REVIEWS

Reconstruction of genetic circuits

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The complex genetic circuits found in cells are ordinarily studied by analysis of genetic and biochemical perturbations. The inherent modularity of biological components like genes and proteins enables a complementary approach: one can construct and analyse synthetic genetic circuits based on their natural counterparts. Such synthetic circuits can be used as simple *in vivo* models to explore the relation between the structure and function of a genetic circuit. Here we describe recent progress in this area of synthetic biology, highlighting newly developed genetic components and biological lessons learned from this approach.

By taking apart an old clock, you could probably come up with a pretty good guess at how it works. But a more concrete understanding of the clock mechanism might be obtained by designing and building one's own clock out of similar parts. Contemporary biology presents us with similar reverse-engineering problems. For example, *Drosophila* cells contain a circadian clock that oscillates with a 24-h rhythm and self-synchronizes to the day/night cycle. Using genetic and biochemical techniques, researchers have isolated genes and proteins involved in interlocked feedback loops of gene expression[1,2] (Fig. 1b) that are necessary for clock function. However, many fundamental questions remain difficult to answer: what sets the period of the oscillation, how does the clock operate reliably in diverse cellular conditions, and what features of its design are responsible for its reliable operation? To gain insight into such questions one could design and build new clock circuits, using similar genes and proteins, and study their dynamics in the organism. In fact, several synthetic genetic clocks have now been constructed in bacteria[3–5] (Fig. 1c). These circuits are much simpler than the *Drosophila* clock. They fail to operate as reliably, but they provide a proof of principle for a synthetic approach to understanding genetic circuits.

As with the clock, many compelling biological questions centre on how interactions among genes and proteins, forming genetic circuits, give rise to specific cellular functions. Genetic and biochemical techniques have successfully identified many circuit components and their interactions. However, in many cases knowledge about these components and their interactions is not sufficient to explain the circuit mechanism. What is missing from the circuit diagram? There are several possible deficiencies: one is that the diagram may be incomplete—interactions may have been missed. The opposite problem also exists: the diagram may be too complete, in the sense that it contains interactions that are not actively involved in the process being investigated (for example, if one of the proteins is not expressed under the relevant conditions). Another problem is ignorance of the effective rules by which proteins and genes interact. For example, *in vivo* values of kinetic parameters such as affinities, binding and degradation rates, and so on, are generally unknown. Finally, the intracellular environment is intrinsically 'noisy', and small copy numbers of molecular species limit the predictability of biochemical reactions[6]. Taken together, these problems reduce our confidence in the combined understanding we get from perturbations, measurements and mathematical modelling.

A reconstructive approach to genetic circuits may offer unique insight into their underlying mechanisms. In this approach, one constructs synthetic replicas of natural genetic circuits out of well-characterized elements, such as genes, proteins, regulatory sequences, and so on, and observes their dynamics in living cells (Fig. 1a). This scheme offers several advantages. First, one can test the sufficiency of an arbitrary circuit for generating a particular function. Second, one may study the circuit mechanism without impairing cellular functions or inducing downstream consequences. Third, different circuit designs with similar functions can be directly compared to determine their relative advantages and disadvantages[7]. Such synthetic circuit reconstruction is made possible by the technologies of molecular biology such as construction of recombinant DNA molecules. Its future progress will benefit from further advances such as recent improvements in longer DNA synthesis[8].

In this way, synthetic genetic circuits can function as physical models of natural genetic circuits. The use of physical models to understand natural phenomena is common throughout the sciences in general and biology in particular. Linus Pauling discovered the α-helix structural motif by building physical ball-and-stick models of proteins[9]. In molecular biology, the synthesis of an artificial chromosome in yeast proved the necessity and sufficiency of particular combinations of sequence elements for chromosome function[10]. Most similarly, in the field of protein design, proteins can be designed *de novo* based on principles and motifs discovered in natural proteins[11,12]. For example, understanding of protein–protein interactions is facilitated by design of synthetic interacting proteins[12].

We describe here how synthetic biology can address biological questions at the level of genetic circuits. We review some tools developed for synthetic biology and a few specific experiments that use these tools to answer fundamental biological questions. Finally, we discuss future directions of this approach. Owing to space limits, we confine ourselves to a small subset of the much larger, and rapidly growing, field of synthetic biology, reviewed elsewhere[13–18] and in this issue[19].

Modular circuit components

In order to build synthetic circuits, one needs genetic components that are well characterized, modular (that is, function similarly in different systems) and act independently of other cellular processes. Early synthetic biology experiments focused on transcriptional regulation components because they are relatively well understood and easy to reconfigure. For example, repressors were used to create feedback loops of various sizes in *Escherichia coli* in

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order to understand noise suppression[20], bistability[21,22] and oscillations[3] in circuits of one, two and three repressors, respectively. Similarly, synthetic cascades without feedback provided information on delays[23], noise propagation[6,24] and sensitivity[25]. More recently, some of these transcriptional circuit designs have been created and analysed in mammalian systems using newly developed transcriptional regulators[26,27]. In one case, a synthetic bistable switch was shown to operate in a mouse[26].

Nevertheless, it is clear that many natural circuits are fundamentally non-transcriptional. An amazing example is the cyanobacterial circadian clock, the operation of which depends on protein phosphorylation but can be independent of transcription and translation (in contrast to its counterpart in *Drosophila*)[28]. Thus, it is critical to appropriate other interaction mechanisms, such as protein modification, regulated degradation, and so on, for use in synthetic circuits.

One class of such non-transcriptional regulatory control mechanism involves *trans*-acting RNA molecules, such as microRNAs. Regulation by RNA offers promising applications in synthetic biology because of the inherent versatility of its sequence-based targeting mechanism[29]. Recently, Bayer and Smolke[30] reported the development of designed 'anti-switches' composed of a modular ligand-binding (aptamer) domain joined to a regulatory domain that inhibits translation of specific target mRNAs in a ligand-dependent fashion (Fig. 2a). Isaacs *et al.*[31] described '*cis*-repressed' riboregulatory molecules containing a translation-repressing hairpin RNA structure. Complementary *trans*-acting RNA molecules relieve inhibition by the hairpin, allowing gene expression. Both types of components should find applications in synthetic circuits that require combinatorial control of gene expression.

Small molecules such as hormones and metabolites carry out many cellular functions—can they be used in synthetic genetic circuits? Recently, Fung *et al.*[5] created the 'metabolator', a synthetic circuit in which transcriptional control of metabolic enzymes generate oscillations between two metabolite pools: acetyl CoA versus acetyl phosphate and acetate (Fig. 2b). The metabolic enzymes that interconvert these two metabolites are under the transcriptional control of acetyl phosphate-sensitive regulators. On a larger scale, metabolic enzymes from plant, yeast and *E. coli* were arranged in synthetic pathways to produce amorpha-4,11-diene[32], a precursor of the highly effective anti-malarial drug artemisinin. Synthetic metabolic pathway construction has the potential to enable synthesis of medically important compounds and may also shed light on fundamental issues of pathway design.

Small diffusible molecules naturally used for intercellular communication have also been appropriated for synthetic circuits. For example, acyl-homoserine lactone (AHL), a signalling molecule in diverse bacteria[33], was recently[34] used to implement a simple multicellular patterning system in *E. coli*, loosely analogous to a morphogen gradient[35] (Fig. 2c). In this work, AHL was produced by a 'sender' strain localized to the centre of a Petri dish. A second set of 'receiver' strains contained synthetic transcriptional circuits that combine positive and negative responses to AHL and thereby serve as 'bandpass' detectors. As a result, the fluorescent output gene was expressed only in cells within a specific range of distances from the source (that is, in a ring). Owing to an unanticipated design feature of the circuit,
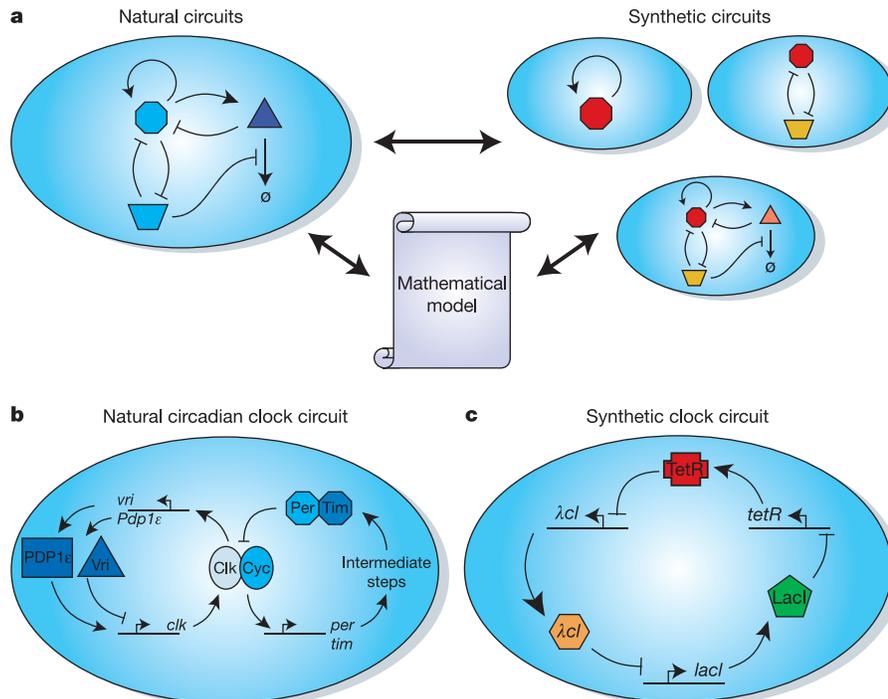

Figure 1 | Natural and synthetic genetic circuits. a, The synthetic biology paradigm. Genetic circuits are composed of interacting genes and proteins (blue shapes, top left). The pointed and blunt arrows represent positive and negative regulation, respectively. Synthetic circuits (top right) based on the natural circuit can be constructed from well-characterized components (red and orange shapes) with similar regulatory effects to form similar or simplified circuits. The dynamics of these synthetic replicas can be compared to the natural system as well as to mathematical models. Analysis of natural circuits, synthetic replicas and models together can help us understand mechanisms used by natural systems. **b**, The *Drosophila* circadian clock is an actively studied natural clock circuit (simplified scheme based on Cyran *et al.*[2]). It contains a negative feedback loop in which Per and Tim, after a delay, repress their own production (via Clk/Cyc, right loop). Interlocked with this negative feedback loop is another loop involving Vri and PDP1ε (left loop). The diagram is highly simplified and many details of the process have been omitted, including post-translational modifications, nuclear transport and active degradation. **c**, The 'repressilator' is a simple synthetic clock circuit consisting of a three-component negative feedback loop that operates in *E. coli*[3]. The three-element loop provides a delayed negative feedback on all components and permits oscillations. In this sense, it models the generation of oscillations by delayed negative feedback. However, as can be seen from the figure this simple synthetic circuit differs markedly from the natural circadian clock in both complexity and design.

the position of this ring did not vary over time as the signal diffused outward from its source. This effect was attributed to a dynamic delay within the circuit: one transcription factor had to be diluted by cell growth to allow expression of the reporter. Thus, the authors uncovered a design strategy for creating stationary patterns from a diffusive gradient that grows with time. We are not aware of evidence for this mechanism in natural morphogen systems; however, similar problems may appear in development, and it will be exciting to see whether synthetic circuits can address them.

One concern when building synthetic genetic circuits is the effects that they might have on endogenous cellular functions. Ideally, one would like circuits to operate independently of the rest of the cell, but the limits to such independence remain unclear. Recently, a step in this direction was taken by Rackham and Chin[36], who generated a set of 'orthogonal' ribosome–mRNA pairs. They used a novel selection-counterselection scheme to select three specifically interacting pairs of ribosome binding sites and their complementary regions on the 16S rRNA (Fig. 2d). These orthogonal ribosomes represent partially independent protein production lines. Their approach might be extended to other steps in gene expression, for example, by creating orthogonal sigma factors for prokaryotic transcription. Circuits based on such orthogonal systems may provide better understanding of the natural regulation of global processes like transcription and translation.
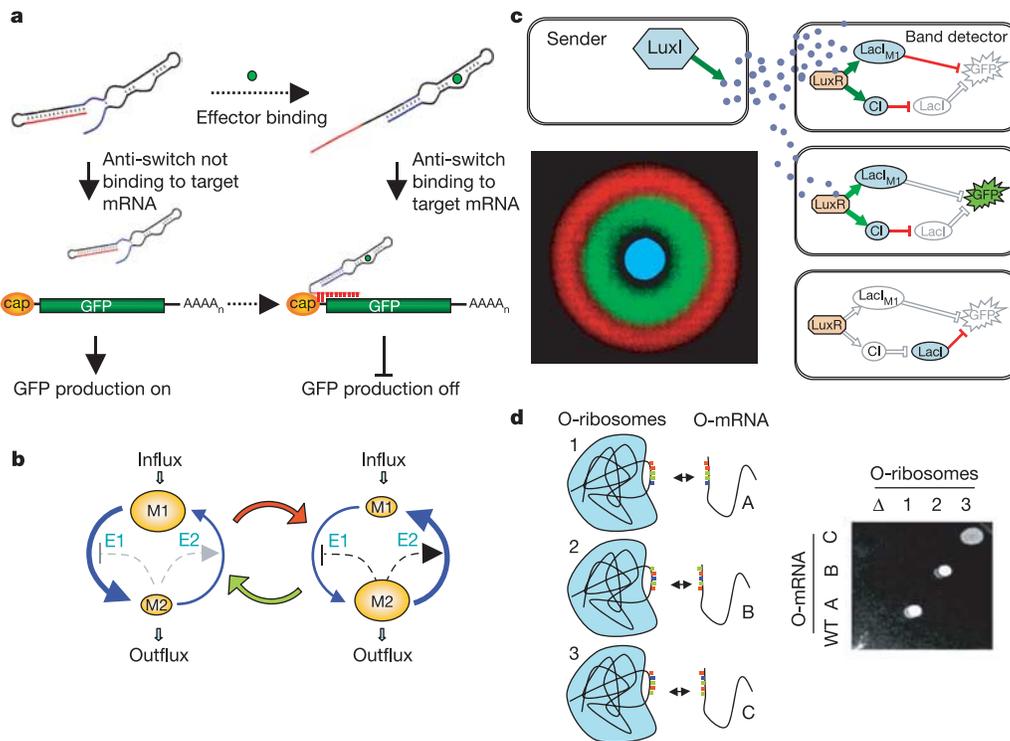
Lessons learned from synthetic circuits

The tools above, and others, will facilitate the development of novel synthetic circuits, but the ultimate value of this approach will depend on what general principles it reveals about circuit design. So far, several important lessons have emerged from the earliest results.

The intracellular environment differs in many ways from the idealized test tubes in which we imagine biochemical reactions occurring and that form the basis for most mathematical models. For example, the cellular environment is highly variable. This variability arises in two ways. First, owing to the low copy number of many cellular molecules, stochastic effects in biochemical reactions can be significant. This stochasticity, or 'intrinsic noise', causes biochemical reactions to be partly unpredictable, and therefore more difficult to model. Second, individual cells differ from one another in many ways. This 'extrinsic noise' causes cell–cell differences in circuit dynamics[37]. There is an interplay between the two types of noise: intrinsic noise in one cellular component, such as a transcription factor, may contribute to extrinsic noise in other components, such as its target genes. Synthetic biology experiments have been instrumental in probing both types of noise and revealing the strong effect they have on the function of genetic circuits.

Rosenfeld et al.[38] used time-lapse movies to analyse a synthetic cascade in which a fluorescent repressor protein and its target gene were monitored simultaneously in individual cells (Fig. 3a). Their

Figure 2 | Modular components in synthetic circuits. a, RNA 'anti-switches.' These molecules contain an aptamer domain that binds to a specific effector (small molecule ligand), as well as a sequence recognition domain complementary to a target mRNA. Binding of the effector changes the conformation of the switch and enables control of target gene expression[30]. **b**, The 'metabolator.' This synthetic oscillatory circuit relies on regulated metabolic fluxes. Oscillations occur between two pools of metabolites, here labelled M1 and M2 (see text). M2 indirectly regulates the metabolic enzymes E1 and E2, corresponding to phosphate acetyltransferase and acetate kinase. Two extreme states of the oscillator are shown in the diagram. On the left, the system has lots of M1 and little M2. In this state, the enzyme activities are small, causing substantial net flux from M1 to M2. On the right is the opposite state in which M2 levels are high and M1 levels are low. Now, E1 and E2 levels increase, reversing the net flux[5]. **c**, Formation of spatial patterns by a synthetic multicellular circuit. Mixed populations of engineered E. coli strains are placed on a Petri dish. Sender cells, localized to the centre (top left), emit AHL, which diffuses away. Band detector strains throughout the plate (right) respond to specific ranges of AHL using a synthetic band detector circuit. The result is concentric ring patterns of gene expression (bottom left), in which two different detector strains (green and red) turn on at specific distances from the source[34]. **d**, Synthesis of orthogonal ribosome–mRNA pairs. The 16S ribosomal subunit and a target ribosome-binding site sequence were coevolved to recognize one another specifically and uniquely (left). A chloramphenicol resistance gene was subcloned together with each of the evolved ribosome-binding sites and co-transformed with each evolved, and one wild-type, 16S subunit, to produce a 'matrix' of strains. As shown on the right, only strains containing matched pairs are able to grow in media containing high levels of chloramphenicol[36].
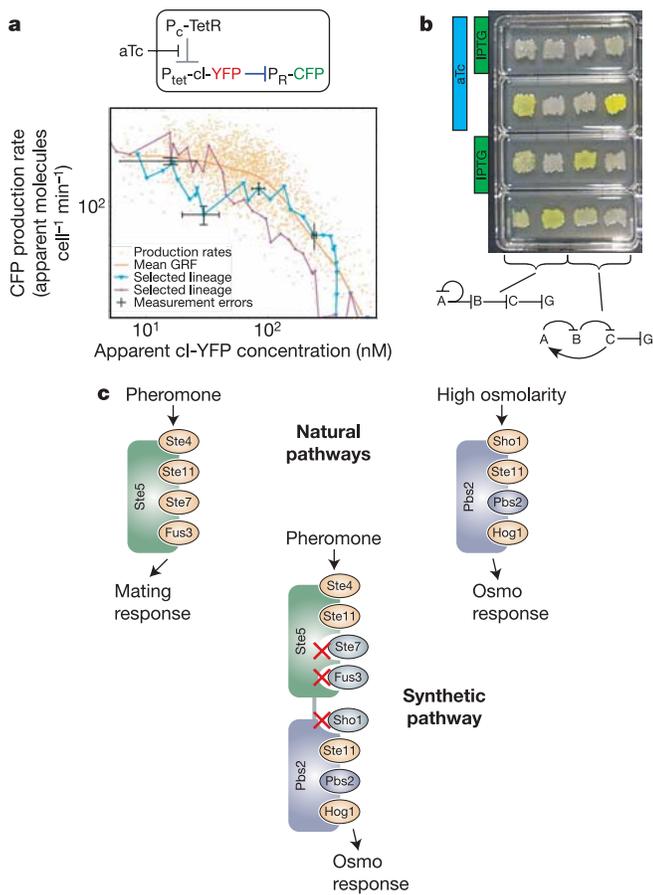
Figure 3 | **Lessons from synthetic biology. a**, Noise in gene regulation[6]. A synthetic transcriptional cascade was constructed to analyse the gene regulation function (GRF) in single cells (top panel). The GRF is the relation between the protein production rate (*y* axis in graph) and the concentration of its repressor protein (*x* axis). The transcriptional cascade comprises reporter gene, cyan fluorescent protein (CFP), under a λ P_R promoter, which can be repressed by CI repressor fused to yellow fluorescent protein (YFP). The CI-YFP fusion protein is under the control of TetR repressor (repression of TetR can be removed through addition of aTc (anhydrotetracycline); TetR is under a constitutive promoter P_C). Each orange point in the graph (bottom panel) represents a measurement made in a single cell at a given time. The average GRF is given by the orange line. Measurements from two selected lineages are indicated in cyan and magenta. The data show significant fluctuations in the GRF that vary slowly, with a timescale of approximately one cell cycle. These results show that the GRF is not well defined at the single cell level. **b**, Exploring the functional potential of transcriptional circuits[40]. Three transcriptional regulators and five promoters were rearranged to produce a small library of random genetic circuits, four of which are shown here (columns). For each circuit, the expression of a green fluorescent reporter gene (G) was measured with and without each of two extracellular inducers (IPTG and aTc). Circuits with four different output functions (yellow–green fluorescence versus four indicated input conditions) are shown here patched onto agar plates; however, each pair shares a single circuit structure, shown below. In these diagrams, A, B and C refer to transcriptional regulatory proteins. Note that different functions are seen with the same diagram (columns 1–2 and 3–4), and similar (but not identical) functions are produced by different diagrams (columns 1 and 4)[49]. **c**, Rewiring signalling pathways. The pheromone and osmolarity response pathways in yeast (top) use protein scaffolds (Ste5 and Pbs2) to avoid crosstalk through the shared component Ste11. By constructing a modified fusion protein of Pbs2 and Ste5, the authors constructed a rewired pathway in which cells produce osmolarity stress responses after pheromone induction[42].

results showed how noise and gene regulation combine to determine the instantaneous rate of expression of a typical gene. The experiments assessed the timescale and amplitude of noise, in the regulation of a specific gene, and showed that the extrinsic component of the noise makes a dominant contribution to the total noise. They also revealed that most fluctuations do not 'average out' over a cell cycle, and therefore the response curve of a repressor–promoter system is not well defined at the single cell level. In related work, Pedraza and van Oudenaarden[24] constructed a linear cascade of three repressors, together with three corresponding fluorescent protein reporters. They showed how fluctuations propagate in a transcriptional cascade. This work used a previously developed theoretical framework[39] to provide an integrated analysis of noise at the circuit level. It remains to be seen how cells and human genetic circuit designers might use such fundamentally noisy components to make reliable circuits.

Synthetic biology relies on the rearrangement of modular elements to create novel circuits. Rather than designing a specific circuit, it is also possible to create combinatorial libraries of many possible circuits. What can such libraries teach us about the computational power and functional diversity of simple biological systems?

Recently, Guet *et al.*[40] addressed this question by creating a synthetic circuit 'slot machine' in which each of three transcriptional regulatory genes was controlled by one of five possible promoters (Fig. 3b). The resulting circuit library was screened for its response to two different chemical inputs that interacted with two of the repressors. A surprisingly large fraction of the diverse circuit designs generated computationally interesting functions, such as NAND and NOR. In this example, the map of known interactions among the components did not uniquely determine the circuit function. Rather, examples were found in which the same circuit architecture produced different functions, as well as others in which the same function was encoded by circuits with different architectures. This synthetic biology experiment demonstrates what a rich functional diversity can be obtained with even a relatively small number of components.

Similarly, Dueber *et al.*[41] recently explored analogous structure–function relations at the level of protein domains. They built a library consisting of a kinase domain fused to various ligand-binding regulatory domains placed in different positions within the protein. They determined the activity of the kinase in the presence and absence of two ligands specific for domains contained in each protein. They found that a large diversity of different functions could be encoded in individual proteins by recombining modular protein domains. The implications at the protein level are similar to those at the circuit level: recombination of modular units can rapidly explore the space of possible functions. It remains to be seen what limits the functional spectrum in these cases.

A final lesson from synthetic circuits involves fidelity in signal transduction. Park *et al.*[42] used a synthetic approach to study how signal fidelity is maintained within pathways that share components. In yeast, both the mating pheromone and osmolarity stress response pathways signal through the mitogen-activated protein (MAP) kinase Ste11, yet they exhibit no crosstalk. This insulation is accomplished by the protein scaffolds Ste5 and Pbs2 that bind Ste11 together with other pathway-specific components (Fig. 3c). Even though Ste11 participates in two pathways (interacts with two scaffolds), it transduces a signal while in a complex with specific upstream and downstream components. Park *et al.*[42] replaced natural tethering interactions with synthetic alternatives that were sufficient to tether together the different components of the MAP kinase cascade and allow signal propagation. To show the sufficiency of tethering, they created a synthetic hybrid scaffold consisting of a modified fusion protein of Ste5 and Pbs2. This construct effectively re-wired the signalling pathway so that pheromone induced osmo-regulatory responses. Here, the synthetic approach involves rewiring, rather than reconstruction per se, and demonstrates that the

principal requirement for signalling through a pathway is proximity of components. It also suggests a simple mechanism by which signal transduction pathways might evolve new capabilities.

Challenges for synthetic circuit design

Constructing a functional synthetic circuit requires assembling diverse genetic elements and getting them to work together. In general, combining disparate components requires the tuning of biochemical parameters such as affinities or rate constants, which is often difficult to implement in biological circuits[3,25]. Characterization of a component may be valid in one context (locus, plasmid, strain, environment, and so on), but not in others. How can one design an operating circuit given these limitations? Several strategies have been applied. First, the use of tunable elements, such as transcription factors derived from *tetR*[43,44], allows external control over some parameters. Second, one can screen libraries of mutated components, or apply directed evolution in the laboratory, to optimize parameters[45]. A third strategy is to use robust circuit designs that are inherently insensitive to unknown or variable parameters. Such designs are particularly interesting because they may have been selected by evolution for the very same property[46].

A related challenge is computational modelling of genetic circuits. Modelling is essential both for analysis of natural systems and also for design of synthetic ones. However, several problems complicate its application to cellular circuits. These include parameter sensitivity, the lack of effective rules to simplify complex circuits, and the difficulty of incorporating extrinsic noise. Because synthetic circuits are simpler and better characterized than their natural counterparts, they will probably offer ideal test systems to develop and refine models. The results should apply both to natural and synthetic circuits.

Future directions

What are the goals of the synthetic circuit paradigm outlined here? One is to better understand natural circuits by building minimal replicas of those circuits, observing their dynamics *in vivo*, and comparing them to one another and to their natural counterparts. The synthetic circuits presented above are highly simplified. However, as we gain confidence and expertise in our ability to build, model and analyse these circuits, we will be able to construct replicas of greater verisimilitude. Possible natural circuits that could be investigated this way include decision making in response to stress and DNA damage, as in the natural p53/mdm2 circuit[47], differentiation in response to extracellular signals, as in oocyte maturation[48], and regulated temporal oscillations, as in the cell cycle and circadian clock[1,10]. Circuits that use the intrinsically noisy nature of the cell to create probabilistic behaviours are particularly compelling examples.

A second goal is to discover what other, non-natural, circuit designs are possible given realistic biological components, and which of those operate reliably *in vivo*. This will be achieved by building and characterizing a variety of alternative circuit designs in living cells. In this way, one may ask what advantages naturally evolved circuits have over synthetic ones. For example, the synthetic clock designs described earlier have not been discovered to occur in nature, suggesting that natural designs may confer better performance. At the same time, non-natural designs may prove useful for biotechnology applications[32].

Perhaps the most intriguing problem is how a circuit operates in the context of a complete organism. There are no dotted lines inside the cell isolating circuits from one another. The ultimate test for this synthetic approach is to delete natural circuits and replace them with synthetic counterparts within organisms. This will require synthetic circuits to interface with the rest of the cell. For example, by replacing the *Drosophila* circadian clock with synthetic versions we could learn more about the interaction of the circadian module with other functional subsystems in the organism. Even the most optimistic synthetic biologist would expect such circuits to be less functional than their natural counterparts. However, perhaps at this stage one can learn more by putting together a simple, if inaccurate, pendulum clock than one can by disassembling the finest Swiss timepiece.

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