Synthesizing Biomolecule-Based Boolean Logic Gates

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ABSTRACT: One fascinating recent avenue of study in the field of synthetic biology is the creation of biomolecule-based computers. The main components of a computing device consist of an arithmetic logic unit, the control unit, memory, and the input and output devices. Boolean logic gates are at the core of the operational machinery of these parts, and hence to make biocomputers a reality, biomolecular logic gates become a necessity. Indeed, with the advent of more sophisticated biological tools, both nucleic acid- and protein-based logic systems have been generated. These devices function in the context of either test tubes or living cells and yield highly specific outputs given a set of inputs. In this review, we discuss various types of biomolecular logic gates that have been synthesized, with particular emphasis on recent developments that promise increased complexity of logic gate circuitry, improved computational speed, and potential clinical applications.

KEYWORDS: synthetic biology, Boolean logic gate, biomolecular devices, chemically inducible dimerization, biocomputers, circuit design
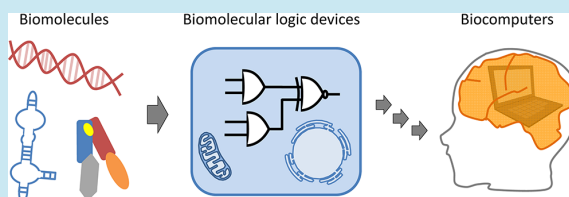
A cell is a sophisticated device that performs three elaborate functions: sensing inputs, processing the input information for decision-making, and executing the outputs. To this end, cells have built-in sensors that can receive the input signals generated by various environmental factors.[1−3] Specifically, the plasma membrane and its integrated receptors can sense pressure, osmotic stress, intracellular contact, temperature, and chemicals. At the same time reactive oxygen species, pH, nutrients, signaling factors, and other indicators of internal state are registered by internal receptors. Varying degrees of a single environmental input or a combination of many of them is presented to the cell at any given time, giving rise to a large array of input information sets. Cells continuously process this multitude of input signals to make decisions about their appropriate responses that lead to changes in gene expression, enzymatic activity, and rewiring of their signaling networks. This decision-making process manifests itself in the form of migration, growth, or division, as well as programmed cell death as the output information. Because this physiological cellular behavior is similar to information processing in a computing device, in the field of synthetic biology, engineering principles have been applied to study fundamental biological components.[4,5]

In a computing device, the input information is mathematically processed into a digital signal. This signal is a code representation of the physical cues and assumes a sequence of discrete values. For instance, in the case of a binary code, the basic unit of information is denoted as a series of "0" and "1" digits. The binary digits indicate the two states of the logic circuit. A threshold is implemented to define the input and output range that can be categorized under each logic set. If the value is either lower or higher than the threshold, the state of

the circuit is defined as either "0" or "1", respectively. Digital circuits make extensive use of logic elements that are interconnected to create logic gates, capable of executing Boolean logic functions including NOT, OR, AND, and all their possible combinations (Figure 1A). In these gates, the sensors read out inputs, and then a computational core assigns them a value of either "0" or "1" depending on the threshold set in place. If the combination of these values meets the system requirements (i.e., in case of an AND gate, if the two different inputs are both "1"), the output is executed. Each gate can be defined by conventional symbols or a truth table (Figure 1A). One remarkable property of these modular logic gates is the potential to network them together to make more complex circuits, making it possible to build integrated circuits that can process versatile inputs.

When these concepts are applied to a living cell, the values of the thresholds of input and output must be defined in a rigorous manner.[5,6] Depending on the precise system used, each set of these "input" and "output" threshold values can be based on the concentration, enzymatic activities, or localization of biomolecules. For example, if two different biomolecules, A and B, can induce apoptosis via the production of biomolecule C, it may be possible to create artificial logic gates that can regulate the induction of apoptosis (Figure 1B). In this device, what sets the threshold values of inputs A and B and output C is defined by the physiological roles of these molecules (concentration, enzymatic activity, etc.). To create an AND gate, the following condition is a prerequisite: if the values of both A and B are above the defined threshold, C is produced,

A

YES gate

INPUT	OUTPUT
0	0
1	1

NOT gate

INPUT	OUTPUT
0	1
1	0

AND gate

INPUT		OUTPUT
A	B	
0	0	0
0	1	0
1	0	0
1	1	1

NAND gate

INPUT		OUTPUT
A	B	
0	0	1
0	1	1
1	0	1
1	1	0

OR gate

INPUT		OUTPUT
A	B	
0	0	0
0	1	1
1	0	1
1	1	1

NOR gate

INPUT		OUTPUT
A	B	
0	0	1
0	1	0
1	0	0
1	1	0

XOR gate

INPUT		OUTPUT
A	B	
0	0	0
0	1	1
1	0	1
1	1	0

XNOR gate

INPUT		OUTPUT
A	B	
0	0	1
0	1	0
1	0	0
1	1	1

IMPLY (A IMPLY B)

INPUT		OUTPUT
A	B	
0	0	1
0	1	1
1	0	0
1	1	1

N-IMPLY (B N-IMPLY A)

INPUT		OUTPUT
A	B	
0	0	0
0	1	1
1	0	0
1	1	0

B

A — Gene Expression — C → Apoptosis
B —

INPUT		OUTPUT	
A	B	A•B = C	
0	0	0	→ No Apoptosis
0	1	0	→ No Apoptosis
1	0	0	→ No Apoptosis
1	1	1	→ Apoptosis

A — Gene Expression — C → Apoptosis
B —

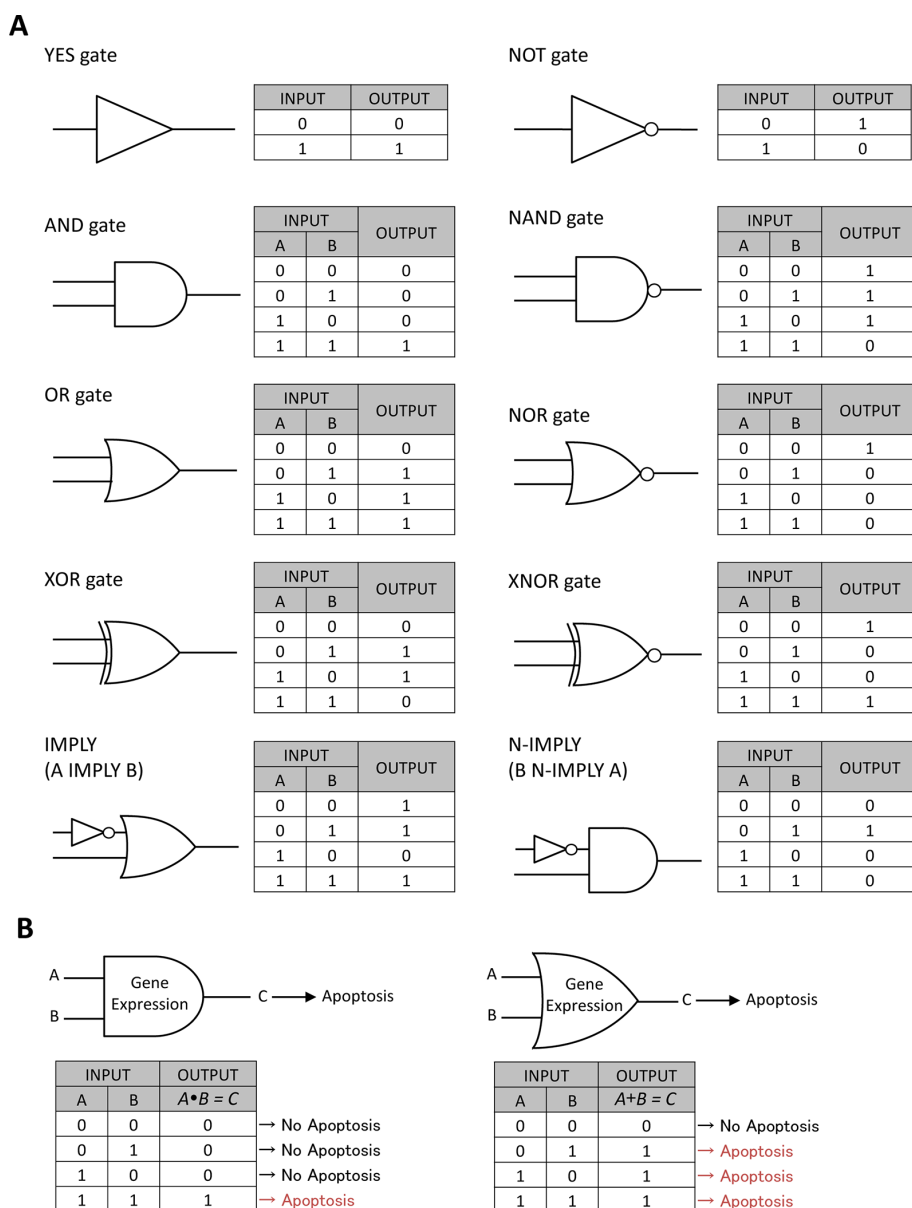| INPUT		OUTPUT	
A	B	A+B = C	
0	0	0	→ No Apoptosis
0	1	1	→ Apoptosis
1	0	1	→ Apoptosis
1	1	1	→ Apoptosis

Figure 1. (A) Traditional symbols and truth table of Boolean logic gates are shown. When the information is sensed or released from the gate, the value is defined as "1". If not, the value is "0". (B) Schematic diagram of apoptosis-controlling device by two-input Boolean logic gates.

and consequently apoptosis is induced. Therefore, only in the case where both inputs are "1" will the output be "1" and apoptosis will occur (Figure 1B, left). In contrast, if either A or B is sufficient to produce C, it is possible to create an apoptosis-inducing device controlled by an OR gate (Figure 1B, right). In reality, the regulatory mechanisms of cellular functions such as apoptosis are far more complex. Therefore for a comprehensive manipulation of cellular functions by an artificial device, it is imperative to control each cellular function via fine-tuning of its constituent logic components.

One of the long-term goals of synthetic biology is the ability to reconstruct the decision-making networks in order to implement them as logic gates in living cells. With such a coveted technology, damaged DNA that may cause tumorigenesis could be repaired immediately in suitably engineered cells. Other applications are in the production of chemicals, biofuels, and food, where engineered eukaryotic cells or bacteria can be used to generate these products more efficiently. Notably, to fulfill their physiological roles, cells such as Purkinje neurons[7] and proteins such as inositol triphosphate receptors[8] or N-WASP,[9] in their native form, all operate as logic gates that assume binary inputs. Therefore, construction of logic gates will help not only in controlling cellular functions but also to enhance our understanding of functional components within cells. In this review we discuss the use of biomolecule-based logic gates in cell-based and cell-free systems (Figure 2) as it
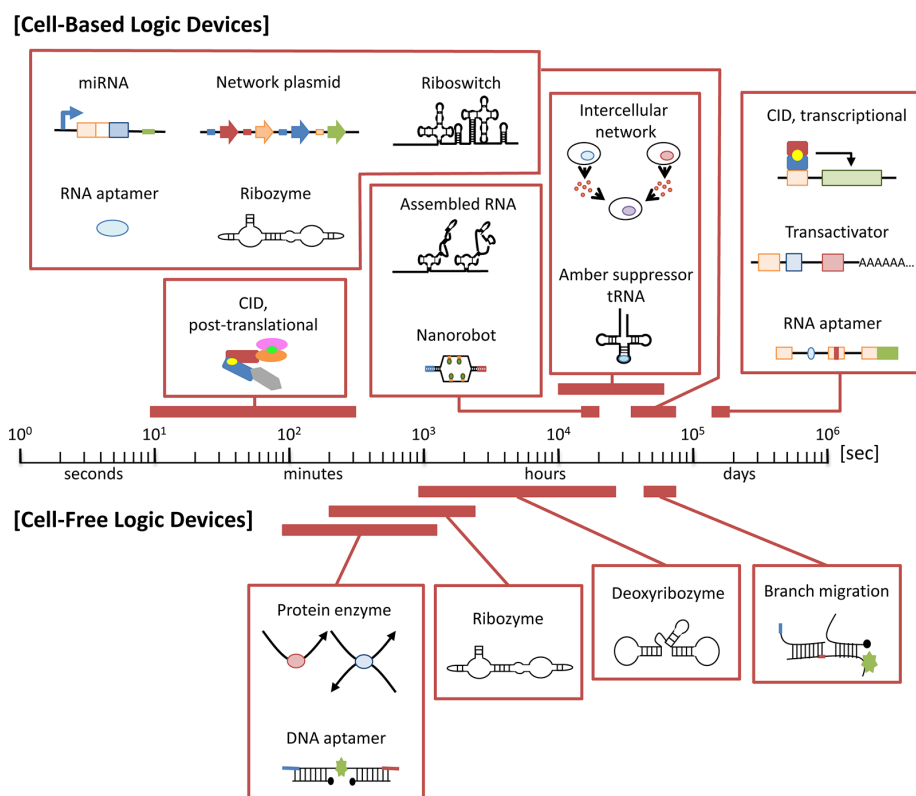
Figure 2. Representative time-scale of the activation time of cell-based and cell-free biomolecule-based logic devices.

applies to computational devices' design. We introduce the *in vitro* biomolecule-based logic devices in sections 1 and 2, and the *in vivo* ones are presented in sections 3 and 4.

1. NUCLEIC ACID-BASED COMPUTATION IN CELL-FREE SYSTEMS

Nucleic acids are biomolecules consisting of sequential phosphodiester-bonded nucleotides that form DNA or RNA, which carry genetic information in cells. These molecules are highly stable and can be easily synthesized to form polynucleotides capable of functioning as molecular cues in information processing. These, together with the inherently predictable base-pairing scheme of such nucleotides, make them an attractive target for many researchers who use nucleic acids as engineering building blocks to create artificial biochemical circuits. Nucleic acid-based computation can be divided into enzyme-based and enzyme-free platforms (Figure 3).

1.1. Nucleic Acid Enzyme-Based Computation Systems. In enzyme-based computational systems, two nucleic acid enzymes, ribozyme (an RNA-based enzyme) and deoxyribozyme (a DNA-based enzyme), are used to design biochemical circuits. Kruger et al. first described ribozyme in 1982 as a naturally occurring RNA catalyst.[10] Subsequently, in 1990 and 1994, Robertson et al. and Breaker et al. identified artificial ribozymes and DNA catalysts known as deoxyribozymes, respectively.[11] To date, many ribozymes and deoxyribozymes have been synthesized in the laboratory, with ribozymes being the only naturally occurring molecule of the two. The potential and versatility of these molecules in

construction of nucleic acid enzyme-based logic gates have been exploited by many groups. For example, Stojanovic et al. reported the design of a deoxyribozyme-based logic gate *in vitro*.[12] Two deoxyribozymes, E6 or 8-17, were used for information processing of an oligonucleotide input and for constructing YES, NOT, AND, and XOR Boolean logic gates (Figure 3A). Similar to the deoxyribozyme-based logic device, ribozymes have also been utilized for *in vitro* computation. For example, allosteric hammerhead ribozyme-based logic gates that process oligonucleotide inputs have been created[13] (Figure 3B).

These enzyme-based devices can constitute relatively fast processing devices (Figure 2). However a major drawback inherent in these devices is that scaling up their network topology causes a major problem: mutual interference between the oligonucleotides that make up the device. Solving this issue by fine-tuning the device should lead to the construction of more complex logic circuits. Indeed, Stojanovic and Stefanovic created a deoxyribozyme-based network of 23 logic gates to encode the game of tic-tac-toe, capable of interactively competing with a human opponent.[14]

1.2. Nucleic Acid Enzyme-Free Computation Systems. For the case of enzyme-free computation, instead of the nucleic acid-based enzymes, Watson–Crick base pairing hybridization rules are exploited. Seelig et al. succeeded in making basic Boolean logic gates (AND, OR, and NOT gate) by using a branch migration scheme.[15] In this system, sequential base pairing triggered by toehold–toehold binding between single strands and subsequent breaks creates a gate function (Figure 3C). Using a similar principle, Qian and Winfree created a "seesaw" gate in which a strictly defined number of
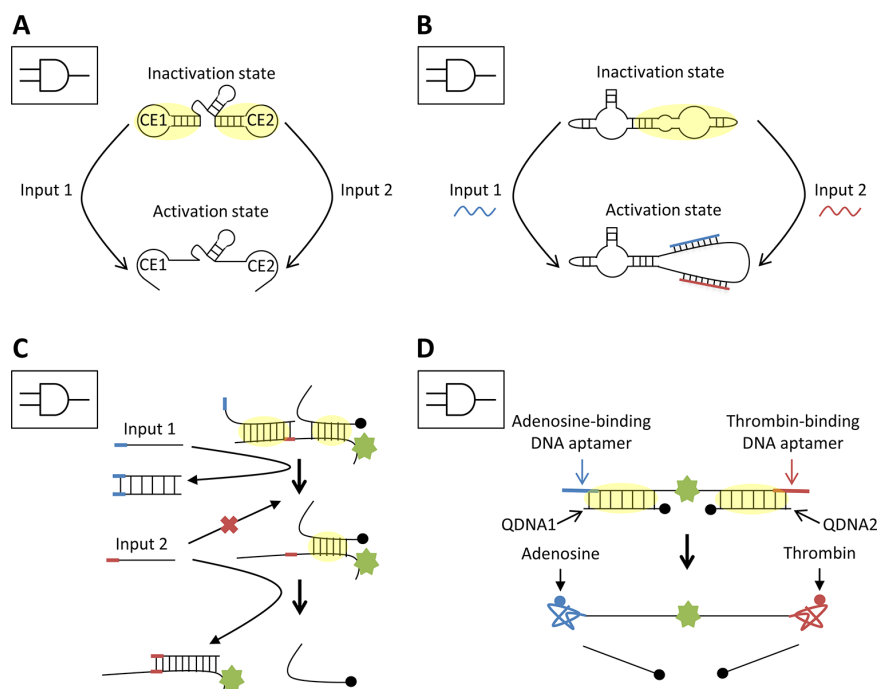
Figure 3. For all of the figures the region(s) that are directly or indirectly affected by the inputs for the device to become activated are highlighted in yellow circles. (A) Schematic diagram of representative deoxyribozyme (8-17)-based logic device (AND gate). Different oligonucleotide inputs (inputs 1 and 2) were hybridized with the corresponding controlling elements (CE1 and 2), leading to the cleavage of the substrate. For the substrate cleavage, both inputs and subsequent conformational change of CEs are required. (B) Schematic diagram of representative ribozyme-based logic device (AND gate). Similar to panel A, simultaneous hybridization of two oligonucleotide inputs with the ribozyme lead to its activation. (C) Schematic diagram of representative branch migration-based logic device (AND gate). For the release of the desired DNA strand (output), sequential branch migration by two different oligonucleotide inputs is required. The green star and the filled black circle represent the fluorophore and quencher moieties, respectively. The toe-hold and its corresponding binding region are both depicted in blue and red for inputs 1 and 2, respectively. (D) Schematic diagram of representative DNA aptamer-based logic device (AND gate). When two inputs are present simultaneously, both QDNAs are released from their complementary strands, leading to the readout of increased fluorescence intensity.

oligonucleotides (DNA signals) form a reversible branch migration-based logic device.[16] Doing so enabled them to create AND or OR gates via precise changes in the concentration of specific DNA signals. Since the input and output in these devices were the same biomolecule, a DNA strand, it is easy to integrate the logic gates' modules to make more sophisticated circuitry.[15,16]

Another example of an enzyme-free computation system, a DNA aptamer-based logic device, was created by Yoshida and Yokobayashi.[17] For the design of their DNA aptamer-based AND gate, a fused adenosine-binding DNA aptamer and a thrombin-binding DNA aptamer containing a fluorescein modification bind to partially complementary fluorescence quencher-modified nucleotides (QDNA1 and 2) (Figure 3D). In this system, both QDNAs are released from the aptamers only when the two inputs (adenosine and thrombin) are added, leading to the enhancement of fluorescence intensity. In the absence of input or if only one input is present, the remaining QDNA(s) attenuate(s) the fluorescence. An OR gate can also be created if the positions of the fluorophore and fluorescence quencher are changed.[17]

Because the principle of Watson−Crick base pairing used in these devices is clearly understood as an immutable one, using such systems bestows great promises for future developments that enable monitoring or controlling cells at the intracellular oligonucleotide level. For instance, a plug-and-play hybrid-

ization-based device that can process endogenous RNA as an input could be installed in living cells to monitor or control cellular behavior. Such plug-and-play systems are modular, and their components can be easily reconfigured to yield the output of interest.

2. PROTEIN-BASED COMPUTATION IN CELL-FREE SYSTEMS

Proteins have also been used to make Boolean logic gates *in vitro*. The landmark was reached in 2006 when Baron et al. constructed an *in vitro* protein-based logic system.[18] In their work a variety of enzymes (glucose oxidase, catalase, glucose dehydrogenase, and horseradish peroxidase) that all operate given two inputs of glucose and hydrogen peroxide were used to construct seven different logic gates (XOR, N-IMPLY, AND, OR, NOR, NOT, and YES gates; Figure 4). Intriguingly, the same group also used the enzyme-based system to develop a half-adder and a half-subtractor circuit by combining either the AND and XOR or XOR and N-IMPLY gates.[19] Scaling up the circuit complexity by concatenating logic gates has also been achieved.[20−22] However, compared with nucleotide-based computational systems, protein-based devices are of limited use in cell-free conditions. This is mainly due to the small repertoire of proteins with orthogonal functionality. This protein-level toolbox of a limited variety sets a restriction in robust logic circuit design. To alleviate this burden, linking
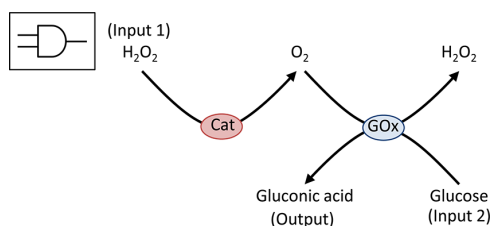
Figure 4. (A) Schematic diagram of the representative protein enzyme-based logic device (AND gate). This experiment was performed under an inert Ar atmosphere in a hermetically sealed vial. For output readout (production of gluconic acid), both glucose (input 2) and O_2 that is released upon addition of H_2O_2 (input 1) are required. Catalase (Cat) and gluconic acid (GOx) are the molecular processors of these reactions.

protein- and nucleic acid-based devices can potentially be achieved, and thus a new paradigm in biological circuit engineering can be introduced.

3. NUCLEIC ACID-BASED COMPUTATION WITHIN CELLS

In order to use biomolecule computers to manipulate and orchestrate cellular functions, devices capable of performing cell-like behavior within living cells should be deployed. In addition, encapsulating these devices in small enclosed spaces, namely, a cell and its compartments, contributes to a higher signal-to-noise ratio. Furthermore, this sequestration of components of logic gates' circuitry attenuates the cross-talk between the circuit constituents. In other words, spatially resolving circuit components enhances the discernment of individual signals. This also reduces the number of orthogonally functioning biomolecules needed in a circuit as compared to *in vitro* biomolecule-based computation. In the following section we introduce a range of functional intracellular nucleic acid-based computers.

3.1. Network Plasmid. Plasmids are undeniably the best-known information carriers that are widely used to express molecules of interest in cells. Guet et al. designed a special plasmid, termed a "network plasmid", composed of genes encoding three transcription factors: LacI, TetR, and λ CI, as well as their corresponding promoters.[23] The readout of this system is the fluorescence of the GFP located downstream of the λ CI-repressible promoter of the plasmid DNA. In this plasmid, for each system three of five promoters that each can be regulated by three transcription factors were chosen. The permutation of these expression conditions results in creation of a total of 125 unique genetic networks given this single plasmid. Indeed, the authors created various GFP expressing devices using a combination of various promoters, small molecule inducers (β-D-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTC)), and host strains of *E. coli*. Among them, logic gates such as YES, NAND, N-IMPLY, and NOR are particularly noteworthy (Figure 5A). These concatenated gene devices can be easily introduced into cells and serve as a powerful tool for creating biocomputers not only in *E. coli* but also in mammalian cells.

3.2. RNA Aptamer. Among other biomolecules, RNA has recently emerged as an attractive material for customized manipulation of cellular functions. An RNA aptamer is a relatively short RNA motif that can interact with other specific target molecules, such as a small drug molecule, another RNA,

or a protein.[24] Major studies have been performed on aptamers since Gold et al. and Szostak et al. first reported a combinatorial repetitive chemical selection process, known as *in vitro* selection for systematic evolution of ligands by exponential enrichment (SELEX), to produce aptamers in 1990.[25,26] Currently aptamers are being studied in various fields ranging from molecular biology to clinical medicine.[27] Among such research efforts, one resulting in the development of a protein-responsive RNA based-regulatory device presents a major landmark in the application of aptamers to the regulation of gene expression in living cells.[28] In this study, a specific protein-binding aptamer was integrated into an intronic sequence between a protein-coding exon and an alternatively spliced exon containing a stop codon, followed by another intron and the next protein-coding exon (Figure 5B). The splicing of the alternatively spliced exon could then be effectively controlled by whether the protein is bound to the aptamer. Failure of the mature mRNA to exclude the alternatively spliced exon leads to early translation termination, which results in synthesis of a nonfunctional peptide. Interestingly, the authors constructed a protein-responsive RNA device in which a disease-associated protein-binding aptamer (for example, β-catenin-binding aptamer) was integrated into the intronic region of an immature RNA that encoded the herpes simplex virus-thymidine kinase (HSV-TK) gene whose product, in turn, is an activator of pro-drug ganciclovir (GCV).[28] The basis of this device is an AND logic gate, so that an interaction between the disease-associated protein and the GCV treatment is required for GCV-induced apoptosis.

Win and Smolke constructed a high-order RNA device comprising three functional components: an RNA aptamer-based sensor and a hammerhead ribozyme-utilizing actuator united by a transmitter component.[29] In the device, theophylline- and tetracycline-responsive RNA aptamers were used to make AND, NAND, NOR, and OR gates (Figure 5C).

3.3. Riboswitch. In 2002, two independent groups, led by Breaker and Nudler, respectively, reported a naturally occurring aptamer-like nucleic acid-based genetic regulatory element, a riboswitch.[30,31] A riboswitch is part of a noncoding region in an mRNA, and gene expression can be modulated via conformational changes in the riboswitch due to the binding of ligands such as free metabolites and small molecules. Many riboswitches have been identified in organisms ranging from bacteria to *H. sapiens* including the cobalamin riboswitch, GlmS ribozyme, and S-adenosylmethionine (SAM)-V riboswitch.[32] As with an aptamer-based device, the riboswitch concept can be applied in the construction of devices for controlling gene expression in living cells. For instance, in such systems, a riboswitch is incorporated into the 5'-UTR of the target transcript and in the presence of a specific ligand regulates transcription. In 2006 a naturally occurring riboswitch that operates on a NOR logic in metE mRNA of *Bacillus subtilis* was identified.[33] For this gate the mRNA is observed to carry two classes of riboswitches in tandem. The first riboswitch binds SAM, whereas the second one has a coenzyme B_{12} (AdoCbl) binding domain. These two riboswitches reside upstream of the gene coding region, and since they function independently of each other, in the presence of either SAM or AdoCbl the metE mRNA is repressed and transcription termination is induced (Figure 5D). In the future, it might be possible to translate such systems in mammalian cells by constructing gene circuits that are composed of integrated riboswitch devices.
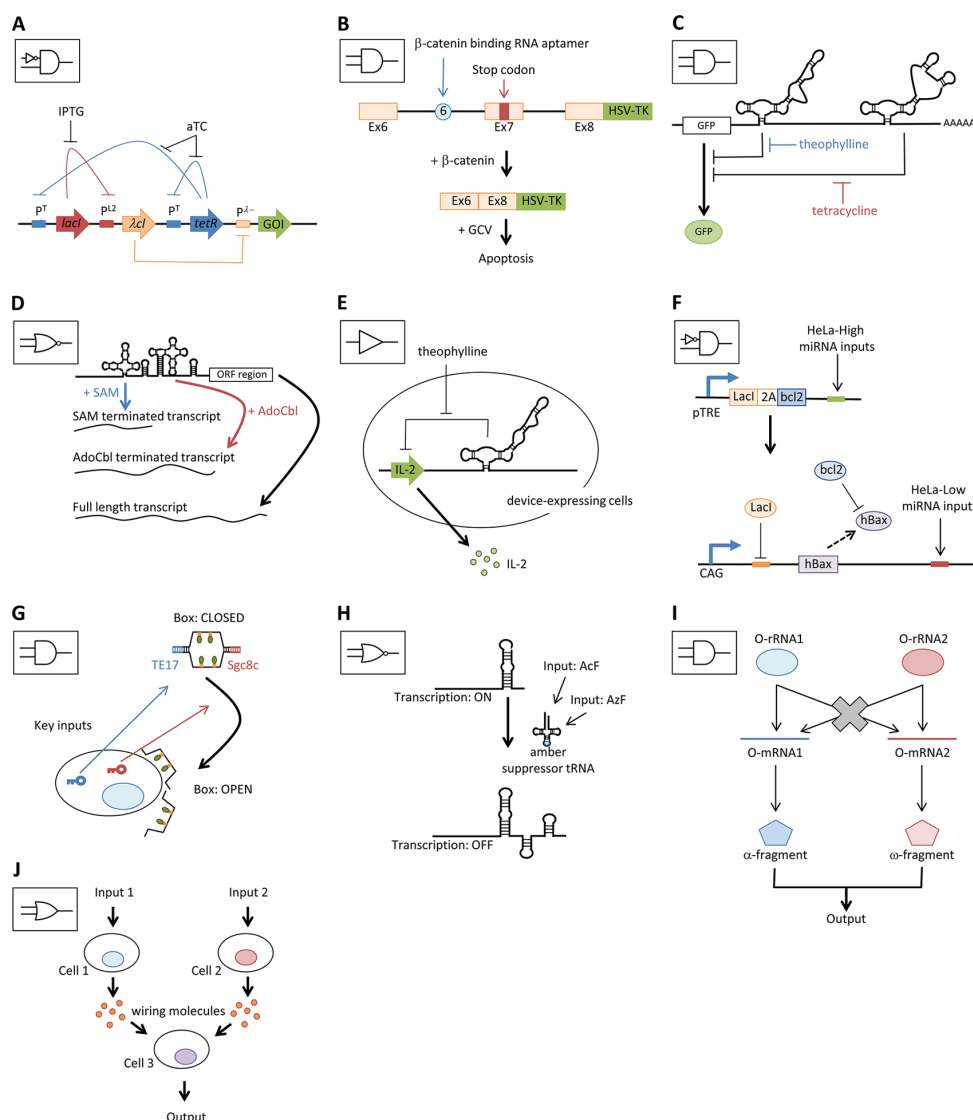
Figure 5. (A) Schematic diagram of representative network plasmid-based logic device (N-IMPLY gate) in lac$^-$ E. coli strain. The device induced the expression of GFP only in the absence of IPTG and in the presence of aTC. P^{L2}, P^T, and P$^{λ-}$ represent promoters repressed by LacI, TetR, and λ CI, respectively. (B) Schematic diagram of representative RNA aptamer-based logic device (AND gate). In the device, β-catenin-binding RNA aptamer was inserted into the intron site 6 position, and interaction of β-catenin with the target RNA aptamer led to the exclusion of exon 7 from mature mRNA, leading to the expression of HSV-TK. For the induction of apoptosis as output, both expression of HSV-TK and the presence of GCV are required. (C) Schematic diagram of representative assembled RNA-based logic device (AND gate) is shown. Translation of gene of interest encoded upstream of the device was allowed only in the presence of both inputs, theophylline and tetracycline. (D) Schematic diagram of the representative riboswitch-based logic device (NOR gate). Two different inputs (SAM and AdoCbl) independently induced the transcription termination of gene of interest through cis-acting corresponding riboswitches. (E) Schematic diagram of representative ribozyme-based logic device (YES gate). Expression of IL-2 is induced in the presence of input, theophylline. (F) Schematic diagram of representative miRNA-based logic device (N-IMPLY gate). Input 1 (a set of miRNAs whose expression level is higher in HeLa cells) suppresses the expression of LacI and bcl2 that repressed the hBax gene expression and hBax function, respectively. The expression of hBax is attenuated by input 2 (a set of miRNAs whose expression level is lower in HeLa cells; therefore input 2 cannot suppress the expression of hBax in HeLa cells). (G) Schematic diagram of representative nanorobot-based logic device (AND gate). To open a nanorobot locked by TE17 lock and sgc8c lock, cells must express the corresponding "key proteins". (H) Schematic diagram of representative amber suppressor tRNA-based logic device (NOR gate). In the device, addition of either AcF or AzF or both allow for the translation of leader peptide region, resulting in the transcriptional attenuation. (I) Schematic diagram of representative orthogonal ribosome/mRNA pair-based logic device (AND gate). The translation of O-mRNA1 and O-mRNA2 is limited by O-rRNA1 and O-rRNA2, respectively. In the device, because α fragment and ω fragment are encoded by O-mRNA1 and O-mRNA2, respectively, expression of both is required for output readout (β-galactosidase activity). (J) Schematic diagram of representative intercellular network-based logic device (OR gate). In the device, the first layer's two different cells containing different sensors process the specific inputs orthogonally and then release the same wiring molecule, leading to the output readout by the second layer cells (marked as cell 3).

3.4. Ribozyme. A ribozyme is a tertiary structure found in noncoding RNA, which self-edits the RNA and eliminates the need for a protein-based enzyme.[34] Like riboswitches, they can control gene expression via mRNA stability,[35,36] which affects the proteome. Using this system, Chen et al. developed an RNA device for inducing T-cell proliferation in a small molecule-dependent manner.[37] Specifically, the catalytic activity of the ribozyme was regulated by a small molecule, theophylline, whose riboswitch was inserted into the 3′-UTR of a target transgene encoding a proliferative cytokine, IL-2 (Figure 5E). The ribozyme was inactivated in the presence of theophylline, allowing IL-2 to be produced and released by RNA-device expressing cells. This led to the proliferation of a T-cell line derived from CTLL-2 mice, which constitutively expressed the IL-2 receptor and was dependent on common γ-chain signaling for survival and proliferation.[38] Without theophylline, the ribozyme self-cleavage of the RNA device resulted in decreased IL-2 production, leading to T cell death. This approach was remarkably successful, and the authors developed an *in vivo* T-cell proliferation system in mice using these engineered cells.[37]

3.5. RNA Interference. RNA interference (RNAi) is arguably the most widespread RNA-based mechanism for gene expression regulation in living cells.[39,40] This post-transcriptional gene-silencing process is based on cytosol-localized short double-stranded RNA, which is recognized and cleaved by an RNA-induced silencing complex (RISC).[41] The induced RNA strand interacts with mRNA containing a subsequence of the full or partial complementary sequence in its coding region or UTR, leading to the subsequent degradation of the target mRNA. In mammalian cells, it has been shown that a small dsRNA (20−24 bp) can specifically knock down target genes without an interferon response and the subsequent apoptosis,[42] thereby extending the use of the RNAi technique to gene silencing. At present, three types of short RNAs are used for RNAi: small interfering RNA (siRNA), short hairpin RNA (shRNA), and micro RNA (miRNA). siRNA and shRNA can be designed and synthesized artificially based on the target gene information and can be delivered exogenously. Unlike siRNA and shRNA, which are exogenous factors, miRNA is an endogenous single RNA strand that regulates gene expression. Many researchers have tried to apply RNAi-based regulation to gene expression to construct a device that can regulate cellular functions as desired. At present, up to five siRNA variables can be integrated into one "plug-and-play" device.[43] miRNA is also used to construct plug-and-play devices. Information about miRNA expression profiles has improved remarkably to the point where the full suite of miRNAs expressed in specific cells is known.[44,45] On the basis of the miRNA profile information, it is possible to construct a miRNA-based device that induces apoptosis when the miRNA expression profile has a defined pattern. Indeed, Xie et al. constructed a miRNA-based plug-and-play device where cancer cells (e.g., HeLa cells) were marked with a unique set of many miRNAs that induced apoptosis only in the specific cancer cells, even if these cells were cocultured with other cell types (e.g., HEK293 cells)[46] (Figure 5F). This concept of "synthetic cell death" is rather promising as it can potentially facilitate precise regulation of therapeutic interventions.

3.6. Nanorobot. Recently, Douglas et al. developed an impressive system called a logic-gated nanorobot.[47] This nanorobot consists of two domains made by a large, single-stranded DNA held in a particular shape by a series of short single-stranded DNA "staples" via a method named DNA

origami.[48] In this case, the "origami" is folded into the two halves of a hexagonal barrel, connected by single-stranded scaffold hinges and two DNA aptamer-based locks. These locks can fasten the two halves of the barrel to bring them into a closed conformation where the molecular payloads can be sequestered. For opening the lock, a specific key recognized by the DNA aptamer is required. If two different locks are used, two specific keys are required to open the barrel; the lock mechanism is equivalent to an AND gate. In the study, by using various locks, the authors succeeded in delivering molecular payloads specifically to different cells lines that expressed the correct combinations of keys, even if these cell lines were mixed (Figure 5G). Combining this logic gated-drug delivery system with the miRNA-based synthetic cell death device described above might lead to novel targeted-therapy strategies.

3.7. Amber Suppressor tRNA. Amber suppressor tRNAs have been used to operate nucleic acid-based computation in *E. coli*.[49,50] Amber suppressor tRNA specifically recognizes the amber codon (UAG) and inserts one of several amino acids instead of inducing translation termination, making it possible to translate subsequent mRNA information. Liu et al. created an artificial amino acid-regulated amber suppressor tRNA-based logic device.[49] In this device, two *trp* operon- and *tna* operon-derived leader peptide mutants carrying a UAG codon in the coding region were placed upstream of a reporter gene encoding GFP, in plasmids named pCCL-006 and pCCL-016, respectively. For the construction of a Boolean logic gate, these plasmids were cotransformed with pEVOL-Dual-pAcF/pAzF encoding a tRNA specific for the UAG codon and two corresponding aminoacyl-tRNA synthetases specific for the artificial amino acids *p*-acetylphenylalanine (AcF) and *p*-azidophenylalanine (AzF). Then the *E. coli* was cultured in a medium containing excess tryptophan. As a result, the device created by pCCL-016 and pEVOL-Dual-pAcF/pAzF functions as an OR gate, while the one using pCCL-006 and pEVOL-Dual-pAcF/pAzF functions as a NOR gate (Figure 5H). In contrast, Anderson et al. used an amber suppressor tRNA, SupD, derived from *E. coli* to create an AND gate in cells.[50] Specifically, SupD gene and T7 mutant gene were placed under the control of a salicylate-activated promoter and an arabinose-inducible promoter, respectively. Because T7 mutant has two amber codons, salicylate-induced SupD expression is a prerequisite for the expression of functional T7, leading to the expression of GFP as the output. Theoretically, the extension of these strategies to a wide range of species including mammalian cells could enable the future construction of a nutrient status-dependent logic device that can regulate various cellular functions.

3.8. Orthogonal Ribosome and Orthogonal mRNA Pair. In 2005, Rackham and Chin developed an orthogonal 16S rRNA (O-rRNA) and an orthogonal mRNA (O-mRNA).[51] Each O-rRNA and O-mRNA pair was designed as a unique translational entity, acting as a unit whose function was mutually independent of the endogenous *E. coli* rRNA and mRNA systems as well as any other synthetic orthogonal pairs. In the O-rRNA and O-mRNA-based logic device, the expression of the α fragment and ω fragment of β-galactosidase was controlled by either the orthogonal or the endogenous translation systems.[52] For output, both α and ω fragment expression is required. The authors created two Boolean logic gates, an AND gate and an OR gate, based on this principle. In the AND gate, the α and ω fragment are encoded by O-mRNA1 and O-mRNA2, respectively, and corresponding O-
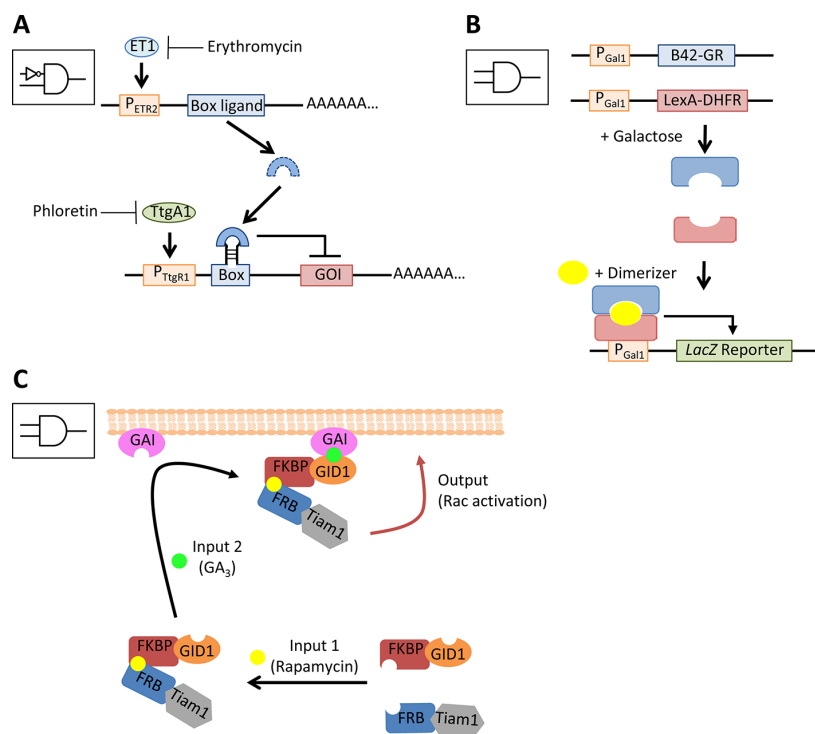
Figure 6. (A) Schematic diagram of representative transactivator-based logic device (N-IMPLY gate). Expression of the gene of interest (GOI) is allowed in the device only in the presence of erythromycin and in the absence of phloretin. (B) Schematic diagram of representative CID-induced transcription-based logic device (AND gate). Dimerizer (e.g., Dex-Mtx)-induced interaction of B42-GR and LexA-DHFR, which in turn induces the expression of GOI. Because the expression of these two proteins is placed under the control of the GAL1 promoter, for the expression of GOI (output), both galactose and dimerizer are required. (C) Schematic diagram of representative CID-regulated transcription-free logic device (AND gate). In the device, both rapamycin-induced and GA$_3$-induced CIDs are required for the translocation of Tiam1 to the plasma membrane, leading to the induction of membrane ruffling.

rRNAs, O-rRNA1 and O-rRNA2, are required for the translation of the α and ω fragments from the O-mRNAs. Only if both O-rRNA1 and O-rRNA2 were present was the output executed (Figure 5I). In the OR gate, the ω fragment was constitutively expressed, and the α fragment was encoded by both O-mRNA1 and O-mRNA2. Thus, if either O-rRNA1 or O-rRNA2 or both were present, the output was executed. Given that these orthogonal biomolecular devices theoretically function independently of the endogenous cellular machinery, such a platform is deemed highly promising in the context of device placement in cell-based systems.

3.9. Intercellular Networks. All of the above-mentioned bimoleculer-based computational systems are processed in a single compartment. *In vitro* this is usually an enclosed chamber, and *in vivo* a cell serves the purpose. Although it is possible to use one distinct bounded space to build a device that consists of multiple logic gates, this type of assembly requires fine-tuning of the components to avoid interference between the signals of each gate. Tamsir et al.[53] and Regot et al.[54] introduced the concept of cellular compartmentalization to build reliably and rationally layered logic gates. A single gate that senses the input was built in a single cell (first layer), and then the readout information (wiring molecules) from this cell was recognized by another cell containing a second gate with actuation as its output function (Figure 5J). Although many challenges remain for the living cell-based computational

system, pursuing this concept still has its allure. This not only makes it possible to reduce the intralayer noise but ultimately allows constructing intercellular communication networks within living cells.

4. PROTEIN-BASED COMPUTATION WITHIN A CELL

The information processing abilities of cells involve cooperation between gene and protein expression. Expressed proteins play an important role in almost all cellular process. They implement most of the input and output signals used during the computational process. Proteins do so by sensing and transducing the input information and executing cellular functions as the output response. In addition, part of the intermediate decision-making process is also achieved by the regulation of protein functions governing the production, destruction, localization, and activities of biochemical molecules. Thereby proteins form an advanced communication and information-dissemination system within cells. Recent studies in the field of synthetic biology have reported the design of protein-based biomolecular devices and have made a case for their application. In this section, we introduce such protein-based computers that function in the context of a living cell.

4.1. Transactivator-Based Gene Circuits. Changes in mRNA levels are critically important for the control of cellular functions. Recently, Ausländer et al. developed a chemically inducible transactivator-based gene circuit with an erythromy-

Table 1. Classification of Representative Biomolecule-Based Logic Devices

	core machinery	inducers	in vitro	in vivo	current networkability	reference
DNA-based logic gates	deoxyribozyme	oligonucleotides	○			12, 14
	hybridization	nucleotides	○		○	15, 16
	DNA aptamer	adenosine, thrombin, nucleotides, protein	○	○		17, 47
	gene expression regulation	IPTG, aTC, arabinose, DOX, NaCl, galactose		○	○	23, 53, 54
RNA-based logic gates	ribozymes	oligonucleotides, theophylline	○	○		13, 37
	hybridization	siRNA, miRNA		○		43, 46
	RNA aptamer	protein, theophylline, tetracycline		○		28, 29
	riboswitch	methionine, AdoCbl		○		33
	orthogonal ribosome/mRNA	orthogonal ribosome		○		52
	amber suppresor tRNA	unnatural amino acids, arabinose, salicylate		○		49, 50
protein-based logic gates	enzyme	glucose, H_2O_2, NADH, acetaldehyde, starch, phosphate, NAD^+, acetylcholine, butyrylcholine, O_2	○		○	18, 19, 20, 21, 22
	transactivator	phloretin, erythromycin, butanolide, pristinamycin, tetracycline		○		55, 56
	CID system	chemical dimerizers		○		59, 60

cin-dependent transactivator and an apple metabolite phloretin-dependent transactivator, which were incorporated into the gene circuit.[55] These transactivators induced the expression of target genes that contained a specific RNA motif-binding protein or a reporter gene. The specific protein could bind to a target motif located upstream of the mRNA target gene, inhibiting the expression of the reporter gene (Figure 6A). Combining this system with other similar ones enables the construction of various logic gates that could be further combined via two-bit processing. Kramer et al. constructed various logic gates in a mammalian cell using chemically inducible transactivator-based gene circuits.[56]

4.2. CID-Based Gene Circuits. Chemically inducible dimerization (CID) systems serve as a promising platform for manipulating cellular functions.[57,58] In such systems, a small molecule induces the dimerization of two different proteins, producing a ternary complex. Bronson et al. used a CID system to create a transcriptional logic device.[59] In their system two different dimerizers (Dex-Mtx or Dex-Tmp) and a dimerization inhibitor (Mtx) were used to regulate the dimerization of an activation domain, B42-glucocorticoid receptor chimera (B42-GR), and a DNA-binding domain, LexA-dihydrofolate reductase chimera (LexA-DHFR). To control the expression of both chimeric proteins, these coding genes were placed downstream of a GAL1 promoter. This configuration enabled the creation of AND, OR, NOT, and NOR gates by choosing the right combination of culture medium nutrient status (presence of either glucose or galactose) and drugs (presence of either Dex-Mtx, Dex-Tmp, or Mtx) (Figure 6B).

4.3. CID-Based Post-translational Circuits. As described above, to date many biomolecule-based logic gates have been created (Table 1). However, one of the major drawbacks of the existing logic gates based on the aforementioned genetic circuits is that they require long periods of time (minutes to hours) to execute the logic function. This weakness is due to the long processing time inherent in transcription and translation machinery (Figure 2). For high-speed *in vivo* computation, non-genetic circuit devices based on CID systems, whose most notable advantage is their time scale, are promising. Recently, Miyamoto et al. constructed a high-speed *in vivo* logic-gate system based on two orthogonal CID

systems, i.e., a rapamycin-induced and a GA_3-induced CID system.[60] In a single living cell, these two orthogonal CID systems induced two distinct proteins of interest (POI) to translocate to different targeted locations with similar kinetics. Within a few seconds after introducing the inputs, the logic was executed by the translocation of POI directly to the target intracellular space. Thus, the output was initiated within the few seconds to minutes that followed. Therefore unlike the genetic circuit-based logic gates, this post-translational level gate had a much faster processing power (Figure 2). They created two basic logic gates, an OR gate and an AND gate, using a Tiam1-based Rac activation probe to induce membrane ruffling (Figure 6C). An advantage of using proteins as the decision-making signal is that each protein displays distinctive features in a spatiotemporally dependent manner, which means the output signal is not limited to a binary code. There exist other plant hormones that dimerize different sets of proteins not only in plants but also in mammalian cells,[61,62] just as is the case with gibberellin. Employing a combination of such plant hormones allows for construction of more sophisticated logic devices capable of accepting several inputs and processing multiple signaling reactions simultaneously.

■ CONCLUSIONS

Since Theodor Schwann, Matthias Jakob Schleiden, and Rudolf Virchow founded the cell theory, many novel insights into these small units of life have been reported. However, despite this progress, it is still difficult to replicate intracellular network topologies, suggesting that many yet unknown phenomena occur in living cells. Since the sequencing of the human genome, rapid developments in microarray and proteomics technology are providing precise information about the function and structure of DNA, RNA, and proteins expressed in cells under specific conditions. In addition, evidence from cell biology is clarifying the intracellular network topology of gene circuits as well as signaling pathways. This information is essential for those who wish to rationally control cells. Based on the concept of logical computation within a living cell, devices to program cellular functions and cell fate have been successfully constructed. Despite its relatively recent emergence in engineering and biology, synthetic biology has allowed us to

alter cellular decision-making processes. This facilitates the implementation of cellular functions that meet our requirements. Indeed, many devices have already been constructed in mammalian cells including logic gates that operate on multi bits (described above), a time-delay circuitry,[63] a synthetic intercellular communication system,[64] bandpass filters,[65] a toggle switch,[66] and oscillators.[67,68] Integrating the transcription and translation machineries has also resulted in the assembly of *in vitro* logic devices that exhibit the advantages of the *in vivo* counterparts as well.[69] All of these devices can be used to rewire intracellular networks and manipulate cellular functions to certain degrees. The applications of such systems in clinical medicine are ever more prominent.[70] There is a long way to go before synthetic biology-based clinical treatments become a reality, but the discipline holds much promise particularly for clinical conditions in which diseased cells exhibit a distinct marker distinguishing them from their healthy counterparts. It is hoped that in the near future libraries of the components that have been used in synthetic devices can be consolidated in one database, easily accessible via computer software. Then the day will come when the synthetic biologist provides the cellular output function of interest to the software and in return will receive a list of the genetic circuit modules with a schematic of the corresponding circuit configuration. Then just another few clicks away the information will be on its way to a facility where the genes of interest are synthesized. Thereafter these gene modules will be available in the lab in only a few days for fast, reliable, and efficient assembly of robust bimolecular computers.

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Notes
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