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Building robust functionality in synthetic circuits using engineered feedback regulation

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The ability to engineer novel functionality within cells, to quantitatively control cellular circuits, and to manipulate the behaviors of populations, has many important applications in biotechnology and biomedicine. These applications are only beginning to be explored. In this review, we advocate the use of feedback control as an essential strategy for the engineering of robust homeostatic control of biological circuits and cellular populations. We also describe recent works where feedback control, implemented *in silico* or with biological components, was successfully employed for this purpose.

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Maintaining homeostasis of metabolites and proteins requires cellular processes to continuously adjust to perturbations imposed by their fluctuating environment. Environmental deviations are often sensed by, and adapted to, using feedback control strategies. As such, feedback control is integral to many homeostatic processes in the cell. Synthetic biology, a discipline that builds novel functional circuits within cells, has sought to mimic the operation of many cellular processes. Strategies employing feedback loops have been effectively used to shape the dynamic behavior of many engineered synthetic circuits, resulting in sophisticated functionalities such as bistability and oscillations. However, the homeostatic potential of feedback control to build robustly operating circuits has remained largely untapped in most of the synthetic circuits built to date.

In this review, we examine the current understanding and implementations of feedback regulation in endogenous and engineered genetic circuits. We also highlight the use of *in silico* feedback, a novel technology for external control of intracellular processes.

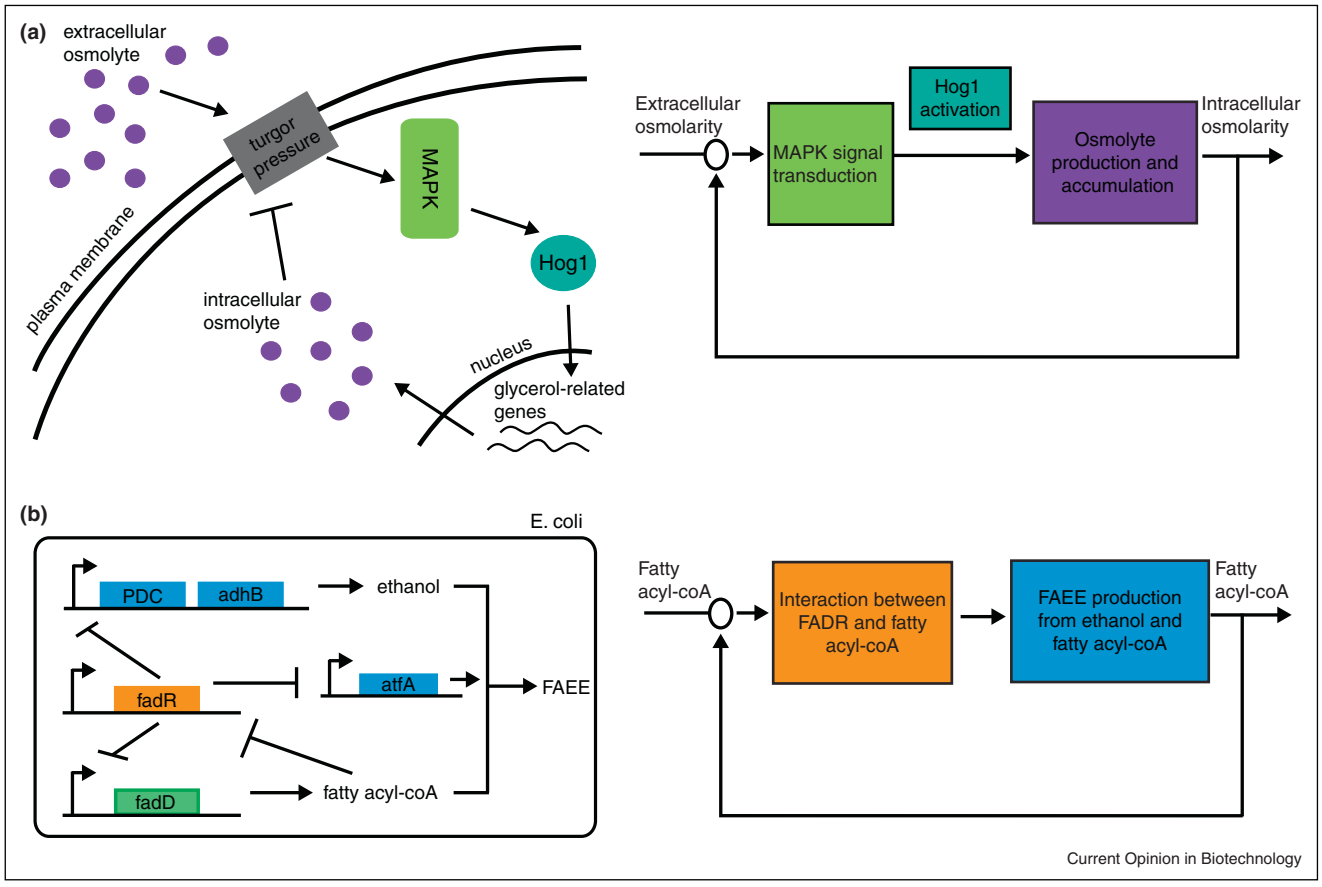
Feedback control using biological components

In diverse processes such as amino acid biosynthesis, chemotaxis, and stress response pathways, feedback loops ensure that any deviation from normal operation is detected and corrected [1–5]. One well-studied example of such homeostatic control occurs in the osmotic stress response, in which cells faced with external fluctuations of osmolyte concentrations actively regulate their turgor pressure. Membrane proteins are thought to sense imbalances between intracellular and external osmolarity, and activate a MAPK pathway that acts to increase the intracellular concentrations of the osmolyte glycerol. Glycerol accumulation then allows the cell to return to its resting turgor pressure, in other words to perfectly adapt (see Figure 1). Control theory analysis showed that such adaptation requires the system to implement integral feedback control [6,7]. Many natural biological systems, from cellular behaviors such as chemotaxis [8] to physiological responses such as calcium regulation [9], also exhibit adaptive feedback control. In these cases, integral control provides a general strategy that performs reliably for a wide range of perturbations and system characteristics, rather than on carefully tuned parameters. In fact, a computational search for 3-node networks capable of perfect adaptation revealed integral feedback control as one of two strategies that are necessary for this behavior [10].

In addition to their key role in homeostatic control, feedback loops also allow cells to generate useful dynamical behaviors. For example, positive feedback loops in the *Xenopus Oocyte* maturation circuit, when layered onto a system which contains an ultrasensitive response, can generate bistability [11]. By contrast, delayed negative feedback loops generate oscillations such as those observed in the Cyclin-CDK circuit that constitutes the engine of cell division cycles [12].

Not surprisingly, the earliest examples of feedback loops in synthetic biology involved the construction of circuits capable of bistability and oscillations [13,14]. Elaborations on these core functionalities also made use of

Figure 1



Feedback control in natural and engineered cellular systems. In systems implementing feedback control, the deviation of a measured output from a desired set-point is assessed. A control algorithm uses this value to compute a corrective action, which when input into the system, regulates its output to the desired set value. Natural and engineered biological systems make use of this type of homeostatic feedback control. **(a)** The yeast osmoregulation system demonstrates feedback control. Disturbances to extracellular osmolarity cause a change in turgor pressure from a steady-state value. Deviation from this value activates MAPK signaling and Hog1 nuclear import, which in turn activates Hog1-dependent synthesis of glycerol. Glycerol accumulation restores the turgor pressure to its pre-perturbation value [6]. **(b)** The FAEE biosynthetic pathway has been engineered in *E. coli* to exhibit homeostatic feedback control. In this system, accumulation of fatty acyl-CoA is sensed by fadR which adjusts the expression of enzymes, allowing for greater production of FAEE. Increased consumption of fatty acyl-CoA during FAEE production reduces fatty acyl-CoA levels, therefore enacting a negative feedback loop [24••].

feedback loops to extend circuit functionality [15–19]. In one example, a synthetic circuit built in *Escherichia coli* used a positive feedback loop implemented by araC auto activation and a negative feedback loop implemented by araC-mediated activation of the lacI repressor. This architecture, consisting of nested positive and negative feedback loops, allowed for robust oscillations with frequencies that are tunable by addition of the lacI inhibitor IPTG or the araC inducer arabinose [18]. Similar oscillatory circuits that exploit sense–anti-sense RNA expression units to build interlaced positive and negative feedback loops have also been implemented in mammalian cells [19].

In addition to building *de novo* functionality, synthetic positive and negative feedback loops have been used to alter the dynamic response of endogenous cellular circuits [20,21]. In *Saccharomyces cerevisiae*, pheromone activates a canonical MAPK cascade that results in a dynamic program of gene expression that prepares the cells for mating. In order to introduce new dynamic responses to pheromone stimulation, feedback loops were engineered into the system by recruiting negative and positive pathway modulators to the MAPK scaffold using complementary leucine zippers [20]. The expression of these pathway modulators is controlled by MAPK signaling, resulting in feedback regulation. Furthermore, adjusting the binding affinity of the zippers tunes the strength of the feedback links, allowing for the creation of various dynamical behaviors such as pulse generation and ultrasensitive switching. This approach was also extended to other MAPK signaling cascades in yeast and mammalian cells, demonstrating its general applicability [22•].

The examples above illustrate the use of feedback loops to implement increasingly sophisticated qualitative behaviors. However, the use of feedback to increase the robustness and quantitative fidelity of synthetic circuits in the face of biological noise and external perturbations is an equally important emerging application, especially in the context of metabolic engineering [23]. The production of industrially relevant molecules in genetically modified organisms is often limited by metabolic imbalances that lead to the accumulation of toxic product intermediates and inefficient use of feedstocks. Metabolic imbalances in endogenous metabolic pathways are corrected using feedback regulation of the enzymatic activity of the pathway, often by end-product inhibition [1]. Mimicking this strategy, feedback control of the metabolic intermediate fatty acyl-CoA was engineered into an *E. coli* strain that produced the diesel fuel replacement fatty acid acyl ester (FAEE) through condensation of acyl-CoA with ethanol [24••] (Figure 1). In order to maintain fatty acyl-CoA at a desired level, the authors engineered a circuit in which the fatty acid-responsive transcriptional repressor FadR regulates the expression of enzymatic components of the biosynthetic pathway. In this system, termed a 'Dynamic Sensor Regulator', buildup of fatty acyl-CoA titrates FadR from its target promoters, resulting in expression of enzymes that catalyze ethanol and FAEE production. As more fatty acyl-CoA is converted to FAEE through the action of these enzymes, FADR repression is restored, therefore implementing a negative feedback loop. This strategy led to a threefold increase in diesel fuel yield. Previous uses of feedback control of metabolic flux also include regulation of the antioxidant lycopene in *E. coli* through engineered responsiveness to the buildup of glucose [25]. Quorum sensing and a genetic toggle switch were also employed as part of a feedback control strategy to dynamically regulate protein expression in response to cell density [26,27].

Monitoring and adjusting the operation of a circuit's output (such fatty acyl-CoA) with feedback control relies on the availability of an endogenous sensor (such as FadR). This type of specialized systems is not necessarily transplantable to general applications. One strategy to circumvent this difficulty is through separating sensing from signal transduction and actuation. Recent work in this direction modularized signal transfer in bacterial two-component phosphotransfer signaling systems [28]. This advancement was made possible through the recruitment of a Histidine Kinase (HK) sensor to its non-native Response Regulator (RR) [29•] through a synthetic scaffold. These modular components have versatile inputs and outputs and can be used, in addition to other circuits that exploit similar strategies [30,31], to create portable 'feedback control modules'. Additional enhancements include the use of components that are orthogonal to the host cell's endogenous pathways, therefore allowing for increased signaling specificity [32].

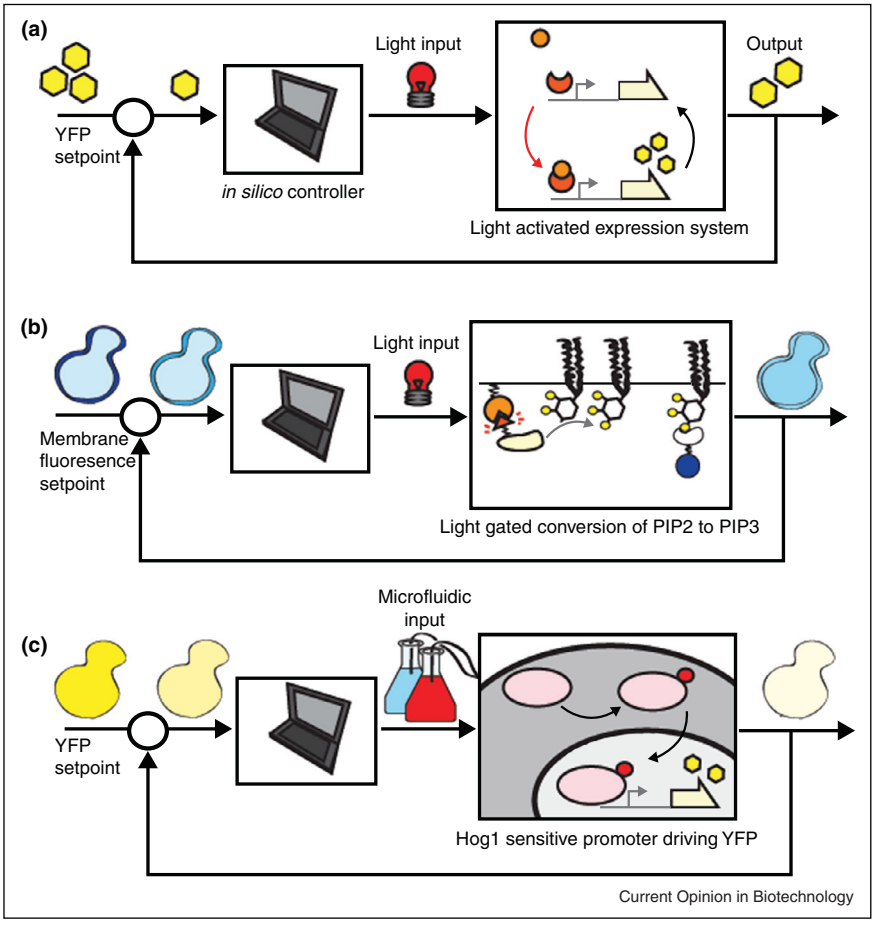
In addition to robust intracellular regulation, feedback control has also been used to achieve inter-cellular control of population behaviors [33–41]. In one example, 'quorum sensing' diffusible small molecules were co-opted to synchronize oscillations across a bacterial population [33]. Here, the oscillatory circuit has the same architecture as the one implemented by Stricker *et al.* [18] but involves the production of acyl homoserine lactone (AHL), a quorum sensing small molecule from the bioluminescent bacteria *vibrio fischerii* that diffuses across cell membranes. Specifically, the main negative feedback loop that drives the oscillations in this circuit involves binding of AHL to luxR. This complex leads to the production of aiiA, a protein that in turn degrades AHL. Diffusion of AHL between neighboring cells allows them to synchronize their oscillations. In subsequent work, the range of synchronization was extended to the millimeter scale by using hydrogen peroxide gas as a fast diffusing signaling molecule in the system [34]. Similar multicellular control has been achieved in yeast cells using diffusible endogenous pathway inducers such as alpha-factor [35]. Orthogonal communication channels were also developed by engineering the diffusible plant hormone cytokinin isopentenyladenine (IP) into a sender yeast strain and the IP responsive cytokinin receptor AtCRE1 into a receiver yeast strain [36,37]. Multicellular control of this nature will be increasingly important in applications where heterogeneous populations need to synergize in order to accomplish useful functions, such as the production of biotechnologically important compounds [42].

Despite these promising successes, the use of biological components to implement feedback control of synthetic circuits remains limited by our current quantitative understanding of these components [43]. Large uncertainties in the homeostatic controller can actually lead to amplification of the perturbations that the feedback is intended to correct. To address this problem, researchers have turned to outsourcing the control of synthetic biological circuits to man-made components. The idea here is that by converting the biological output to some experimentally measurable quantity that can be read by a computer (e.g. expression level of a fluorescent protein), an appropriate control strategy can be devised *in silico* and then administered in real time to the cell via an engineered input. In this way, more precise external controllers can be used to achieve robust homeostatic regulation of an underlying cellular circuit.

Feedback control *in silico*

Exerting external control to regulate the growth rate and other physiological properties of cellular populations has a long history, particularly in the context of culture growth in chemostats [44]. Chemostats constantly monitor culture variables such as the quantity of nutrients and use this information as inputs into an external control scheme,

Figure 2



In silico feedback enables quantitative control of cellular circuits. In this scheme, a circuit output such as a fluorescent protein is measured by microscopy or flow cytometry. The output is compared to a desired set-point and a regulation error is computed. This error is used to devise an appropriate control strategy *in silico*, which is then applied to the cellular circuit using an engineered input. **(a)** *In silico* feedback regulation of gene expression through a light-activated transcription factor [45••]. **(b)** *In silico* feedback regulation of PIP3 levels in the membrane through light gated recruitment to the membrane of the enzyme that catalyzes the conversion of PIP2 to PIP3 [47••]. **(c)** *In silico* feedback regulation of a HOG pathway responsive promoter using a microfluidic device that administers pulses of sorbitol to the growth chamber [53•].

implemented using electronic circuits or a computer, to maintain constant growth by adjustment of culture dilution rate. This idea of external 'closed loop control' of a biological system has seen renewed interest in the context of synthetic biology, specifically for the regulation of intracellular variables. Such a control scheme relies on the ability first, to measure the appropriate variable continuously in real-time, second, to assess the deviation of this variable from a desired value and compute a corrective action using an appropriate algorithm, and finally third, to administer the corrective action back to the biological circuit.

Successful implementations of external feedback control have been achieved in *S. cerevisiae* and mammalian cells (see Figure 2). Milias-Argeitis *et al.* controlled expression of a fluorescent protein (Venus) from a *GAL1* promoter using this strategy [45••]. To assess deviation from desired protein expression, fluorescence is measured using a flow cytometer and then input into a computer algorithm. The algorithm relies on a model of *GAL1* gene expression and established techniques of Model Predictive Control [46] to compute the corrective input. This input is then administered to the biological circuit using a light gated interaction between the photoreceptor chromoprotein PhyB and Phytochrome Interacting Factor PIF. Synthetic constructs of these two *Arabidopsis Thaliana* proteins were expressed in *S. cerevisiae* to allow light-driven regulation of the GAL1 promoter. Optogenetic tools therefore allowed for real-time *in silico* control of this biological circuit.

Using the same Phy/PIF light gated interaction, Toettcher *et al.* were able to use a similar strategy to

control fast post-translational modifications such as membrane recruitment of proteins [47]. In their system, the light gated interaction of Phy/PIF proteins leads to the membrane recruitment of the enzyme PIK3, which catalyzes the conversion of the phospholipid 2′ phosphoinositide (PIP2) to 3′ phosphoinositide (PIP3). A fluorescent protein fused to a PIP3 binding domain allows PIP3 membrane concentration to be measured using microscopy. Deviations from a desired PIP2 concentration value are corrected by light inputs, whose intensities are determined by an *in silico* proportional integral derivative (PID) controller. Importantly, this work succeeded in robustly controlling the output of a natural system both in the presence of its endogenous feedbacks and pharmacological modulators of PIP3 levels. These results, combined with advances of optogenetics technologies [48–52], introduce the intriguing possibility of addressable control of individual cells for technological applications including the production of biofuels or small molecule drugs. These same strategies also offer exciting therapeutic opportunities, such as the possibility of using real-time external feedback control to achieve, for example, deep brain stimulation in the treatment for Parkinson's disease or neuron de-synchronization to control epileptic seizures.

Microfluidic approaches have also been used to implement *in silico* control of cellular systems in the context of the yeast HOG pathway [53•]. Expression from an osmo-responsive promoter was measured and Model Predictive Control used to compute the duration of sorbitol treatment necessary to achieve desired promoter activity. To implement this strategy, the group constructed a microfluidic device capable of changing the osmolyte concentration precisely and with high temporal resolution. When the output of a single cell was used to design the sorbitol input, the variability of this cell relative to the variability seen in the population was reduced. Microfluidic technologies have also been used to devise control strategies that do not require an underlying model of the system [54]. This is useful for controlling complex biological systems that contain multiple time scales and modes of endogenous regulation. Overall, advances in microfluidic technologies have increased the time periods over which cells can be studied and manipulated [41,55,56], allowing for increasingly sophisticated control of cellular populations.

Conclusion

Feedback control, implemented using either biological components or external *in silico* strategies, presents a unique opportunity to introduce robustness into the operation of synthetic circuits. *In silico* control of biological systems offers several advantages, such as the ability to compensate through sophisticated computational algorithms for an incomplete understanding of the underlying biology and the possibility to reprogram different behaviors in the controlled cellular circuit without the need to re-engineer it *de novo*. The flexibility afforded by *in silico* control makes it ideal for circumventing the extensive tuning often required to control the operation of synthetic circuits using only biological building blocks. However, future advances in our understanding of biological components and their rules of composition and operation might soon level this playing field.

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- •• of outstanding interest

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