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Computational design of synchronous sequential structures in biological systems

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ABSTRACT

Numerous applications of synthetic biology require the implementation of scalable and robust biological circuits with information processing capabilities. Basic logic structures, such as logic gates, have already been implemented in prokaryotic as well as in eukaryotic cells. Biological memory structures have also been implemented either *in vitro* or *in vivo*. However, these implementations are still in their infancy compared to their electronic equivalents. Their response is mainly asynchronous. We may learn from electronic computer systems that robust and scalable computing devices can be implemented only with edge-triggered synchronous sequential structures. Implementation of such structures, however, has yet to be performed in the synthetic biological systems even on the conceptual level.

Herein we describe the computational design and analysis of *edge-triggered D flip-flop* in *master–slave* configuration based on transcriptional logic. We assess the robustness of the proposed structure with its global sensitivity as well as parameter sweep analysis. Furthermore, we describe the design of a robust *Johnson counter*, which can count up to $2n$ cellular events using a sequence of n flip-flops. Changing the state of the counter is edge-triggered either with synchronization, i.e. clock signal, or with a pulse, which corresponds to the occurrence of observed event within the cellular environment. To the best of our knowledge this represents the design of the first biological synchronous sequential structure on such level of complexity.

1. Introduction

First successful implementations of logic structures in *Escherichia coli* cells, such as logic gates [1] and oscillators [2], mark the beginning of the field of synthetic biology [3]. Further development of these structures received large attention in the last 15 years [4,5]. More complex processing systems, such as a simple biological computer, are together with one of their vital parts, i.e. scalable, robust and reliable memory structures, however, yet to be implemented. Memory structures are also of significant importance for the wider scope of synthetic biology applications. For example, memory could be used in order to identify cell populations responsive to specific events and track their progression through the cellular response [6,7]. Moreover, logic structures can be used in a combination with biological memory to select and maintain one of the possible states of the system with fundamentally different biological functions, e.g., to implement an effective multi-state treatment from inhibition of inflammatory processes (state 1) to tissue regeneration (state 2).

Attention towards the implementation of robust and scalable biological memory has therefore been increasing in recent years. In this context we can divide the current implementations of biological memory structures in two groups, i.e. *long-term memory circuits* with high density, which are based on DNA recombination, on one hand [8–11], and *transcriptional memory devices* with short-term storage capabilities, which are based on bistable genetic response, on the other hand.

Several *in-vitro* implementations of long-term memory circuits have been reported in the last decade [10,11]. Few realizations of *switchable and reversible long-term memory circuits* have also been reported recently [8,12,13]. These are able to interface the logic functions with the long-term memorisation of their outputs [9]. The complexity of such circuits together with relatively long access times, i.e. read and especially write times, make them however unsuitable for current information processing applications.

Transcriptional memory devices are on the other hand less complex and have significantly shorter access time in comparison to DNA recombination based circuits. Their implementations are based on a bistable transcriptional response of gene

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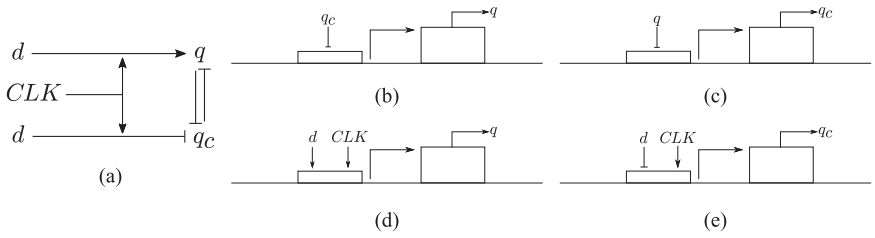

Fig. 1. Design of the basic biological clocked D flip-flop. (a) represents its logic scheme and (b–e) genes in its transcriptional implementation. In all figures *d* represents the data protein and *CLK* the clock protein, while *q* and q_c represent the complementary output proteins. Sharp end arrows indicate transcriptional activation, whereas blunt end arrows indicate transcriptional repression. In (b–e) small boxes indicate transcription factor binding sites, bent arrows indicate promoters and large boxes indicate protein coding regions.

regulatory interactions [6], which can be implemented in several ways, e.g. using single self-activating transcriptional factor (TF) [14], or either double-positive or double-negative feedback loop between two interacting TFs. An example of natural system which reflects bistable behaviour, and involves both positive autoregulation and double-negative feedback loop, is *phage lambda switch system* [15]. The first synthetic implementation of short-term storage, i.e. *toggle switch*, was performed in a similar manner using double-negative feedback loop [1]. This was later extended to a so called *push-on push-off switch* [16]. Short-term memory using autoregulatory transcriptional positive feedback was implemented in yeast cells [17]. Maintenance of active state of a signal pathway was achieved with the integration of autoregulatory positive feedback in the MAP kinase system [18]. Recently, yeasts cells have been used to implement multicellular short-term memory [19]. Short-term memory structures have also been implemented in mammalian [20] and even in human cells [7].

In spite of significant progress in the field of synthetic biological memory, implementation of *synchronous sequential structures* in biological systems is yet to be performed. These are, however, essential for the implementation of more complex biological information processing structures, since they provide synchronisation between the logic elements, which results in a robust behaviour of the system. Herein we present the computational design and analysis of biological *master–slave D flip-flop*, which is *edge-triggered* by a *synchronisation (clock) signal*. We apply the proposed structure to the design of a *Johnson counter*, which reflects robust behaviour, and can count up to 2*n* events using a sequence of *n* flip-flops. Changing the state of the counter can be triggered either with synchronization, i.e. clock signal, or with a pulse which corresponds to the occurrence of the observed event. Correctness of the proposed counter's performance is not affected by the pulse length as is the case of the *state-of-the-art* biological counters [21]. All the code used in this paper is available at http://lrss.fri.uni-lj.si/bio/material/counter.zip under the Creative Commons Attribution license.

2. Computational design of biological D flip-flop

Edge-triggered memory structures are of a significant importance for the implementation of complex processing structures, but are yet to be implemented in biological systems. In this section we describe the computational design of the robust biological D flip-flop in a master–slave configuration.

2.1. Clocked D flip-flop

We propose the design of the biological clocked D flip-flop, which is based on a double-negative feedback loop as is also the case in its electronic equivalent [22]. Negative feedback loop is as in the toggle switch regulatory circuit [1] implemented with two complementary proteins (*q* and q_c) that serve also as outputs of the flip-flop. Their expression is additionally controlled by two input proteins, i.e. *data protein* (*d*) and *clock protein* (*CLK*), for which we presume an oscillatory behaviour. The design of proposed clocked D flip-flop is presented in Fig. 1. The first two genes expressing proteins *q* and q_c (see Fig. 1(b) and (c)) are active, when the complementary proteins (*q* for q_c and vice versa) are absent. The second two genes expressing proteins *q* and q_c (see Fig. 1(d) and (e)) are active only when the clock protein *CLK* is present. If the data protein *d* is active at the same time, *q* is expressed, otherwise q_c is expressed. Proteins *q* and q_c provide the state maintenance with the double-negative feedback loop while *d* and *CLK* enable the switching of the state. Expression of output proteins *q* and q_c is described in Table 1.

Table 1
Expression of output proteins *q* and q_c. (a) The expression of the protein *q* and (b) the expression of the protein q_c. The value 0 represents absence, i.e. low concentration, and 1 respectively presence, i.e. high concentration, of specific protein. Symbol x is used as a *don't care* value.

(a)

CLK	*d*	q_c	*q*
0	x	0	1
0	x	1	0
1	0	x	0
1	1	x	1

(b)

CLK	*d*	*q*	q_c
0	x	0	1
0	x	1	0
1	0	x	1
1	1	x	0

2.2. Master–slave D flip-flop

The clocked flip-flop as described in the previous section assumes that the switching of the state is triggered with a high level of clock signal. The duration of the high level clock pulses therefore needs to be precisely tuned with the dynamic response of each gene. The pulse needs to be long enough to perform the switch, but at the same time short enough to prevent more than one change of the flip-flop state [21]. The implementation thus requires a stable and robust clock signal, which is usually not the case in biological systems. The problem can be avoided using edge-triggered structures in which the state is never changed more than once in the same clock signal period, i.e. with a single pulse. Edge-triggered flip-flop can be implemented with a master–slave configuration using two clocked flip-flops as presented in Fig. 2. Proposed biological D flip-flop again uses two input proteins, i.e. *CLK* and *d*, and an additional pair of complementary proteins, i.e. *a* and a_c. We assume that each protein in the scheme is expressed by two independent genes as in the clocked flip-flop described in Section 2.1. The enhanced flip-flop also includes two additional negative feedback loops that

Table 2
Expression of proteins a, a_c, q and q_c. (a) The expression of the protein a, (b) the expression of the protein a_c, (c) the expression of the protein q and (d) the expression of the protein q_c. The value 0 represents absence and 1 respectively presence of specific protein. Symbol x is used as a *don't care* value.

(a)

CLK	d	a_c	a
0	0	x	0
0	1	x	1
1	x	0	1
1	x	1	0

(b)

CLK	d	a	a_c
0	0	x	1
0	1	x	0
1	x	0	1
1	x	1	0

(c)

CLK	a	q_c	q
0	x	0	1
0	x	1	0
1	0	x	0
1	1	x	1

(d)

CLK	a_c	q	q_c
0	x	0	1
0	x	1	0
1	0	x	0
1	1	x	1

prevent the overexpression of output proteins q and q_c and thus perform *jitter reduction*. Expression of observed proteins, i.e. a, a_c, q and q_c, is presented in Table 2.

2.3. Modelling the master–slave flip-flop

The proposed master–slave flip-flop can be verified with the establishment of its mathematical model upon which simulations can be performed. We established a deterministic model based on *ordinary differential equations* (ODEs), which describes the system with the following equations

$$\frac{da}{dt} = \alpha_1 \cdot \Theta(d - K_{d_1}) \cdot \Theta(K_{d_2} - CLK) + \alpha_2 \cdot \Theta(K_{d_3} - a_c) - \delta_1 \cdot a, \quad (1)$$

$$\frac{da_c}{dt} = \alpha_1 \cdot \Theta(K_{d_1} - d) \cdot \Theta(K_{d_2} - CLK) + \alpha_2 \cdot \Theta(K_{d_3} - a) - \delta_1 \cdot a_c, \quad (2)$$

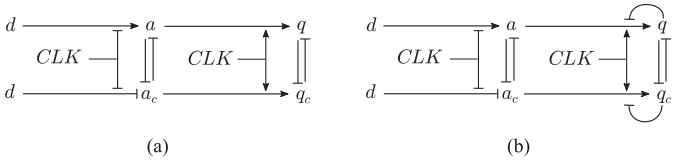

Fig. 2. Design of the biological D flip-flop in a master–slave configuration. The scheme uses two sequentially connected clocked flip-flops introduced in Section 2.1 – (a) The *jitter* of the output protein concentrations can be avoided with additional autoregulatory negative feedback loops for output proteins q and q_c – (b). In both figures d represents the data protein and CLK the clock protein, a and a_c represent the complementary output proteins of the master D flip-flop, while q and q_c represent the complementary output proteins of the slave D flip-flop.

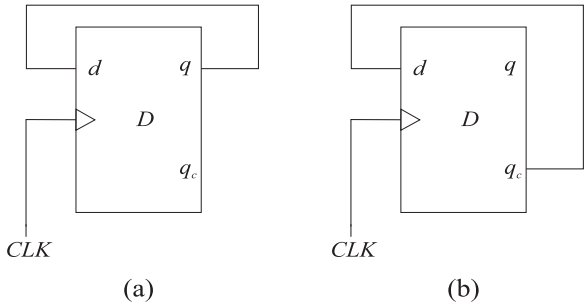

Fig. 3. Topologies used for evaluating the performance of biological master–slave D flip-flop. (a) The topology in which the state of the flip-flop (q) should be maintained, i.e. output protein q is fed back to the input data protein d. (b) The topology in which the state of the flip-flop (q) should be switched after each positive edge of the clock signal (CLK), i.e. complementary output protein q_c is fed back to the input data protein d.

$$\frac{dq}{dt} = \alpha_3 \cdot \Theta(a - K_{d_4}) \cdot \Theta(CLK - K_{d_5}) \cdot \Theta(K_{d_7} - q) + \alpha_4 \cdot \Theta(K_{d_6} - q_c) \cdot \Theta(K_{d_7} - q) - \delta_2 \cdot q, \quad (3)$$

$$\frac{dq_c}{dt} = \alpha_3 \cdot \Theta(a_c - K_{d_4}) \cdot \Theta(CLK - K_{d_5}) \cdot \Theta(K_{d_7} - q_c) + \alpha_4 \cdot \Theta(K_{d_6} - q) \cdot \Theta(K_{d_7} - q_c) - \delta_2 \cdot q_c, \quad (4)$$

where Θ is a *unit step function*, α_1, α_2, α_3 and α_4 the maximal expression rates of proteins a, a_c, q and q_c, K_{d_1}, K_{d_2}, K_{d_3}, K_{d_4}, K_{d_5}, K_{d_6} and K_{d_7} dissociation constants that present the threshold concentrations of specific TFs to activate, respectively inhibit, the expression of regulated proteins, and δ_1 and δ_2 the degradation rates of observed proteins. The background for the derivation of described mathematical model is explained in the Supplementary text.

2.4. Tuning the master–slave flip-flop

Our goal was to tune the dynamic response of the established model with the desired behaviour, i.e. correct and robust performance, of edge-triggered D flip-flop. Tuning was performed on the basis of simulation results of established mathematical model in a combination with *genetic algorithm* (GA) (see Supplementary text for its detailed description). GA was applied to the calibration of the kinetic parameters' values, for which biologically plausible ranges according to [23] were used (see Supplementary text).

Dynamic response of the system was evaluated using two different functionalities of the flip-flop, i.e. *maintaining the state* and *switching the state*. While state maintenance needs to be achieved when the input signal is fixed, switching needs to be performed on the positive clock edge together with the change of data input signal concentration levels. Another aspect we additionally considered is the *modularity* of the topology. Flip-flops can be modularly connected in a sequence if the data input protein concentration levels comply with the output protein concentration levels. We thus performed the simulations on two different topologies for each set of kinetic parameter values. The first topology uses an output protein (q) as a data input (see Fig. 3(a)), which should cause the state maintenance. The second topology uses a complementary output protein (q_c) as a data input (see Fig. 3(b)), which should cause the state switch after each positive edge of clock protein (CLK). Both topologies use a protein with an oscillatory behaviour as a clock protein.

The convergence of GA was achieved in solely 16 iterations with only 40 individuals and random initial parameter values, which

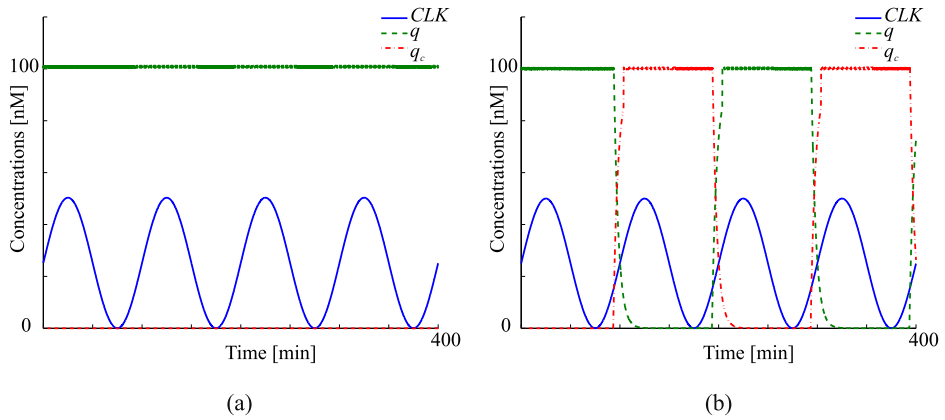

Fig. 4. Simulation results of the master–slave D flip-flop model using the parameter values obtained with described GA. (a) The simulation results of the topology in which state should be maintained. (b) The simulation results of the topology in which state should be switched after each positive edge of clock signal. The parameter values used in the simulation are as follows: $\alpha_1 = 0.8508\,s^{-1}$, $\alpha_2 = 1.5299\,s^{-1}$, $\alpha_3 = 0.3431\,s^{-1}$, $\alpha_4 = 1.5299\,s^{-1}$, $K_{d_1} = 99.0481$ nM, $K_{d_2} = 12.4672$ nM, $K_{d_3} = 34.9188$ nM, $K_{d_4} = 99.0481$ nM, $K_{d_5} = 14.6698$ nM, $K_{d_6} = 11.7473$ nM, $K_{d_7} = 99.8943$ nM, $\delta_1 = 0.0036\,s^{-1}$ and $\delta_2 = 0.0036\,s^{-1}$.

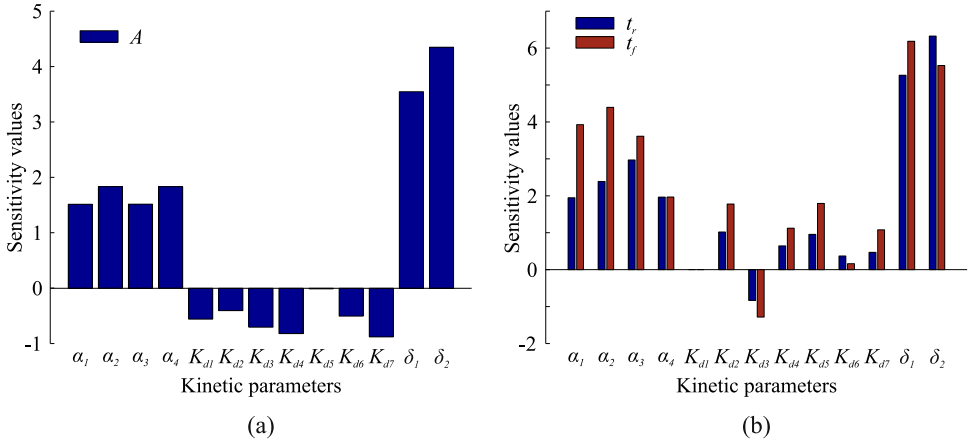

Fig. 5. Results of the global sensitivity analysis of proposed master–slave D flip-flop topology. (a) The analysis of the flip-flop amplitudes, i.e. difference between low (0) and high (1) logic state of the flip-flop (A). (b) The analysis of rise (t_r, blue colour) and fall (t_f, red colour) times. The values are given in the logarithmic scale (negative values denote values between 0 and 1 in the linear scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

represents the first indicator for the robustness of established topology. We used a sinusoidal clock signal with the period of 100 minutes, which can be implemented with a simple genetic oscillator, such as e.g. repressilator described in [2] and recently upgraded in [24]. The simulations were performed in the MATLAB environment. More detailed descriptions of parameter tuning process are available in Supplementary text. Simulation results of one amongst many valid solutions obtained with the described procedure are presented in Fig. 4.

3. Sensitivity analysis of proposed flip-flop topology

One of the main problems of the robust design of synthetic biological systems is the variation of parameter values due to different environmental factors [25]. We performed a global sensitivity analysis to evaluate the effects of each kinetic parameter value variations on the overall dynamics of the system. Since we are dealing with a high-dimensional and poorly connected parameter space it is not possible to perform the analysis with a straightforward adaptation of existing global sensitivity analysis methods. We developed a computational framework that is able to evaluate the global sensitivity of observed topology. The proposed framework (1) generates viable solutions using GA described in Section 2.4, (2) clusters the solutions into different parameter regions on the basis of distances between the parameter values (4 regions were obtained in our scenario), (3) resamples the parameter values within each region using *orthogonal sampling* (32 samples were generated for each of the regions) [26], (4) evaluates the sensitivities of each of the regions using *Morris sensitivity analysis method* (8 trajectories were evaluated for each sample) [27–29], and (5) combines the sensitivity measures for all samples into sensitivity values describing the influence of each of the parameters on the dynamics of the system.

Morris sensitivity analysis, as many other sensitivity analysis methods, perturbs the kinetic parameter values from the nominal values, for which the system exhibits desired behaviour. Qualitative behaviour was maintained in all of the $4 \cdot 32 \cdot 8$ experiments we performed, i.e. both topologies still reflected desired behaviour, which indicates the robustness of proposed topology. We additionally observed quantitative effects of parameter perturbations, i.e. influences that each of the parameters has on the *difference between low (0) and high (1) state of the flip-flop* (A), time needed to perform the switch from low state to high state, i.e. *rise time* (t_r), and time needed to perform the switch from high state to low state, i.e. *fall time* (t_f). For each of the observed characteristics we calculated the *absolute mean elementary effect* (μ) of the parameter values as a sensitivity measure (for details see [29]). The results of the sensitivity analysis are shown in Fig 5. Results show that all three characteristics are mostly affected by parameters δ_1 and δ_2 (note that the

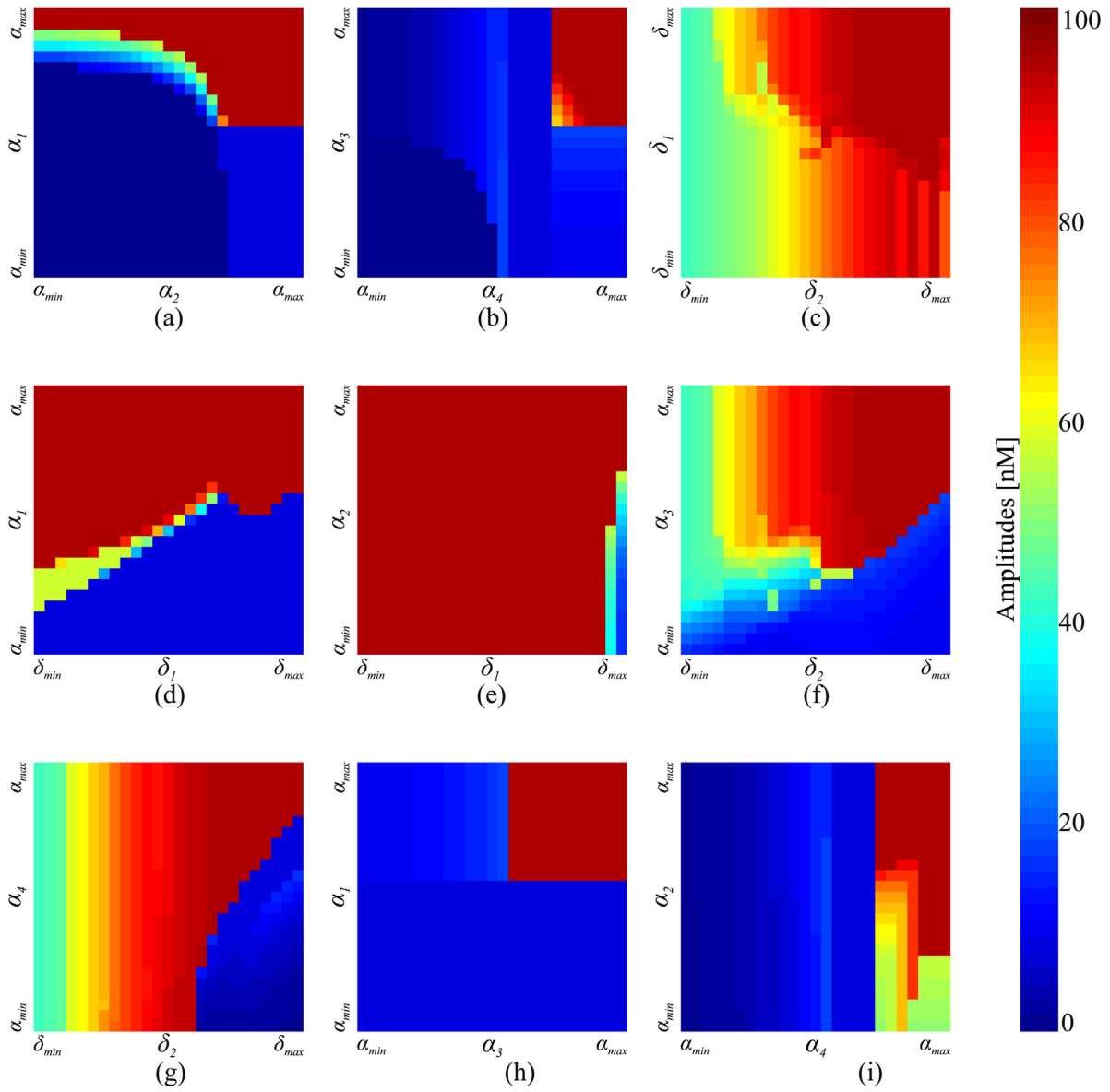

Fig. 6. Results of the 2-dimensional parameter sweep analyses of counter amplitudes performed on the combinations of parameters with the largest sensitivity values (see Fig. 5(a). The nominal parameter values used in the analyses were obtained from the central point of the largest cluster in the viable solution space. In each analysis two different parameters were perturbed throughout their whole feasible regions (see Supplementary text). Each interval between the minimal and the maximal parameter values was sampled logarithmically with 25 samples. (a) The parameters α_1 and α_2 were perturbed, in (b) α_3 and α_4, in (c) δ_1 and δ_2, in (d) α_1 and δ_1, in (e) α_2 and δ_1, in (f) α_3 and δ_2, in (g) α_4 and δ_2, in (h) α_1 and α_3, and in (i) α_2 and α_4. Red colour denotes the maximal ($\geq$100 nM) and blue colour the minimal amplitude (incorrect counter behaviour). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sensitivity values are given in logarithmic scale). The fact is, that these two parameters can be tuned with relatively large accuracy even *in vivo* using, e.g., protein degradation tags [30].

Since the clock periods are relatively long in comparison to maximal rise as well as fall times, we dedicated our further analyses solely to the flip-flop amplitude, i.e. difference between low (0) and high (1) logic state of the flip-flop. The additional analyses were performed on the combinations of parameters with the largest effect on the flip-flop amplitude to obtain additional insights into the system's dynamics. We used a 2-dimensional parameter sweep analysis (PSA) to observe the influences of pairwise parameter perturbations on the response of the system. Parameters were perturbed throughout their whole feasible regions (see Supplementary text). The central point of the largest cluster in the viable solution space, which can be interpreted as the most robust solution, was used as a nominal point, i.e. non-perturbed solution, of the analyses. The results of the analyses are shown in Fig. 6. The results of the PSA analyses can be used to define the quantitative effects of each parameter combination on the counter amplitudes as well as to further investigate the regions for which the system reflects requested behaviour (note that the Morris sensitivity analysis was performed only on the viable solution spaces with perturbations that did not have an effect on a qualitative response of the system). As expected, correct flip-flop behaviour was lost with large perturbations in some cases. The gene expression rates of the master flip-flop (i.e. α_1 and α_2) and of the slave flip-flop (i.e. α_3 and α_4) do not seem to have much influence on the amplitudes of the counter. On the other hand, gene expression rates comprise the combinations of the parameters that affect the system most significantly in a qualitative manner. The results show that for low values of parameter α_1 the system does not work correctly (see Fig. 6(a), (d), (h)), while α_2 does not seem to have so large influence

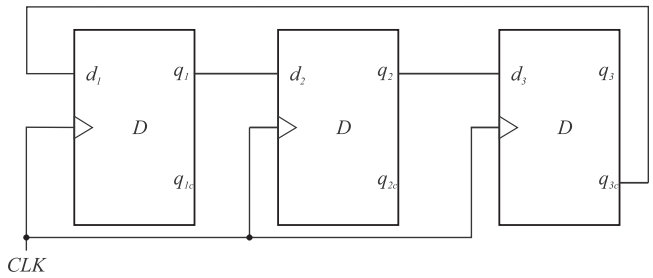

Fig. 7. Three-bit Johnson counter using a sequence of D flip-flops. The output q of each flip-flop is fed to the data input d of the next flip-flop in the sequence. The complementary output q_c of the last flip-flop is fed to the data input d of the first flip-flop in the sequence to obtain a circular scheme.

on the qualitative response (see Fig. 6(e) and (i)) except in a combination with low α_1 values (see Fig. 6(a)) or high δ_1 values (see Fig. 6(e)). Additionally, the flip-flop only works correctly for high α_3 and α_4 values (see Figs. 6(b), (h) and (i)), with the exception when degradation rates are lower. In this case high amplitudes and correct flip-flop response can be obtained also with lower values of α_3 and α_4 (see Fig. 6(f) and (g)). While different combinations of degradation rates δ_1 and δ_2 do not seem to influence the correctness of the flip-flop behaviour (see Fig. 6(c)), they significantly affect the amplitude values (see also Fig. 5(a)). Moreover, they define the qualitative response of the system when combining their perturbations with the perturbations of other parameters. They especially affect the space of the viable gene expression rates when their values are high (see Fig. 6(d), (e), (f), (g)), which is also the case in the solution we obtained with the GA – degradation rate values need to be high in order to obtain larger counter amplitudes (see Fig. 6(c)).

4. Computational design of Johnson counter

Implementation of a robust and scalable biological counter capable of counting up to hundreds of cellular events would have several promising applications in the field of synthetic biology. For example, robust and scalable biological counter would substantially increase the progress in study and prevention of cancer development [8]. Currently implemented counters are unfortunately able to count only up to three events, whereas changing the state is triggered with a high level of input signal which needs to be long enough to perform the switch, and at the same time short enough to prevent more than one change of the state [21] as was also the case of the clocked D flip-flop. A scalable and reliable biological counter can be on the other hand implemented with the application of the proposed master–slave flip-flop, which as we have shown, reflects robust and modular behaviour.

It is possible to implement a digital logic counter which counts up to 2^n events using n flip-flops. These counters however require a substantial amount of additional logic elements, i.e. logic gates. Johnson counter, on the other hand, presents a compromise between the complexity of the circuit and number of events that can be traced, since it can count up to $2n$ events using n D flip-flops without any additional logic elements. The basic design of a three-bit Johnson counter ($n = 3$) is presented in Fig. 7. The flip-flops in the Johnson counter topology are connected in a sequential manner, whereas the output of a preceding flip-flop (q_i) is connected to the data input of the next flip-flop in the sequence (d_{i+1}). The data input of the first flip-flop (d_1) is on the other hand connected to the complementary output of the last flip flop (q_{3c}). All flip-flops are synchronised with the same clock signal (*CLK*). Such scheme will lead to a counter that is able to count up to $2n$ events using n flip-flops. Basic counting sequence of the three-bit counter presented in Fig. 7 is a sequence of states (000, 100, 110, 111, 011, 001), whereas

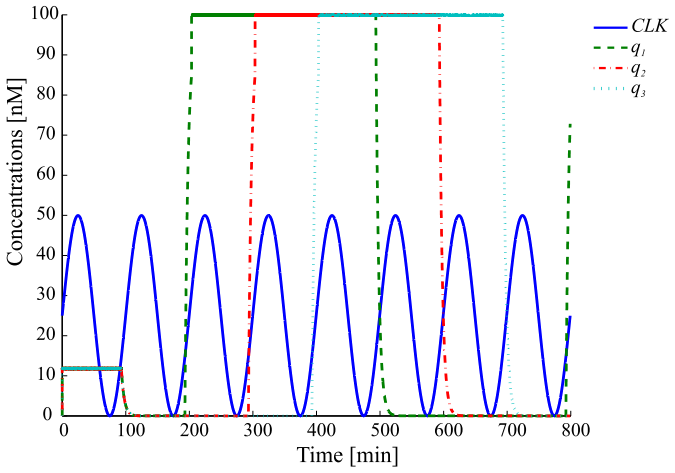

Fig. 8. Simulation results of proposed biological Johnson counter. Simulations were performed using the parameter values describes in Section 2.4. Results demonstrate a valid counting sequence, i.e. (000, 100, 110, 111, 011, 001).

flip-flop i has a logic state 1 when the concentration of protein q_i is high and the concentration of protein q_{ic} is low, and logic state 0 when the concentration of protein q_i is low and the concentration of protein q_{ic} is high.

We simulated the dynamics of proposed biological counter with a straightforward extension of the Eqs. (1)–(4) using the parameters obtained with GA described in Section 2.4. We assumed that all flip-flops in the topology share the same kinetic parameter values for the sake of their logic compatibility. Simulation results are presented in Fig. 8. Performed simulations demonstrate a valid counting sequence of six permitted states of Johnson counter. Following the initialisation, the output protein levels are low in the beginning of the simulation. The concentration of protein q_1 starts increasing together with the positive clock edge at time $t = 200$ min while concentrations of proteins q_2 and q_3 remain low, which leads the counter to state 100. At the next positive clock edge the concentration of protein q_2 increases, which represents the state 110. At time $t = 400$ min the concentration of protein q_3 increases and the counter changes its state to 111. At the next positive clock edge, the concentration of protein q_1 decreases, which brings the counter to state 011. The concentration of protein q_2 decreases at time $t = 600$ min, which represents state 001. Finally, the concentration of protein q_3 decreases at $t = 700$ min which brings the counter to the initial state, i.e. 000.

5. Conclusion

Synthetic biology scientific community has devoted large attention towards the implementation of robust and scalable biological memory structures in recent years. The design of edge-triggered synchronous sequential structures, which are vital for the implementation of complex information processing systems, however, has not been performed yet. We described a computational design of an edge-triggered D flip-flop based on transcriptional logic. Edge-triggered flip-flop was designed upon a master–slave configuration of the basic clocked D flip-flop. Furthermore, we applied GA to tune the response of the topology with the calibration of kinetic parameter values. Values used in the optimisation process were derived from the literature and correspond to biological relevant data, which means that the parts used in the topology, i.e. promoters, protein coding sequences, etc., could be constructed in the near future, which would bring the design to its *in vivo* implementation. We additionally confirmed the robustness of the topology using a global sensitivity analysis framework developed specifically

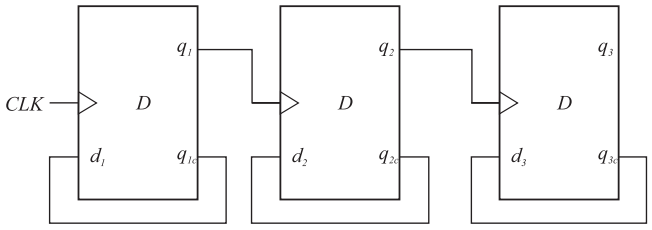

Fig. 9. Three-bit ripple counter using a sequence of D flip-flops. The output q of each flip-flop is fed to the clock input (represented by a triangle) of the next flip-flop in the sequence. The complementary output q_c of each flip-flop is fed to the data input d of the same flip-flop.

to cope with high-dimensional and poorly connected parameter spaces as is the case in the proposed system. We analysed the relations between selected parameters that need to be fulfilled for the system to work correctly using PSA.

The proposed flip-flop was used in the design of a Johnson counter, which is able to count up to $2n$ events using n flip-flops. The complexity of the proposed counting scheme is comparable to the complexity of a *ripple counter* with counting module 2^n (see Fig. 9), which however requires relatively small delays of the signal propagation through logic gates in comparison to clock signal periods. While this does not hold for transcriptional logic circuits, the described ripple counter proves to be unsuitable to be used in biological systems. Johnson counter on the other hand reflects appropriate dynamics even in the case of large gate propagation delays. This is achieved with the use of shared clock signal among all flip-flops in the topology. Moreover, all segments in the counter are composed of the same number of logic layers, which provides an equal delay for all parts of the circuit that need to be stable at the same time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jocs.2016.11.010.

References

[1] T.S. Gardner, C.R. Cantor, J.J. Collins, Construction of a genetic toggle switch in *Escherichia coli*, Nature 403 (2000) 339–342, http://dx.doi.org/10.1038/35002131.
[2] M.B. Elowitz, S. Leibler, A synthetic oscillatory network of transcriptional regulators, Nature 403 (6767) (2000) 335–338, http://dx.doi.org/10.1038/35002125.
[3] D.E. Cameron, C.J. Bashor, J.J. Collins, A brief history of synthetic biology, Nat. Rev. Microbiol. 12 (2014) 381–390, http://dx.doi.org/10.1038/nrmicro3239.
[4] M.C. Kevin, P.D. Samuel, K.L. Timothy, Foundations and emerging paradigms for computing in living cells, J. Mol. Biol. 428 (5 Pt B) (2016) 893–915, http://dx.doi.org/10.1016/j.jmb.2016.02.018.
[5] M. Inniss, P. Silver, Building synthetic memory, Curr. Biol. 23 (17) (2013) R812–R816, http://dx.doi.org/10.1016/j.cub.2013.06.047.
[6] D. Burrill, P. Silver, Making cellular memories, Cell 140 (1) (2010) 13–18, http://dx.doi.org/10.1016/j.cell.2009.12.034.
[7] D. Burrill, M. Inniss, P. Boyle, P. Silver, Synthetic memory circuits for tracking human cell fate, Genes Dev. 26 (13) (2012) 1486–1497, http://dx.doi.org/10.1101/gad.189035.112.
[8] J. Bonnet, P. Subsoontorn, D. Endy, Rewritable digital data storage in live cells via engineered control of recombination directionality, Proc. Natl. Acad. Sci. U. S. A. 109 (23) (2012) 8884–8889, http://dx.doi.org/10.1073/pnas.1202344109.
[9] P. Siuti, J. Yazbek, T. Lu, Synthetic circuits integrating logic and memory in living cells, Nat. Biotechnol. 31 (5) (2013) 448–452, http://dx.doi.org/10.1038/nbt.2510.
[10] G.M. Church, Y. Gao, S. Kosuri, Next-generation digital information storage in DNA, Science 337 (2012) 1628, http://dx.doi.org/10.1126/science.1226355.
[11] N. Goldman, P. Bertone, S. Chen, C. Dessimoz, E.M. LeProust, B. Sipos, E. Birney, Towards practical, high-capacity, low-maintenance information storage in synthesized DNA, Nature 494 (2013) 77–80, http://dx.doi.org/10.1038/nature11875.
[12] A. Padirac, T. Fujii, Y. Rondelez, Bottom-up construction of in vitro switchable memories, Proc. Natl. Acad. Sci. U. S. A. 109 (47) (2012) E3212–E3220, http://dx.doi.org/10.1073/pnas.1212069109.
[13] L. Yang, A. Nielsen, F. Jesus, M. Conor, M. Laub, T. Lu, C. Voigt, Permanent genetic memory with > 1-byte capacity, Nat. Methods 11 (12) (2014) 1261–1266, http://dx.doi.org/10.1038/nmeth.3147.
[14] A. Tiwari, J.C. Ray, J. Narula, O.A. Igoshin, Bistable responses in bacterial genetic networks: designs and dynamical consequences, Math. Biosci. 231 (1) (2011) 76–89, http://dx.doi.org/10.1016/j.mbs.2011.03.004.
[15] M.A. Ptashne, Genetic Switch (Third Edition): Phage Lambda Revisited, Cold Spring Harbor Laboratory Press, New York, USA, 2004.
[16] C. Lou, X. Liu, M. Ni, Y. Huang, Q. Huang, L. Huang, L. Jiang, D. Lu, M. Wang, C. Liu, D. Chen, C. Chen, X. Chen, L. Yang, H. Ma, J. Chen, Q. Ouyang, Synthesizing a novel genetic sequential logic circuit: a push-on push-off switch, Mol. Syst. Biol. 6 (1) (2010) 350, http://dx.doi.org/10.1038/msb.2010.2.
[17] A. Caroline, D. Drubin, J. Eskin, E. Gee, D. Landgraf, I. Phillips, P. Silver, Rational design of memory in eukaryotic cells, Genes Dev. 21 (18) (2007) 2271–2276, http://dx.doi.org/10.1101/gad.1586107.
[18] N. Ingolia, A. Murray, Positive-feedback loops as a flexible biological module, Curr. Biol. 17 (8) (2007) 668–677, http://dx.doi.org/10.1016/j.cub.2007.03.016.
[19] J. Sardanyés, A. Bonforti, N. Conde, R. Solé, J. Macia, Computational implementation of a tunable multicellular memory circuit for engineered eukaryotic consortia, Front. Physiol. 6 (2015), http://dx.doi.org/10.3389/fphys.2015.00281.
[20] T. Lebar, U. Bezeljak, A. Golob, M. Jerala, L. Kadunc, B. Pirš, M. Stražar, D. Vučko, U. Zupančič, M. Benč ina, V. Forstnerič, R. Gaber, J. Lonzarič, A. Majerle, A. Oblak, A. Smole, R. Jerala, A bistable genetic switch based on designable DNA-binding domains, Nat. Commun. 5 (5007) (2014), http://dx.doi.org/10.1038/ncomms6007.
[21] A.E. Friedland, T.K. Lu, X. Wang, D. Shi, G. Church, J.J. Collins, Synthetic gene networks that count, Science 324 (5931) (2009) 1199–1202, http://dx.doi.org/10.1126/science.1172005.
[22] A.P. Godse, D.A. Godse, Digital Electronics, A Conceptual Approach, Technical Publications, 2014.
[23] U. Alon, An Introduction to Systems Biology, Chapman & Hall/CRC, 2007.
[24] L. Potvin-Trottier, N.D. Lord, G. Vinnicombe, J. Paulsson, Synchronous long-term oscillations in a synthetic gene circuit, Nature (2016), http://dx.doi.org/10.1038/nature19841.
[25] B.-S. Chen, C.-H. Wu, A systematic design method for robust synthetic biology to satisfy design specifications, BMC Syst. Biol. 3 (66) (2009), http://dx.doi.org/10.1186/1752-0509-3-66.
[26] A.G. Garcia, Orthogonal sampling formulas: a unified approach, SIAM Rev. 42 (3) (2000) 499–512.
[27] Z. Zi, Sensitivity analysis approaches applied to systems biology models, IET Syst. Biol. 5 (6) (2011) 336–346, http://dx.doi.org/10.1049/iet-syb.2011.0015.
[28] A. Saltelli, S. Tarantola, F. Campolongo, M. Ratto, Sensitivity Analysis in Practice, John Wiley & Sons Ltd, 2004.
[29] M.D. Morris, Factorial sampling plans for preliminary computational experiments, Technometrics 33 (2) (1991) 161–174, http://dx.doi.org/10.1080/00401706.1991.10484804.
[30] M. Butz, M. Neuenschwander, P. Kast, D. Hilvert, An N-terminal protein degradation tag enables robust selection of highly active enzymes, Biochemistry 50 (40) (2011) 8594–8602, http://dx.doi.org/10.1021/bi2011338.

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