Toward in vivo digital synchronous sequential circuits

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Abstract: We present the model of a first digital synchronous sequential circuit, i.e. 1-bit synchronous counter, which is about to be realized within the living cell. Mathematical model was constructed upon gene expression based logic with ordinary differential equations (ODEs), particularly Hill equations. The behaviour of the counter was simulated in `Matlab/Simulink` environment. With the promising simulation results presented in the paper physical realization of the circuit described within the living cell can be initiated. Introduction of synchronization with a special signal, i.e. clock signal, brings many benefits to the field. Realization of such circuit would therefore present an important step toward the construction of complex biological information processing capable systems.

Key–Words: Synthetic biology, Protein-based computing, Sequential logic, Synchronization, Ordinary differential equations, Biological modelling, Circuits, Master-Slave flip-flop

1 Introduction

Structures that represent basic primitives for the construction of information processing capable systems were up until recently based on electronic components. We are facing many problems with their continuous miniaturization and requirements for faster circuit responses. Even more, we are starting to reach the limit in the meaning of their sizes and their response times. Therefore alternative information processing platforms need to be found in order to satisfy the future processing demands [1].

Synthetic biology is an emerging field that is rapidly evolving and is promising many applications in the near future. Information processing capable systems based on engineered transcriptional networks as biological circuits are certainly one of them [2]. Dynamics of these circuits is based on the presence (respectively absence) of specific DNA-binding proteins, i.e. transcription factors. We can manipulate these circuits with genetic engineering (i.e. DNA recombination technique) to achieve their desired behaviour. Basic biological circuits that function whether as combinatoric logical gates [3, 4], latches [6, 7, 8, 9] or oscillators [10] have already been realized in living cells. On the other hand fusion of these primitives into a functional synchronous sequential logic circuit has not been performed yet.

Synchronization of logic circuits with a special signal, i.e. clock signal, has many benefits. Among others main benefits are [11]

- The ability to memorize is gained with the introduction of synchronization with dedicated signal, i.e. *clock signal*.
- Behaviour of synchronous circuits can be predicted more precisely if we have a stable clock signal. Therefore, performance analysis is easier to be made.
- Design of circuits with bigger complexity is easier.

Here we present the model of synchronous biological counter that is about to be realized in the living cell. In the remainder of this paper we describe basic primitives used to manipulate biological systems (i.e.

gene expression based logic), we present the model of 1-bit biological counter based on ordinary differential equations (ODEs) and its behaviour simulated in `Matlab/Simulink` environment. We conclude the article with our remarks and future directions.

2 Gene Expression Based Logic

Gene Expression Based Logic derives from the presumption that cells can be programmed by introducing synthetic DNA containing new commands that instruct the cell to perform a set of artificial tasks [4]. DNA strand is basically composed of regulatory region (i.e. *promoter*) and of structural genes that define instructions for the construction of output proteins, i.e. gene expression. Gene expression is carried out in two phases, namely *transcription* and *translation*.

In transcription phase *messenger RNA* (mRNA) molecules are synthesized. Transcription of specific structural genes into mRNA molecules is initiated when an enzyme *RNA polymerase* (RNAP) binds to the belonging promoter. Binding rate of RNAP to the promoter and thus rate of transcription can be regulated by certain DNA-binding proteins called *transcription factors*, which bind to the *operator site* of the promoter. They can be divided in two main groups, namely *repressors* and *activators*. Repressors decrease the rate of transcription with the binding to the operator site. Even more, effective repressors prevent the binding of RNAp to the promoter and thus inhibit the transcription. On the other hand activators increase the rate of transcription of mRNA molecules. Even more, sometimes transcription is not initiated without the presence of protein that has a function of an activator. We are able to manipulate the properties of promoter's operator site and thus define which transcription factors affect the expression of a certain gene and how do they affect the expression. Proteins expressed can onward regulate the transcription of other genes, i.e. they have a role of transcription factors for some other or their own operator site. The situation where protein regulates its own expression is called *autoregulation*.

In translation phase *ribosomes* bind to mRNA in order to compose the correct sequence of amino acids into a target protein. The record of amino acids sequence which defines the target protein is included in mRNA molecules. Ribosomes therefore travel alongside mRNA molecules constructing the target protein. Because the degradation of mRNA molecules is mostly very fast, the gene expression rate can be equalized with the transcription rate of mRNA.

We can take advantage of the behaviour described to build our own biological circuits with desired functionalities, i.e. with the modifications of operator sites of the promoter and with the modifications of structural genes in order to code the amino acid sequences of desired proteins [5]. The basic primitives and the notation used in the construction of such circuits and also in the remainder of this paper is presented in Fig. 1.

We can construct a basic biological circuit that has a functionality of *NOT gates* with the use of input protein (i.e. repressor) which effectively represses the transcription of output protein (see Fig. 1(a)). On the other hand *driver gates* can be constructed with the use of input protein (i.e. activator) which activates the transcription of output protein (see Fig. 1(b)), i.e. transcription is not initiated without the presence of the input protein. Gates that have a functionality of logical implication (i.e. *implies gates*) can be constructed with two input proteins, where first input functions as a repressor (see Fig. 1(c), input protein X), but only when not bound with the second input, i.e. in the absence of the second protein (see Fig. 1(c), input protein A). Logical conjunction circuit (i.e. *AND gates*) can also be constructed with two input proteins, where first input functions as an activator (see Fig. 1(d), input protein X), but only when bound with the second input, i.e. in the presence of the second protein (see Fig. 1(c), input protein A). Joint denial circuit (i.e. *NOR gates*), can be constructed with the generalization of NOT gates in the meaning of input number. We can thus have arbitrary number of inputs, whereas each input represses the transcription of output protein (see Fig. 1(e)).

3 Biological counter model

3.1 Basic model

Counter is a synchronous sequential circuit with only one mandatory input (i.e. *clock signal*) which synchronizes its behaviour - internal value (state) of the counter is changed (i.e. increased) only when clock signal is in a specific state. Current state of the counter can be obtained with counter outputs. An n-bit counter therefore needs to have n outputs and optionally also their complements. Graphical representation of *positive edge-triggered* (i.e. its state is changed on the rising edge of clock signal) 1-bit counter is presented in Fig. 2. The *Lookup table* of the counter is presented in Table 1.

Circuit described can be realized with the use of standard electronic components connected in accordance with logical scheme presented in Fig. 3. Logical scheme where only the basic logical gates are used (see Fig. 1) is presented in Fig. 3. Counter pre-

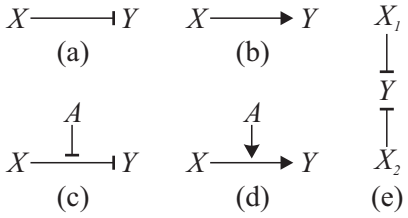

Figure 1: The basic primitives and the notation used to describe the effects of input protein (namely X) on the expression rate of structural gene Y. Circuit (a) presents the situation where X represses the transcription of protein Y. Circuit (b) presents the situation where X activates the transcription of protein Y. In circuit (c) X is unable to effectively repress the expression of structural gene when bound with ligand A (A inactivates the repressive effect of protein X on transcription of structural gene Y). In circuit (d) X is capable of activating the expression of structural gene Y only when bound with ligand A (A activates the inducible effect of protein X on transcription of structural gene Y). Circuit (e) differs from (a) only in the number of inputs - expression is initiated only when both inputs are absent.

sented is based on *RS flip-flop*. We can construct a model of gene expression based 1-bit counter with a straightforward transition from the circuit representing a classical counter based on electronic components to a circuit which is based on biological components described in Fig. 1.

Based on the notation presented in section 2, Fig. 5 presents the biological equivalent of the 1-bit counter presented in Fig. 4.

The circuit has only one input (namely CLK) which represents protein that synchronizes our counter, i.e. clock signal. Oscillations of CLK protein concentration can be realized using the repressi-

q	$\overline{q}$	D^1q	$D^1\overline{q}$
0	0	X	X
0	1	1	0
1	0	0	1
1	1	X	X

Table 1: Lookup table of 1-bit counter where q presents the current state of the counter, $\overline{q}$ its complement, D^1q state of the counter in the next time step and $D^1\overline{q}$ complement of the state in the next time step. Label X denotes an invalid combination.

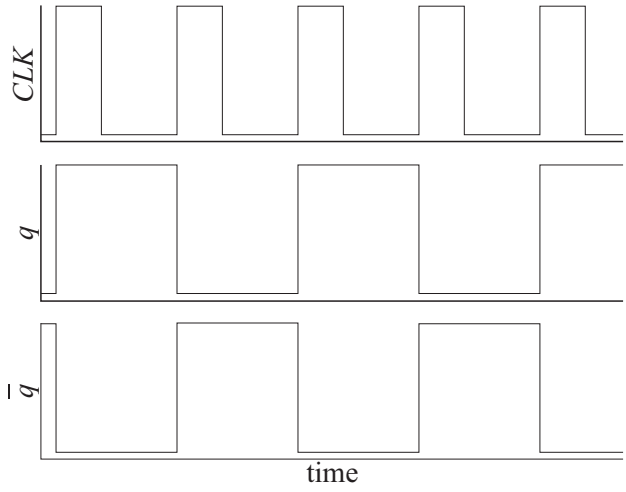

Figure 2: Behaviour of 1-bit counter circuit, where CLK is an input signal representing clock, q is an output signal representing current state of the counter and $\overline{q}$ representing complement of the counter's current state.

lator circuit described in [10]. It is evident that other proteins used have the following functionalities:

- q: its presence inhibits the transcription of protein $\overline{q}$ and when in active state for the promoter of gene R_1 (when protein CLK is present) it also induces the transcription of protein R_1,
- $\overline{q}$: its presence inhibits the transcription of protein q and when in active state for the promoter of gene R_2 (when protein CLK is present) it also induces the transcription of protein R_2,
- R_1: its presence inhibits the transcription of protein q,

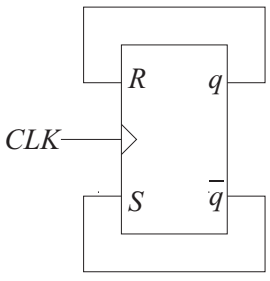

Figure 3: Logical scheme of 1-bit counter based on RS flip-flop, where CLK is an input signal representing clock, q is an output signal representing current state of the counter and $\overline{q}$ representing complement of the counter's current state.

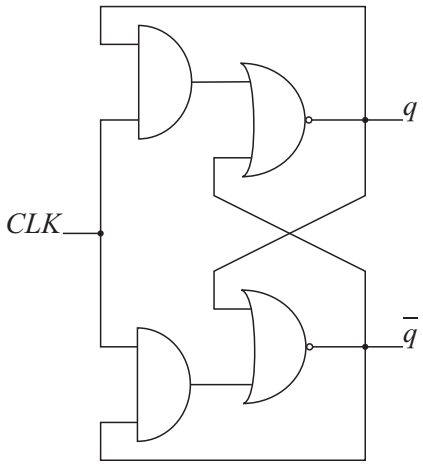

Figure 4: Logical scheme of 1-bit counter based on RS flip-flop implemented only with the basic logical gates.

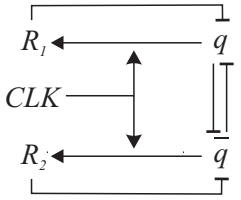

Figure 5: Biological scheme of 1-bit counter constructed on the basis of gene based expression logic.

- R_2: its presence inhibits the transcription of protein $\overline{q}$,
- CLK: when present it binds to protein q (respectively $\overline{q}$) and transforms it to its active state for the promoters R_1 and R_2.

R_1 (respectively R_2) is transcribed when sufficient concentration of protein denoted as CLK (i.e. *ligand*) binds to sufficient concentration of protein denoted as q (respectively $\overline{q}$) and changes its shape thus it can bind to R_1 (respectively R_2) promoter. Protein denoted as q (respectively $\overline{q}$) is unable to bind the promoter and thus activate the transcription of repressor R_1 (respectively R_2) in its primary shape. We can interpret this behaviour as AND function, whereas CLK and q (respectively $\overline{q}$) present the inputs and R_1 (respectively R_2) presents the output of AND logic gates.

As certain amount of intermediate protein R_1 (respectively R_2) is generated (i.e. certain concentration is reached) transcription of output protein q (respectively $\overline{q}$) is ceased and its concentration decreases due to protein degradation rate. In order to hold the state of the counter when input protein (i.e. CLK) is not present, output proteins repress each other - stronger protein (i.e. the one with the larger concentration) represses the weaker one (i.e. the one with the smaller concentration).

We can interpret this behaviour in the following way: if output protein is present in sufficient amount then it represses itself in the presence of clock pulse. Therefore its transcription is ceased and eventually its concentration starts decreasing due to the protein degradation rate. On the other hand if output protein is not present in sufficient amount in the same time as clock pulse, transcription is activated while it cannot repress itself. Its concentration therefore starts increasing due to transcription activation. We can expect that each output will change from active to inactive state (respectively from inactive to active state) in the presence of clock pulse.

When clock pulse is not present intermediate proteins are not expressed and their concentrations start decreasing due to protein degradation rate. As their concentration ceases their effect on outputs becomes negligible. On the other hand output proteins repress each other and therefore sustain (memorize) output state as it was when clock pulse was present. Note that the oscillatory behaviour described reflects the functionality of 1-bit counter.

We can construct the structure using DNA segment with 4 promoter regions. DNA segment illustration of 1-bit biological counter with mutual influences among different proteins is presented in Fig. 6, where $\rightarrow$ presents a promoter region and $\underline{label}$ presents coding sequence (structural gene) of protein denoted as $label$. Other symbols are used in accordance to section 2.

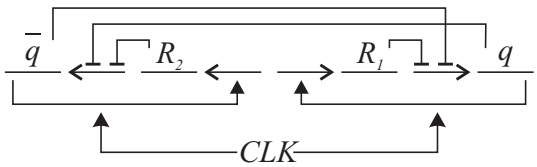

Figure 6: Illustration of DNA segment presenting basic version of 1-bit counter.

Deterministic model based on *ordinary differential equations* (ODEs) was constructed (see Section 4.1) and used to simulate the circuit in `Matlab/Simulink` environment. The model was able to achieve the proper behaviour only with restrained input signal (i.e. clock signal) properties - input signal had to be tuned regarding the model response. Model also reflected great sensitivity to noise and various delays (i.e. gene expression and binding

delays) introduction. Unstable model was unable to switch the states of the outputs in correct intervals. Switches of the outputs were to frequent and are at the first sight made independently of clock pulse (see Fig. 7). The main problem of basic counter lies in the great sensitivity to the length of clock pulse. Counter would not reach its proper state in a given time if pulse was too short. On the other hand counter would start oscillating before pulse inactivity and would therefore indicate invalid behaviour as well if pulse was too long. In order to solve these problems extended model was constructed.

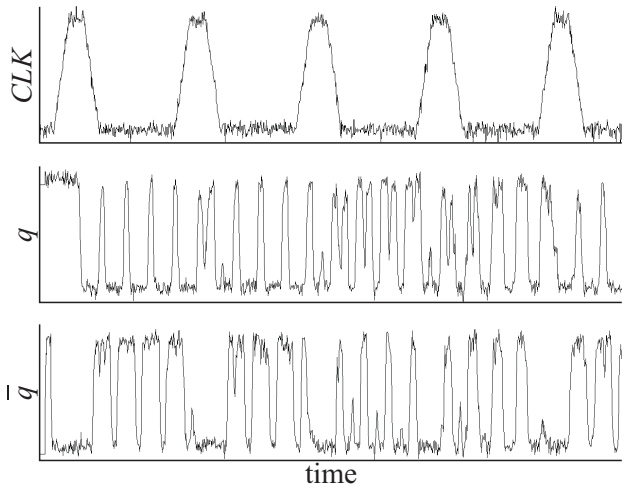

Figure 7: Unstable behaviour of basic model of 1-bit counter constructed on the basis of gene expression based logic, where y axes present the concentrations of each protein in a certain time and x axes present time.

3.2 Extended model

We can solve problems of the basic counter presented in section 3.1 with the introduction of additional phase, which is active when clock signal is in the inactive state - protein denoted as CLK is not present in sufficient amount to affect the behaviour of the basic circuit (see Fig. 8). Two phases used are therefore complementary (when one is active the other one is inactive and vice versa). The construction offered would solve the problem of the clock pulse length, but only in the situation when the pulse is too long. Invalid behaviour in the situation when clock pulse is too short still remains, but can be ignored while it is trivial to provide a pulse that is long enough. On the other hand shortening of the pulse that is too long presents a big problem which is solved with the introduction of extended model.

Logical scheme of the extended version of biological counter is presented in Fig. 8. Note that both inputs denoted as CLK present the same protein i.e. clock signal. Complementary phases were achieved with the use of basic circuits presented in Fig. 1(c) (i.e. implies gates) and Fig. 1(d) (i.e. AND gates).

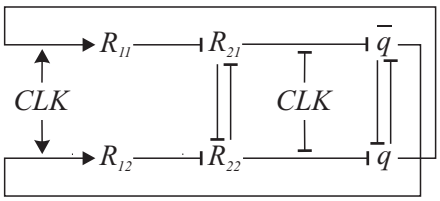

Figure 8: Extended biological scheme of 1-bit counter constructed on the basis of gene expression based logic.

It is evident from the figure that proteins used have the following functionalities:

- q: its presence inhibits the transcription of protein $\overline{q}$ and when in active state for the promoter R_{12} (when protein CLK is present) it also induces the transcription of protein R_{12},
- $\overline{q}$: its presence inhibits transcription of protein q and when in active state for the promoter R_{11} (when protein CLK is present) it also induces the transcription of protein R_{11},
- R_{11}: its presence inhibits the transcription of protein R_{21},
- R_{12}: its presence inhibits the transcription of protein R_{22},
- R_{21}: its presence inhibits the transcription of protein R_{22} and when in active state for the promoter $\overline{q}$ (when protein CLK is absent) it also inhibits the transcription of protein $\overline{q}$,
- R_{22}: its presence inhibits the transcription of protein R_{21} and when in active state for the promoter q (when protein CLK is absent) it also inhibits the transcription of protein q,
- CLK: when present it transforms protein q (respectively $\overline{q}$) to its active state for the promoter R_{12} (respectively R_{11}) and protein R_{21} (respectively R_{22}) to its inactive state for the promoter $\overline{q}$ (respectively q).

First part of the model is obviously similar to the model presented in previous section. The main difference is in additional phase of extended model. In

the basic model output started changing immediately as there was a change in the input. On the other hand extended model works in two phases. Intermediate proteins R_{11}, R_{12}, R_{21} and R_{22} change their logical states when input signal is active (i.e. sufficient concentration of protein CLK is present). But unlike the basic model output is not affected until clock is in the inactive state. Introduction of this functionality allows us to have an input signal that is more flexible. Presence of input protein (namely CLK) inactivates repressive effect of protein R_{21} (respectively R_{22}) on output protein q (respectively $\overline{q}$) after binding process (note that this functionality of input protein is opposite from the one described in previous section, but has a similar effect). When the concentration of input protein ceases it is not able to bind to protein R_{21} (respectively R_{22}). Therefore proteins R_{21} and R_{22} become active and can start repressing output proteins. Logical states of the outputs change only when clock pulse is not present (i.e. counter is triggered on falling edge of the clock signal). Structure described is analogous to *Master-Slave flip-flop circuit* [12]. Although the circuit seems easy to optimise at first glance intermediate phases cannot be skipped while any simplified version suffers from instability (similar to the circuit described in previous section) The logical scheme of the circuit is presented in Fig. 9.

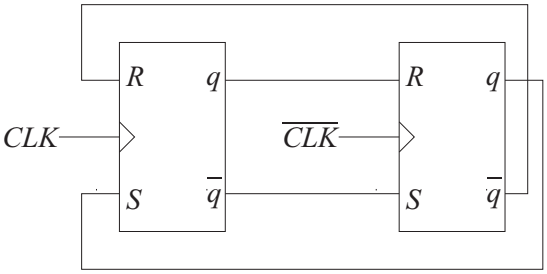

Figure 9: Logical scheme of an extended 1-bit counter implemented with two RS flip-flops where the first flip-flop is positive and the second one is negative edge triggered (Master-Slave hierarchy).

We can construct the structure using DNA segment with 6 promoter regions. DNA segment illustration of extended version of biological counter with mutual influences among different proteins is presented in Fig. 10, where $\rightarrow$ presents a promoter region and $\underline{label}$ presents coding sequence (structural gene) of protein denoted as $label$. Other symbols are used in accordance to section 2. Note that both inputs denoted as CLK present the same protein i.e. clock signal.

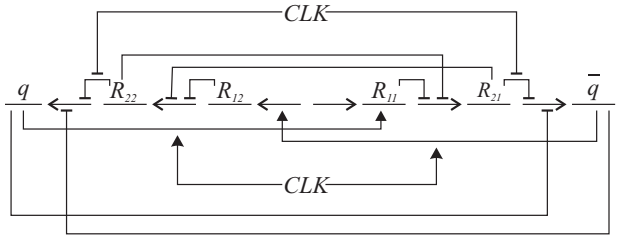

Figure 10: Illustration of DNA segment presenting extended version of 1-bit counter.

4 Modelling of biological counter

4.1 Mathematical model of basic counter

Deterministic model based on ordinary differential equations (ODEs) particularly Hill equations was used in order to model the behaviour of biological counter [13, 14]. Model can thus be described as

$$\frac{dR_1}{dt} = \beta_0 + \frac{\beta \cdot q_F^n}{K^n + q_F^n} - \delta \cdot R_1, \tag{1}$$

$$q_F = \frac{q \cdot CLK^n}{K^n + CLK^n}, \tag{2}$$

$$\frac{dR_2}{dt} = \beta_0 + \frac{\beta \cdot \overline{q}_F^n}{K^n + \overline{q}_F^n} - \delta \cdot R_2, \tag{3}$$

$$\overline{q}_F = \frac{\overline{q} \cdot CLK^n}{K^n + CLK^n}, \tag{4}$$

$$\frac{dq}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_1 + \overline{q}}{K})^n} - \delta \cdot q, \tag{5}$$

$$\frac{d\overline{q}}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_2 + q}{K})^n} - \delta \cdot \overline{q}, \tag{6}$$

where CLK, R_1, R_2, q and $\overline{q}$ present specific protein concentrations, q_F, $\overline{q}_F$ present specific protein concentrations after binding with inducer CLK (i.e. in active state for certain promoters), β_0 *leakiness coefficient* (i.e. minimal transcriptional rate coefficient), β *maximal transcriptional rate coefficient*, K *activation coefficient* (i.e. the concentration of active input protein needed to significantly activate, respectively repress expression), n *cooperativity coefficient* and δ *protein degradation rate*.

4.2 Mathematical model of extended counter

Extended counter can be described in the similar way as a basic counter using Hill equations.

$$\frac{dR_{11}}{dt} = \beta_0 + \frac{\beta \cdot q_F^n}{K^n + q_F^n} - \delta \cdot R_{11}, \tag{7}$$

$$q_F = \frac{q \cdot CLK^n}{K^n + CLK^n}, \tag{8}$$

$$\frac{dR_{12}}{dt} = \beta_0 + \frac{\beta \cdot \overline{q}_F^n}{K^n + \overline{q}_F^n} - \delta \cdot R_{12}, \tag{9}$$

$$\overline{q}_F = \frac{\overline{q} \cdot CLK^n}{K^n + CLK^n}, \tag{10}$$

$$\frac{dR_{21}}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_{11}+R_{22}}{K})^n} - \delta \cdot R_{21}, \tag{11}$$

$$\frac{dR_{22}}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_{12}+R_{21}}{K})^n} - \delta \cdot R_{22}, \tag{12}$$

$$\frac{dq}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_{22F}+\overline{q}}{K})^n} - \delta \cdot q, \tag{13}$$

$$R_{21F} = \frac{R_{21}}{1 + (\frac{CLK}{K})^n}, \tag{14}$$

$$\frac{d\overline{q}}{dt} = \beta_0 + \frac{\beta}{1 + (\frac{R_{21F}+q}{K})^n} - \delta \cdot \overline{q}, \tag{15}$$

$$R_{22F} = \frac{R_{22}}{1 + (\frac{CLK}{K})^n}, \tag{16}$$

where CLK, R_{11}, R_{12}, R_{21}, R_{22}, q and $\overline{q}$ present specific protein concentrations, q_F, $\overline{q}_F$, R_{21F}, R_{22F} present specific protein concentrations after binding with inducer CLK, β_0 leakiness coefficient, β maximal transcriptional rate coefficient, K activation coefficient, n cooperativity coefficient and δ protein degradation rate.

5 Simulation and results

5.1 Simulation of basic counter

Deterministic model based on ordinary differential equations (see Section 4.1) was used to simulate the behaviour of the basic 1-bit counter in `Matlab/Simulink` environment.

Based on the literature the following values were assigned to the coefficients in order to simulate counter behaviour [15, 16]

$$\beta_0 = 0.2\frac{\mu M}{min}, \tag{17}$$

$$\beta_1 = 4\frac{\mu M}{min}, \tag{18}$$

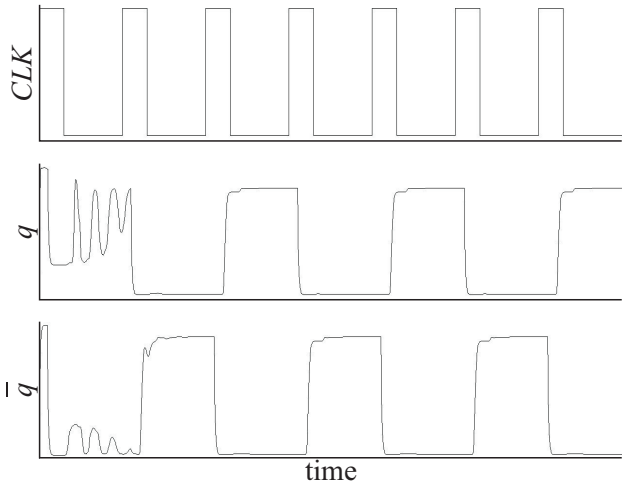

Figure 11: Correct behaviour of the basic counter in ideal environment, where y axes present the concentrations for each protein in a certain time and x axes present time. Note that initial perturbation was needed in order to achieve the desired oscillations (concentration of both output proteins is large in the beginning of the simulation).

$$K = 1\mu M, \tag{19}$$

$$n = 3, \tag{20}$$

$$\delta = 1min^{-1}, \tag{21}$$

where M stands for molar concentration unit, i.e. $\frac{mol}{L}$.

The simulation of the counter behaviour was first conducted in ideal environment, i.e. without noise and with slew rate which presented an ideal clock signal, i.e. slew rate of the signal equalled infinity. Even in the ideal environment counter would reflect the correct behaviour only when the length of the clock pulse was properly tuned with transcription and binding delays used in the simulation. An example of correct behaviour is presented in Fig. 11.

When noise signal and unideal clock pulse were introduced correct behaviour was even harder to achieve. Most configurations would therefore reflect the unstable behaviour (see Fig. 7). As mentioned earlier problems of the basic counter were solved with its extended version.

5.2 Simulation of extended counter

Extended model described in section 4.2 was used to simulate the behaviour of the extended 1-bit counter in

`Matlab/Simulink` environment (see Fig. 12 and Fig. 13). The same values as in the simulation of basic counter (see Section 5.1) were assigned to the coefficients β_0, β, K, n and δ.

Different simulations of extended counter reflected the correct behaviour using different clock parameters (different slew rates, duty cycles and periods). The real environment behaviour was approximated with noise introduction. Various delays were used in order to model time needed for binding, activation, repression and transcription. Representable results of the simulation are shown in Fig. 14. Initial perturbation of the counter was needed in order to achieve the desired oscillations (note that concentration of both output proteins is large in the beginning of the simulation).

As can be seen the results of the simulation are in accordance with expected behaviour (see Fig. 2). The only difference is that counter presented in Fig. 2 is positive edge triggered while biological counter presented in the preceding section is negative edge triggered (because of the Master-Slave hierarchy). Therefore the state of the counter is changed on each negative front of clock signal and the counter is thus negative edge triggered.

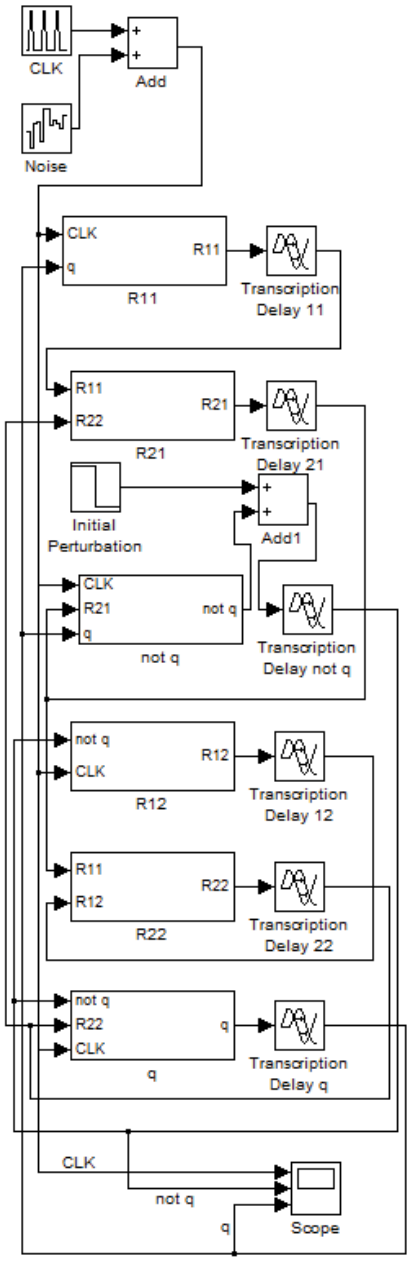

Figure 12: Model of extended 1-bit counter in `Matlab/Simulink` environment, where subsystems for calculation of certain protein concentrations are presented by `Simulink` blocks labeled as $R11$, $R12$, $R21$, $R22$, q and $not\ q$, CLK presents the concentration of input protein, *Noise* presents the white-noise generator and *Delays* present time delays in transcription/translation processes.

6 Conclusion

First model of synchronous sequential circuit with the use of gene expression based logic was presented in the article. Although simulation results are promising the circuit has yet to be realized within the living cell to prove that it is possible to use synchronous sequential logic within gene transcriptional networks in the manners described. The transition to complex synchronous sequential logic circuits would be straightforward if our presumptions are proved to be correct. Thus our future work is directed toward in vivo realization of the circuit presented here. Our future work also includes the transition of models based on the gene transcriptional networks like the one presented here to signal transduction networks, which have many benefits over the gene transcriptional networks when used as information processing systems [17, 18].

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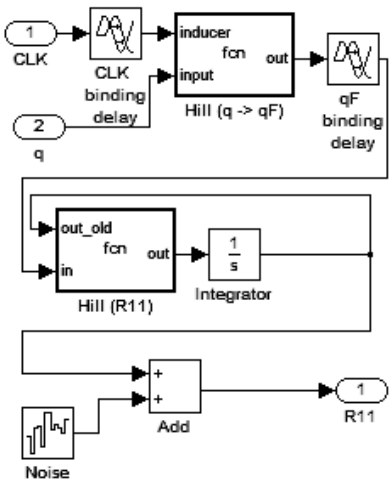

Figure 13: An example of a subsystem which defines certain protein concentration in a given time point. Subsystem presented is responsible for calculation of protein R_{11} concentration. Delay blocks present binding delays of protein CLK to protein q and protein q_F to promoter site, blocks denoted as *Hill* present equations for activation of protein q (See Eq. 8) and for transcription of protein R_{11} (See Eq. 7). Integrator presents the integration of Eq. 7.

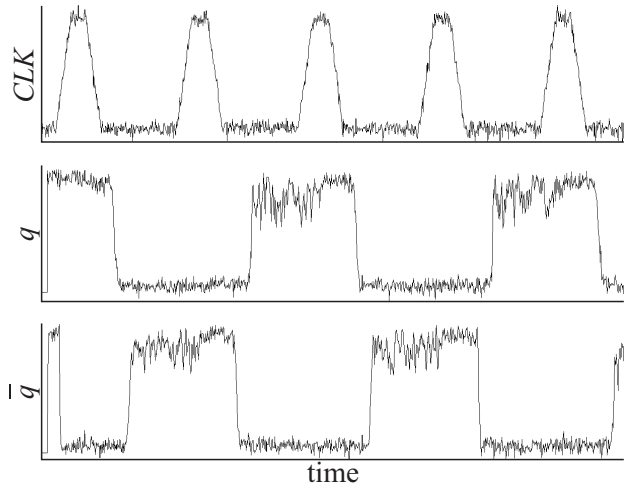

Figure 14: Representable simulation of extended version of 1-bit counter constructed with gene based expression logic, where y axes present the concentrations for each protein in a certain time and x axes present time.

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