Laboratory of Biobased Chemistry and Technology

Thesis Report BCT

Design of an *in vivo* controller

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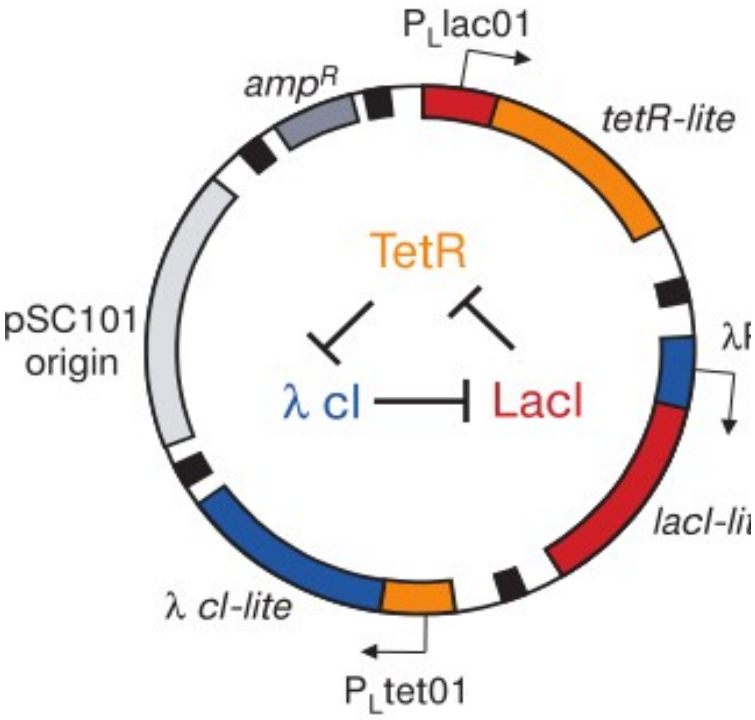

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Design of an *in vivo* controller

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Summary

Much of the cellular machinery interacts with each other, making it difficult to obtain accurate measurements of reactions happing *in vivo*. Synthetic biologists try to circumvent this problem by forward-engineering new biological systems that interact only minimally with the existing machinery. This allows for the design of an *in vivo* controller, a subsystem capable of pushing a system to a desired state. Such a controller can be useful for making a system less sensitive to noise.

We report the design of an *in vivo* controller, one specific for the chosen test system, the repressilator. We aimed to induce oscillations in a stable steady state repressilator using our controller.
The repressilator is a synthetic delay oscillator constructed of three transcription factors that inhibit each others expression [1]. A sensor module is designed that is capable of sensing the frequency of the repressilator. This sensor takes the form of a type 3 incoherent feed-forward loop, which is able to attenuate high frequency oscillations [2]–[5]. Thus, at low (or zero) frequency oscillations the sensor protein concentration is high, inducing the controller. At high frequency oscillations the sensor protein concentration is low and the controller is not activated.
Simulations of the designed repressilator-sensor-controller system show that when the repressilator is stable, high sensor concentration induces oscillations. When the repressilator is already in an oscillatory regime the sensor protein concentration is low and the controller is not active.

Table of Contents

1. Introduction

Synthetic biology is a relatively new field in biological science. It approaches biology from an engineer's point of view. Synthetic biology aims to enhance the understanding of life by creating new biological systems and studying their properties. This approach aids our understanding of biology, because natural biological systems are, through all their interconnections, too complex to observe the behavior of a single system. By constructing new modules that do not interact with other modules in the cell, and observing their behavior we can learn new things about the functioning of biochemical systems.

In biology it is commonplace to start working on a large system and then zoom-in on a smaller part of the system. This approach is called top-down and is accompanied by reverse-engineering, describing an already existing system. Synthetic biology, however, is about creating (*forward-engineering*) new biological systems, consisting of a single gene to a system of hundreds of genes. This approach is called bottom-up.
Forward-engineering of biological systems is often achieved by rational design, in which a system is designed and tested theoretically using mathematical modeling before it is constructed in the wet lab, see Figure 1. This approach avoids costly lab work and potential alterations can be implemented fast as the theoretical side is fully developed.

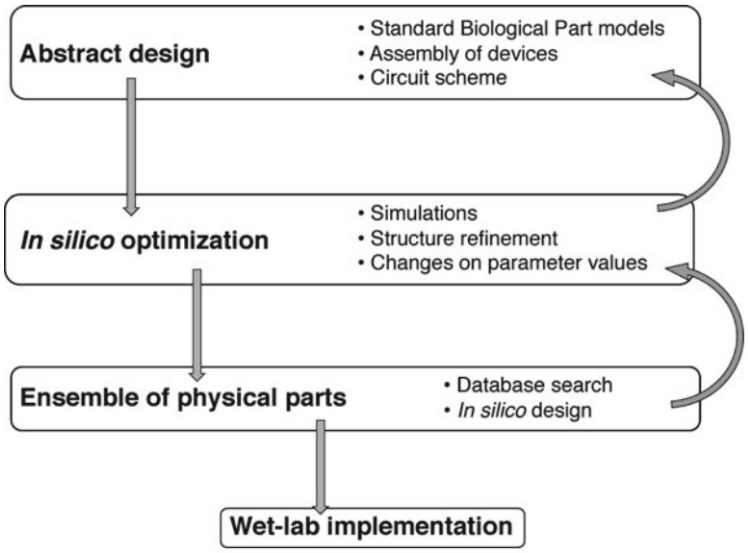

Figure 1: Schematic representation of the rational design process [20]

Forward-engineering and rational design of systems has become more efficient over time. In the past synthetic biologists focused on the creation of genetic modules that introduced one or two new features in an organism [6]. Now developments in the field allow us to engineer whole synthetic systems [7].
Although new systems that do not to interact with any natural systems can be designed, they will encounter interference from the existing biological machinery, as for example these new systems will use the existing transcription and translation machinery.

This allowed us to play with the idea of a biological module that can reduce the influence of perturbations. Hence, the aim of this study is to design an *in vivo* controller, based on

control theory. This module must be able to steer the system into a certain direction, but it should be able to do this automatically, i.e. without human interference.
Next to achieving the aim, we hope to answer to following questions:

- How can we couple the system to the controller?
- What would be a suitable biochemical representation of the controller?
- Is an *in vivo* controller feasible?

In order not to make the project too difficult we will consider a simple artificial biological system as the system to be controlled: the repressilator. This is a biological oscillator and for some parameter sets it oscillates and for some it does not. We aim to induce the repressilators oscillations when it is not oscillating using a new controller module. Coupling the system to the controller presents a significant challenge, as we will need a biological module that can interpret the frequency of oscillations. A possible solution to this problem will be presented in the sensor design chapter.

2. Background

Control theory

Control theory is a field of applied mathematics that focuses on controlling and steering dynamical systems. A controller is a module or device that pushes a system to some required or desired behavior. Control theory has been around since the eighteenth century, when James Watt developed a regulator that could control the speed of a steam engine. Since then the field of engineering has greatly improved and control systems are present everywhere [8].
Control systems are not only present in man-made machines, but are naturally present in many biological systems, as in for instance temperature and sugar concentration regulation in the human body [8]–[10]. Theories for modeling such biological control systems are in development [11], [12].

There are basically two main types of control structures: Open-loop control systems and closed-loop control systems. In closed-loop control (Figure 2), also called feedback control, the output of a system is the input of the controller. In turn, the output of the controller is the input for the original system. In open-loop control (Figure 3) the output of the system does not influence the controller, hence there is no closed loop.
The control strategy deployed in this study is closed-loop control.

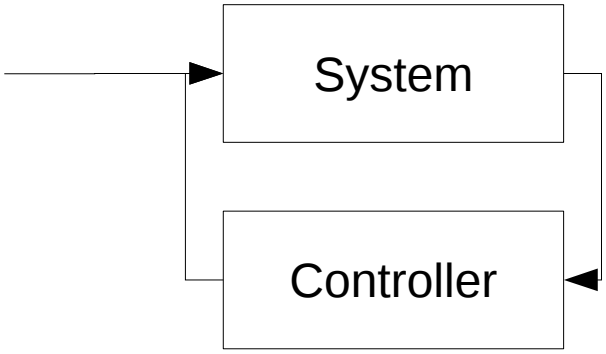

Figure 2: Schematic representation of a closed-loop control system

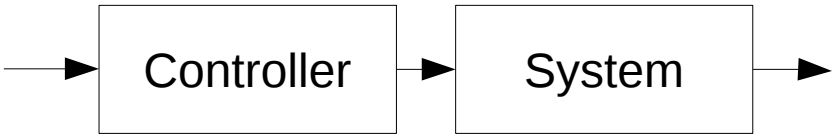

Figure 3: Schematic representation of an open-loop control system

The Repressilator

The system that we will use to design and test the control system is the repressilator by Elowitz and Leibler [1]. It is an artificial oscillating network, resembling a ring oscillator in electrical engineering. It is a delay oscillator, realized by a negative feedback loop.
As presented by Elowitz and Leibler the system comprises three genes, coding for transcription factors (LacI, TetR, cI) which are engineered to repress each other, see Figure 4.

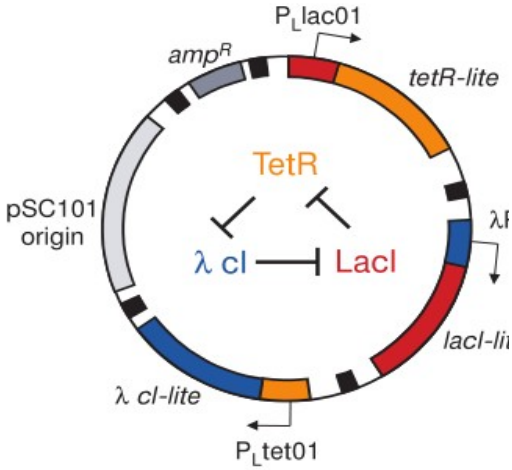

Figure 4: The repressilator plasmid as presented in [1]

The repressilator can be generalized to more genes, where a system with an odd number of repressors has other characteristics than one with an even number of repressors [13]. Elowitz and Leibler described the repressilator system with the following (dimensionless) ODEs:

$$\frac{dm_i}{dt} = -m_i + \frac{\alpha}{(1+p_j^n)} + \alpha_0 \qquad \frac{dp_i}{dt} = -\beta(p_i - m_i) \tag{1}$$

with i = LacI, TetR, λ cI and j = λ cI, LacI, TetR.

Furthermore, *m* is the concentration of mRNA [-] and *p* [-] is the protein concentration. α_0 is the transcription rate at full repression. $\alpha + \alpha_0$ is the transcription rate without full repression. β is the ratio of protein decay rate to mRNA decay rate, *n* is the Hill coefficient. Time is rescaled in units of mRNA lifetime (and thus unitless), protein concentrations are scaled in units of K_m, the dissociation constant, and mRNA concentrations are rescaled by their translation efficiency.
Using this information and the constants given in the paper, the following parameter values are obtained, see [14]:

Table 1: Parameters of the repressilator model, with their values

Parameter	α_0	α	K_m	β	n
Value [units]	0.2164 [proteins cell^{-1} K_m^{-1}]	216.4 [proteins cell^{-1} K_m^{-1}]	40 [monomers cell^{-1}]	0.2 [-]	2 [-]

This model relies on the following assumptions [1], [13]:
a) Genes are present in constant amounts.
b) Proteins binding to the regulatory regions of the genes either enhance or inhibit their expression. Binding reactions are in equilibrium.
c) Transcription and translation are operating under saturated conditions.
d) mRNAs and free proteins are degraded by first order reactions.

e) All three genes are identical, except for their DNA-binding specificities, i.e. the proteins bind to different promoters.
This system exhibits three kinds of behavior, depending on the parameters and the initial conditions. It can be in a stable steady state, exhibit damped oscillations or have sustained oscillations, see Figure 5.

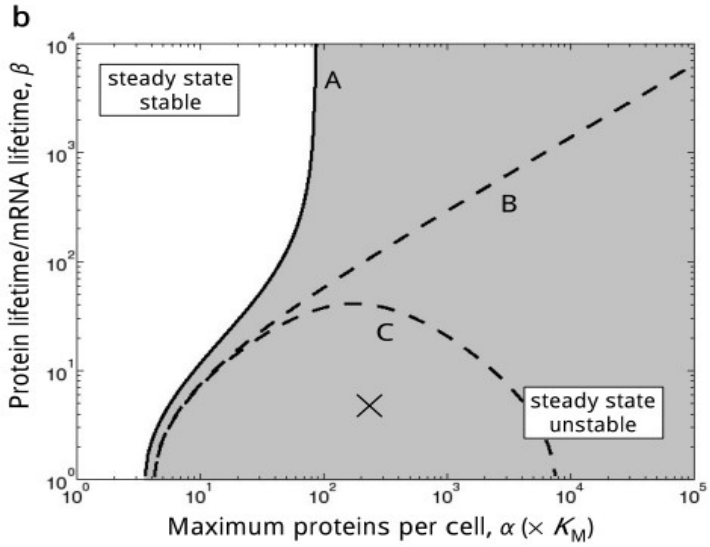

Figure 5: Stability diagram as presented in [1]

The repressilator is in stable steady state in the white part of the graph. In the grey part the system is unstable. The three different sections of the grey part correspond to different parameter sets:

A) $n=2.1$, $\alpha_0 = 0$; B) $n = 2$, $\alpha_0 = 0$; C) $n= 2$, $\alpha_0/\alpha = 10^{-3}$

The transition from the stable steady state to sustained (limit-cycle) oscillations (Figure 6) occurs via a supercritical Hopf bifurcation [13], [15]. A Hopf bifurcation occurs when an eigenvalue or complex conjugate pair of eigenvalues of the system's Jacobian is purely imaginary [16]. A Hopf bifurcation is supercritical when the appearing limit-cycle is stable. Bifurcations, in general, depend on the parameters of a system. The major control parameter in the repressilator model is the maximal synthesis rate of proteins, α.

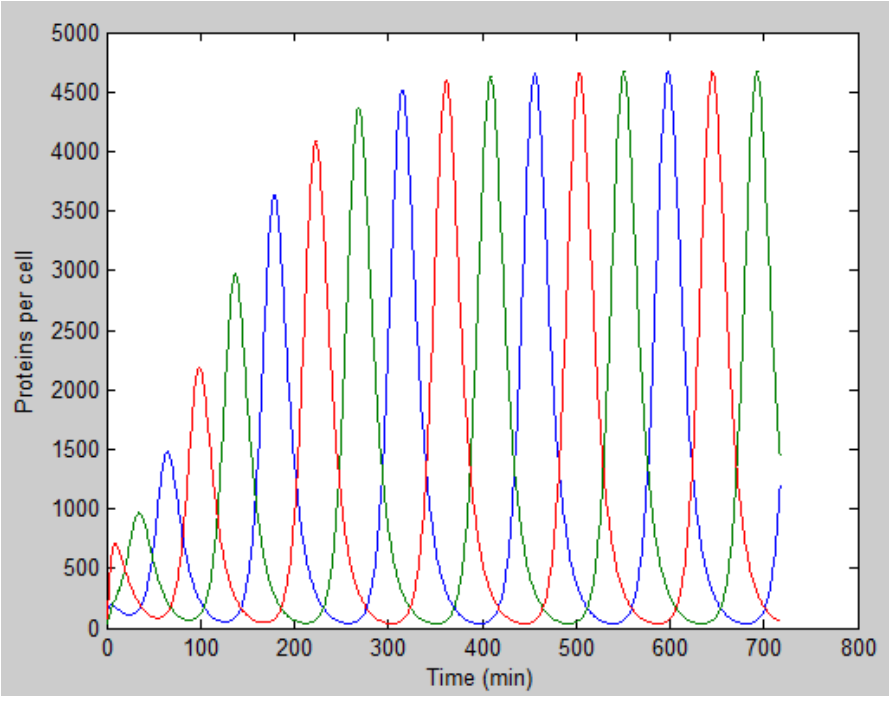

Figure 6: Dynamic behavior of the repressilator proteins

3. Sensor design

The sensor is a bridge between the system and the controller. It recognizes the behavior of the original system, in this case the oscillations or steady state of the repressilator. It can then pass this information to the controller.
This extra module is necessary, because the controller should only interfere when the frequency of the repressilator is around zero. Thus, the output signal of the system needs to be interpreted in order to give a signal to the controller.
The most straightforward design is to link the sensor to the frequency of the repressilator, i.e. the output signal is 0 at ω = 0 or 1 when ω ≠ 0. However, the sensor should be a physically realizable, biological module and thus it should be possible, at least theoretically, to express a working system in an organism.
To this end two design principles are presented for a sensor: averaging and resonance.

Averaging effect
The averaging effect is based on the assumption that the uncontrolled system oscillates with such a high frequency that the sensor cannot cope with the signal and will average out the signal, resulting in a constant output.

Consider a system

$$\dot{x}=f(x,u) \tag{2}$$

with $u(t+T)=u(t)$.
Let τ be a typical time scale of the system. Assume that the time scale of the input is much smaller than the system's time scale.
It can be shown (Appendix I) by integrating over one period (τ) that

$$\dot{x}=\langle f(x)\rangle \tag{3}$$

meaning that at high input frequencies the system behavior will be approximately constant.

Applied to the repressilator-sensor system, it means that the decay rate of sensor proteins is magnitudes lower than that of repressilator proteins. Consequently the repressilator oscillates relatively fast with respect to the sensor which cannot keep up. To the sensor the input will seem constant, the average of the signal.

I will further demonstrate the averaging principle using the following system:

$$\begin{aligned}\dot{x}&=\alpha u(\omega t)-\lambda x\\ \dot{y}&=\frac{\beta x^2}{K_y^2+x^2}-\mu y\\ \dot{z}&=\frac{\sigma y^2}{K_z^2+x^2}-\rho z\end{aligned} \tag{4}$$

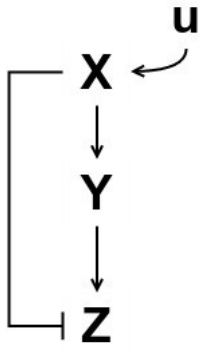

Figure 7: Schematic representation the averaging sensor system, an incoherent type 3 feed-forward loop

with β σ growth rates [concentration/time], λ μ ρ decay rates [1/time], K_z & K_y dissociation constants [concentration/time].
System (4) is a type 3 incoherent feed-forward loop, see Figure 7 [2]. It is activated by the input *u* at the subsystem *x. x* then activates *y,* which in turn activates *z. z* is also inhibited by *x.*

The system was nondimensionalized into

$$\begin{aligned}
&\frac{dX}{d\tau} = a\,U(\Omega\tau) - X \\
&\frac{dY}{d\tau} = b\,\frac{X^2}{1+X^2} - mY \\
&\frac{dZ}{d\tau} = s\,\frac{Y^2}{K^2+X^2} - r\,Z \\
&\text{with } \tau = t\ \lambda,\ X = \frac{x}{K_y},\ Y = \frac{y}{K_y},\ Z = \frac{z}{K_y} \\
&a = \alpha\,\lambda^{-1},\ b = \frac{\lambda^{-1}\beta}{K_y},\ s = \frac{\lambda^{-1}\sigma}{K_y},\ K = \frac{K_z}{K_y} \\
&m = \lambda^{-1}\mu,\ r = \lambda^{-1}\rho,\ \Omega = \lambda^{-1}\omega,\ U = \frac{u}{K_y}
\end{aligned} \tag{5}$$

Using system (5) for the averaging approach:

$$f = \begin{bmatrix} a\,U(\Omega\tau) - X \\ b\,\frac{X^2}{1+X^2} - mY \\ s\,\frac{Y^2}{K^2+X^2} - r\,Z \end{bmatrix} \Rightarrow \langle f \rangle = \begin{bmatrix} a\langle U\rangle - X \\ b\,\frac{X^2}{1+X^2} - mY \\ s\,\frac{Y^2}{K^2+X^2} - r\,Z \end{bmatrix}$$

$$where \langle U \rangle = \frac{1}{\epsilon}\int_0^{\epsilon} U(\Omega t')\,dt' \; and \; \epsilon = \frac{1}{\Omega}$$

$$Let, for\ instance,\ U(\Omega\tau) = \cos^2(2\pi\Omega\tau), then$$

$$\Omega\int_0^{\frac{1}{\Omega}} \cos^2(2\pi\Omega t')\,dt' \Rightarrow \frac{1}{2\pi}\int_0^{2\pi} \cos^2(y)\,dy = \frac{1}{2}$$

$$Thus\ for\ U = \cos^2(.):$$
$$a\,U(\Omega\tau) \approx a\ for\ \Omega \to 0$$
$$a\,U(\Omega\tau) \approx \frac{a}{2}\ for\ \Omega \gg 1$$

The subsystem X in (5), with input $U=\cos^2(\omega t)$, will go to *a* at no/low frequencies or to *a/2* at high frequencies. This implies that system (5) acts as a low-pass filter.

For a maximal effect the difference between *a* and *a/2* should be maximized.
Consider a steady state of the system for $U_{ss} = 1$:

$$
\begin{aligned}
X_{ss} &= a \\
Y_{ss} &= \frac{b}{m}\frac{a^2}{1+a^2} \\
Z_{ss} &= \frac{s}{r}\frac{\left(\frac{b}{m}\frac{a^2}{1+a^2}\right)^2}{K^2+a^2} = \frac{\theta a^4}{(1+a^2)^2(K^2+a^2)} \\
with\ \theta &= \frac{b^2 s}{m^2 r}
\end{aligned}
\tag{6}
$$

If θ is a constant, Z_{ss} is a function of *a* and *K*. So in order to find the optimal *Z* the parameters *a* and *K* need to be varied.

Constructive interference, i.e. resonance

In this section an alternative to the sensor module described by Eq. 4 is described. This alternative sensor is based on resonance. When the input is not oscillating there is no resonance, thus (almost) no sensor activity. However, when the input is oscillating, the sensor will resonate and the resulting gain (i.e. sensor protein concentration increase) can be used to induce or repress the controller.

The sensor system that we propose here, Eq. 7, is a feed-forward loop like in the averaging approach, but with a feedback from *z* to *x*, see Figure 8. This feedback should result in an increase in x when z is increased, whereas an increase in x decreases z. Let the resonance sensor system be described by:

$$
\begin{aligned}
\dot{x} &= \alpha u(\omega t) + \frac{\beta z^2}{K_x^2+z^2} - \kappa x \\
\dot{y} &= \frac{\gamma x^2}{K_y^2+x^2} - \lambda y \\
\dot{z} &= \frac{\zeta y^2}{K_z^2+x^2} - \mu z
\end{aligned}
\tag{7}
$$

In this sensor design the gain resulting from the resonance is used for repressing the controller. It is key that the peaks of the sensor signal are much higher than the steady state, such that the steady state level can be neglected.

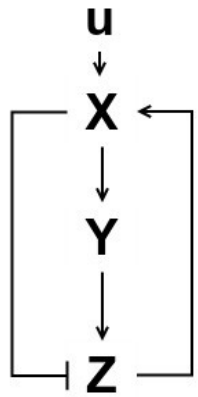

Figure 8: Schematic representation of the proposed resonance system

Because the frequency of the repressilator depends on the parameters, the sensor should be capable to resonate in response to different driving frequencies.

A system that is capable of resonating is the Hasty oscillator [17], [18].

4. Controller

As mentioned in the background chapter, a controller is a module that manages system behavior, and pushes it into a desired direction. Often this is achieved with state or output feedback control, where a state or output is the input of the controller. This principle is often used in mechanical systems. As we would like to control the behavior of a biological system, the controller should be simple enough to realize a biochemical representation of this controller.
Next to simplicity, our goal is to control the oscillatory behavior of my system (1), which depends on a bifurcation. This means that we should control the bifurcation parameters, instead of the states of the system.
To summarize: The controller should invoke oscillations when there are none, and do nothing when there are oscillations.
In order to realize this behavior the controller will control either of two system parameters: α, the maximal transcription rate and β, the ratio between protein decay and mRNA decay, which both can influence the bifurcation, see Figure 5. A large α is preferred for oscillations [16].
The simplest option is to increase α when the system is not oscillating, because this can be a simple linear increase. Therefore the focus will be on increasing α when the system is not oscillating.

There are two options:

1. couple the sensor protein concentration to α directly: α := α+z or α := α*z
   in which z is the sensor protein concentration.
   However, in this scheme α is always modified, and a function of time.

2. Make the activation of the controller concentration dependent:
   If the average z of the system is bigger than the average of z of the oscillating system the controller kicks in and oscillations are induced. Here the controller is bistable, where hysteresis occurs at a certain threshold of z protein concentration, effectively using on-off control.

   if $\bar{z} > \bar{z}_{oscillating}$: $\alpha = \alpha * \bar{z}$
   else: $\alpha = \alpha$.

   However, this approach uses an if statement to model a high concentration transcriptional activation, which is not biologically feasible. Instead a toggle switch might be used to add bistability.

5. Results

This section reports the results of the averaging and resonance sensor design approaches and of the controller, and how these integrate with the repressilator system, i.e.: does the repressilator oscillate when it is coupled to the sensor and the controller? The first section describes the averaging sensor and how the parameters were optimized. In the next section the behavior of the resonance sensor is described. Then the results of simulations with the coupled system, repressilator-sensor-controller, are described.

Averaging sensor

The optimal value of Z was calculated by maximizing the difference between $Z(a,K)$ and $Z(a/2,K)$, iteratively. For system (5) with U=10 the optimal value of a is 0.077 and the optimal value of K is 0.3, see Figure 9.

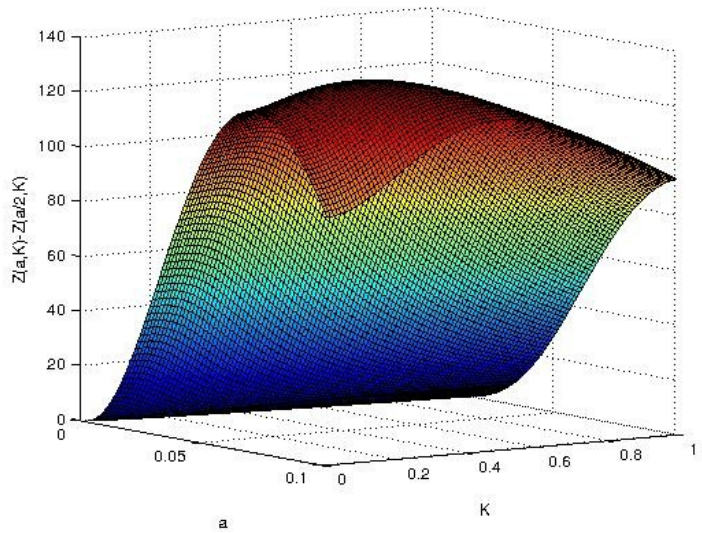

Figure 9: Maximizing the difference between Z(a,K) and Z(a/2,K)

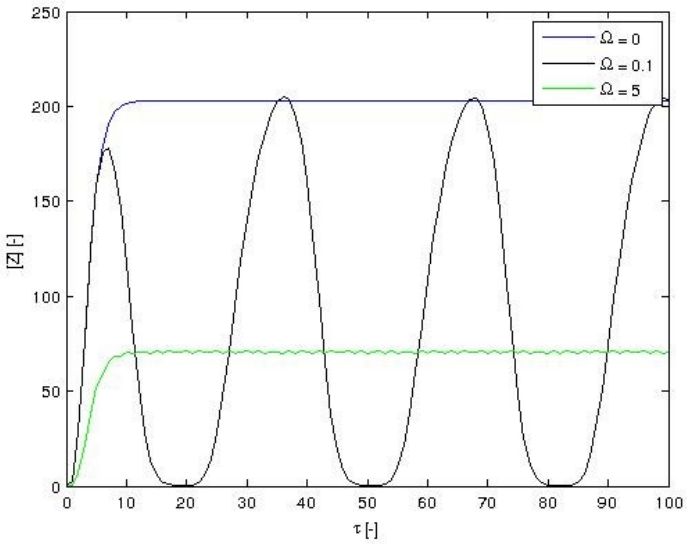

Figure 10: Time responses of the example system with $U=\cos^2(\Omega\tau)$ *for different values of* Ω*. At the highest frequency Z goes to the steady state value of* Z(a/2)

Using the calculated values of *a* and *K* in system (5) gives the time response depicted in Figure 10. It shows an example of the averaging effect. At Ω = 0, a steady state, the system response is *Z(a).* At Ω = 5, the system response resembles a steady state response of *Z(a/2)*.

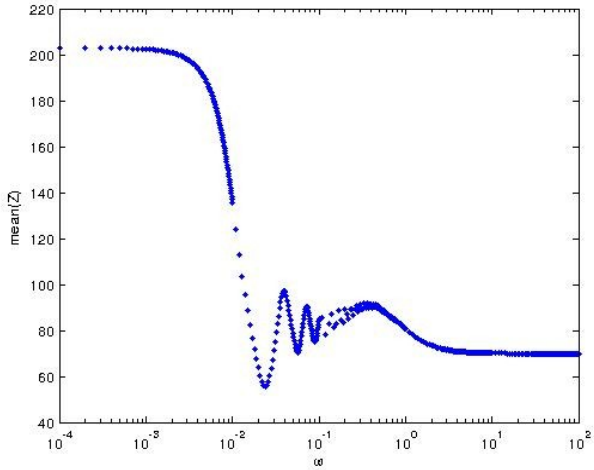

Figure 11: Frequency vs the average of Z

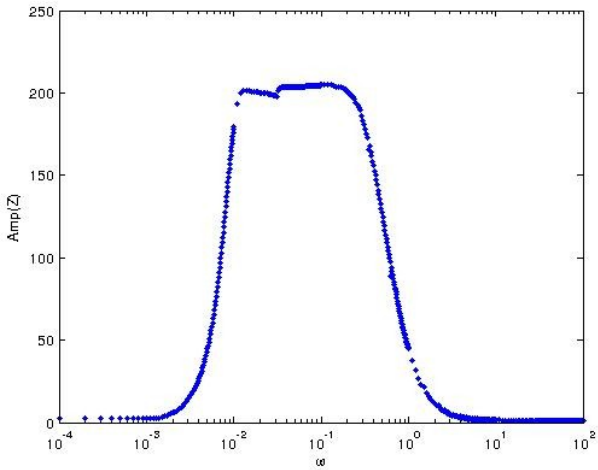

Figure 12: Frequency vs the amplitude of Z

In Figure 11 and 12 the relation between Z and ω(= Ω/10) is plotted. The mean Z concentration is a discrete approximation of $\frac{1}{T}\int_0^T Z\,dt$. At low frequencies the mean Z concentration is that at the *a* steady state and the amplitude is around zero. At high frequencies the amplitude is also around zero and the mean Z concentration is that at the *a/2* steady state. In between there is a passband for frequencies between around

0.02 to around 1 where the sensor can keep up with the input, indicating the sensor acts as a band-pass filter. This is not the low-pass filter behavior that was initially aimed for. However, at the passband frequencies the mean concentration is still significantly lower than the mean concentration at low or zero frequency, meaning that the sensor should still work although an integrating step is required.

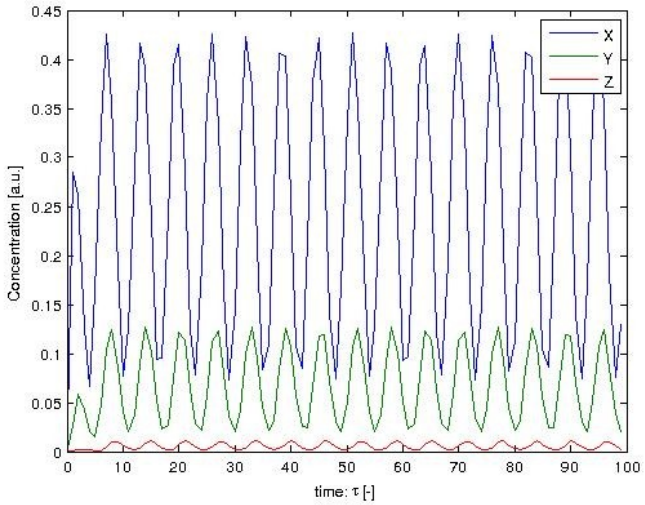

Figure 13: response of the resonance sensor, with u = $cos^2(\omega t)$

Resonance sensor

The resonance system as considered in section 3 does not increase the amplitude of z in respect to x, it is decreased, see Figure 13.

The coupled system

Until now we have only collected results from the separate modules. Now it is time to couple them together and see whether the sensor works with the repressilator and whether the controller is able to induce oscillations.

The repressilator system (1) is a dimensionless system. Therefore it was coupled to the dimensionless sensor model (5). This resulted in either a too low or too high concentration of Z, as the decay rate of X could not be modified. Hence, we used the full sensor model (4), where modification of the decay rate is possible.
The coupling of the repressilator to the sensor means that the values of a and K should be optimized again, as the repressilator behaves differently from a $\cos^2(\omega t)$ input. However, because we used the dimensional model, the parameters now have dimensions, and therefore different values. We used α and K_z as the parameters to be optimized, using the same approach as with the dimensionless model. The result is shown in Figure 14.

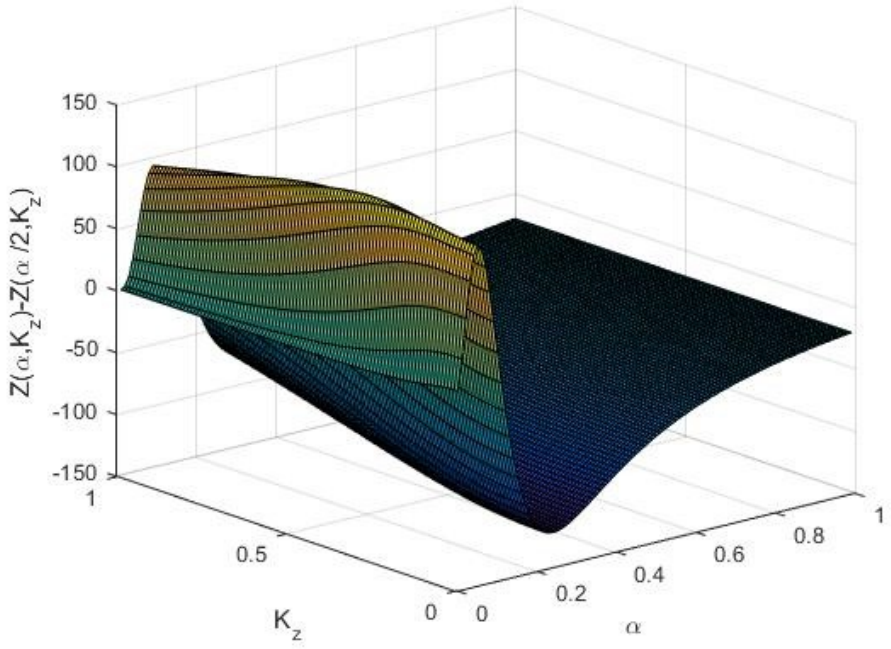

Figure 14: Maximizing the difference between $Z(\alpha,K_z)$ and $Z(\alpha/2,K_z)$

In Figure 14 two peaks are visible, a maximum and a minimum. The maximum corresponds to $\alpha = 0.094$ and $K_z = 0.7$. The minimum corresponds to $\alpha = 0.3$ and $K_z = 0.1$.

The figures shown below show the averaging sensor coupled to the repressilator. In Figure 15a the steady state is shown using the parameters at the maximum in Figure 14, in Figure 15b the corresponding oscillating state is shown. As can be seen from Figure 15b, the repressilator does not oscillate fast enough for the averaging effect to work, using this parameter set. However, since the sensor protein level is significantly lower than in steady state the sensor can function correctly.

a ***b***

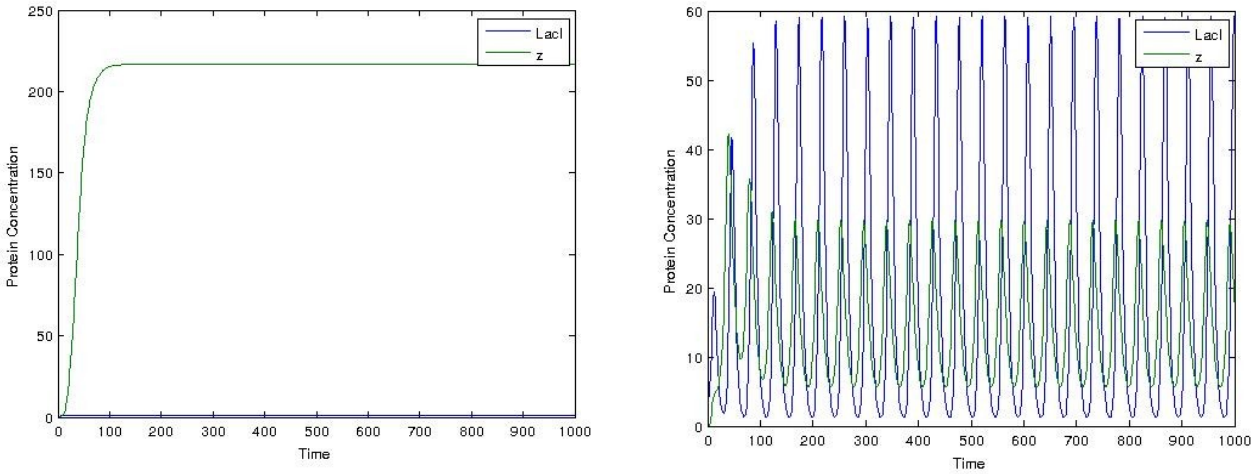

Figure 15: Behavior of the repressilator protein LacI concentration and the sensor z protein concentration using the parameter set at the maximum. Time scaled to mRNA lifetime, protein concentration is in units of repressilator K_m.
a) Repressilator in steady state, α = 2.164 b) repressilator oscillating, α = 216.4

When the controller is added to the model the following figures are obtained:

a ***b***

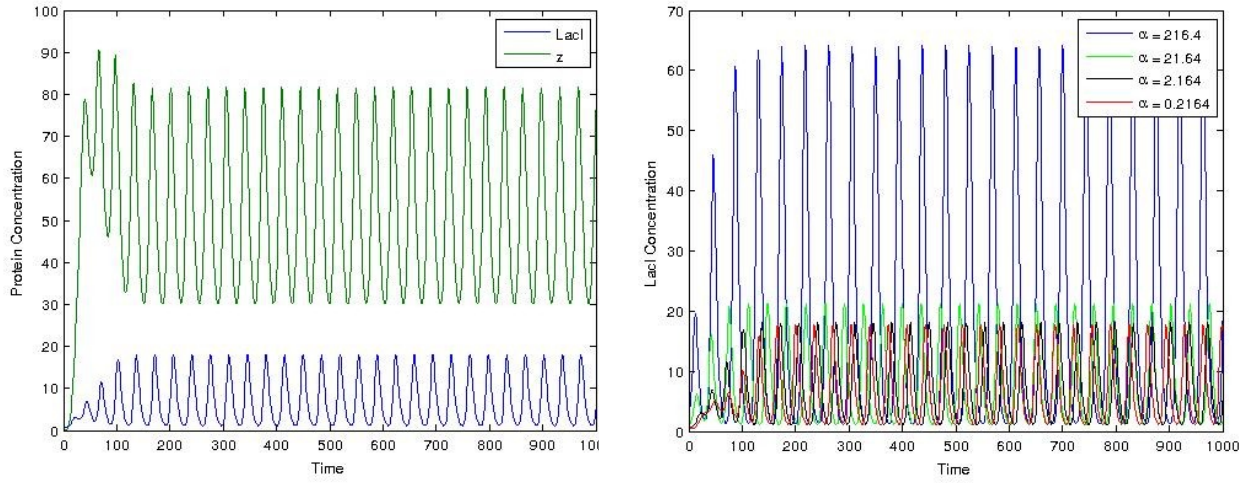

Figure 16: Behavior of the (a&b) repressilator LacI protein concentration and (b) the sensor z protein concentration, with controller (α := α+z), parameters at the maximum (Figure 9).

a) LacI and z protein concentrations, α = 2.164 initially. b) LacI concentration for different values of α.

In Figure 16a it can be observed that z is going to a steady state as in Figure 15, but as α gets larger, LacI begins to oscillate and z starts to oscillate too.

a ***b***

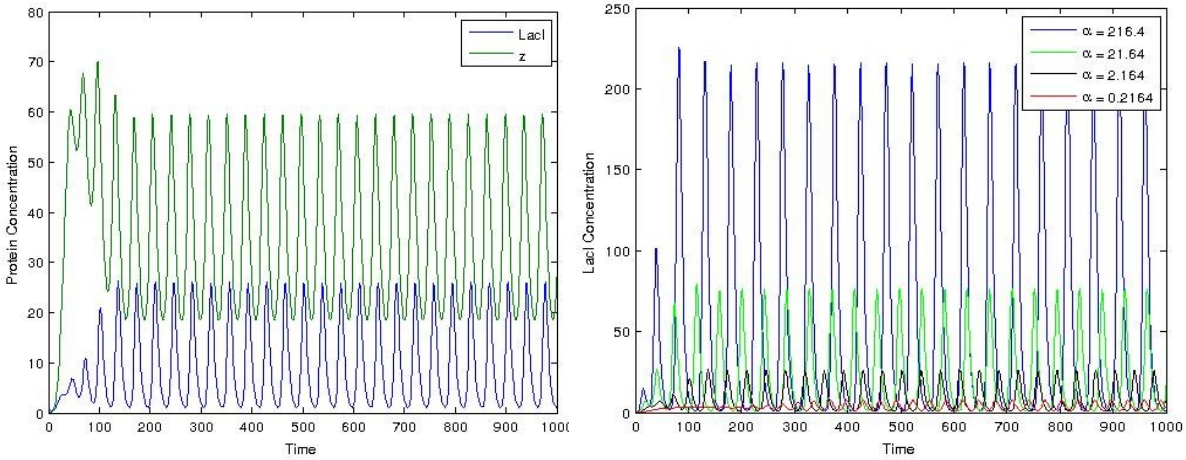

*Figure 17: Behavior of: (a&b) the repressilator LacI protein concentration and (a) the sensor z protein concentration, with controller (α := α*z), parameters at the maximum.*

a) LacI and z protein concentrations, α = 2.164 initially, b) LacI concentration for different values of α.

In Figure 16 an additive controller was used; in Figure 17 a multiplicative controller was used. After the addition of the controller to the model, both the sensor protein concentration and the repressilator protein concentration began to oscillate.

When the parameters from the minimum in Figure 14 are used, the z protein concentration is lower than when using the other parameter set (Figure 18a), but it is also lower when the repressilator is oscillating (Figure 18b), around 1. The averaging effect does also not work using this parameter set.

a ***b***

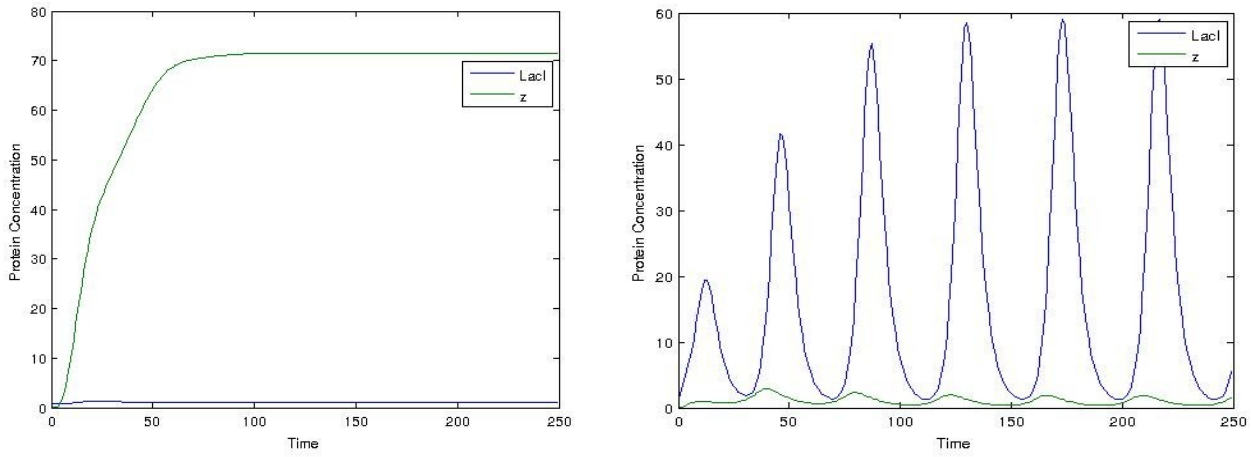

Figure 18: Behavior of the repressilator LacI protein concentration and the sensor z protein concentration using the parameter set at the minimum (Figure 9). Time scaled to mRNA lifetime, protein concentration is in units of repressilator K_m.
a) Repressilator in steady state, b) repressilator oscillating

a ***b***

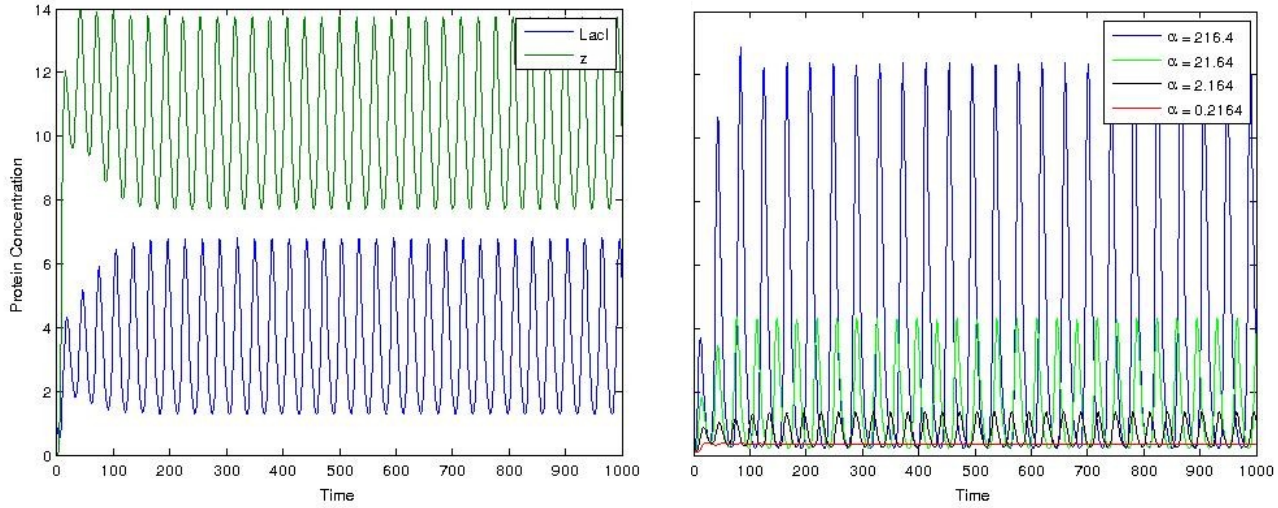

*Figure 19: Behavior of: (a&b) the repressilator LacI protein concentration and (a) the sensor z protein concentration, with controller (α := α*z), using the parameter set at the minimum. Time scaled to mRNA lifetime, protein concentration is in units of repressilator K_m.*

a) LacI and z protein concentrations, α = 2.164 initially, b) LacI concentration for different initial values of α.

a ***b***

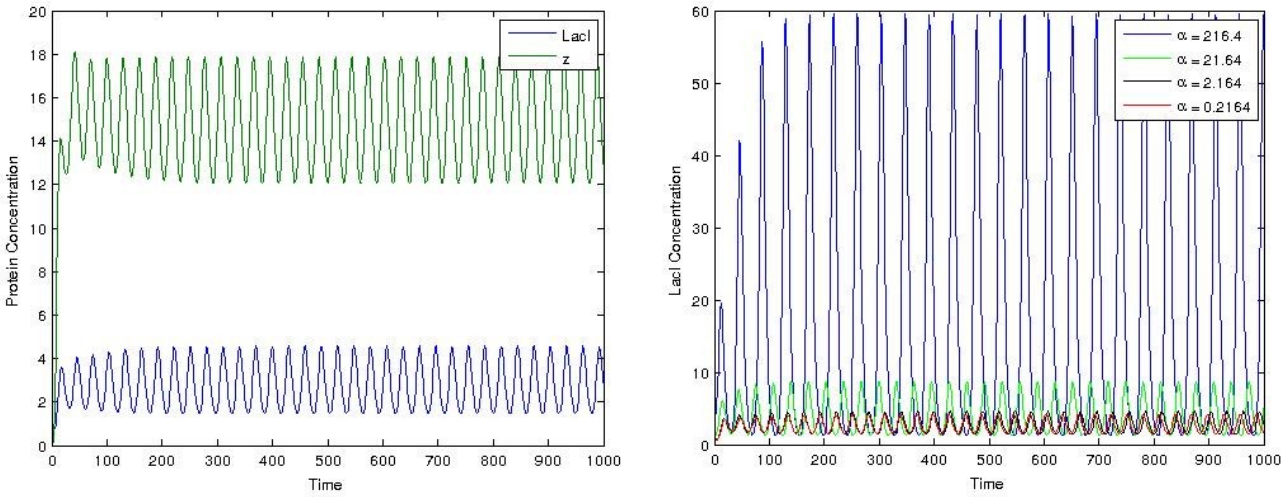

Figure 20: Behavior of: (a&b) the repressilator LacI protein concentration and (a) the sensor z protein concentration, with controller (α := α+z), using the parameter set at the minimum. Time scaled to mRNA lifetime, protein concentration is in units of repressilator K_m.

a) LacI and z protein concentrations, α = 2.164 initially, b) LacI concentration for different initial values of α.

Figure 19 and Figure 20 show the behavior of LacI and sensor z protein concentrations, with a multiplicative controller (Figure 19) and a additive controller Figure 20 using the α and K_z values from the minimum in Figure 14.
These figures show a lower amplitude for controller-induced LacI oscillations, in Figure 19b the lowest value for α does not induce oscillations at all.

6. Feasibility

This chapter concerns the feasibility of constructing the proposed repressilator-sensor-controller system *in vivo*. The feasibility concerns biological parts, the physical meaning of the parameters, and whether parameter values are realistic and assumptions are correct.

Averaging sensor

The genes used in the repressilator are commonly used when creating synthetic circuits. Therefore they cannot be used in the sensor, as this would cause interference between the sensor and the repressilator. The sensor genes should not occur naturally or be active in the chosen organism, e.g. E. coli, as this could also cause interference.
The z sensor protein needs to activate the controller. Therefore z protein lifetime is an important factor in the functioning of the sensor and of the controller: when protein lifetime is too short, the averaging effect can be canceled. So the z protein needs to be a stable protein that activates the transcription of a gene and is the final gene in an inconsistent 3 FFL.

Controller

The design for the used controller is quite simple. The controller increases the parameter α, which is the total protein concentration in the cell. The controller should thus be a gene that codes for a (or any) protein. Ideally, this gene is transcribed and translated fast and the lifetime of the protein is relatively long, in order to increase the impact of the controller. The protein should be fairly small, in order to increase the response time and resource (amino acid, GTP) efficiency. It should be a redundant protein, since the only function of the protein is to increase the overall protein concentration.
A drawback of the used controller is the fact that α itself now oscillates, making it a function of time, α(t). It behaves the same as sensor z protein concentration, but multiplied by the initial α value. The sensor is necessary, as using LacI instead of z protein does not give oscillations (data not shown).
The controller is also not an external input, but a state of the system, fed back into the system. It thus could be argued that the controller is not in fact a controller, but a change to the system itself.
It should be noted that increasing α cannot be endlessly repeated, because of the appearance of a heteroclinic cycle when α gets too large [19].
In order to activate the controller only when the z protein concentration is at a certain level the use of toggle-switches has to be investigated. Toggle-switches are gene cascades of two activators and/or inhibitors through which bistablity is achieved. Combined with ultrasensitivity this yields a system that can quickly switch from one stable state to another (hysteresis). I have thus far been unable to obtain a functional concentration sensitive sensor.

Parameters

The repressilator does not oscillate fast enough for the averaging effect to kick in when using the same parameters as described in [1], [16]. This should not be a problem as the sensor protein concentration is sufficiently low. It is possible to influence the frequency of the repressilator though. Of the two control parameters β has the biggest influence on the frequency of the repressilator, see eq. (82) in [13].

According to Brian Ingalls [16] maintaining β near one keeps the repressilator in the oscillating regime. This implies controlling the decay rate of either/both proteins and mRNA. This requires the controller to activate a specific protease and/or RNAase. However, in (16) he uses a different, dimensional, repressilator system thus this does not apply to system (1). Increasing α seems a simpler option.
Until now I mentioned two control parameters, α and β, whereas the Hill coefficient is also a control parameter. However, as Elowitz and Leibler found that a Hill coefficient of 2 approximates reality well [1], it will not be elaborated upon.

Resonance sensor
In this case the sensor z protein could be a multimerizing protein that does not multimerize at low concentrations (steady state), but does at higher concentrations (when oscillating). The multimer is then a repressor, which will repress transcription of the controller. In this case the sensor z protein and controller should be encoded by different genes than in the averaging approach.
As can be seen in Figure 13 the proposed resonance sensor is unable to amplify the received signal, it is reduced.
It is possible that a relaxation oscillator might work, or one of the feed-forward loop variants.

7. Conclusion

It is possible to change the behavior of the repressilator from a stable steady state to an oscillatory regime using a controller. This controller needs a sensor module to receive state information from the repressilator. The averaging sensor is functional, but the proposed resonance sensor is unable to amplify the received signal.
It should be able to realize this system *in vivo*, but I was unable to find the biological parts to do so.

8. Future work

In order to find out if the designed system works *in vivo* appropriate biological parts have to be chosen. With the biological parts the wet lab work can be carried out.
It would be interesting to find a resonance system that will actually work. As mentioned, a relaxation oscillator might have promising qualities.
In order to make the controller concentration dependent the use of toggle-switches has to be investigated.

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Appendix I

$$\textit{Consider}:\ \dot{x}=g(x,u)$$
$$u(t+T)=u(t);\ u=u(\omega t)$$

$$\textit{Let } \tau \textit{ be a typical time scale of the system}$$
$$\textit{Rescale time}:\ \tilde{t}=\frac{t}{\tau}$$
$$\Omega=\omega\tau;\ \epsilon:=\frac{1}{\Omega}$$

$$\textit{Assumption}:\ \Omega\gg 1$$

$$\frac{dx}{dt}=g(x,u(\Omega\tilde{t}))$$
$$x(t+\epsilon)-x(t)=\int_t^{t+\epsilon} g(x,u)\,dt'$$
$$x(t+\epsilon)=\sum_{n=0}^{\omega} x^n(t)\frac{\epsilon^n}{n!}$$
$$\sum_{n=1}^{\omega} x^n(t)\frac{\epsilon^n}{n!}=\int_t^{t+\epsilon} g(x(t'),u(t'))\,dt'$$
$$\int_t^{t+\epsilon} g\Big(\sum_{n=0}^{\omega} x^n(t)\frac{(t'-t)^n}{n!},u(t')\Big)dt'=\int_0^{\epsilon} g\Big(x(t)+\sum_{n=1}^{\omega} x^n(t)\frac{t_1^n}{n!},u(t_1)\Big)dt_1$$
$$\int_0^{\epsilon} g\Big(x(t)+\sum_{n=1}^{\omega} x^n(t)\frac{t_1^n}{n!},u(t_1)\Big)dt_1=\int_0^{\epsilon} g(x(t),u(t+t_1))\,dt_1+\int_0^{\epsilon} J\,\gamma\,dt_1+\ldots$$
$$\textit{with } J=\textit{Jacobian}\wedge\gamma=\sum_{n=1}^{\omega} x^n(t)\frac{t_1^n}{n!}$$
$$\int_0^{\epsilon} J\,\gamma\,dt_1=\int_0^{\epsilon} J(x(t),u(t+t_1))\sum_{n=1}^{\omega} x^n(t)\frac{t_1}{n!}dt_1$$
$$\int_0^{\epsilon} J\,\gamma\,dt_1=\sum_{n=1}^{\omega}\epsilon^{n+1}\int_0^1 J(x(t),u(\frac{t}{\epsilon}+t_2))x^n(t)\frac{t_2^n}{n!}dt_2\Rightarrow$$
$$\int_0^{\epsilon} J\,\gamma\,dt_1=O(\epsilon^2)$$

$$\textit{Collecting terms of } O(\epsilon):$$
$$\dot{x}(t)\epsilon=\int_0^{\epsilon} g(x(t),u(\frac{t+t_1}{\epsilon}))dt_1$$
$$\dot{x}(t)\epsilon=\epsilon\int_0^1 g(x(t),u(\frac{t}{\epsilon}+t_2))\,dt_2;\ \textit{with } t_2=\frac{t_1}{\epsilon}$$
$$\dot{x}(t)\epsilon=\epsilon\int_0^1 g(x(t),u(t_2))dt_2\Rightarrow$$

$$\dot{x}=\int_0^1 g(x(t),u(t_2))\,dt_2+O(\epsilon)\Rightarrow$$
$$\langle g(x(t))\rangle=\int_0^1 g(x(t),u(t_2))\,dt_2\Rightarrow$$
$$\dot{x}=\langle g(x)\rangle$$