INVITED PAPER

Synthetic Gene Circuits: Design, Implement, and Apply

This article gives a broad overview of the field of synthetic biology, focusing on the engineering of genetic circuits.

By Andrew Lezia, Arianna Miano, and Jeff Hasty

ABSTRACT | Synthetic biologists engineered the first genetic toggle switch and clock at the turn of the 21st century. Since the development of these first gene circuits, methods for circuit design and construction have improved dramatically, narrowing the gap between concept and proof-of-principle implementation. Engineered genetic constructs have started to leave the lab for the real world where they are being used in applications, including medicine, biosensing, and industrial chemical production. The field of synthetic gene circuits has also grown from focusing on single, isolated circuits to designing complex systems that operate across multiple populations in carefully engineered consortia. In addition, design methods have progressively moved toward including detailed models of the interactions between the host genome and the synthetic gene circuits that it contains in order to better predict circuit dynamics. This article will review some of the most recent advances in gene circuit design and implementation, with a focus on synthetic gene circuits being applied to address real-world problems.

KEYWORDS | Computational design; genetic engineering; synthetic biology; synthetic gene circuits.

I. INTRODUCTION

Over a few billion years, life has evolved solutions to problems at the limit of human comprehension. In this period, natural selection has driven the emergence of innumerable molecular machines in the form of proteins and ribonucleic acid (RNA) that facilitate diverse chemical reactions with astounding specificity and efficiency. Equally as fascinating as nature's endless toolbox are the complex networks that these tools operate within, which span individual molecular pathways to entire ecosystems. Until relatively recently, this breadth of parts and devices seemed impervious to human tinkering, shrouded in the complexity of the parts themselves and their encompassing networks. In the last 100 years or so, human innovation and research have cleared much of this fog of complexity allowing us to see biological systems not as magic but as decomposable and comprehensible networks. In addition to uncovering the basic flow of information at the molecular level, we have identified many small motifs of genetic interactions that occur much more often than random and contribute to massive biological networks [1]. This relatively newfound understanding of biological networks and their components has grown at a rapid pace leading to researchers constructing their own synthetic gene circuits over the last 20 years [2]–[4]. The first synthetic gene networks mimicked simple electronic circuits and showed that biological systems could be created with a bottom-up design approach centered around principles, such as abstraction and modularity typically seen in traditional engineering fields [5], [6]. While these initial forays into synthetic gene circuits helped shine a light on the fact that the biological systems could be engineered, they also brought into focus a slew of characteristics that kept biology distinct from engineering, including the stochasticity of gene expression and context-dependent behavior of genetic parts. Now, the field of genetic circuits is in its adolescence, with a multitude of engineered circuits leaving the lab to solve real-world problems. Given the advances in constructing and implementing genetic circuits, there has been no better time for scientists and engineers from other fields

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Andrew Lezia and **Arianna Miano** are with the Department of Bioengineering, University of California at San Diego, La Jolla, CA 92093 USA (e-mail: andrewlezia@gmail.com).

Jeff Hasty is with the Department of Bioengineering, the Division of Biological Sciences, Molecular Biology Section, and the BioCircuits Institute, University of California at San Diego, La Jolla, CA 92093 USA.

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to contribute to the field. A circuit that would have taken researchers through an arduous gauntlet of molecular cloning two decades ago can now be constructed with relative ease due to major advances in deoxyribonucleic acid (DNA) assembly methods and standardization of genetic parts [7]–[9].

In biology, the central dogma describes the general flow of information through biological networks from DNA, to messenger RNA (mRNA), to protein. In designing genetic circuits, each of the connections in the central dogma (e.g., DNA to mRNA) represents a point where regulation can be implemented to achieve the desired behavior, such as a digital logic gate. Simple genetic circuits consist of one or more transcriptional units (TUs) that are connected together to achieve a specific input–output transfer function. In bacteria, a typical TU consists of the following DNA sequence regulatory elements in order: a promoter that controls the rate of mRNA transcription by an RNA polymerase from a coding DNA sequence (CDS), a ribosome binding site (RBS) that controls the rate of translation initiation of a protein from an mRNA template by a ribosome, one or more CDSs that typically encode instructions for building proteins (or regulatory RNA molecules), and a terminator that stops transcription from continuing past the terminator sequence.

One of the simplest types of regulation to use in a circuit is transcriptional regulation or, in other words, regulating the rate of mRNA production from different promoters. For instance, one way to engineer the logical AND function in a cell is using three distinct TUs and promoters. Promoters P1 and P2 are activated by external inputs A and B, respectively, and each promoter makes a transcription factor that is necessary to activate transcription from promoter P3. Thus, in this simple example, the CDS driven by promoter P3 is only expressed if both the external inputs A and B are present (i.e., AND gate behavior). While thinking strictly in terms of the central dogma and TU's allows for the construction of many circuits via the combination of different logic gates, there are many additional regulatory strategies outside of this framework that can be used to create more complex, dynamic gene circuits.

In this article, we review advances in the design, implementation, and application of synthetic gene circuits. For design, we focus on the breadth of computational tools currently being used to design and test genetic circuits *in silico*. For implementation, we describe some of the new biological parts available for researchers to construct new genetic circuits, highlight advances in the standardization of genetic circuit assembly methods, and discuss methods for tuning genetic circuits to achieve the desired behavior. Finally, for application, we look at a few examples where genetic circuits are being deployed outside of the lab for therapeutics, biosensing, and biomanufacturing. Throughout the review, we try to focus on analogies between synthetic gene circuits, and electrical and computer engineering while acknowledging some of the limits of using a traditional engineering approach for biological circuits.

II. DESIGN APPROACHES

Despite great advances in creating complex genetic circuits, circuit design remains one of the most challenging aspects of genetic engineering [10]. The design of efficient genetic circuits can be time-consuming and unreliable, and is often based on trial-and-error analysis and testing. While simple circuit modules, such as switches [6], logic gates [11], and clocks [12], have been designed and characterized in a moderately quantitative fashion, the slow pace of progress toward increased complexity necessitates new tools and approaches. In this section, we review quantitative approaches for designing robust genetic circuits and describe the utility of different design methods for modeling different types of genetic circuits, such as analog and digital systems.

One of the initial attempts at standardizing the design process for genetic circuits was inspired by the principles of electronic design automation (EDA) [13]. As EDA was initially created to help engineers in the design of semiconductor-based electronics, *Cello* was designed to automatically suggest a genetic circuit design from a researcher's description of the circuit function. The desired function is specified using the hardware-independent, descriptive language Verilog together with a *user constraint file* that specifies the organism and strain, the layout of the genetic system, and the operating conditions for logic gates used to build the system. In order to facilitate design standardization, Cello supports the exchange of genetic designs in the Synthetic Biology Open Language (SBOL) file format [14]. This format was originally created to promote global data exchange between laboratories and between software programs. In terms of genetic parts, *Cello* combines multiple NOT/NOR logic gates that are implemented using transcriptional repressors, proteins that downregulate gene expression by binding the gene's promoter region and either preventing RNA polymerase machinery from transcribing DNA or slowing it down. Fig. 1(a) shows the different steps involved in circuit design using *Cello*.

Cello has been shown to be particularly effective in designing genetic circuits in prokaryotes due to the availability of insulator parts that maximize context independence of gates performance [16]. In particular, Cello was shown to be key in the design of genetic circuits for therapeutic bacteria using native strains from the human gut [17]. On the other hand, the predictability of gate performances is undermined in eukaryotes due to their complex mechanisms of transcription and translation. In order to overcome this challenge, a study proposed the combination of minimal constitutive promoters, operators for DNA-binding proteins, and ribozymes that allowed the construction of NOT/NOR gates in yeast [18].

Although digital logic gates have been widely used to build circuits using a small selection of parts [19], [20],

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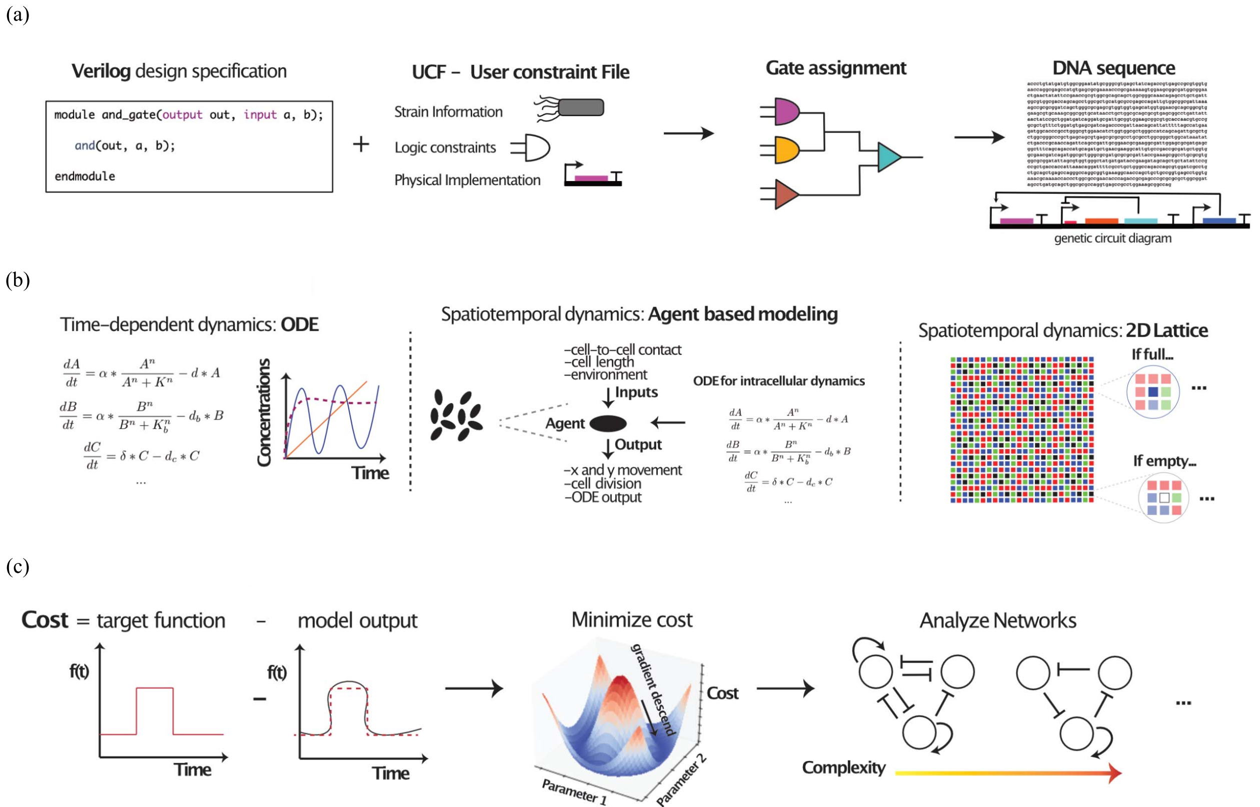

Fig. 1. *Circuit design approaches. (a) Different steps involved in genetic circuit design using Cello from the Verilog design specification to the DNA output to be synthesized [automated logic gates (Cello)]. (b) Three mathematical modeling approaches to circuit modeling are illustrated. From the left, ODE modeling, 2-D lattice modeling, and agent-based modeling, respectively. (c) Main steps involved in circuit design using machine learning are illustrated, starting from the cost function definition that is minimized through gradient descent algorithm to eventually generate potential network motifs to be implemented [15].*

they have the disadvantage of being subject to unwanted crosstalk between other genetic systems, and they can also be inefficient in the resource-limited environments of cells [21], [22]. On the other hand, analog behavior is often found in natural, living systems and can inspire the design of synthetic analog circuits that can carry out sophisticated computational functions using a small number of genetic parts. In particular, analog circuits were successfully shown to perform logarithmically linear sensing, addition, and power-law and ratiometric computations [23]. An example of an analog circuit with a large dynamic range is a positive-feedback-based circuit constructed using genetic parts from natural quorum sensing (QS) systems [23]. Table 1 summarizes the main differences between analog and digital genetic circuits.

Such circuits that display nonlinear dynamics cannot be achieved by only combining logic gates as previously described and require a different approach for circuit design. In this case, mathematical modeling using ordinary differential equations (ODEs) is preferred [see Fig. 1(b)]. The first step in this approach consists of creating a qualitative model of the genetic circuit using a directed graph with signed edges that indicate the causal interactions between pairs of genes. The second step consists of translating this diagram into a system of differential equations, which quantitatively describes the time-varying concentrations of mRNA and proteins in the cell [24], [25]. This approach has been used to successfully predict the time-dependent behavior of synthetic gene circuits at both the single cell and population level [12], [26]–[29]. In particular, visualizing the parameter space can be used to direct design changes by pointing to the genetic parts (e.g., RBSs, promoters, and transcription factors) that need tuning in order to achieve the desired output.

Although ODEs are a very effective tool for modeling analog circuit behavior when model variables can be assumed to only change with time, they do not suffice if the circuit's dynamics are also dependent on the distribution

Table 1 Summary of the Differences Between Analog and Digital Genetic Circuits

Analog	Digital
Continuous variables	Boolean logic
Large variability of parts	Small variability of parts
Ad hoc design	Modular design
High parameters sensitivity	Low parameter sensitivity
Energy efficient	Inefficient and resource-intesive

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of cells and molecules in space. One example where this is particularly true is for synthetic circuits based on cell-to-cell communication. These systems often rely on the production and sensing of small, chemical-signaling molecules that freely diffuse in the surrounding culture environment and across the cell membrane [30]. Another example where spatial dynamics need to be considered is for genetic circuits used in synthetic ecologies that are characterized by multiple, distinct, engineered, populations interacting in a 2-D or 3-D environment [31], [32]. In order to model the spatiotemporal behavior of these synthetic communities and improve circuit design, multiple methods have been adopted including partial differential equations (PDEs), agent-based models, and lattice models.

In agent-based modeling, a system is described as a collection of autonomous decision-making entities referred to as agents. Each agent can assess its surroundings and make decisions according to a set of user-defined rules. In the context of genetic circuits, each agent is representative of a single cell that can be modeled as a spherocylinder (for *E. coli*) of unit diameter that grows linearly along its axis and divides equally after reaching a critical length. This cell can move along a 2-D plane due to forces generated by interactions with other cells, including inelastic cell-to-cell contact forces, which can be computed using a standard spring-dashpot model. In order to describe the intracellular dynamics of each cell, a system of ODEs describing the biochemical interactions among the circuit parts can be used [32]. In order to help scientists build agent-based models, there is a wide range of frameworks, such as NetLogo [33] and FLAME [34], which provides the user with a minimum set of functions, which can be extended as desired. Although these frameworks are easy to use and adaptable to many cell types, they require an extensive effort from the user if more complex behaviors, such as chemotaxis and environmental interaction, need to be modeled. In order to address this need, researchers developed BSim, an agent-based tool that enables the simulation of realistic 3-D and complex environments, as well as single cells and population dynamics [35].

In contrast to agent-based models, lattice models take a high-level approach where each cell in a population is modeled as a pixel in a 2-D grid with a finite number of states (such as present/absent). Similar to agent-based models, each cell follows a set of rules and makes decisions based on the states of neighboring pixels. This simple strategy has been effective for predicting complex dynamics of synthetic ecologies composed of multiple populations and can be used to guide circuit design by predicting how each genetic part affects the population dynamics over time [31], [36]. Overall, agent-based modeling is better suited to describe systems where cell-to-cell interactions, environmental perturbations at the single-cell level, and chemotaxis are considered important. On the other hand, since 2-D lattice models solely rely on a simple set of rules to update state depending on neighborhood states, it is better suited to simulate high-level population dynamics.

The modeling approaches described so far have the advantage of being able to describe the qualitative spatiotemporal behavior of entire synthetic populations and communities of cells using a small set of specifications. On the other hand, if the main objective of the design is to achieve output robustness in spite of environmental perturbations and intracellular noise, a different modeling approach based on control engineering principles should be used.

Adopting control engineering principles to genetic circuit design has helped researchers realize predictable and reliable circuit designs [37]. This approach has been particularly successful for circuits used to maintain the production of a certain protein at a constant level, even in the presence of external perturbations, a phenomenon known as homeostasis. In nature, living systems can maintain homeostasis using integral control, a system based on negative feedback that performs mathematical integration to achieve robust regulation. Researchers were able to engineer an integral control system in bacteria by first mathematically deriving the biomolecular controller topology to be used. Then, they implemented this topology in living cells and demonstrated its utility as a robust growth-rate controller [38]. One of the main challenges associated with this design approach is the significant metabolic burden imparted on the host cell due to the high number of genetic components needed.

A possible solution to reduce the metabolic burden of genetic control systems is to use multicellular systems where different system functions are distributed across multiple members of microbial consortia [39]. In this approach, one consortia member can act as a “controller” that senses and acts to regulate the phenotype of the “target” population. In turn, the “target” responds by producing an external signal (such as an inducer molecule), which can be sensed from the “controller” population and used to implement the feedback control. This distributed control system was recently demonstrated experimentally with the design of a two-strain gene circuit that senses and responds to which strain is in the majority [40]. Control engineering is not the only design approach from engineering and physics that has been a source of inspiration for synthetic gene circuit designs.

A decade ago, researchers proposed a new design framework based on Bayesian statistics, which uses reverse engineering to estimate the most probable design that could give rise to a set of empirical data [41]. In detail, the approach starts by first defining the input and output characteristics of the system. Then, several competing designs are specified together with prior probabilities (priors) on the different design parameters. In order to quantify the difference between the simulated output and the target one, a distance function is defined. The system is then evolved using a sequential Monte Carlo algorithm so that each population more accurately approximates the desired

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behavior. Finally, the model posterior probability gives information on the ability of each design to achieve the target behavior, giving a clear ranking of the most promising designs to further investigate experimentally. Furthermore, the parameter posterior gives insights into the parameter's sensitivity to the input–output specification, which can further guide experimental implementation. The statistical nature of this approach allows it to address problems, which would be particularly challenging with other design methods previously described. In particular, it can easily handle stochastic models, take into account kinetic parameter uncertainty, and incorporate stochastic environmental perturbations.

Following similar principles to Bayesian approaches, an innovative circuit design workflow based on machine learning was recently proposed [15] [see Fig. 1(c)]. The main goal of this approach is to accelerate the computational screening of genetic circuits to enable the design of larger circuits for complex tasks. Hiscock *et al.* [15] designed a python module called "GeneNet" that consists of three parts. The first part defines the cost function, which defines the error between the model output and the target output. The starting model function is defined as a vector y that comprises N transcription factors whose concentrations are represented by the vector components. Assuming that all genes can interact with each other, a matrix of $N \times N$ parameters is generated. If a parameter is positive, the interaction represents gene activation, while, if negative, it represents repression. In addition, each gene is assumed to degrade with a certain rate k, which can be different for each gene. The cost function is then defined as the mean squared error with respect to the target function. Step two consists of choosing model parameters that minimize the cost function using gradient descent. Finally, the last step consists of analyzing the gene networks proposed by the algorithm and choosing the most promising ones to implement. The main advantages of this method compared to previous approaches are speed of execution and scalability to larger and more complex designs. On the other hand, one of the main drawbacks is that this approach only selects a single genetic circuit out of many possible alternatives, which might also function well. Therefore, a future improvement might involve the classifications of multiple circuits allowing researchers to consider several options. Another disadvantage is the "black box" nature of the machine learning algorithm, which can hide the intuition behind the final circuit topology, making it difficult to match it to its mechanistic explanation.

Another software toolbox that was developed to aid the design of complex genetic circuits is iSYNBADm [42]. This software runs under the popular MATLAB computational environment, and it relies on multicriteria (Pareto optimal) design to find circuit designs from a library of biological components.

Coming up with plausible and quantitative biological blueprints for engineering cell behavior using computational tools is only one challenge in genetic circuit design. Equally as demanding is identifying appropriate biological parts with parameters that approximate those suggested by computational models. This idea is analogous to the advances in semiconductor material science that enabled new integrated circuit designs to be realized by having well-characterized parts (e.g., appropriately doped silicon nanocrystals) [43]. For instance, in creating logic gate-based circuits using transcriptional repression, it is crucial that repressors are chosen with input–output functions that closely match those suggested by the design method. As an example, the previously discussed automated design workflow for genetic circuits, Cello, makes use of a library of 16 distinct transcriptional repressors that act on unique, synthetic promoter sequences [44]. Importantly, each repressor exhibits strong repression of its target promoter while having relatively little crosstalk with the other promoters, which is key for successful implementation of the circuit designs suggested by Cello. In general, the context-dependent behavior of different genetic parts makes it challenging to choose the correct part, and characterizing the performance of different components in the context that they are used is an active area of research [45], [46]. This is particularly true when modeling genetic circuits in mammalian hosts in which competition for the cell's resources between exogenous circuits and endogenous processes can make the circuit behavior highly unpredictable. Compared to the design and implementation of genetic circuits in prokaryotes, the design of genetic networks in mammalian cells is still very slow and very prone to unpredictable failures. Recently, a study proposed a design strategy based on the use of a toolkit of transcription factors, tunable promoter, and parts for posttranslational regulation together with a computational model [47]. Although this framework offers a great approach to tackle the complexity of mammalian circuit design, this field is still in its infancy, and it has the potential to significantly grow in the next years.

In Section III, we will give a broad overview of recent developments in creating, discovering, and tuning new biological parts to implement genetic circuit designs.

III. CIRCUIT IMPLEMENTATION

Much like electrical engineers must choose components, such as resistors, inductors, capacitors, and semiconductors to implement electrical circuit designs, synthetic biologists must select biological parts in order to implement genetic circuit designs. While electrical circuit components are responsible for regulating the flow of electricity through a larger system, biological parts can loosely be thought of as regulating the flux of RNA, protein, and metabolites within single cells and among populations of cells. As the field of synthetic gene circuits has grown, so has the number and type of parts available to researchers to program their desired host cell or organism. Implementation of genetic circuit design can generally be broken down into two components: 1) the selection of regulatory parts to carry out the circuit function and 2) the

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synthesis and assembly of those parts into the organism of choice. In this section, we will discuss the types of components that researchers use to implement gene circuit designs. We will touch on the more basic components that researchers used to construct some of the first gene circuits and focus in more detail on new components being used for synthetic gene circuits, such as CRISPR-Cas9 (CRISPR-associated protein 9) expression systems and RNA-based regulatory parts. Finally, we will review some of the recent advancements in assembling these parts for insertion into a desired host and strategies for tuning part parameters by directed evolution.

The earliest synthetic gene circuits utilized transcription factors from well-studied bacterial operons, such as the ubiquitous IPTG inducible system from the lac operon of *E. coli*. For instance, the repressilator, one of the first synthetic oscillator circuits, utilized three transcriptional repressors: TetR, LacI, and cl from phage λ [5]. While native, protein-based transcriptional regulation has been an invaluable tool for synthetic gene circuit creation, there are some challenges associated with using these transcription factors, such as lack of orthogonality from one another and the host genome. To address many of these challenges, a number of alternative transcription regulators have been developed that are programmable and can be more easily tailored to control the expression of multiple genes simultaneously. Zinc-finger proteins and transcription activator-like effectors (TALEs) were some of the first tools used to create synthetic transcription factors that could, in theory, act on any chosen DNA sequence. Produced by pathogenic bacteria in the genus Xanthomonas, naturally occurring TALEs can enter plant cell nuclei to activate gene expression [48]. TALE proteins contain a central domain composed of a series of tandem amino acid repeats that allow the protein to recognize and bind specific DNA sequences. This central DNA-binding domain can be engineered to recognize specific, user-defined DNA sequences to create orthogonal transcriptional activators. Garg *et al.* [49] developed an algorithm to computationally design synthetic TALEs with little to no off-target activity and used their algorithm to create a set of eight TALEs that were tested in human cells. Li *et al.* [50] recently developed a library of 26 transcription activator-like effector repressors (TALERs) that repress gene expression from specific hybrid promoters through the steric hindrance of components needed for transcriptional initiation. They then used these TALERs to construct cascade circuits and genetic sensory switches in mammalian cells. Zinc fingers are DNA-binding domains found in many eukaryotic transcription factors. By modifying and combining multiple zinc finger domains, zinc finger proteins that target specific DNA sequences can be engineered. Utilizing artificial zinc fingers, Khalil *et al.* [51] created a library of synthetic transcription factors and used these transcription factors to make genetic circuits in yeast.

Following these approaches to programmable transcription regulators came clustered regularly interspaced short palindromic repeat (CRISPR)-based gene expression systems. Originally discovered as a natural immunity system in bacteria, CRISPR has rapidly become one of the most widely used and studied tools in molecular biology. In CRISPR, short RNA molecules called guide RNA (gRNA) are transcribed by a cell to guide a CRISPR-associated (Cas) protein to a DNA target complementary to the gRNA sequence. The most commonly used Cas protein is Cas9 from *Streptococcus pyogenes*, which triggers double-stranded DNA breakage in DNA targeted by the gRNA. While wild-type Cas9 has revolutionized the field of gene editing, a mutant form of the protein lacking its typical nuclease activity called dCas9 ("dead Cas9") has proven to be valuable for synthetic gene circuit implementation. Like the original Cas9 protein, dCas9 can still be targeted to precise DNA sequences where it can impact gene regulation in a myriad of ways. For instance, alone, dCas9 can efficiently inhibit transcription [CRISPR interference (CRISPRi)] in yeast and bacteria when targeted to a gene's promoter sequence [52], [53]. dCas9 can also be fused to specific activation and repression domains, which has allowed researchers to generate dCas9 fusion proteins that can efficiently repress (CRISPRi) or activate [CRISPR activation (CRISPRa)] gene expression in organisms ranging from *E. coli* to human cells [53]–[56] [see Fig. 2(a)].

The ability to separate gRNA and dCas9 fusion protein expression from one other makes CRISPR-based regulation a great method to engineer digital logic circuits. For instance, a basic AND gate can be constructed by driving expression of the gRNA and dCas9 genes with two different inducible promoters so that the output of a third promoter (which requires the gRNA and dCas9 fusion for activation) is only ON when both inducer molecules are present. Demonstrating the power of this type of approach, Liu *et al.* [57] implemented a CRISPR-Cas9-based AND gate to identify bladder cancer cells. In this circuit, Cas9 and gRNA expressions were driven by two different promoters associated with bladder cancer. The guide-RNA targeted a constitutively expressed *lacI* gene that, in turn, repressed expression of an output luminescence gene. Thus, only when both bladder-cancer-associated promoters were activated, the Cas9/gRNA complex could form and cleave the *lacI* gene DNA sequence leading to expression of the output gene.

One of the major potential advantages of using CRISPR-based gene expression is that it is modular in nature. For instance, in theory, any different number of genes can be activated or repressed by a particular dCas9 fusion protein by simply modifying the gRNA sequence to target the gene of interest. This differs from traditional transcriptional regulation where a different transcription factor must be used for each different promoter sequence. As an impressive demonstration of this modularity, Reis *et al.* [58] recently used CRISPR interference to simultaneously repress up to 13 genes in a single cell. Although a powerful tool for synthetic biology, the Cas9 protein has significant limitations including off-target

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activity [59] and potential toxicity to host cells [60]. Decreasing off-target activity and reducing the toxicity of Cas9-based gene expression systems are currently active areas of research. For instance, researchers recently developed a dCas9 variant with reduced toxicity for use in bacterial gene circuits [61]. Similarly, many groups are actively investigating strategies to limit the off-target activity of Cas9, such as using truncated gRNA sequences [62]. While more work is needed to fully overcome challenges associated with Cas9-based gene expression systems, current progress suggests that these systems will continue to improve rapidly and be used in larger, more complex genetic constructs.

Another class of genetic parts that have grown in usefulness over the last few years is RNA-mediated genetic switches. In natural systems, microRNAs (miRNAs) have been recognized as important regulatory molecules that can modulate gene expression in a variety of ways but most commonly inhibit translation by cleaving mRNA or blocking mRNA access to a ribosome [63]. For synthetic gene circuits, RNA-based regulatory parts have multiple potential advantages over protein-based parts. For instance, the expression of RNA parts is thought to be less burdensome on the host cell compared to protein expression [64]. In addition, since many RNA regulation strategies involve posttranscriptional control, RNA switches are particularly useful for eukaryotic organisms as they do not have to enter the nucleus to be effective.

One group of engineered RNA-based parts for synthetic gene circuits is short RNA sequences, known as riboswitches, which respond to small molecules [see Fig. 2(a)]. These parts consist of two domains: 1) an aptamer domain that recognizes a specific small molecule and 2) an actuator domain or expression platform that undergoes a conformation change when the small molecule binds the aptamer. The actuator domain can potentially regulate either transcription or translation, and there have been numerous regulatory mechanisms found for riboswitches in natural systems [65]–[68]. For instance, a riboswitch can regulate gene expression at the posttranscriptional level by inhibiting ribosome binding to an RBS sequence on an mRNA transcript in one conformation and permitting ribosome binding in the other conformation, allowing translation to be controlled by the small molecule inducer. Dixon *et al.* [69] developed a set of riboswitches that respond to nonnaturally occurring molecules and are orthogonal to one another, demonstrating how riboswitches can be engineered as components to control cellular behavior with exogenous molecular inputs.

While riboswitches rely on interactions between small molecules and RNA to regulate translation, another class of RNA-based regulatory parts called riboregulators relies on RNA-RNA interactions to control gene expression [70]. Much like riboswitches, riboregulators can control mRNA translation in response to external input. Specifically, a typical riboregulator consists of a cis-repressed mRNA molecule that cannot be translated unless a transactivating RNA regulator is present to bind the mRNA and expose an RBS on the RNA. Green *et al.* [71] developed a class of riboregulators called toehold switches that greatly improved upon traditional riboregulators by permitting greater flexibility in the design of the RNA molecules composing the regulator. Specifically, toehold switches utilize an RNA toehold to facilitate binding between the target mRNA and the activating trigger RNA, and this toehold-facilitated binding strategy essentially eliminates any sequence constraints in switch design [see Fig. 2(a)]. The sequence flexibility afforded by toehold switches leads to improved dynamic range, orthogonality between regulators, and the ability to rationally program switch behavior compared to previous riboregulators. Putting the impressive characteristics of toehold switches to use, Green *et al.* [72] created "ribocomputing" systems capable of evaluating complex logic functions with as many as 12 inputs. Implementation and application of riboswitches and toe-hold switches for molecular biosensing are covered in more detail in a recent review article [73].

The creation and characterization of new genetic parts for circuit construction are only able to further the field of synthetic gene circuits if researchers can seamlessly select and combine parts in novel ways. Ideally, DNA parts would be modular and standardized to allow different groups to quickly choose parts from a collection to implement their circuit design. One of the first, widely adopted attempts to standardize the construction of genetic circuits from a library of parts was the BioBrick Assembly Standard. In this system, parts (e.g., promoters, RBSs, CDSs, and terminators) called BioBricks exist on part plasmids where they are flanked by multiple restriction sites [74]. Two parts can be combined by digesting each part plasmid with unique restriction enzymes that have compatible sticky ends with one another. In the original BioBrick standard, when two genetic parts are assembled, restriction enzyme cut sites flanking the assembled parts are preserved. This enables additional parts to be readily added in further assembly rounds. A significant downside of the original standard was the formation of an eight base pair scar between combined parts, which prevented the creation of fusion proteins using the standard. This limitation was addressed in some of the advances on the original standard [75].

One of the biggest advancements in the construction of genetic circuits came from the discovery of multiple techniques to assemble many pieces of DNA in simple, single reactions. One technique, Gibson Assembly, made possible the combination of many pieces of DNA in a single reaction by taking advantage of the mutual activity of T5 exonuclease, Phusion polymerase, and Taq ligase at 50C [76]. With this method, different genetic parts could be rapidly combined once they had been polymerase chain reaction (PCR)-amplified with overhangs containing overlapping sequences with the adjacent part.

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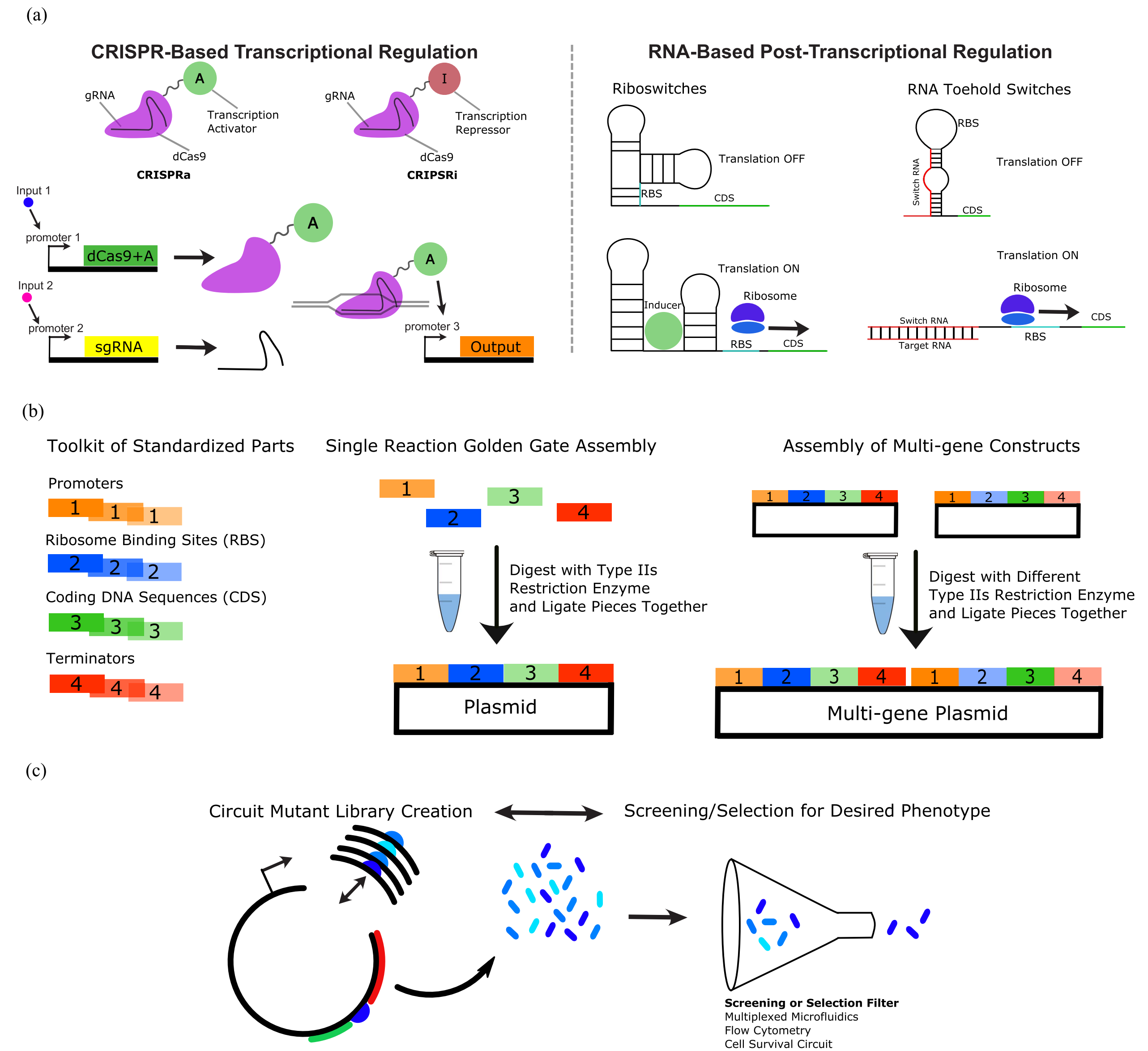

Fig. 2. ***Genetic circuit implementation. (a) Examples of regulatory circuit parts that exhibit modularity and orthogonality and can control gene expression via control of transcription or translation (parts for genetic circuits). Left: examples of how CRISPR and dCas9 can be used to activate (CRIPSRa) or repress (CRISPRi) transcription from user-defined promoter sequences using targeting gRNAs and dCas9 fused to transcriptional repressor or activator domains. Right: basic principle by which riboswitches and RNA toe-hold switches can be used to regulate translation of a given mRNA. Of particular note is that, for toe-hold switches, the design of the switch and target RNA is independent of the RBS and CDS sequence. (b) Overview diagram of MoClo kits and genetic circuit construction via Golden Gate Assembly (modular assembly of genetic circuits). This strategy enables the assembly of multiple different TUs from a library of standardized parts in a single plasmid construct using a series of simple one-pot reactions. (c) Simple workflow for using directed evolution to tune the behavior of a genetic circuit (genetic circuit tuning by directed evolution). A library of circuit mutants is made by pseudorandomly mutating one or more circuit components and assembling these components in a single construct. After library creation and transformation into the desired organism, the library can be screened for the desired behavior. Multiple rounds of mutagenesis and selection can be done to further tune and optimize circuit behavior.***

While an incredibly powerful tool overall, Gibson Assembly, does have some weaknesses, such as the need to PCR amplify each part, which could lead to undesirable mutations and the difficulty of assembling very small pieces of DNA due to the exonuclease activity. Another cloning strategy, Golden Gate Assembly, developed around the same time dramatically accelerated the creation of standardized, easy-to-use, genetic part kits [see Fig. 2(b)]. Golden Gate Assembly takes advantage of type IIS restriction endonucleases that cut DNA at a distinct point away from their recognition sequence. This allows parts to be designed, which are digested with the same enzyme, but that have unique overhangs after digestion allowing many parts to be digested and ligated in a single reaction

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mixture [77], [78]. By standardizing the user-defined overhangs and utilizing different type IIS restriction enzymes, a significant number of modular cloning (MoClo systems) have been developed for use in organisms spanning bacteria [79]–[81], yeast [82], [83], plants [84], [85], and mammalian cells [86].

As an example of a typical MoClo workflow, we look at the yeast MoClo Toolkit developed by Lee *et al.* [82] in the laboratory of John Dueber. This toolkit exemplifies many of the characteristics researchers need to take into account in order for their MoClo platform to be used by other researchers. For example, in this yeast toolkit, the authors identify eight unique part types (types 1–8) with predefined flanking overhangs. Each part type has as a recommended function associated with it (e.g., type 3 parts are CDSs and type 4 parts are transcription terminators), but, in theory, a researcher could create a new part of any type by adding the correct overhangs to the part. The kit employs a GFP-dropout strategy to facilitate screening of correctly assembled constructs, and their system uses alternating type IIS restriction enzymes and selection markers to make the construction of multigene constructs simple and easy. Importantly, the kit is readily available on Addgene and has 100+ basic parts making it accessible. The utility of this kit has further been improved by the creation of compatible part libraries by other groups. Specifically, Shaw *et al.* [83] created 42 parts for building tunable GPCR-based biosensors that are designed for use with the yeast MoClo toolkit.

Despite these advances in DNA part standardization, a major challenge in the creation of synthetic gene circuits is that genetic parts that have been characterized in one context will not necessarily behave the same in a different context (i.e., different host cells, media compositions, temperatures, and so on) One way synthetic biologists have combated this problem is by applying directed evolution techniques from protein engineering to synthetic gene circuits [see Fig. 2(c)]. For challenging problems with many difficult to predict nonlinear interactions, mimicking the process of natural selection in biology can help researchers to find approximate solutions. For instance, computer scientists occasionally use genetic algorithms inspired by evolutionary biology to find numerical solutions to challenging nonlinear problems [87].

To apply directed evolution to the optimization of synthetic gene circuits, researchers can use the computational circuit-design methods discussed in Section II to identify circuit components that may need to be tuned to achieve the desired behavior. For instance, in developing a synthetic switch that is able to transition reliably between ON- and OFF-states at desired inducer concentrations, researchers may choose the promoter sequence for one of the genes in the circuit as a target for mutant library creation based on circuit modeling predictions. Recently, Tominga *et al.* [88] developed a workflow for the directed evolution of synthetic switches in yeast, demonstrating the power of evolution approaches for synthetic gene circuits. One of the main obstacles in applying directed evolution to synthetic gene circuits has been difficulty screening for circuits with complex dynamic phenotypes. For example, screening a large mutant library of circuits for AND gate behavior is easier than screening a library of circuits for oscillatory behavior. A workflow was recently developed for screening mutant libraries of dynamic gene circuits that operate at the population level, such as synchronized oscillators, using multiplexed microfluidics to simultaneously monitor gene expression over time in many library strains at once [89]. In another advance in screening libraries of synthetic gene circuits, Luro *et al.* [90] recently developed a method for isolating single cells after high-throughput time-lapse microscopy. Using their technology, they were able to identify multiple precise synthetic gene oscillators from a mutant library, showcasing the utility of their technology for directed evolution approaches in synthetic biology. Together rational, computational design approaches combined with standardized genetic part kits and supplemented by directed evolution approaches for tuning circuit behavior will continue to accelerate the implementation of more complex synthetic gene circuits.

IV. CIRCUIT APPLICATIONS

Due to the advances in circuit design and implementation discussed so far, the number of gene circuits being developed for real-world applications is burgeoning. While the specific application areas for synthetic gene circuits are incredibly diverse and often overlap each other in complex ways, there are a few broad categories that encompass much of the progress that has been made in taking gene circuits out of the lab and into the real world. In this section, we present examples of synthetic gene circuits being designed for: 1) living therapeutics for cancer therapy; 2) whole-cell biosensors; and 3) biomanufacturing cell factories. As some of these application areas, such as biomanufacturing, predate the formal acknowledgment of synthetic gene circuits as a field, we focus on examples where the use of more complex regulatory behavior enabled by advances in circuit design and implementation has led to innovative strategies in these areas.

A. Synthetic Gene Circuits for Living Therapeutics

Compared to traditional, chemical-based therapeutics for disease, therapies made from and delivered by engineered living organisms offer many advantages due to their potential for dynamic, environment-specific behavior. For instance, if we consider a system with two states, diseased and healthy, synthetic gene circuits can enable an engineered cell to distinguish between these two states and activate a specific program of gene expression depending on the state, much like a computer [91].

One area where synthetic gene circuits are making considerable progress is cancer therapy. Both engineering of human cells (e.g., T-cells) and nonpathogenic strains of bacteria have recently shown considerable promise in

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treating certain cancer types. Both of these therapeutic strategies can stimulate the immune system to selectively attack cancerous cells. To start, we describe work from our group and others on using engineered bacteria for cancer therapy that demonstrates: 1) why bacteria may be good vehicles for delivering therapy to solid tumors; 2) how synthetic gene circuits can be used to dynamically control the release of a therapeutic and the size of the bacterial population delivering the therapy; and 3) how additional circuitry could help solve challenges related to gene circuit stability and exogenous control of therapeutic release.

The identification of tumor-colonizing bacteria and their potential antitumor effects dates back over a century [92], and recent research has further reinforced the observation that many different types of bacteria can colonize and grow within a range of solid tumor types [93]–[96]. Given this abundance of research on the ability of bacteria to colonize solid tumors and challenges associated with some chemotherapeutics penetrating into solid tumors, multiple groups identified the potential for bacteria to be used as vehicles for therapy delivery.

One system was recently developed that enables bacteria to release different therapeutic payloads within tumors while preventing unchecked growth of the bacterial population [see Fig. 3(a)]. This system, the synchronized lysis circuit (SLC), utilizes a naturally occurring bacterial phenomenon, QS, to synchronize therapeutic production and release from a tumor-colonizing population of bacteria [97]. Specifically, the circuit consists of three primary genes: 1) an N-Acyl homoserine lactone (AHL) synthase, LuxI; 2) a lysis gene derived from a phage; and 3) a gene encoding an anticancer therapeutic. Each of these genes is driven by the pLux promoter, which is activated by the AHL-LuxR transcription factor complex. At low population levels, cells produce low levels of AHL due to leaky expression from the pLux promoter. AHL is free to diffuse among the cells, and as the population grows, the local concentration of AHL increases in proportion to the number of cells. Once the population size reaches a quorum threshold size, AHL concentrations become sufficient to fully activate the pLux promoter via LuxI-mediated positive feedback, which leads to significant production of the lysis gene and therapy gene. Production of the lysis gene causes the majority of cells in the population to lyse and release the therapy molecule, while the few remaining cells are able to grow up again perpetuating cells of growth, therapy production, and lysis.

As this mechanism of delivery relies on bacteria lysing and releasing their intracellular contents, it is readily amenable to releasing diverse types of therapeutic molecules as potentially difficult to engineer secretion mechanisms that are unnecessary. In addition, since this circuit regularly culls the bacterial population size, it addresses some of the major safety concerns associated with the containment of engineered therapeutics. Since its original development, the SLC has been utilized to deliver nanobodies targeting an anti-phagocytotic receptor that is commonly overexpressed in cancer [98] and immune checkpoint-inhibiting nanobodies [99].

While the initial design of the SLC has proven success expressing different types of therapeutics in mouse models, there are multiple challenges associated with this approach including the propensity of the lysis circuit to mutate over time and the lack of exogenous control over the lysis circuit behavior. To address stability issues of the SLC and extend its functional lifetime, Liao *et al.* [29] recently developed new genetic circuitry across multiple engineered strains of bacteria [see Fig. 3(a)]. In this rock-paper-scissors (RPS) circuit, three strains of *E. coli* are engineered to each express a unique bacterial toxin known as a colicin. Each strain is also designed to express immunity to two out of the three colicins in the circuit while remaining susceptible to the third colicin. This creates a simple network of interactions where three strains can be cycled by sequentially introducing a strain that kills the previous strain. This circuit was used to extend the functional lifetime of the SLC by introducing a copy of the SLC into each strain making up the RPS circuit. As soon as the SLC becomes nonfunctional in one strain due to mutation, this strain can be replaced by the next strain in the RPS circuit and so on, allowing the SLC to function over much longer time scales than possible in a single strain alone. While this RPS circuitry has yet to be applied with the SLC *in vivo*, it represents a promising strategy for extending the lifetime of genetic circuits in real-world applications.

In another advancement on the original SLC circuitry, Miano *et al.* [28] recently developed a mechanism to control the dynamics of the SLC using an external inducer molecule [see Fig. 3(a)]. This circuit, the inducible SLC (iSLC), makes use of the QS machinery from the photosynthetic bacterium *Rhodopseudomonas palustris*, which relies on the external, plant-derived inducer molecule, p-coumaric acid (pCA) to produce its QS molecule, p-coumaroyl-HSL (pC-HSL). When this pCA-inducible QS system was used for the SLC instead of the original Lux system, the dynamic behavior of the SLC could be controlled in one of three different states depending on the concentration of pCA. For low pCA concentrations, insufficient pC-HSL is produced to activate lysis, and the population grows normally. For intermediate levels of pCA, the SLC functions as described previously, with synchronized cycles of growth, therapy production, and lysis. Finally, for high pCA concentrations, the circuit acts as a kill switch, with lysis protein production high enough to prevent significant growth of the population. The ability to control the dynamics of an engineered therapy strain with a nontoxic inducer, such as pCA, along with the kill-switch control capability to quickly eliminate the engineered strain with high levels of pCA, makes this circuit a promising strategy for bacterial-based cancer therapeutics.

Engineered chimeric antigen receptor (CAR) T-cells represent another example where synthetic gene circuits have been successfully applied to combat cancer. In brief, CAR T-cell therapy approaches work by modifying a cancer

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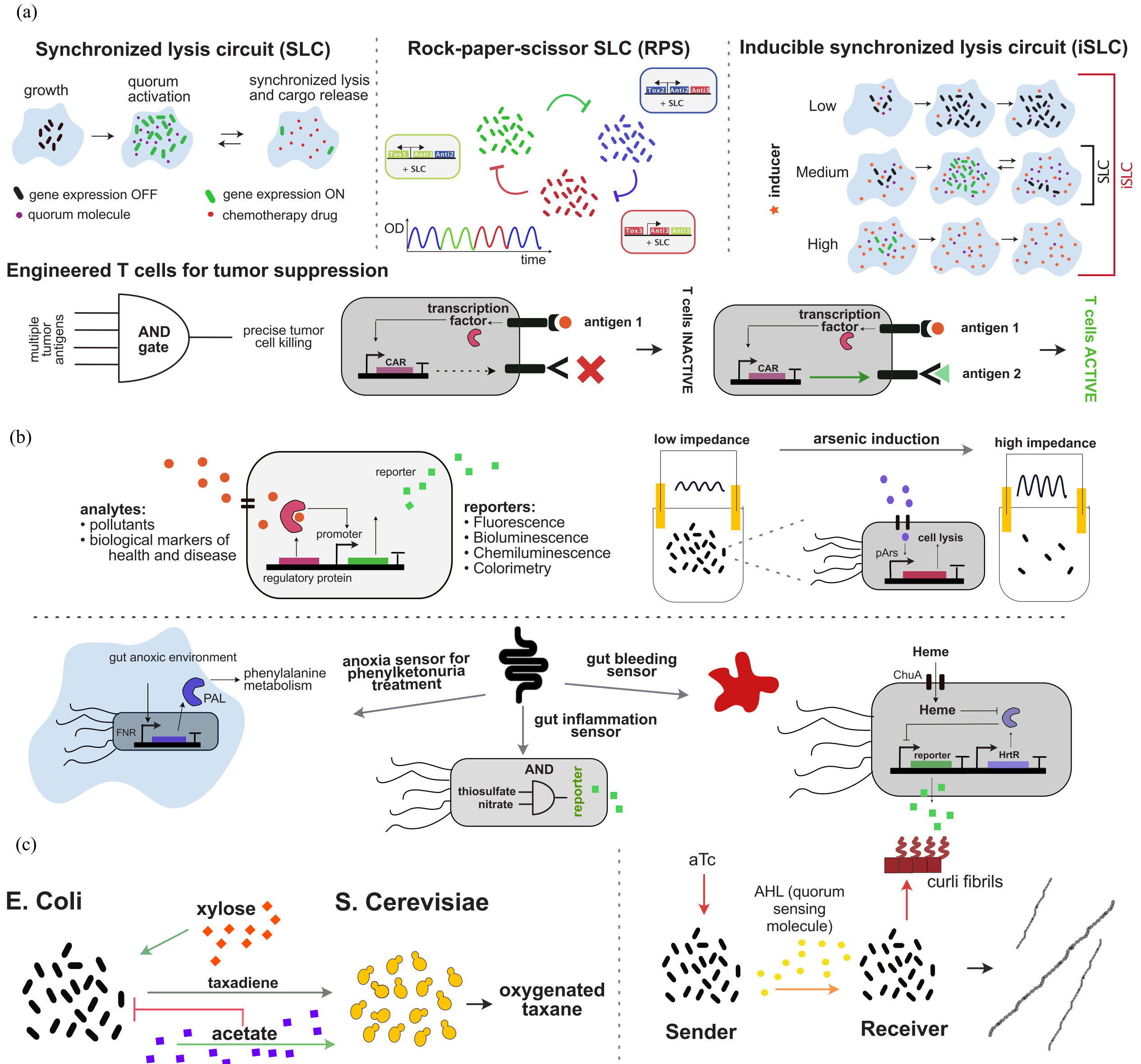

Fig. 3. *Genetic circuit applications. (a) Illustrations of different genetic circuits for the creation of living therapeutics for cancer treatment (living therapeutics for cancer treatment) (living therapeutics for cancer treatment). The top left figure shows the behavior of the SLC that can deliver bursts of therapy payload release in an oscillatory fashion [97]. The following figure shows a synthetic community of three populations that can kill each other with toxins in an RPS pattern [29]. Each population is also equipped with the SLC for cargo release. The last figure shows an iSLC that has different dynamics as a function of inducer concentration [28]. The bottom figure shows a combinatorial antigen sensing genetic circuit for selective activation of T-cells for tumor suppression [100]. (b) Top left figure shows the general circuit design for the creation of whole-cell biosensors. The top right figure shows an example of a whole-cell sensor combined with impedance detection for arsenic sensing. The bottom figure shows three examples of whole-cell biosensors for biomarker sensing in the gut: anoxia (left [101], gut inflammation (center) [102], and heme (right) [103] (whole-cell biosensors). (c) Two examples of whole-cell biosensors for biomanufacturing. Left: engineered two strains ecology for the production of oxygenated taxane using a cross-feeding design [104]. Right: two strain community that communicates through QS for the production of curli fibrils biofilms [105].*

patient's native T-cells with a specific receptor that allows the T-cells to identify and attack cancerous cells [106], [107]. One major challenge associated with CAR T-cell-based therapy is ensuring specificity for cancer cell killing to ensure safety.

The ability to create relevant genetic circuits for improving the specificity of CAR T-cell therapy was dramatically accelerated by the creation of synthetic Notch (syn-Notch) receptors. Syn-Notch receptors are engineered proteins based on the naturally occurring Notch receptor. The wild-type Notch receptor consists of an extracellular domain that recognizes Delta family proteins on other cells and an intracellular domain that acts as a transcriptional regulator. Upon binding of the appropriate ligand to the extracellular domain, an intramembrane proteolytic domain cleaves part of the Notch receptor, freeing the

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intracellular component of the protein, so it can enter the nucleus and activate transcription. Morsut *et al.* [100] found that both the extracellular and intracellular domains on Notch could be replaced with user-defined domains to create receptors that bind relevant targets and drive desired programs of gene regulation following proteolysis of the intramembrane domain [see Fig. 3(a)]. Since the extracellular receptor domain and intracellular regulator domain can be swapped independently, different syn-Notch receptors orthogonal to one another can be expressed within the same cell. Roybal *et al.* [108] demonstrated the utility of syn-Notch receptors for genetic circuits by using them to implement AND gate circuits in CAR T-cells. Specifically, they used a syn-Notch receptor that recognized one antigen and activated the expression of a CAR that recognized a separate antigen. Thus, the T-cells would only kill cells where both antigens were present (AND gate behavior).

While syn-Notch receptors represent a way to use immune cells to distinguish between normal and cancerous cells, there is also research into directly modifying cancer cells with genetic circuits that trigger cell death. A genetic classifier circuit is one that executes the desired program of gene expression in a cell if and only if a user-defined set of endogeneous inputs is present in that cell. It is now well-documented that many cancer types have specific, aberrant expression of miRNA, small noncoding RNA molecules that impact gene expression by impacting stability and translation of specific, complementary mRNA molecules [109], [110]. Given this difference in miRNA expression profile between healthy and cancerous tissue, miRNAs are a promising endogeneous input for classifier circuits that selectively identify cancer cells. Xie *et al.* [111] developed a multi-input miRNA-based logic circuit to identify cancer cells and showed that it could be used to trigger apoptosis (i.e., programmed cell death) in the HeLa cancer cell line without affecting non-HeLa cells. To further advance the ability to design miRNA-based classifier circuits, Mohammadi *et al.* [112] developed a computational workflow for developing biologically feasible classifier circuits. They validated their approach by showing that they could, in theory, design accurate cancer cell classifiers for numerous cancer subtypes based on publicly available miRNA datasets. In a different advancement in computational methods for classifier circuit construction, Becker *et al.* [131] developed an approach for finding optimal classifier circuits using miRNA datasets and Answer Set Programming, a declarative programming strategy used for challenging search problems. Showing the type of work that will be needed to translate these circuits to the clinic, Dastor *et al.* [113] demonstrated an experimental workflow for introducing and testing miRNA classifier circuits in preclinical, animal models of cancer. Specifically, they delivered an miRNA classifier into an animal model of disseminated colorectal cancer metastasis using an Adeno-associated virus (AAV) vector delivery platform and highlighted the importance of detailed *in vivo* characterization of synthetic gene circuits in moving toward clinical translation of these circuits.

Synthetic gene circuits in human cells have also been applied to address many diseases outside of cancer, such as diabetes. For instance, human cells have been engineered to release the blood-glucose regulating hormones insulin and GLP-1 in response to stimulation with radio wave frequencies or light, respectively [114], [115]. In a recent advancement in genetic circuits for diabetes, Xie *et al.* [116] engineered a human cell line that could sense blood glucose levels and respond to abnormal levels by producing insulin and GLP-1 in a dose-dependent fashion. This closed-loop circuit was able to ameliorate glucose dysregulation in a mouse model of type 1 diabetes and demonstrates an important step toward creating circuits that are able to tune their output in response to physiologically relevant levels of an input molecule.

B. Synthetic Gene Circuits for Whole-Cell Biosensors

A growing field in synthetic biology is the creation of whole-cell biosensors for the detection of environmental pollutants or as affordable point-of-care diagnostic technology. The general mechanism of a whole-cell biosensor consists of the detection of a specific analyte of interest, followed by signal amplification and conversion into an electrical or optical readout [117]. The use of whole-cell biosensors, compared to *in vitro* diagnostics, has several advantages, such as low production cost due to the fast growth and self-replicating properties of microorganisms and the possibility of constant monitoring for an extended period of time without interruption. In addition, microorganisms can be found in a variety of ecosystems, making them a rich natural resource for specific genes and pathways that can detect a wide variety of analytes.

One of the most promising application areas for whole-cell biosensors is the real-time detection of relevant compounds within the human body [118]. For this purpose, the probiotic bacteria strain *Escherichia coli* Nissle was engineered to detect bleeding during gastrointestinal transit by expressing a luminescent protein in the presence of heme. The cells were packaged in a capsule together with miniaturized electronics to detect the bioluminescent readout and then wirelessly transmit sensor information to an external device [103]. The same probiotic strain was also used to design a whole-cell biosensor for gut inflammation by engineering an AND gate genetic circuit, which can simultaneously detect thiosulfate and nitrate biomarkers, greatly improving diagnosis specificity [102] [see Fig. 3(b)]. The use of *Escherichia coli* Nissle has also been recently demonstrated to create synthetic living therapeutics for the treatment of phenylketonuria, which is characterized by the inability to metabolize phenylalanine [see Fig. 3(b)]. The strain was engineered to express phenylalanine-metabolizing enzymes in response to the detection of anoxic conditions found in the mammalian gut [101], demonstrating the power of whole-cell

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biosensors for living therapeutics [see Fig. 3(b)]. While probiotic bacteria have been widely used as the host strains for whole-cell biosensor applications in the gut, genetically engineered human cells have been used for personalized treatments of allergies. In particular, mammalian cells were engineered to detect histamine production from basophils extracted from patients' whole-blood cells. The genetic circuit is composed of a membrane receptor, which responds to the presence of extracellular histamine by activating a signaling pathway, which results in the production of a fluorescent readout [119].

Another area where whole-cell biosensors have demonstrated remarkable results is pollutants detection. Currently, the most common methods to investigate soil and water pollution rely on gas/liquid chromatography or spectroscopy approaches that are time-consuming and expensive [120]. Therefore, the use of whole-cell biosensors is particularly appealing due to their simplicity and low cost. A recent study developed a tunable array of whole-cell biosensors for the detection of phenols, benzene, and toluene that are carcinogenic xenobiotics found in polluted water [121]. The authors designed this biosensor based on the natural ability of the MopR genetic system to degrade phenol, giving a concrete example of how the variety of microorganisms in nature can be exploited for the design of biosensors. Heavy metal contamination represents one of the most widespread pollutants found in soil and water across the world, making it a popular target for whole-cell biosensors [117]. The simplest type of genetic circuit for heavy metal detection consists of using a heavy-metal inducible promoter that drives the expression of a reporter protein, such as GFP. The main challenge associated with this design is the low signal-to-noise ratio. In order to solve this problem, researchers have been focusing on three strategies: lowering the background noise by insulating and uncoupling the expression of the output from the basal expression of the other modules, increasing the output by signal amplification, and digitalizing the signal using toggle switches and logic gates [122]–[124]. Recently, more creative and complex circuit designs have been proposed for the construction of whole-cell biosensors. One example consists of a population-based genetic circuit that induces the cells to lyse when arsenic is detected in the surrounding medium. The drop in population density is detected by monitoring the cell culture impedance over time, without the need for fluorescence detection [125] [see Fig. 3(b)]. In order to translate these designs into practical technologies in the field, multiple challenges need to be addressed, including signal noise due to the population phenotypical heterogeneity and stochastic protein expression, crosstalk in multiple analytes' sensors, improved monitoring approaches (microfluidics and microelectronics), and biosafety.

C. Synthetic Gene Circuits for Biomanufacturing

One of the most promising areas of synthetic biology is engineering synthetic microbial consortia where multiple organisms, from the same or different species, are engineered to interact with each other to perform complex behaviors [126].

Engineering an entire community, instead of a single population, is particularly advantageous in bioproduction applications where microorganisms are used to produce metabolites of interest by reconstructing complex metabolic pathways. In this scenario, one major obstacle is the significant metabolic burden associated with expressing many enzymes from a pathway, which can make engineering full pathways in single cells unfeasible. This is demonstrated by Zhou *et al.* [104] who divided the pathway for synthesizing the acetylated diol paclitaxel precursor in two engineered populations of *Escherichia coli* and *Saccharomyces cerevisiae*, allowing them to take advantage of the fast growth of prokaryotes while also benefiting from the more advanced protein expression machinery of eukaryotes [see Fig. 3(c)]. In this study, the authors also described a strategy to achieve long-term coexistence of the two strains by engineering a mutualistic interaction between the two populations. This is achieved by providing xylose as the sole carbon source in the media, which can be metabolized by *E. coli* but not *S. cerevisiae*. When *E. coli* uses xylose, it excretes acetate, which, in turn, inhibits its own growth. On the other hand, *S. cerevisiae* cannot use xylose directly but can metabolize acetate; therefore, it relies on the presence of *E. coli* in the media for its own growth and survival.

The same concept of dividing a pathway across multiple strains was demonstrated in another study where the authors engineered four populations of yeast for the saccharification and fermentation of cellulose to ethanol [127]. Cellulose is considered one of the most abundant renewable carbon sources that can be enzymatically degraded for ethanol production, but its usage is currently limited by the high cost of the cellulases needed to complete the degradation process. Therefore, the authors proposed directly using genetically modified microorganisms by engineering each strain to carry out a reaction in the cellulose degradation process, leading to a modular system, which could easily be tuned by adjusting the ratio of different populations in the consortium.

Another key advantage of using microbial consortia is the ability to engineer precise spatial interactions through cell-to-cell communication, allowing for the production of complex functional biomaterials of different sizes and patterns. This was demonstrated in a previous study in which an *E. coli* consortium was engineered to produce curli fibrils of predetermined patterns by controlling their expression with QS and inducible systems [105] [see Fig. 3(c)]. The system consisted of a "sender" population, which produced the signaling molecule if the tetracycline inducer was present, and a "receiver" population, which could detect the signaling molecule and produce curli fibrils in response. The authors demonstrated that this system was able to produce environmentally switchable conductive biofilms and cofibrils assembled with quantum dots.

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Overall, although many promising applications of microbial consortia for bioproduction and bioremediation have been demonstrated, the field is still in its infancy. Current progress in sequencing technologies and gene mining will enable us to access the richness of natural communities of microorganisms to discover new pathways and enzymes, which will greatly expand the tools for designing synthetic consortia.

V. DISCUSSION

Since synthetic biology's outset, quantitative, math-based approaches to genetic circuit design have been crucial to the field's success. With the increased number of well-characterized genetic parts and a better understanding of gene regulation, computational approaches are more poised to reliably inform gene circuit design than ever before. Automated circuit design software programs, such as *Cello*, have made great strides in the abstraction and standardization of circuit design, while other differential equation-based approaches, such as agent-based modeling, have helped researchers understand the behavior of dynamic intercellular circuits. As the field moves forward, tools from machine learning are likely to become more prevalent in the circuit design workflow, as they have the potential to integrate large experimental datasets and rapidly test many large circuit variants *in silico* to facilitate the construction of larger circuits with more distinct, integrated components. The future of circuit design methods will most likely be a synthesis of more coarse-grained black-box approaches and fine-grained, detail-oriented approaches that come together to provide meaningful predictions of circuit behavior.

While analogies to electrical engineering were crucial in solidifying the field of synthetic biology, continued progress in the field will be spurred on by expanding focus to what makes biological systems different from systems in other engineering fields. Much like Maxwell's equations that provide the basic framework for engineering electrical systems, there seem to exist similar biological "laws" that need to be recognized to successfully engineer more complex biological systems in different organisms. For instance, "growth laws" describing how *E. coli* gene expression is predictably impacted by growth rate can serve to improve the design of circuit architectures that exhibit growth-rate independent behavior [128], [129]. Similarly, increased focus on models that take into account interactions between synthetic genetic constructs and the host organism genome will be crucial for predicting genetic circuit behavior [130]. The discovery of new regulatory "laws" across different organisms and conditions has and will continue to rely heavily on quantitative analysis of biological systems, making this an excellent area for data scientists and physicists to engage in.

In the past, one of the largest barriers to entry in synthetic biology was the difficulty of implementing genetic circuit designs. Often, the required genetic parts for a design did not exist, and if they did, they behaved unpredictably or were too difficult to efficiently assemble and place in a host organism. While still far from perfect, the field has made incredible strides in strategies for circuit implementation. Now, many modular, predictable orthogonal circuit parts have been developed to engineer circuits in many different host organisms. Similarly, modular molecular cloning strategies and accessible part toolkits have dramatically reduced the time and effort needed to assemble more complex genetic constructs. Together, these innovations have made genetic circuit research more accessible to scientists from other disciplines than ever before. Importantly, these advances also allow researchers, such as computer scientists and electrical engineers, more time to apply their expertise to this field rather than getting bogged down in molecular biology troubleshooting.

With the vast improvements in the design and implementation of genetic circuits, real-world applications for these circuits continue to blossom in different areas. While increasing circuit complexity in the lab will continue to inform us on how to better engineer living systems, going forward, it is likely that the most useful circuits for real-world applications will eschew complexity for stability and predictability. As many synthetic biologists can confirm, the last two decades of research have revealed countless reasons why a circuit might not work how it should. The lessons learned from challenges in engineering predictable behavior in living systems should be synthesized to continue developing methods to make circuits more robust. Rather than using the array of knowledge obtained from decades of tinkering in the lab to develop more and more complex circuits to address real-world problems, genetic engineers should focus on using this knowledge to make simple circuits extremely reliable. It is obvious that some real-world problems will require genetic circuits that encode complex multilayered logic or intricate dynamic behaviors, but all industrial and medical applications will require genetic circuits that behave with the utmost predictability.

VI. CONCLUSION

In summary, advances in the ability to readily assemble synthetic genetic constructs have dramatically lowered the barrier for scientists and engineers outside of biology to get involved in this field. Furthermore, with increasing focus on engineering cells to perform more complex functions and a desire to further expand synthetic biology to mammalian cells, state-of-the-art *in silico* modeling techniques from computer scientists, physicists, and engineers will be essential for the further advancement of synthetic genetic systems. Finally, while many scientists have begun using synthetic gene circuits to tackle real-world problems in the past decade, it is clear that the full potential of synthetic biology is far from tapped. In the coming years, increased accessibility to genetic engineering should further permit researchers traditionally outside of the field of biology to tackle existing challenges in the design of genetic circuits, which will, in turn, increase the range of real-world problems that these circuits can solve.

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APPENDIX
GLOSSARY OF BIOLOGICAL TERMS

The following is a list of definitions for key biological terms used throughout the article.

1) *Deoxyribonucleic Acid (DNA):* A molecule that consists of a double helix composed of two polynucleotide chains that carry the genetic instructions for the function and reproduction of all organisms.
2) *Messenger Ribonucleic Acid (mRNA):* A single-stranded molecule of RNA that corresponds to the sequence of a gene and is read by a ribosome in order to synthesize the corresponding protein.
3) *Promoter:* A sequence of DNA where proteins bind to initiate transcription of the downstream region into RNA.
4) *Transcription:* The process of RNA polymerase creating an RNA molecule from a DNA template.
5) *Translation:* The process of creating a protein from an mRNA template carried out by ribosomes.
6) *Central dogma:* The central dogma defines the flow of information in living organisms. It states that DNA contains the instructions that are first transcribed into mRNA and eventually translated into proteins. In particular, one triplet of base pairs is translated to a specific amino acid.
7) *RNA polymerase:* An enzyme that synthesizes RNA from a DNA template.
8) *Coding DNA Sequence (CDS):* DNA sequences encoding the instructions to make proteins or regulatory RNA molecules.
9) *Transcription terminator:* A DNA sequence that marks the end of a gene and causes transcription to terminate by interfering with RNA polymerase.
10) *Genetic circuit:* A natural or synthetic regulatory network of interacting RNA, proteins, and metabolites whose components and interactions are specified by instructions encoded as DNA sequences. Synthetic gene circuits often use biological parts to implement logic functions in living cells similar to the logical functions carried out in electronic circuits.
11) *Transcription factor*: Proteins that play a role in controlling the rate of mRNA production by RNA polymerase from specific genes. Some transcription factors (activators) increase the rate of mRNA production for a gene, while others (repressors) decrease the rate of mRNA production by a gene.
12) *Operator:* In prokaryotes, a DNA sequence where a specific transcription repressor can bind to prevent mRNA transcription by RNA polymerase.
13) *Ribozyme:* An enzyme made of RNA instead of protein that can catalyze specific reactions within cells.
14) *Ribosomes:* Cellular components made of protein and RNA where the translation of all cellular proteins is carried out.
15) *Quorum Sensing (QS):* A cell-to-cell communication mechanism in bacteria where small molecules that can diffuse freely between cells coordinate gene expression in cellular populations.
16) *Plasmid:* An extra-chromosomal, circular piece of DNA that replicates independently. Plasmids typically exist in prokaryotic cells and are important tools for synthetic gene circuit construction in all classes of organisms.
17) *Polymerase Chain Reaction (PCR):* A method for exponentially amplifying specific DNA sequences using specific oligonucleotide primers that flank the DNA region of interest. Advancements in using PCR have dramatically improved the ability to engineer genetic constructs.
18) *Gibson assembly:* A common method used for the isothermal assembly of many DNA pieces in a single reaction.
19) *Golden gate assembly:* A method for assembling multiple DNA pieces in a single reaction that takes advantage of type II restriction enzymes. As this method easily allows for standardization of different biological part types, it has been crucial to the development of MoClo toolkits for making genetic circuits.
20) *Restriction enzyme:* An enzyme that cuts DNA at specific sequences. Restriction enzymes have been pivotal to genetic engineering since the field's inception.
21) *Zinc-finger proteins:* Proteins consisting of DNA-binding domains called zinc fingers. Importantly, zinc-finger domains can be modified to target specific DNA sequences enabling their use in engineered transcription factors.
22) *Transcription Activator-like Effectors (TALEs):* Proteins secreted by some pathogenic bacteria to infect plants and activate gene expression. These proteins can be engineered to target user-defined sequences for use as versatile transcription activators in genetic circuits.
23) *Ribosome Binding Site (RBS):* A short nucleotide sequence in prokaryotic mRNA transcripts recognized by ribosomes to initiate translation.
24) *Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR):* A family of DNA sequences found in prokaryotes that play a role in natural defense against viruses and other foreign DNA. The discovery and characterization of natural CRISPR systems led to the development of biological tools for targeted DNA editing and gene expression silencing.
25) *Guide RNA (gRNA):* A piece of RNA responsible for guiding Cas enzymes to particular DNA sequences that are complementary to the gRNA.
26) *Cas9:* A protein with nuclease activity that cuts DNA at specific DNA sequences and is guided to its target by a gRNA molecule.
27) *dCas9:* An altered version of the Cas9 protein without DNA-cutting activity. By fusing dCas9 with proteins that impact gene regulation, dCas9 has been used as a tool to regulate gene expression in biological circuits.
28) *CRISPRi:* A technique for using dCas9 either alone or fused to a transcriptional repressor to repress gene expression from a user-defined DNA sequence.

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29) *CRISPRa:* A technique for using dCas9 either fused to a transcriptional activator to increase gene expression from a user-defined DNA sequence.
30) *Riboswitch:* A regulatory sequence in some mRNA molecules that changes structure upon binding to a specific molecule, which can either turn off or turn on gene expression.
31) *Riboregulator:* An RNA-based regulatory part that can control gene expression. Riboregulators typically consist of a cis-repressed mRNA molecule that cannot be translated unless a transactivating RNA regulator is present to bind the mRNA.
32) *RNA toe-hold switch:* A type of riboregulator with improved sequence flexibility that enabled genetic circuits to be created with many RNA inputs.
33) *MicroRNA (miRNA):* Nonprotein coding RNA molecules that regulate gene expression by interacting with specific, complementary mRNA sequences.
34) *CAR T-cells:* Engineered immune cells (T-cells) that have been modified with a receptor designed to target specific, diseased cells in immunotherapy. ■

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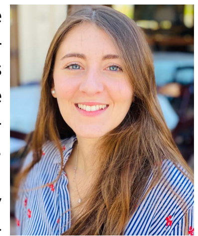
ABOUT THE AUTHORS

Andrew Lezia received the B.S. degree in biomedical engineering with a minor in electrical engineering from Washington University in St. Louis, St. Louis, MO, USA, in 2017. He is currently working toward the Ph.D. degree in bioengineering at the University of California at San Diego, La Jolla, CA, USA, in Dr. Jeff Hasty's Lab, with a focus on synthetic biology.

His current research interests include designing and creating population-level synthetic gene circuits, developing technology for screening-engineered cellular dynamics, and exploiting phenotypic heterogeneity in microbial populations.



Arianna Miano received the M.Eng. degree in biomedical engineering from Imperial College London, London, U.K., in 2018. She is currently working toward the Ph.D. degree at the Bioengineering Department, University of California at San Diego, La Jolla, CA, USA.

Her research in the Hasty Lab, University of California at San Diego, focuses on engineering population dynamics in bacterial communities.

Jeff Hasty is currently a Physicist with the Department of Bioengineering and the Division of Biology, University of California at San Diego, La Jolla, CA, USA. His research focuses on the development of engineering principles for the design of gene circuits in synthetic biology.

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