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Advances in synthetic biology: on the path from prototypes to applications

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Synthetic biology combines knowledge from various disciplines including molecular biology, engineering, mathematics and physics to design and build novel proteins, genetic circuits and metabolic networks. Early efforts aimed at altering the behavior of individual elements have now evolved to focus on the construction of complex networks in single-cell and multicellular systems. Recent achievements include the development of sophisticated non-native behaviors such as bistability, oscillations, proteins customized for biosensing, optimized drug synthesis and programmed spatial pattern formation. The *de novo* construction of such systems offers valuable quantitative insight into naturally occurring information processing activities. Furthermore, as the techniques for system design, synthesis and optimization mature, we will witness a rapid growth in the capabilities of synthetic systems with a wide-range of applications.

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Introduction

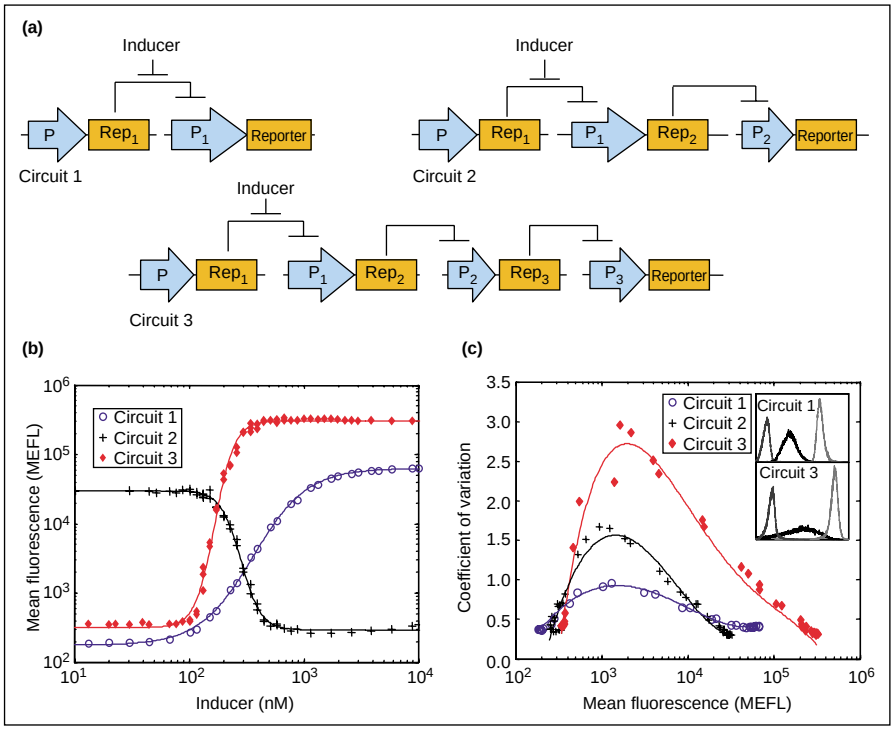
Synthetic biology aims to create novel behaviors through the engineering of genetic elements and the integration of basic elements into circuits that implement more complex functions. Scientists have long viewed the behavior of complex biological systems as a function of the behavior of their constituent parts; for example, hybrid Boolean networks that consist of digital and analog logic elements [1–3]. Initial efforts in engineering synthetic elements focused on the development of novel transcriptional activators and repressors, often through the incorporation of foreign transcription factor binding sites into promoter sequences [4,5]. Modifications to regulatory kinetics, such as transcription factor cooperativity and operator binding affinity, were used to develop elements with step-like digital responses that are robust to input noise [6]. In addition to having a collection of components that approximate digital behavior, synthetic biologists have also developed and utilized elements with fine-tuned analog characteristics (e.g. rheostat-like control). These engineering efforts have both helped to characterize how specific residues or DNA-binding sites can alter biological activity and have created a library of useful components.

To elicit more complex regulatory behaviors, elements can be combined into circuits, the simplest of which is the transcriptional cascade. Here, genes are arranged in series whereby each gene product regulates the expression of one downstream target (Figure 1a). In natural systems, evolution has already optimized the regulatory interactions between network elements in cascades and other network motifs to work cohesively towards achieving a particular behavior. In synthetic biology, networks are typically assembled from unrelated elements that have not been optimized by this evolutionary process. Hence, one of the main challenges in engineering synthetic circuits is altering the kinetics of individual elements until they are impedance-matched such that they function correctly within the context of the new network [7,8•]. Through the use of computational and directed evolution techniques that overcome such difficulties, several functional cascades have been built and analyzed as described below.

Network motifs in synthetic systems

Cascades are useful for studying the fundamental mechanisms of information flow in regulatory networks. They have a simple topology and, to a first approximation, their steady-state output is a direct monotonic function of the input. One useful property of certain signal transduction cascades, such as the mitogen-activated protein kinase (MAPK) cascade in *Saccharomyces cerevisiae*, is that their steady-state behavior approximates digital logic with an ultrasensitive step-like dosage-response function [9]. Transcriptional cascades can also possess similar response properties. The analysis of synthetic transcriptional cascades of various lengths has shown that, under certain conditions, increasing the depth of the cascade sharpens the ultrasensitive response to a stimulus and makes the input/output function more digital [10] (Figure 1b). Another important property of cascades is their dynamic behavior in response to both internal and environmental changes [11]. Through the construction of one- and two-step cascades, it was shown that the delayed response in transcriptional cascades is a function of cascade depth. In addition, long cascades can be robust to input noise by

Figure 1



Ultrasensitivity and noise propagation in transcriptional cascades. **(a)** Synthetic transcriptional cascades of different lengths. Repressors (Rep_1, Rep_2 and Rep_3) inhibit expression of genes from target promoters (P_1, P_2 and P_3), but the gene product of Rep_1 can be inactivated by an inducer. **(b)** Mean fluorescence (MEFL) of circuits 1–3 as a function of the inducer. Circuit 3 is more sensitive and transitions from low to high output over a smaller range of inducer concentrations. **(c)** Coefficient of variation (CV) in fluorescence determined from fluorescence-activated cell sorting (FACS) as a function of mean fluorescence. Circuit 3 shows the highest CV in the transition region (noise amplification), but all circuits show the same noise levels for high and low outputs (noise attenuation). (Figure adapted from [10].)

acting as low-pass filters, (i.e. transient fluctuations in input have no effect on the output) [10,12]. Synthetic transcriptional cascades have also been useful for studying noise propagation, including amplification, attenuation and the contribution of extrinsic and intrinsic factors to phenotypical variations [10,13,14] (Figure 1c). The study of cascades has also been extended to eukaryotes [15] where long cascades are more common than in prokaryotes [11]. Analysis of the fundamental properties of cascades through synthetic construction continues to yield important insight into this ubiquitous network motif.

Feed-forward motifs involve a master regulatory gene that regulates downstream target genes through multiple non-circular pathways. In networks where a feed-forward loop is added to a cascade, the steady-state output remains a direct function of the input; however, the dynamics of reaching a steady state can involve non-monotonic transitions. For example, in response to a single change in the input stimulus from low to high, a pulse-generator network displayed a transient non-monotonic change in the output from low to high and then back to low [16]. The magnitude of the pulse in response to a change in input was engineered by mutating regulatory elements to have increased DNA-binding affinity or transcription rates. Furthermore, the pulse amplitude and delay also conveyed information about the rate of increase in the input signal, roughly acting as a numerical integrator. The coherent feedforward loop may add delay to a circuit response when the input changes from low to high, but will not affect the response time on an input change from high to low [17].

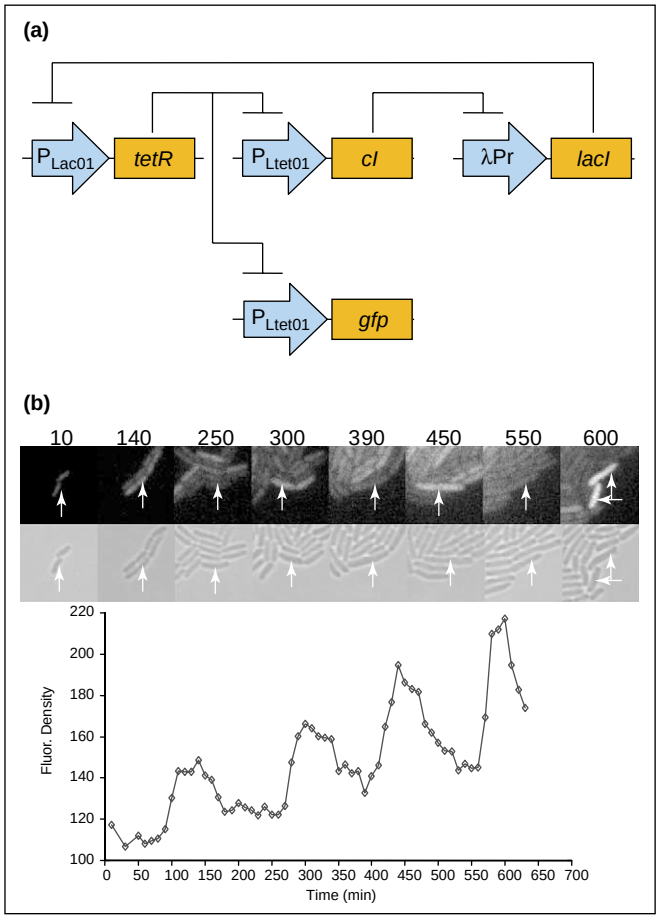
Regulatory feedback has been integrated into cascades to create networks with more sophisticated properties where the steady-state output is no longer a simple function of the input. In the case of a single promoter without an input, the addition of negative autoregulation has been shown to reduce gene expression noise [18]. Single-element negative feedback also speeds the response time for a promoter to reach its maximum steady-state level after induction [19]. Using a feedback system comprising two promoters whose gene products cross-repress each other, a bistable toggle switch was constructed in *Escherichia coli* [20]. The stable state of the network could be switched through the transient application of exogenous stimuli that interferes with the DNA-binding activity of one of

the two repressors. Using a similar feedback-driven network, an epigenetic toggle switch was also constructed in mammalian cells [21]. A gene network designed to produce autonomous limit-cycle oscillations in gene expression was constructed by linking a trio of repressors in a ring topology [22] (Figure 2). Importantly, the observed phenotypical variations of the oscillations motivated subsequent studies of gene expression noise using several synthetic systems [10,13,14,23•]. A different feedback-based network was also used to demonstrate oscillations where components of the lactose- and nitrogen-regulated response systems were fused together to produce a relaxation oscillator [24]. In addition, positive feedback was incorporated into eukaryotic transcriptional cascades, creating a bimodal expression profile in response to a graded input [25]. In theory, essentially any artificial regulatory scheme can be achieved through the proper combination of the motifs discussed above (cascades, feed forward, and feedback), although there are real physical limits on the speed and complexity that these networks can attain *in vivo*.

Engineering multicellular systems

Equipped with the ability to develop a variety of single-cell networks, synthetic biologists have recently focused their attention on engineering multicellular systems that utilize cell–cell communication to achieve coordinated behaviors. Such systems are useful for studying multicellular phenomenon ranging from synchronized gene expression in homogenous populations to spatial patterning in developmental processes. There are also many cases where coordinated cell behaviors can either optimize network operation or enable an entirely new class of capabilities. As with single-cell synthetic gene networks, initial steps focused on the fabrication of basic transgenic communication capabilities. The quorum sensing mechanism from *Vibrio fischeri* [26] was partitioned into sender and receiver components that were integrated into

Figure 2



Oscillatory network. **(a)** Circuit diagram of the repressilator circuit. The network includes destabilized versions of the tetracycline repressor (*tetR*), lambda repressor (*cI*), and Lac repressor (*lacI*) that are regulated by the promoters P_{Llac01}, P_{Ltet01}, and λP_R, respectively. In addition, a green fluorescent protein gene (*gfp*) that is regulated by a second P_{Ltet01} promoter is used to monitor the state of the network. Under certain conditions the network will exhibit limit-cycle oscillations. **(b)** Microscope images showing fluorescent and corresponding bright field images of a growing microcolony with arrows pointing to a single cell whose oscillatory fluorescence is shown in the graph. (Figure adapted from [22].)

two separate *E. coli* populations. The resulting multicellular system was used to verify and analyze the liquid-phase and solid-phase behavior of engineered cell–cell communication using acyl-homoserine lactone (AHL) synthesis and response [27]. As a demonstration for integrating a single-cell network with cell–cell communication, quorum sensing was used to activate a toggle switch upon reaching high-cell density [28•]. In this system, a toggle switch memory element in each cell maintained information about whether the cell had ever reached a critical threshold density, even if the population was subsequently diluted. To improve single-cell oscillatory behavior, computational work suggested that communication among a population might synchronize oscillatory periods and create mass-action behavior that makes the individual networks more robust to gene expression noise [29,30].

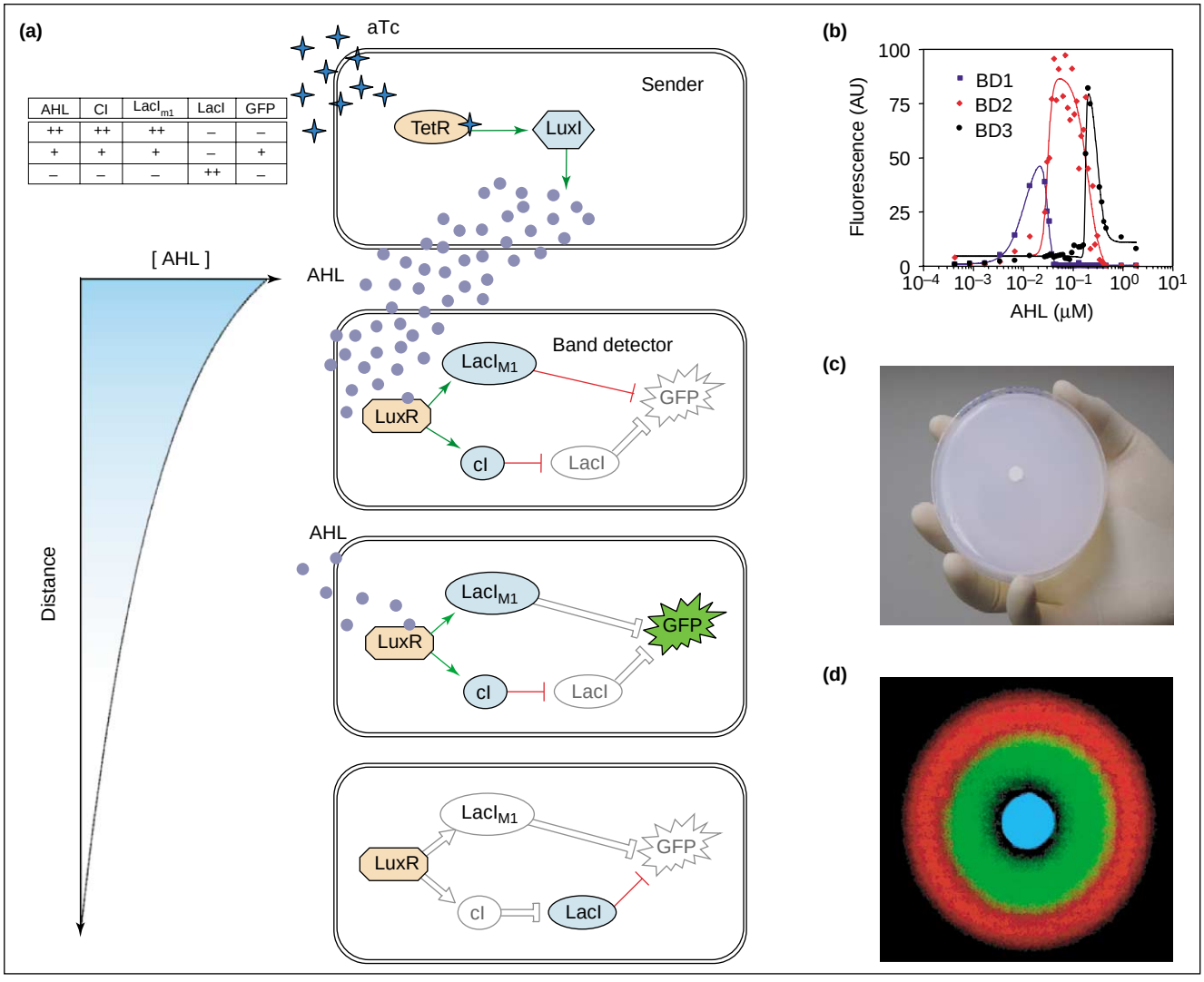
An important feature of multicellular systems is the ability to endow them with interesting spatiotemporal dynamics. The pulse generator network discussed above was integrated into a two-population system where in solid-phase experiments the pulse in receiver cells was triggered by signals from deliberately arranged sender cells [16]. Because the pulse amplitude reflects the rate at which the signal accumulates and signal accumulation occurs more rapidly near the senders, only receiver cells proximal to the senders produced an appreciable response to the signal. Such signal processing capability is beneficial in designing systems with fine-grained localized responses using communication mechanisms based on coarse-grained chemical diffusion. Recently, programmed pattern formation was demonstrated using a band-detect network that integrates communication modules with intracellular regulatory elements that have differing repression capabilities [31•] (Figure 3). The network produces non-monotonic dosage responses that are high only when the signal is within a user-defined concentration range. By deliberately arranging sender cells on solid-phase media containing a mixture of receiver cells, this band-detect network generated a variety of spatial patterns including bullseyes, ellipses, hearts and clovers. The design of more sophisticated multicellular behaviors will require the integration of multiple signaling systems. Analogous to the problem of impedance-matching with intracellular regulatory elements, the response sensitivity of intercellular communication elements must be balanced to produce functional multicellular networks. Recently, a signal amplification network was used to improve the sensitivity of one such communication system from *Pseudomonas aeruginosa* [32] so that it will better match the *V. fischeri* elements used in all the multicellular systems described above. The signal amplifier network was also used to analyze the cross-talk between several quorum-sensing systems. Another system that could prove beneficial in the development of multicellular systems employs the metabolite acetate to accomplish quorum-sensing behavior [33]. This system is likely to exhibit minimal cross-talk with AHL-based communication modules.

Applications of synthetic systems

For future biotechnology applications, the synthetic regulatory modules described above must be interfaced with sensory inputs and biological response outputs. These inputs allow the engineered circuitry to monitor external environmental conditions and internal cell state, while the outputs enable the engineered circuitry to control a range of processes such as metabolism, cell-cycle, growth, differentiation, and synthesis of pharmaceutical products. A synthetic gene network was used to maintain *E. coli* cell cultures at artificially tunable low densities by employing the exogenous quorum-sensing system described above to regulate cell death [34]. One possible application of this population-based gene expression system is in the creation of cost-effective long-term fermentation processes for the self-regulated production and subsequent harvesting of exogenous cytotoxic proteins. In a different system, a novel interface was constructed between the SOS pathway and genes regulating biofilm formation through a toggle switch [28•] (Figure 4). Exposing these engineered cells to transient UV irradiation (the input) permanently flipped the state of a toggle switch and caused cells to form biofilms (the output). A third system re-routed the α-factor signal transduction pathway that normally regulates mating in yeast to regulate the high osmolarity response instead [35••]. Research has also focused on the development of sensor proteins that respond to a variety of extracellular ligands. For example, using a computational approach, *E. coli* periplasmic binding proteins were modified to bind trinitrotoluene (TNT), L-lactate or serotonin in place of the wild-type sugar or amino acid ligands [36]. The conformational changes resulting from binding a foreign ligand activated a synthetic signal transduction pathway with readily observable output.

Synthetic biologists have also used engineered enzymes and metabolic pathways that interface with the endogenous cellular biochemical machinery to produce compounds with pharmaceutical and environmental applications. A synthetic amorpha-4,11-diene synthase and the mevalonate isoprenoid pathway from *S. cerevisiae* were integrated into a strain of *E. coli* to produce a precursor for the antimalarial terpenoid artemisinin [37••]. After careful engineering of the expression levels of the ten exogenous genes, the system was able to synthesize significant amounts of the target compound. This work could lead to inexpensive and effective means for large-scale artemisinin production with important implications for the world-wide treatment of malaria. In a separate study, the same research team integrated the parathion hydroxylase into *E. coli* and the enzymes for *p*-nitrophenol mineralization into *Pseudomonas putida*,

Figure 3



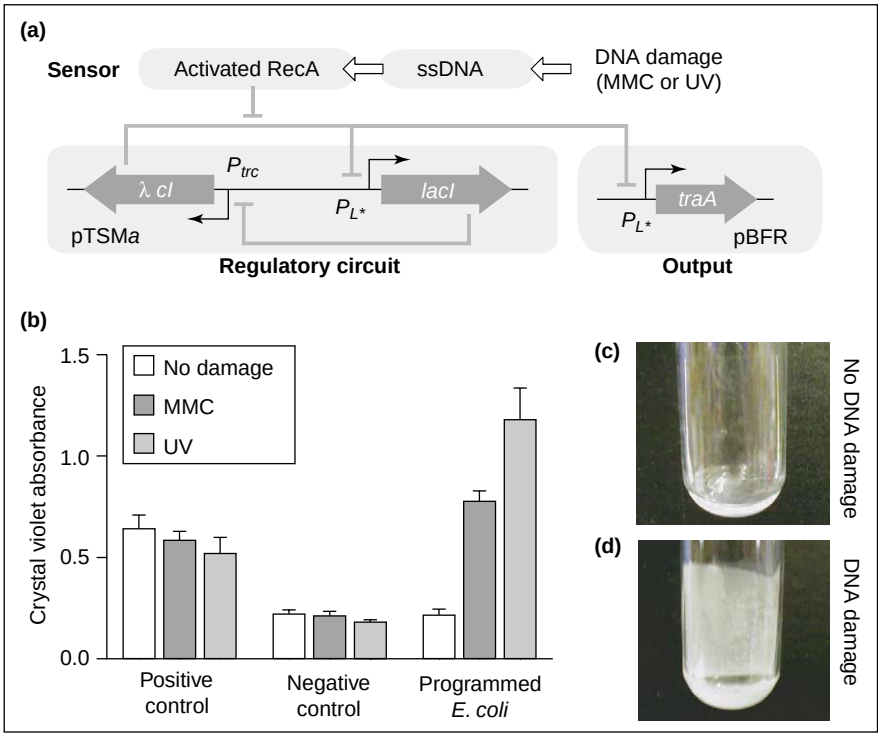
Spatial patterning of gene expression with the band detect network. **(a)** Band detect network design and activity as a function of distance from a sender cell. aTc serves to initiate production of LuxI in the sender cells by binding the tetracycline repressor (TetR) and disabling it from repressing the tetracycline promoter. AHL, the product of the LuxI enzyme, diffuses from sender cells. At high concentrations, AHL binds to its cognate R protein, LuxR, to activate expression of both the lambda repressor (cI) and a codon-optimized Lac repressor ($LacI_{M1}$), which represses expression of green fluorescent protein (GFP). At medium AHL concentrations the cells produce cI and $LacI_{M1}$, and while cI levels are sufficiently high to repress LacI expression the weaker $LacI_{M1}$ repressor does not inhibit expression of GFP. At low AHL levels, $LacI_{M1}$ and cI are not expressed and GFP is repressed by LacI expression. Constitutively expressed response proteins are shown in orange and regulated proteins in blue; green and red arrows designate transcriptional induction and repression, respectively. The table in the top left shows the expected network behavior as a function of AHL input. By altering the sensitivity of LuxR to AHL, three band detect (BD) variants of the system were built (BD1, BD2 and BD3). Each variant responds to a distinct range of AHL concentration. **(b)** Fluorescence response of three BD network variants in response to different AHL concentrations. **(c)** Experimental setup includes senders in a white paper disk placed in the center of an M9 agarose Petri dish containing a homogenous mixture of two band detect strains (BD2-Red and BD3-Green). **(d)** The corresponding fluorescence image after overnight incubation showing the emergence of two distinct bands. The red band and green bands correspond to the fluorescence of two distinct BD networks. Senders in the middle are expressing a cyan fluorescent protein (CFP). (Figure adapted from [31•].)

forming a multispecies system capable of biodegrading the insecticide parathion [38]. Computer-aided protein engineering has also been used for creating novel input-output systems, whereby a ribose-binding protein was retargeted to exhibit triose phosphate isomerase activity [39]. This work demonstrated that catalytic functions could be integrated into sensory proteins that normally lack enzymatic activity.

Circuit engineering

Concurrent with the development of prototype systems, general purpose techniques and approaches for building

Figure 4



Novel input-output systems: sensing DNA damage and biofilm formation. **(a)** Genetic network connecting DNA damage (input) and biofilm formation (output). In the circuit cI inhibits expression from promoter P_{L^*}. Sustaining DNA damage, however, causes activation of RecA, which cleaves cI, making it inactive. This allows expression of *lacI*, which inhibits *cI* expression. Without cI, the cell also produces *traA*, which results in biofilm formation. **(b)** Crystal violet absorbance of control strains and engineered strains as a result of exposure to DNA-damaging agents (MMC and UV). An increase in crystal violet absorbance in response to exposure to DNA-damaging agents indicates the formation of biofilms in the exposed cultures. **(c)** A test tube containing engineered cells that have not been exposed to DNA damaging agents shows no biofilm formation. **(d)** Test tube containing engineered cells exposed to DNA-damaging agents. These cells do form biofilms. (Figure adapted from [28•].)

and refining synthetic networks have emerged [40]. Even in simple systems, intuition is rarely sufficient to build functional synthetic networks. Thus, the experimental construction of synthetic circuits is almost always guided by computational models [41]. These models use a variety of mathematical abstractions to provide valuable insight into the behavior of a system by considering both element kinetics and network connectivity *in silico* [42]. Owing to the high costs and delays associated with fabricating physical systems, rapid prototyping using computational design tools is common throughout most engineering disciplines. One important difference between established quantitative engineering disciplines and synthetic biology is that state-of-the-art biological modeling tools still do not offer the same level of precision and predictive power. Nevertheless, models using relative kinetic rates have proven useful in approximating system behaviour, despite the lack of exact information regarding the kinetic rates of many components [24]. Most initial attempts at constructing functional systems do not result in the desired behavior. One strategy to optimize such systems is to employ sensitivity analysis [43]. In this computational approach, randomly chosen kinetic rates are assigned to the elements of the system and run in separate trials. Information regarding the contribution of each element's kinetics to overall system behavior can be gleaned from analyzing data from a large number of runs. This data can then guide the genetic manipulation of system elements towards achieving the desired goal *in vivo*.

Another approach to optimizing non-functional circuit design is to employ directed evolution, which does not require detailed knowledge of component kinetics or information on how their interactions produce an overall behavior [44]. The rational design of mutations that alter component behavior works well when properties dictating the component activity are well established, as is often the case with ribosome-binding sites and operators. However, when the principles that dictate component behavior are not well known (e.g. protein folding) or the effect of specific mutations on overall systems are unclear, desired alterations may be best realized using directed evolution [8•]. Directed evolution subjects a given component or circuit to random mutagenesis, followed by a screen or selection to isolate mutants that meet the

desired behavioral criteria. In addition, it provides an efficient method for considering a large number of mutations. This technique was demonstrated in the optimization of a non-functional transcriptional cascade [8•] and cell–cell communication elements [45]. Entire pathways can also be optimized using directed evolution. For example, exposing the arsenate detoxification pathway to random DNA shuffling created a 12-fold increase in resistance to arsenic [46]. In a related study, combinatorial synthesis of various network topologies revealed how different connectivities of the same basic elements can yield a wide range of behaviors [47]. The analysis of evolved components and pathways along with the observed behavior of the randomly generated network topologies offers valuable insight into individual component properties and the rules of system composition.

One of the critical bottlenecks in this field is the cost and time-consuming nature of building new circuits. Recent *de novo* DNA synthesis and error-correction techniques [48,49•] that refine earlier oligonucleotide-based DNA assembly approaches [49•,50] offer significant promise in the rapid and inexpensive development of large and complex synthetic systems and even whole organisms. The important problem of frequent errors with *de novo* synthesis of DNA was addressed using either protein-mediated error correction or special hybridization techniques [48,49•]. The latter approach used photo-programmable microfluidic chips to synthesize a large library of oligonucleotides, which are PCR-amplified and assembled into longer segments. Using these methods, both plasmids and linear DNA in excess of 10 kb have been rapidly synthesized with acceptable error rates for a fraction of the current cost of commercial synthesis. The combination of computer-aided circuit design with efficient synthesis will facilitate substantial growth in the number and complexity of synthetic gene networks in the near future.

Conclusions

Synthetic biology currently offers the ability to study cellular regulation and behavior using *de novo* networks, while future applications of synthetic systems will also extend to the fields of medicine and biotechnology. Engineered *E. coli* populations have been programmed to form spatial patterns with various shapes. Such patterning systems will serve as templates for building user-defined physical structures, with implications for programmed tissue regeneration and the formation of complex biomaterials. Bacteria, like those that degrade parathion, will be engineered to treat biological wastes or to aid in the clean-up of toxic spills. In the future, engineered protein biosensors will enable the creation of organisms with exquisite detection capabilities that can identify internal and external hazards such as pathogens or explosives. As exemplified by the development of artemisinin-producing *E. coli*, synthetic networks will also be utilized to supplement and potentially supplant current methods for the production of medicines and simple polymers. Although the ability to program cell behaviors is still in its infancy, it is clear that the power to freely manipulate the set of instructions governing the behavior of organisms will have a tremendous impact on our quality of life and our ability to interact with and control the physical world surrounding us.

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