Design and Control of Synthetic Biological Systems

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Abstract. In the field of synthetic biology, genetic networks are designed by combining well-characterized genetic parts, similar to electronic circuits. Such gene networks are called synthetic genetic circuits. The design approach for synthetic genetic circuits is based on mathematical modeling and numerical simulation. The approach allows the realization of various cellular functions. However, unavoidable differences in the initial states or fluctuations of the gene expression in cells have prevented the precise prediction and control of cellular behavior. Therefore, the design of synthetic genetic circuits is not sufficient, and the dynamic control of the circuits is also required. In this report, we provide examples of synthetic circuit designs and the control of synthetic biological systems, as well as perspectives on design and control.

Keywords: Synthetic biology, Control engineering, Synthetic genetic circuits.

1 Introduction

Industrial and clinical uses of cells have recently gained attention as an important theme [1, 2]. To achieve a desired cellular behavior, the traditional (trial and error) method, in which genes are changed one by one, is inefficient. Instead, a systematic method is required, because gene networks are composed of complex combinations of genes. In the field of synthetic biology, a systematic method including numerical simulation is used to engineer gene networks that program cells to perform desired behaviors, and such gene networks are referred to as synthetic genetic circuits.

Synthetic genetic circuits are engineered by combining well-characterized genetic parts, similar to electronic circuits. The design approach of synthetic genetic circuits consists of the following three steps (Figure 1). First, a synthetic genetic circuit is designed to implement the desired cellular function in cells. Second, a mathematical model of the dynamic cellular behavior is built. Third, through numerical analysis using the model, the range of the modifiable parameter values for the desired behavior is analyzed. If the range is acceptable, then the designed synthetic genetic circuit is constructed in cells, and if not, the above three steps are repeated. If the synthetic genetic circuits constructed through the approach do not work well, we can fine-tune various parameters, such as the protein synthesis rate, which can be adjusted by mutations of the ribosome binding site (RBS) sequence, by reference to the numerical

analysis results. Therefore, the design approach allows the realization of novel cellular functions, such as oscillation [3-6], counts of the input numbers [7], or optimization of a metabolic pathway to produce desired materials [8]. In addition, synthetic genetic circuits that include a cell-cell communication mechanism can program cells to perform population-level behaviors, such as pattern formation [9-11] and synchronized oscillation [6].

While a synthetic circuit is useful to program cells to perform a certain behavior, a synthetic circuit cannot precisely modify the behavior, because the behavior is affected by unavoidable differences in the initial states or fluctuations of gene expression in cells. Thus, the design approach is not sufficient for applications to complex purposes, such as the induction of cell differentiation for tissue engineering, and dynamic control is also required.

In this report, we introduce examples of synthetic circuit design in Section 2 and the control of synthetic biological systems in Section 3. We will discuss the perspectives of the future integration of the design and control of synthetic biological systems in Section 4.

Fig. 1. Three-step approach for genetic networks to program cells to perform desired functions

2 Introduction of Synthetic Circuit Design

In this section, we introduce several synthetic circuits classified into three functional categories: switches, oscillators, and population-level behavior.

2.1 Switches

Small molecule-responsive switch-like functions are important for the rational induction of cell phenotypes in tissue engineering. Such switch-like functions have

been achieved by the bistable structures of genetic networks. For example, a genetic toggle switch [12], which involved the mutual inhibition of the LacI and CIIs genes (Figure 2a), was constructed in *E. coli*. Numerical analysis of the genetic toggle switch, in terms of the expression rates of the two genes, revealed that the balance of the expression rates is important for the stability. The analysis showed that the cells with the genetic toggle switch exhibited bistability *in vivo* when the expression rates of the genes were balanced. On the other hand, the cells exhibited monostability when the expression rates were imbalanced. This work was an important milestone for an integrated *in silico* and *in vivo* approach. In mammalian cells, a similar genetic network structure, named an epigenetic toggle switch [13], was constructed and consisted of the mutual inhibition of the *E. coli* erythromycin-resistance gene repressor E and the pristinamycin-induced protein PIP (Figure 2b).

A state transition in response to induction was implemented in *E. coli*. The riboregulated transcriptional cascade (RTC) counter (Figure 2c) involved the repression of RNA polymerase production, by cr binding to the RBS sequence and forming a stem-loop structure [7]. The stem-loop structure was unraveled by the non-coding RNA taRNA, driven by chemical input. A three-step counter was constructed, in which gene transcription was initiated by RNAP, which was translated by the previous count of the chemical.

Fig. 2. Synthetic circuits programming cells to act with switch-like behavior. (a) Genetic toggle switch with a mutually inhibitory topology of the LacI and CIIs. (b) Epigenetic toggle switch with a mutually inhibitory topology of the E-KRAB and PIP-KRAB proteins. (c) The RTC counter has two regulation timings. One is taRNA-regulated translation. In the absence of taRNA, the RBS on the mRNA sequence forms a stem-loop structure with cr. This structure prevents the 30S ribosomal subunit from binding to the RBS, and results in transcription inhibition. When taRNA binds to cr, cr dissociates from the RBS, and transcription occurs. The other is polymerase-regulated transcription. Sequences downstream of the T7 and T3 promoters are transcribed in the presence of the T7 and T3 polymerases, respectively.

2.2 Oscillators

Many organisms exhibit oscillatory behaviors, such as circadian rhythms and cardiac functions. The first synthetic oscillator, repressilator, consisted of three repressor proteins, LacI, TetR, and CI. LacI represses TetR production, TetR represses CI production, and CI represses LacI production [3] (Figure 3a). The three repressors are destabilized by the presence of destruction tags, because numerical simulations revealed that oscillation is likely to appear under conditions with high repressor production and a comparable degradation rate of the repressors. The period of the oscillation was longer than the doubling time of the cells, indicating that the oscillation did not occur as a consequence of cell division, but as a result of the synthetic circuit.

The dual feedback circuit includes the genes encoding the activator AraC and the repressor LacI, with expression activated by the AraC-arabinose complex and repressed by LacI. The period of the oscillation was tunable by arabinose, which drives AraC activation, and IPTG, which inhibits LacI repression [4] (Figure 3b). A theoretical report predicted that the time-delay drives oscillatory behavior [14]. Further elegant experiments then revealed that the circuit consisting of only the self-repression of LacI can oscillate, because of the time-delay of LacI folding and multimerization.

Fig. 3. Synthetic oscillators. (a) Repressilator has a three-way standoff structure composed of LacI, TetR, and CIts. (b) The dual feedback oscillator consists of positive feedback by AraC and negative feedback by LacI. (c) Synthetic mammalian clock positive feedback by tTA and transcription repression by antisense tTA mRNA. The tTA-antisense production is driven by PIT, with production positively regulated by tTA.

A synthetic oscillator for mammalian cells, a synthetic mammalian clock (Figure 3c), was achieved with the transcription activator tTA, which is translationally repressed by binding the complementary mRNA for tTA [5] (Figure 3c). The transcription of the complementary mRNA is induced by the pristinamycin-dependent transactivator, PIT, with production positively regulated by tTA. This synthetic mammalian clock, based on sense-antisense transcriptional repression and time-delay, is a representative of the molecular mechanism and the expression dynamics of a mammalian circadian clock, which presently has too many components to accurately define the molecular network.

2.3 Population Behaviors

Population behaviors by synthetic genetic circuits require cell-cell communication mediated by intercellular signaling molecules [15]. Population level behaviors in both liquid and solid cultures have been reported. Synchronized oscillation in liquid culture was implemented in *E. coli* by a quorum oscillator [6], which consisted of positive feedback by an intercellular signaling mechanism mediated by acyl-homoserine lactone (AHL), and increased AHL degradation by the AHL catabolic enzyme AiiA [16], with production induced by the AHL-LuxR complex (Figure 4a).

Synthetic phenotypic diversification in liquid culture was realized in *E. coli* by a diversity generator [17], which was designed by integrating the mutual inhibitory structure by LacI and CIs and the intercellular signaling mediated by AHL (Figure 4b). The protein synthesis rates in the mutual inhibitory structure are balanced in the presence of sufficient AHL, but they become imbalanced in the absence of AHL. The cells with the diversity generator diversified into two distinct cell states, depending on their initialization to a certain state. The ratio of the cell states after the diversification was tuned by changing the AHL-accumulation speed, which was adjusted by two experimental parameters: the cell density at the initial time and the AHL-production activity of LuxI. The initial cell density was controlled by the inoculation volume of the cell culture. The LuxI activity was modified by mutations of the gene encoding LuxI, and the mutation sites were reported previously [18].

An edge detector to detect the boundary between red light and darkness in solid culture was constructed, by combining a dark sensor and genetic logic gates [10] (Figure 4c). The dark sensor includes the P_{ompC} promoter and Cph8, which phosphorylates the OmpR protein to induce transcription from the P_{ompR} promoter under dark conditions and dephosphorylates the OmpR-P complex. The genetic logic gates have the $P_{lux-\lambda}$ promoter, with transcription activated by AHL and repressed by CI. The genes encoding the AHL production enzyme LuxI and CI are downstream of the P_{ompR} promoter in the dark sensor, and the lacZ gene is downstream of the $P_{lux-\lambda}$ promoter, as the reporter gene. Therefore, the edge detector cells in red light and far from the light did not produce LacZ, and the cells at the boundary produced LacZ.

3 Control

Unavoidable differences in the initial states or fluctuations of gene expression in cells prevent the precise manipulation of cellular behavior. Therefore, the conventional static control method called “following a recipe” is not sufficient to manipulate the higher cellular functions programmed by a synthetic genetic circuit. To achieve higher functions, such as the induction of cell differentiation for tissue engineering, a dynamic control method, in which the input amount is updated based on an observed value, is required. Thanks to the development of fluorescent microscopy for real-time measurement and micro-fluidic devices for cell incubation and chemical input, control theory is now being experimentally applied to synthetic biological systems.

Toettcher et al. achieved the control of the membrane recruitment of a protein in individual mammalian cells [19]. They constructed a red light responsive circuit, in which the fluorescent protein-fused PIF6 (BFP-PIF) binds to the membrane protein-fused PhyB (PhyB-mCherry-CAAX) under 650 nm light, and dissociates from PhyB under 750 nm light, in mammalian cells (Figure 5a). They controlled the recruitment of BFP-PIF, which could be quantified by the fluorescence intensity, by the ratio of the intensities of the 650 nm and 750 nm light inputs. The ratio was calculated by a PI controller, which is simple and powerful. The BFP fluorescence intensities of cells incubated by micro-fluidics were measured by microscopy. The cellular responses to the light inputs differed from each other, because the cells produced different amounts of BFP-PIF and PhyB-mCherry-CAAX. However, the feedback control compensated for the cell-to-cell differences.

Furthermore, feedback control of gene expression by light input has been achieved by Argeitis et al. They constructed a light-switchable gene system, in which Venus YFP is regulated by the P_{Gal1} promoter, in yeast [20]. The P_{Gal1} promoter is activated by Gal4, which consists of a binding domain (Gal4BD) and an activation domain for the P_{Gal1} promoter (Gal4AD). In the system, PhyB-fused Gal4BD (PhyB-GBD) and PIF3-fused Gal4AD (PIF3-GAD) are constitutively produced. In the presence of PCB, PhyB is converted to the Pr state under 650 nm light irradiation, and PIF3-GAD can then bind to the PhyB-GBD (Figure 5b). This binding leads to promoter activation, and results in Venus YFP production. On the other hand, when PhyB converts to the Pfr state, the bound PIF3-GAD dissociates under 750 nm light irradiation (Figure 5b). The YFP fluorescence intensity of the cells was measured by flow cytometry every 30 minutes. The intensities between the measurements were thus estimated by the use of a Kalman filter. Based on the estimated intensities, model predictive control [21] was performed to determine the input (dark, 650 nm light or 750 nm light) every 15 minutes. This feedback-control strategy achieved target intensity from various initial states, in contrast to the nonfeedback-control strategy. The feedback control of gene expression in more complex synthetic circuits seems to be challenging, because the high nonlinearity and the time delay in the relationship between an input and gene expression may prevent successful control. Indeed, Menolascina et al. tried to control CBF1-GFP production in the synthetic circuit IRMA [22], which consists of five genes, in yeast by chemical input [23], and reported open-loop preliminary experimental results.

Fig. 4. Synthetic circuits for population-level behavior. (a) AHL in the population of cells with the synchronized oscillator induces the production of the AHL synthesis enzyme LuxI and the AHL catabolic enzyme AiiA. Therefore, the synchronized oscillator consists of the positive and negative feedback structures of AHL. (b) The presence of AHL in the population of cells with the diversity generator induces Clts production. In the absence of sufficient AHL, the mutual inhibitory network of LacI and Clts is bistable, whereas the network is monostable with LacI production in the absence of sufficient AHL. (c) In a dark environment, Cph8, with PCB produced by *ho1* and *pcyA* in the edge detector, phosphorylates OmpR, which is originally produced in *E. coli*. The phosphorylated OmpR binds to the P_{OmpC} promoter and activates transcription from the promoter located upstream of the *luxI* and *cl* genes. The synthesis of the reporter protein LacZ is activated by the AHL-LuxR complex, and is repressed by Cl. The edge detector cells in the dark area do not produce LacZ, because of repression by Cl. The cells in the dark area produce AHL, which diffuses in the plate. Therefore, if the cells in the red light illuminated area are located near a dark area, they produce LacZ, and if not, they do not produce LacZ.

Fig. 5. Synthetic circuits that utilized dynamic control. (a) PhybB fused with CAAX-mCherry is localized to the cell membrane by the C-terminal CAAX motif of Kras. The conformation of PhyB changes from the Pr state to the Pfr state by 650-nm light irradiation, and the change can be reversed by 750 nm light irradiation. PIF6 can bind to the Pfr-state PhyB, resulting in PIF6-BFP recruitment to the cell membrane. (b) PhyB fused with GAL4BD binds to the P_{Gal1} promoter, regulating Venus YFP production. Under 650 nm light, the conformation of PhyB changes from the Pr state to the Pfr state, and can be reversed by 750 nm light irradiation. GAL4AD-PIF3 can bind to the Pfr state PhyB, and results in Venus YFP production.

4 Conclusion and Perspectives

Various synthetic genetic circuits have been developed for use in investigations of the design principles of genetic networks governing diverse biological phenomena [24] or for manipulations of biological systems, such as metabolites [1]. Since synthetic genetic circuits for higher functions require more genetic parts, the strong limitations on the number of genetic parts have hampered the development of new synthetic genetic circuits. Therefore, greater numbers of species with available genetic parts are required for larger and more complex synthetic biological systems. To increase the components of a synthetic circuit, not only repressor or activator proteins, but also non-coding RNAs and riboswitches should be developed. Indeed, non-coding RNAs [5, 7] and a riboswitch [25] have recently been used for synthetic circuits. Especially, the riboswitch will be a powerful tool for small molecule-responsive gene regulation. With an enlarged library of genetic parts, more complex functions in higher organisms will be achieved. After combining the genetic parts, tuning their parameter values, such as protein activities or protein synthesis rates, will be very important to realize a desired function. For example,

the cells with the diversity generator introduced in section 2-3 cannot diversify if the enzymatic activity of LuxI is too high or low [17]. Since the parameter value of a genetic part is determined by its DNA sequence, directed evolution [26, 27] is an efficient approach to enlarge the library for tuning parameter values. In traditional directed evolution, a gene encoding the desired protein activity or the desired transcription rate of a promoter is selected from a library of the randomly mutated gene, by the application of selective pressure. Directed evolution has been applied to increase the activities of various proteins, such as enzyme [18] or signal receptor [28], and has also been utilized to increase the protein synthesis rate [29]. Further advances in directed evolution strategies, such as library construction [30], will allow us to more easily tune the parameter values of genetic parts and result in the successful construction of more complex synthetic circuits.

The computational analysis of synthetic circuit design, based on a mathematical model, provides a guide for tuning the parameters when a constructed synthetic genetic circuit does not work. Indeed, the design of the synthetic genetic toggle switch was evaluated by using parameter phase diagrams from the mathematical model [12]. Numerical simulation based on a mathematical model is frequently used for predictions of dynamic behavior, such as oscillation [3]. The selection of the information to include in a mathematical model is determined by the type of predicted desired cellular behavior. For example, to predict the formation of synthetic patterns, spatial information is included in their mathematical models [9, 10].

In addition to the design of synthetic circuits, the control of cells with the synthetic circuits is important for reliable cellular behavior. However, very few studies have applied control theory to synthetic biological systems, because control engineering has not been used for the design of genetic circuits. To apply control theory, controllability, measurability and stability must be considered in genetic circuit design. To achieve the controllability of complex genetic circuits, genetic parts such as riboswitches should be developed. Technologies such as the electroactive microwell array [31] are also required to assess more states of cells, for measurability. Furthermore, for systematic control, a linear approximation of a mathematical model of cells with a synthetic circuit, such as piecewise affine approximation [32], would be required, because a non-linear system is difficult to control systematically. The integration of synthetic biology and control engineering will generate technological breakthroughs in the fields of chemical plants and regeneration therapy.

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