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Understanding and exploiting feedback in synthetic biology

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HIGHLIGHTS

- There are important distinctions between feedback in process control and in biology.
- Negative, positive, and combined feedback exhibit unique properties.
- Feedback can be readily applied to advance the construction of biological systems.

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ABSTRACT

Synthetic biology employs traditional engineering concepts in the construction of cells and organisms. One of the most fundamental concepts is feedback, where the activity of a system is influenced by its output. Feedback can imbue the system with a range of desirable properties such as reducing the rise time or exhibiting an ultrasensitive response. Feedback is also commonly found in nature, further supporting the incorporation of feedback into synthetic biological systems. In this review, we discuss the common attributes of negative and positive feedback loops in gene regulatory networks, whether alone or in combination, and describe recent applications of feedback in metabolic engineering, population control, and the development of advanced biosensors. The examples principally come from synthetic systems in the bacterium *Escherichia coli* and in the budding yeast *Saccharomyces cerevisiae*, the two major workhorses of synthetic biology. Through this review, we argue that biological feedback represents a powerful yet underutilized tool that can advance the construction of biological systems.

1. Introduction

The field of synthetic biology aims to construct biological systems in order to better understand nature and to address pressing challenges facing society. However, the inherent complexity of biology impedes the construction of predictable and robust systems. Fortunately, concepts in traditional engineering disciplines offer approaches to reduce biological complexity and subsequently advance biological design.

One engineering discipline that has strongly influenced synthetic biology is process control. This discipline seeks to automate industrial processes in order to maintain system specifications with limited oversight. One prevalent tool in process control is feedback. Feedback serves multiple purposes, including driving the output of a system toward a desired setpoint, countering disturbances in system inputs, filtering measurement noise, and decoupling relationships between multiple inputs and multiple outputs. The physical link between system inputs and outputs is called a feedback loop. Process control principally focuses on negative feedback loops that drive the system output toward the setpoint. Positive feedback loops, which drive the system output away from the setpoint, are adopted less frequently (Horowitz and Hill, 1989).

Both negative and positive feedback loops can be found throughout the architecture of gene regulatory networks. Extensive studies, particularly in the field of systems biology, have revealed that these biological feedback loops shape the dynamics, variability, and steady-state response of the system. These influences in turn have been implicated in the adaptability and robustness of biological systems. The mechanisms of feedback vary widely and can be combined, resulting in overlaid negative and positive feedback loops with unique properties.

Despite the many benefits of feedback in biology and its prevalence in other engineering disciplines, synthetic biology has been slow to implement feedback in the design of biological systems. Arguably, the field is still grappling with how to construct large-scale systems that behave predictably and has not yet advanced to the point of including additional layers of control. As a result, recent work has centered on the construction of logic gates with either higher-order processing or the ability

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to interpret multiple signals (Rinaudo et al., 2007; Friedland et al., 2009; Tamsir et al., 2011; Moon et al., 2012). Feedback has been integrated into a handful of synthetic biological systems, but often only in the cases in which feedback was essential for the desired system behavior (e.g. genetic oscillators (Elowitz and Leibler, 2000; Atkinson et al., 2003; Fung et al., 2005; Stricker et al., 2008)). However, feedback offers a wealth of attributes that can generate more desirable behaviors and improve the robustness of biological systems.

In this review, we discuss the known properties of negative and positive feedback loops – both in isolation and in combination – in biological systems. Examples principally are drawn from bacteria and yeast, the current workhorses of synthetic biology. Many of the insights into feedback were drawn from synthetic systems, demonstrating how synthetic biology informs our understanding of nature. We then discuss recent applications of feedback loops in synthetic biology. Finally, we conclude by describing how feedback can be further implemented to advance current applications in the field.

2. Modes of feedback

Feedback can occur at multiple steps of gene expression or through the interactions between organisms. Fig. 1 illustrates representative mechanisms through which feedback can be introduced. We touch on many of these mechanisms in discussing the properties and application of feedback in biology. Note that even simpler examples of feedback often engage multiple mechanisms at one time.

2.1. Transcriptional regulation

In the first step of gene expression, messenger RNA is transcribed from the DNA of a gene. This step begins with transcription factors recruiting RNA polymerase to the gene's promoter and ends with termination and release of the polymerase. Both steps offer opportunities for both negative and positive feedback, by controlling the accessibility or methylation of the DNA (Lim and Van Oudenaarden, 2007; Octavio et al., 2009), the availability of RNA polymerase or transcription factors, or transcriptional termination (Winkler et al., 2002; Lucks et al., 2011). The simplest form of transcriptional feedback, called auto-regulation, involves a transcription factor regulating its own transcription. Auto-regulation is one of the most common transcriptional architectures found in bacteria (Shen-Orr et al., 2002) and has undergone the most extensive characterization out of all modes of feedback.

2.2. Post-transcriptional regulation

Following transcription, the messenger RNA is translated into protein. In microorganisms, this step can be regulated by modulating RNA stability and translation. In most cases, the responsible mechanisms involve the interaction of a trans-acting factor. This factor could be a protein, such as a ribonuclease or the RNA binding protein CsrA; an RNA, such as Hfq-binding small RNAs or synthetic riboregulators; or a small molecule, such as cofactors recognized by translational riboswitches (Waters and Storz, 2009). Many of the RNA-based mechanisms are still undergoing characterization and have not been quantitatively studied in the context of feedback.

2.3. Post-translational regulation

Once a protein is formed, feedback can be introduced by modulating the protein's stability, localization, or activity. Stability can be modulated by attaching a degradation tag or altering protease activity, influencing protein levels. Next, localization can be modulated by targeting the protein to the cell membrane or to an organelle, affecting whether the protein can access its target. Finally, protein activity can be modulated through chemical modifications or reversible binding of small molecules, RNAs, or proteins ((Liu et al., 1997; Wassarman and Storz, 2000; Buchler and Cross, 2009; Hunsicker et al., 2009), altering the ability of the protein to carry out its normal functions. Modulating protein activity is the most common post-translational mechanism of

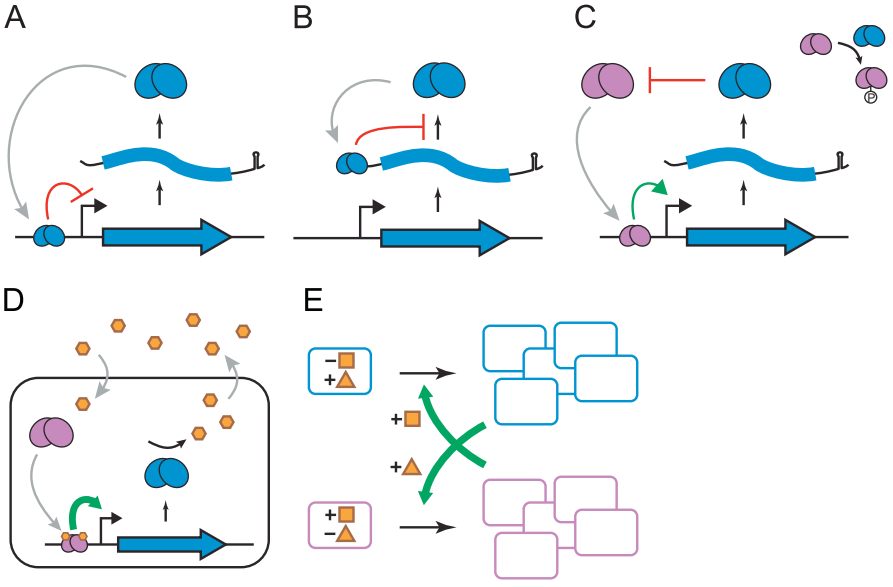

Fig. 1. Modes of feedback in microorganisms. Feedback can be introduced at the different steps of gene expression (A)–(C) and through the interactions between cells (D) and (E) Red block lines designate negative regulation while green arrows designate positive regulation. (A) Transcriptional regulation, as shown for auto-repression. (B) Post-transcriptional regulation, as shown for an RNA-binding protein inhibiting its own translation. (C) Post-translational regulation, as shown for phosphorylation of a transcription activator that inhibits DNA binding. (D) and (E) Cell–cell interactions, as shown for the synthesis and secretion of a diffusible molecule that activates the expression of the synthesis enzyme (D), and for a mutualistic interaction where one strain produces an essential metabolite not produced in the other strain (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

feedback. One prominent example involves a small molecule reversibly binding to a transcription regulator, altering the ability of the regulator to bind DNA. Small-molecule responsive regulators, such as TetR, LacI, and AraC, are the most common components used in the construction of synthetic biological systems and are featured prominently in this review. Regardless of the exact mechanism, the protein inevitably influences its own activity or abundance. For instance, tetracycline binding to the transcription repressor TetR de-represses the expression of the TetA transporter protein, which reduces the intracellular concentration of tetracycline by pumping it out of the cell.

2.4. Cell–cell interactions

The final mode of feedback occurs between cells, influencing gene expression and/or cell growth. One common means of cell–cell interactions is through the secretion of diffusible factors. The secreted factors, either small molecules or peptides, interact with a surface receptor or an intracellular transcription regulator. In natural systems, one of these genes often is responsible for the synthesis of the signaling molecule, forming a positive feedback loop formed between all members of the population (Waters and Bassler, 2005). Induction and feedback only occur at high cell densities, granting cells a way to coordinate gene expression with their neighbors. This phenomenon is called quorum sensing (Ng and Bassler, 2009).

Quorum sensing is a standard tool in synthetic biology to elicit communication between engineered cells. The most common quorum sensing system, adapted from the marine bacterium *Vibrio fischeri*, combines the LuxI enzyme that synthesizes the *N*-acyl homoserine lactone (AHL) signaling molecule and the LuxR regulator that activates gene expression when bound to AHL. This system and other similar systems have been employed in a range of synthetic biology applications, including spatial patterning (Basu et al., 2005; Sohka et al., 2009), controlling the cell density of a bacterial culture (You et al., 2004), and engineering communication between bacteria and eukaryotes (Weber et al., 2007; Biliouris et al., 2012). As discussed later in this review, quorum sensing also offers a unique opportunity to introduce feedback across length scales well beyond that of a single cell.

Cell–cell interactions also can elicit feedback by modulating cellular health and survival. The interactions can be beneficial, such as complementary metabolic pathways, or harmful, such as predator-prey relationships. Many of these interactions can be readily engineered into a population of otherwise non-interacting organisms. Examples include linking quorum sensing to the activation or repression of a toxin (You et al., 2004; Balagaddé et al., 2008) and overproducing an essential metabolite that cannot be synthesized in other cells (Shou et al., 2007). The interacting cells form key components of feedback by ensuring the survival or death of their neighbors. This stands in contrast to the other modes of feedback that begin and end with gene expression.

3. Properties of negative feedback

Process control commonly employs negative feedback among other control strategies to maintain chemical processes. A feedback controller calculates the difference between the measured output and a desired output of the system, the setpoint, such as product purity or temperature. The controller then adjusts the process to minimize this difference (proportional control). Aside from using the deviation from setpoint, the controller can take into account how long the difference has persisted (integral control) and how quickly the difference is changing (differential control). These different modes of feedback can be used depending on the required speed and stability by which the controller responds to setpoint deviations (Seborg et al., 2011).

Natural biological systems also implement negative feedback, but in ways that can differ from negative feedback in process control. First, in process control, negative feedback is often separate from the process itself through the actions and programming of an installed control system. In biological systems, the feedback is imbedded within the genetics and biochemistry of the process. Second, in process control, the principal goal of negative feedback is to maintain the system output within desired specifications. In biology, this goal can be more varied. Negative feedback alters the dynamics, steady-state behavior, and cell–cell variability of biological systems (Fig. 2), where the benefit of each contribution likely depends on the cellular context (Thomas et al., 1995; Wall et al., 2003). Fortunately, we can exploit these attributes in the rational design of synthetic biological systems as discussed below. Note that all of the attributes we describe were observed for negative auto-regulation (Alon, 2007a), although other regulatory mechanisms have been shown to exhibit similar properties (Bashor et al., 2008).

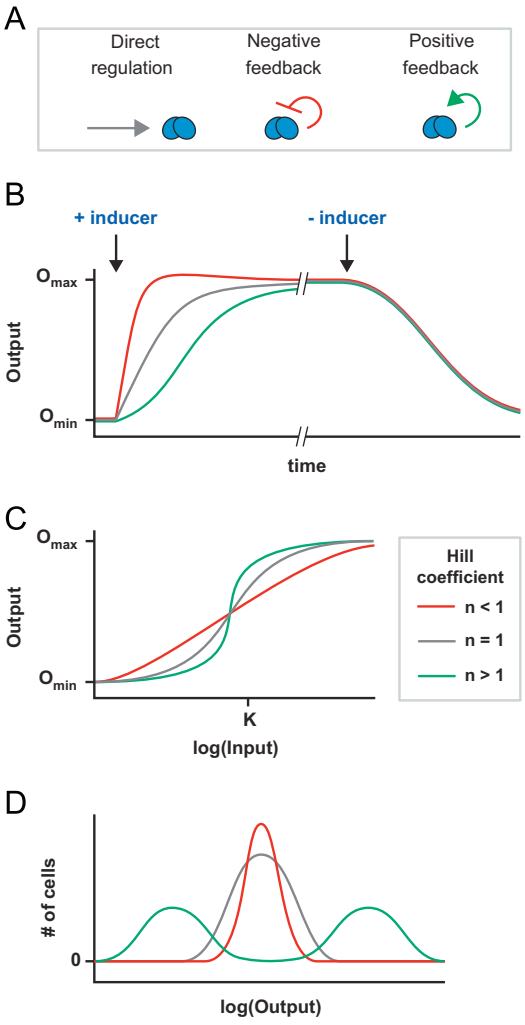

Fig. 2. Contrasting characteristics of negative and positive feedback. These characteristics are also discussed in (Alon, 2007a). (A) Comparing direct regulation (gray), negative feedback (red), and positive feedback (green) according to (B) response dynamics, (C) input-output relationship at steady-state, and (D) cell–cell variability for an intermediate input value. In comparison to direct regulation, negative feedback and positive feedback exhibit opposite behaviors. The only exception is the settling time, where all three are predicted to exhibit similar responses times. The indicated Hill coefficients assume no cooperativity in the feedback loop. The Hill coefficient and K in (C) are part of the Hill Eq. (1). Adapted from (Alon, 2007b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1. Dynamics: Shorter response time

Microorganisms constantly face unpredictable and fluctuating environments. How quickly microorganisms adapt to new environmental conditions could mean the difference between life and death. Negative feedback offers one approach to influence the overall dynamics by shortening the rise time (Savageau, 1974). Rise time can be defined as the time required for the gene product to proceed halfway between an initial steady-state concentration and a final, elevated steady-state concentration. Rosenfeld et al. (2002) experimentally demonstrated the shorter rise time with a synthetic auto-repression circuit in *Escherichia coli*. They tested two genetic circuits: one composed of the TetR transcription repressor controlling the expression of the GFP fluorescent reporter protein (direct regulation), and a second circuit composed of a TetR–GFP fusion that represses its own expression (auto-repression). Time course experiments following addition of the inducer, anhydrotetracycline (aTc), demonstrated that the rise time was up to five times smaller for auto-repression than for direct regulation. The reason for the decrease in the rise time is the following: for promoters of equal strength, both GFP and the TetR–GFP fusion accumulate at the same rate. However, the TetR–GFP fusion begins repressing its own synthesis, lowering the final steady-state concentration. The result is an apparent acceleration toward the final steady-state. Accordingly, use of lower aTc concentrations further reduced the response time, because TetR still can repress its own expression. Note that the settling time, the time required for the gene product to proceed halfway between an initial steady-state concentration and a final, lowered steady-state concentration (e.g. through the removal of aTc), is expected to be similar for auto-repression and for direct regulation. Despite this, the shorter response time afforded to negative feedback may be beneficial in the design of synthetic systems that must act rapidly, such as medical diagnostics.

3.2. Steady-state: Linearized response

The steady-state relationship between the input and output values of a negative feedback loop is just as important as the approach to steady state. This relationship can be illustrated for the bacterial transcription regulator TetR, which represses the expression of itself and the tetracycline exporter TetA. The antibiotic tetracycline binds TetR, relieving repression of both TetR expression and TetA expression. TetA then exports tetracycline, alleviating the inhibitory effect of this antibiotic on protein translation (Biliouris et al., 2011). While negative feedback should shorten the response time of the system to tetracycline, negative feedback also impacts the relationship between TetA levels and tetracycline concentrations. This relationship is important because it determines how well cells can negate the deleterious effects of tetracycline without investing too many resources in TetA production.

Nevozhay et al. (2009) investigated the steady-state attributes of negative feedback through the construction of two synthetic gene circuits in the model yeast *S. cerevisiae*. One circuit constitutively expressed TetR (direct regulation) while the other circuit auto-repressed TetR (auto-repression). Each circuit was tested by incubating the cells in varying concentrations of the tetracycline analog doxycycline and by measuring GFP under the control of a TetR-repressible promoter. They found that auto-repression extended the range of doxycycline concentrations that yield intermediate levels of GFP. In other words, auto-repression linearized the steady-state response to doxycycline. An accompanying mathematical model predicted this behavior, where negative feedback dampened the induction of the repressor. Madar et al. (2011) made similar observations for auto-repression of the AraC transcription regulator as part of L-arabinose utilization in *E. coli*. The benefit of linearization is that it allows the output to be finely tuned by varying the input signal. The ability to fine tune expression would be extremely useful in synthetic biology, such as when optimizing enzyme levels in an engineered metabolic pathway or when accurately predicting the concentration of a measured analyte with engineered biosensors.

3.3. Cell–cell variability: Noise reduction

Biological systems are inherently noisy (Balázsi et al., 2011). Noise, as measured by cell–cell variability in protein levels, arises from the small number of molecules involved in gene expression (intrinsic noise) and variation in the abundance and activity of the gene expression machinery (extrinsic noise). The result of such noise is large variations in protein levels between genetically identical cells in the same environment. These differences can interfere with the reliable processing of biological signals. In addition, these differences can introduce heterogeneity that promotes survival of a fraction of a cell population even in unpredictable and changing environments. Correspondingly, gene circuits have been shown to suppress or enhance noise, which will be critical for the future design of synthetic biological systems.

The impact of negative feedback on noise was first assessed experimentally by Becskei and Serrano (2000). They evaluated a synthetic system in *E. coli* in which a TetR-repressed promoter controlled the expression of a TetR–GFP fusion. Using fluorescence microscopy to measure GFP levels in individual cells, they found that the variability of GFP levels across the population was two to three times higher for direct regulation than for auto-repression. Nevozhay et al. (2009) further confirmed this finding in yeast. The physical explanation is that auto-repression drives GFP levels to the same average value. When repressor levels are below this value, expression increases due to reduced feedback; when repressor levels are above this value, expression decreases due to enhanced feedback. Implementing negative feedback to reduce noise would be critical for any synthetic system that must faithfully convert an input signal into a defined output value. Reducing noise would be especially important when developing devices composed of only a few engineered cells, such as those for in vivo disease diagnostics in which population averaging cannot be applied.

One critical feature of negative feedback is the inherent delay between the altered input signal and the change in the system output (Seborg et al., 2011). Substantial delays in feedback, such as from the slow loss of a stable protein or from a cascade of regulators, can cause the system output to oscillate (Thomas, 1978; Bratsun et al., 2005). Stricker et al. (2008) experimentally demonstrated that even auto-repression, the simplest form of negative feedback, can exhibit sufficient delays in gene expression to cause oscillations in the measured output. Maithreye et al. (2008) separately demonstrated that a delay in feedback increases instability of the system output, although systems with or without delayed feedback eventually converged on the same output value. The impact of delays begs the question: what is the propensity of feedback in an experimental biological system to exhibit oscillations and how much effort must be exerted to either avoid or promote this behavior? Further investigations will help clarify when engineers need to be concerned about the potential for oscillatory behavior while introducing feedback into a biological system.

4. Properties of positive feedback

While process control inherently employs negative feedback, it tends to avoid positive feedback because of its destabilizing effect

on the system output. The most common example in chemical processes is thermal runaway, where heat emitted by an exothermic reaction increases the reaction rate, releasing more heat. Thermal runaway poses a major safety threat in the chemical industry, and, when left uncontrolled, has led to numerous instances of catastrophic explosions and the release of chemical pollutants and toxins into the environment (Stoessel, 2008).

In contrast, positive feedback is widely employed in biology. For instance, bacteria employ positive feedback in sugar utilization, where the imported sugar induces the expression of sugar-specific transporters, leading to further sugar import. What distinguishes positive feedback in process control and in biological systems is that the latter always has an upper limit, preventing the biological equivalent of thermal runaway. Below we discuss the properties of positive feedback in biological systems in the same contexts as negative feedback: dynamics, steady-state behavior, and cell–cell variability (Alon, 2007a). Overall, the properties of positive feedback and negative feedback are opposites (Kaufman and Thomas, 2003), offering contrasting tools in the construction of synthetic biological systems (Fig. 2).

4.1. Dynamics: Longer response time

In contrast to negative feedback, positive feedback exhibits a longer rise time in comparison to direct regulation (Savageau, 1974). The extended rise time was first demonstrated experimentally in *E. coli* using the transcription activator cI, which is involved in the lysis-lysogeny decision in lambda bacteriophage (Maeda and Sano, 2006). To generate positive feedback, Maeda and Sano (2006) tested a gene encoding a cI–GFP fusion under the control of a cI-activated promoter. Binding by the LacI repressor downstream of the promoter prevented initial auto-activation, which could be relieved with the addition of the LacI inducer IPTG. For direct regulation, GFP was expressed from a LacI-repressed promoter. The rise time following the addition of IPTG was up to four times greater for positive feedback than for direct regulation. The explanation, as supported by mathematical modeling, was that positive feedback increased the steady-state output because more cI leads to greater transcription. Note that the settling times for direct regulation and auto-activation (e.g. removal of IPTG) are expected to be similar, paralleling deactivation of a negative feedback loop. A delayed response could be useful in the construction of synthetic systems that require a sustained input signal or a timed series of cellular events (Temme et al., 2008), although other genetic circuits can generate delays following circuit activation or deactivation (Mangan and Alon, 2003). Furthermore, positive feedback imbues a system with other critical behaviors as described below. The availability of other delay-generating circuits and the additional properties of positive feedback argue against the use of positive feedback in biological design with the sole intent of altering the response dynamics.

4.2. Steady-state: Ultrasensitivity

While negative feedback linearizes the response of a system to its input, positive feedback can give rise to an ultrasensitive response. Under this type of response, a small change in the input signal results in a much larger change in the output. This relationship is captured by the Hill coefficient (n), a constant in the following empirical equation relating the system input (I) and the system output (O)

$$O = O_{\min} + (O_{\max} - O_{\min}) \frac{I^n}{K^n + I^n}, \quad (1)$$

where K is the input value yielding an output halfway between the maximal output ($O_{\max}$) and the minimal output ($O_{\min}$). This equation can be fit to experimental data in order to estimate a value of the Hill coefficient. For a system lacking any feedback or cooperativity, the Hill coefficient is one. For Hill coefficient values greater than one, the response curve becomes steeper, indicating that the response has become more sensitive.

One of the best-studied examples of ultrasensitivity in positive feedback is the lactose utilization pathway. This pathway exhibits positive feedback when induced with the lactose analog IPTG. IPTG relieves repression by the transcription repressor LacI, which induces expression of the lactose transporter LacY and leads to the further import of IPTG. Jensen et al. (1993) first demonstrated a sharp response of the activity of a LacI-repressed promoter to IPTG, which became shallower in the absence of *lacY*. A Hill coefficient of 4.5 later was measured for this same system, compared to 2.6 in the absence of *lacY* (Kuhlman et al., 2007). To put this into perspective, for a Hill coefficient of one, the IPTG concentration must increase by a factor of 81 to go from 10% to 90% of the maximal output. For a Hill coefficient of 4.5, the IPTG concentration must increase by a factor of 2.7 to achieve the same relative change in output, underscoring how positive feedback can render the relationship between the system input and output more switch-like. The reason for this behavior is that positive feedback amplifies the input signal, further increasing the system output.

Positive feedback may be beneficial for a range of synthetic biology applications requiring switch-like responses, such as the development of digital cellular devices or the threshold detection of disease biomarkers. It is worth noting that other circuit configurations and modes of regulation can produce ultrasensitivity, such as protein allostery (Koshland et al., 1966), regulatory cascades (Huang and Ferrell, 1996; Hooshangi et al., 2005), or sequestration or stoichiometric action of a regulator (Levine et al., 2007; Buchler and Cross, 2009). However, none of these other configurations have a propensity for bistability as described next for positive feedback.

4.3. Cell–cell variability: Increased noise and bistability

Positive feedback increases cell–cell variability in a similar fashion to other circuit configurations exhibiting ultrasensitivity (Hooshangi et al., 2005; Mehta et al., 2008). The increased variability emerges from noise in the input leading to large changes in the output. However, in the presence of strong positive feedback, a biological system can become bistable (Guespin-Michel and Kaufman, 2001). Bistability was observed experimentally over fifty years ago in the lactose utilization pathway (Novick and Weiner, 1957). Intermediate concentrations of the lactose analog thio-methylgalactoside (TMG) induced the pathway either fully or negligibly, a phenomenon that has been called an ‘all-or-none’ response. The ‘all-or-none’ response emerges from the import of TMG inducing the expression of the transporter LacY, which drives further TMG import and LacY expression. This cycle of import and induction continues until LacY levels are maximized. Similar bistable behavior was observed in a natural MAPK signaling cascade that induces the expression of the MAPK components (Ferrell and Machleder, 1998) and in synthetic transcriptional auto-activation systems in *E. coli* (Isaacs et al., 2003) and in budding yeast (Becskei et al., 2001; Ajo-Franklin et al., 2007).

One common feature of bistable systems with rare exception is hysteresis (Guidi and Goldbeter, 1997). Hysteresis represents a phenomenon where the input–output relationship is influenced by the history of the system. In the case of the lactose utilization pathway, pre-incubating the cells with TMG lowered the TMG concentration associated with the transition between induced and uninduced states (Novick and Weiner, 1957; Ozbudak et al.,

2004). The basis of hysteresis is that positive feedback maintains the induced state even at lower inducer levels. Only through stochastic fluctuations do the cells switch states (Choi et al., 2008). When positive feedback is sufficiently strong, the cell can be 'locked' into the induced state even upon complete loss of the input signal. Members of the Silver laboratory exploited this feature to generate synthetic 'memory' devices that capture a transient cellular state such as DNA damage (Ajo-Franklin et al., 2007; Burrill and Silver, 2011).

Mathematical modeling has shown that non-linearity in the positive feedback loop—whether through non-linear kinetics or regulator cooperativity—is essential for bistability and hysteresis (Keller, 1995; Ozbudak et al., 2004). However, there are exceptions to this rule. To and Maheshri (2010) demonstrated in yeast that auto-activation by a non-cooperative regulator can generate a steady-state bimodal response as long as the regulator is unstable and expressed stochastically. However, hysteresis was not explored, leaving the unanswered question of whether their system exhibits bistability. Separately, Tan et al. (2009) demonstrated in bacteria that auto-activation of a non-cooperative regulator can generate bistability and hysteresis as long as overexpression of the regulator slows cellular growth. Note that the decreased cellular growth introduced an additional positive feedback loop, which improved the robustness of bistability as discussed in Section 5.2.

Bistability is arguably the most advantageous attribute of positive feedback for synthetic biology because it ensures that biological systems either are fully induced or uninduced—a digital readout of an input signal. The ability to establish one of two fixed states would benefit a wide range of synthetic biology applications, including programmed cell differentiation, analog-to-digital conversions for cellular computing, and generation of synthetic microbial consortia from a single starter strain.

5. Properties of combined negative and positive feedback

While negative feedback and positive feedback offer contrasting attributes, combining these forms of feedback can augment these attributes or introduce entirely new ones (Demongeot et al., 2000). We next discuss the ramifications and potential applications of combining feedback loops in biology. Our focus is on the most salient features of each combination. Fig. 3 illustrates features of representative loops. Overall, each combination can offer improved properties over a single positive or negative feedback loop, although the improvements come at the cost of additional system components and the potential for additional emergent behaviors.

5.1. Multi-negative feedback loops

In the two seminal examples of synthetic genetic circuits, negative regulatory events were combined in series into a single feedback loop. In the first example, Gardner et al. (2000) constructed a double-negative feedback loop in *E. coli*. This 'toggle switch' was comprised of the transcription repressors LacI and TetR, where LacI repressed the expression of TetR and TetR repressed the expression of LacI. Because a double-negative feedback loop is equivalent to a single positive feedback loop, the toggle switch similarly exhibited ultrasensitivity, bistability, and hysteresis. However, unlike positive feedback, the toggle switch could fix each stable state—in this case either high LacI

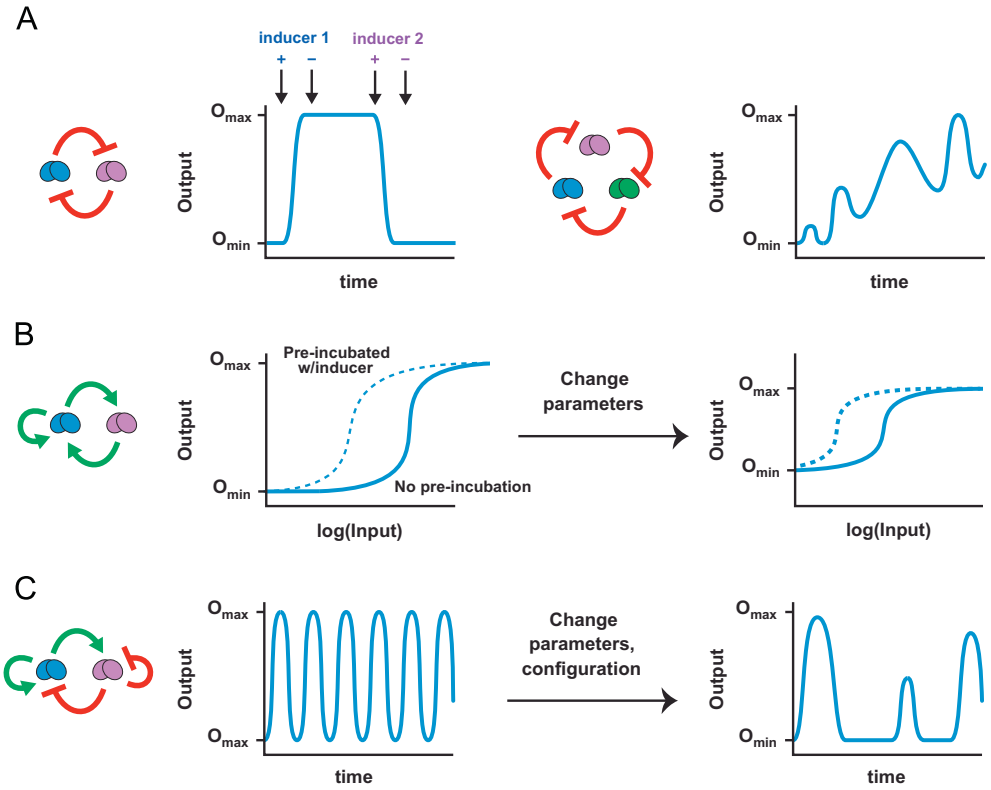

Fig. 3. Combining negative and positive feedback. The presence of multiple regulatory interactions imparts enhanced or even unique properties. (A) Double-negative and triple-negative feedback loops. A double-negative feedback loop (left) functions similarly to a single positive feedback loop, only the double-negative loop 'locks' the induced state and the uninduced state. Transient exposure to the appropriate inducer drives the system from one state to another. A triple-negative feedback loop (right) generates oscillations, although the oscillations are inconsistent. (B) Layered positive feedback loops. These loops exhibit the same general properties of ultrasensitivity and bistability as a single positive feedback loop. However, both properties in the layered loops are more robust to changes in system parameters such as feedback strength or expression strength. (C) Nested negative and positive feedback loops. Depending on the exact configuration and system parameters, the system can exhibit a range of behaviors including stable oscillations (left) and excitability (right).

levels or high TetR levels. Furthermore, a pulse of inducer, either IPTG or aTc, was sufficient to drive the system into the opposite state. The ability to drive the system irreversibly into either state offers a clear advantage over auto-activation, where only the induced state is reinforced.

In the second example, Elowitz and Leibler constructed and tested a triple-negative feedback loop in *E. coli* that has been dubbed the 'repressilator' (Elowitz and Leibler, 2000). This loop was comprised of three transcription regulators, TetR, cI, and LacI, where TetR repressed the expression of cI, cI repressed the expression of LacI, and LacI repressed the expression of TetR. This loop resembles a single-negative feedback loop with a delay in feedback. Accordingly, mathematical modeling predicted that this circuit would exhibit oscillations under a broad parameter range (Elowitz and Leibler, 2000). While the circuit did exhibit oscillations, the oscillations were short-lived with ranging amplitudes and periods. This unsatisfactory performance was attributed to noise in gene expression. The properties of the 'repressilator' argue against the use of triple-negative feedback loops to generate oscillations, especially in comparison to other oscillatory circuits that combine negative and positive feedback (see Section 5.3).

5.2. Layered positive feedback loops

Unlike multi-negative feedback loops, any number of positive regulatory steps results in positive feedback. The properties described above for a single positive feedback loop thus apply to multiple positive feedback loops. The notable difference is that the presence of multiple positive feedback loops can improve the robustness of these properties. Shah and Sarkar (2011) comprehensively investigated the properties of multiple feedback loops by modeling the behavior of all possible interactions between two or three regulators. In particular, they evaluated the extent to which each possible circuit exhibits ultrasensitivity and bistability across a range of parameter values, which can be described as the robustness of the behavior. Both enzyme and transcription regulators were included in the analysis to account for differences in the regulatory properties. Modeling revealed that the presence of multiple positive feedback loops increased the robustness in both ultrasensitivity and bistability. Furthermore, the most robust circuits were hybrid circuits of both enzyme regulators and transcription regulators. Part of the rationale for the improved robustness was that enzyme regulators such as kinases and phosphatases can exhibit zero-order ultrasensitivity (Goldbeter and Koshland, 1981) that contributes to both ultrasensitivity and bistability.

It is worth noting that regulatory cascades, regulator sequestration, and stoichiometric action by a regulator can also generate ultrasensitive responses. When coupled with positive feedback, these mechanisms have been shown to enhance the robustness of bistability (Ferrell and Machleder, 1998; Bashor et al., 2008; Levine and Hwa, 2008). Chen and Arkin (2012) most recently demonstrated this effect in *E. coli* using a repressor (the anti-sigma factor *rsiW* from *Bacillus subtilis*) that sequesters an auto-activated regulator (the sigma factor *sigW* from the same bacterium expressed from a SigW-activated promoter). Titrating the anti-sigma factor tuned the input threshold that separates the induced and uninduced states, offering a means to readily tailor the quantitative properties of this system. These characteristics would be beneficial in synthetic biology for the construction of bistable systems without exhaustive component optimization.

5.3. Nested negative and positive feedback loops

Combining both negative and positive feedback intertwines otherwise opposing attributes, potentially creating altogether unique and flexible responses. One response is oscillations, where competition between negative and positive feedback forces the system to oscillate between induced and uninduced states. The oscillations are more stable than those associated with a multi-negative feedback loop (e.g. the 'repressilator'), arguing for one utility of nested negative and positive feedbacks. Atkinson et al. (2003) first demonstrated this behavior in *E. coli* by combining positive feedback (auto-activation by the transcription regulator NtrC) and negative feedback (NtrC-activated expression of LacI that represses the expression of NtrC). The resulting synthetic circuit exhibited oscillations, although these oscillations dampened within a few cycles. Fung et al. (2005) demonstrated that similar oscillations could be generated by combining transcriptional and metabolic regulation in *E. coli*. In their system, dubbed the 'metabolator,' the conversion between two metabolic pools was controlled by two enzymes that were regulated either positively or negatively by one of the metabolites. This system also exhibited oscillations, although the amplitude and persistence of the oscillations varied.

Advancing on these previous efforts, Stricker et al. (2008) generated stable oscillations in *E. coli* using the transcription activator AraC and the transcription repressor LacI. Specifically, they designed a system with one positive feedback loop (AraC auto-activation) and two negative feedback loops (LacI auto-repression and AraC-activated expression of LacI that represses the AraC expression). Their design showed remarkable consistency in the amplitude and period of the oscillations, where the period could be tuned by changing temperature, media conditions, or the concentration of the inducers L-arabinose and IPTG. The improved persistence and uniformity of the oscillations may be attributed to the additional feedback loops built into the circuit. As an extension of this design, members of the same research group constructed a similar circuit using quorum sensing (Danino et al., 2010). This system also contained both positive feedback (activation of the AHL synthase LuxI by AHL) and negative feedback (activation of the AHL-degrading enzyme AiiA by AHL) in a similar configuration to the previous design. The new design synchronized oscillations across the entire population, resulting in macroscopic oscillations in otherwise microscopic cells.

Coupling positive and negative feedback loops can also lead to excitability, a distinct response not observed for positive or negative feedback loops in isolation. In this response, the system undergoes stochastic and transient induction. Stochastic fluctuations in the mediator of positive feedback drive induction until negative feedback returns the system to the uninduced state. Excitable behavior was observed directly in DNA uptake in *B. subtilis* during nutrient starvation (Süel et al., 2006). Two feedback loops control ComK, the master regulator of the uptake machinery. ComK undergoes auto-activation (positive feedback) and also represses ComS, which otherwise inhibits the degradation of ComK (negative feedback). In roughly 4% of the population of starved cells, stochastic fluctuations in ComK led to an increase in ComK levels driven by auto-activation. Eventually, reduction in ComS levels caused the increased degradation of ComK, returning ComK levels to the original uninduced state. Through the construction of synthetic circuits, Cağatay et al. (2009) revealed that other regulatory configurations of the same components exhibit excitability, although the natural circuit exhibited the greatest and most variable duration of induction. The distinct induction characteristics of the natural circuit allowed for greater variability in the uptake of DNA between cells, perceivably balancing the risk and reward of random DNA uptake (Cağatay et al., 2009). Synthetic biology could benefit from stochastic and transient induction in the development of altruistic biological systems (Lee et al., 2010) or of systems requiring a consistent subpopulation regardless of growth conditions.

The combination of negative and positive feedback can lead to a range of behaviors depending on the exact circuit configuration and system parameters. Tian et al. (2009) performed modeling to assess how the relative strength of negative and positive feedback impact the resulting response. They found that the relative strength of negative and positive feedback is the predominant determinant of the observed response. Bistability emerges when positive feedback dominates, whereas a graded, unimodal response emerges when negative feedback dominates—exactly paralleling what was observed for each type of feedback in isolation. When the strengths of feedback are balanced, the system can exhibit excitability or oscillations. The varied responses demonstrate that coupling negative and positive feedback offers a flexible motif for system design and may be the best approach for generating stable oscillations or excitability.

6. Current applications of feedback in synthetic biology

Clearly, negative feedback and positive feedback offer diverse advantages in the construction of synthetic biological systems. However, there are only a few examples of feedback being applied in synthetic biology to address scientific or technological challenges. We discuss representative examples below, which fall into three categories: metabolic control, biosensor design, and population control (Fig. 4).

6.1. Metabolic control

Metabolic engineering has overwhelmingly relied on static control for the expression of heterologous pathways (Tyo et al., 2007). Static control tunes the levels of each enzyme to maximize product yields, although the levels can be changed only through further genetic manipulations. In contrast, cells are dynamical systems that undergo constant perturbations even in a well-controlled environment, similar to chemical processes. Perturbations such as variability in enzyme levels or in metabolite levels can have a detrimental impact on the overall yield of the final product. Typically, these perturbations result in the diversion of too much metabolic flux away from cellular growth or in the build-up of toxic intermediates in the culture medium. One solution is the use of metabolic feedback to dynamically regulate enzyme levels. Farmer and Liao (2000) first demonstrated the benefits of metabolic feedback in *E. coli* in order to regulate the microbial conversion of glucose to the carotenoid lycopene. They employed the transcription activator NtrC, which is phosphorylated by the metabolic intermediate acetyl phosphate. Because acetyl phosphate levels increase as excess flux from glucose

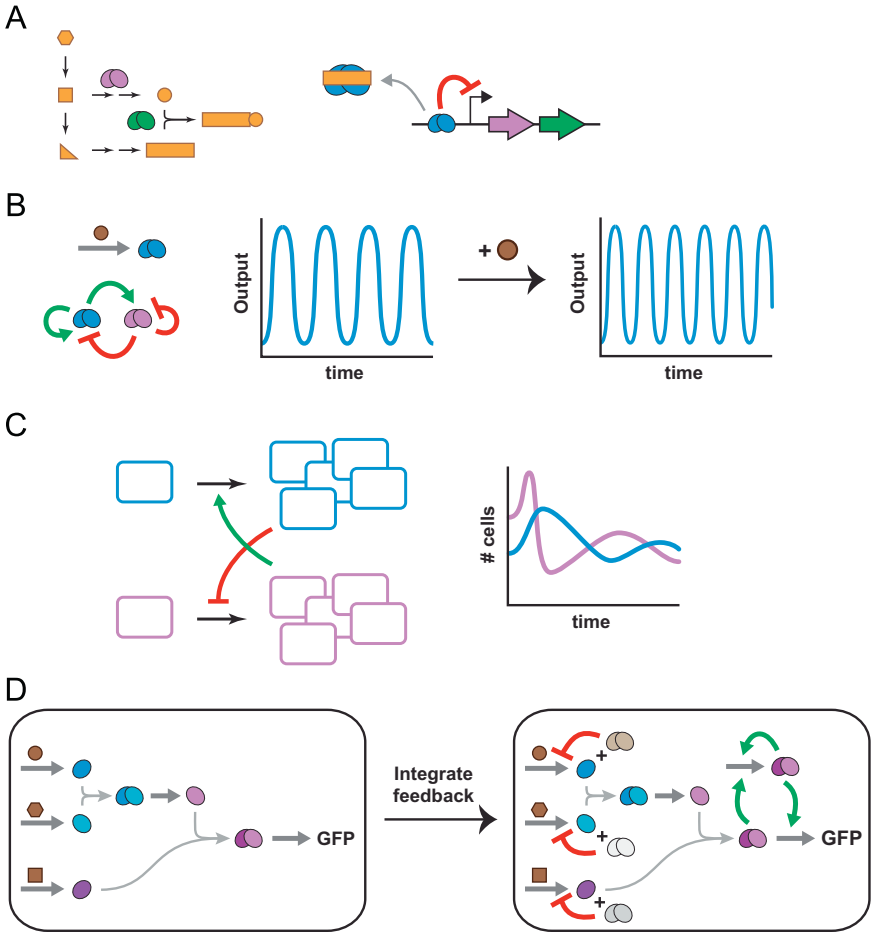

Fig. 4. Current and future applications of feedback in synthetic biology. (A) Metabolic control. Feedback can be used to control enzyme expression and activity based on the concentration of pathway intermediates or metabolic flux toward cell growth and homeostasis. The illustrated example controls enzyme expression for two branches of a metabolic pathway. Control limits both excessive enzyme expression and the toxic buildup of the intermediate. Adapted from (Zhang et al., 2012). (B) Advanced biosensors with accurate and measurable outputs. This example couples negative and positive feedback for stable oscillations. The sensed molecule controls the expression of one component of the loop, thereby modulating the frequency of oscillations. Adapted from (Prindle et al., 2012). (C) Population control. Feedback can be engineered into the interactions between different organisms. The interaction can help restrain the relative number of cells even if one organism grows faster than the other. Adapted from (Balagaddé et al., 2008). (D) Robust and rapid logic devices. Feedback can be introduced to accelerate processing by logic gates or to generate a more switch-like response. A three-input AND gate serves as an example. Adapted from (Moon et al., 2012).

catabolism is directed toward acetate production, NtrC activity could be employed to divert excess metabolic flux from acetate production to product synthesis. To create this link, Farmer and Liao placed genes involved in lycopene synthesis under an NtrC-activated promoter. This genetic manipulation created a negative feedback loop that reduced lycopene production when too much metabolic flux was diverted away from cell growth and increased lycopene production when metabolic flux was being wasted through acetate production. Introduction of this loop improved cell growth and increased the yield of lycopene by at least 10-fold, offering a generalized approach to balance growth demands and product synthesis in a wide range of metabolic engineering applications.

Zhang et al. (2012) adopted a different approach to introduce dynamic metabolic control into microbial chemical synthesis, specifically for the conversion of glucose to fatty acid ethyl esters in *E. coli*. Rather than balancing cell growth and product synthesis, they controlled two convergent branches of the fatty acid ethyl ester synthesis pathway with fatty acid, a key pathway intermediate. The genes encoding the pathway branches were placed under the control of the transcription repressor FadR, which is deactivated when bound to fatty acids or fatty acyl–CoA. The result was a negative feedback loop that increased fatty acid conversion when the concentration of fatty acid was elevated. This loop increased the yield of fatty acid ethyl esters by 3-fold and improved the stability of pathway genes. It is worth noting that one of the inhibitors of FadR, fatty acyl–CoA, is synthetized from fatty acid, inadvertently introducing a positive feedback loop that may generate bistability or oscillations. Observing these behaviors would require single-cell analyses, which are rarely performed in metabolic engineering. Overall, these studies demonstrate the utility of negative feedback in the dynamic control of heterologous metabolic pathways. Because metabolite-responsive transcription regulators and riboswitches are available in nature and can be engineered, dynamic control could be applied broadly in metabolic engineering (Tang et al., 2008; Carothers et al., 2011; Michener et al., 2012). Applying these regulators for metabolic control will advance metabolic engineering by consistently improving product yields and making microbial chemical synthesis a more competitive alternative to traditional routes of industrial chemical synthesis.

6.2. Biosensor design

Feedback was an essential feature of the next category of applications. Building on previous work from the Hasty group, Prindle et al. (2012) developed a biosensor that modulates its oscillatory frequency in response to a sensed molecule. The underlying genetic circuit coupled negative and positive feedback through two diffusible signaling molecules: AHL and hydrogen peroxide. AHL induced the expression of the AHL synthase LuxI (positive feedback) and the AHL-degrading enzyme AiiA (negative feedback). Hydrogen peroxide activated the expression of NADH dehydrogenase II responsible for hydrogen peroxide production (positive feedback). A hybrid promoter controlled by both AHL and hydrogen peroxide regulated the circuit output GFP. *E. coli* cells harboring the engineered circuit were grown in a microfluidic device that collected individual micro-colonies or 'biopixels.' A permeable wall and gas-filled cavity separated adjacent micro-colonies. AHL coordinated oscillations within the micro-colony, while hydrogen peroxide coordinated oscillations between adjacent micro-colonies by diffusing through the cavity. This setup synchronized oscillations between cells in the same micro-colony as well as adjacent colonies, leading to synchronous oscillations over millimeter-length scales—over 1000 times the size of an individual bacterial cell.

Prindle and coworkers expanded this engineered circuit to sense arsenic. An additional copy of the *luxI* gene was placed under the control of an arsenic-responsive promoter, which is activated when soluble arsenite binds the transcription regulator ArsR. The inducible expression of additional LuxI modulated the period of the oscillations from 68 min in the absence of arsenite to 80 min in the presence of 1 μM arsenite. Importantly, the period closely correlated with intermediate arsenite concentrations, offering a macroscopic, frequency-based readout of toxin concentrations. The range of periods admittedly was limited, although insights from the group's previous work (Stricker et al., 2008) coupled with riboswitches or other post-transcriptional regulatory mechanisms (Liang et al., 2011) could expand the observed range, thereby improving the accuracy and sensitivity of frequency-based biosensors.

6.3. Population control

In the final category of applications, feedback was applied to engineer interactions between cells that impact growth and viability. This category can be split into two types: antagonistic interactions, which restrain population growth, and mutualistic interactions, which promote population growth. In the simplest example of antagonistic interactions, You et al. (2004) engineered a negative feedback loop in *E. coli* that induces cell death at high cell densities. The loop connected quorum sensing to the expression of the toxic protein CcdB. At low cell densities, the expression of the *ccdB* gene was low and growth rate of the cells was unperturbed. At high cell densities, the expression of the *ccdB* gene was activated, preventing the further accumulation of cells. The plateau in cell density exhibited damped oscillations in line with a mathematical model and could be tuned by modulating the stability of AHL.

Balagaddé et al. (2008) extended this work to construct a synthetic predator-prey system composed of two engineered strains of *E. coli*. This system combined two orthogonal quorum sensing systems and either induction or repression of the *ccdB* gene. The engineered 'predator' *E. coli* cells secreted one AHL molecule and repressed the expression of *ccdB* in the presence of a different AHL signaling molecule. The engineered 'prey' *E. coli* cells secreted the second AHL molecule and activated the expression of *ccdB* in the presence of the first AHL signaling molecule. This configuration generated a negative feedback loop composed of two cells where the prey increased the growth of the predator, while the predator reduced the growth of the prey. The synthetic circuit replicated common behaviors associated with natural predator-prey systems, including coexistence, oscillations, and extinction, that depended on the experimental conditions. Aside from providing insights into natural systems, the two antagonistic circuits conferred control over the density of an isogenic or mixed population. The major limitation is that the antagonistic interactions exert selective pressure on the cells, potentially driving the loss of the *ccdB* gene in the above examples (Balagaddé et al., 2005). This limitation would pose challenges for the long-term maintenance of a population density.

Mutualistic interactions offer a distinct approach to control cellular populations. These interactions promote the growth of all participating members of the population, which is a common attribute of microbial consortia found in nature (Wintermute and Silver, 2010). In one example of an engineered mutualistic system, Shou et al. (2007) engineered two yeast strains to form a positive feedback loop through cross-feeding. One strain was engineered to overproduce the nucleobase adenine and to not produce the essential amino acid lysine, while the other strain was engineered to overproduce lysine and to not produce adenine. Engineering each strain to produce one metabolite and not

the other interlinked their growth. Following inoculation of the two strains into a single culture, the cell density of both strains showed large initial swings. Interrogation of the large swings revealed that each strain had to undergo lysis in order to release the essential metabolite, an intriguing feature of the system. Following the swings in cell density, the systems showed persistent stability even after large dilutions, which the authors attributed to the selection of beneficial mutations in either strain.

In a more recent example of engineered mutualistic interactions, Kerner et al. (2012) generated two strains of *E. coli* that excrete a metabolite required by the opposing strain. In contrast to the circuit developed by Shou and coworkers, excretion of the metabolites did not require cell killing and was under inducible control. Growth of the strains with different concentrations of the exogenous inducers tuned the growth rate of the co-culture and the ratio of the two strains. The resulting relationship was highly non-linear and did not lend to a simple mathematical model. We attribute this relationship to the competing benefits (utilizing the essential metabolite) and costs (gene over-expression and metabolite shedding) of the mutualistic interactions, potentially imparting nested positive and negative feedback loops.

These two circuits demonstrate that positive feedback, through mutualistic interactions can stabilize the ratios of participating strains. These interactions are expected to be more stable than antagonistic interactions by promoting rather than restraining growth. The limitation to this approach is that participating strains will continue growing until the resources in the medium are depleted. Future work within this application could focus on combining both mutualistic and antagonistic interactions to control population density and composition and to achieve novel population dynamics afforded to coupling positive and negative feedback loops. The design of engineered communities undoubtedly will become a central thrust of synthetic biology with widespread applications in metabolic engineering, human health, and biomanufacturing.

7. Future directions

The above examples illustrate how feedback has been integrated into the design of biological systems. However, feedback has been absent in one of the largest thrusts of synthetic biology: the design of logic devices. These devices are being pursued with the goal of constructing increasingly large genetic circuits that perform more complex logic operations. Recent examples have marked major milestones in engineering biology, including the development of circuits that count (Friedland et al., 2009), circuits that process up to four different input signals (Moon et al., 2012), and circuits that detect edges separating regions in the light and in the dark (Tabor et al., 2009). These circuits can be described as first-pass 'prototypes,' where more advanced versions perceivably would integrate control strategies, fail-safes, and other more complex operations. However, the prototypes tended to require extensive optimization to function properly even under defined laboratory conditions. Some of the optimization may be attributed to typical complications such as poor expression, which can be relieved through recently developed approaches (Salis et al., 2009; Lou et al., 2012; Qi et al., 2012). However, the need for optimization could be relaxed through the integration of feedback in the initial design. For instance, logic devices could be constructed with multiple positive feedback loops to introduce robust and tunable bistability or with negative feedback loops to reduce cell–cell variability in the sensory components (Fig. 4D). Integration of feedback may even reduce the optimization time, leading to the faster development of a functional system. To facilitate the widespread use of feedback, a library of portable feedback modules could be developed that exhibit a range of feedback properties and can readily be inserted into existing genetic circuits (Nistala et al., 2010). These modules could be as simple as auto-repression or as complex as layered positive feedback. Modules also could offer a range of feedback properties through genetic modifications or use of allosteric regulators that can be tuned exogenously (Kerner et al., 2012). Finally, these modules could be extended to feature other modes of feedback, such as allosteric enzymes used in the optimization of metabolic pathway design. The resulting collection of modules could be broadly utilized for the construction of biological systems with relaxed design requirements.

Another potential hurdle to integrating feedback is the predominant use of transcriptional regulation in logic devices. Promoters have a limited capacity for regulator binding sites, restricting how many inputs a single promoter can receive. This restriction could be alleviated through the further development of post-transcriptional regulators such as regulatory RNAs. Ongoing work in the design and construction of synthetic regulatory RNAs should contribute significantly to the construction of large-scale circuits and the accompanying ability to introduce feedback (Win and Smolke, 2008; Lucks et al., 2011). For this idea to be fully realized, we must better understand how regulatory RNAs shape the properties of feedback. Recent work has highlighted how regulatory RNAs and transcription regulators can display different regulatory properties, even in the context of genetic circuits (Levine and Hwa, 2008; Mehta et al., 2008; Beisel and Storz, 2011). Further efforts to understand the properties of both synthetic and natural RNA-based genetic circuits will lay the foundation to engineer feedback with diverse mechanisms of regulation.

8. Conclusions

Biological feedback offers numerous advantages for synthetic biology that extend beyond the typical use of feedback in process control. Many of these advantages were elucidated through the characterization of synthetic biological systems. Even though synthetic biology has lent critical insight into the attributes of feedback, the field has been slow to adopt these attributes. As constructed systems become larger and more complex, feedback will become an essential feature that must be integrated even in the prototype stage. Doing so will facilitate future efforts in biological design, potentially matching or even surpassing the prevalence of feedback in nature.

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