Design strategy for an initial state-independent diversity generator

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Abstract

Initial state-independent phenotypic diversification will be a powerful tool for directing cells to multiple phenotypes in practical situation, in which initial cellular states are unknown. In this study, we designed Symmetric Diversity Generator (SDG) for the initial state-independent phenotypic diversification, in which homogenous cells diversify into two phenotypes and the ratio of the phenotypes do not depend on the initial cellular state. The SDG consists of two mechanisms: an intracellular mutual inhibition by repressors and an intercellular activation of the repressor productions by intercellular activators that are expected to compensate imbalance of repressor concentrations and of intercellular activator concentrations. We computationally evaluated the initial state dependence of the SDG in terms of the ratio of the two phenotypes after the diversification, and found the SDG still has initial state dependence. For lower dependence, we designed two kinds of symmetric diversity generator focusing on degradation rate of activators and responsiveness of repressor productions to transcription factors, activators and repressors. Our computational evaluation suggests that the latter approach is much more promising than the former one because the intercellular activators can compensate the imbalance of the transcription factors in advance of response of repressor productions. The former approach would be used for improvement of robustness of other synthetic genetic circuits already designed.

Key Words: synthetic biology, phenotypic diversification, catabolic enzyme, dissociation constant

Area of Interest: others
1. Introduction

Cellular phenotypic diversification, which plays a crucial role for regeneration of tissues, by synthetic genetic circuit, will be a powerful tool for engineering tissues in which several phenotypes co-exist. For practical use of the phenotypic diversification, the ratio of phenotypes in a cell population after the phenotypic diversification should be predictive. Because cellular state is generally unknown, the ratio should not depend on the cellular state at initial time. In this study, we designed synthetic circuits to program a cell population to diversify into two phenotypes from homogenous population and the phenotypic ratio after the diversification do not depend on initial cellular state.

Our design strategy for the low initial state dependence of the phenotypic diversification is based on the experience of intercellular activator mediated Escherichia coli cell diversification, in which cells diversify into two distinct phenotypes represented by the intracellular mutual inhibition of repressors. This intercellular activator stabilizes one phenotype whereas the other phenotype is always stable [1]. The uniqueness of our design strategy in this study is in its use of “neutral phenotype” by intercellular activators-dependent stabilities of the both distinct phenotypes (Figure 1a). The cells firstly transit to the neutral phenotype due to low concentration of the intercellular activator molecules, and then it starts phenotypic diversification through appearance of the two phenotypes by accumulation of intercellular activator molecules (Figure 1b).

According to the above design strategy, we firstly designed a synthetic circuit, named Symmetric Diversity Generator (SDG) followed by two derivatives of the synthetic circuits named a Fast Activator Degradation Symmetric Diversity Generator (FAD-SDG) and Low Responsive Repressor production Symmetric Diversity Generator (LRR-SDG). SDG makes use two kinds of intercellular activators which respectively stabilize the two phenotypes represented by the intracellular mutual inhibition of repressors. FAD-SDG incorporates catabolic enzymes for the intercellular activators to increase the degradation of the intercellular activators. LRR-SDG incorporates different promoters for repressor and intercellular activator productions to weaken the sensitivity of only repressor productions to the intercellular activators and repressors.

Population based numerical simulation study based on differential equations enables us to evaluate the effectiveness of the circuits in advanced to time-consuming and expensive experimental study. In addition, we can predict the sensitivity of the intercellular activators if appropriate physiological and kinetic parameters are used. Indeed, such process is generally used and allows the realization of various complex cellular functions, such as bistability [2][3], pattern formation [4][5] and oscillators [6][7][8][9].

The rest of the paper is as follows. In section 2, we show the design of the SDG. We also show the design of the FAD-SDG and LRR-SDG by the suggestions from the observation of the numerical simulation of the SDG. In section 3, we show analysis of the initial state dependence of the SDG, FAD-SDG, and LRR-SDG to evaluate the synthetic genetic circuits. In section 4, we conclude the study.
Figure 1. Intercellular activator dependent stabilities of phenotypes and desired cellular behavior. Open square shows stable steady state. (a) Intercellular activator 1 stabilizes phenotype P1, cells of which have intracellular repressor 1 (bottom right). Intercellular activator 2 stabilizes phenotype P2, cells of which have intracellular repressor 2 (upper left). Neutral phenotype, cells of which have neither repressor proteins, is stable under low activator concentrations (bottom left), whereas phenotype P1 and P2 are stable under high intracellular activator concentrations (upper right). (b) Desired dynamical behavior of cell population. Initially, due to low concentration of intercellular activators, the cells transit to neutral phenotype. Accumulation of the intercellular activators makes appearance of the two phenotypes, and then the cells diversify into two phenotypes.

2. Symmetric Diversity Generators

2.1 The Symmetric Diversity Generator (SDG)

We designed the SDG, which consists of an intracellular mutual inhibition by repressors R1 and R2 and the intercellular activation of the promoter strengths for R1 and R2 production by intercellular activators A1 and A2 respectively produced by the EA1 and EA2 enzymes (Figure 2a).
In this paper, we defined the P1 phenotype as the phenotype where the R1 and EA2 productions are dominant and the P2 phenotype as that where the R2 and EA1 productions are dominant. Figure 3 shows the predicted dynamic behavior of the cell population in the two-dimensional protein number space. All of the parameters used for Figure 3 are provided in Table 1. When the cells with the SDG were set to the P1 phenotype and the concentration of both intercellular activators in the medium was zero at the initial time (Figure 3a, 0 min), the number of the all proteins of the cells started to transit to zero, because of the lack of both intercellular activators. Sufficient accumulations of the intercellular activators caused the bistability and the cell population started to divide (Figure 3a, 180 min), and finally completely diversified (Figure 3a, 600 min). Even if the cells were the P2 phenotype at the initial time, the similar behavior was predicted (Figure 3b).

For improvement of the initial state dependence of the phenotypic ratio, we proposed two approaches suggested from Figure 3 to modify the SDG. These approaches involve with the concentration balance in the period from the transition of the repressor protein numbers to zero to the sufficient accumulation of the intercellular activators to cause the bifurcation. The imbalance of the initial numbers of enzymes EA1 and EA2 results in imbalanced accumulation of the activators A1 and A2. Therefore, Figure 3a suggests that one of reasons for the initial state dependence results from the concentration balance of the both activators in the period from the transition of the repressor protein numbers to zero to the sufficient accumulation of the intercellular activators to cause the bifurcation. Other than the two approaches, there is third approach that fastens the transition of the repressors to zero, expected that the time of the imbalanced activator accumulation becomes short. However, because the activator enzymes will remain and keep producing intercellular activators after the transition, the third approach will be nonsense.

Figure 2. The Symmetric Diversity Generator (SDG) and two synthetic genetic circuits based on the SDG to further improve the initial state dependence.

a. The SDG consists of an intracellular mutual inhibition of repressor proteins R1 and R2 and intercellular activation of the strengths of the promoters regulating the repressor enzymes by intercellular activators A1 and A2. b. The Fast Activator Degradation Symmetric Diversity
Generator (FAD-SDG) has the genes encoding catabolic enzymes for the two kinds of intercellular activators, in addition to the genes for the SDG. The Low Responsive Repressor production Symmetric Diversity Generator (LRR-SDG) consists of the same kinds of genes as the SDG, but the responsiveness of repressor production to transcription factors is lower than that of intercellular activator production. To generate the differences in responsiveness, each gene was regulated by a different promoter. The responsiveness can be weakened by mutating the transcription factor binding site.

Figure 3. The diversification of the SDG.

a, b. Diversifications from the phenotype P1 \((R1 = EA2 = 2000, R2 = EA1 = 0)\) and the phenotype \(P2 (R1 = EA2 = 0, R2 = EA1 = 2000)\), respectively. The open squares, open circles, and open triangles indicate the stable P1 state, stable P2 state and unstable steady state, respectively.

2.2 Fast Activator Degradation Symmetric Diversity Generator (FAD-SDG)

The simplest idea to improve the imbalanced activator accumulation is the increase of the degradation of the intercellular activators. From the idea, first approach is the relief of the imbalanced activator accumulation during the transition of the repressor numbers to zero. By the first approach, we designed a FAD-SDG, which adds the catabolic enzymes for A1 and A2 to the SDG (Figure 2b).

2.3 Low Responsive Repressor production Symmetric Diversity Generator (LRR-SDG)

To overcome the imbalanced activator accumulation, the enhancement of the robustness against the imbalanced activator accumulation is another possible idea. From the idea, second approach is the elongation of the period for sufficient time to adjust the imbalanced activator concentrations. By the second approach, we then designed the LRR-SDG, in which the responsiveness of the production of both R1 and R2 to the transcriptional factors is lower than that of the production of both EA1 and EA2 (Figure 2c). The responsiveness of gene expression to a transcription factor is defined as the dissociation constant between the factor and the binding site on the promoter driving the gene expression. Therefore, for the difference of the responsiveness, a promoter driving a repressor coding gene expression on the LRR-SDG is different from a promoter driving an enzyme coding gene expression (Figure 2c).
3. Evaluation of the initial state dependence of the designed circuits

We computationally analyzed the phenotype P1 ratio after the diversification from various initial protein numbers to evaluate the initial state dependence of the SDG, FAD-SDG and LRR-SDG (Figure 4). In the case of the SDG, the P1 ratio was more imbalanced as the initial number was more imbalanced (Figure 4a). These ratios were especially imbalanced when the initial numbers of R1 and EA2 were high/medium and the initial numbers of R2 and EA1 were medium/high respectively. This imbalance is because the production of high initial number of repressor is activated by the activator produced by medium initial number of activator enzyme before the transition of this repressor to zero. The FAD-SDG did not improve the initial state dependence from the SDG (Figure 4b). The results of the FAD-SDG are because under conditions with high catabolic enzyme activity, the activator accumulation was not sufficient to achieve bistability, and the imbalance of the activator accumulation could not be resolved under the low activity conditions. Finally, the initial state dependence of LRR-SDG was much lower than those of the SDG and the FAD-SDG (Figure 4c). The lower responsiveness of repressor production to the cognate intercellular activator in the LRR-SDG provides the sufficient time to modify the accumulation of the opposite activator to a type at the initial time.

To experimentally realize the desired function, the sensitivity of the function to the parameter values, such as the maximum protein synthesis rates, is preferably low, because promoters and genes with precise parameter values are difficult to be obtained. Therefore, we analyzed the initial state dependence, changing the maximum synthesis rate of R1 ($\alpha_{R1}$) as representative parameters, and showed the initial state dependence is low under the either 0.5 or 2 folds analysis (Figure 5a, b). We also showed the low initial state dependence when the maximum synthesis rate of EA1 ($\alpha_{EA1}$) was the either 0.5 or 2 folds (Figure 5c, d).

![Figure 4](image_url) The initial state dependence of the P1 ratio of the SDG, FAD-SDG and LRR-SDG.

The color-coded P1 ratios, in which the color scale ranges from blue (low) through green and yellow to red (high), as a function of the initial numbers with the SDG (a), FAD-SDG (b) and LRR-SDG (c), respectively. All parameter values are listed in Table 1. The P1 ratio values are averages of five simulations.
Figure 5. The initial state dependence of the P1 ratio of the LRR-SDG under changed parameter values.

The color-coded final P1 ratios, in which the color scale ranges from blue (low) through green and yellow to red (high). The maximum R1 synthesis rate was changed to 0.5 folds ($\alpha_{R1} = 25$) (a) and 2 folds ($\alpha_{R1} = 100$) (b) from the value listed in Table 1. The maximum EA1 synthesis rate was changed to 0.5 folds ($\alpha_{EA1} = 25$) (c) and 2 folds ($\alpha_{EA1} = 100$) (d) from the value listed in Table 1. The P1 ratio values are averages of five simulations.

4. Conclusion

In this study, for tunable diversification in situations where cellular states are unknown, we started by designing the SDG. Our numerical simulation showed that the SDG cells diversify into the two distinct phenotypes from either phenotype (Figure 3). To further improve the initial state dependence, we designed two synthetic circuits by small modification of the SDG, focusing on the imbalanced accumulation of the intercellular activators. One is the FAD-SDG, in which the intercellular activator degradation rates are increased. We did not observe big improvement of the initial state dependence of the FAD-SDG from the SDG (Figure 4a, b). The other is the LRR-SDG, in which the responsiveness of the repressor productions to the transcriptional factors is lower than that of the activator enzyme productions. The initial state dependence is lower than that of the other two circuits (Figure 4c). The modification approach of the second circuit can be widely applied to any other synthetic genetic circuits, because the dissociation constant for a transcription factor could be easily decreased by the mutation of its binding site. In spite of this advantage, the design method has not been frequently used. Therefore, further improvement of the performance of existing synthetic genetic circuits can be achieved by the modification approach of the second circuit.

The computational analysis of a designed synthetic genetic circuit before its construction provides a guide for tuning its parameters, such as protein production rates. Our computational analysis by the biologically reasonable range of parameter values [10], showed that the range of the
ratio of the maximum repressor rates of the LRR-SDG for the diversification is sufficiently wide to be tuned (Figure 5). The parameters will be tuned by mutations in the DNA sequence around a target gene, called directed evolution [11]. Increases in the dissociation constants of the transcription factors to a target promoter are achieved by mutating the binding sites of the factors on the DNA sequence of the promoter, while decreases in the constants would be more difficult to engineer. The selection of appropriate intercellular activator systems is an important challenge in the construction of the LRR-SDG. For an activator molecule, autoinducer 1 systems (AI-1s) are frequently used in synthetic biology. Generally, when there are two or more intercellular activator systems in a synthetic genetic circuit, then cross-talk, which is activation by non-cognate intercellular activators, will appear. Since cross-talk would weaken the modification effect by the intercellular activators during the diversification, we will introduce the effect of cross-talk to the LRR-SDG model, to analyze the allowable range of the cross-talk strength, and select the two activator systems of the LRR-SDG in future work.

Our design of the LRR-SDG could be used for a synthetic approach toward tissue engineering. To apply the design to mammalian cells, a synthetic mutual inhibitory network and an intercellular activator mechanism for mammals are required. Mutual inhibitory networks in mammals have already been realized [3]. Intercellular activator systems for eukaryotes have been developed [12][13]. The biggest experimental challenge to apply the LRR-SDG to mammalian cells is combination of the synthetic mutual inhibitory network and intercellular activator systems described above. To overcome the issue, we should construct new promoters whose each transcription is inhibited by an intracellular repressor and activated by an intercellular activator. Such promoters can be constructed by reference to the construction of BioLogic gates for mammalian cells reported by Kramer et al. [14]. The LRR-SDG for mammals would be efficient to maintain cellular diversity of a tissue shaped by cell scaffold or bio-printing technology. By the maintenance of phenotypic diversity, the engineered tissue will be robust against cell loss caused by shaping technologies, especially cell schaffo ld technology [15]. Therefore, our synthetic genetic circuit for tunable diversification from any cellular state is a novel tool for directing cell types at the population level, because the ratio of phenotypes is important for embryonic development.

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Appendix. Mathematical models for simulations

To predict the dynamic behavior of cell populations of the SDG (Figure 2a) and the LRR-SDG (Figure 2c), we built mathematical models based on the following simplified biochemical reactions.

\[ \emptyset \xrightarrow{k_{i1} \cdot (R2_i \cdot A1)} R1 \]
\[ \emptyset \xrightarrow{k_{i2} \cdot (R2_i \cdot A1)} EA2 \]
\[ \emptyset \xrightarrow{k_{i3} \cdot (R_i \cdot A2)} R2 \]
\[ \emptyset \xrightarrow{k_{i4} \cdot (R_i \cdot A1)} EA1 \]
\[ \emptyset \xrightarrow{k_{i5} \cdot (E4_{i1} \ldots \cdot E4_{iN_a} \cdot N_a)} A2 \]
\[ \emptyset \xrightarrow{k_{i6} \cdot (E1_{i1} \ldots \cdot E1_{iN_a} \cdot N_a)} A1 \]
where \( R_1 \) and \( R_2 \) (\( i = 1, 2, \ldots, N \)) denote the number of molecules of repressor proteins \( R_1 \) and \( R_2 \) in the \( i \)-th cell, respectively. \( A_1 \) and \( A_2 \) denote the number of intercellular activators \( A_1 \) and \( A_2 \) in the culture containing the cells. \( EA_1 \) and \( EA_2 \) denote the number of enzymes required to produce the intercellular activators \( A_1 \) and \( A_2 \), respectively. We considered the following two assumptions. The time course of \( N_{tot} \), which denotes the number of cells in \( V_m \) (liter) of culture, conforms to the logistic curve. The intercellular activators diffuse immediately through the cell membrane. Additionally, for simplicity, we always focused on \( N \) cells as representatives for samples of increasing \( N_{tot} \) cells, and considered them as separate \( N \) chemical reactors, except for the intercellular activators. The propensity functions of the production/degradation of each chemical species are \( k_\mu^+ (\gamma = +, - \text{ and } \mu = R_1, R_2, EA_1, EA_2, A_1, A_2) \), defined by the following equations:

\[
k_{R_1}^+(R_2, A_1) = \alpha_{R_1} \frac{R_2^{n_{R_2}}}{K_{R_2}^{n_{R_2}} \Omega_{R_2}^{n_{R_2}}} \frac{A_1^{n_{A_1}}}{K_{A_1}^{n_{A_1}} \Omega_{A_1}^{n_{A_1}}} + \varepsilon
\]

\[
k_{R_2}^+(R_2, A_2) = \alpha_{R_2} \frac{R_1^{n_{R_1}}}{K_{R_1}^{n_{R_1}} \Omega_{R_1}^{n_{R_1}}} \frac{A_2^{n_{A_2}}}{K_{A_2}^{n_{A_2}} \Omega_{A_2}^{n_{A_2}}} + \varepsilon
\]

\[
k_{EA_1}^+(EA_1, A_1, N_{tot}) = \frac{N_{tot}}{N} \lambda_{EA_1} \sum_{i=1}^{N} EA_1_i
\]

\[
k_{EA_2}^+(EA_2, A_2, N_{tot}) = \frac{N_{tot}}{N} \lambda_{EA_2} \sum_{i=1}^{N} EA_2_i
\]

\[
k_{R_1}(R_1) = d_{R_1} R_1
\]

\[
k_{R_2}(R_2) = d_{R_2} R_2
\]

\[
k_{EA_1}(EA_1) = d_{EA_1} EA_1
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k_{EA_2}(EA_2) = d_{EA_2} EA_2
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\[
k_{A_1}(A_1) = d_{A_1} A_1
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\[
k_{A_2}(A_2) = d_{A_2} A_2
\]

\[
\frac{dN_{tot}}{dr} = r N_{tot} (N_{max} - N_{tot})
\]

where \( \alpha_\mu \) (\( \mu = R_1, R_2, EA_1, EA_2 \)) denote the maximum production rate of each protein; \( \varepsilon \) denotes the leaky production of each chemical species; \( \lambda_\mu \) (\( \mu = A_1, A_2 \)) are the rate constants for intercellular activator production from the corresponding enzymes; and \( d_\mu \) (\( \mu = R_1, R_2, EA_1, EA_2 \),...
$A1$, $A2$) are the degradation rates. $K_{\mu,j}$ ($\mu = R1, R2, A1, A2, j=1, 2$) denote the dissociation rates of each chemical species, respectively. $\Omega_c = 1.66 \times 10^{-15}$ (liter) $\times 6.02 \times 10^{23}$ (mol)$^{-1}$ $\approx 1.0 \times 10^3$ (molecules/µM) is the conversion constant of molecules into the concentration in the cell volume. $\Omega_m = V_m$ (liter) $\times 6.02 \times 10^{23}$ (mol)$^{-1} \approx 6.0 \times V_m \times 10^{17}$ (molecules/µM) is the conversion constant of molecules into the concentration in the $V_m = 0.1$ (liter) culture.

To predict the dynamic behavior of populations of FAD-SDG cells (Figure 2b), we added the effect of a catabolic enzyme to the intercellular activator degradation terms [9], as follows:

$$
\dot{k}_{A1}(A1) = d_{A1} A1 + \gamma_{A1} \left(\frac{A1/\Omega_m}{1 + g_{A1} C41}\right) \Omega_c N_{tot}
$$

$$
\dot{k}_{A2}(A2) = d_{A2} A2 + \gamma_{A2} \left(\frac{A2/\Omega_m}{1 + g_{A2} C42}\right) \Omega_c N_{tot}
$$

where $CA1$ and $CA2$ denote the concentrations of catabolic enzymes for intercellular activators $A1$ and $A2$; and $\gamma_{A1}$ and $\gamma_{A2}$ denote the maximum catabolic rates of intercellular activators $A1$ and $A2$. For all stochastic simulations, we implemented the Poisson τ-leap algorithm [16]. Unless otherwise noted, the parameter values used in the simulations are listed in Table 1.

**Table 1. Parameters used for the simulations in this study**

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