

Computational design of digital and memory biological devices

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Abstract The use of combinatorial optimization techniques with computational design allows the development of automated methods to design biological systems. Automatic design integrates design principles in an unsupervised algorithm to sample a larger region of the biological network space, at the topology and parameter levels. The design of novel synthetic transcriptional networks with targeted behaviors will be key to understand the design principles underlying biological networks. In this work, we evolve transcriptional networks towards a targeted dynamics, by using a library of promoters and coding sequences, to design a complex biological memory device. The designed sequential transcription network implements a JK-Latch, which is fully predictable and richer than other memory devices. Furthermore, we present designs of transcriptional devices behaving as logic gates, and we show how to create digital behavior from analog promoters. Our procedure allows us to propose a scenario for the evolution of multifunctional genetic networks. In addition, we discuss the decomposability of regulatory networks in terms of genetic modules to develop a given cellular function. *Summary.* We show how to use an automated procedure to design logic and sequential transcription circuits. This methodology will

allow advancing the rational design of biological devices to more complex systems, and we propose the first design of a biological JK-latch memory device.

Keywords Computational design · Digital behavior · Synthetic biology · Systems biology · Transcriptional networks

Introduction

In the last years, there has been a tremendous work on inferring the topologies of transcription networks (Babu and Teichmann 2003) with the hope of elucidating their design principles. Studies of recurrent network motifs showed that their dynamics could provide useful functions (Mangan and Alon 2003; Brandman et al. 2005). These reverse-engineering studies have uncovered those principles and they have provided the framework to plan the design of synthetic circuits. The exponential decrease of DNA synthesis cost, together with the new development of standardized genetic parts (Endy 2005), will provide the technological means to design large genetic networks. The easiest way to construct such genetic parts is to use the available genomic knowledge, although there has also been some work on creating synthetic parts such as promoters with modified or added operator sites (Buchler et al. 2003; Bintu et al. 2005a, b), modified ribosome binding sites (Basu et al. 2004), or codon-optimized coding regions (Basu et al. 2005). Protein engineering has also been providing new coding sequences for several decades now, corresponding to new designed proteins with specified functions that sometimes have even no similarity with any known natural protein sequence (Kuhlman et al. 2003; Looger et al. 2003; Jaramillo et al. 2002).

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The construction of transcriptional networks could also take advantage from the design of promoter sequences having a combinatorial regulation. In fact, there are already some examples on the design of synthetic promoters regulated by two transcriptional factors (Joung et al. 1994; Mayo et al. 2006). Currently, most of the designed transcriptional networks (Elowitz and Leibler 2000; Gardner et al. 2000; Atkinson et al. 2003) have been engineered using rational design techniques (Hasty et al. 2002), limiting the use of computational modeling to the solving of dynamical equations. The design of such networks would highly benefit from the use of computational methods to automatically design new circuits. For instance, we could evolve our circuits by replacing elementary parts, such as promoters, from a catalogue of these. Furthermore, the design of genetic circuits from modular components allows designing a panoply of transcriptional circuits from a few elementary components. This is exemplified by the combinatorial generation of 125 transcriptional networks, some of them working as logical devices, using only a library of five promoters controlling three transcriptional factors (Guet et al. 2002). They characterized the behavior of their three-gene networks under two inductors and discovered that some of the networks behaved like a logic gate of type NAND, NOR or NOT. Sometimes transcriptional networks having the same topology would give completely different behavior. Each promoter only contained one operator site, so it could only receive one transcription factor. Their results suggested that a given circuit topology could show several behaviors. In this work we perform a computational evolution that will allow us to generate similar transcriptional circuits to those found in that previous work (Guet et al. 2002). An important question in understanding the mechanism of living systems is the design principle of regulatory networks having a given behavior. Analyzing our functional genetic networks, designed following a combinatorial optimization procedure, we can unveil new of such principles.

The combination of computational and combinatorial techniques has already been used in other disciplines to generate complex behaviors. In particular, an essential aspect of networks is the evolution and design or their dynamical behavior. Work on Artificial Life or on hardware evolution (Taubes 1997; Raichman et al. 2003) shows how automated methods could be used to design emergent complexity (Kahtan and Alon 2005). In biology, an evolutionary method to engineer electronic circuits was suggested for the design of genetic circuits with oscillatory behavior (Mason et al. 2004), although the used model was purely Boolean. A more accurate model introduced protein species together with biochemical reactions, and allowed to design networks with a specified function (Francois and Hakim 2004). They obtained switches and oscillators

mostly based on protein–protein interactions but without external inputs to control its behavior. Recently, it has been proposed a methodology to evolve circuits with a targeted asymptotic behavior by computing the steady state of the system (Paladugu et al. 2006). Here, we propose to go beyond those results by considering a general time response and addressing transcription regulation to implement genetic networks that could be eventually synthesized. In addition, our networks can be controlled by external signals.

An interesting step towards the design of complex biological circuits is the ability to design networks with digital behavior, where the output gene concentrations could be described as Boolean functions of the inputs. Moreover, genetic circuits sharing the same topology could behave very differently depending on their kinetic parameters. Therefore, rational design techniques that only take into account network topology considerations are not sufficient to ensure a given dynamics. This would force us to enumerate all possible parameter combinations to design gene networks with a targeted behavior, which is impossible due to the exponential explosion of possible models. To address this issue, we have developed an algorithm that searches the space of artificial transcriptional networks to find the optimal circuits with a targeted temporal behavior. During our simulation, we add or subtract genes, change kinetic constants or the combinatorial regulation logic at promoters. We evolve in time each generated circuit and we use the average deviation to an expected temporal function as a scoring function. We use Monte Carlo Simulated Annealing (Kirkpatrick et al. 1983; Metropolis et al. 1953) method to do an optimization in the space of all possible genetic circuits.

A toggle switch was presented in (Gardner et al. 2000) by implementing a two-gene core repressor–repressor. In fact, this topology implements a RS-Latch. A further step was designing a D-Latch based on the previous circuit with a rational approach (Fritz et al. 2007). In this work, we propose to go beyond those results by designing a more complex memory device: a JK-Latch. This device is richer than a D-Latch because it has two inputs to control its behavior, and it is more predictable than a RS-Latch because all the entries of the truth table are defined. In addition, we have applied our methodology to design some motifs by targeting digital behaviors such as logic gates in order to compare our results with the ones found in Nature.

For simplicity, we have focused on the design of transcription networks, neglecting post-translation regulation. Already those networks can generate an arbitrary complex behavior, as they have recently been suggested to form a programmable computing machine, belonging to the class of Boltzmann machines (Foster 2001). Importantly, one inconvenient of those networks is their slow response,

making difficult to use cascades of many stages (Hooshangi et al. 2005), as it is usually done when designing electrical circuits. Therefore, the implementation of a transcription logic in the promoter region will play an important role in generating complex behavior.

Methods

Mathematical model

Multiple approaches are available to describe biological networks, particularly transcriptional circuits. We consider for this work a deterministic and continuous model to describe the regulatory interactions between genes. Further works will take into account stochastic effects on the system. A whole description of these networks considers all the species involved in the processes of transcription, translation, and regulation, such as DNA, mRNA and proteins (see Fig. 1). In general, a regulation between a transcription factor (repressor or activator) and a gene can be modeled according the following differential equations

$$\frac{d}{dt}[V] = \rho[U]^n - \sigma[V], \tag{1}$$

$$\frac{d}{dt}[D] = \mu[V : D] - \theta[V][D], \tag{2}$$

$$\frac{d}{dt}[R] = \varphi[D] + \psi[V : D] - \lambda[R] - \delta[R], \tag{3}$$

$$\frac{d}{dt}[Y] = \lambda[R] - \beta[Y], \tag{4}$$

where U is the non-active form of the regulator and V its active form (i.e., transcription factor). ρ and σ are the kinetic coefficients for n -merization. If a regulator does not n -merize, this description is also valid with $U = V, n = 1, \rho = \sigma$. D is the DNA, R the mRNA, and Y the folded protein from that regulated gene. θ and μ are the kinetic coefficients for binding and unbinding between the

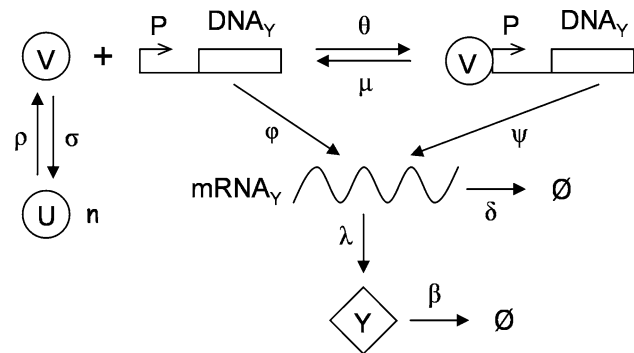


Fig. 1 Biological scheme of a transcriptional regulation. Greek letters denote the kinetic constants of the different biochemical reactions

transcription factor and the promoter, respectively. φ and ψ are the transcriptional kinetics from free and occupied DNA, respectively. We assume that DNA is not degraded, mRNA does it with kinetics δ , and protein with β . λ is the translational kinetics. We have considered that the cellular resources, such as RNA-Polymerases, Ribosomes, nucleotides and amino-acids, are sufficient to sustain a synthetic system and could be assumed constant. Thus, the transcription and translation rates only depend on the amounts of DNA and mRNA, respectively. We add an additional mass balance equation,

$$[D_t] = [D] + [V : D], \tag{5}$$

as the amount of DNA within the cell is conserved. Those equations can be rewritten for more than one transcription factor.

However, we simplify the model to get a more reduced formulation, despite of introducing more non-linearity. We suppose that the intermediate species have low temporal rates (i.e., $d[V]/dt = d[D]/dt = d[R]/dt = 0$), and we define the following parameters

$$K^n = \frac{\mu\sigma}{\theta\rho}, \tag{6}$$

$$a = \frac{\lambda}{\delta + \lambda} \varphi[D_t], \tag{7}$$

$$c = \frac{\lambda}{\delta + \lambda} \psi[D_t]. \tag{8}$$

Thus, we can write the following effective differential equation for the protein dynamics

$$\frac{d}{dt}[Y] = a \frac{1}{1 + \left(\frac{[U]}{K}\right)^n} + c \frac{1}{1 + \left(\frac{[U]}{K}\right)^{-n}} - \beta[Y], \tag{9}$$

where a and c are the rate of protein synthesis at low and high U concentrations, respectively. Therefore, the temporal evolution of a transcription factor concentration ($[Y_i]$) of the network can be written as

$$\frac{d}{dt}[Y_i] = \alpha_i R_i - \beta_i[Y_i] + \gamma_i, \tag{10}$$


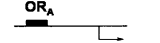

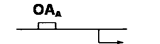

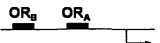

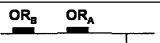

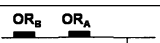

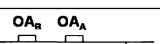

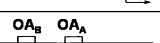



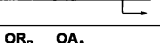
where α_i is the synthesis (transcription–translation) rate of gene i , β_i its degradation rate, and γ_i its basal rate. The function R_i is the regulatory factor that contains the information about the regulation of a given promoter by other transcription factors (see Table 1).

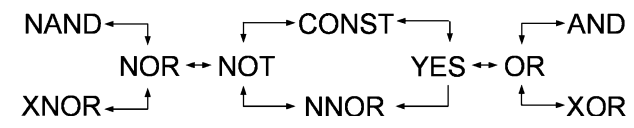
We have chosen the following expression for the regulatory factor of gene i :

$$R_i = c_{00} + c_{10}R_{iA} + c_{01}R_{iB} + c_{11}R_{iA}R_{iB}, \tag{11}$$

where c_{pq} are a set of coefficients defined to match a given asymptotic behavior (see Table 1) and R_{iA} and R_{iB} are given by

Table 1 List of promoters used in this study

TYPE	REGULATORY LOGIC	REGULATORY FUNCTION	PROMOTER WITH OPERATORS
0	-	1	CONSTITUTIVE
1 NOT	A  out	R^r_A	
2 YES	A  out	R^a_A	
3 NOR	A, B  out	$R^r_A R^r_B$	
4 XNOR	A, B  out	$1 - R^r_A - R^r_B + 2R^r_A R^r_B$	
5 NAND	A, B  out	$R^r_A + R^r_B - R^r_A R^r_B$	
6 AND	A, B  out	$R^a_A R^a_B$	
7 OR	A, B  out	$R^a_A + R^a_B - R^a_A R^a_B$	
8 XOR	A, B  out	$R^a_A + R^a_B - 2R^a_A R^a_B$	
9 NNOR	A, B  out	$R^a_A R^r_B$	



The first column shows the promoter’s label together with its name. The second column shows the standard logic symbol for the transcriptional activation as a function of the input concentrations of transcription factors *A* and *B*. The third column shows the corresponding value of the regulatory function R_i used in Eq. 10. The fourth column schematically shows the operator sites where the repressors (black boxes) or activators (white boxes) bind to the promoter. Bottom: Possible promoter transitions during the optimization procedure

$$R^r_{iA} = \frac{1}{1 + \left(\frac{[Y_A]}{K_{iA}}\right)^{n_{iA}}}, \tag{12}$$

in case of having a protein that represses transcription and by

$$R^a_{iA} = \frac{\left(\frac{Y_A}{K_{iA}}\right)^{n_{ij}}}{1 + \left(\frac{[Y_A]}{K_{iA}}\right)^{n_{iA}}}, \tag{13}$$

in case of activating transcription. K_{iA} is the activation or repression coefficient and n_{iA} is a Hill coefficient. In Table 2 we show the different ranges for the parameter values and their units used this work. We have only considered the c_{pq} corresponding to the choice of promoters in Table 1, although we will later show the use of another choice of c_{pq} . Those promoters asymptotically implement digital behaviors. The parameters c_{pq} could also be fitted against experimental data. In addition, we could also have

Table 2 Units and range values for the kinetic parameters considered in our mathematical model (see Eq. 10)

Parameter	Units	Max value	Min value
α	$\mu\text{M}/\text{min}$	10	0.1
β	1/min	2	0.001
γ	$\mu\text{M}/\text{min}$	0.01	0.0002
K	μM	5	0.01
n	-	3	1

considered promoters regulated by more than two transcription factors.

In addition, we choose to design transcriptional networks composed of promoters regulated by less than three transcriptional factors. We can find promoters with synergistic regulation by two transcriptional factors in *E. coli* and in *S. cerevisiae*: in *E. coli*, the promoter controlling gene *fucAO* is synergistically activated by *CRP* and *fucPIKUR* (Bronsted and Atlung 1996), the promoter controlling gene *cirA* is activated by *CRP* and repressed by *fur* (Griggs et al. 1990), and the transcription factors *fnr* and *arcA* repress gene *sdhCDBA* (Park et al. 1997). On the other hand, in yeast, the promoter controlling gene *PDR5* is activated by *PDR1* and *PDR3* (Katzmann et al. 1994), and the promoter controlling gene *GALI* is activated by *GAL4* and repressed by *MIG1* (Nehlin et al. 1991), and the transcription factors *MOT3* and *ROX1* repress gene *DANI* (Sertil et al. 2003).

Optimization procedure

We have developed a software to computationally design transcriptional networks with a target behavior (Rodrigo et al. 2007). We design circuits with a targeted dynamics by solving an optimization problem where we generate different circuits that are scored according to a distance from the expected time-behavior. We start from a circuit of random topology and random parameters. We use Simulated Annealing (Kirkpatrick et al. 1983) as an optimization procedure, which is implemented through the use of Monte Carlo algorithm with Metropolis updating and an exponential cooling schedule (Metropolis et al. 1953).

We randomly consider two different types of moves during a Monte Carlo step. In the first type of move, we randomly modify a parameter in the network model by a constant amount (positive or negative). For each parameter, the shift depends on its current value. For simplicity we did not consider the possibility of having different transition probabilities for every parameter. Moreover, this shift is adjusted to have an acceptance probability of 50%, which improves the convergence. For the second type of moves we modify the network topology by randomly choosing among

five possibilities: (i) to replace a promoter (which amounts to replacing the regulatory function in Eq. 11), (ii) to add a new regulation between a gene and a promoter, (iii) to remove a regulation on a given promoter, (iv) to add a new gene and (v) to remove a gene. In all moves, we randomly choose a gene and perform the corresponding modification.

To improve the convergence, in move (i) not all transitions are possible (see bottom of Table 1). For simplicity, in this work we do not consider promoters regulated by more than two transcription factors. In move (iii) we select randomly a regulation of the circuit to be suppressed. To avoid isolated genes, we also remove genes that do not take part of the circuit after this move. In move (iv) we also add a new regulation between the new gene and another (selected randomly) in the previous circuit. Notice that by adding genes we increase the circuit’s ability to reproduce a given behavior, which will produce an evolution towards large networks. One way to limit the corresponding explosion in network size is to either add a size penalty in the scoring function or reduce the probability to add a new gene. If a gene does not regulate any promoter, we remove it because it does not affect the behavior of the circuit. That gene could be regulated or not. In addition, for convergence purposes we take a much higher probability to do a parameter move (0.99 by default using our algorithm). Like that, for each generated circuit during the optimization, we can explore the possible behaviors according to the fixed range of the parameter values.

For each constructed genetic network, we solve the dynamics for the protein concentrations and then we compute a scoring function. If $z(t)$ gives the targeted behavior and $y(t)$ is the dynamics of the corresponding circuit, we define as score

$$\Phi = \int_{t_i}^{t_f} |y - z| \chi dt, \tag{14}$$

where χ is a weighting factor that takes 0 or 1 values to only score a region of interest.

Results

Design of a memory device

We have applied our methodology to design a sequential circuit implemented as a transcriptional network. Such network is able to store information resulting in a genetic memory. We have targeted a JK-Latch (Vingron 2003) which is the preferred type of flip-flop for most logic circuit designs because its behavior is very rich and completely predictable under all conditions. For this circuit, there are two inputs (J and K), which are the setters of each stable state. When both inputs are activated for a brief time (producing a pulse) the system switches of state. In fact, if this situation is prolonged the system could behave as an oscillator. Essentially, this circuit works as the toggle switch, already implemented with transcription factors (Gardner et al. 2000), which could be seen as a RS-Latch, except for the case in which both inputs are present.

We have designed two biological implementations of JK-Latches using transcription factors. The first one is guided by the electronic implementation (see Fig. 2a), where we have used as logic gates promoters with combinatorial regulation (see Fig. 2b). This rational approach is applied to engineer a JK-Latch by implementing on a RS-Latch two positive feedbacks from the outputs of the system (A and B). In that case, like in the electronic design, these feedbacks are integrated in two synergistically activated promoters. Unfortunately, it is not always possible to perform such rational designs, and in this article we aim to show a new technique to create alternative designs. As described in the previous section, we propose to use an unsupervised computational design technique. We have imposed the desired specifications (as shown in Fig. 3a) to design a novel circuit (see Fig. 3b) that has no resemblance to the previous rational design (see Fig. 2b). We have constructed Φ by adding the eight scores constructed by using the four possible entries of the truth table shown in Fig. 3a for each of the two possible initial conditions ($A = 1, B = 0 \mu\text{M}$ and $A = 0,$

Fig. 2 (a) Electronic implementation of a JK-Latch which is a sequential circuit. (b) Rational design of a biological memory device implementing a JK-Latch. The parameters and corresponding SBML file with the model can be found in the supplementary material

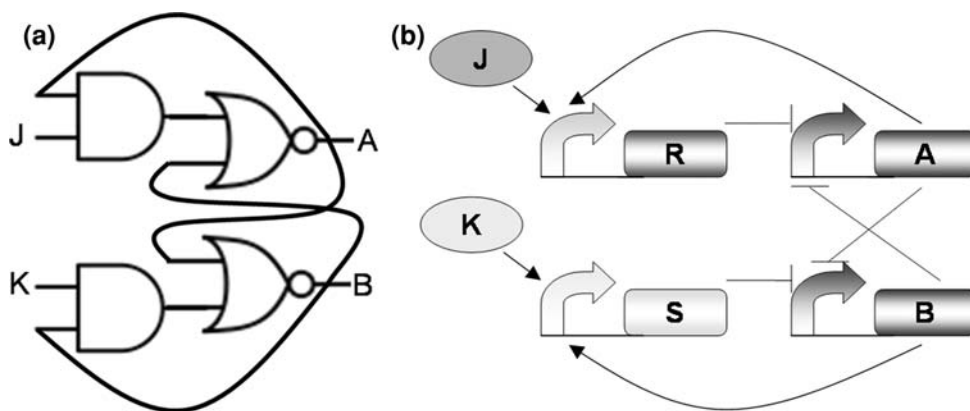
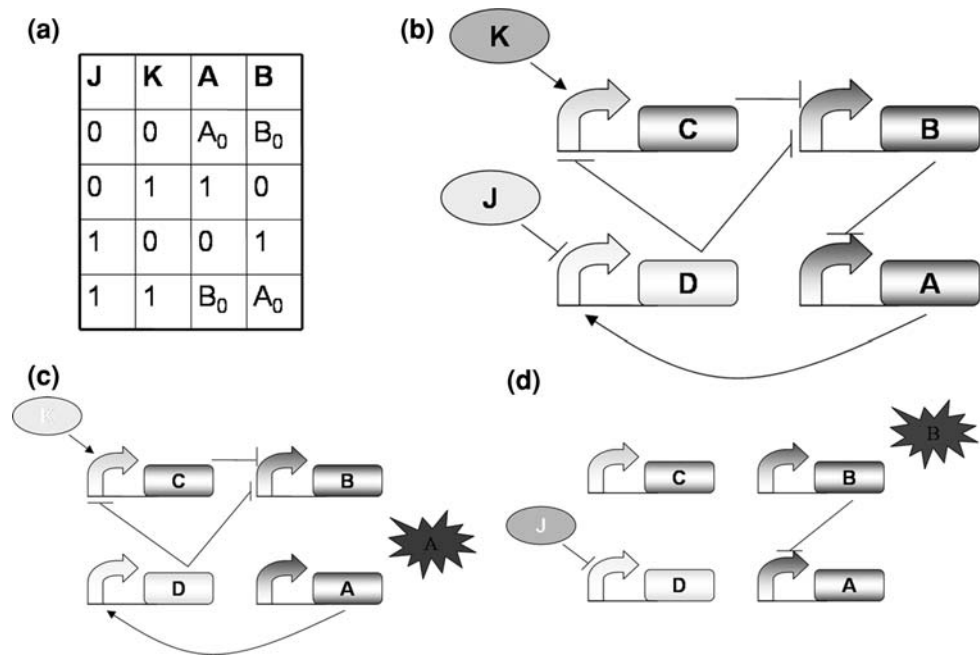


Fig. 3 (a) Truth table for a JK-Latch. (b) Computational design of a biological memory device implementing the truth table of a JK-Latch. (c) First stable state of this device in which protein *A* is expressed and *B* is repressed. (d) Second stable state in which protein *B* is expressed and *A* is repressed. The parameters and corresponding SBML file with the model can be found in the supplementary material



$B = 1 \mu\text{M}$). We provide as supporting information the corresponding SBML (Hucka et al. 2003) file containing the parameter values of the designed circuit. Our design was obtained by an in silico evolution optimizing both the topology and parameters of the network, without relying on an analogy between biology and electronics.

The work principle of this circuit relies on the fact that there are two stable states and the behavior fluctuates as a flip-flop from one to another according to the external inputs. In the first state (or state *A*), protein *A* is expressed activating *D*, which is in charge of repressing *B* and *C* (see Fig. 3c). In the second state (or state *B*), protein *B* is expressed repressing *A* (see Fig. 3d). In this case, *C* and *D* remain inactivated. According to the specifications of this circuit, the system remains in its current state when no inputs are present. When *K* is present the system is set to the state *A* (i.e., no changes are observed if the system is already in that state, and a flip-flop is produced if the system is in the state *B*). On the other hand, the transcription factor *J* is the setter for the state *B*. A switch of state is always produced when the two inputs pulse simultaneously. In addition, our system has the property to oscillate when both inputs are continuously present. We can see this dynamics in Fig. 4 in which we show different input conditions during a simulation of 1,000 min.

Conditional systems show hysteresis effects. We have computed the steady state of *A* after a pulse of *K* for several constant values of *J*. In Fig. 5a, b we show the hysteresis diagrams for the rationally and computationally designed JK-Latches, respectively. At low levels of *J*, the system behaves as a memory device. However, for $J = 0.5$ the system is not appropriate as a memory device because it

relaxes back to its previous state when the input disappears. In our computational design, when $J = 0$ the switch point occurs at $K \simeq 0.7 \mu\text{M}$. For higher values of *J*, this point increases and the system loses its ability to store information when we consider a constant *J*. For *J* close to $1 \mu\text{M}$, the switching dynamics disappears. Similar results are obtained based on the rational design. In this case, for $J = 0$ the switch point is $K \simeq 0.45 \mu\text{M}$, and a constant leakage of *J* avoids reaching $A = 1 \mu\text{M}$ (e.g., $A = 0.25 \mu\text{M}$ for $J = 0.5 \mu\text{M}$) but still allows the memory function.

Also, we have performed a stochastic simulation (Gillespie 1977) of this circuit to see its robustness under molecular noise (see Fig. 6). We have simulated several input conditions from different initial conditions. To perform such simulation we define a set of effective reactions involving the whole process of transcription and translation. We consider two possible reactions: protein synthesis and protein degradation, neglecting the fluctuations due to the mRNA dynamics. Hence, their fluxes are Hill functions of the transcription factors. We provide a MatLab file to perform this simulation as supporting information. We can see how the average behavior of the system is maintained as in Fig. 4. Further simulations could be performed, by detailing the reaction map and proposing typical parameter values to complete it, to obtain more accurate stochastic dynamics as the molecular noise can induce errors in the dynamics (Kepler and Elston 2001).

Design of digital devices

We have applied our methodology to design genetic devices behaving as logic gates. Our devices consist on

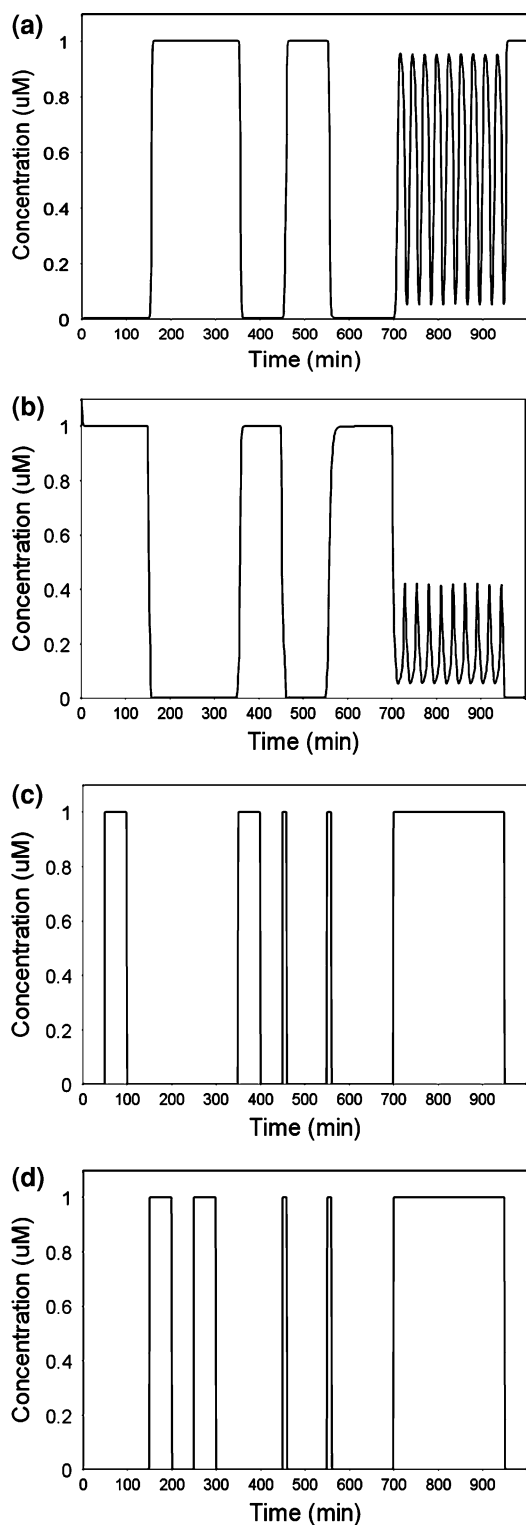


Fig. 4 Dynamics for our biological memory device implementing a JK-Latch. In (a) dynamics of A, in (b) dynamics of B, in (c) dynamics of J, and in (d) dynamics of K

genetic circuits having the concentration of two and one transcription factors as input and output, respectively. We have targeted AND, OR, NAND and NOR gates, and in

Fig. 7 we show the resulting circuits. To compute the objective function we have averaged the score obtained with each transfer function corresponding to every entry of the truth table. u_1 and u_2 are the input transcription factors and y is the output corresponding to the concentration of gene a 's product. We have evaluated the score during 100 min. However, to better appreciate the behavior under different input conditions, we have chosen to plot a temporal dynamics where the input transcription factors concentrations u_1 and u_2 take all possible Boolean values of a two-input truth table. Inputs can be activators or repressors according to the chosen promoter during the simulation. In the inset of Fig. 7 we also show the equivalent digital circuits according to the interaction of each transcription factor with its corresponding promoters. We provide the parameters of those circuits in the supporting information.

For the AND device (see Fig. 7, circuit I), the algorithm selected inducible promoters at the input genes, which implies that the u_1 and u_2 would correspond to the concentrations of two activator transcription factors. At $t = 200$ min we appreciate a little peak due to the simultaneous change of the inputs, which it is not unexpected as the device was evolved using the scores of steady states. This device could be easily understood in terms of its regulations. Initially the output gene a is off as it is controlled by a synergistic AND promoter (type 6 in Table 1) that requires b and c to be at high concentrations to start transcription. When the two inputs increase their concentration b and c start to build up and then a is produced. If either u_1 or u_2 are at low concentrations then b or c cannot be produced in enough quantities to induce the transcription of a . For the OR circuit (see Fig. 7, circuit II) we notice that the output almost fits the targeted behavior, being insensitive to the input changes at $t = 200$ min and at $t = 300$ min. This device has more latency when the output concentration shifts down because there is a self-activation on gene a . For the NAND network (see Fig. 7, circuit III), we obtain two repressors as inputs and the circuit has symmetry. In the NOR circuit (see Fig. 7, circuit IV) we have got a repressor and an activator as inputs. The latencies at $t = 0$ min and at $t = 400$ min are different. In the first case, the system starts from the initial condition and follows a spontaneous evolution with no inputs actions. However, the state of the system with the two inputs takes more time to grow up when their effects stop. We could reduce this latency, but compromising the steady state value. Actually, we have seen that we could get gene networks with small latencies as a result of our optimization, although sometimes we could not decrease the latency (circuits II and IV in Fig. 7) without compromising the score. In fact, we could lower the latency of circuit IV when we decreased the activation coefficient corresponding to

Fig. 5 Hysteresis cycles for two different JK-Latches between the steady state of A and the pulsing amplitude of K for several constant values of J . In (a) computationally designed network (see Fig. 3). In (b) rationally designed network (see Fig. 2) with parameter optimization using our algorithm

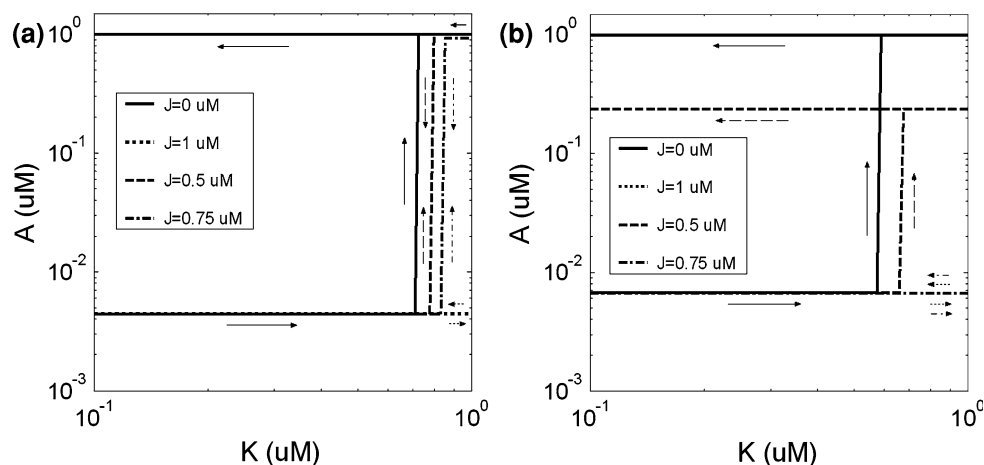
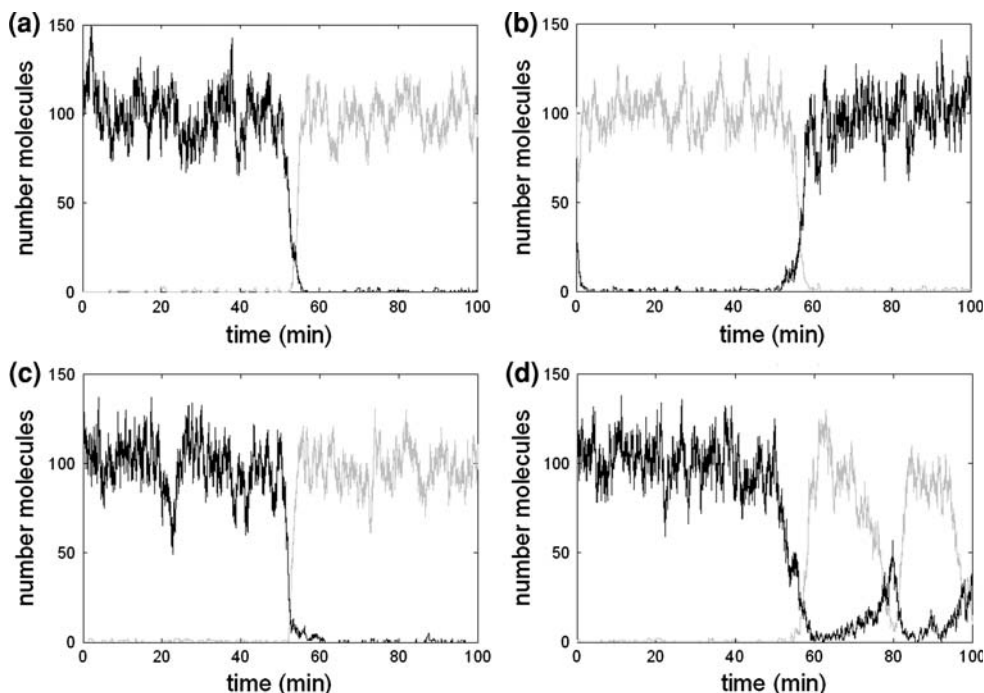


Fig. 6 Stochastic simulations (Gillespie 1977) for the memory device implementing a JK-Latch. We have considered 100 molecules as 1 unit in terms of concentration. A is shown in gray and B in black. In (a) K is set to high at 50 min while J is always low starting with the state B , in (b) J is set to high at 50 min while K is low starting with the state A , in (c) J and K are set to high at 50 min for just 10 min starting with the state B , and in (d) J and K are set to high at 50 min starting with the state B



the regulation of gene d by gene c (K_{dc}), but the height of maximum output expression also decreased.

Sensitivity analysis

To study the robustness of our designed memory devices, we have performed a sensitivity analysis for the transcription (α) and degradation (β) rates. For that, we plot the relative score variation ($(\Phi - \Phi_0)/\Phi_0$) versus the relative parameter value (α/α_0 and β/β_0) for the different circuit genes. In Fig. 8a, b we show the robustness of the computationally designed JK-Latch under perturbations on C and D at the transcription and degradation level. Oppositely, in Fig. 8c, d we show the robustness of the rationally designed JK-Latch under perturbations on R and S at the

transcription level and on R at the degradation level. We note that as our designs were optimized by Simulated Annealing, they are located in local minima being robust in a neighborhood of the optimized value.

On the other hand, as it is difficult to map a given network topology to a function, we have studied the parameter sensitivities for the circuit II from Fig. 7. Figure 9 allows us to show this fact by performing a functional evolution just by changing one kinetic parameter. In Fig. 9a we show how varying the transcription–translation rate of gene c we could modify the circuit behavior between OR and AND. In this case, the OR state is more robust as we can observe in the figure. In Fig. 9b, the evolution is between OR and NOR playing with the self-activation coefficient of gene a . In this case, we observe that the NOR state is more robust.

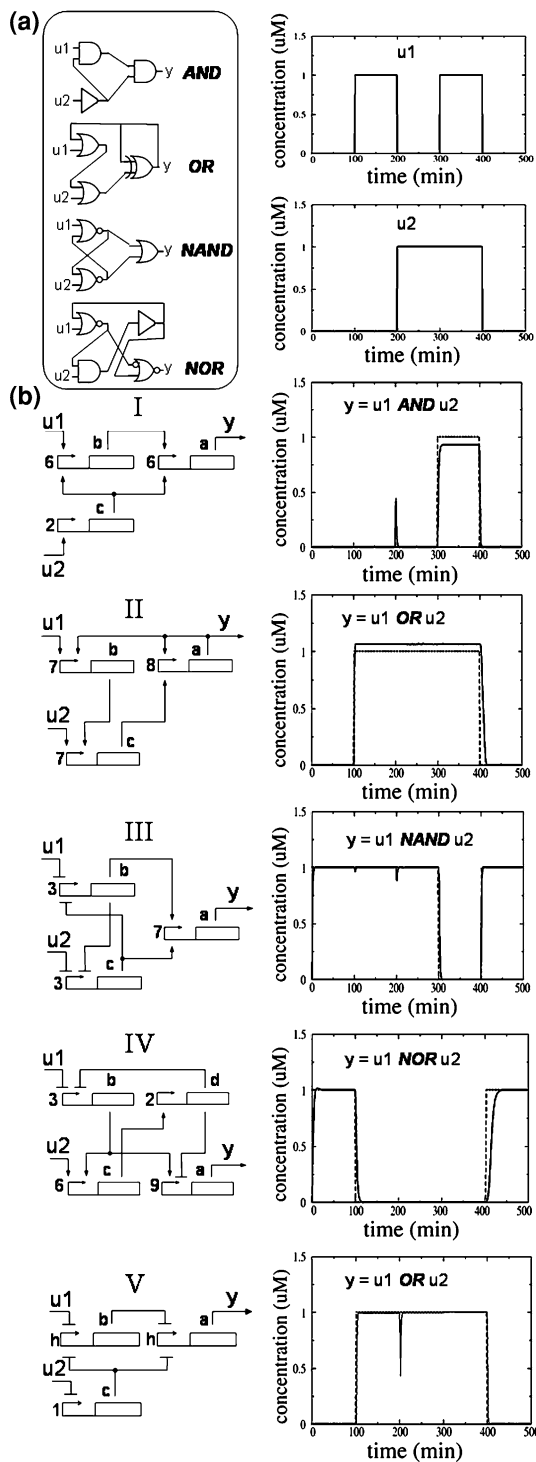


Fig. 7 Biological devices designed with digital targeted behavior. (a) Digital electronic circuit diagrams corresponding to the designed genetic networks. (b) On top, the time variation of the concentration of two genes, $u1$ and $u2$, chosen to be the device input genes. Below there are the resulting optimal regulatory genetic networks that more closely follow a given targeted behavior. Genes are noted with letters, and promoters with numbers according to their type (see Fig. 1). Two promoters in circuit V are noted with h (considered as hybrids), because they do not belong to the default set from Fig. 1. We targeted devices showing an AND, OR, NAND and NOR logic in terms of the input ($u1$ and $u2$) and output (a) genes. On the right, there are the corresponding time-variation of the reporter output gene concentration y . Dashed line represents the targeted behavior, and solid line the obtained evolution from the optimal genetic network. The parameters can be found in the supplementary material

Discussion

Circuit implementation and functionality

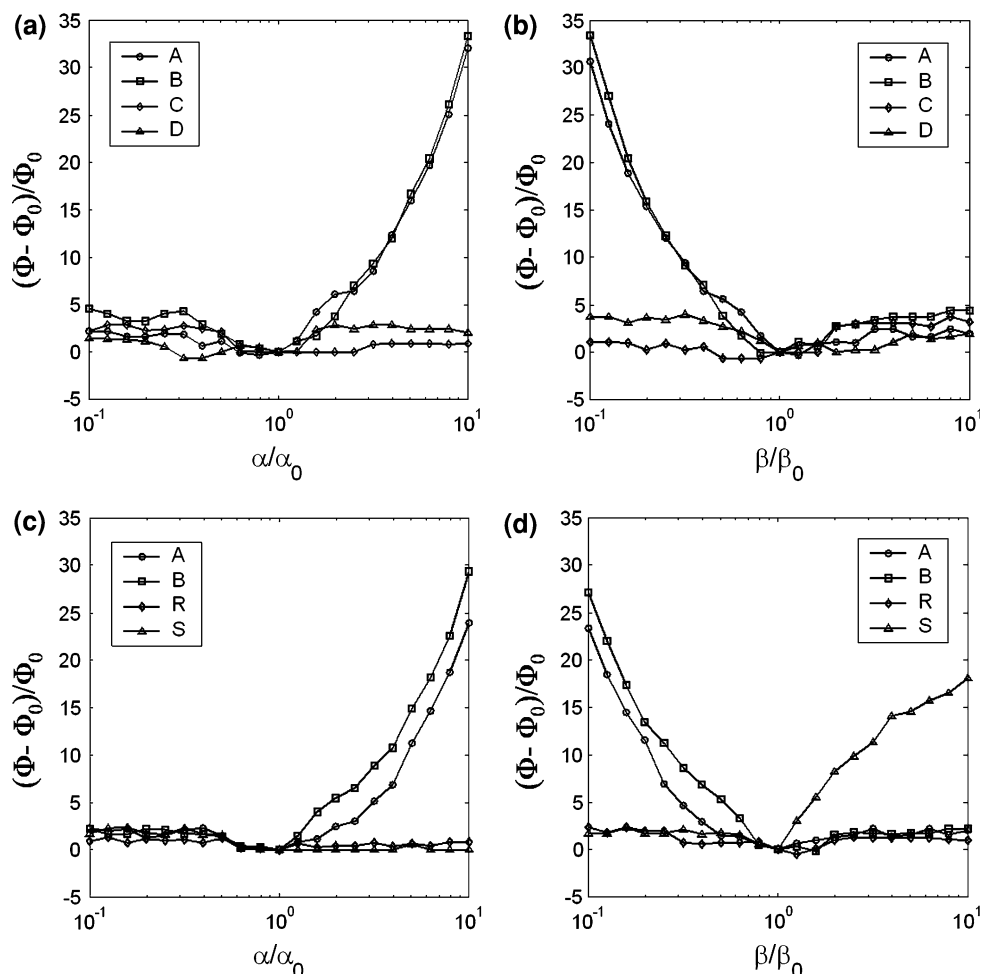
Our designs of digital devices and of a JK-Latch show that we can use automatic computational design to engineer networks without relying on design principles inspired in the electronic analogy. Our computational approach not only is able to design combinatorial but sequential circuits. The design of such memory systems has a big relevance in biology because the cell is able to react against a given external stimulus taking into account the previous conditions. We notice that natural-occurring promoters do not behave as perfect step functions but have a smoother behavior. This requires a careful fine-tuning of parameters during the design process. We have shown how to use our procedure by considering promoters with such smooth response (as in Fig. 7 circuit V).

We could understand the working principle of our computational JK-Latch by considering the relevant sub-circuits. Firstly, we find a toggle switch between D and B (as B represses D by means of A), which will become the core of our biological memory. Nevertheless, the ability to switch when both inputs are present, but independently of the previous state, requires a more complex topology. In fact, the oscillatory behavior when both inputs are present is a non-trivial consequence of such specification, since we targeted a switch that changed state when pulsing with both inputs at the same time. This behavior can be reached in a small region of the parameter space though. In addition, we note that, instead of using two positive feedbacks as the rational design proposes, our design relies on the feedback of A on D . Furthermore, our circuit just requires the use of three two-regulated promoters, instead of four as the rational design proposes, which is convenient for the experimental implementation.

However, the in vivo implementation of a circuit which has been designed in silico usually requires many experiments in order to tune the desired behavior (Weiss 2001) and to avoid possible cross talks (Buchler et al. 2003).

We could also perform a functional evolution between AND and NOR by modifying the two considered parameters at the same time. Notice that we could then engineer an AND function from the OR device by simply doing directed mutagenesis on the ribosome binding site of gene c , decreasing then its expression rate. Or engineer an NOR function by decreasing the transcription factor affinity of a on its promoter region.

Fig. 8 Parameter sensitivity analysis for the computationally designed JK-Latch (a, b), and for the rationally designed JK-Latch (c, d). Subindex 0 refers original values



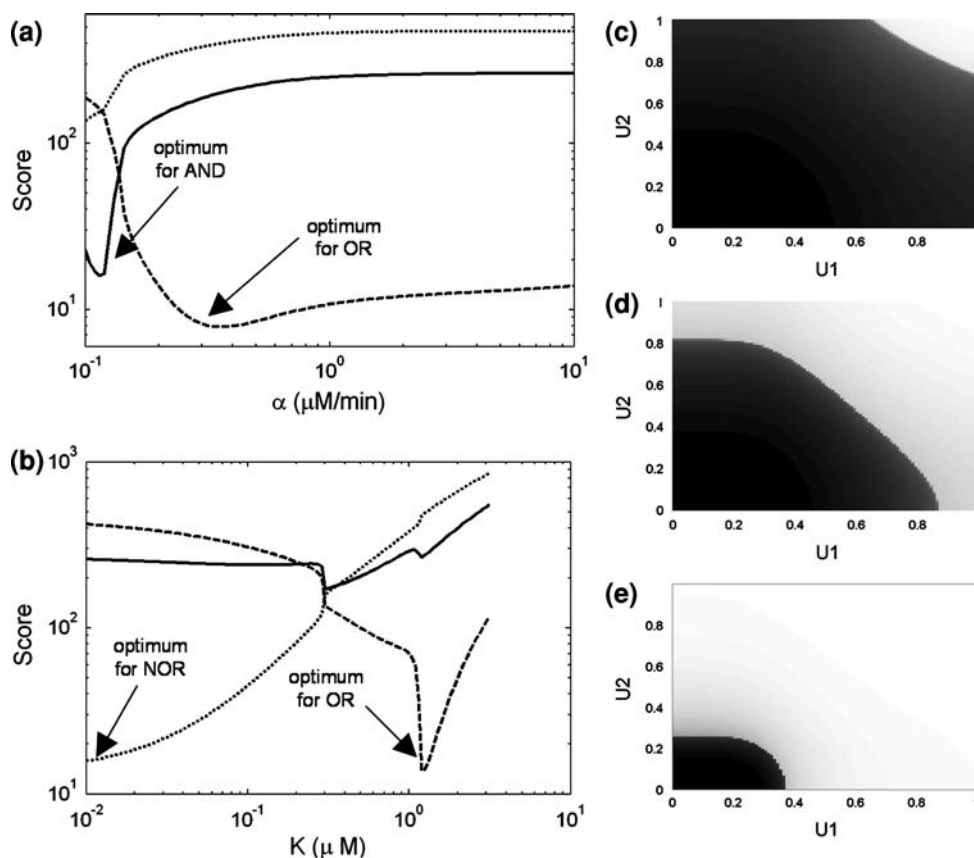
Moreover, a constant fine-tuning of the model by using experimental feedbacks will introduce more accuracy even with in vitro environments (Kim et al. 2006). It could be possible to incorporate this experimental feedback into our automatic design procedure by developing a new algorithm that would design circuits by assembling models of biological parts (Rodrigo et al. 2008). This will require a repository of already characterized synthetic or natural parts. Fortunately, recent combinatorial work has shown the easiness of creating large amounts of promoter parts by generating a set of promoters together with their experimental characterization (Cox III et al. 2007). Our computational design of a JK-Latch could be implemented by using *araC* as *K*, and λ -*cI* as *J* for the external controlling signals. Subsequently, *luxR-luxI* could work as *A*, using *luxP_RcI-O_R1* as promoter of *D* (Basu et al. 2004), and λ -*cro* as *B* with the *P_{RM}* as promoter of *A* (Hasty et al. 2002). Finally, *lacI* could be *C* and *tetR* be *D*, implementing the *lacI* and *tetR* operator sites on the promoter region of *B*, and the *araC* and *tetR* sites on the promoter of *C* (Cox III et al. 2007). Nevertheless, the use of such biological parts will require experimental fine-tuning to obtain the specified behavior. In fact, previous works

(Weiss 2001) needed to use site-directed mutagenesis in the ribosome binding sites or in the operator sites to evolve the whole system. In addition, directed evolution techniques can evolve the behavior of the system by fine-tuning the kinetic parameters (Yokobayashi et al. 2002; Nomura and Yokobayashi 2007).

Could natural genetic networks be understood as systems of devices?

Could natural circuits have a selective pressure for a given network motif or for a given dynamical function? We have found several circuits with targeted AND behavior. If a selective pressure existed for a given network module behavior, then some circuits within a module would get rewired by evolution while maintaining their functionality. For instance, it could be that some AND circuits would occasionally appear in evolution substituted by another AND circuit. We notice that several of our results in Fig. 7 showing digital behaviors appeared very often in natural gene networks. It is interesting that two of them (*I* and *V* in Fig. 7b) were found very often in *E. coli* and *S. cerevisiae* as network motifs. Circuit *I* was found 28 and 26 times in

Fig. 9 We plot the scores of circuit *II* (see Fig. 7) by computing them using AND (solid line), OR (dashed line) and NOR (dotted line) behaviors. We have performed a parameter scan of (a) the transcription–translation rate of gene *c* (α) taking the activation coefficient of promoter of gene *a* (K) equal to 1.2 μM , and (b) the activation coefficient of promoter of gene *a* (K) taking the transcription–translation rate of gene *c* (α) equal to 10 $\mu\text{M}/\text{min}$. Behavior of the system versus the input concentrations when α is (c) 0.1 (the value of the optimum for AND in (a)), (d) 0.15, and (e) 0.4 $\mu\text{M}/\text{min}$ (the value of the optimum for OR in (a)), remaining K constant



E. coli and *S. cerevisiae*, respectively, as a coherent feed-forward loop (FFL) system (Mangan and Alon 2003) where it was suggested this Circuit could function as an AND gate able to filter out pulses in the input. For instance, the topology of circuit *I* appears in the genes (corresponding to our genes *a*, *b* and *c*) *appCBA*, *appY* and *arca* in *E. coli*, and in the genes *PDR5*, *PDR3*, and *PDR1* of *S. cerevisiae*. Likewise the topology of the circuit *V* is found in the genes *CYC7*, *ROX1* and *TUPI* in *S. cerevisiae*, but it is not present in *E. coli* (Mangan and Alon 2003).

On the other hand, it could happen that natural gene circuits would not rely on functional modules (Hartwell et al. 1999) but on a complex intertwined network of interactions, as it would usually happen with evolutionary design (Bosl and Li 2005). In this later case, maybe the only way to design a system of devices would be by using an evolutionary design procedure. We could then use directed evolution of gene circuits (Yokobayashi et al. 2002) or in a combination with a computational procedure. More work is needed to elucidate this point.

Functional evolution of genetic networks

Our optimization procedure also allows us to analyze the evolvability of genetic devices. Here we consider the circuit

II of Fig. 7, where we have modified a single parameter, α , in order to change the device function to behave as an AND gate (see Fig. 9a). This parameter was related to the expression of gene *c* and this sensitivity provides an evolvable mechanism for device function, as mutations affecting its expression will have a dramatic effect in the device function. Therefore, the transcription–translation efficiency of that gene would be under a strong selection pressure, which could be the general rule in biology, according to the experimental evidence from recent work where protein expression levels were found to be shaped by evolution (Dekel and Alon 2005). Moreover, by changing a few parameters we could get a NOR gate as well, inverting completely the initial behavior (see Fig. 9b).

We could propose a scenario for the evolution of device function by considering the situation *d* in Fig. 9. This circuit shows an intermediate behavior between AND and OR digital devices (when α is around 0.15 $\mu\text{M}/\text{min}$), but rapidly converged to OR (when α is higher) or AND (when α is lower). Then, if we consider the case where the input concentrations always remain below a threshold (such as 0.7 μM) then the device would work as a perfect AND gate. On the other hand, if the concentrations were always above another threshold (such as 0.5 μM) then the device would work as a perfect OR gate. Hence, by shifting the expression level of the input transcriptions factors, either

dynamically or by evolution (Dekel and Alon 2005), we could change the function of the device while maintaining all the circuit properties. This promiscuity in system function could play a mayor role in the evolution of biological systems. In fact, a device could simultaneously have several functions and a change in the environment could switch between them. In this way, if during evolution the environment would not change enough then the alternative function could be lost.

Further applications

Our methodology could also be adapted to the inference of regulatory networks provided a specific device function is known. Another application is to train parameters for given in vivo genetic circuits (Feng et al. 2004). In future applications we could refine the mathematical model to include stochastic dynamics and an optimization that would also score for robust circuits. We could also use our methodology to understand and derive new types of control systems in transcriptional and metabolic networks (El-Samad et al. 2005). Finally, our work shows how to design genetic networks without the constraint of using components adopting Boolean values, often used to design transcription networks. Gene concentrations can adopt a continuous range of values and only a computational procedure can simultaneously optimize the design of a circuit and its parameters to fit a given function. Future automatic design work will have to incorporate experimental data to build the models to be explored in the network optimization.

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