Gene regulation: **Towards a circuit engineering discipline**

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Genetic circuits can now be engineered that perform moderately complicated switching functions or exhibit predictable dynamical behavior. These 'forward engineering' techniques may have to be combined with directed evolution techniques to produce robustness comparable with naturally occurring circuits.

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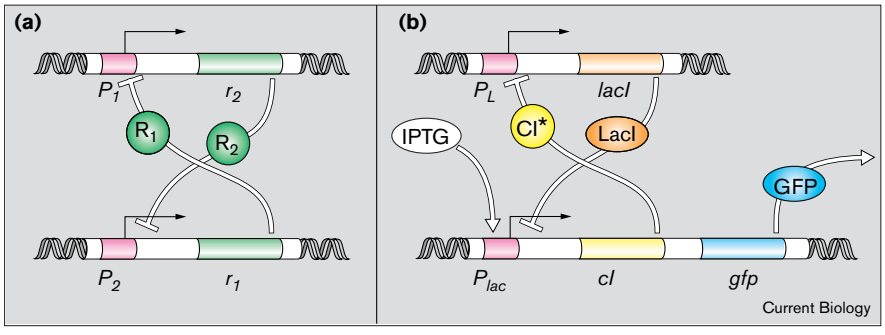
In a summary of the general conclusions of a 1961 Cold Spring Harbor Symposium, Monod and Jacob comment that "it is obvious from the analysis of these [bacterial genetic regulatory] mechanisms that their known elements could be connected into a wide variety of 'circuits' endowed with any desired degree of stability" [1]. It is remarkable that now, nearly 40 years later, with near universal acceptance of the validity of Monod and Jacob's vision, the practice of genetic circuit engineering is still in its infancy. Two papers published recently in *Nature* [2,3] provide a snapshot of the current state of the discipline. Elowitz and Leibler [2] describe a genetic circuit engineered into *Escherichia coli* cells that oscillates asynchronously with regard to the cell-division cycle. Gardner *et al.* [3] describe a toggle-switch circuit that can be switched between two stable states by transient external signals. In both studies, the circuits' qualitative performance is consistent with the predictions of relatively simple differential equation models that characterize the dynamics of production, degradation and genetic regulation.

The central feature of the toggle switch is a bistable, two-promoter configuration, involving constitutively active and repressible promoters (Figure 1a, P_1 and P_2), that each control transcription of a repressor of the other when activated. It is intuitively clear that there are likely to be two stable situations, P_1 ON or P_2 ON, given comparable promoter kinetics. Gardner *et al.* [3] demonstrate how this inherently bistable configuration can be engineered so that external signals can flip the system from one state to the other. In one design, for example, P_2 is an IPTG-inducible *lac* promoter, R_1 is a thermally unstable CI repressor, and the state of the switch is sensed by addition of a reporter gene coding for green fluorescent protein (GFP) directly downstream of the *cI* gene (Figure 1b). Gardner *et al.* [3] found that all cells in colonies grown for six hours in the presence of IPTG under conditions for CI stability remained in the P_2 ON state after removal of the IPTG signal. Transiently raising the temperature so that CI was unstable for seven hours caused the cell population to switch completely to the alternative P_1 ON state.

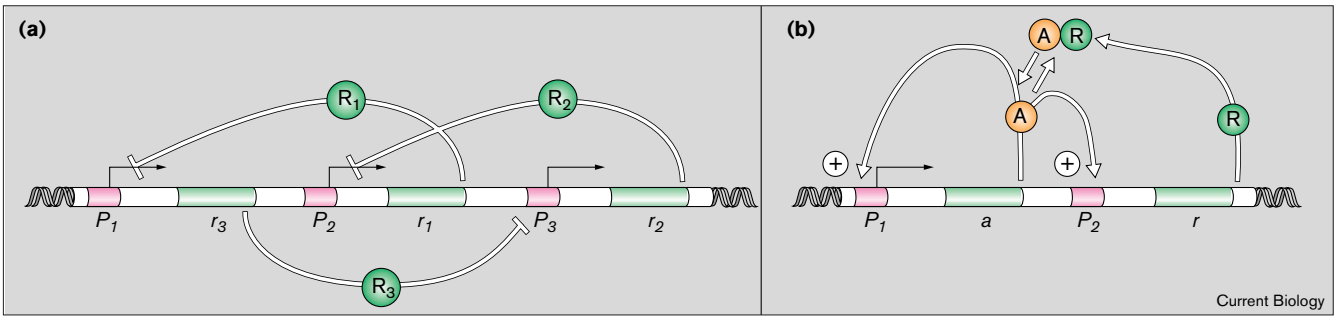
Sophisticated engineering of artificial genetic switches is not at all new [4], but this is perhaps the first engineered design that exploits bistability to produce a switch with the capability of *reversibly* switching between two alternative stable expression states. One can envision variations in the design where, say, IPTG is replaced by a signal transmitted by a 'two-component' system — a kind of signal transduction mechanism commonly found in bacteria consisting of transmembrane receptor and response-regulator components — so that the cells would continuously express the GFP after one extended encounter with the receptor's activating ligand. Or genes for two alternative regulon-controlling proteins could be inserted downstream to the genes for R_1 and R_2, enabling toggling between alternative metabolic or morphological states.

Figure 1



(a) Inherently bistable configuration of mutually antagonistic repressors. **(b)** One of two genetic toggle switch designs based on this bistable switch concept implemented in [3]. Exposure to IPTG switches the cell population to the P_{lac} ON state. In the absence of IPTG, exposure to the temperature range of CI instability toggles the switch to the P_L ON state. The asterisk indicates that the CI used in this genetic switch is thermally unstable.

Figure 2



(a) With suitable choices of component kinetics and favorable gene dosage conditions, this circular genetic cascade of three repressor–promoter links creates an oscillator circuit termed a 'repressilator' [2]. In this specific case, the repressors are as follows: R_1, LacI; R_2, CI; R_3, TatR. **(b)** A genetic circuit predicted to exhibit relatively robust hysteresis-based oscillations [5]. Protein R, when present, binds strongly to A, so that P_2 is no longer activated. Resulting alternating periods of R production and degradation create the oscillatory behavior.

The synthetic oscillator circuit described by Elowitz and Leibler [2] has less immediately evident technical applications. What possible use is there for a bacterial population engineered to glow intermittently and erratically like a crowd of drunken fireflies? Well, probably not much. But pursuing the effort to design an artificial oscillator with a robust, predictable frequency that is reliably inherited at cell division could lead to deep insights into the design rules underlying natural oscillatory systems. Furthermore, resolving differences between the behavior predicted from quantitative simulation and the experimentally observed behavior should greatly improve our understanding of the intracellular environment.

The design of the oscillatory circuit, referred to as a 'repressilator', involves a daisy-chain of promoter–repressor pairs (Figure 2a), producing a cyclic negative feedback loop, engineered onto a low copy number plasmid. Elowitz and Leibler [2] modeled the configuration's protein production and degradation dynamics using a system of coupled differential equations, and identified molecular characteristics of the components favoring limit-cycle oscillations (a limit-cycle oscillator undergoes periodic transitions between two alternative extreme states of the system). Following design insights from the model, the circuit was constructed with relatively strong promoters and with repressor molecules engineered for short half-lives. Oscillations were monitored by a separate, higher copy plasmid containing a TetR-repressible promoter fused to the gene for a short-lived GFP. Some 40% of the cells containing the oscillator and monitor plasmids exhibited oscillatory behavior. Simulations of the predicted circuit behavior, using stochastic kinetics for the genetic mechanisms of transcription and translation, predicted highly variable oscillations, and the observed oscillations were indeed quite erratic.

Stochastic phenomena undoubtedly are the cause of the failure of oscillations in 60% of the cells and for the erratic changes in behavior observed in the oscillatory 40% at cell septation times. The repressilator design has additional sources of stochastic variation beyond stochastic gene expression kinetics. For example, the number of plasmids containing the oscillator circuit and the number of plasmids containing the GFP reporter construct will each vary independently from cell to cell; there will be random variations in the partitioning of both the plasmids and the repressor protein populations at the time of septation; and the GFP protein folding times will also exhibit statistical variation.

While cells have solved the problem of construction of robust and predictable circuits from inherently noisy components, there is currently only limited understanding of how these natural circuits are designed to achieve stability and predictability. In a related paper in the same journal issue, Barkai and Leibler [5] model a different hypothetical oscillator circuit design, shown in Figure 2b. Their modeling results suggest that this design would have stable oscillations and be relatively insensitive to global changes in the cell. It will be interesting indeed to see if cells containing a circuit of this design actually exhibit the predicted increased stability. Rational circuit designs for many applications, therapeutic applications for example, will have to have qualities of robustness and reliability comparable to natural circuits. As we do not yet know the design principles of natural circuits, this presents a considerable challenge for design *de novo*. The many frustrations of metabolic engineers seeking to reengineer metabolic pathways for novel functions illustrate this problem.

Traditionally, the engineering of cellular circuitry has been an industrial practice. The most developed aspect of this field is in metabolic engineering [6–9]. Metabolic engineering generally involves the modification of cells, usually bacteria, to overproduce endogenous or heterologous natural products. Two approaches have been taken to this challenge: first, 'rational analysis' to determine

changes to engineer into the pathways; and second, 'directed evolution' to produce strain improvement by selective pressure (for example [9]). Rational analysis techniques include metabolic control and flux analysis, thermodynamic analysis and full kinetic modeling [10–12]. The goal is usually to maximize output of a valuable product, to minimize feedstock costs, or to increase reliability. The complexity of the cellular environment is a limiting factor in the rational design approach, so that additional selection-driven evolution is almost always necessary [13].

It does seem that ambitions for the size, complexity and type of circuit and pathway changes have outstripped our ability to predict their behavior quantitatively, and perhaps even our ability to select for the required behavior. It will be a challenge, for example, to design selection schemes that can be used to drive cells containing artificially engineered oscillator circuits toward robust, reliable, noise-resistant oscillation by successive selections. The emerging paradigm for genetic circuit engineering is to use relatively accurate simulation techniques to design cellular circuits that operate within a 'selection distance' of target specifications. The engineered cells containing circuits that are in weak compliance with the desired specification will then have an enhanced probability of producing more compliant mutants that can be captured in a 'directed evolution' selection screen.

What types of engineered circuits will be useful? Engineering tasks that are central now include the following examples: the design of pathways for production of industrial or medically important molecules such as detergent proteins or antibiotics; the design of cells and viruses that perform complex tasks, such as multistep degradations and conversions in bioremediation or cell-specific activity for gene therapy; and the design of circuits to probe the cellular environment or to aid in molecular biological research. In the near term, we can expect a variety of relatively simple, but useful, types of cellular switches, transducers, signal processors, sensors and actuators built out of more or less standard parts.

The engineering challenge is to design for reliable performance over a wide range of time-scales and under a wide range of environmental conditions. Undoubtedly, this will require moving beyond circuits solely consisting of genetic elements to circuit designs incorporating engineered protein–protein or protein–metabolite interactions to achieve the speed necessary for certain functions. Surely genetic circuit engineering will emulate the older engineering disciplines and progress to production of well-characterized components with good quality control and the capability to accurately predict the behavior of proposed circuits with future bioengineering computer-aided design tools. The ability to rationally engineer a system is perhaps the most stringent indicator of understanding. By that criterion, it seems we are still near the very beginning of a long and fruitful scientific and engineering journey.

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