

Optimal Design of Phosphorylation-Based Insulation Devices

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Abstract—We seek to minimize both the retroactivity to the output and the retroactivity to the input of a phosphorylation-based insulation device by finding an optimal substrate concentration. Characterizing and improving the performance of insulation devices brings us a step closer to their successful implementation in biological circuits, and thus to modularity. Previous works have mainly focused on attenuating retroactivity effects to the output using high substrate concentrations. This, however, worsens the retroactivity to the input, creating an error that propagates back to the output. Employing singular perturbation and contraction theory tools, this work provides a framework to determine an optimal substrate concentration to reach a tradeoff between the retroactivity to the input and the retroactivity to the output.

I. INTRODUCTION

Understanding modularity is one of the most pressing matters in systems biology. Modularity is the property according to which the input/output behavior of a system does not change upon interconnection and has been proposed as one of the possible levels of biological organization [1]. It was suggested, however, that biomolecular systems are not always modular because impedance-like effects at the interconnections, called retroactivity, alter the system's behavior [2][3] [4]. Fig. 1 shows the system model introduced in [3] to explicitly account for retroactivity. System II, with input u and output y , is subject to retroactivity to the output s , due to interconnection to a downstream system, and applies retroactivity to the input r to its upstream system. In a biological circuit, this occurs, for example, when a protein is used as a transcription factor. The downstream process uses the protein in its reactions, directly affecting its dynamics. Retroactivity has also been related to fan-out [5], which is defined as the maximum regulation capacity of a transcription factor.

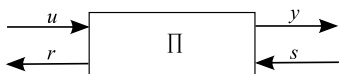


Fig. 1. System II with input u , output y , retroactivity to the input r and retroactivity to the output s .

From an engineering point of view, an insulation device can be used to decouple the dynamics of interconnected components. An insulation device is analogous to an insulating amplifier in electrical circuits, where a signal is transmitted to a downstream system while minimizing the impedance effects. It has been suggested that signaling pathways, such

as the mitogen-activated protein kinase (MAPK) cascade, can be used as amplifiers and placed in negative feedback loops to obtain insulation from downstream loading [6]. It was shown in [3] that phosphorylation cycles can attenuate retroactivity to the output through a mechanism similar to high gain feedback even without an explicit negative feedback. And indeed, *in vitro* implementations have confirmed this theoretical prediction [7]. Other implementations of insulation devices have also been realized *in vitro*, where the dynamics of a biological oscillator were successfully decoupled from the dynamics of DNA tweezers using a genelet amplifier circuit [8].

An ideal insulation device has the retroactivity to the input r in Fig. 1 close to zero and the effect of the retroactivity to the output s on y is completely attenuated. A fundamental question is whether these two requirements are in conflict with each other. Here, we study this problem when the insulation device is realized with a phosphorylation cycle. It was shown before [3] that as the amounts of cycle substrate and phosphatase are increased, the effect of the retroactivity to the output on the cycle output protein could be attenuated. However, increased amounts of cycle substrate result in an increased retroactivity to the input. In this paper, we propose to parameterize the error of the insulation device output with the cycle substrate and phosphatase concentrations to determine an optimal amount that minimizes both retroactivity effects.

Our approach to characterize this tradeoff is based on singular perturbation and contraction theory. We determine an upper bound on the steady state error between the output of the insulation device under study and an ideal insulation device. This upper bound is a function of the substrate and phosphatase concentrations and can be minimized with respect to these variables. We then show through simulation that the upper bound that we have calculated is tight.

This paper is organized as follows. In Section II, the mathematical tools needed for the problem solution are provided. In Section III, the system model and problem are presented in terms of the chemical reactions and differential equations describing the phosphorylation cycle. Also, the definition of the input error and output error of the insulation device are given. In Section IV, a general solution approach using model reduction techniques is presented. Sections V and VI provide the input and output error in terms of the cycle substrate and phosphatase concentrations. In Section VII, the total error of the insulation device is provided.

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II. MATHEMATICAL TOOLS

Theorem 1. (*Contraction Theorem*): Adapted from [9]. Consider the n -dimensional deterministic system $\dot{x} = f(x, t)$, where f is a smooth nonlinear function. The system is said to be contracting if any two trajectories, starting from different initial conditions, converge exponentially to each other. A sufficient condition is the existence of some matrix measure, m , such that there is a $\lambda > 0$ with $m\left(\frac{\partial f(x, t)}{\partial x}\right) \leq -\lambda$ for all x and for all $t \geq 0$. The scalar λ defines the contraction rate of the system.

Throughout this paper, the vector norm $|\cdot|$ will refer to the ℓ^2 -norm given by $|x|_2 = (\sum_{j=1}^n |x_j|^2)^{1/2}$ and $m_2(A)$ the induced matrix measure given by $m_2(A) = \max_i \left(\lambda_i \left\{ \frac{A+A^*}{2} \right\} \right)$ where λ_i denotes the matrix's i^{th} eigenvalue.

Lemma 1. (*Robustness*): Adapted from [9]. Assume that the system

$$\dot{x} = f(x, t) \quad (1)$$

is contracting, with contraction rate λ , and consider the perturbed system

$$\dot{x}_p = f(x_p, t) + d(x_p, t), \quad (2)$$

where $d(x_p, t)$ is bounded, so there is a $\bar{d} \geq 0$ such that $|d(x_p, t)| \leq \bar{d}$ for all x_p and for all $t \geq 0$. Then, the trajectory of the perturbed system satisfies

$$|x_p(t) - x(t)| \leq e^{-\lambda t} |x_p(0) - x(0)| + \frac{\bar{d}}{\lambda}. \quad (3)$$

Lemma 2. Adapted from [10]. Assume that the system $\dot{x} = f(x, z(t))$ is partially contracting in x with contraction rate λ_x so that the solution of the system $f(x_s, z(t)) = 0$ can be written as $x_s = \gamma(z)$, i.e., there is a unique global mapping between x and z . Assume further that there exists a $\bar{d} \geq 0$ such that $\left| \frac{\partial \gamma(z)}{\partial z} \dot{z} \right| \leq \bar{d}$ for all x , for all z and for all $t \geq 0$. Then, any trajectory $x(t)$ satisfies

$$|x(t) - \gamma(z(t))| \leq e^{-\lambda_x t} |x(0) - \gamma(z(0))| + \frac{\bar{d}}{\lambda_x}. \quad (4)$$

Proof. Let x be the solution of $\dot{x} = f(x, z(t))$ while $x_s = \gamma(z(t))$ is the solution of the ‘‘perturbed’’ system $\dot{x}_s = f(x_s, z(t)) + \frac{\partial \gamma(z)}{\partial z} \dot{z}$ with disturbance $\frac{\partial \gamma(z)}{\partial z} \dot{z}$. Applying the result (3) from Lemma 1 yields bound (4). \square

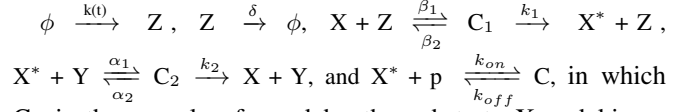
Proposition 1. Consider the system (1)-(2) in Lemma 1 and let $|d(x_p, t)| \leq C_0 + \sum_{k=1}^n C_k e^{-\lambda_k t}$. Having $x_0 = |x(0) - x_p(0)|$, the upper bound on $|x(t) - x_p(t)|$ is given by

$$|x(t) - x_p(t)| \leq x_0 e^{-\lambda t} + \frac{C_0}{\lambda} + \sum_{k=1}^n \frac{C_k}{\lambda - \lambda_k} e^{-\lambda_k t}. \quad (5)$$

Proof. Let $X(t) = |x_p(t) - x(t)|$, then $\frac{dX}{dt} + \lambda X \leq |d(x_p, t)| \leq C_0 + \sum_{k=1}^n C_k e^{-\lambda_k t}$ as in [9]. Multiplying by the integrating factor $e^{\lambda t}$ we can re-write the differential equation as $\frac{d}{dt}(e^{\lambda t} X) \leq e^{\lambda t} (C_0 + \sum_{k=1}^n C_k e^{-\lambda_k t})$. The differential equation can now be integrated to obtain (5) where the terms with negative coefficients were neglected for the approximation. \square

III. SYSTEM MODEL AND PROBLEM

A phosphorylation cycle consists of a set of two reversible enzymatic reactions, where the activation and deactivation of a substrate through the addition/removal of a phosphate group is used to transmit information to a downstream system [11]. Throughout this work, for a given species X its concentration is denoted by X (italics). In a phosphorylation cycle, a kinase labeled Z , regulated by $k(t)$, activates substrate X through a phosphate transfer reaction to form X^* , while Y deactivates X^* to form X . Protein X^* also regulates a downstream system by binding to sites p forming complex C . These sites can be DNA promoter sites if X^* is a transcription factor or they can belong to a substrate if X^* is an active kinase. The chemical reactions for the system are:



C_1 is the complex formed by the substrate X and kinase Z and C_2 is the complex formed by the protein X^* and phosphatase Y . The assumed conservation laws are: $X_T = X + X^* + C_1 + C_2 + C$, $Y_T = Y + C_2$, and $p_T = p + C$.

The ODE model of the phosphorylation cycle is given by:

$$\begin{aligned} \frac{dZ}{dt} &= k(t) - \delta Z \\ &\quad - \underbrace{\beta_1 Z X_T \left(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{C}{X_T} \right)}_r + (\beta_2 + k_1) C_1, \\ \frac{dC_1}{dt} &= \beta_1 Z X_T \left(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{C}{X_T} \right), \\ &\quad - (\beta_2 + k_1) C_1 \\ \frac{dC_2}{dt} &= -(k_2 + \alpha_2) C_2 + \alpha_1 Y_T X^* \left(1 - \frac{C_2}{Y_T} \right), \\ \frac{dX^*}{dt} &= k_1 C_1 + \alpha_2 C_2 - \alpha_1 Y_T X^* \left(1 - \frac{C_2}{Y_T} \right), \\ &\quad + \underbrace{k_{off} C - k_{on} X^* (p_T - C)}_s, \\ \frac{dC}{dt} &= -k_{off} C + k_{on} X^* (p_T - C). \end{aligned} \quad (6)$$

Here, r represents the retroactivity to the input and s represents the retroactivity to the output. One can abstract the signal flow in (6) using system Σ in Fig. 2. Signal Z drives the X^* dynamics through complex C_1 while the binding and unbinding reaction of Z with X creates retroactivity r in the Z dynamics. Similarly, X^* drives the C dynamics downstream, while being affected by the retroactivity s . An ideal insulation device should behave as system Σ_I in Fig. 2 where the terms under brace r and s in (6) were set to zero.

The key tunable parameters in this system are X_T and Y_T , which will be kept at a constant ratio $Y_T/X_T = \rho$ throughout the analysis. We seek to adjust the values of these parameters in such a way that the behavior of the system is close to that of an ideal insulation device. This can be better appreciated in Fig. 3, where different substrate concentrations are tested using a sinusoidal input $k(t)$ on system (6). The black line describes the ideal behavior X_I^* given by $r, s = 0$. The red line is the behavior of X^* in the system having $r, s \neq 0$.

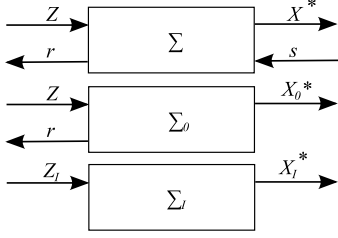


Fig. 2. Top: system Σ with input Z , output X^* , retroactivity to the input r and retroactivity to the output s . Middle: System Σ_0 with input signal Z subject to retroactivity r , while output signal X_0^* has retroactivity to the output $s = 0$. Bottom: system Σ_I is the ideal realization of system Σ , where both signals Z_I and X_I^* are not subject to retroactivity.

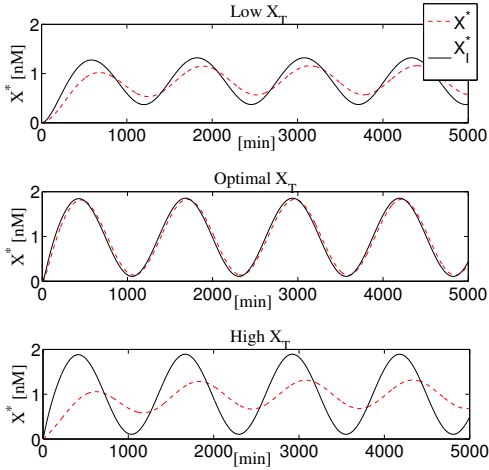


Fig. 3. The red line is output X^* of system Σ in Fig. 2 and the black line is output X_I^* of system Σ_I , with different substrate concentrations. In all simulations, the parameters are taken from [12]: $k_{off} = 10 \text{ min}^{-1}$, $k_{on} = 1 \text{ (nM min)}^{-1}$, $\delta = 0.01 \text{ min}^{-1}$, $k(t) = \delta(1 + \sin(\omega t))$, and $\omega = 0.005 \text{ min}^{-1}$. Also $k_1 = k_2 = 0.6 \text{ min}^{-1}$, $\alpha_1 = \beta_1 = .006 \text{ (nM min)}^{-1}$, $\beta_2 = \alpha_2 = 6 \text{ min}^{-1}$, $p_T = 10 \text{ nM}$ and $\rho = 1$. Low $X_T = 10 \text{ nM}$, intermediate $X_T = 100 \text{ nM}$ and high $X_T = 1000 \text{ nM}$.

As we can see from Fig. 3, having too high or too low values of X_T leads to an error between the actual X^* and the ideal X_I^* device responses. The reason being that a large X_T concentration applies a load to the $Z(t)$ dynamics changing the nominal signal $Z_I(t)$, while attenuating the effect of p_T on the $X^*(t)$ dynamics. This tradeoff between minimizing the effect of r and s is studied in this paper. Specifically, the total output error $\Delta X_{TOT}^*(t) := X^*(t) - X_I^*(t)$, is quantified by determining positive functions, $A(X_T)$, $B(X_T)$, $\lambda(X_T)$ such that $|\Delta X_{TOT}^*| \leq A(X_T)e^{-\lambda(X_T)t} + B(X_T)$.

IV. SOLUTION APPROACH

This problem will be solved by quantifying the errors in X^* due to r and s in (6) to find $A(X_T)$, $B(X_T)$ and $\lambda(X_T)$. To this end, the virtual system Σ_0 in Fig. 2 is obtained from Σ by setting $s = 0$. The output error is defined as $\Delta X_0^*(t) := X^*(t) - X_0^*(t)$, which accounts for the error in $X^*(t)$ only due to retroactivity s . Likewise, the virtual system Σ_I in Fig. 2 is obtained from Σ by setting $r = 0$ and $s = 0$. The input error, defined as $\Delta X_I^*(t) := X_0^*(t) - X_I^*(t)$, accounts for the error in $X_0^*(t)$ due only to retroactivity

to the input r . It will be shown that the total output error, given by $\Delta X_{TOT}^* = X^*(t) - X_I^*(t)$, can be upper bounded by $|\Delta X_{TOT}^*(t)| \leq |\Delta X_0^*(t)| + |\Delta X_I^*(t)|$. We proceed to separately determine the output error and input error.

First, a system order reduction will be performed through singular perturbation to obtain a two-state variable model for $Z(t)$ and $X^*(t)$. Processes in system (6) occur in three timescales [13]. The slowest timescale is that of the kinase dynamics due to protein production and decay and the intermediate timescale is that of phosphorylation. The fastest timescale is that of the binding and unbinding reactions to form complexes C_1 , C_2 and C . Thus, singular perturbation parameters $\epsilon_1 := \delta/k_1$ and $\epsilon_2 := \delta/k_{off}$ are selected so that $\epsilon_2 \ll \epsilon_1 \ll 1$. We define the parameters: $k_d := k_{off}/k_{on}$, $b_1 := \beta_1\epsilon_2/\delta$, $a_1 := \alpha_1\epsilon_2/\delta$, $b_2 := \beta_2\epsilon_2/\delta$, $a_2 := \alpha_2\epsilon_2/\delta$, and $k_x := k_2/k_1$. The transformation $w := Z + C_1$ and $y := X^* + C_2 + C$ is also performed on system (6), converting it to standard singular perturbation form [14]:

$$\begin{aligned} \frac{dw}{dt} &= k(t) - \delta(w - C_1) \\ \epsilon_1 \frac{dy}{dt} &= \delta C_1 - k_x \delta C_2 \\ \epsilon_2 \frac{dC_1}{dt} &= \delta b_1(w - C_1)(X_T - y - C_1) - \delta(b_2 + \epsilon_2/\epsilon_1)C_1 \end{aligned} \quad (7)$$

$$\begin{aligned} \epsilon_2 \frac{dC_2}{dt} &= \delta a_1(Y_T - C_2)(y - C_2 - C) - \delta(a_2 + k_x\epsilon_2/\epsilon_1)C_2 \\ \epsilon_2 \frac{dC}{dt} &= \frac{\delta}{k_d}(y - C_2 - C)(p_T - C) - \delta C. \end{aligned}$$

We let $Z(t, \epsilon_1, \epsilon_2)$ and $X^*(t, \epsilon_1, \epsilon_2)$ denote the Z and X^* trajectories of system (7) when transformed back to the original coordinates. This system is the same as described in Example 1 of [13]. Since it satisfies all the required conditions, one can use Lemma 2 (Case 1) of [13], which performs a nested application of Tikhonov's singular perturbation Theorem, to determine the reduced order dynamics.

V. INPUT ERROR

To determine the input error $\Delta X_I^* = X_0^*(t) - X_I^*(t)$, the effect of r in the reduced order dynamics of $Z(t)$ will be analyzed. The error produced by r will be written as $\Delta Z(t) := Z(t) - Z_I(t)$, which acts as a disturbance in the dynamics of $X_0^*(t)$ leading to the error $\Delta X_I^*(t)$.

The kinase dynamics evolve in the slowest timescale, thus singular perturbation is performed by setting $\epsilon_1 = 0$ and $\epsilon_2 = 0$. Defining the phosphorylation and dephosphorylation dissociation constants as $k_{d1} = \beta_2/\beta_1$ and $k_{d2} = \alpha_2/\alpha_1$, respectively, and assuming $X^* \ll k_{d2}$, k_d , and $p_T \ll X_T$ (thus working in the linear regime of the Michaelis-Menten functions), the slow manifold is given by $\tilde{X}^* = \psi_x(\tilde{Z}) := \frac{\tilde{Z}X_T k_{d2}}{\tilde{Z}[k_{d2} + (k_x + 1)Y_T] + k_x k_{d1} Y_T}$, $C_2 = \psi_2(\psi_x(\tilde{Z})) := \frac{Y_T}{k_{d2}} \psi_x(\tilde{Z})$, $C_1 = \psi_1(\psi_x(\tilde{Z})) := k_x \frac{Y_T}{k_{d2}} \psi_x(\tilde{Z})$, $C = \psi_c(\psi_x(\tilde{Z})) := \frac{p_T}{k_d} \psi_x(\tilde{Z})$. Variables \tilde{X} and \tilde{Z} denote the approximation of X and Z in system (7) once $\epsilon_1 = 0$ and $\epsilon_2 = 0$. The

reduced order dynamics of \tilde{Z} are obtained by differentiating the slow variable w with respect to time. We have that $\frac{dw}{dt} = \frac{d\tilde{Z}}{dt} + \frac{dC_1}{dt} = \frac{d\tilde{Z}}{dt} + \frac{d\psi_1}{d\psi_x} \frac{d\psi_x}{d\tilde{Z}} \frac{d\tilde{Z}}{dt}$, so that $\frac{d\tilde{Z}}{dt} = \frac{1}{1 + \frac{d\psi_1}{d\psi_x} \frac{d\psi_x}{d\tilde{Z}}} \frac{dw}{dt}$, which, employing the first equation in (7), can be expanded as

$$\frac{d\tilde{Z}}{dt} = (1 - R_z(\tilde{Z}))f_z(\tilde{Z}, k(t)), \quad (8)$$

where

$$R_z(\tilde{Z}) := \frac{k_{d1}X_T}{\left\{ \tilde{Z} \left[\frac{k_{d2}}{Y_T k_x} + \frac{(k_x+1)}{k_x} \right] + k_{d1} \right\}^2 + k_{d1}X_T}, \quad (9)$$

$$f_z(\tilde{Z}, k(t)) := k(t) - \delta \tilde{Z}.$$

By proof of Case(1) in Lemma 2 of [13], one has that $|\tilde{Z}(t) - Z(t, \epsilon_1, \epsilon_2)| = O(\epsilon_1) + O(\epsilon_2/\epsilon_1)$, so for $\epsilon_1, \epsilon_2 \rightarrow 0$, $\tilde{Z}(t)$ will be taken as a good approximation of $Z(t, \epsilon_1, \epsilon_2)$, and be denoted by $Z(t)$ with abuse of notation.

A. Bound for $\Delta Z(t)$

From (8), it is notable that the reduced input dynamics have the form of a nominal contracting system with an additive disturbance. The nominal or isolated system is given by setting $R_z = 0$ in (8), that is,

$$\frac{dZ_I}{dt} = f_z(Z_I, k(t)). \quad (10)$$

The connected or perturbed Z dynamics are given by

$$\frac{dZ}{dt} = f_z(Z, k(t)) + h_z(Z, k(t)), \quad (11)$$

where the expression $h_z(Z, k(t))$ has been defined as

$$h_z(Z, k(t)) := -R_z(Z)f_z(Z, k(t)). \quad (12)$$

In order to apply the robustness result given in Lemma 1 to find a bound on ΔZ , we first need a bound on the perturbation $h_z(Z, k(t))$.

Claim 1. Define $\bar{k} := \max_{t \geq 0} |k(t)|$ and assume that $Y_T > \max \left\{ \frac{2\bar{k}k_{d2}/k_x}{\delta k_{d1}/2 - 2\bar{k}(k_x+1)}, \frac{\bar{k}k_{d2}/\delta}{k_x k_{d1} - \bar{k}(k_x+1)/\delta} \right\}$ and $k_{d1} > \max \left\{ \frac{4\bar{k}(k_x+1)}{\delta k_x}, \frac{\bar{k}(k_x+1)}{\delta k_x} \right\}$.

Also let $z = \gamma_z(k)$ denote the globally unique solution of $f_z(z, k) = 0$ and define $V_z := \max_{t \geq 0} |\dot{k}(t)|$. Then, the upper bound on $h_z(Z, k(t))$ is given by

$$|h_z(Z, k(t))| \leq C_0^z e^{-G_z t} + C_1^z, \quad (13)$$

where $C_0^z := \delta \left(\frac{X_T}{X_T + k_{d1}} \right) \gamma_0^z$ and $C_1^z := \left(\frac{X_T}{X_T + k_{d1}} \right) \frac{V_z}{G_z}$, defining $\gamma_z^0 := |Z(0) - \gamma_z(Z(0))|$ and $G_z := \frac{\delta k_{d1} X_T / 2 + \delta k_{d1}^2}{(k_{d1} + X_T)^2}$.

Proof. See Appendix A-1. \square

Claim 2. Let $\lambda_z = \delta$ be the contraction rate of system (10) and $\Delta Z_0 = |Z(0) - Z_I(0)|$, then we have

$$|\Delta Z(t)| \leq \Delta Z_0 e^{-\lambda_z t} + \frac{C_0^z}{\lambda_z - G_z} e^{-G_z t} + \frac{C_1^z}{\lambda_z}. \quad (14)$$

Proof. In order to apply Lemma 1 to system (10) - (11), the contraction rate λ_z of the isolated system (10) is obtained.

This is a positive number such that $m_2 \left(\frac{\partial f_z(Z_I, k(t))}{\partial Z_I} \right) \leq -\lambda_z$, and it is given by $\lambda_z = \delta$.

From Claim 1, since (13) satisfies Proposition 1, we have (14). \square

The assumptions on Claim 1 are satisfied for Y_T and k_{d1} sufficiently large. Also, after a transient, the input error is bounded by

$$\lim_{t \rightarrow +\infty} |\Delta Z(t)| \leq \frac{C_1^z}{\lambda_z} = \frac{X_T(k_{d1} + X_T) \frac{V_z}{\delta}}{\delta k_{d1} \left(\frac{X_T}{2} + k_{d1} \right)} =: \Delta Z_\infty. \quad (15)$$

Taking the derivative with respect to X_T , one has $\frac{\partial \Delta Z_\infty}{\partial X_T} = \frac{V_z}{\delta^2 k_{d1}} \frac{X_T^2 / 2 + 2X_T k_{d1} + k_{d1}^2}{(X_T / 2 + k_{d1})^2} > 0$, for all X_T . Therefore, as X_T increases the error ΔZ_∞ increases.

B. Bound for $\Delta X_I^*(t)$

The activated substrate dynamics X^* evolve in the intermediate timescale. The singular perturbation analysis for the intermediate timescale is performed by setting only $\epsilon_2 = 0$ in (7). Let $C = \gamma_c(\hat{X}^*) := \frac{p_T}{k_d} \hat{X}^*$, $C_2 = \gamma_2(\hat{X}^*) := \frac{Y_T}{k_{d2}} \hat{X}^*$, $C_1 = \gamma_1(\hat{Z}, \hat{X}^*) := \frac{\hat{Z}}{\hat{Z} + k_{d1}} (X_T - \hat{X}^* - \gamma_2(\hat{X}^*))$. Variables \hat{X}^* and \hat{Z} denote the dynamics of X and Z in the intermediate timescale. The reduced order dynamics of \hat{X}^* are now obtained by differentiating the slow variable y with respect to time and employing the second equation of (7): $\frac{dy}{dt} = \frac{d\hat{X}^*}{dt} + \frac{dC_2}{dt} + \frac{dC}{dt}$, then $\frac{dy}{dt} = \frac{d\hat{X}^*}{dt} + \frac{\partial \gamma_2}{\partial \hat{X}^*} \frac{d\hat{X}^*}{dt} + \frac{\partial \gamma_c}{\partial \hat{X}^*} \frac{d\hat{X}^*}{dt}$, yielding $\frac{d\hat{X}^*}{dt} = \frac{1}{1 + \frac{\partial \gamma_2}{\partial \hat{X}^*} + \frac{\partial \gamma_c}{\partial \hat{X}^*}} (k_1 \gamma_1 - k_2 \gamma_2)$. Further assuming $Z \ll k_{d1}$, and defining $k'_1 := k_1/k_{d1}$ and $k'_2 := k_2/k_{d2}$, we can write

$$\frac{d\hat{X}^*}{dt} = (1 - R_x) f_x(\hat{X}^*, \hat{Z}), \quad (16)$$

where

$$R_x = \frac{p_T/k_d}{p_T/k_d + 1 + Y_T/k_{d2}}, \quad (17)$$

$$f_x(\hat{X}^*, \hat{Z}) = \frac{k'_1 X_T \hat{Z} \left(1 - \frac{\hat{X}^*}{X_T} - \frac{Y_T}{k_{d2}} \frac{\hat{X}^*}{X_T} \right) - k'_2 Y_T \hat{X}^*}{1 + Y_T/k_{d2}}.$$

The reduced order dynamics of \hat{Z} can be obtained by differentiating the slow variable w with respect to time, thus by proof of Case(1) in Lemma 2 of [13], one has that $|\hat{Z}(t, \epsilon_1) - Z(t, \epsilon_1, \epsilon_2)| = O(\epsilon_2/\epsilon_1)$, and $|\hat{X}^*(t, \epsilon_1) - X^*(t, \epsilon_1, \epsilon_2)| = O(\epsilon_2/\epsilon_1)$.

Here, $\hat{X}^*(t, \epsilon_1)$ will be taken as a good approximation of $X^*(t, \epsilon_1, \epsilon_2)$ and we denote it by $X^*(t)$ with abuse of notation. Also, since $\hat{Z}(t, \epsilon_1)$ and $\tilde{Z}(t)$ are both good approximations of $Z(t, \epsilon_1, \epsilon_2)$, we will use $\tilde{Z}(t)$ in (16), given in (8), as a good approximation of $\hat{Z}(t)$.

From (16), it is notable that the reduced input dynamics have the form of a nominal system with an additive disturbance. The nominal or isolated X^* dynamics are given by setting the retroactivity term $R_x = 0$ ($p_T = 0$) in (16) and using (10), that is,

$$\frac{dX_I^*}{dt} = f_x(X_I^*, Z_I). \quad (18)$$

The dynamics of X_0^* can now be treated as the perturbed version of (18) with an input $Z(t) = Z_I(t) + \Delta Z(t)$, where $\Delta Z(t)$ satisfies (14):

$$\frac{dX_0^*}{dt} = f_x(X_0^*, Z_I) + h_{\bar{x}}(X_0^*, \Delta Z) \quad (19)$$

and $h_{\bar{x}}(X_0^*, \Delta Z)$ is defined as $h_{\bar{x}}(X_0^*, \Delta Z) := \frac{k'_1 X_T \Delta Z \left(1 - \frac{X_0^*}{X_T} - \frac{Y_T}{k_{d2}} \frac{X_0^*}{X_T}\right)}{1 + Y_T/k_{d2}}$.

Claim 3. Having $\Delta X_{I0}^* = |X_0^*(0) - X_I^*(0)|$, the input error satisfies

$$|\Delta X_I^*(t)| \leq \Delta X_{I0}^* e^{-\lambda_x t} + \frac{C_0^T}{\lambda_x - \lambda_z} e^{-\lambda_z t} + \frac{C_1^T}{\lambda_x - G_z} e^{-G_z t} + \frac{C_2^T}{\lambda_x}, \quad (20)$$

where $C_0^T := \left(\frac{k'_1 X_T}{1 + Y_T/k_{d2}}\right) \Delta Z_0$, $C_1^T := \left(\frac{k'_1 X_T}{1 + Y_T/k_{d2}}\right) \frac{C_0^z}{\lambda_z - G_z}$, $C_2^T := \left(\frac{k'_1 X_T}{1 + Y_T/k_{d2}}\right) \frac{C_1^z}{\lambda_z}$, and $\lambda_x := \frac{k'_2 Y_T}{1 + Y_T/k_{d2}}$.

Proof. Recalling $\Delta Z(t)$ from (14), the disturbance $h_{\bar{x}}(X_0^*, \Delta Z)$ satisfies $|h_{\bar{x}}(X_0^*, \Delta Z)| \leq \frac{k'_1 X_T}{1 + Y_T/k_{d2}} \left(\Delta Z_0 e^{-\lambda_z t} + \frac{C_0^z}{\lambda_z - G_z} e^{-G_z t} + \frac{C_1^z}{\lambda_z}\right)$. The contraction rate of the isolated system (18) is found as a positive λ_x such that $m_2 \left(\frac{\partial f_x(X_I^*, Z_I)}{\partial X_I^*}\right) \leq -\lambda_x$, for all X_I^* , and Z_I , which results in $\lambda_x = \frac{k'_2 Y_T}{1 + Y_T/k_{d2}}$. Recalling the constants defined in Claim 3 and using Proposition 1 yields (20). \square

The steady state input error can be found as

$$\lim_{t \rightarrow +\infty} |\Delta X_I^*(t)| \leq \frac{C_2^T}{\lambda_x} = \frac{\Delta Z_\infty}{\rho k'_2/k'_1} =: \Delta X_{I\infty}^*, \quad (21)$$

which increases as X_T increases. This behavior is captured by Fig. 4, where the steady state error is also shown as obtained from simulation with a periodic input $k(t)$. One can also see that it lies below the calculated upper bound (15) for all values of X_T .

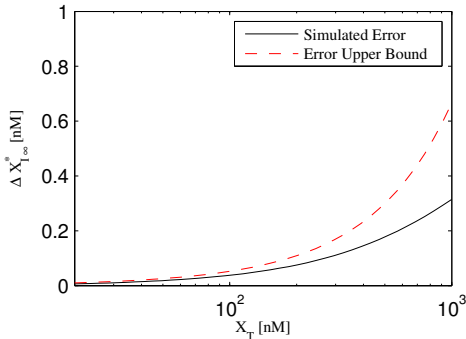


Fig. 4. Simulated input error from model (18) - (19) and estimated error upper bound from (21).

VI. OUTPUT ERROR

The dynamics of X^* , subject to both retroactivity effects r and s , will be compared to the dynamics of X_0^* which

only subject to the retroactivity effect r in order to obtain the output error ΔX_0^* . The dynamics of X^* are given by

$$\frac{dX^*}{dt} = f_x(X^*, Z) + h_x(X^*, Z), \quad (22)$$

where the expression $h_x(X^*, Z)$ has been defined as

$$h_x(X^*, Z) := -R_x f_x(X^*, Z). \quad (23)$$

Equation (22) will be treated as the perturbed version of the $X_0^*(t)$ dynamics given in (19). In order to apply the robustness result given in Lemma 1 to find a bound on ΔX_0^* , we first need a bound on the perturbation $h_x(X^*, Z)$.

Claim 4. The disturbance $h_x(X^*, Z)$ satisfies

$$|h_x(X^*, Z)| \leq C_0^x e^{-G_x t} + C_1^x, \quad (24)$$

where $C_0^x := \left\{ \frac{p_T}{k_d} \left[k'_1 W_x (1 + \frac{Y_T}{k_{d2}}) + k'_2 Y_T \right] \right\} \gamma_x^0$ and $C_1^x := \left\{ \frac{p_T}{k_d} \left[k'_1 W_x (1 + \frac{Y_T}{k_{d2}}) + k'_2 Y_T \right] \right\} \frac{V_x}{G_x K_x \rho}$, defining $\gamma_x^0 := |X(0) - \gamma_x(Z(0))|$, $W_x := \gamma_z(\bar{k}) + \gamma_z^0 + \frac{V_z}{G_z \delta}$, $G_x := \frac{k'_2 Y_T}{1 + Y_T/k_{d2} + p_T/k_d}$, $K_x := \frac{k'_2/k_{d2}}{k_1/k_{d1}}$, and $V_x := \delta |Z(0) - \gamma_z(k(0))| + \frac{V_z}{G_z}$.

Proof. See Appendix A-3. \square

Claim 5. Having λ_x as the contraction rate of (18) and $X_0 := |X^*(0) - X_0^*(0)|$, $|\Delta X_0^*(t)|$ satisfies

$$|\Delta X_0^*(t)| \leq X_0 e^{-\lambda_x t} + \frac{C_0^x}{\lambda_x - G_x} e^{-G_x t} + \frac{C_1^x}{\lambda_x}. \quad (25)$$

Proof. Recalling λ_x from Claim 3, one can apply Proposition 1 to get (25). \square

Assuming $Z(0) = \gamma_z(k(0))$, after a transient the output error is bounded by $\lim_{t \rightarrow +\infty} |\Delta X^*(t)| \leq \frac{C_1^x}{\lambda_x} =: \Delta X_{0\infty}^*$, where

$$\Delta X_{0\infty}^* = m_x \left[\frac{(k_{d1} \rho + Y_T)^2 2k_1 V_z (k_{d1} \rho)^2}{(2k_{d1} \rho + Y_T)^2} + \frac{\delta^2 k_{d1} \rho [k_2 k_{d1} Y_T + \gamma_z k_1 (k_{d2} + Y_T)]}{Y_T^2 (k_{d2} + Y_T) (2k_{d1} \rho + Y_T)} \right], \quad (26)$$

$$m_x = \frac{2k_{d2}^2 p_T V_z}{\delta^3 k_2^2 k_d k_{d1}^2 K_x \rho^2}.$$

Differentiating with respect to Y_T , one has

$$\frac{\partial \Delta X_{0\infty}^*}{\partial Y_T} = -m_x \left[\frac{(k_{d1} + Y_T) [8k_1 k_{d1} \rho V_z (k_{d1} \rho + Y_T)^2]}{Y_T^3 (2k_{d1} \rho + Y_T)^3} + \frac{\delta^2 \gamma_z k_1 (4k_{d1}^2 \rho^2 + 3k_{d1} \rho Y_T + Y_T^2)}{Y_T^3 (2k_{d1} \rho + Y_T)^2} + \frac{\delta^2 k_2 k_{d1} [Y_T^2 3k_{d1} \rho + Y_T^3 + 2k_{d1} \rho^2 (k_{d2} + 2Y_T)]}{Y_T^2 (k_{d2} + Y_T) (2k_{d1} \rho + Y_T)^2} \right]$$

making $\frac{\partial \Delta X_{0\infty}^*}{\partial Y_T} = \rho \frac{\partial \Delta X_{0\infty}^*}{\partial Y_T} < 0$. Thus, the error decreases as X_T is increased. Fig. 5 captures this behavior. As it is expected, the calculated upper bound lies above the system error simulation for all values of X_T . Furthermore, without downstream clients ($p_T = 0$), $\Delta X_{0\infty}^* = 0$ since the error

only accounts for the retroactivity to the output. In (26), since $Y_T = X_T \rho$, $\Delta X_{0\infty}^*$ converges to a value different from zero as $X_T \rightarrow \infty$. This is due to the presence of the phosphatase term Y_T/k_{d2} in the R_x expression, which accounts for the phosphatase also placing a load on the output protein X^* . This is different from [3], where the phosphatase term was not accounted for in the retroactivity expression.

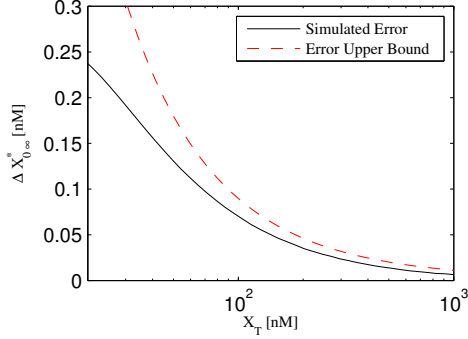


Fig. 5. Simulated output error from model (19)–(22), and estimated error upper bound from (26).

VII. TOTAL OUTPUT ERROR

The total output error can be upper bounded using the triangle inequality $|\Delta X_{TOT}(t)| \leq |\Delta X_0^*(t)| + |\Delta X_T^*(t)|$. Assuming the initial conditions are the same for connected and isolated systems, and having $A(X_T) = \max\left\{\frac{C_0^x}{\lambda_x - G_x}, \frac{C_0^T}{\lambda_x - \lambda_z}, \frac{C_1^T}{\lambda_x - G_z}\right\}$, $\lambda(X_T) = \min\{G_x, \lambda_z, G_z\}$, and $B(X_T) = \Delta X_{I\infty}^* + \Delta X_{0\infty}^*$, the total error takes the form $|\Delta X_{TOT}(t)| \leq A(X_T)e^{-\lambda(X_T)t} + B(X_T)$. Evaluating the limit at infinite time we have,

$$\lim_{t \rightarrow +\infty} |\Delta X_{TOT}(t)| \leq \Delta X_{I\infty}^* + \Delta X_{0\infty}^*. \quad (27)$$

The optimal selection of X_T , which minimizes both the effects of r and s on signal $X^*(t)$, is given by the minimum of the above expression, which is shown in Fig. 6. The first term $\Delta X_{I\infty}^*$, defined in (21), increases with X_T since it comes from the input error, while the second term, defined in (26), decrease as X_T increases since it comes from the output error. This illustrates a tradeoff between the input and output errors. This expression also predicts no error for constant inputs, meaning $V_z = 0$, making retroactivity for the presented model a purely dynamical effect. A final remark on Fig. 6 is that the bound is tight about the minimum.

Note that if the calculated bounds $\Delta X_{I\infty}^*$ and $\Delta X_{0\infty}^*$ fall in the order of ϵ_2/ϵ_1 , then the approximation error due to singular perturbation is not negligible anymore and should be accounted for in the calculations of the error bounds.

VIII. CONCLUSIONS AND FUTURE WORK

This work presents the application of tools from nonlinear systems analysis such as contraction theory and singular perturbation to analyze the tradeoff between input and output retroactivity of a phosphorylation-based insulation device. The analysis shows that while increasing the substrate concentration of the phosphorylation cycle reduces the effect of

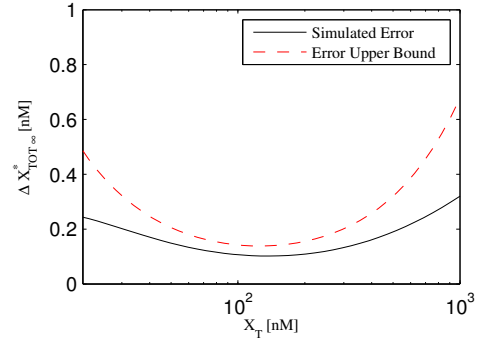


Fig. 6. Simulated input error from model (22) and (19), with no retroactivity to the input, and estimated error upper bound from (27).

retroactivity to the output, the cycle becomes a load itself to the upstream system. The error produced by the retroactivity to the input is then propagated to the output, attenuating the output signal. Thus, an optimal substrate concentration was found to be the minimum of a weighted sum of the input and output errors. The results from this work can be used to estimate the minimum upper bound on the error given by the insulation device. This work will be extended to generalized interconnection structures, providing a methodology for the combined minimization of input and output retroactivities.

REFERENCES

- [1] U. Alon. An introduction to systems biology, design principles of biological circuits. *CRC Press*, 2006.
- [2] J. Saez-Rodriguez, S. Gayer, M. Ginkel, and E. Dieter Gilles. Automatic decomposition of kinetic models of signaling networks minimizing the retroactivity among modules. *Bioinformatics*, 24:i213–i219, 2008.
- [3] D. Del Vecchio, A. J. Ninfa, and E. D. Sontag. Modular cell biology: retroactivity and insulation. *Molecular Systems Biology*, (4):1–16, 2008.
- [4] S. Jayanthi, K. Soli Nilgiriwala, and D. Del Vecchio. Retroactivity controls the temporal dynamics of gene transcription. *ACS Synthetic Biology*, 2013.
- [5] K. H. Kim and H. M. Sauro. Fan-out in gene regulatory networks. *Journal of Biological Engineering*, 4(16):1–14, 2010.
- [6] H. M. Sauro and B. Ingalls. Mapk cascades as feedback amplifiers. *Tech. Rep.*, <http://arxiv.org/abs/0710.5195>, 2007.
- [7] P. Jiang, A. C. Ventura, E. D. Sontag, S. D. Merajver, A. J. Ninfa, and D. Del Vecchio. Load-induced modulation of signal transduction networks. *Sci. Signal*, 4(194):ra67, 2011.
- [8] E. Franco, E. Friedrichs, R. Jungmann J. Kim, R. Murray, E. Winfree, and F. C. Simmel. Timing molecular motion and production with a synthetic transcriptional clock. *Proceedings of the National Academy of Science*, 108(40):E784–E793, 2011.
- [9] W. Lohmiller and J. J. E. Slotine. On contraction analysis for nonlinear systems. *Automatica*, 34(6):683–696, 1998.
- [10] D. Del Vecchio and J.J. Slotine. A contraction theory approach to singularly perturbed systems. *IEEE Trans. Aut. Control*, 58(3):752–757, 2013.
- [11] E. Klipp, W. Liebermeister, C. Wierling, A. Kowald, H. Lehrach, and R. Herwig. *Systems biology*. Wiley VCH, 2009.
- [12] W. Chen, M. Niepel, and P. Sorger. Classic and contemporary approaches to modeling biochemical reactions. *Cold Spring Harbor Laboratory Press*, pages 1861–1875, 2010.
- [13] S. Jayanthi and D. Del Vecchio. Retroactivity attenuation in biomolecular systems based on timescale separation. *IEEE Transactions on Automatic Control*, 56(4):748–761, 2011.
- [14] H. K. Khalil. *Nonlinear systems* (3rd edition). Prentice Hall, 2002.
- [15] J. E. Marsden and M. J. Hoffman. *Elementary classical analysis*. W. H Freeman and Company, 1993.

APPENDIX

A-1 Consider the system

$$\frac{dZ}{dt} = (1 - R_z(Z))f_z(Z, k(t)) =: g_z(Z, k(t)). \quad (28)$$

The upper bound on $h_z(Z, k(t))$ from (12) is determined by bounding Z using Lemma 2, then using Lipschitz continuity. Let Z_s be the solution of $g_z(Z_s, k(t)) = 0$, which is given by $Z_s = \gamma_z(k(t)) = k(t)/\delta$. To apply Lemma 2 we need: the bound \bar{d} in (3) such that $\left| \frac{\partial \gamma_z(k)}{\partial k} \dot{k}(t) \right| < \bar{d}$, and the contraction rate of (28). The bound can be given by

$$\left| \frac{\partial \gamma_z(k)}{\partial k} \dot{k}(t) \right| \leq V_z/\delta. \quad (29)$$

The contraction rate G_z of (28) is a positive number such that $m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right) \leq -G_z$, for all Z , and for all $k(t)$. We have shown in Appendix A-2 that $m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right) \leq m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right)\Big|_{Z=0}$, making

$$m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right) \leq \frac{X_T \{2\bar{k}[\frac{k_{d2} + Y_T(k_x + 1)}{Y_T k_x} - \delta k_{d1}] - \delta k_{d1}^2\}}{(k_{d1} + X_T)^2} \Big|_{Z=0}.$$

Selecting a Y_T as in the assumption of Claim 1, the X_T factor is smaller than $-\delta k_{d1} X_T/2$, so the contraction rate can be set to

$$G_z := \frac{\delta k_{d1} X_T/2 + \delta k_{d1}^2}{(k_{d1} + X_T)^2}. \quad (30)$$

Now Lemma 2 can be applied directly using (29) and (30). Letting $\epsilon_z(t) := Z(t) - \gamma_z(k(t))$, it follows from Lemma 2 that $|\epsilon_z(t)| \leq e^{-G_z t} |Z(0) - \gamma_z(k(0))| + \frac{V_z}{G_z \delta}$. Finally, recalling (12), one has $|h_z(Z, k(t))| \leq |R_z(Z)| |f_z(Z, k(t))|$. From (9), since $\frac{\partial R_z}{\partial Z} < 0$, it follows that

$$|R_z(Z)| \leq \frac{X_T}{X_T + k_{d1}}. \quad (31)$$

One has that $|f_z(Z, k(t))| = |f_z(\gamma_z(k(t)) + \epsilon_z(t), k(t))| \leq |f_z(\gamma_z(k(t)), k(t))| + |\Delta_z(t)|$, in which $\Delta_z(t) := f_z(\gamma_z(t) + \epsilon_z(t), k(t)) - f_z(\gamma_z(k(t)), k(t))$. Since $f_z(Z, k(t))$ is Lipschitz continuous in Z with constant $\alpha_z = \delta$, it follows that $|\Delta_z(t)| \leq \alpha_z \epsilon_z(t)$. Also $f_z(\gamma_z(k(t)), k(t)) = 0$, so that

$$|f_z(Z, k(t))| \leq \alpha_z \epsilon_z(t). \quad (32)$$

From (31) and (32) we have $|h_z(Z, k(t))| \leq \frac{X_T (\delta |Z(0) - \gamma_z(k(0))| e^{-G_z t} + \frac{V_z}{G_z})}{X_T + k_{d1}}$. Recalling constants C_0^z and C_1^z in Claim 1, we have $|h_z(Z, k(t))| \leq C_0^z e^{-G_z t} + C_1^z$.

A-2 Claim: From system (28), $m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right) \leq m_2\left(\frac{\partial g_z(Z, k(t))}{\partial Z}\right)\Big|_{Z=0}$.

Proof. From (9) we see that $(1 - R_z) \frac{\partial f_z}{\partial Z} \leq (1 - R_z) \frac{\partial f_z}{\partial Z} \Big|_{Z=0} \leq 0$, since $\frac{\partial(1-R_z)}{\partial Z} \geq 0$ and $\frac{\partial f_z}{\partial Z} < 0$.

Thus it suffices to prove that $\frac{\partial}{\partial Z} [f_z \frac{\partial(1-R_z)}{\partial Z}] < 0$. Defining $a = k_{d1} X_T (Y_T k_x / k_{d2})^2$, $b = [1 + Y_T(k_x + 1)/k_{d2}]$, and $c = Y_T k_x k_{d1} / k_{d2}$ we have that

$$\begin{aligned} \frac{\partial}{\partial Z} \left[f_z \frac{\partial(1-R_z)}{\partial Z} \right] &= - \frac{2ab(c + bZ)^2 (c\delta + 3b\bar{k} - 2b\delta Z)}{[a + (c + bZ)^2]^3} \\ &\quad - \frac{a(c\delta - b\bar{k} + 2b\delta Z)}{[a + (c + bZ)^2]^3} \end{aligned}$$

which is always negative if $c > b\bar{k}/\delta$ as in the assumptions of Claim 1 and noting that $c^2 < a$. \square

A-3 Consider the system

$$\frac{dX^*}{dt} = (1 - R_x) f_x(X^*, Z) =: g_x(X^*, Z), \quad (33)$$

The upper bound on $h_x(X^*, Z)$ from (23) can be obtained by bounding X^* using Lemma 2 and using Lipschitz continuity. and let X_s be the globally unique solution of $g_x(X_s, Z) = 0$ and denote it $X_s = \gamma_x(Z)$. In order to apply Lemma 2, we need: the bound \bar{d} in (3) given by $\left| \frac{\partial \gamma_x(Z)}{\partial Z} \frac{dZ}{dt} \right| < \bar{d}$ and the contraction rate of (33). To obtain bound \bar{d} , it was shown in Appendix A-4 that $\left| \frac{\partial \gamma_x(Z)}{\partial Z} \right| \leq \frac{1}{K_x \rho}$, also that the bound on $\left| \frac{dZ}{dt} \right| \leq V_x$ where $V_x := \delta |Z(0) - \gamma_x(k(0))| + \frac{V_z}{G_z}$. Thus $\left| \frac{d\gamma_x(Z)}{dt} \right| \leq \left| \frac{\partial \gamma_x(Z)}{\partial Z} \right| \left| \frac{dZ}{dt} \right| \leq \frac{V_x}{K_x \rho}$. To complete Lemma 2, the contraction rate G_x of (33) is determined as a positive number such that $m_2\left(\frac{\partial g_x(X^*, Z)}{\partial X^*}\right) \leq -G_x$, for all X^* and for all Z . One has $m_2\left(\frac{\partial g_x(X^*, Z)}{\partial X^*}\right) \leq -\frac{k_2' Y_T}{1 + Y_T/k_{d2} + p_T/k_d}$. Thus, G_x can be defined as $G_x := \frac{k_2' Y_T}{1 + Y_T/k_{d2} + p_T/k_d}$. Letting $\epsilon_x(t) := X^*(t) - \gamma_x(Z(t))$ and using Lemma 2, one has

$$|\epsilon_x(t)| \leq e^{-G_x t} |X(0) - \gamma_x(Z(0))| + \frac{V_x}{G_x K_x \rho}. \quad (34)$$

Now, recalling (23) one has $h_x(X^*, Z) := -R_x f_x(X^*, Z)$, so that $|h_x(X^*, Z)| \leq |R_x| |f_x(X^*, Z)|$. Given that $X^* = \gamma_x(Z) + \epsilon_x(t)$, we have that $|f_x(X^*, Z)| = |f_x(\gamma_x(Z) + \epsilon_x(t), Z)| \leq |f_x(\gamma_x(Z), Z)| + |\Delta_x(t)|$, in which $\Delta_x(t) := f_x(\gamma_x(Z) + \epsilon_x(t), Z) - f_x(\gamma_x(Z), Z)$. Recalling W_x from Claim 4, and since $f_x(X^*, Z)$ is Lipschitz continuous with constant $\alpha_x = \frac{k_1' W_x (1 + Y_T/k_{d2}) + k_2' Y_T}{1 + Y_T/k_{d2}}$, one has $|\Delta_x(t)| \leq \alpha_x \epsilon_x(t)$. Also $f_x(\gamma_x(Z), Z) = 0$, so that $|f_x(X^*, Z)| \leq \alpha_x |\epsilon_x(t)|$. It follows that

$$|h_x(X^*, Z)| \leq R_x \alpha_x |\epsilon_x(t)|. \quad (35)$$

Recalling variables C_0^x and C_1^x in Claim 4, it follows from (34) and (35) that $|h_x(X^*, Z)| \leq C_0^x e^{-G_x t} + C_1^x$.

A-4 Claim: From system (33), $\left| \frac{d\gamma_x(Z)}{dt} \right| \leq \frac{V_x}{K_x \rho}$.

Proof. Using the Implicit Function Theorem [15], one has $\frac{\partial \gamma_x(Z)}{\partial Z} = -\frac{\partial g_x}{\partial Z} \left(\frac{\partial g_x}{\partial X^*} \right)^{-1}$. It follows that $\frac{\partial \gamma_x(Z)}{\partial Z} = \frac{k_1' X_T (1 - \frac{\gamma_x}{X_T} - \frac{Y_T}{k_{d2}} \frac{\gamma_x}{X_T})}{k_1' X_T Z (\frac{1}{X_T} + \frac{Y_T}{k_{d2}} \frac{1}{X_T}) + k_2' Y_T}$, thus $\frac{\partial \gamma_x}{\partial Z} < \frac{\gamma_x}{\partial Z} \Big|_{Z=0}$. Recalling K_x in Claim 4 and the definition of ρ , we have that $\frac{\partial \gamma_x}{\partial Z} \leq \frac{1}{K_x \rho}$.

Now, from (11), one has $\left| \frac{dZ}{dt} \right| \leq |1 - R_z| |f_z|$. Using (9), $|1 - R_z| \leq 1$. From (32), $|f_z(Z, k(t))| \leq \alpha_z e^{-G_z t} |Z(0) - \gamma_z(k(0))| + \alpha_z \frac{V_z}{G_z \delta}$. Defining $V_x := \delta |Z(0) - \gamma_z(k(0))| + \frac{V_z}{G_z}$, the upper bound on the time derivative of $\gamma_x(Z)$ is given by

$$\left| \frac{d\gamma_x(Z)}{dt} \right| \leq \left| \frac{\partial \gamma_x(Z)}{\partial Z} \right| \left| \frac{dZ}{dt} \right| \leq \frac{V_x}{K_x \rho}. \quad \square$$