics have been carried out on system level (70, 76, 80). Developments in this direction are largely hindered by the lack of analytical tools to characterize the stochastic properties of systems through the CME, 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, (82–84)).

3.2. Feedback control for robustness to uncertainty

Robustness to parametric uncertainty is a defining feature of negative feedback systems (85). Since most biological parameters are either 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.

Transcriptional negative feedback reduces sensitivity of biofuel production to

parameter uncertainty. As illustrated in Figure 2f, genetic controllers can be applied to improve productivity of biofuel in engineered microbes. In (38), the authors propose to apply negative feedback to improve output from a synthetic biofuel production circuit. A major design trade-off in this circuit is the fact that, while increased number of efflux pumps (p) (i.e. biofuel transporters) improves microbial tolerance to biofuel toxicity, overexpression of the pumps can lead to reduction in cell growth, reducing population-wide biofuel output. As a consequence, in order to maximize biofuel production, expression of efflux pumps must be regulated to an optimal level (38). Although this theoretical optimal pump expression level can be computed numerically, due to uncertainty in system parameters and implementation, reaching it through fine-tuning of parameters is impractical. The authors thus numerically investigate whether a closed loop (CL) circuit, where pump gene transcription is activated by the intracellular biofuel level (x), can outperform an open loop (OL) circuit, where pumps are constitutively expressed, in the face of parametric uncertainty. Pump expression in the two circuits are modeled respectively by:

$$\text{OL circuit}: \quad \frac{\mathrm{d}}{\mathrm{d}t}p = k_p - \gamma p, \qquad \text{CL circuit}: \quad \frac{\mathrm{d}}{\mathrm{d}t}p = k_p \frac{x}{x+\lambda} - \gamma p, \tag{7}$$

where k_p is the basal pump production rate constant, and λ describes a threshold biofuel level at which pump production is 50% activated. The negative feedback in the CL circuit functions in the following way: 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. The authors found that the CL circuit is much less sensitive than the OL circuit to uncertainty in almost all system parameters. As illustrated in Figure 4b, the CL circuit can tolerate a much larger 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.

Realizing integral controllers in living cells. In control design, parametric uncertainty is most effectively addressed using integral controllers (85). Assuming that the CL system is stable, integral controllers can drive an unknown plant to reach a constant set-point without steady state error regardless of constant disturbances. These properties are particularly appealing to synthetic biology applications due to the unavoidable presence of disturbances and uncertainties. In fact, integral control motifs have been identified in many natural biomolecular control systems, including bacterial chemotaxis (86), calcium homeostasis (87), and yeast osmoregulation (88).

Recently, there has been increasing interest to synthesize integral controllers *in vivo* to increase a genetic circuit's robustness to uncertainty and disturbances (65, 89, 90). In a theoretical study by Briat et al.(65), the authors propose a type of integral controllers realizable through simple biomolecular mechanisms (see Figure 4c). 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 $\mathrm{z}_1 + \mathrm{z}_2 \xrightarrow{\theta} \emptyset$, where θ is the degradation rate constant. Biomolecular controllers of this type are named an "antithetic integral controller", and their dynamics follow:

$$\frac{\mathrm{d}}{\mathrm{d}t}z_1 = u - \theta z_1 z_2, \qquad \frac{\mathrm{d}}{\mathrm{d}t}z_2 = x_o - \theta z_1 z_2. \tag{8}$$

A linear transformation leads to the memory variable $z := z_1 - z_2$, whose dynamics is the integration of tracking error: $\mathrm{d}z/\mathrm{d}t = u - x_o$. Under the assumption that the closed loop system is stable, and that the set-point u is reachable by the closed loop dynamics, the output of the regulated biomolecular process (x_o) can reach reference input u independent of parameters and constant disturbances (Figure 4c). Physically, the dynamics in (8) can be realized through, for example, RNA interactions (91) and σ/anti-σ factor interactions (65). This design circumvents many physical difficulties in the implementation of an *in vivo* controller, including the lack of direct methods to generate negative signals and to realize signal subtraction in cells. Furthermore, it is shown in (65) that, even when the system operates with a small number of molecules (i.e. noisy environment), the expectation of x_o is guaranteed to converge to the desired set-point.

Another type of theoretically proposed biomolecular controllers approximates integral action through saturation of certain Hill-type or Michaelis-Menten-type kinetics (89, 90). One such circuit proposed in (89) consists of activation of protein z (a memory variable) by transcriptional activator $\mathrm{x_o}$ (output protein), and a saturating amount of protease that degrades z, resulting in the following dynamics:

$$\frac{\mathrm{d}}{\mathrm{d}t}z = \alpha\frac{x_o}{x_o + k} - \gamma_{\max}\frac{z}{z + k_{\deg}} \approx \alpha\frac{x_o}{x_o + k} - \gamma_{\max}, \tag{9}$$

where α is the maximum production rate of z, k is the dissociation constant between $\mathrm{x_o}$ and the promoter of z, $\gamma_{\max}$ is the maximum degradation rate constant with saturating amount of protease, and $k_{\deg}$ is the dissociation constant between the protease and protein z. The approximation in (9) 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.

Implementing integral controllers *in vivo* has tremendous potential to increase robustness of genetic circuits and to modularize their steady state responses (92). While experimental characterization of both types of integral controllers in equations (8) and (9) are still in progress, further theoretical studies to explore the fundamental performance limitations and design constraints of these biomolecular controllers are still required (e.g., (91)).

3.3. Robust tracking

Less work has been devoted to design 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 very limited to date, and, as a consequence, most of the current research in this field has been 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.

Design and implementation of a biomolecular concentration tracker is presented by Hsiao et al. in (66). As demonstrated in Figure 4d, 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 anti-scaffold protein. A synthetic two-component system is utilized to actuate expression of the output. The two-component system consists of a histidine kinase (HK) donating a phosphate to the response regulator (RR) to become active RR^* in the presence of scaffold r. The active response regulator (RR^*) can then activate expression of the anti-scaffold

(output y), which binds with the scaffold (input r). The anti-scaffold (output) thus reduces the ability of the scaffold (input) to sequester HK and RR to activate gene expression, closing the negative feedback loop. In (66), the authors demonstrate 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 on this circuit has revealed that this sequestration-based negative feedback mechanism contains an approximate signal subtractor (93).

4. Feedback control to maintain modularity

Performing modular/layered design is a convenient way to systematically create larger and more sophisticated systems (12, 94). 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. Although exceptions exist (95), this modularity property is at the basis of much of systems and control theory since feedback control has been widely used to preserve a system's I/O behavior upon changes in context. Modularity allows to design 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 I/O properties depend on context, which includes both connectivity to and presence of other systems. Direct connectivity creates loading effects captured by the concept of retroactivity. The pure presence of a system can affect the I/O properties of a different system because these 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

Retroactivity. Referring to Figure 5a, when an upstream system is connected to a downstream one, a "signaling molecule" generated in the upstream system becomes involved in chemical reactions with species of 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 and a change in 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 (96).

As an example, consider the interconnection of an upstream genetic clock (46) to a downstream genetic circuit (Figure 5a). Letting A and R represent the concentrations of the activator and repressor proteins of the clock, the *isolated* clock dynamics can be written as:

$$\frac{\mathrm{d}}{\mathrm{d}t}A = f_A(A,R) - \gamma A, \qquad \frac{\mathrm{d}}{\mathrm{d}t}R = f_R(A) - \gamma R, \tag{10}$$

where f_A and f_R are Hill functions describing transcriptional regulations between A and R, and γ is the protein decay rate constant assumed to be the same for both transcription factors for simplicity (59). 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 $\mathrm{P_D}$ in the downstream system. As a consequence, it is no longer available to the reactions constituting the clock's dynamics. Assuming that A binds with $\mathrm{P_D}$ according

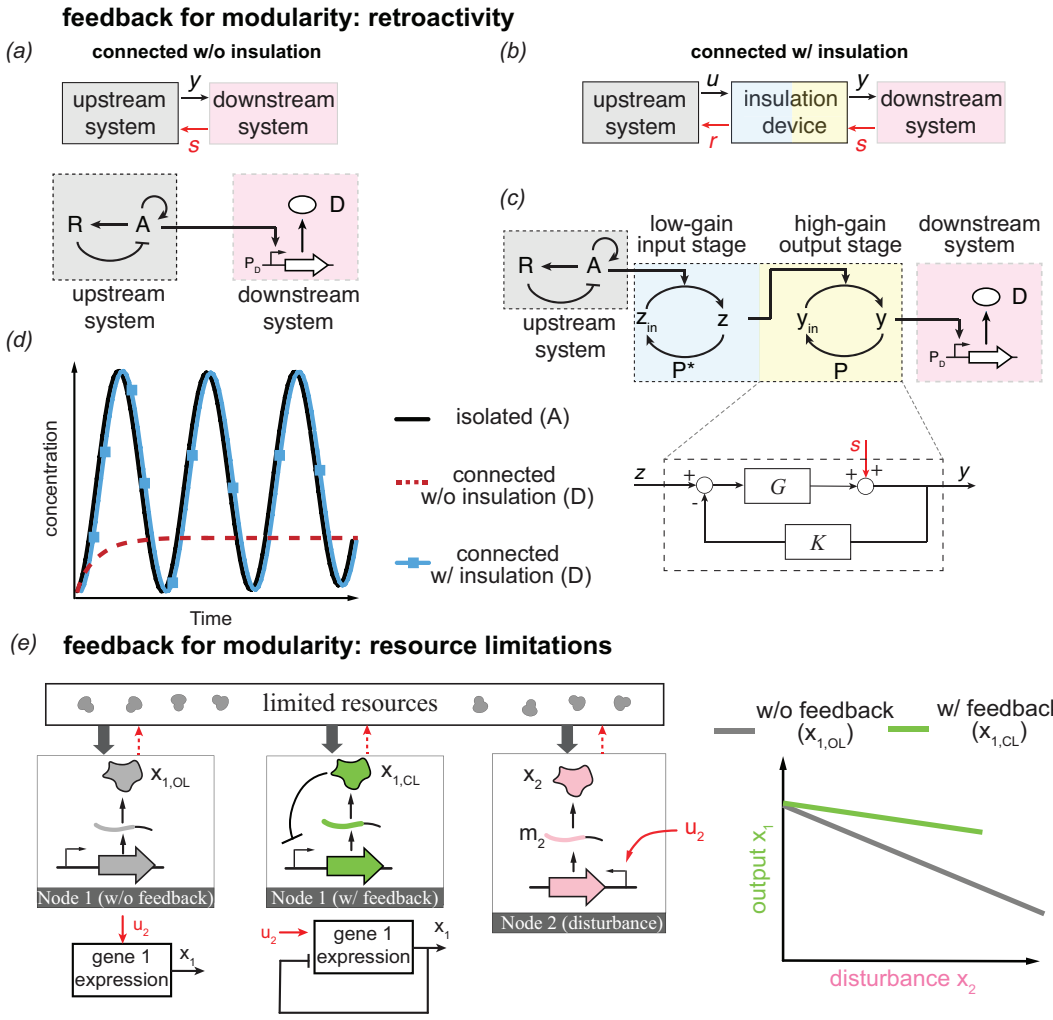

Figure 5

Feedback control modularizes genetic circuits. (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 the downstream system to allow faithful transmission of signals, despite retroactivity. 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. By increasing the concentration 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 time-scale separation to mitigate potential loading effects imparted by the high-gain stage while ensuring low retroactivity to the input (obtained with low protein z concentration). (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 production of protein x_2 uses ribosomes, reducing their availability to the expression of gene 1. Production of x_1 is thus affected by u_2. A gene with negative autoregulation is less affected by such non-regulatory interactions arising from resource competition.

to $\mathrm{A} + \mathrm{P_D} \underset{\mathrm{k^-}}{\overset{\mathrm{k^+}}{\rightleftharpoons}} \mathrm{c}$, the dynamics of the *connected* clock become

$$\frac{\mathrm{d}}{\mathrm{d}t}A = f_A(A,R) - \gamma A + \overbrace{k^- c - k^+ A P_D}^{s}, \qquad \frac{\mathrm{d}}{\mathrm{d}t}R = f_R(A) - \gamma R, \tag{11}$$

where $s := k^- c - k^+ A p_D$ is the *retroactivity to the output*, which, comparing to equation (10), represents the effect of binding between A and the downstream promoter on the clock dynamics. As illustrated in Figure 5c, 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) (97). Retroactivity therefore breaks modularity and renders layered design difficult.

Effects of retroactivity have been experimentally demonstrated in both genetic circuits (17, 43, 98) and in signaling circuits reconstructed *in vitro* (99) and *in vivo* (100). In these experiments, depending on the biomolecular processes involved, retroactivity can appreciably slow-down upstream dynamics or change the steady state I/O response.

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 5b). 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 of y tracking u independent of s can be formulated as a disturbance attenuation problem. A well-known control theoretic solution is to use high-gain negative feedback (101). 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 5c). This diagram leads to

$$y = \frac{G}{1+KG}z + \frac{s}{1+KG}, \tag{12}$$

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 5c can be realized through a phosphorylation cycle (96). As shown in Figure 5c, the cycle takes kinase z as an input to convert the inactive substrate $\mathrm{y_{in}}$ into active substrate y that regulates the downstream system. Phosphatase P converts y back into $\mathrm{y_{in}}$. In this system, the negative feedback is realized by the phosphatase P and the gain G is proportional to the total concentrations of phosphatase and substrate (P and $\mathrm{y_{in}}$, respectively). This design has been experimentally validated in (43).

Implementing high-gain negative feedback through the aforementioned phosphorylation cycle requires y_{in} to be present in large amounts. This design requirement creates a major trade-off as large y_{in} imparts a significant load to the input kinase, creating large retroactivity to the input (r) (102). To overcome this limitation, one can design a cascade of two phosphorylation cycles (Figure 5c). The output stage is designed as before and is a *high-gain stage*. The input stage, in 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 z binding to a large amount of y_{in}. This is because the dynamics of the phosphorylation cycle are much faster than protein expression, which determines the time-scale of the input to the insulation device (e.g., A in Figure 5c). In fact, a general theoretical result in (103) states that the temporal effects of retroactivity can be attenuated by any biomolecular system with sufficiently fast dynamics compared to that of the input, which is consistent with fundamental studies on the relationship between high-gain feedback and time scale separation (101). In (17), a two-stage insulation device designed based on the above principle was constructed in yeast and called *the load driver*, which results in complete retroactivity attenuation (Figure 5d).

4.2. Mitigation of resource competition effects

Resource competition introduces non-regulatory interactions among genes. An important source of context dependence that has received much attention recently is the competition for transcriptional and translational resources/machinery, chiefly for RNAPs and ribosomes. These resources are produced by the host cell and their total concentrations can be regarded as conserved under constant growth conditions (104). While in general, determining the exact limiting factor (e.g., RNAPs or ribosomes or both) for a genetic circuit depends largely on the type of host cells and their growth conditions (105), many experiments reveal that resource competition effects are most significant at the translational level in bacteria *E. coli*, and, consequently, ribosome competition is the major limiting factor (106–108). Non-regulatory interactions due to ribosome competition can be exemplified by considering the simple genetic circuit in Figure 4e composed of two nodes: node 1 producing protein x_1 and inducible node 2 producing protein x_2 under the control of transcription factor u_2. On the transcription level, the dynamics of mRNAs in the two nodes (m_1 and m_2) can be written as

$$\frac{\mathrm{d}}{\mathrm{d}t}m_1 = \alpha_1 - \delta m_1, \qquad \frac{\mathrm{d}}{\mathrm{d}t}m_2 = \alpha_2 F_2(u_2) - \delta m_2, \tag{13}$$

respectively, where δ is the decay rate constant, α_i ($i = 1, 2$) is the transcription rate constants for node i. The extent to which node 2 is activated by input u_2 is captured by increasing Hill function $F_2(u_2)$. Translation rate of m_1 and m_2 is proportional to the amount of free ribosomes R available in the host cell, leading to the following dynamics:

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \beta_1 \frac{m_1 R}{\kappa_1} - \gamma x_1, \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \beta_2 \frac{m_2 R}{\kappa_2} - \gamma x_2, \tag{14}$$

where for $i = 1, 2$, β_i is the translation rate constant, κ_i is the dissociation constant between m_i and free ribosomes, and γ is the protein decay rate constant. Taking into account the conservation of ribosomes $R_t = R + m_1 R/\kappa_1 + m_2 R/\kappa_2$, where R_t represents the total concentration of ribosomes, and $m_i R/\kappa_i$ represents the concentration of ribosomes bound to node i, the free concentration of ribosomes can be found to be $R = R_t/(1 + m_1/\kappa_1 + m_2/\kappa_2)$. If we further assume that mRNA dynamics are much faster than protein dynamics (19, 64), and set equations (13) to quasi-steady state, the state of each node under ribosome competition can be represented by the following protein dynamics:

$$\frac{\mathrm{d}}{\mathrm{d}t}x_1 = \frac{T_1}{1 + J_1 + J_2 F_2(u_2)} - \gamma x_1, \qquad \frac{\mathrm{d}}{\mathrm{d}t}x_2 = \frac{T_2 F_2(u_2)}{1 + J_1 + J_2 F_2(u_2)} - \gamma x_2, \tag{15}$$

where lumped parameters $T_i := \alpha_i \beta_i R_t/(\delta \kappa_i)$ represents the maximum production rate constant of protein i, and $J_i := \alpha_i/(\kappa_i \delta)$ is the *resource demand coefficient* of node i, representing its capability to sequester ribosomes. By equation (15), the dynamics of node 1 (x_1) becomes coupled to the input of node 2 (u_2), and, as a consequence, expression of two nodes becomes coupled. In addition, from equation (15), steady state concentrations of the two proteins $\bar{x}_1(u_2)$ and $\bar{x}_2(u_2)$ follows a linear relationship (see Figure 4b), called an "isocost line", a phenomenon that has been verified experimentally (107, 109). Such unintended coupling between nodes in a genetic circuit largely hinders our capability to predict design outcomes.

More generally, the dynamics of node i in an n-node genetic circuit can be written as

$$\frac{\mathrm{d}}{\mathrm{d}t} x_i = \frac{T_i F_i(u_i)}{1 + \sum_{k=1}^{n} J_k F_k(u_k)} - \gamma x_i, \tag{16}$$

and as a consequence of equation (16), expression of every node is coupled to one another, largely demolishing circuits' modularity. This model has been experimentally validated in (110) and illustrates how the effective interactions in a genetic circuit can be determined by the "superposition" of intended regulatory interactions and non-regulatory interactions due to resource competition. In particular, the experiments of (110) illustrate this superposition of regulatory and non-regulatory interactions on a genetic activation cascade, whose I/O response can significantly change due to resource competition.

Mitigation of resource competition effects through negative feedback. For each node i in model (16), 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. Similar to its function in electrical engineering (42), negative feedback can be exploited to modularize the I/O response of node i. This idea has been theoretically explored in (111) and experimentally investigated in (109) for the simple circuit in Figure 5e. In particular, by engineering the product of gene 1 (x_1) to repress itself, at steady state, the extent to which $\bar{x}_1$ is coupled to $\bar{x}_2$ decreases. It is yet unclear if other biomolecular feedback controllers, such as the integral controllers in Section 3.2, can mitigate the effects of resource competition more efficiently (92, 111), and whether the controller can be used to robustify genetic circuits to other forms of competition such as competition for degradation machinery (112). More importantly, since feedback controllers do not increase a host cell's capability to produce proteins, but instead increase demand by the regulated genes in face of resource limitations, scaling up this strategy to include multiple nodes with feedback may reach fundamental design trade-offs that remain to be explored (92).

Circuit-host interaction. When resource demand by a synthetic circuit becomes too large, the physiology of the host cell may be affected (106, 108, 113), resulting in another form of context dependence known as host-circuit interaction, which is not accounted for in equation (16). Host-circuit interaction arises from growth-modulated feedback, where synthetic circuit expression retards host cell growth and this, in turn, affects synthetic circuit expression, leading to unexpected circuit behaviors (113, 114). While phenomenological models describing the effect of synthetic circuit expression on host cell growth exist, and preliminary experiments using negative feedback to robustfy circuits' response to changes in cell physiology are promising (106), the mechanistic link between host cell growth and synthetic circuit expression remains largely unexplored. Answering this question in the

future may allow implementation of a central controller that interacts with the host cell to optimize resource production, distribution and utilization (115, 116), a strategy often used to solve resource allocation problems in engineering (117).

5. Feedforward control for compensation and temporal response shaping

In a classical control design set-up, feedforward compensators are commonly designed to complement feedback controllers, especially when a model of the plant to be controlled and/or the disturbance to be attenuated is known (85). One way this is accomplished is by the incoherent feedforward loop motif (Figure 6). There is strong evidence that natural systems may use feedforward control to compensate for uncertainty since incoherent feedforward loops are highly prevalent in natural systems in bacteria and yeast cells (118). The incoherent feedforward loop has been shown to be used by natural biological systems in a variety of settings including microRNA degradation of mRNA (119), insulin release in beta cells (120), and robustness to temperature disturbances (121).

The standard topology of an incoherent feedforward 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. These opposite signed gains give the incoherent feedforward loop its name. Due to this incoherent nature, under constant input disturbances, one path compensates for the input transmitted by the other path, allowing the output of the motif to approximately reject constant disturbances (Figure 6). This incoherent feedforward motif is much more

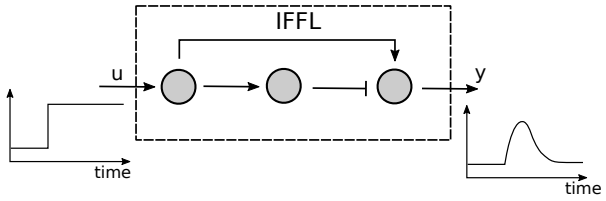

Figure 6

The incoherent feedforward loop. If the two branches are well balanced, the system rejects a step distrubance input u.

frequent in natural systems than the expected prevalence in a random network (11). This discovery prompted further research into motifs highly prevalent in natural systems (122) and, specifically, into special properties of incoherent feedforward loops that help explain this prevalence (123–125). For example, the incoherent feedforward loop acts as a pulse generator in response a step input (122). 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, it has been shown that the response of an incoherent feedforward loop may only be sensitive 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 (125). It has also been shown that incoherent feedforward loops that perfectly adapt to constant or step inputs contain an integrator which may be made explicit through a change of coordinates (126). These studies represent a reverse engineering approach for natural systems for increased understanding of the biological processes.

Due to the investigation of feedforward motifs in natural systems, researchers have used these motifs to forward engineer synthetic systems with improved disturbance compensation. For example, synthetic DNA encoding a circuit design is usually introduced in the host cell in the form of a plasmid (refer to Figure 1a); however, the copy number of the plasmid can be highly variable from cell to cell, leading to high variability in the concentration of expressed proteins. The use of an incoherent feedforward loop architecture has been shown to mitigate the effect of plasmid copy number variability on the concentration of proteins encoded by the plasmid (45). In particular, in this circuit design, the output protein expressed by the plasmid was placed under negative regulation by a regulator protein, leading to an incoherent feedforward loop with input the plasmid copy number and output the concentration of the plasmid protein.

The design of an incoherent feedforward loop for disturbance compensation is appealing as it is often simpler to build than a feedback regulation mechanism. However, for compensation to occur, the two branches of the feedforward motif need to be "well balanced". This translates into specific choices of parameters which are hard so set in practice. Novel designs that combine feedback with feedforward may be particularly useful for enhancing the robustness of incoherent feedforward architectures to parameter variations.

6. Coordination of multi-cellular behavior

In recent years, multi-cellular coordination has become a new frontier in synthetic biology. Multi-cellular coordination can be realized through cell-cell communication, in which small molecules synthesized in "sender" cells diffuse through the cell membrane to regulate expression of genetic circuits in "receiver" cells, a mechanism well-known in bacterial quorum sensing (127). 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 the "states" of its neighbors and then adjust its own activity accordingly to affect the collective population behavior. The system level architecture of multicellular coordination highly resembles that of cooperative control (128).

The capability to program cellular behvaiors collectively leads to genetic circuits with novel spatiotemporal functionalities, including population controllers (129, 130), edge detectors (131), synchronized oscillators (63) and spatial pattern generators (132, 133). These circuits can benefit future applications of synthetic biology. In addition, multi-cellular coordination, combined with intracellular feedback control, can reduce heterogeneity of gene expression in the population (134). Finally, multi-cellular coordination among different cell strains allows us to engineer "distributed genetic circuits", where the burden of sensing, computing and actuation are distributed to multiple cell strains (135–137). In biological terms, the symbiotic coexistence of multiple cell strains is called a *consortium*, which can be used to increase productivity in biosynthesis applications (135, 138). A distributed genetic circuit can also circumvent, in principle, the lack of modularity often found in circuits that operate at the single-cell level, such as unwanted structural interactions (16), retroactivity (96) and resource competition (110). In the following sections, we review these aspects of multicellular coordination in more detail.

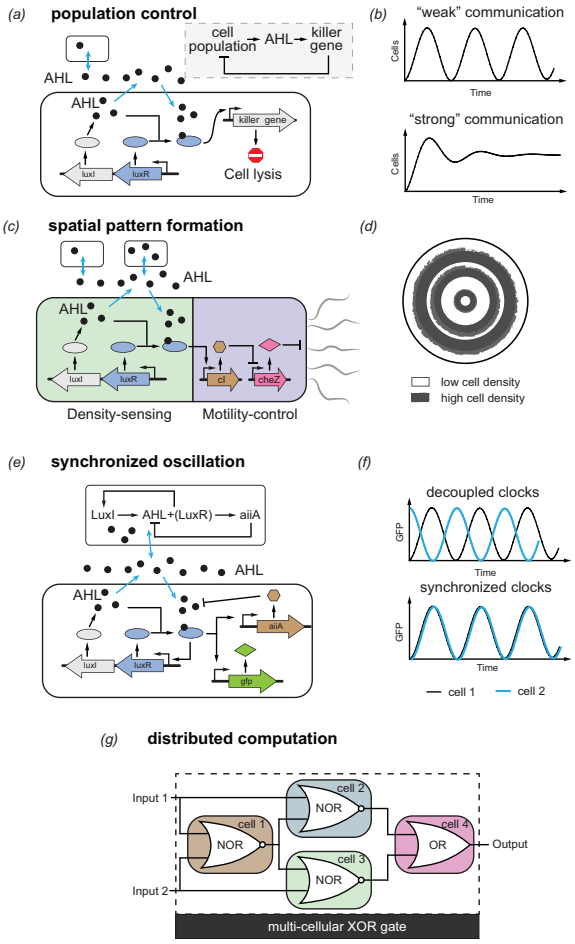

Figure 7

Multi-cellular coordination circuits. (a) The population control circuit introduced in (129). Since each synthesize diffusible small molecule AHL, an increase in cell density results in an increase in intercellular AHL concentration. Increased AHL triggers 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, leading to oscillatory population dynamics. (c)-(d) By coupling the population sensing circuit with bacteria motility control, a population of engineered bacteria can form spatial patterns autonomously (133). (e)-(f) Cell-cell communication synchronizes a population of genetic clocks (63). (g) Multi-cellular coordination enables distributed computation in genetic circuits (137).

6.1. Population control

You et al. constructed one of the earliest genetic circuits that uses multi-cellular coordination to maintain the density of *E. coli* at a desired level (129) (see Figure 7a). The circuit realizes cell-cell communication through the well-characterized quorum sensing system in the marine bacterium *Vibrio fischeri* consisting of the proteins LuxI and LuxR (127). The LuxI protein is constitutively produced to catalyze the synthesis of small diffusible 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 intracellular AHL concentration, activating the killer gene to decrease population size and closing the feedback loop. Experimental results in (129) demonstrate that population size settles to a constant level under various growth conditions. In a more recent study (130), Scott et al. constructed a similar population control circuit in *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 7b, when LuxI degrades rapidly, the amount of AHL produced in each individual cell is small, leading to weak "cell-cell communication strength" and oscillatory population dynamics. Conversely, communication strength is strong when LuxI degradation is slow and more AHL is produced in each cell, enabling the population to reach a consensus (i.e., population size reaches steady state).

While the aforementioned circuit regulates population of a single cell strain, a number of studies have emerged that attempt to control the population dynamics of multiple cell strains/types (e.g., microbial consortia) (130, 139, 140). These studies increase our understanding of natural ecosystems (130, 139), and are critical to the implementation of distributed genetic circuits (140), which is a promising research direction that we shall discuss in Section 6.4. Maintaining a population of metabolically competing species remains a grand-challenge, however, 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 (141, 142). Promising results have recently appeared in (130), in which the authors experimentally demonstrated that the population ratio of two strains can be maintained by a "decentralized" population control strategy, where each cell strain is equipped with a population control circuit (similar to that in Figure 7a). This control strategy, as well as others that involve communication and regulation among strains (130, 140), can significantly improve our ability to construct multi-cellular genetic circuits with more functionalities. However, an understanding of the feedback control mechanisms that may overcome current challenges in stably and robustly maintaining a consortium with two or more strains is largely lacking. A control theoretic approach may be critical to mature such an understanding.

6.2. Pattern formation

Synthetic pattern formation systems could lay the foundation for future biomaterials that self-organize into patterns of biological entities (143). One of the earliest pattern formation circuits was developed by Basu et al. (132). The pattern forms on a plate containing a spatially homogeneous population of "receiver cells" surrounding "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" contains an incoherent feedforward circuit that takes AHL as input and produces a fluorescence reporter as output. The feedforward circuit is tuned to produce a biphasic I/O dose response curve, and therefore, fluorescence output is produced at intermediate AHL concentrations, forming a fluorescence ring on the plate.

The circuit in (132), 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. (133) constructed an autonomous pattern formation circuit by coupling a LuxR/LuxI population density-sensing module with a motility-control module, which includes the gene CheZ in the *E. coli* chemotaxis pathway (see Figure 7c). In low cell density regions, the concentration of AHL is low, and consequently, *cheZ* expression drives the flagellar motor to enable the cell to move roughly in a straight line. Alternatively, in high cell density regions (high AHL concentration), the flagellar motor spins in clockwise direction, causing the cell to tumble (19), which disables its motility. As a result, spatial oscillation occurs, and cells aggregate into stripe patterns.

6.3. Reduction of cell-cell variability

Through population averaging, multi-cellular coordination can serve as an effective tool to reduce population level heterogeneity in gene expression. In (134), Vignoni et al. theoretically studied a circuit consisting of negative autoregulation and cell-cell communication. In particular, *luxI* gene expression is under transcriptional repression by LuxR:AHL complex. A saturating amount of LuxR is produced constitutively, and AHL synthesis is catalyzed by protein LuxI, forming an effective negative feedback loop around the LuxI production processes. Since AHL diffuses freely across the membrane, *luxI* expression depends, in principle, on the intercellular concentration of AHL, which reflects average LuxI expression level across the population. The authors demonstrate through a combination of analytical study and numerical simulations, 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 (34).

Reducing population heterogeneity is especially crucial for multi-stable and oscillatory circuits. Cell-cell variation may lead to noise-induced transition among phenotypes (i.e. stable steady states) in a multi-stable circuit (32, 71), jeopardizing its desired functionality. Koseska et al. numerically studied a population of genetic toggle switches, and found that coupling of switches through small molecules enhances precision of cell decision (144). Similarly, Danino et al. used cell-cell communication to reduce heterogeneity in a population of genetic clocks (i.e. clock synchronization) (63). As shown in Figure 7e, each cell in the population contains a synthetic genetic activator/repressor clock (46). The diffusible molecule AHL has dual functions: enabling intracellular transcriptional activation that gives rise to oscillatory dynamics on single cell level, and mediating cell-cell communication to synchronize the genetic clocks. Experimental results in (63) demonstrated that the synchronized genetic clocks can produce sustained oscillation at the population level (Figure 7f). This contrasts earlier experiments of decoupled genetic clocks, where population level oscillation is damped out as cells in the population become progressively out of phase due to noise (46).

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 strain 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 (136, 137). For example, in (137), Tamsir et al. built a genetic XOR gate using a composition of four NOR and OR gates that are distributed into four different *E. coli* strains (Figure 7g). 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 (135, 145).

Nevertheless, a number of technical challenges remain before this technology turns to maturity (146). A major system level hurdle lies in the fact that "communication strength" (i.e. concentration of communicating small molecules) is dependent 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 for discussion on population control). Secondly, an appreciable amount of delay may occur during signal transmission (i.e. diffusion), which may deteriorate circuits' temporal response or even cause instability (85). The solution to both problems, may benefit significantly from a control theoretic approach, since closely related problems, such as multi-agent coordination in the presence of communication delay, have been addressed in other engineering contexts (128). Meanwhile, exploration and characterization of orthogonal cell-cell communication modules in bacteria (147) and eukaryotic cells (148, 149) remain preliminary, and more inputs from the biological engineering community is required to expand the tool box.

We envision that at least two control layers are required in future genetic circuits. "Low level" intracellular controllers modularize 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 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 design and implementation of more sophisticated circuits.

7. Summary and outlook

In this review, we discussed how control design principles have permeated synthetic biology to tackle fundamental problems encountered when programming cells to work for us: designing circuit's dynamics (Section 2), improving circuit's robustness to unknowns (Sections 3,5), aiding modular and layered design (Section 4), and programming the emergent behavior of cell populations (Section 6). While the field of synthetic biology has gone through quick progress and has clearly demonstrated its remarkable potential in ground-breaking applications (Section 1), a number of significant challenges still 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 multi-stable systems), the spectrum of functions that can be realized is still unclear, and especially, the extent of achievable precision for more quantitative design. Imposing strict analogies with engineering, chiefly with electrical engineering, may be misleading due to a number of 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, RNA-RNA interactions) that are used in synthetic biology are plagued by 10x-100x uncertainty in key parameters. These may also dramatically change with temperature, pressure, cell metabolism, and the specific circuit's context (Section 4), 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 that there is rapid development of new biological tools that continuously expands the set of core processes that can be used for design (e.g., CRISPR-based regulators (14)). Compared to this rapid pace, *theory is lagging behind* and new processes become used before they are systematically characterized. There are significant challenges to systematic characterization, which include system identification techniques that can handle nonlinear parameterizations typical of biomolecular processes, the lack of fast and precise sensors, and the small set of sensors that can be used in a single experiment. 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 this respect to obtain, for example, repeatable performance of amplifiers despite 5x variations in their components (42). The key to obtain this is to compare the actual output of the system to the desired one, 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 that we face is much larger than that found in engineered 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 due to context dependence (16, 94). Context dependence leads to I/O characteristics of core processes that widely change 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 capability to scale up circuit's size and sophistication. In addition to the remarkable advances in the biological engineering community towards minimizing interference among basic parts (for example, DNA promoters and terminators) (16), engineering *in vivo* biomolecular controllers provides a promising path towards making the I/O behavior of genetic circuits independent of context (Section 4).

At the multi-cellular level, programming the emergent behavior of a bacterial population is still 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 our guts), communication delay due to diffusion, nonlinearity in "agent dynamics" and spatial heterogeneity make traditional control theoretic formulations inapplicable. Interestingly, and as illustrated in Section 6, experimentalists are already implementing multi-cellular computation and extensively using feedback control for coordination. However, these designs often miss theoretical guarantees, have poor robustness properties, and are not reliable. More generally, the key question in any multi-cellular computation of how to robustly maintain desired cell populations in multi-strain consortia remains largely open.

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