Review

Customizing cell signaling using engineered genetic logic circuits

Baojun Wang[1,2] and Martin Buck[2]

[1] Department of Mathematics, Imperial College London, London, UK
[2] Department of Life Sciences, Imperial College London, London, UK

Cells live in an ever-changing environment and continuously sense, process and react to environmental signals using their inherent signaling and gene regulatory networks. Recently, there have been great advances on rewiring the native cell signaling and gene networks to program cells to sense multiple noncognate signals and integrate them in a logical manner before initiating a desired response. Here, we summarize the current state-of-the-art of engineering synthetic genetic logic circuits to customize cellular signaling behaviors, and discuss their promising applications in biocomputing, environmental, biotechnological and biomedical areas as well as the remaining challenges in this growing field.

Engineering genetic logic gates from cellular components to rewire cell signaling

There is a long-standing interest in the ability of the cell to sense various extracellular signals with extreme specificity, and decide precisely what response should be initiated to adapt to the ever-changing environment. Like an electronic biosensor, cells typically have three interconnected modules to accomplish the task, that is, the input sensors, the internal gene regulatory networks and the output actuators (Figure 1) [1,2]. The input sensors are receptors either embedded in the cell membrane or inside the cytoplasm, which the cell uses to detect what and how much of the input signals are present, e.g., small molecules, heat, light, nucleic acids and antigens, and transduce them into differential gene transcriptional levels or post-translational modifications. Next, the transduced information is processed combinatorially by the downstream gene regulatory network, mimicking the logic circuit in electronic circuitry [3], and integrated logically before an output decision is made. According to the decision, the gene expression machineries are then altered to produce different levels of cognate proteins and biochemicals as the output actuators, resulting in final phenotypic changes in motility, growth, morphology, metabolism and so on.

In this sense, cells can be viewed as replicating living computers but with biological inputs and outputs. The internal gene regulatory networks could be viewed as assemblies of Boolean logic gates which cells use to cascade and integrate multiple environmental and cellular signals [3–5]. Biologists have spent many years analyzing the genetic, biochemical, biophysical and structural biology behind the logic relationships which exist among the myriad of biological components. Now we have a long list of biological components with diverse known functions, particularly enlarged following the sequencing and characterization of many microbial genomes as well as the available vast omics datasets. Thus, from an engineering point of view, these discrete characterized components can, in principle, be assembled into synthetic integrated gene circuitries with degrees of predictable human designed functions. Through such bottom-up circuit construction, not only are we now able to test and improve our understanding of the natural cell signaling and gene networks but we can also extend and modify the behavior of organisms, engineering them to perform new customized tasks, such as detecting toxic pollutants, diagnosing and treating diseases in the long term, producing drugs and chemicals.

As with designing silicon-based electronic circuitry, customized genetic logic circuits can be constructed to link the various cellular sensors and actuators and to program living cells to generate precise desired behaviors in response to specific extra- or intracellular signaling inputs [2,6] (Figure 1). In particular, genetic circuits that can integrate multiple input signals, for example, AND (the output is high only when all inputs are high), NAND (the output is low only when all inputs are high) and NOR (the output is low when either input is high) logic gates, are essential for cells to recognize complex conditions, for example, a disease-specific microenvironment, normally specified by a combination of several signals, to enhance the sensing specificity and increase the accuracy of biological control. To date, several synthetic gene circuits [7–18] have been constructed to perform various digital logic functions (Table 1) and have demonstrated the great potential of using biological logic computational circuits to customize cell signaling for many useful applications. For example, several logic AND gates were constructed to link the inputs to pathogenicity related signal responsive promoters [19], microRNAs [18] or proteins [20] and the output to a therapeutic suicide gene to achieve highly specific *in vivo* targeting and killing of diseased cells. Furthermore, multiple logic gates have been combined to build larger information processing circuits with advanced cellular functions, such as projected image edge detection [16] and robust multicellular biocomputing [21,22].

There is also great recent progress in the area of biomolecular computing which typically applies enzymes [23,24], DNA [25,26] or RNA [27] in an *in vitro* biochemical

Corresponding authors: Wang, B. (b.wang06@imperial.ac.uk); Buck, M. (m.buck@imperial.ac.uk)
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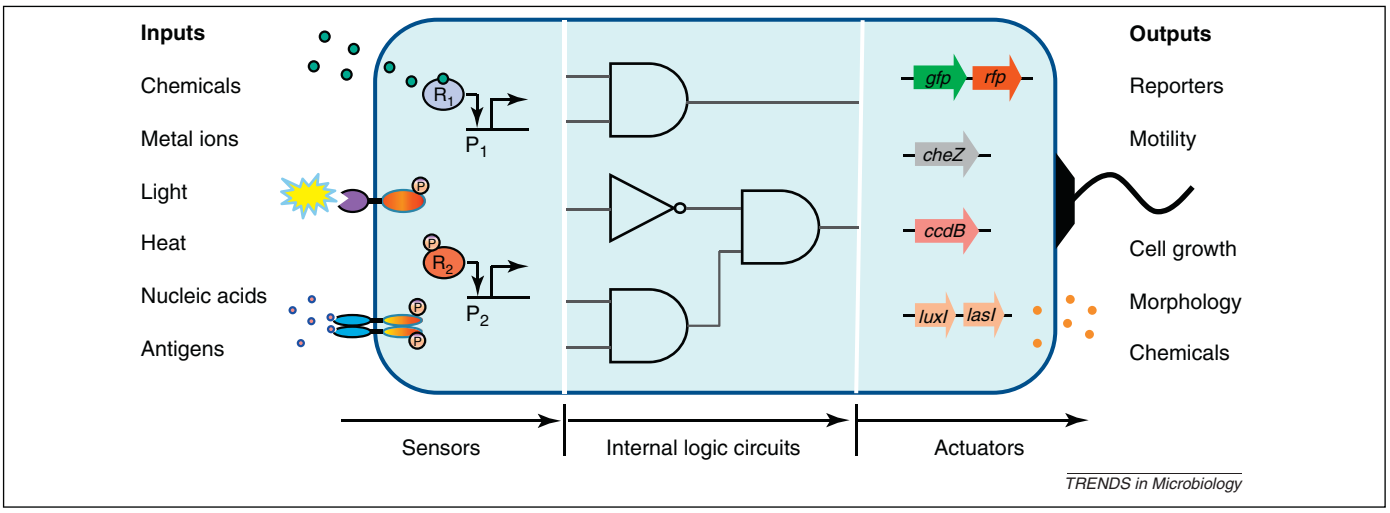

Figure 1. Designing customized cell signaling responses by harnessing the inherent modularity of cell signaling and gene regulatory networks. Genetically engineered cells typically comprises a cascade of three exchangeable modules, that is, the sensors such as sensor kinases or intracellular receptor proteins (R) for detecting surrounding chemicals, nucleic acids, proteins, light, pH and heat, etc., the internal information processing circuits for integrating these multiple input signals to make a logic decision, and finally the output actuators for producing reporters, toxins, chemicals, electrons, motility, growth changes, etc. By designing different synthetic logic circuits to connect the various natural or synthetic sensors and the selected output genes, we can program cells to carry out novel customized tasks.

system to execute complex logic computing tasks. For example, AND gates, OR gates and even a four-bit square root circuit have been constructed from 130 single DNA strands followed by the execution of computing through multilayer reversible DNA strand displacement cascades where both inputs and outputs are DNA [26]. A DNA and restriction enzyme-based *in vitro* diagnostic automaton was constructed to detect multiple biochemical disease indicators where the final positive state only occurs under a predesigned logic combination of the inputs [28]. However, given the vast array of functions available to a living cell, in this review we focus on how to control and rewire signaling responses in living organisms using various engineered genetic logic circuits. In the following sections, we discuss the relevant background, current progress and challenges in this rapidly growing field from three aspects, that is, engineering synthetic genetic logic circuits at transcriptional, RNA or protein levels to program cells to sense various input signals and to execute different novel tasks in a designed logical manner.

Customizing signaling by synthetic transcriptional-based logic circuits

The most common and diverse genetic logic circuits constructed so far are based on gene transcriptional modules. This is not surprising as gene regulation in cells is mostly known at the transcriptional level and now we have a wealth of knowledge on the transcriptional activation and repression modules in many prokaryotes and eukaryotes. Typically, a transcriptional module comprises a transcription factor (TF) protein and a cognate regulatory promoter. The TF binds specifically to its operator sites on the promoter to activate (activator) or inhibit (repressor) its transcription through a series of complex interactions with the promoter bound RNA polymerase, sigma factors and other cofactors such as ATP and DNA looping facilitators [29]. Often, the activities of the TFs themselves are subject to control by other proteins, such as sensor kinases in two component systems, or directly regulated by small molecules. For instance, the repressive effects of the LacI repressor and the tetracycline repressor TetR on the P_{lac} and P_{tet} promoters, respectively, can be relieved by their cognate inducer molecules isopropyl β-D-thiogalactopyranoside (IPTG) and tetracycline [30]. Similarly, activation of P_{luxI} by the LuxR activator is achieved only when the latter is bound to its cognate quorum signaling molecule 3O6HSL [31]. In this sense, a transcriptional module could be thought of simply as a logic switch with discrete on and off states. Hence, activation and repression modules correspond to BUFFER and NOT gates, respectively, that transform a molecular input into increased or decreased gene expression output, with the tightness and cooperativity of the underlying regulation determining the switching characteristics of the logic gates.

By harnessing these diverse transcriptional building blocks, researchers have constructed several genetic Boolean logic gates or circuits (assemblies of logic gates) coupled with various inputs and outputs (Table 1). In one example, a population density or oxygen level dependent cell invader was constructed in *Escherichia coli* simply by using a Boolean BUFFER gate with an input of either the quorum sensing module *luxR*–P_{luxI} or anaerobic *fdhF* promoter and the *inv* gene encoding invasin as the output, resulting in malignant cell invasion under high cell densities or hypoxia [32]. By changing the input to the transcriptional regulator LasR and its P_{lasI} promoter and the outputs to lysis E7 (a self-destruction protein) and pyocin S5 (a *Pseudomonas aeruginosa* bacteriocin), an *E. coli*-based pathogen detector was able to sense and destruct *P. aeruginosa* efficiently [33]. Utilizing a phage lambda *cI*–P_{λ} repression module based NOT gate coupled to a synthetic quorum sensing sensory module, a periodic stripe pattern of high and low densities can be formed autonomously by *E. coli* on a solid agar plate (Figure 2c) by controlling the cell motility with an output *cheZ* gene [34].

Despite the diverse capabilities of systems comprising only single input logic gates, multi-input logic gates, such as AND, NAND and NOR gates, are necessary when a

Table 1. Selected examples of using engineered genetic logic circuits to customize cell signaling responses

Input signals	Logic circuits	Output actuators	Refs
Rewiring signaling by transcriptional-based logic circuits			
Chemicals, AHLs[a], signaling promoters	Two or three input-AND, NAND, NOR gates	*gfp*, *lacZ*, *TK1* (cell death), *phz* operon (electron mediator phenazine)	[7,12,19,21,66]
Light and AHLs	NOT and AND gates	*lacZ* (pigment for light/dark edge detection)	[16]
AHL, oxygen, light	BUFFER gates	Lysis E7 (destruction) and pyocin S5 (toxin), *inv* (invasion), *DsRed* (red fluorescence)	[32,33,70]
AHL (cell density), chemicals, light	NOT gates	*cheZ* (motility controlled stripes), *gfp*, DsRed	[7,34,70]
Chemicals, UV, heat	Toggle switches	*gfp* and *mrfp* (push-on push-off switch), *traA* (biofilm), *FLO1* (yeast flocculation)	[1,8,17,71]
Cell strains	Consortium-based AND gate	*gfp* (consensus output)	[35]
Chemical pulses	Toggle switch and counter	*minC* (cell division), *gfp*	[36,37]
Metal ions (arsenite)	Synchronized oscillator	*gfp* (oscillating biosensor array)	[38]
Rewiring signaling by RNA-based logic circuits			
Chemical ligands	Riboswitch-based BUFFER gates	*gfp*, *cheZ* (chemotaxis), *atzA* (atrazine catabolism)	[41,42]
Chemicals	tRNA-mediated AND gate	*gfp*, *inv* (cell invasion)	[11]
Ligands, antisense RNAs	Ribozyme-mediated AND, NAND, NOR gates	*gfp*	[43,72]
Proteins and chemical	RNA splicing-mediated AND gate	*gfp*, *HSV-TK* (apoptosis)	[20]
microRNAs	RNAi-mediated AND gates	*ZsYellow*, *hBax* (cell suicide)	[18,45]
Rewiring signaling by protein-based logic circuits			
Light, signaling proteins	Chimeric or heterologous protein-based BUFFER gates	*lacZ* (pigment production), controlled protein interaction (morphology), *gfp*, smUOX (urate homeostasis)	[53–55,73]
Ligand peptides	Allosteric protein-based AND gate	Actin polymerization	[52]
Ligand (pheromone)	Scaffold-mediated feedback controller	Altered yeast mating response dynamics	[56]
O-ribosomes, O-mRNAs	Translational AND gate	*gfp*, β-galactosidase (orthogonal translation)	[57,74]

[a]AHLs: acylhomoserine lactones, a family of small molecules used for communication in bacterial quorum sensing, such as 3O6HSL, 3OC12HSL and C4HSL synthesized by LuxI, LasI and RhlI, respectively.

highly specific and accurate response is needed. In fact, any arbitrary complex logic system can be built with universal NAND or NOR gates alone. Bronson *et al.* constructed a three-input AND gate with specific chemicals as inputs and *lacZ* (encoding β-galactosidase) as the output reporter but the device lacked modularity, that is, the inputs are specific and not exchangeable [12]. Other non-modular logic AND gates were also built using hybrid promoters embedded with multiple repressor (TetR, LacI, CI) or activator (LuxR) binding sites [13,14]. To solve the problems of modularity (having exchangeable inputs and outputs to increase the reusability) and orthogonality (no crosstalk with the host genetic context to increase the robustness and stability) associated with these logic gates, logic AND, NOT and NAND gates have recently been engineered (Figure 2a), and these gates are both modular and orthogonal to the host *E. coli* chassis. The two-input AND gate comprises two heterologous genes, *hrpR* and *hrpS*, and one σ^{54}-dependent output promoter, *hrpL*, from *Pseudomonas syringae* [7]. *hrpR* and *hrpS* encode regulatory enhancer binding proteins that act synergistically to coactivate the tightly regulated *hrpL* promoter. Both the inputs and output of these gates were designed to be promoters to facilitate their connection to different upstream and downstream transcriptional modules. Owing to modularity, the inputs can be rewired to different input sensors and the output can be used to drive various cellular responses. Furthermore, these gates were forward-engineered from well-characterized components in various contexts with predictable behaviors and were shown to behave robustly in different cellular backgrounds. In one example, inspired by the yeast two-hybrid system, a modular transcriptional-based AND gate was constructed in mammalian cells using selected inherent disease response-related promoters as the inputs and the TK1 killer gene as the output to achieve highly specific identification and killing of diseased tumor cells *in vivo* [19]. A cell consortium-based AND gate was also constructed using two mutually activating cell populations as the inputs [35]. The two cell strains each produce a different quorum sensing signal required for the output activation of the other. Thus, a fluorescent output was seen only when the two populations were cultured together to high cell densities, illustrating the combinatorial logic character of the system.

Whereas single logic gates enable cells to integrate multiple input signals, much higher-level tasks can be achieved by the combination of multiple logic gates in one genetic circuit. An elegant example is the bacteria-based dark/light edge detection program built by combining a synthetic dark light sensor (Cph8) to one NOT and AND gate pair with *lacZ* as the output (Figure 2b) [16]. Several other versatile logic systems have been engineered employing biostable toggle switches by either interfacing to the native signaling network or customized input signals of the host. For example, Kobayashi *et al.* have coupled an engineered genetic toggle switch to the native SOS signaling pathway of the cell to induce biofilm formation in response to DNA-damaging agents such as UV [1]. Ellis *et al.* used a similar toggle switch circuit, but operated it in a monostable state, to construct a genetic timer to control

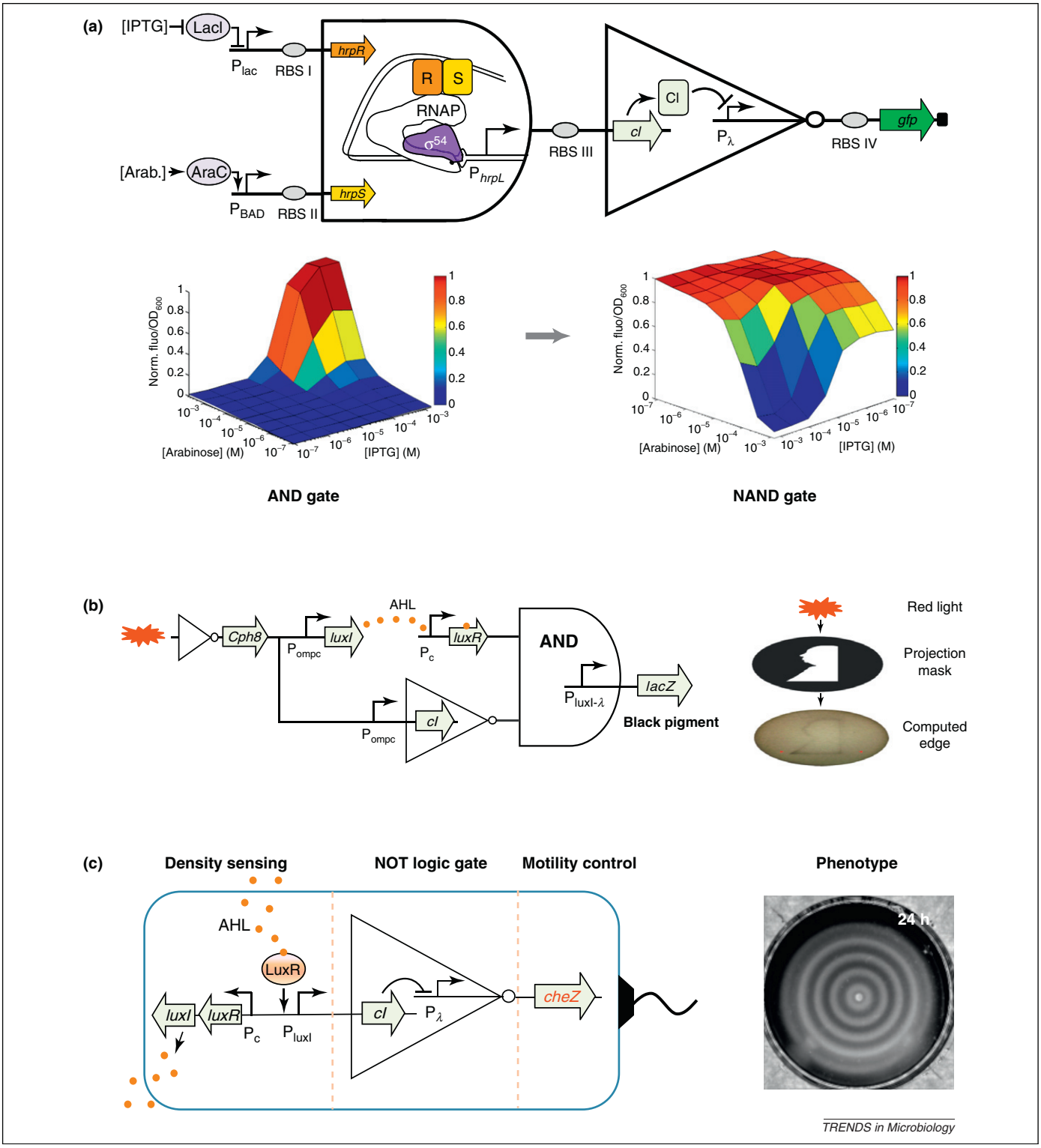

Figure 2. Using engineered transcriptional-based genetic logic gates to program cellular behaviors. **(a)** The modular and orthogonal genetic AND, NOT gates and the composite NAND gate were implemented in *Escherichia coli*. The AND gate is designed harnessing the σ^{54}-dependent HrpR/HrpS heteroregulation module in the *hrp* regulatory system of the *Pseudomonas syringae* plant pathogen. The output *hrpL* promoter is activated only when both input genes are expressed under the two environment-responsive promoters (P_{lac} and P_{BAD}). The NAND gate is assembled by directly coupling the modular *cI*–P_λ based NOT gate to the AND gate. The devices were fully characterized with robust digital-like logic functions in various contexts. The bottom are characterized responses of the AND and NAND gates under various combinations of the two input signals. Adapted from [7]. **(b)** The bacteria-based dark/light edge detection program was created by combining a synthetic dark sensor (Cph8) to one NOT gate and one AND gate with *lacZ* (encoding β-galactosidase) as the output. The imposed red light inhibits Cph8 activation on the P_{ompc} promoters. Thus, only cells in the dark can synthesize acylhomoserine lactones (AHLs) via *luxI* and, because of the lamda dominated repression system, only cells in the light will have output from the hybrid promoter ($P_{luxI-\lambda}$). As a result, only the cells in the light that are closest to the dark region will produce black pigment, due to the sensing of diffusible AHLs secreted by the cells in the dark, that is, at the dark/light edge. Adapted from [16]. **(c)** A sequential stripe pattern of high and low cell densities was autonomously established on an agar plate by *E. coli*, seeded initially in the plate center, by connecting a cell density sensory module (based on the quorum sensing *luxRI* system) to the input of a NOT gate and the output to the motility control gene *cheZ*. The periodicity of the stripe can be adjusted simply by varying the copy number of the CI repressor. Adapted from [34]. Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; Arab., arabinose; RBS, ribosome binding site.

yeast sedimentation by connecting the output to the native *FLO1* gene promoter [17]. By rewiring the toggle switch output to the cell division-related *minC* gene, a chemical pulse nanorecorder was built in *E. coli* which can record if the host has been exposed to a certain chemical inducer, with elongation size being proportional to the exposed length of the chemical pulse [36]. Other complex tasks were also demonstrated using programmed logic circuits in *E. coli*, such as a cellular event counter [37] and the recent arsenic-modulated cell population oscillator with long-range synchronization [38]. Hence, the myriad transcriptional regulatory modules in many prokaryotes and eukaryotes provide the research community with an extremely rich resource for characterizing and harnessing their specialized functions to design synthetic genetic logic circuits that can tailor cell signaling behavior for various prescribed applications.

Customizing signaling by synthetic RNA-based logic circuits

In the past few years, there has been a growing trend of using RNA-based post-transcriptional regulation to control gene expression within genetic circuit construction to program cellular behaviors. This is largely due to the fact that there have been an increase in RNA-mediated gene regulation mechanisms and components found in nature as well as the relative ease in predicting and manipulating the secondary structure of RNAs to impose customized regulatory functions on them. In gene circuit engineering, RNA-mediated regulation currently employs three major mechanisms of RNA regulation – *cis*-acting RNA structure related modulation of translation, catalytic RNA or ribozyme-mediated cleavage of target transcripts and *trans*-acting antisense small RNAs-mediated regulation of translation [39]. Both negative and positive regulation can be generated from these mechanisms and the activities are often subject to the regulation of other environmental signals such as small molecules, nucleic acids and proteins. For example, a strong hairpin structure at the 5′ end of an mRNA can block the access of the ribosome to the ribosome binding site to initiate translation [40], whereas a ribozyme cleavage can lead to fast degradation of the target transcripts by RNase. In higher eukaryotes, RNA interference (RNAi) pathways employ microRNAs to silence their target genes.

Several representative pieces of works have already shown that these RNA regulatory components can be applied to engineer genetic logic gates to program various cellular behaviors. In one example, a small molecule responsive riboswitch has been developed [41], which included aptamer domains in the mRNA 5′ untranslated region (UTR) to control translation initiation in a ligand-dependent manner. The specific binding of the ligand to the aptamer domain of the riboswitch induces a conformational change in the 5′ UTR of its own mRNA, thereby regulating gene translation. The selected theophylline-dependent riboswitch can control chemotactic *cheZ* gene expression in *E. coli* and guided the bacteria to move towards the theophyl-

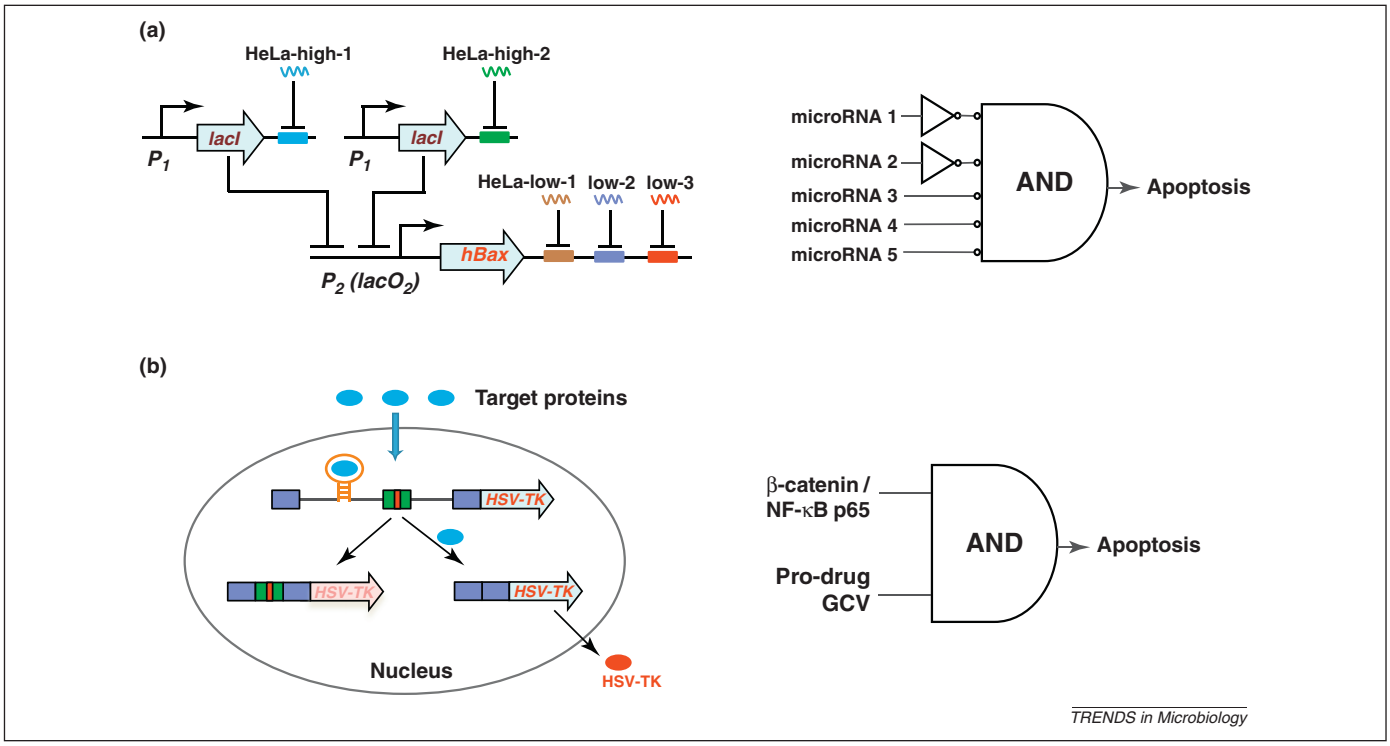

Figure 3. Controlling cell signaling using RNA-based genetic logic circuits. **(a)** A multi-input RNA interference (RNAi)-based AND logic circuit was designed to selectively identify and kill cervical cancer cells. The AND logic gate is able to detect five selected microRNA (colored squiggles) characteristics of the HeLa cervical cancer cell line and produce a toxic protein to kill the classified cells under the predefined logic profile of the five input microRNAs (three low and two high). *lacI*/*P2*(*lacO2*) inverter module is used for detecting the high profile of two microRNAs and *hBax* is the output suicide gene. Adapted from [18]. **(b)** A protein-responsive alternative RNA splicing-mediated AND logic device was constructed to detect protein biomarkers and induce apoptosis in human kidney cells. Disease signaling pathway protein, such as β-catenin and nuclear factor-κB (NF-κB) p65, responsive aptamers was embedded in the 5′ introns of the output pre-mRNA containing two introns and three exons and the gene *HSV-TK*. The binding of the target protein to the aptamer alters the slicing pattern of the output mRNAs, resulting in high level gene expression (HSV-TK expressed) compared with nonbinding state (HSV-TK not expressed due to a stop codon within the middle alternatively spliced exon). HSV-TK triggers cell apoptosis when the prodrug ganciclovir (GCV) is also present. When both the target protein and the prodrug inputs rise to threshold levels, the host cell is programmed to commit suicide. Adapted from [20].

line signal in a synthetic gradient-dependent way. Subsequently, this group selected out an apatamer-mediated riboswitch responsive to the pesticide atrazine and engineered cells to seek atrazine on an agar plate and then destroy it with the atrazine catabolism gene *atzA* as the output [42]. In another example, a modular two input AND gate has been constructed by including amber stop codons into the T7 RNA polymerase gene under an inducible promoter [11]. Thereby, the transcribed T7 mRNAs are not translated to polymerase until the nonsense amber codon suppressor tRNA *supD* is also transcribed from another inducible promoter. The AND gate was connected to *inv* to trigger cellular invasion under two inducible inputs. Higher level signal processing RNA logic devices such as AND, NAND and NOR gates were also demonstrated using ligand-responsive ribozyme-mediated cleavage in the 3′ UTR of the target output gene [43]. Subsequently, a theophylline-responsive ribozyme-based switch was engineered by the same group to regulate the expression of cytokines for controlling T cell proliferation in mice [44].

More recently, small regulatory microRNAs were exploited to control the transcription of the mRNA of interest via an RNAi pathway in a programmable logical manner in mammalian cells [45]. Figure 3a shows a proof-of-concept application of a logic AND gate constructed on the basis of this mechanism [18]. The researchers selected five specific microRNAs from the microarray databases to identify the cervical cancer cell line and designed a classifier AND logic circuit to integrate the profiles of these five inputs. Usually, microRNAs bind their corresponding microRNA target sequences in the 3′ UTR of the output gene transcript to cause its degradation [46]. Three microRNA target sequences were placed in the 3′ UTR of the output suicide gene *hBax* to detect their low level states in the cell. A double-inversion circuit was then constructed to detect the high level states of the other two microRNAs. Hence, the output gene will not trigger apoptosis until the predefined AND logic (three low and two high) combination of the five microRNAs appears in a single cell. This five-input AND logic gate greatly enhances the specificity and accuracy of biological sensing and control and holds great promise for applications in molecular therapy. Figure 3b shows another elegant example of using RNA-based logic circuits to detect protein biomarkers and then trigger apoptosis in human cells. By putting the protein-responsive aptamer in the specific intron regions of the target pre-mRNA, the splicing pattern of the output mRNA can be altered according to the intracellular protein levels, resulting in changes of the output gene expression [20]. The researchers designed disease pathway related β-catenin and nuclear factor-κB (NF-κB) responsive AND logic devices that trigger apoptosis together with a prodrug as the second input (Figure 3b). Thus, versatile genetic logic systems can be engineered to rewire cell signaling and sensing by exploiting the diverse regulatory mechanisms that RNA has in gene regulation, especially so in eukaryotes, and hence poses very promising applications in disease diagnosis and treatment.

Customizing signaling by synthetic protein-based logic circuits

Owing to the myriad activities and functionalities of proteins in cells, researchers have also long been engineering proteins to render them with various customized characteristics. For example, both directed evolution and structure-based rational design approaches are applied to modify enzymes [47], scaffold [48] and regulatory proteins [49] to either increase reaction rates in metabolic pathways or respond to new input signals. Nevertheless, it is much more challenging to predictably engineer protein-based logic circuits as compared with transcriptional- and RNA-based ones. This is largely due to the complex nature of protein folding and tertiary structures as well as the less independent characteristics of the amino acids within proteins [50]. Yet, the inherent modularity of many allosteric signaling and transcriptional activator proteins indicates that we might be able to alter their regulatory input and actuator output specificities through rational homologous or heterologous domain recombination [51,52]. Although it has not become a routine to build protein circuits to rewire cell signaling, several encouraging examples have demonstrated the feasibility and great promise of using synthetic protein based logic gates to program cellular behaviors. To date, various signaling and regulatory proteins have been modified by domain combination, swapping or evolution to render them responsive to novel input signals or able to act on noncognate target substrates.

In a proof-of-principle bacterial photographing system, a synthetic light sensitive chimeric protein switch was made in *E. coli* by fusing the photoreceptor domain of the phytochrome Cph1 protein from the cyanobacterium *Synechocystis* to the intracellular signal transduction domain of the *E. coli* EnvZ kinase [53]. The engineered bacteria have a *lacZ* reporter fused to the response regulator OmpR regulated promoter expressing β-galactosidase and turning the media black when exposed to red light. A synthetic light-controlled reversible protein–protein interaction device was also demonstrated to be able to recruit cytoskeleton related proteins to the cellular membrane and thus spatiotemporally reshape cell morphology [54]. Skerker *et al.* showed that the specificity of a histidine kinase to its response regulator in a two-component system (TCS) can be rewired by rationally mutating several amino acids in the DHp domain of the kinase [55]. They established several cases where the *E. coli* EnvZ kinase can be rationally altered to phosphorylate other noncognate response regulators, such as CpxR and PhoP, instead of the cognate OmpR both *in vitro* and *in vivo*. This work indicates the possibility that we may rewire the native cellular TCS signaling pathway by rationally designing the signaling kinase to act on a novel response regulator and thus initiate a programmed response.

In one study, Dueber *et al.* constructed synthetic protein gates to integrate two non-native physiological signals through modular domain recombination and reorganization of the allosteric N-WASP (neuronal Wiskott–Aldrich syndrome protein) [52]. The autoinhibitory N-WASP functions as a signaling switch for actin polymerization in cells, and responds to two native signals corresponding to the two input inhibitory domains. Based on the modular architecture of this allosteric signaling protein, two heterologous input domains (PDZ and SH3) were fused to the

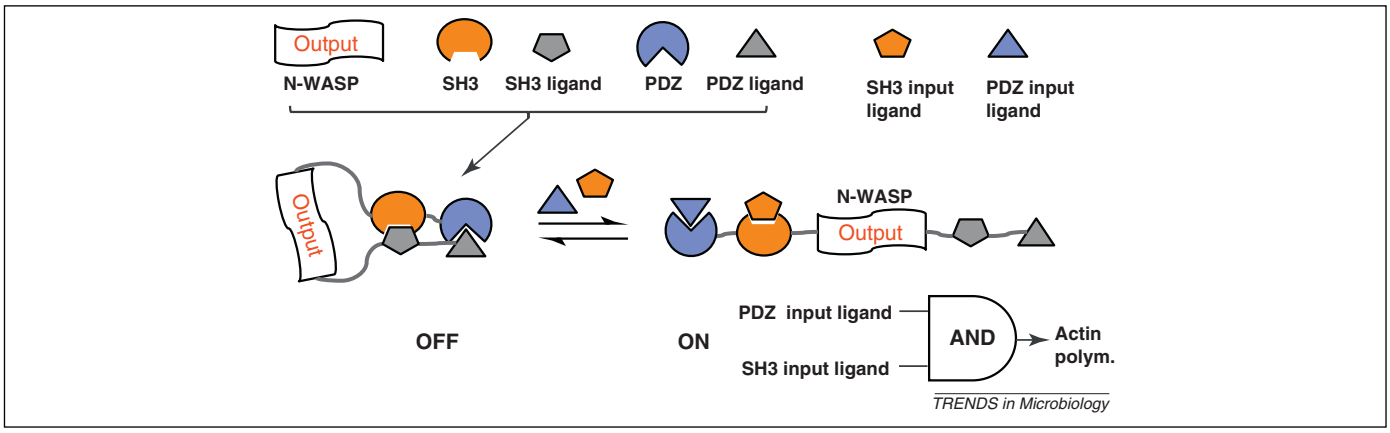

Figure 4. Rewiring cell signaling using synthetic protein-based logic circuits. A synthetic protein logic gate was constructed to integrate two nonphysiological input signals by modular domain recombination. The researchers engineered variants of the allosteric actin regulatory protein N-WASP by combing two heterologous autoinhibitory input domains (PDZ and SH3) with the output domain of N-WASP. The autoinhibition can be released by two competing ligand peptides that are cognate to the two input domains to trigger actin polymerization in a *Xenopus oocyte* extract. By varying the locations of the two input domains as well as their intramolecular ligand binding affinities, different output gating behaviors have been obtained including AND and OR gates. Adapted from [52].

constitutive output domain of N-WASP to mimic the wild-type autoinhibitory state (Figure 4). However, when both the cognate ligand peptides to PDZ and SH3 are added, the output domain inhibitory effect was released to trigger actin polymerization, that is, a protein AND logic gate. By changing the positions and gaps between the three domains, other types of gating behaviors were also generated. This work shows that simple modular domain recombination can generate functional synthetic protein gates to rewire cell signaling and a modular framework will facilitate the evolution of these gates.

In another study, Bashor *et al.* constructed a synthetic scaffold protein device to systematically reshape response dynamics of the yeast mating MAPK (mitogen-activated protein kinase) pathway [56]. The Ste5 scaffold protein in the pathway was engineered with an artificial leucine zipper binding domain to be able to recruit a positive or negative pathway modulator to the native signal transduction process. With this synthetic feedback loop, diverse signaling dynamics such as ultrasensitive, accelerated and delayed mating responses were generated using different combinations of feedback strengths and binding decoys. Rackham and Chin [57] designed orthogonal ribosome–mRNA pairs by evolving the natural ribosome (mutating the 16S rRNA sequence) to new versions with altered mRNA-binding sites (orthogonal ribosomes) which exclusively translate the cognate mRNAs that are not substrates for the endogenous ribosome (orthogonal mRNAs). Two mutually orthogonal pairs, orthogonal as well as to the endogenous pair, were found and used in a circuit to generate AND cellular logic behavior in *E. coli* with the orthogonal ribosomes as the inputs and β-galactosidase as the output. Clearly, regulatory and signaling proteins provide us with an extra layer with which to customize cell signaling by designing various synthetic protein-based logic gates and regulatory pathways through rational protein domain combinations and domain swapping as well as through directed evolution.

Concluding remarks and future directions

Synthetic genetic logic circuits hold great promise to rewire cell signaling and gene networks to engineer designer organisms for carrying out human designed functions. Many proof-of-principle examples have been showcased by the pioneers in this field and demonstrated basic design principles and potential applications of genetic logic circuits. Starting from the transcriptional-based logic circuits to the more recent RNA and protein-based logic circuits, different layers and methods of gene regulation have been employed to control gene expression and to build various functional cellular logic circuits that can sense multi-input cognate or noncognate cellular signals, integrate them in a combinatorial logical manner and then launch desired cellular actuations. The roles of these circuits are diverse: ranging from monitoring environmental signals as biosensors, detecting disease indicators and triggering autonomous treatment to controlling metabolic pathways to produce high value chemicals in an industrial fermentor.

To realize the full potential of customized genetic logic circuits, however, several important challenges need to be addressed in this field. Here, we outline the key questions to encapsulate the many hurdles during the whole cycle of gene circuit design, construction, measurement, modeling and delivery (Box 1). In particular, the current scale of functional circuits is small and usually contains no more than two or three logic gates with few regulatory components [58]. This is partly because there are only a limited number of orthogonal building blocks in the current toolkit of circuit engineering, preventing us from building large-scale circuits with complex logic functions. Yet, to construct even this type of small circuits, it is still often a trial-and-error process with much tweaking and many rounds of fine-tuning. Circuit designs often lack sufficient performance predictability at present, even though the component parts are well characterized individually. In addition, the circuits are often characterized with limited parameters only in a selected context. We do not know how they would behave even in a slightly different context (both abiotic and biotic). The unpredictability is possibly due, in a large part, to the noise and context dependent effect pertaining to biochemical components. Genetically identical cells in the same environment show variable phenotypes, attributed in part to stochastic gene expression [59]. Variability in messenger RNA and protein levels between cells will for

Box 1. Outstanding questions

- How can we rapidly diversify current circuit building blocks to solve orthogonality and scale-up circuit design for more complex applications?
- How can the context-dependent variability of biological components be controlled or harnessed in circuit construction to circumvent noise propagation and unpredictability?
- How can we assay *in situ* the many circuit and cellular properties (e.g., mRNAs, proteins and metabolites) in parallel with both high temporal and spatial resolution?
- How can we reduce the DNA cloning effort and increase circuit building efficiency through novel fast and low cost gene synthesis, assembly and screening techniques?
- What type of models can be constructed to abstract discrete component characteristics and to automate the whole circuit design process with predictability?
- How can we best interface synthetic circuits with the host native signaling and gene networks without unintended crosstalk and overburdening, and also interface with the accessible electronic world?
- How can synthetic circuits and organisms be stably maintained in the delivered context with minimized risk of unintended evolution?

some applications of synthetic circuits place a constraint on their usefulness, but may be advantageous in other contexts where greater 'physiological space' is explored by the cell or microorganism. Clearly, being able to predict and manipulate gene expression noise and fully understanding its origins is crucial to refine circuit design and application.

Therefore, new effort and methods, for example, directed evolution [60,61] or harnessing genetically distinct natural modules [7,62], are required to rapidly diversify our limited toolbox including new orthogonal transcriptional modules, RNA regulators and protein switches. In particular, complexly regulated promoters with multiple control inputs in nature provide a rich resource from which to engineer multi-input orthogonal logic gates [63,64]. New sensors and actuators are also needed to better interface the cells with our real world, for example, sensors that are sensitive to electricity [65] and actuators that can generate direct digital output such as electric current [66]. New design principles and strategies that can integrate multilayer gene regulation need to be developed to increase the predictability and scale of circuit construction including new modeling paradigms [67]. Other supporting technologies and platforms, such as high throughput and low cost gene synthesis, efficient DNA assembly standards and methods (e.g., the BioBrick [68] and Gibson assembly [69]), phenotype screening and characterization, will be of great value to facilitate the next generation of synthetic gene circuits to achieve advanced cellular functions for biocomputing, bioremediation, biomanufacturing and biotherapy.

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