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Rational design of memory in eukaryotic cells

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The ability to logically engineer novel cellular functions promises a deeper understanding of biological systems. Here we demonstrate the rational design of cellular memory in yeast that employs autoregulatory transcriptional positive feedback. We built a set of transcriptional activators and quantitatively characterized their effects on gene expression in living cells. Modeling in conjunction with the quantitative characterization of the activator-promoter pairs accurately predicts the behavior of the memory network. This study demonstrates the power of taking advantage of components with measured quantitative parameters to specify eukaryotic regulatory networks with desired properties.

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The field of synthetic biology aims to design biological systems to perform tasks to better understand analogous natural systems and for direct applications in research and medicine (e.g., see Andrianantoandro et al. 2006; Drubin et al. 2007). Currently our ability to design biological systems is limited by the difficulty of predicting the behavior of even simple genetic circuits because often a given network topology will show qualitatively different behavior depending on the quantitative features of the underlying components. While advances have been made with networks composed of well-studied modules in bacteria (Guido et al. 2006), this challenge is acute in more complex eukaryotic systems where the components rarely have measured characteristics. Therefore, to make rapid progress in designing eukaryotic systems, synthetic biologists require both a pool of quantitatively annotated biological parts and the knowledge that these parts can be logically engineered into more complex networks with predictable function.

One such network, which carries intrinsic value and tests a bottom-up design approach, is a network that cannot store information. Memory, which can be defined as a protracted response to a transient stimulus, is exemplified in differentiation where a precursor cell makes a permanent and heritable cell fate decision in response to transient signals. Two major feedback motifs characterized in natural systems that exhibit memory are mutual inhibition and autoregulatory positive feedback (e.g., see Xiong and Ferrell 2003; Ptashne 2004; Huang et al. 2006; Zordan et al. 2006). Initial work in building eukaryotic cellular memory has demonstrated a bistable transcriptional mutual repression switch, a “toggle switch,” in bacteria and mammalian cells [Gardner et al. 2000; Kramer et al. 2004]. Others have executed autoregulatory positive feedback designs to varying levels of success [Beceksi et al. 2001; Atkinson et al. 2003; Kramer and Fussenegger 2005; Vilaboa et al. 2005; Ingolia and Murray 2007]. However, none of the eukaryotic synthetic network studies succeed at demonstrating predictable behavior of a system.

In this study we describe the rational design and construction of a high fidelity, modular memory device in yeast based on transcriptionally controlled autoregulatory positive feedback. This device heritably retains an induced state in individual cells in response to a transient stimulus. The rational design approach used here employs an extensive in vivo quantitative characterization of a set of synthetic transcription factors and the prediction of system behavior via network models incorporating these measured parameters. By successfully constructing this memory device, we established the essential parameters for maintaining an autoregulatory positive feedback loop in a dividing cellular system. Most importantly, we demonstrated predictability of system behavior in eukaryotes when the system is built from well-understood components.

Results and Discussion

Functional activators based on a modular architecture

To rationally engineer a memory device, we designed a set of fluorescently labeled synthetic transcription factors and their corresponding reporter genes to serve as candidate components. Each activator gene consists of a DNA-binding domain (DBD), two tandem copies of the monomeric red fluorescent protein (RFP) mCherry (Shaner et al. 2004), the viral activation domain VP64 [Bearli et al. 1998], and the SV40 nuclear localization sequence (NLS) [Kalderon et al. 1984; Lanford and Butel 1984], all under control of the GAL1/10 promoter [Fig. 1A]. Each reporter gene has multiple copies of the DNA-binding sites corresponding to its given transcription factor upstream of the minimal CYC1 promoter, and its protein coding region encodes two tandem copies of the yellow fluorescent protein variant [YFP] Venus [Fig. 1A; Nagai et al. 2002]. The DBDs used were the LexA DBD [Hurstel et al. 1986, 1988], an engineered variant of the murine zinc-finger Zif268 [ZifH] [Hurt et al. 2003], and three human zinc fingers [ERG2, Gli1, and YY1] [Chavrier et al. 1990, Kinzler and Vogelstein 1990; Shi et al. 1991].

To evaluate the function of these constructs in yeast, we constructed two types of strains—one type carrying...
Quantitative characterization of a subset of transcriptional activators

With this set of functional activators, we had components to construct a positive autoregulatory feedback loop. Modeling (see the next section, Creating a Cellular Memory Device) indicates that only feedback loops with particular quantitative features will result in systems that will switch between two stable states upon transient induction. Specifically, the production rate of a reporter as a function of activator concentration constrains which function of activator concentration for individual cells (red, blue, light-blue lines). Only s and \( \beta \) could be robustly determined in B. (C) Time-lapsed RFP and YFP fluorescence images of cells containing the ZifH activation cascade 0, 75, and 150 min after induction. (D) Rate of change of reporter concentration as a function of activator concentration for individual cells (red, blue, green, and light-blue lines).

Figure 1. Synthetic transcriptional activators in yeast. (A) Schematic diagram of an activator cascade composed of an activator gene and a reporter gene. (B) DIC and fluorescence images of live cells containing the ZifH activator cascade in the absence (−galactose) and presence (+galactose) of the inducer or only the ZifH reporter gene in the presence of the inducer. (C) Dot plots of YFP versus RFP fluorescence intensity for single cells grown as in B.

Figure 2. Steady-state and dynamic behavior of activation cascades. The mean steady-state reporter versus activator concentrations for the ZifH [A] and LexA [B] activator–reporter strains. The solid lines show best fits to the data. Only s and \( \beta \) could be robustly determined in B. (C) Time-lapsed RFP and YFP fluorescence images of cells containing the ZifH activation cascade 0, 75, and 150 min after induction. (D) Rate of change of reporter concentration as a function of activator concentration for individual cells (red, blue, green, and light-blue lines).
Table 1. *Best-fit Hill parameters for the activators*

<table>
<thead>
<tr>
<th>Strain and method</th>
<th>s ± SE</th>
<th>β ± SE</th>
<th>K ± SE</th>
<th>n ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZifH, SS</td>
<td>0.23 ± 0.14</td>
<td>2.7 ± 0.2</td>
<td>13 ± 3</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>ZifH, dyn</td>
<td>0.11 ± 0.03</td>
<td>3.6 ± 1.0</td>
<td>18 ± 9</td>
<td>2.7 ± 1.5</td>
</tr>
<tr>
<td>LexA, SS</td>
<td>0.01 ± 0.005</td>
<td>9.3 ± 0.6</td>
<td>40–60</td>
<td>ND</td>
</tr>
<tr>
<td>LexA, dyn</td>
<td>0.01 ± 0.007</td>
<td>12.5 ± 3.0</td>
<td>60 ± 33</td>
<td>3.4 ± 0.8</td>
</tr>
</tbody>
</table>

s and β are in the units of 10^9 reporter molecules cell^-1 min^-1. K is in 10^9 activator molecules cell^-1. Bold values were used for predictions. [SS] Steady state; [dyn] dynamic; [ND] not determined.

Creating a cellular memory device

We next set out to construct a transcriptional positive feedback loop intended to confer “memory” of a stimulus to a yeast cell and its progeny [Fig. 3A]. In our proposed memory device, a signal induces synthesis of a “sensor” transcription factor, which triggers the expression of an “autofeedback” transcription factor. This autofeedback activator binds to its own promoter and, under appropriate circumstances, will continue to activate its own expression even in the absence of stimulus, resulting in memory. This synthetic network can exist in three different steady states: never exposed to stimulus (“off”), stimulus present (“on”), and previously exposed to stimulus (“memory”). In our implementation, the sensor activator is labeled with RFP, the autofeedback activator is labeled with YFP, and the stimulus is galactose.

For this device to exhibit memory, the autofeedback loop must contain two stable states. In terms of our mathematical model, the production rate of the autofeedback activator must be balanced by its decay rate at three activator concentrations (Eq, 5, Fig. 3B). The lowest and highest of these concentrations correspond to the “off” and “memory” stable states, while the intermediate concentration is an unstable steady state (“switch”) where the system switches between the stable states. In the off stable state, the basal levels of activator stimulate low levels of autofeedback synthesis that decrease rapidly due to the comparatively high rate of autoactivator decay. In contrast, in the memory state, the high concentration of autoactivator catalyzes a fast rate of its own synthesis that balances the decay rate and perpetuates its own production. Under this model the growth rate of the yeast cells becomes important, since the effective dilution of the activator during cell growth dictates its decay rate (Supplementary Fig. S3). Bistability of the autofeedback circuit can only be achieved if the synthesis of the autoactivator is a cooperative process (n > 1). For the memory state to respond specifically to a transient stimulus two more conditions must be met.

![Figure 3](image-url)
First, the sum of the noninduced level of sensor activator and autoactivator must be less than the switch concentration; otherwise, the system will always be in the memory state. Second, in the presence of the stimulus, the sensor activator concentration must be greater than the switching concentration in order to jump start autoactivator production and shift the system into the memory state (Fig. 3B).

We used the measured Hill parameters, together with several assumptions, to predict the behavior of an auto-feedback loop in which either LexA or ZifH were used as the DBD. First, we assume that the transcriptional activator–reporter pair dictates the 

\[ K \]

and 

\[ s \]

such that these parameters are comparable in an activator–reporter strain and its corresponding memory device strain. Since \( \beta \) depends on the protein being synthesized, we also assume that \( s \) and \( \beta \) for autoactivator synthesis can be scaled from \( s \) and \( \beta \) for reporter synthesis by a proportionality constant. This constant is set to the ratio of the RFP intensity from a strain carrying a tandem set of mCherry modules under control of the GAL1/10 promoter to the RFP fluorescence from the LexA or ZifH activator strain (0.10 and 0.043, respectively). Our model, which has no free parameters with these assumptions, indicates that the LexA activator can be used to make a switchable bistable system at certain growth rates (Fig. 3B). However, this model predicts that the basal levels of ZifH activator will be greater than the “switch” concentration for the ZifH system, and thus cause a switch into the memory state independently of exposure to a stimulus (data not shown). This effect is predicted for the ZifH memory loop because of the higher leakiness of the ZifH promoter (i.e., its higher value of \( K \)), coupled with the more potent effect of ZifH activator on gene expression (i.e., its lower value of \( \beta \)) (Table 1). We tested the model predictions by building both networks and characterizing their responses.

The LexA memory network indeed responds specifically to induction and shows bistability, creating a high-fidelity in vivo three-state memory device. Cells with the LexA network never exposed to the induction stimulus show no significant RFP or YFP fluorescence (Fig. 3C,D); i.e., are in the “off” state. Cells grown to steady-state in the presence of the induction stimulus are in the “on” state, showing significant fluorescence for both RFP and YFP. After the stimulus is removed and cells re-establish a steady-state, the sensing gene is no longer expressed, but the feedback activator continues to be present at the same level (Fig. 3C,D). These cells are in the “memory” state. Observation over approximately eight cell divisions by microscopy shows that ~90% of cells retain the memory state continuously (data not shown). Bistability of the loop was verified by flow cytometry, which shows that in all three states, individual cells fall into two well-separated populations based on their fluorescence intensity (Fig. 3D). In contrast, the ZifH memory network is, as predicted, fixed in the “memory” state before induction (Supplementary Table S1).

The above results for the LexA system were obtained in raffinose with a doubling rate of 240 min. As discussed, the effective degradation rate of the activator is determined by the doubling rate of the cells. In glucose liquid culture the doubling time of the culture decreases to ~90 min. Under these growth conditions, our model suggests that the LexA loop is just on the border between a mono- and bistable system (Fig. 4A), and would likely lose the memory state: The production of the autoactivator cannot keep up with the increased dilution of the autoactivator, so in the absence of the “sensor” activator, the system relaxes to the “off” state. In fact, we observed no RFP or YFP fluorescence from the LexA memory strain after transient induction, indicating it cannot hold the memory state when grown in glucose liquid culture (Fig. 4B). Our model also suggests that a modest change in growth rate—i.e., from a doubling time of 90 min to 120 min—would return the autofeedback loop to a distinctly bistable region of phase space (Fig. 4A); such a change would sufficiently slow the decay of the autoactivator so that the production rate can keep pace with autoactivator dilution. This change in growth rate was achieved by growing the cells in glucose on solid media rather than in liquid culture, and indeed, the cells with a slower doubling time retain memory; i.e., maintain YFP fluorescence after transient induction (Fig. 4B). Thus, we can reliably tune the functionality of our memory and control our growth rate.

As a further test of our model, we attempted to modify the memory system to hold the memory state even under rapid growth conditions. Possible alterations include increasing the Hill coefficient (\( n \)), decreasing the concentration of activator required for half maximal activation.
(K), or increasing the maximum production rate [β]. Increasing n or decreasing K would require alterations to the transcriptional activator or promoter and would require recharacterization of the system. We therefore modified β by simply adding an identical autofeedback gene at a second locus. Assuming the two copies act identically, this change doubles s and β of autoactivator, without affecting n or K. We predicted that the basal expression rate of two autofeedback genes would still be low enough to maintain the “off” state in the absence of inducer, but the increased production rate would maintain the memory state at high growth rate [Fig. 4C]. Indeed, the dual autofeedback memory device switches specifically and remains in the memory state even in liquid glucose culture [Fig. 4D].

Here we demonstrated a memory device composed of synthetic transcription factors in an autoregulatory positive feedback motif that retains an induced state in a heritable fashion in response to a transient stimulus. This result supports previous studies showing that autoregulatory positive feedback loops are capable of a switch-like output in both yeast and mammalian cells [Becskei et al. 2001; Kramer and Fussenegger 2005; Vila–boa et al. 2005; Ingolia and Murray 2007]. However, the device presented here integrates high-fidelity memory, with both potential interchangeability with regard to stimulus and a quantitative, single-cell description of the system. Such a defined memory module can be exploited in research applications to identify cells that experience specific events and determine whether this correlates with later behavior, or could be incorporated into a more complex network that causes a cell to “differentiate” in a certain fashion after experiencing a defined event. The memory module also potentially fills a direct need in industrial biotechnology: It permits high induction of recombinant proteins without the high cost of shuttle vectors [Sikorski and Hieter 1989]. Yeast strains are single-site integrations of the strain PSY580A [MATα, ura3-52, trp1Δ63, leu2Δ1]. Activator and reporter genes were integrated into the LEU2 and URA3 loci, respectively. The autofeedback gene was inserted in the URA3 locus, the second at TRP1. Yeast were grown for at least 24 h at 30°C in media for induction [2% galactose, 2% raffinose], noninduction [2% raffinose], and repression [2% glucose]. For measurements, cells were grown to mid-log phase (approximate OD600 of 0.3–0.6).

Flow cytometry and fluorescence microscopy
For flow cytometry analysis, we used a Dako MoFlo [Glostrup, Denmark] with 488- and 568-nm tunable lasers.

For live-cell imaging, a drop of cell suspension was placed on an agarose pad with media, and placed cell-side down on a glass-bottom dish. Images were acquired on an Eclipse TE2000-E equipped with a 60x objective [Nikon], Orca 285 CCD camera, Metamorph 6.3r7 software, and JLP [YFP] and HeRed [RFP] filter sets (Chroma).

YFP and RFP intensity values were normalized such that a control strain had the same intensity between experiments. Reported intensity values are normalized, background-corrected averages.

Modeling of reporter and autoactivator synthesis
We use a single mathematical framework to describe the relationship between the activator concentration and the production rate of either the reporter or autoactivator activator. We assume that the rate of production of either the reporter or autoactivator species, (dX/dt)producer, can be described by the sum of a basal rate and a Hill function:

\[ \frac{dX}{dt}_{\text{produce}} = s + \frac{\beta A^n}{K^n + A^n} \]

where s is the basal rate of production of activator, n is the Hill cooperativity, K is the concentration of activator that yields half the maximal production rate, β is the maximal rate of protein production, and A is the activator concentration [Alon 2006].

The decay rate of either the reporter or autoactivator concentration is affected by both dilution due to cell growth and protein degradation. However, since the reporter protein and the autoactivator are degraded at a rate that is many times slower than their dilution rate (Supplementary Fig. S3), the decay in the species concentration can be simplified to

\[ \frac{dX}{dt}_{\text{decay}} = - \frac{\ln 2}{\tau} X \]

where τ is the time required for the number of cells to double by growth. The overall change in the concentration of the reporter or autoactivator activator can then be written as

\[ \frac{dX}{dt} = s + \frac{\beta A^n}{K^n + A^n} - \frac{\ln 2}{\tau} X \]

At steady state, the production rate is balanced by the decay rate, so the relationship between the steady-state activator concentration, \( A_s \), and steady-state reporter concentration, \( R_s \), can be written as

\[ R_s = \frac{\ln 2}{\tau} \left( s + \frac{\beta A^n_s}{K^n + A^n_s} \right) \]

In the case of the positive feedback loop, we assume that the autofeedback gene is activated identically by the sensor-derived activator and the autofeedback activator, so we rewrite Equation 3 as

\[ \frac{dA_s}{dt} = s + \frac{\beta (A_s + A_s)^n}{K^n + (A_s + A_s)^n} - \frac{\ln 2}{\tau} A_s \]

where \( A_s \) is the autoactivator concentration and \( A_s \) is the sensor-derived activator concentration.

All mathematical modeling was performed using MATLAB (The MathWorks).

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Materials and methods
Plasmid cloning and yeast strains
Constructs were made via a BioBrick assembly method [Knight 2003; Phillips and Silver 2006]. Genes were cloned into yeast integrating
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References


