Published in final edited form as:
Nat Rev Genet. 2010 May ; 11(5): 367–379. doi:10.1038/nrg2775.

Synthetic Biology: Applications Come of Age

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Abstract

Synthetic biology is bringing together engineers and biologists to design and build novel biomolecular components, networks and pathways, and to use these constructs to rewire and reprogram organisms. These re-engineered organisms will change our lives in the coming years, leading to cheaper drugs, “green” means to fuel our cars, and targeted therapies to attack “superbugs” and diseases such as cancer. The *de novo* engineering of genetic circuits, biological modules, and synthetic pathways is beginning to address these critical problems and is being used in related practical applications.

The circuit-like connectivity of biological parts and their ability to collectively process logical operations was first appreciated nearly 50 years ago[1]. This inspired attempts to describe biological regulation schemes with mathematical models[2–5] and to apply circuit analogies from established frameworks in electrical engineering[6, 7]. Meanwhile, breakthroughs in genomic research and genetic engineering (e.g., recombinant DNA technology) were supplying the inventory and methods necessary to physically construct and assemble biomolecular parts. As a result, synthetic biology was born with the broad goal of engineering or “wiring” biological circuitry—be it genetic, protein, viral, pathway, or genomic—for manifesting logical forms of cellular control. Synthetic biology, equipped with the engineering-driven approaches of modularization, rationalization, and modeling, has progressed rapidly and generated an ever-increasing suite of genetic devices and biological modules.

The successful design and construction of the first synthetic gene networks—the genetic toggle switch[8] and the repressilator[9] (Box 1)—demonstrated that engineering-based methodology could indeed be applied to build sophisticated, computing-like behaviour into biological systems. In these two cases, basic transcriptional regulatory elements were designed and assembled to realize the biological equivalents of electronic memory storage and timekeeping (Box 1). Within the framework provided by these two synthetic systems, biological circuits can be built from smaller well-defined parts according to model blueprints, they can be studied and tested in isolation, and their behaviour can be evaluated against model predictions of the system dynamics. This methodology has subsequently been applied in the synthetic construction of additional genetic switches[8, 10–18], memory elements[8, 14, 15, 19], and oscillators[9, 10, 20–23], as well as of other electronics-inspired genetic devices, including pulse generators[24], digital logic gates[25–30], filters[31–33], and communication modules[23, 31, 34, 35].

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Box 1

Early synthetic biology designs: switches and oscillators

Switches and oscillators that occur in electronic systems are also seen in biology and have been engineered into synthetic biological systems.

Switches

In electronics, one of the most basic elements for storing memory is the reset-set (RS) latch based on logical NOR gates. This device is bistable, in that it possesses two stable states that can be toggled with the delivery of specified inputs. Upon removal of the input, the circuit retains memory of its current state indefinitely. These forms of memory and state switching have significant implications in biology, such as in the differentiation of cells from an initially undifferentiated state. One means by which cellular systems can achieve bistability is through genetic mutual repression. The natural P_R/P_{RM} genetic switch from bacteriophage lambda, which uses this network architecture to govern the lysis/lysogeny decision, consists of two promoters that are each repressed by the gene product of the other (that is, by the Cro and CI repressor proteins). The genetic toggle switch[8], constructed by our research group, is a synthetically engineered version of this co-repressed gene regulation scheme. In one version of the genetic toggle, the P_L promoter from lambda phage was used to drive transcription of *lacI*, whose product represses a second promoter Ptrc-2 (a *lac* promoter variant). Ptrc-2, on the other hand, drives expression of a temperature-sensitive λ CI repressor protein (cI-ts), which inhibits the P_L promoter. The activity of the circuit is monitored through the expression of GFP. The system can be toggled in one direction with the exogenous addition of the chemical inducer isopropyl-β-D-thiogalactopyranoside (IPTG) or in the other with a transient increase in temperature. Importantly, upon removal of these exogenous signals, the system retains its current state, creating a cellular form of memory.

Oscillators

Timing mechanisms, much like memory, are also fundamental to many electronic and biological systems. Electronic timekeeping can be achieved with basic oscillator circuits, such as the LC circuit (inductor L and capacitor C), which act as resonators for producing periodic electronic signals. Biological timekeeping, which is widespread among living organisms[120], is achieved with circadian clocks and similar oscillator circuits, such as the one responsible for synchronizing the critical processes of photosynthesis and nitrogen fixation in cyanobacteria. The circadian clock of cyanobacteria is based on, among other regulatory mechanisms, intertwined positive and negative feedback loops on the clock genes *kaiA*, *kaiB*, and *kaiC*. Elowitz and Leibler constructed a synthetic genetic oscillator based, not on clock genes, but on standard transcriptional repressors (the repressilator)[9]. Here, a cyclic negative feedback loop composed of three promoter–gene pairs, in which the ‘first’ promoter in the cascade drives expression of the ‘second’ promoter’s repressor and so on, was used to drive oscillatory output in gene expression.

Now, ten years after the demonstration of synthetic biology’s inaugural devices[8, 9], engineered biomolecular networks are beginning to move into the application stage and yield solutions to many complex societal problems. While much work remains on elucidating biological design principles[36], this foray into practical applications signals an exciting coming-of-age time for the field.

Here, we review the practical application of synthetic biology to biosensing, therapeutics, and the production of biofuels, pharmaceuticals and novel biomaterials. Many of the examples herein do not fit exclusively or neatly into only one of these three application categories;

however, it is precisely this multivalent applicability that makes synthetic biology platforms so powerful and promising.

BIOSENSING

Cells have evolved a myriad of regulatory circuits—from transcriptional to post-translational—for sensing and responding to diverse and transient environmental signals. These circuits consist of exquisitely tailored sensitive elements that bind analytes and set signal detection thresholds, and transducer modules that filter the signals and mobilize a cellular response (Box 2). The two basic sensing modules must be delicately balanced: this is achieved by programming modularity and specificity into biosensing circuits at the transcriptional, translational and post-translational levels, as described below.

Box 2

Synthetic biosensor architectures and examples

Biosensors consist of two basic modules (top panel in figure): sensitive elements for recognizing and binding analytes, and transducer modules for transmitting and reporting signals.

Transcriptional

Transcriptional biosensors (panel a) are built by linking environment-responsive promoters to engineered gene circuits for programmed transcriptional changes. In the example shown, a transcriptional AND gate was designed to sense and report only the simultaneous presence of two environmental signals (e.g., salicylate and arabinose)25. At one gate input, the researchers encoded an environment-responsive promoter (e.g., P_{BAD}) that activates transcription of a T7 RNA polymerase gene in response to a single environmental signal (e.g., arabinose). The gene, however, carries internally encoded amber stop codons (red asterisks) that function to block translation of its transcript. Activation of the second gate input is the key to unlocking translation; specifically, translation can be induced when a second promoter (e.g., P_{sal}) activates transcription of the SupD amber suppressor tRNA in response to a second unique signal (e.g., salicylate). In other words, only when the two environmental signals are simultaneously present can the T7 RNA polymerase be faithfully expressed and used to activate an output T7 promoter. This is an example of how sophisticated specificity can be programmed into a transducer module by creatively linking the sensory information of multiple sensitive elements. Furthermore, the design is transcriptionally modular in that different sets of environment-responsive promoters can be interfaced to the AND gate.

Translational

Translational biosensors (panel b) are typically built by linking RNA aptamer domains to RNA regulatory domains. The example shown is an OFF “antiswitch”. Here, the small molecule theophylline is recognized and bound by the aptamer stem of the RNA biosensor. This causes a conformational change in the molecule that liberates the antisense domain from its sequestering stem loop and allows it to inhibit translation of an output reporter[11].

Post-translational

Post-translational biosensors (panel c) consist of membrane-bound protein receptors that trigger signal transduction cascades through signaling proteins, such as response regulators of two-component systems. In the example shown, a synthetic protein scaffold was engineered to physically localize the pathway components of the yeast mating mitogen-activated protein (MAP) kinase pathway, which here is being triggered by the alpha mating factor[79]. By recruiting pathway positive and negative modulators (+/−) to the scaffold, the

modular system can be tuned to enable desired responses to upstream signals (e.g., accelerated, delayed, ultrasensitive responses).

Hybrid

The hybrid example (panel d) shows a synthetic genetic edge detection circuit[82]. The sensitive element is a light-dark sensor, Cph8, made as a chimera of the photoreceptor domain of the cyanobacteria phytochrome Cph1 and the kinase domain of *E. coli* EnvZ. This synthetic sensor activates an engineered gene circuit that combines cell-cell communication (genes and promoters of the Lux operon) with a logical AND gate ($P_{lux\text{-}\lambda}$) in order to trace the edges of an image. Specifically, the absence of light triggers Cph8 kinase activity, which correspondingly activates the *ompC* promoter. Cells not receiving light will therefore produce a cell-cell communication molecule 3-oxohexanoyl-homoserine lactone (AHL)—via expression of its biosynthetic enzyme LuxI—and the transcriptional repressor CI. AHL binds to the constitutively expressed transcription factor LuxR to activate expression from the $P_{lux\text{-}\lambda}$ promoter, which is simultaneously and dominantly repressed by CI. The result is that *only* cells that receive light and are nearby to AHL-producing dark cells will activate the final gate and produce pigment through β-galactosidase activity.

Transcriptional

As the first dedicated phase of gene expression, transcription serves as one method by which cells mobilize a cellular response to an environmental perturbation. As such, the transcription machinery, which includes the genes to be expressed, their promoters, RNA polymerase and transcription factors, all serve as potential engineering components for transcriptional biosensors. Most synthetic designs have focused on the promoters and their associated transcription factors, given the abundance of known and characterized prokaryotic and eukaryotic environment-responsive promoters, which include the well-known promoters of the *Escherichia coli* (*E. coli*) *lac*, *tet*, and *ara* operons.

Both the sensory and transducer behaviours of a biosensor can be placed under synthetic control by directly engineering environment-responsive promoter sequences. In fact, this was the early design strategy adopted to establish inducible expression systems[37–40]. By introducing, removing, or modifying activator and repressor sites, a promoter's sensitivity to a molecule can be tuned. Synthetic mammalian transactivation systems are generic versions of this strategy, in which an environmentally-sensitive transcription factor is fused to a mammalian transactivation domain to cause inducer-dependent changes in gene expression. Synthetic mammalian biosensors based on this scheme have been created for sensing signals such as antibiotics[41–43], quorum-sensing molecules[44, 45], gases and metabolites[46–49], and temperature changes[50, 51]. Fussenegger and colleagues have even incorporated this transgene design into mammalian circuits, creating synthetic networks that are responsive to electrical signals[52].

While the engineering of environment-responsive promoters has been valuable and useful, additional control over modularity and specificity can be achieved by embedding environment-responsive promoters within engineered gene networks. Achieving true modularity with genetic parts is inherently difficult because of unintended interference among native and synthetic parts, and therefore requires careful decoupling of functional modules. One such modular design strategy was employed by Kobayashi *et al.*[34] to develop whole-cell *E. coli* biosensors that respond to signals in a programmable fashion. In this design, a sensory module (i.e., an environment-responsive promoter and associated transcription factor) was coupled to an engineered gene circuit that functions like a central processing unit. *E. coli* cells were programmed to respond to a deleterious endogenous input, specifically, DNA-damaging stimuli such as UV radiation or mitomycin C. The gene circuit, which was chosen to be the

toggle switch described above (see Box 1), processes the incoming sensory information and flips from an 'off' to an 'on' state when a signal threshold is exceeded. Because the biosensor has a decoupled, modular nature, it can be wired to any desired output, from the expression of a standard fluorescent reporter to the activation of natural phenotypes, such as biofilm formation (e.g., via expression of *traA* gene) or cell suicide (e.g., via expression of *ccdB* gene).

Sometimes a single signal may be too general to characterize or define an environment. For such situations, Anderson *et al.*[25] devised a transcriptional AND gate that could be used to integrate multiple environmental signals into a single genetic circuit (Box 2a), thus programming the desired level of biosensing specificity. Genetic biosensors of this sort could be useful for communicating the state of a specific microenvironment (e.g., in an industrial bioreactor) within a "sea" of environmental conditions such as temperature, metabolite levels, or cell density.

Translational

RNA molecules have a diverse and important set of cellular functions[53]. Noncoding RNAs can splice and edit RNA, modify ribosomal RNA, catalyze biochemical reactions, and regulate gene expression at the level of transcription or translation[53–56]. This last regulatory subset of noncoding RNAs[57–59] is well-suited for rational design[60] and, in particular, for biosensing applications. Many regulatory RNA molecules are natural environmental sensors[61–69], and because of their ability to take on complex structures defined by their sequence, these molecules can mediate diverse modular functions across distinct sequence domains. Riboswitches[70], for instance, bind specific small molecule ligands via aptamer domains and induce conformational changes in the 5′ untranslated region (UTR) of an mRNA, thus affecting its expression. Aptamer domains that are modeled after riboswitches represent versatile and widely used sensitive elements for RNA-based biosensing. The choice and number of aptamer domains can provide control over specificity. Building an entire RNA-based biosensor typically requires coupling the functionalities of an aptamer domain (the sensitive element) and a post-transcriptional regulatory domain (the transducer module) on a modular RNA molecule scaffold.

Antisense RNAs[59, 71] are one such class of natural regulatory RNAs that can control gene expression through post-transcriptional mechanisms. By linking a riboswitch aptamer to an antisense repressor on a single RNA molecule, Bayer and Smolke engineered *trans*-acting, ligand-responsive riboregulators of gene expression in *S. cerevisiae*[11] (Box 2b). Binding of the aptamer to its ligand (e.g., the small molecule theophylline) induces a conformational change in the RNA sensor that either sequesters the antisense domain in a stable stem loop (ON switch) or liberates it to inhibit translation of an output gene reporter (OFF switch). As a result of the cooperative dependence on both ligand and target mRNA, this biosensor exhibits binary-like switching at a threshold ligand concentration, similar to the genetic toggle design. Importantly, this detection threshold can be adjusted by altering the RNA sequence and thus the thermodynamic properties of the structure. In principle, the "antiswitch" framework is modular, in other words, aptamers for different ligands and antisense stems targeting different downstream genes could be incorporated into the scaffold to devise new sensors. In practice, and as in many current synthetic biology designs, developing new sensors by aptamer and antisense replacement would likely involve re-screening compatible secondary structures to create functioning switches. In the future, this platform could be combined with rapid, *in vitro* aptameric selection techniques[72–75] for generating a suite of RNA biosensors that report on the levels of various mRNA species and metabolites within a cell. However, here it should also be noted that aptamers show specificity for a relatively biased ligand space, and as a result aptamers for a target ligand cannot always be found.

Yet another method for transducing the sensory information captured by aptamer domains is to regulate translation through RNA self-cleavage[69, 70]. RNA cleavage is catalyzed by ribozymes, some of which naturally possess aptameric domains and are responsive to metabolites[69]. Yen *et al.*[76] took advantage of this natural framework and encoded ligand-sensitive ribozymes within the mRNA sequences of reporter genes. In the absence of its cognate ligand, constitutive autocleavage of the reporter mRNA resulted in little or no signal. The RNA biosensor is flipped when cognate ligand is present to inhibit the ribozyme's activity. Similar to the "antiswitch" framework (and with the same looming technical challenges), these engineered RNAs could potentially be used as endogenous sensors for reporting on a variety of intracellular species and metabolites.

Post-translational

The diversity and complexity of signal transduction pathways are vast. Factors such as the nature of the molecular interactions, the number of interconnected proteins in a cascade, and the use of spatial mechanisms dictate which signals are transmitted, whether a signal is amplified or attenuated, and the dynamics of the response. Despite the multitude of factors and interacting components, signal transduction pathways are, in the simplest sense, hierarchical schemes based on sensitive elements and downstream transducer modules, and can be rationalized as such for engineering protein-based biosensors.

The primary sensitive element for most signal transduction pathways is the protein receptor. Whereas environment-responsive promoters and RNA aptamers are typically identified from nature or selected with high-throughput combinatorial methods, protein receptors can be designed *de novo* at the level of molecular interactions. Looger *et al.*[77], for instance, devised a computational method for redesigning natural protein receptors to bind new target ligands. Starting with a "basis" of five proteins from the *E. coli* periplasmic binding protein (PBP) superfamily, the researchers replaced each of the wild-type (WT) ligands with a new, non-native target ligand and then used an algorithm to combinatorially explore all binding pocket residue mutations and ligand-docking configurations. This procedure was used to predict novel receptors for a carcinogen and explosive (trinitrotoluene, TNT), a medically-important metabolite (L-lactate), and a chemical associated with psychiatric conditions (serotonin). The predicted receptor designs were experimentally confirmed to be strong and specific *in vitro* sensors, as well as *in vivo* cell-based biosensors.

Protein receptors, such as the ones discussed above, are typically membrane-bound; they trigger protein signaling cascades that ultimately result in a cellular response. However, several synthetic methods can be employed to transmit captured sensory information in a tunable and desirable manner. Skerker *et al.*[78] rationally rewired the transmission of information through two-component systems, by identifying rules governing the specificity of a histidine kinase to its cognate response regulator. Alternatively, engineered protein scaffolds can be designed to physically recruit pathway modulators and synthetically reshape the dynamical response behaviour of a system[79] (Box 2c). This constitutes a modular method for programming protein-based biosensors to have any desired response, including accelerated, delayed or ultrasensitive responses, to upstream signals.

Hybrid approaches

Combining synthetic transcriptional, translational, and post-translational circuits into hybrid solutions, and harnessing desired characteristics from each, could lead to the creation of cell-based biosensors that are as robust as those of natural organisms. Using a synthetic hybrid approach, Voigt and colleagues developed *E. coli*-based optical sensors[80–82]. A synthetic sensor kinase was engineered to allow cells to identify and report the presence of red light. As a result, a bacterial lawn of the engineered cells could faithfully "print" a projected image, in

the biological equivalent of photographic film. Specifically, a membrane-bound photoreceptor from cyanobacteria was fused to an *E. coli* intracellular histidine kinase to induce light-dependent changes in gene expression[80] (Box 2d). In a clever example of its use, the bacterial optical sensor was applied in image edge detection[82]. In this case, by wiring the optical sensor to transcriptional circuits that perform cell-cell communication (quorum sensing system from *Vibrio fischeri*) and logical functions (Box 2d), the researchers programmed only the cells that receive light *and* directly neighbor cells that do not receive light to produce a pigment, thus tracing the edges of a projected image. Indeed, this work shows that complex behaviour can emerge from properly wiring together smaller genetic programs, with unique advantages to real-world applications.

THERAPEUTICS

Human health is afflicted by new and old foes, including emergent drug-resistant microbes, cancer, and obesity. All the while, progress in medicine is faced with challenges at each stage of the therapeutic spectrum, ranging from the drying up of pharmaceutical pipelines to limited global access to viable medicines. In a relatively short amount of time, synthetic biology has made promising strides in reshaping and streamlining this spectrum (Box 3). Indeed, the rational and model-guided construction of biological parts is enabling new therapeutic platforms, from the identification of disease mechanisms and druggable targets to the production and delivery of small molecules.

Box 3

Synthetic biology's impact on the therapeutic spectrum

a. **Drug discovery**. A synthetic mammalian gene circuit that enabled drug discovery for anti-tuberculosis compounds[90]. The antibiotic ethionamide is rendered cytotoxic to *M. tuberculosis* by the enzyme EthA within infected cells. Because EthA is natively repressed by EthR, resistance to ethionamide treatment is common. In the gene circuit, a fusion of EthR and the mammalian transactivator VP16 binds a minimal promoter with a synthetic EthR operator site, and activates expression of a reporter gene SEAP (human placental secreted alkaline phosphatase). This platform allows for the rapid screening of EthR inhibitors within mammalian cells.

b. **Treatment & delivery**. A synthetic mammalian genetic switch for tight, tunable, and reversible control of a desired gene for therapeutic or gene delivery applications. In the OFF configuration, expression of the gene of interest (green) is repressed both at the levels of transcription and translation. Constitutively expressed LacI repressor (red) binds to the *lac* operator sites in the transgene module of the gene of interest, thus repressing its transcription. Any transcriptional leakage is repressed at the level of translation by an interfering RNA (blue), which targets the gene's 3′ UTR. The system is switched ON by addition of IPTG, which binds LacI repressor proteins and consequently relieves both forms of repression.

c. **Drug production & access**. The discovery of drugs does not always translate to the people who need them the most, because drug production processes can be difficult and costly. Antibiotics are industrially produced from microbes and fungi, and are therefore widespread and cheap. Conversely, many other drugs are isolated from hosts that are not as amenable to large-scale production and are therefore costly and in short supply. Such drugs include the antimalaria drug artemisinin and the anticancer drug taxol. Fortunately, global access to drugs is being enabled by hybrid synthetic biology and metabolic engineering strategies for the microbial production of rare natural products. In the case of artemisinin, there exist two

biosynthetic pathways for the synthesis of the universal precursors to all isoprenoids, the large and diverse family of natural products of which artemisinin is a member. The native pathway found in *E. coli* (deoxyxylulose 5-phosphate, DXP, pathway) has been difficult to optimize, so the researchers synthetically constructed and tested in piece-wise fashion (e.g., "Top" and "Bottom" operons) the entire *S. cerevisiae* mevalonate-dependent (MEV) pathway in *E. coli*. The researchers initially used a simple, orthogonal host platform to construct, debug, and optimize a large metabolic pathway[105]. They then linked the optimized heterologous pathway to a codon-optimized form of the plant terpene synthase, *ADS*, in order to funnel metabolic production to the specific terpene precursor to artemisinin. This work allowed them to build a full, optimized solution that could be ultimately and seamlessly deployed back into *S. cerevisiae* for cost-effective synthesis and purification of industrial quantities of the immediate drug precursor of artemisinin[106]. Pathway intermediate abbreviations: FPP, farnesyl pyrophosphate.

Disease Mechanism

An electrical engineer is likely to prototype portions of a circuit on a breadboard before printing it as an entire integrated circuit. This allows for the rigorous testing of submodules in an isolated, well-characterized environment. Similarly, synthetic biology provides a framework for synthetically reconstructing natural biological systems to explore how pathological behaviours may emerge. This strategy was employed to lend mechanistic insight into a primary immunodeficiency, known as agammaglobulinemia, in which patients cannot generate mature B cells and as a result are unable to properly fight infections[83]. The researchers developed a synthetic testbed by systematically reconstructing the various components of the human B cell antigen receptor (BCR) signaling pathway in an orthogonal environment. This allowed them to identify network topology features that trigger BCR signaling and assembly. A rare mutation in the Igβ-encoding gene, identified in one patient, was then introduced into the synthetic system and shown to abolish assembly of the BCR on the cell surface, thereby linking this faulty pathway component with disease onset. Pathogenic viral genomes can similarly be reconstructed to study the molecular underpinnings of infectious disease pandemics. For instance, synthetic reconstruction of the Severe Acquired Respiratory Syndrome (SARS) coronavirus[84] and the 1918 Spanish influenza virus[85] helped to identify genetic mutations that may have conferred human tropism and increased virulence.

Drug Target Identification

Building up synthetic pathways and systems from individual parts is one way of identifying disease mechanisms and therapeutic targets. Another is to deploy synthetic biology devices to systematically probe the function of individual components of a natural pathway. Our group, for instance, has engineered modular riboregulators that can be used to tune the expression of a toxic protein or any gene within a biological network[86]. To achieve post-transcriptional control over a target gene, the mRNA sequence of its 5′-UTR was designed to form a hairpin structure that sequesters the ribosomal binding site (RBS) and prevents ribosome access to it. Translational repression of this *cis*-repressed mRNA could then be alleviated by an independently regulated *trans*-activating RNA that targets the stem-loop for unfolding. Engineered riboregulators were used in a subsequent study to tightly regulate the expression of CcdB, a toxic bacterial protein that inhibits DNA gyrase, so as to gain a better understanding of the sequence of events leading to induced bacterial cell death[87]. These synthetic biology studies, in conjunction with systems biology studies of quinolones (antibiotics that inhibit gyrase)[87], led to the discovery that all major classes of bactericidal antibiotics induce a common oxidative damage cellular death pathway[88, 89]. This work provided new insights into how

bacteria respond to lethal stimuli, and paved the way for the development of more effective antibacterial therapies.

Drug Discovery

Once a faulty pathway component or target is identified, whole-cell screening assays can be designed using synthetic biology strategies for drug discovery. As a demonstration of this approach, Fussenegger and colleagues[90] developed a synthetic platform for screening small molecules that could potentiate a *Mycobacterium tuberculosis* antibiotic (Box 3a). Ethionamide, currently the last line of defense in the treatment of multidrug-resistant tuberculosis, depends on activation by the *M. tuberculosis* enzyme EthA for efficacy. Due to transcriptional repression of *ethA* by the protein EthR, however, ethionamide-based therapy is often rendered ineffective. To address this problem, the researchers designed a synthetic mammalian gene circuit, featuring an EthR-based transactivator of a reporter gene, and used it to screen for and identify EthR inhibitors that could abrogate resistance to ethionamide. Importantly, because the system is a cell-based assay, it intrinsically enriches for inhibitors that are nontoxic and membrane-permeable to mammalian cells, which are key drug criteria as *M. tuberculosis* is an intracellular pathogen. This framework, in which drug discovery is applied to whole cells that have been engineered with circuits that highlight a pathogenic mechanism, could be extended to other diseases and phenotypes.

Therapeutic treatment

Synthetic biology devices have additionally been developed to serve as therapies themselves. Entire engineered viruses and organisms can be programmed to target specific pathogenic agents and pathological mechanisms. In two separate studies[91, 92], for instance, engineered bacteriophages were deployed to combat antibiotic-resistant bacteria, by endowing them with genetic mechanisms that target and thwart bacteria's antibiotic evasion techniques. The first study was prompted by the observation that biofilms, in which bacteria are encapsulated in an extracellular matrix, have inherent resistance to antimicrobial therapies and are implicated sources of persistent infections. To more effectively penetrate this protective environment, T7 phage was engineered to express the biofilm matrix-degrading enzyme dispersin B (DspB) upon infection[91]. The two-pronged attack of phage-induced lysis fueling the creation and spread of matrix-degrading enzyme resulted in 99.997% removal of biofilm bacterial cells.

In the second study[92], it was hypothesized that inhibition of certain bacterial genetic programs could help current antibiotic therapies achieve more effective activity. In this case, bacteriophages were deliberately designed to be non-lethal so as not to elicit resistance mechanisms; instead, non-lytic M13 phage was used to suppress the bacterial SOS DNA damage response by overexpression of its repressor, *lexA3*. The engineered bacteriophage significantly enhanced killing by three major classes of antibiotics in traditional cell culture and in *E. coli*-infected mice, potentiated killing of antibiotic-resistant bacteria, and importantly reduced the incidence of antibiotic-induced resistant cells.

Synthetically engineered viruses and organisms that are able to sense and link their therapeutic activity to pathological cues may be useful in the treatment of cancer, where current therapies often indiscriminately attack tumors and normal tissues. Adenoviruses, for instance, were programmed to couple their replication to the state of the p53 pathway in human cells[93]. Normal p53 production would result in inhibition of a critical viral replication component, whereas a defunct p53 pathway, which is characteristic of tumor cells, would allow viral replication and cell killing. In another demonstration of translational synthetic biology applied to cancer therapy, Voigt and colleagues[94] developed cancer-targeting bacteria and linked their ability to invade the cancer cells to specific environmental signals. Constitutive expression of the heterologous *inv* gene (from *Yersinia pseudotuberculosis*) can induce *E. coli* cells to invade

both normal and cancer human cell lines. So, to preferentially invade cancer cells, the researchers placed *inv* under the control of transcriptional operons that are activated by environmental signals specific to the tumor microenvironment. These engineered bacteria could be made to carry or synthesize cancer therapies for the treatment of tumors.

Therapeutic delivery

In addition to engineered therapeutic organisms, synthetic circuits and pathways can be used for the controlled delivery of drugs as well as for gene and metabolic therapy. In some cases, sophisticated, kinetic control over drug release in the body may yield therapeutic advantages and reduce undesired side effects. Most hormones in the body are released in time-dependent pulses. Glucocorticoid secretion, for instance, has a circadian and ultradian pattern of release, with important transcriptional consequences for glucocorticoid-responsive cells[95]. Faithfully mimicking these patterns in the administration of synthetic hormones to patients with glucocorticoid-responsive diseases, such as rheumatoid arthritis, may decrease known side effects and improve therapeutic response[95]. Periodic synthesis and release of biologic drugs can be autonomously achieved with synthetic oscillator circuits[9, 10, 20–22] or programmed time-delay circuits[96]. In other cases, one may wish to place a limit on the amount of drug released by programming the synthetic system to self-destruct after a defined number of cell cycles or drug release pulses. Our group has recently developed two variants of a synthetic gene counter[14] that could be adapted for such an application.

Gene therapy is beginning to make some promising advances in clinical areas where traditional drug therapy is ineffective, such as in the treatment of many hereditary and metabolic diseases. Synthetic circuits offer a more controlled approach to gene therapy, such as the ability to dynamically silence, activate, and tune the expression of desired genes. In one such example[12], a genetic switch was developed in mammalian cells that couples transcriptional repressor proteins and an RNA interference (RNAi) module for tight, tunable, and reversible control over the expression of desired genes (Box 3b). This system would be particularly useful in gene silencing applications, as it was shown to yield > 99% repression of a target gene.

Additionally, the construction of non-native pathways offers a unique and versatile approach to gene therapy, such as for the treatment of metabolic disorders. Operating at the interface of synthetic biology and metabolic engineering, for instance, Liao and colleagues[97] recently introduced the glyoxylate shunt pathway into mammalian liver cells and mice to explore its effects on fatty acid metabolism and, more broadly, whole-body metabolism. Remarkably, the researchers found that when transplanted into mammals the shunt actually increased fatty acid oxidation, evidently by creating an alternative cycle. Furthermore, mice expressing the shunt showed resistance to diet-induced obesity when placed on a high-fat diet, with corresponding decreases in total fat mass, plasma triglycerides, and cholesterol levels. This work offers a new synthetic biology model for studying metabolic networks and disorders, and for developing treatments for the increasing problem of obesity.

The discovery of drugs and effective treatments may not quickly or ever translate to the people who need them the most, because drug production processes can be difficult and costly. As reviewed below, here synthetic biology is bringing more cost-effective manufacturing processes to the production of such rare and costly drugs (Box 3c).

BIOFUELS, PHARMACEUTICALS AND BIOMATERIALS

Recent excitement surrounding the production of biofuels, pharmaceuticals and biomaterials from engineered microorganisms is matched by the challenges that loom in bringing these technologies to production scale and quality. The most widely used biofuel is ethanol produced

from corn or sugar cane[98]; however, the heavy agricultural burden combined with the suboptimal fuel properties of ethanol make this approach to biofuels problematic and limited.

Microorganisms engineered with optimized biosynthetic pathways to efficiently convert biomass into biofuels are an alternative and promising source of renewable energy. These strategies will succeed only if their production costs can be made to compete with, or even out-compete, current fuel production costs. Similarly, there are many drugs whose production processes preclude their capacity for a wider and more cost-effective therapeutic reach. New synthetic biology tools would also greatly advance the microbial production of biomaterials and the development of altogether novel materials.

Constructing biosynthetic pathways

One of the first design decisions when engineering for biofuels, drugs, or biomaterials is which biosynthetic pathway(s) to construct around and, further to that, which host organism to use. Typically, this decision originates with searching for organisms innately capable of achieving some desired biosynthetic activity or phenotype[99]. For biofuel production, for instance, microorganisms have evolved to be proficient in converting lignocellulosic material to ethanol, biobutanol, and other biofuels. These native isolates possess unique catabolic activity, heightened tolerances for toxic materials, and a host of enzymes designed to break down the lignocellulosic components. Unfortunately, these highly desired properties exist in pathways that are tightly regulated according to the host's evolved needs and therefore may not be suitable in their native state for production scale. A longstanding challenge in metabolic and genetic engineering is determining whether to improve the isolate host's production capacity or whether to transplant the desired genes/pathways into an industrial model host, such as *E. coli* or *S. cerevisiae*; these important considerations and tradeoffs are reviewed elsewhere[99].

The example of the microbial production of biobutanol, a higher energy density alternative to ethanol, provides a useful glimpse into these design tradeoffs. Butanol is converted naturally from acetyl-coenzyme A (acetyl-coA) by *Clostridium acetobutylicum*[100]. However, it is produced in low yields and as a mixture with acetone and ethanol, thus requiring significant cellular engineering of a strain for which most standard molecular biology techniques do not apply[101, 102]. On the other hand, importing the biosynthetic genes into an industrial microbial host can lead to metabolic imbalances[103]. In an altogether different approach, Liao and colleagues[104] bypassed standard fermentation pathways and recognized that a broad set of the 2-keto acid intermediates of *E. coli* amino acid biosynthesis could be synthetically shunted to achieve high-yield production of butanol and other higher alcohols in two enzymatic steps.

Complementary to efforts in traditional metabolic and genetic engineering is the use of engineering principles for constructing functional, predictable, and non-native biological pathways *de novo* to control and improve microbial production. In an exemplary illustration of this, Keasling and colleagues engineered the microbial production of precursors to the antimalarial drug artemisinin to industrial levels[105, 106] (Box 3c). There are now many such examples of the successful application of synthetic approaches to biosynthetic pathway construction, for instance in the microbial production of fatty-acid-derived fuels and chemicals (e.g., fatty esters, fatty alcohols, and waxes)[107], methyl halide-derived fuels and chemicals [108], polyketide synthases that make cholesterol-lowering drugs[109], and polyketides made from megaenzymes that are encoded by very large synthetic gene clusters[110].

Optimizing pathway flux

Once biosynthetic pathways are constructed, the expression levels of all the components need to be orchestrated to optimize metabolic flux and achieve high product titers. A standard approach is to drive the expression of pathway components with strong and exogenously

tunable promoters, such as the $P_L$tet, $P_L$lac, and P_{BAD} promoters from the *tet*, *lac*, and *ara* operons of *E. coli*, respectively. To this end, there are ongoing synthetic biology efforts to create and characterize more reusable, biological control elements based on promoters for predictably tuning expression levels[111, 112]. Further to this, synthetic biologists have devised a number of alternative methods for biological pathway balance, ranging from reconfiguring network connectivity to fine-tuning individual components. A richer discussion of these topics, including the fine-tuning of parts, model-guided approaches, and the development of next-generation interoperable parts, is presented elsewhere[113]. In Box 4, we detail a few such synthetic biology strategies that specifically pertain to the optimization of metabolic pathway flux. These strategies span a spectrum from those driven by evolutionary techniques to those driven by rational design and *in silico* models, to those that combine both approaches.

Box 4

Synthetic biology approaches to controlling metabolic flux

a. **Evolutionary strategies**. In the production of artemisinin precursors, the native *E. coli* isoprenoid pathway (DXP) was eschewed in favor of a heterologous pathway so as to circumvent the complex regulatory control imposed by the host (see Box 3c). In an alternative method of relieving regulatory control over the large number of DXP pathway components, Wang *et al.*[121] rapidly diversified and evolved, and thus optimized, the native DXP biosynthetic pathway in *E. coli.* The researchers developed a rapid, automated method for the *in vivo*directed evolution of pathways, which they termed multiplex automated genome engineering (MAGE); they then applied it to evolve the translational efficiencies of DXP pathway components to achieve maximal lycopene production. Specifically, cells were subjected to cycles of genetic modifications (via oligo-mediated allelic replacement) in an automated fashion to explore sufficient genomic diversity for optimizing biosynthetic pathways in laboratory time scales. Pathway intermediate abbreviations: G3P, glyceraldehydes 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate.

b. **Rational design**. At the other end of the spectrum are strategies that rely on quantitative models and blueprints for the rational design of optimized networks and pathways. Typically, a component of interest (e.g., an engineered promoter, P*, or RBS sequence, RBS*) will be built into a simple test network. The network and its input/output data will then be fed into a model, which attempts to determine a parameter set that optimally describes the component's dynamics within the framework of the model. Finally, the optimized parameter set will be used to forward-engineer altogether new networks and components. For example, stochastic biochemical models have been developed to capture the expression dynamics of synthetically engineered promoters; these models were subsequently employed to predict the correct, *in vivo* behaviour of different and more complex gene networks built from the modeled components[122, 123]. Similarly, at the level of translation, thermodynamic models that predict the relative translational initiation rates of proteins can be used to rationally forward-engineer synthetic RBS sequences to give desired expression levels[124]. Such techniques harness modeled genetic parameters (transcriptional or translational) to predict the level of expression of proteins and enzymes within a network.

c. **Hybrid approaches**. In a hybrid rational-combinatorial approach, Dueber *et al.* [125] hypothesized that metabolic flux could be controlled by spatially recruiting the enzymes of a desired biosynthetic pathway using synthetic protein scaffolds. To

construct the enzyme scaffolding, the researchers tethered protein-protein interaction domains from metazoan signaling proteins (e.g., GBD, SH3, PDZ domains) that recognize and bind cognate peptides fused to the enzymes to be recruited (atoB, HMGS, HMGR). By varying the number of repeats of an interaction domain, they could additionally control the stoichiometry of the enzymes recruited to the complex. Once again, using the heterologous MEV pathway in *E. coli* as a model, they combinatorially (albeit, at a significantly smaller scale) optimized the stoichiometry of the three enzymes responsible for producing mevalonate from acetyl-coA. Finally, they showed that the optimized synthetic scaffold could significantly increase product titer while also reducing the metabolic load on the host; in other words, their high product titers did not require the overexpression of biosynthetic enzymes in the cell. Gene symbol abbreviations: *atoB*, acetoacetyl-CoA thiolase from *E. coli*; *HMGS*, HMG-CoA synthase from *S. cerevisiae*; *HMGR*, HMG-CoA reductase from *S. cerevisiae*.

Programming novel functionality and materials

Beyond facilitating metabolic tasks, synthetic systems can infuse novel functionality into engineered organisms for production purposes or for building new materials. Early work in the field laid the groundwork for constructing basic circuits that could sense and process signals, perform logic operations, and actuate biological responses[114]. Wiring these modules together to bring about reliable, higher-order functionality is one of synthetic biology's next major goals[113], and an important application of this objective is the layering of "smart" control mechanisms over metabolic engineering. For instance, circuits designed to sense the bioreactor environment and shift metabolic phases accordingly would further improve biofuel production. Alternatively, autonomous timing circuits could be used to shut down metabolic processes after a prescribed duration of time. Biological timers of this sort have been developed using genetic toggle switches that had been deliberately rendered imbalanced through model-guided promoter engineering[112]. These genetic timers were used to program the time-dependent flocculation of yeast cells to facilitate the separation of cells from, for instance, the alcohol produced in industrial fermentation processes.

Synthetic control systems can also be employed to extract and purify the synthesized product. This is particularly important in the production of recombinant proteins, bioplastics, and other large biomaterials, whose high titer can lead to intracellular accumulation, the formation of inclusion bodies, and cell toxicity. For instance, to export recombinant spider silk monomers, Widmaier *et al.*[115] searched for a secretion system that would enable efficient and indiscriminate secretion of proteins through *both* bacterial membranes. The *Salmonella* type III secretion system (T3SS) not only fulfills these criteria but also possesses a natural regulatory scheme that ties expression of the protein to be secreted to the secretion capacity of the cell; as a result, the desired protein is only expressed when sufficient secretion complexes have been built. The researchers needed only to engineer a control circuit that hitches their heterologous genes to the innate genetic machinery for environmental sensing and secretion commitment to obtain superior secretion rates of recombinant silk protein.

Finally, there is an emerging branch of synthetic biology that seeks to program coordinated behaviour in populations of cells, which could lead to the fabrication of novel biomaterials for a variety of applications. The engineering of synthetic multicellular systems is typically achieved with cell-cell communication and associated intracellular signal processing modules, as was elegantly employed by Hasty and colleagues to bring about synchronized oscillations in a population of bacterial cells[23]. Weiss and colleagues have similarly done pioneering work in building biomolecular signal processing architectures that are able to filter communication

signals originating from 'sender' cells[24, 31]. These systems, which can be programmed to form intricate multicellular patterns from a solid-phase cellular lawn, would aid the development of fabrication-free scaffolds for tissue engineering.

FUTURE CHALLENGES AND CONCLUSIONS

The future of translational synthetic biology is dependent on and limited by the development of reliable means for connecting smaller functional circuits to realize higher-order networks with predictable behaviours. In a previous perspective[113], we outlined four research efforts aimed at improving and accelerating the overall design cycle and allowing more seamless integration of biological circuitry (Box 5).

Box 5

Recommendations for improving the synthetic biology design cycle

1. Scaling up to larger and more complex biological systems while simultaneously minimizing interference among parts will require an expanded synthetic biology toolkit and, in particular, libraries of interoperable parts. Eukaryotic systems are fertile grounds for discovering such parts, as many synthetic biology devices are based on a small repertoire of prokaryotic regulatory elements.
2. Modeling and fine-tuning of synthetic networks should be emphasized, particularly as the network size and complexity increases. This will facilitate proper matching of input/output behaviours (i.e., transfer functions) when distinct modules are connected.
3. There is a need to develop new probes and high-throughput methods for the *in vivo* measurement of circuit dynamics in order to rapidly characterize parts and debug networks.
4. The development of cellular testing platforms are needed to quicken the pace of identifying problematic network nodes and ease failure-prone jumps associated with either building a more complex network or deploying a network in a more complex organism. These testing platforms may be cells engineered to have minimal genomes[126–129] or lower model organisms that have been equipped with specific machinery from higher organisms.

Beyond the challenge of improving the design cycle, applied synthetic biology would benefit from once again summoning the original inspiration of biocomputing. The ability to program higher-level decision-making into synthetic networks would yield more robust and dynamic organisms, including ones that can accomplish many tasks simultaneously. Furthermore, as adaptive and predictive behaviours are naturally present in all organisms (including microbes)[116, 117], synthetic learning networks built from genetic and biological parts[118, 119] would infuse engineered organisms with more sophisticated automation for biosensing and related applications.

Finally, the majority of synthetic biology is currently practiced in microbes. Yet, many of the most pressing problems, and in particular those of human health, are inherently problems with mammalian systems. Therefore, a more concerted effort towards advancing mammalian synthetic biology is critical to next-generation therapeutic solutions, including engineering synthetic gene networks for stem cell generation and differentiation.

By addressing such challenges, we will be limited, not by the technicalities of construction or the robustness of synthetic gene networks, but only by the imagination of researchers and the number of societal problems and applications for which synthetic biology can resolve.

Acknowledgments

We thank members of the Collins Lab for helpful discussions and K.M. Flynn for help with artwork. We also thank the Howard Hughes Medical Institute and the NIH Director's Pioneer Award Program for their financial support.

GLOSSARY

Memory element	A device that is used to store information about the current state of a system
Pulse generator	A circuit or device used to generate pulses. A biological version has been implemented in a multicellular bacterial system, whereby receiver cells respond to a chemical signal with a transient burst of gene expression, whose amplitude and duration depends on the distance from the sender cells
Digital logic gates	A digital logic gate implements Boolean logic (such as AND, OR, NOT) on one or more logic inputs to produce a single logic output. Electronic logic gates are implemented using diodes and transistors and operate on input voltages or currents, whereas biological logic gates operate on cellular molecules (chemical or biological)
NOR gate	A digital logic gate that implements logical NOR, or the negation of the OR operator. It produces a HIGH output (1) only if both inputs to the gate are LOW (0)
Filters	An algorithm or device for removing or enhancing parts or frequency components from a signal
Modularity	Refers to the capacity of a system or component to function independently of context
Environment-responsive promoters	Promoters that directly transduce environmental signals (e.g., heavy metal ions, hormones, chemicals, temperature) that are captured by their associated, sensory transcription factors
Quorum sensing	A cell-to-cell communication mechanism in many species of bacteria, whereby cells measure their local density (via the accumulation of a signaling molecule) and subsequently coordinate gene expression
Aptamer	Oligonucleic acids that bind to a specific target molecule, such as a small molecule, protein, or nucleic acid. Nucleic acid aptamers are typically developed through *in vitro* selection schemes but are also found naturally (e.g., RNA aptamers in riboswitches)
Antisense RNA	Antisense RNAs bind segments of mRNA in *trans* to inhibit translation
Two-component system	Among the simplest types of signal transduction pathways. In bacteria, they consist of two domains, a membrane-bound histidine kinase (sensitive element) that senses a specific environmental stimulus and its cognate response regulator (transducer domain) that triggers a cellular response
Orthogonal environment	A cellular environment or host into which genetic material is transplanted to avoid undesired, native host interference or regulation.

	Orthogonal hosts are often organisms with sufficient evolutionary distance from the native host
Riboregulators	Small regulatory RNAs found in prokaryotes that can activate or repress gene expression by binding segments of mRNA in *trans*. They are typically expressed in response to an environmental signaling event
DNA gyrase	A type II DNA topoisomerase that catalyzes the ATP-dependent supercoiling of closed-circular dsDNA by strand breakage and rejoining reactions. Control of chromosomal topological transitions is essential for DNA replication and transcription in bacteria, making gyrase an effective target for antimicrobial agents (e.g., the quinolone class of antibiotics)
Biofilms	The surface-associated communities of bacterial cells encapsulated in an extracellular polymeric substances (EPS) matrix. Biofilms are an antibiotic-resistant mode of microbial life found in natural and industrial settings
Ultradian	Periods or cycles that are repeated throughout a 24-hour circadian day
Glyoxylate shunt pathway	A two-enzyme metabolic pathway unique to bacteria and plants that is activated when sugars are not readily available. This pathway diverts the tricarboxylic acid (TCA) cycle so that fatty acids are not completely oxidized but, instead, converted into carbon energy sources
Metabolic flux	Rate of flow of metabolites through a metabolic pathway, which is regulated by the enzymes in the pathway
Flocculation	A specific form of cell aggregation in yeast triggered by certain environmental conditions, such as the absence of sugars, e.g., once the sugar in a beer brew has been fermented into ethanol

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ONLINE SUMMARY

- Early synthetic biology designs, namely the genetic toggle switch and repessilator, demonstrated that regulatory components can be characterized and assembled to bring about complex, electronics-inspired behaviors in living systems (e.g., memory storage and timekeeping).
- Through the characterization and assembly of genetic parts and biological building blocks, many more devices have been constructed, including switches, memory elements, oscillators, pulse generators, digital logic gates, filters, and communication modules
- Advances in the field are now allowing the expansion beyond small gene networks, to the realm of larger biological programs that hold promise for a wide range of applications, including biosensing, therapeutics, and the production of biofuels, pharmaceuticals, and biomaterials.
- Synthetic biosensing circuits consist of sensitive elements that bind analytes and transducer modules that mobilize cellular responses. Balancing these two modules involves engineering modularity and specificity into the various circuits.
- Biosensor sensitive elements include environment-responsive promoters (transcriptional), RNA aptamers (translational), and protein receptors (post-translational).
- Biosensor transducer modules include engineered gene networks (transcriptional), non-coding regulatory RNAs (translational), and protein signal transduction circuits (post-translational).
- Synthetic biology’s contributions to therapeutics have included engineered networks and organisms for disease mechanism elucidation, drug target identification, drug discovery platforms, therapeutic treatment, therapeutic delivery, and drug production and access.
- In the microbial production of biofuels and pharmaceuticals, synthetic biology has supplemented traditional genetic and metabolic engineering efforts by aiding in the construction of optimized biosynthetic pathways.
- Optimizing metabolic flux through biosynthetic pathways is traditionally accomplished by driving the expression of pathway enzymes with strong, inducible promoters. New synthetic approaches include the rapid diversification of the various pathway components, the rational and model-guided assembly of pathway components, as well as hybrid solutions.

Biographies

James J. Collins is an Investigator of the Howard Hughes Medical Institute, and a William F. Warren Distinguished Profesor, University Professor, Professor of Biomedical Engineering, and Co-Director of the Center for BioDynamics at Boston University. He is also a core founding faculty member of the Wyss Institute for Biologically Inspired Engineering at Harvard

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Ahmad S. Khalil is a Howard Hughes Medical Institute Postdoctoral Fellow training in the lab of Jim Collins at Boston University. His research interests are in the advancement of systems and synthetic biology through novel technologies, and in particular through integrated microfluidic devices and automation. He received his doctorate in mechanical engineering from the Massachusetts Institute of Technology, where he worked in the labs of Angela Belcher and Matt Lang on single-molecule studies of biological systems. While at MIT, he was awarded a Charles Stark Draper Laboratory Fellowship in Biomedical Engineering.

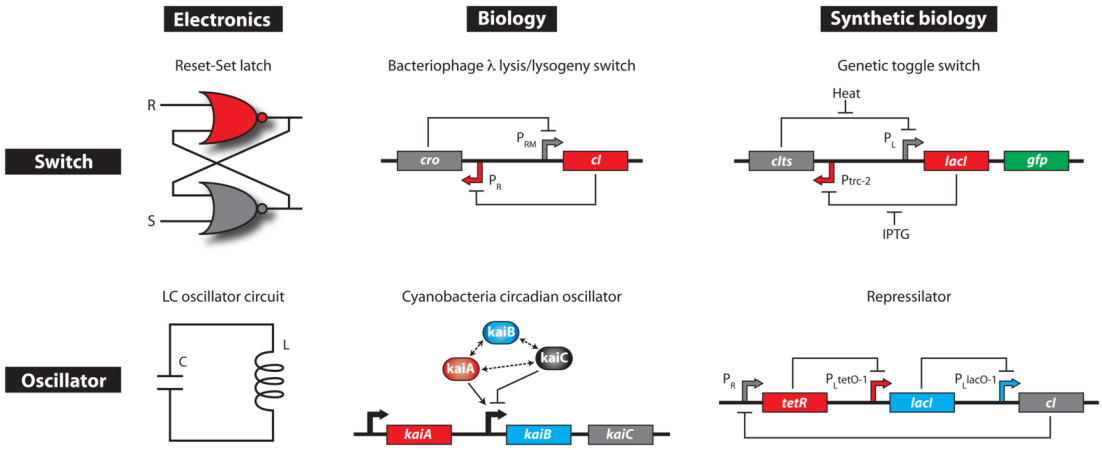

FIGURE 1.

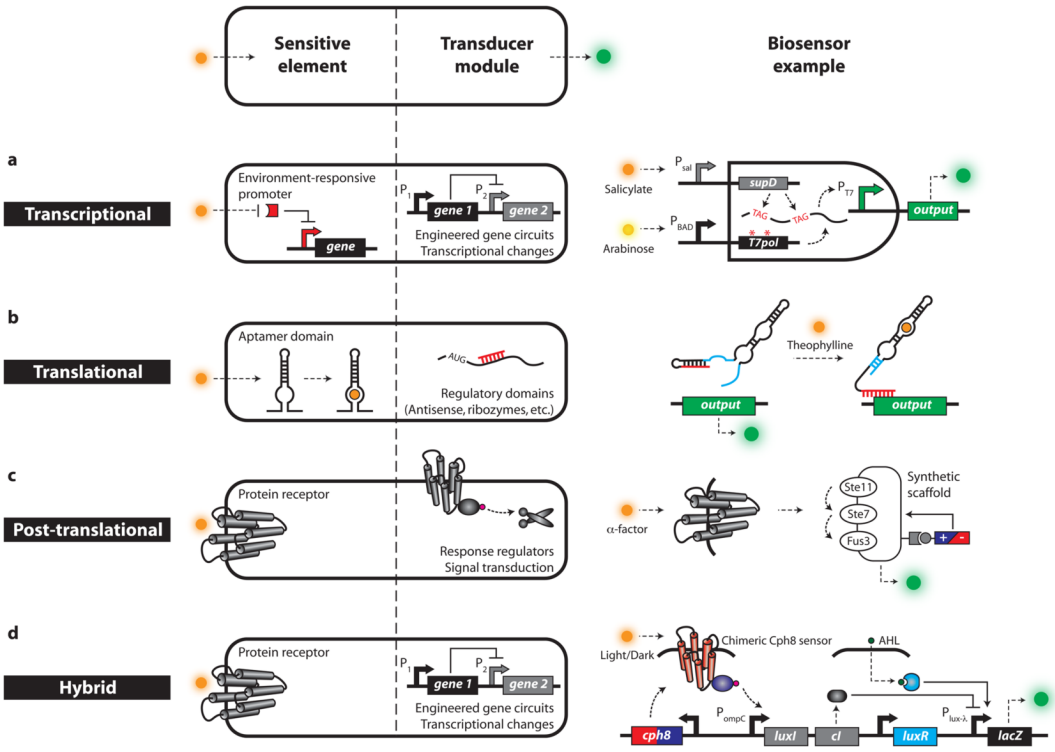

FIGURE 2.

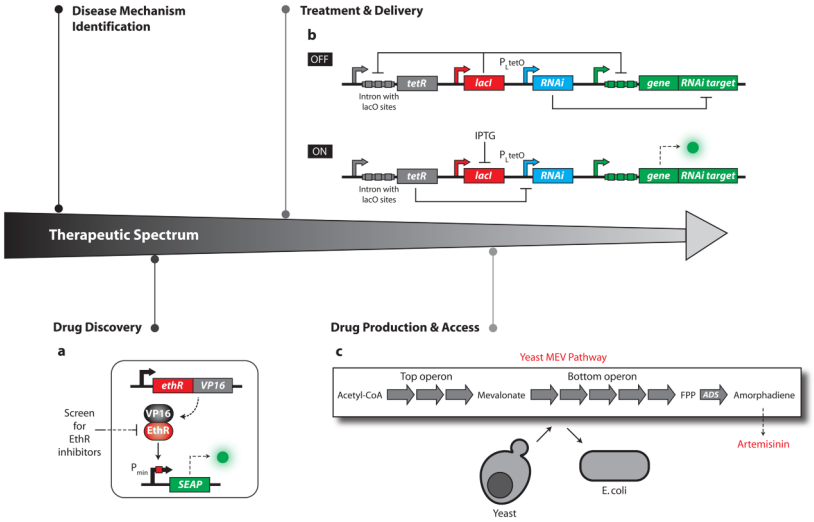

FIGURE 3.

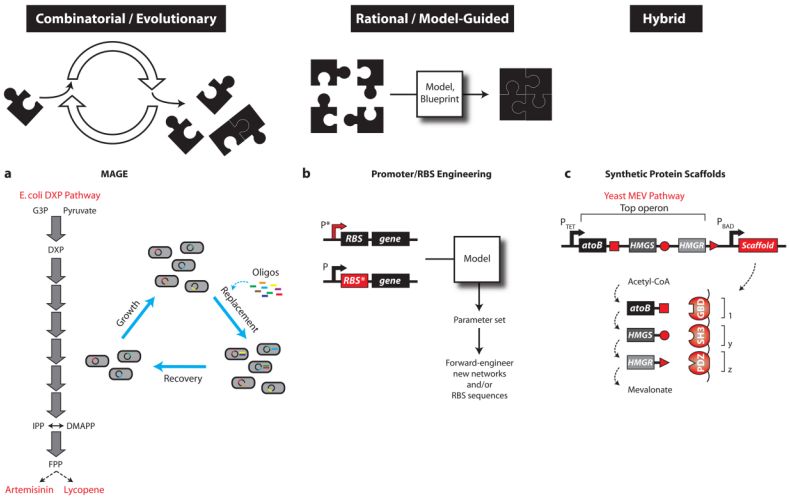

FIGURE 4.