Advances and Future Challenges in Synthetic Gene Circuits: Postprint

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Abstract

Genetic circuits are dynamic regulatory systems that organisms utilize to control their own life processes. Guided by engineering design principles, artificial genetic circuits involve the simplification and reprogramming of natural gene regulatory circuits, as well as the introduction of artificial rules that do not exist in nature. Artificial genetic circuits are composed of genetic switches, biological oscillators, logic gates, and other components to execute various regulatory functions. The diverse design and construction of artificial genetic circuits have not only greatly advanced our understanding of the fundamental principles underlying life regulation, but have also further enriched the toolkit for transforming and recreating natural biological systems, providing novel solutions for practical demands in fields such as medicine and health, agricultural environment, and industrial fermentation. Although substantial research achievements have been made in the field of artificial genetic circuits over the past two decades, the numerous complex biochemical reactions and signal transduction pathways inherent in cells present challenges for designing and assembling genetic circuits with more sophisticated functions. Accordingly, achieving predictable design and assembly of complex genetic circuits within microscopic cells and ensuring robust functionality of these circuits under complex in vivo and in vitro environments will become the key central questions and significant challenges that must be overcome in artificial genetic circuit research in the forthcoming years.

Full Text

Abstract

Genetic circuits are dynamic regulatory systems that control life processes in living organisms. Guided by engineering design principles, synthetic genetic circuits simplify and reprogram natural genetic regulatory networks, and even introduce artificial rules that do not exist in nature. These circuits consist of various components such as genetic switches, biological oscillators, and logic

gates to execute diverse regulatory functions. The design and construction of a wide variety of synthetic genetic circuits have not only greatly advanced our understanding of the fundamental principles of life regulation, but have also enriched our ability to modify and recreate natural biological systems, providing novel solutions for practical needs in medicine, agriculture, environmental management, and industrial fermentation. Although the past two decades have witnessed fruitful research achievements in synthetic genetic circuits, the numerous complex biochemical reactions and signal transduction pathways within cells pose significant challenges for designing and assembling circuits with more sophisticated functions. Consequently, achieving predictable design and assembly of complex genetic circuits in microscopic cells and ensuring robust circuit performance under complex in vivo and in vitro environments will be the key core issues and major challenges that must be overcome in synthetic genetic circuit research in the coming years.

Keywords: synthetic biology, synthetic genetic circuits, reprogramming, environmental adaptation, modularization
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Introduction

Synthetic biology is a discipline that uses engineering principles to modify and create life. It has demonstrated enormous potential in bioenergy, new materials, environmental pollution control, and cancer therapy, and can play a major role in national scientific development and industrial upgrading, supporting the "Made in China 2025" initiative. Compared with traditional bioengineering, the greatest advancement of synthetic biology lies in the systematic application of engineering design principles: simplifying and modularizing naturally existing enzymes and regulatory molecules according to these principles to design components with various basic functions. Synthetic genetic circuits utilize such components, designed based on ideas similar to electronic circuit programming, to execute control functions.

Programming life, like circuit programming, requires a large number of engineered components such as counters, pulse generators, logic gates, and signal filters to achieve control from low-level to high-level and from simple to complex. To construct such components, we must simplify life systems according to engineering design principles based on traditional biology's understanding of life regulation laws, and then combine them in different ways. Although both genetic circuits and electronic circuits are information processing systems for signal collection and processing, they are fundamentally different in many aspects. Genetic circuits operate in dynamically growing living cells—a mixture of numerous molecules—whereas electronic circuits operate in solid metal and semiconductor materials where insulation between components is easily achieved. These differences determine that genetic circuit design and assembly must explore new approaches rather than simply copying successful electronic circuit solutions.

Over the past 20 years, synthetic biology has produced a number of foundational works, achieving a leap from "zero to one" in synthetic genetic circuit design, regulatory elements, and assembly methods. Correspondingly, synthetic genetic circuits have also evolved from basic to composite types, beginning to possess the ability to simulate and explore advanced life processes.

Basic Synthetic Genetic Circuits

Basic synthetic genetic circuits are fundamental biological control devices designed, simulated, and constructed in an electronic engineering manner based on biological understanding of life systems. These include genetic switches, biological oscillators, counters, pulse generators, logic gates, signal filters, etc. In 2000, Collins' group at Boston University designed the first synthetic biology functional module—a transcriptional bistable switch (Figure 1a [Figure 1: see original paper]). This module successfully achieved the bistable effect predicted by mathematical models in *Escherichia coli* and could be used as a basic genetic switch [3]. In the same year, Elowitz and Leibler at Princeton University realized a more complex functional module—a gene expression oscillator (Figure 1b). This device utilized three gene modules that mutually inhibit and derepress each other to achieve regular oscillation of the output signal [2].

These two works demonstrated the possibility of rationally designing biological components at both theoretical and experimental levels, providing significant guidance for the development of synthetic biology, and are therefore called "milestones of synthetic biology." As basic elements of logic circuits, various logic gates have also emerged over the past 20 years, including "AND gates," "NOT gates," and "OR gates." For example, one "AND gate" uses T7 bacteriophage RNA polymerase with amber mutations and tRNA that can rescue amber mutations to construct independent input signals, enabling integration and processing of any two environmental signals and providing corresponding downstream output (Figure 1c) [8]. Similar logic gates, genetic switches, and oscillators can be assembled not only from various basic regulatory elements in prokaryotes, but also constructed from more complex regulatory and signal transduction elements to function in eukaryotes and even human cells [9-12].

Composite Synthetic Genetic Circuits

Using basic synthetic genetic circuits as fundamental devices, complex composite circuits can be built to simulate advanced life processes. In 2014, Ouyang Qi's group at Peking University designed a synthetic genetic circuit with Pavlovian classical conditioning behavior, recreating the learning function of higher organisms' neural networks in *E. coli*. This circuit consisted of two logic "AND gates," two logic "OR gates," and one memory module. It could receive one environmental signal molecule A that did not cause output response and one environmental signal B that could trigger output response. Repeated co-stimulation with these two signals could change the memory state of *E. coli*, eventually enabling signal A alone to trigger output response (Figure 2a [Figure 2: see original paper])

[13]. In 2012, Voigt's group at MIT assembled three binary logic "AND gates" into a large quaternary logic "AND gate," realizing a synthetic genetic network that could simultaneously sense four different environmental signals (Figure 2b) [14]. In addition to these two works, many other useful composite circuits have been developed, such as adders, edge detectors, and multi-input logic circuits.

To achieve more advanced control functions, synthetic genetic circuits inevitably become increasingly complex, leading to rapidly escalating design challenges. The field of electronic circuit design encountered similar problems in the 20th century, with solutions based on computer-aided automated circuit design and simulation. Therefore, in 2016, Voigt's group developed a computer program called "Cello" (meaning "cellular logic") for automated design of composite synthetic genetic circuits. This program can automatically provide genetic circuit designs that perform Boolean logic operations according to user requirements, achieving functions similar to electronic circuit design software. The program integrates extensive characterization data of transcriptional regulatory elements, experience in biological component assembly, known biological constraints of components, and automatic logic circuit compilation tools. After users select input signals, output signals, host cells, and other information, the program selects appropriate components from a standardized biological element characterization database, performs simulation and optimization from perspectives such as dynamic range and biological toxicity, and outputs the DNA sequence of the circuit and quantitative prediction results [15]. Researchers can directly synthesize and load the DNA sequence into host cells for functional execution. This program can significantly improve the design efficiency of synthetic genetic circuits.

Applications of Synthetic Genetic Circuits

Synthetic genetic circuits have already played important roles in both basic scientific research and practical applications. In basic research, the ability of synthetic biology to interfere with, reconstruct, and even recreate natural biological systems has become a powerful tool for biologists to explore the rules of complex biological operation. In practical applications, synthetic genetic circuits have also demonstrated enormous potential in metabolism engineering, medicine, agriculture, energy, and other fields, greatly promoting development in these areas.

"Building to Understand" – Applications in Basic Research

Using synthetic genetic circuit components, natural genetic circuits can be reprogrammed to construct artificial life processes beyond evolutionary rules, which can be used to explore basic scientific questions that are difficult to study with traditional biology. This approach is called "building to understand." Currently, this method has opened broader space for research on the origin of life, biological evolution, and life network regulation.

For example, cells specifically utilize certain regulatory network topologies in stress regulation. Therefore, by replacing certain specific elements in the topology network to reprogram it, we can gain deeper understanding of some characteristics of natural genetic circuits. In 2009, Elowitz's group at Caltech constructed a "positive-first then negative" artificial negative feedback genetic circuit in *Bacillus subtilis*, replacing the natural "negative-first then positive" negative feedback network (Figure 2a). They found that this artificial negative feedback network produced competence responses with short duration and low noise, while the natural negative feedback network produced competence responses with highly variable duration and wide distribution. This discovery revealed a survival strategy for organisms in adapting to environmental changes—using amplified gene expression noise to adapt to environmental variability and uncertainty [16].

Compared with bacteria, regulatory networks in higher organisms are more complex and contain more unknown areas, which can give the "building to understand" research method greater room for application. As early as 2003, Lim's group at UCSF studied the response strategies of yeast cells to environmental signals. By reprogramming two completely different signal pathways—the mating response and high osmolarity response—they successfully achieved rewiring of input and output signals. This work proved that scaffold proteins in MAPK signal transduction pathways are spatially localized signal nodes. At the same time, it also demonstrated that as long as hierarchical protein-protein interactions based on scaffold proteins are integrated, artificial signal pathways that are completely dependent on protein-protein interactions and can be reprogrammed can be obtained (Figure 2b) [17]. There are many similar works on regulatory topology replacement and signal pathway grafting, such as artificially altered regulatory order of lambda phage switches and two-component regulatory systems with grafted input and output signals [5,18]. These studies not only deepen our understanding of the physiological functions of natural genetic circuits, but also provide high-quality basic regulatory elements for the de novo design of genetic circuits.

Practical Applications

Computer chips are indispensable core components in various electrical devices, controlling their functions and bringing "intelligent" responses. Similarly, synthetic genetic circuits, as programmable control components for various synthetic biology applications, can often achieve "intelligent" control methods that are difficult to realize with traditional technologies.

In tumor therapy, CAR-T technology has demonstrated remarkable therapeutic effects, but there is still room for improvement in T cell activation level regulation, target specificity, and signal pathway control. To address these issues, Wong's group at Boston University proposed a novel CAR-T design scheme called "SUPRA CAR," which has been called the next-generation CAR-T therapy by many technology media and has attracted widespread attention [19]. The

SUPRA CAR scheme splits the fixed extracellular scFv single-chain antibody and intracellular CD3z signaling domain of CAR-T into two parts connected by a universal structure such as Leucine-Zipper, enabling modular design and programmability of both parts separately and creating possibilities for synthetic genetic circuit design. Synthetic genetic circuits based on this scheme can generate logical responses to multiple antigen signals and regulate signal pathways of different immune cell types. Through appropriate circuit design, it also enables modulation of T cell activation response intensity to reduce therapeutic side effects.

Furthermore, in metabolic engineering, synthetic genetic circuits have also demonstrated potential for "intelligent" control, providing better solutions. Synthetic genetic circuits designed based on bacterial quorum sensing can dynamically regulate target gene expression according to bacterial population density, enabling genes that negatively affect bacterial growth to be expressed only after bacteria reach a certain density. This avoids the contradiction between microbial growth and product formation in traditional fermentation processes, achieves dynamic regulation of "grow first, then produce," and also avoids the use of expensive inducers [20].

Challenges in Synthetic Genetic Circuit Design

When using synthetic genetic circuits to perform control functions, the circuits need to be loaded into different chassis organisms. The diversity of chassis requirements across different application fields demands adaptability of synthetic genetic circuits to chassis organisms. To achieve this goal, synthetic genetic circuits require "modular" design. Modularity is one of the core attributes of synthetic biology components. The design goal is to decompose biological systems into functionally independent modules and ensure that assembly between modules does not alter module functions. Modular design enables constructed biological systems to be scaled and amplified like electronic systems, so extensive work in synthetic biology focuses on developing modular components. However, the reality is far more complex than envisioned. Genetic circuits cannot be strictly isolated from host cells but instead couple with cellular physiological states to form an integrated whole. Synthetic genetic circuits can produce unpredictable interfering effects on cell physiology, and these interfering effects also cause theoretically "modular" biological elements and genetic circuits to lose predictability and no longer be "modular." This means that finely characterized component properties in one organism cannot be directly applied to another, because once components leave the physiological state in which they were characterized, their behavior may deviate from expectations. If this problem cannot be overcome, synthetic biological components cannot be used to build complex circuits step by step like electronic components, but instead require point-to-point optimization of components in individual chassis organisms, consuming substantial time and effort. Currently, challenges in synthetic genetic circuit design mainly manifest in two aspects: (1) overexpression of synthetic genetic

elements triggers cellular growth burden and toxicity; (2) special physiological mechanisms within cells affect synthetic genetic circuit function.

Overexpression-Induced Cellular Burden and Toxicity

Cells use limited resources to complete numerous physiological processes such as nutrient uptake, energy metabolism, DNA replication, and cell division. To optimize their own growth, cells must balance resource allocation according to environmental conditions. When the addition of synthetic genetic circuits disrupts this balance, it may cause cellular burden that affects normal cell growth. Cellular burden mainly manifests in two aspects: First, synthetic genetic circuits occupy RNA polymerases, ribosomes, and related cofactors and energy resources of the chassis cells during protein expression. Second, overexpressed proteins may also trigger cellular stress responses and activate stress pathways such as ppGpp. In addition to growth burden, synthetic genetic circuits may also cause cellular toxicity. Unlike growth burden, toxicity arises from off-target effects of synthetic genetic circuits interfering with normal physiological activities of chassis cells during regulation, rather than from occupying basic intracellular resources.

When chassis cells experience growth slowdown due to burden or toxicity, it in turn negatively affects the predictability and genetic stability of internal synthetic genetic circuits. For example, among elements designed based on TetR family repressors, some repressors may bind to non-specific target sites in the chassis cell genome, affecting chassis cell growth while also reducing the predictability of these elements [21]. Furthermore, during cell culture, sequences of synthetic genetic circuits may undergo various random mutations. Usually, the impact of these mutations is minimal, but if a mutant provides growth advantages to its cell, it will quickly dominate the population, causing the synthetic genetic circuit to fail at the population level. For example, a population size control element designed based on quorum sensing failed after 3-6 days of subculture due to massive proliferation of escape mutants [22].

Many widely used high-quality elements are also limited in their application and promotion due to their own cellular toxicity. For example, the CRISPRi-dCas9 (CRISPR interference-dCas9) regulatory system is considered an excellent programmable regulatory element and has been widely used in genetic circuit construction. However, as dCas9 expression level increases, significant growth retardation occurs. Some researchers believe this toxicity originates from off-target effects of CRISPR. Therefore, in practical use of CRISPRi-dCas9, researchers need to expend considerable effort to balance the positive effects of the transcriptional regulatory switch with its negative effects on cell growth. Synthetic biology component design must ultimately be adjusted for downstream application problems, while downstream applications require healthy, fast-growing host cells. Therefore, component toxicity will be one of the bottlenecks for synthetic biology to move toward applications.

Special Physiological Mechanisms Affecting Circuit Function

Some physiological mechanisms within cells may also unexpectedly affect component function, such as queueing-up effects and retroactivity. Synthetic biological elements and chassis cells jointly utilize limited intracellular resources such as enzymes and ribosomes. Resource competition can cause functionally unrelated genetic circuit elements to interfere with each other. For example, when yellow fluorescent protein and cyan fluorescent protein with LAA degradation tags are overexpressed in the same *E. coli*, they may overload the ClpXP degradation machinery, forcing the two different fluorescent proteins to queue for entry into the ClpXP degradation channel, thereby creating interference between two unrelated protein degradation processes [23]. This mechanism is called the queueing-up effect. Similarly, when the number of ribosomes in cells is insufficient, translation of mRNA also competes for ribosomes, causing queueing effects among unrelated mRNA translation processes. Retroactivity refers to the effect where downstream systems in a signal pathway provide feedback to upstream systems, thereby affecting upstream system function. Specifically, the degree to which a gene is regulated in a synthetic genetic circuit may be affected by the number of other genes receiving similar regulation, similar to how the voltage difference across a resistor depends on the number of other resistors in parallel. Both queueing effects and retroactivity can seriously impact synthetic genetic circuit function but are difficult to consider comprehensively during design, thus posing challenges for predictable design of synthetic genetic circuits.

Conclusions and Future Perspectives

The development of synthetic genetic circuit design, regulatory element toolboxes, and assembly methods has experienced tremendous progress over the past decade. However, various interactions between synthetic genetic circuits and chassis cells have hindered further enhancement of the complexity of artificially designed life systems. To break through this bottleneck, we need to focus on some fundamental engineering scientific questions in synthetic biology, such as the impact of cellular physiological systems on synthetic genetic circuits and related component design principles, to gain insights into future research directions. We believe future research should focus on the following issues: (1) Emphasize characterization of component functions and behaviors under different culture conditions, and standardize experimental result description and quantification methods to facilitate data integration and sharing; (2) Develop high-throughput technologies based on transcriptomics, proteomics, and other multi-level approaches to reduce the monetary and time costs of obtaining global phenotypic databases of synthetic organisms, and perform targeted data mining to provide data support for understanding the mechanisms of component-host interactions; (3) Develop new whole-cell models as needed to describe cellular physiological rules such as resource allocation and enhance the predictability of synthetic biological systems; (4) Research and develop component-host isolation

technologies and strategies, and summarize design principles to eliminate the influence of their interactions. In-depth research in these areas will enable us to truly achieve precision in artificial life system design and promote efficient translation of synthetic biology achievements in application fields.

References

1. Nandagopal N, Elowitz M B. Synthetic biology: integrated gene circuits. Science, 2011, 333(6047): 1244-1248.
2. Elowitz M B, Leibler S. A synthetic oscillatory network of transcriptional regulators. Nature, 2000, 403(6767): 335-338.
3. Gardner T S, Cantor C R, Collins J J. Construction of a genetic toggle switch in Escherichia coli. Nature, 2000, 403(6767): 339-342.
4. You L, Cox R S, 3rd, Weiss R, et al. Programmed population control by cell-cell communication and regulated killing. Nature, 2004, 428(6985): 868-871.
5. Levskaya A, Chevalier A A, Tabor J J, et al. Synthetic biology: engineering Escherichia coli to see light - These smart bacteria 'photograph' a light pattern as a high-definition chemical image. Nature, 2005, 438(7067): 441-442.
6. Anderson J C, Clarke E J, Arkin A P, et al. Environmentally controlled invasion of cancer cells by engineered bacteria. Journal of Molecular Biology, 2006, 355(4): 619-627.
7. Balagadde F K, Song H, Ozaki J, et al. A synthetic Escherichia coli predator-prey ecosystem. Molecular Systems Biology, 2008, 4: 187.
8. Anderson J C, Voigt C A, Arkin A P. Environmental signal integration by a modular AND gate. Molecular Systems Biology, 2007, 3: 133.
9. Tigges M, Marquez-Lago T T, Stelling J, et al. A tunable synthetic mammalian oscillator. Nature, 2009, 457(7227): 309-312.
10. Greber D, Fussenegger M. An engineered mammalian band-pass network. Nucleic Acids Research, 2010, 38(18): e174.
11. Rinaudo K, Bleris L, Maddamsetti R, et al. A universal RNAi-based logic evaluator that operates in mammalian cells. Nature Biotechnology, 2007, 25(7): 795-801.
12. Muller M, Auslander S, Spinnler A, et al. Designed cell consortia as fragrance-programmable analog-to-digital converters. Nature Chemical Biology, 2017, 13(3): 309-316.
13. Zhang H Q, Lin M, Shi H D, et al. Programming a Pavlovian-like conditioning circuit in Escherichia coli. Nature Communications, 2014, 5: 3102.

14. Moon T S, Lou C, Tamsir A, et al. Genetic programs constructed from layered logic gates in single cells. Nature, 2012, 491(7423): 249-253.

15. Nielsen A A, Der B S, Shin J, et al. Genetic circuit design automation. Science, 2016, 352(6281): aac7341.

16. Maamar H, Raj A, Dubnau D. Noise in gene expression determines cell fate in Bacillus subtilis. Science, 2007, 317(5837): 526-529.

17. Park S H, Zarrinpar A, Lim W A. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. Science, 2003, 299(5609): 1061-1064.

18. Sprinzak D, Elowitz M B. Reconstruction of genetic circuits. Nature, 2005, 438(7067): 443-448.

19. Cho J H, Collins J J, Wong W W. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell, 2018, 173(6): 1426-1438.

20. Gupta A, Reizman I M, Reisch C R, et al. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nature Biotechnology, 2017, 35(3): 273-279.

21. Stanton B C, Nielsen A A, Tamsir A, et al. Genomic mining of prokaryotic repressors for orthogonal logic gates. Nature Chemical Biology, 2014, 10(2): 99-105.

22. Scott S R, Din M O, Bittihn P, et al. A stabilized microbial ecosystem of self-limiting bacteria using synthetic quorum-regulated lysis. Nature Microbiology, 2017, 2: 17083.

23. Cookson N A, Mather W H, Danino T, et al. Queueing up for enzymatic processing: correlated signaling through coupled degradation. Molecular Systems Biology, 2011, 7: 561.

Figure 1. Basic Synthetic Genetic Circuits
(a) Synthetic genetic circuit with toggle switch function; (b) Synthetic genetic circuit with oscillator function; (c) Synthetic genetic circuit with logic "AND gate" function

Figure 2. Rewired Synthetic Genetic Circuits
(a) Comparison of natural and artificial negative feedback networks and dynamic responses in the *Bacillus subtilis* stress competence regulatory network; (b) The mating signaling network and high osmolarity response network in *Saccharomyces cerevisiae* were artificially rewired to obtain mating signals that activate high osmolarity response gene expression

Note: Figure translations are in progress. See original paper for figures.