Quantifying Crosstalk in Biochemical Systems

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***Abstract*— Recent work has introduced biocircuit architectures that exhibit robust oscillatory behavior in organisms ranging from cyanobacteria to mammals. Complementary research in synthetic biology has introduced oscillators *in vivo* and *in vitro* suggesting that robust oscillation can be recapitulated using a small number of biochemical components. In this work, we introduce signaling crosstalk in biocircuits as a consequence of enzyme-mediated biochemical reactions. As a motivating example, we consider an *in vitro* oscillator with two types of crosstalk: crosstalk in production and degradation of RNA signals. We then pose a framework for quantifying crosstalk and use it to derive several dynamical constraints and suggest design techniques for ameliorating crosstalk in *in vitro* biochemical systems. We demonstrate that the effects of crosstalk can be attenuated through the effective tuning of two key parameters in order to recover desired system dynamics. As an example, we show that by changing the balance between production and degradation crosstalk, we can tune a system to be stable or exhibit oscillatory behavior.**

I. Introduction

High-throughput technologies have led to the era of systems biology as an integrative approach to connect molecular-level mechanisms to cell-level behavior [1]. While the systems biology approach generated complex wiring diagrams for analyzing biological systems on a large scale, a complementary approach in synthetic biology focused on creating networks to elucidate design principles to achieve desired functions [2]. However, it has often been overlooked that biological systems are under continuous selection pressure to maximize performance while minimizing cost, i.e., maintaining only the minimal amount of critical machinery [3], [4]. Consequently, there exists a potential to overload important pathways during times of stress and to place unanticipated burden on central cellular enzymes [5], [6]. Therefore, it is critical to have a detailed understanding of the underlying molecular mechanisms that capture the loading effects on different components — indeed, such understanding is a necessary first step in further development of systems and synthetic biology.

A biocircuit, synthetic or natural, can be conceptually divided into two (intertwined) layers — a layer that constitutes molecular architecture reflecting designed or known interactions and a layer that consists of uncertain or hidden interactions, referred to as crosstalk, arising from either background processes or byproducts of circuit processes. This crosstalk layer is not easily avoided and often is fundamental to circuit operation [7], yet only a few studies have examined how to overcome crosstalk [8] or to exploit it as a mechanism for tuning circuit performance [9].

In engineering, crosstalk is traditionally viewed as the interaction between two signal relays. Old electronic circuits suffered various problematic effects such as ringing, crosstalk, and noise. Thus, essentially all modern circuits must be designed with signal integrity in mind [10]. However, in biology, crosstalk seems to be a ubiquitous feature that can take multiple forms, including hidden reactions [11], non-specific binding [12], waste management [13], and enzyme loading [5] possibly with different types of impact on system dynamics. In this work, we consider crosstalk arising from enzyme loading, i.e., the saturation effects that occur from substrates competing for the same enzyme.

We organize this paper as follows: In Section II we consider the simplified oscillator model for a circadian clock proposed by Barkai and Leibler which is robust to stochastic noise in simulation studies [14], [15] — unlike other circadian clock models [16]. We introduce an *in vitro* model reconstructing the Barkai-Leibler oscillator and analyze the impact of the saturation effects of competing substrates for production and degradation enzymes. We show how these competitive effects lead to undesired interactions between system components — a dynamical effect we call crosstalk. In Section III we introduce the mathematical background for studying crosstalk in biochemical systems and propose a measure for quantifying crosstalk between components and for the system as a whole. Finally, in Section IV we introduce conceptual approaches towards designing systems that are robust to crosstalk. We discuss two ways to ameliorate crosstalk in the context of robust biochemical system design and illustrate how these techniques can be used to recover oscillations in the Barkai-Leibler oscillator.

II. Motivating Example

A. In Vitro Barkai-Leibler Oscillator

The model for circadian clock proposed by Barkai and Leibler take the form of Figure 1 inset [14], [15]. It involves two genes, D_A and D_R, coding for an activator A and a repressor R, which are transcribed into mRNA and subsequently translated into protein. The activator A binds to the promoters for genes D_A and D_R to increase their transcription rates, while R acts as the negative element by sequestering the activator A. It is noteworthy that the oscillation did not rely on the cooperative binding of A to the promoters of genes or cooperative binding of R to A; the activation mechanism was simple binding and the repression mechanism is a stochiometric sequestering. On the other hand, a key requirement for oscillation was the time-scale separation for A and R dynamics — production

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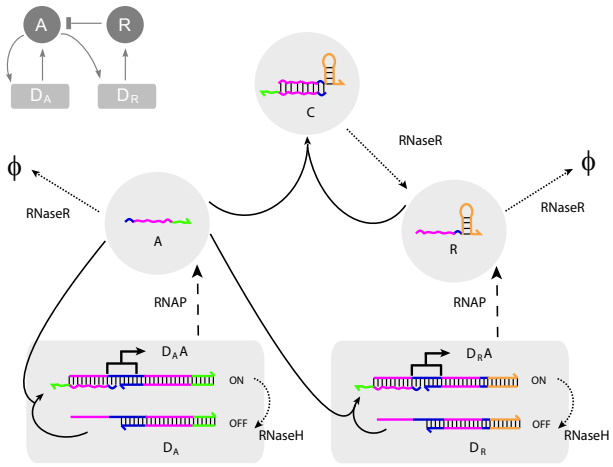

Fig. 1. A schematic diagram of the Barkai-Leibler oscillator (top left) and the detailed molecular reactions for an *in vitro* genelet oscillator. There are two synthetic templates, D_A and D_R, producing an activator A and a repressor R using RNAP. The activator A binds to the promoter sites of the two templates to enhance transcription rates, while the repressor R sequesters A into a complex C. The RNA signals A, R, and C are degraded selectively by RNase R, while A bound to DNA templates are degraded by RNase H. The sequence domains are color-coded to indicate identical or complementary sequences.

and degradation of A were on a much faster time scale than those of R.

Here, we propose a model that captures the essential features of the Barkai-Leibler oscillator in a simplified *in vitro* setting (Figure 1). We do so for three reasons: 1) to introduce a novel synthetic *in vitro* switch design, 2) to lay a groundwork for general circuit construction methods, and 3) to propose a model of an *in vitro* system without crosstalk and examine the effects of adding crosstalk on the system's oscillatory performance. Finally, our results in Section IV also suggest approaches for tuning crosstalk and thus, actual experiments to validate model predictions.

The circuit is constructed as follows: the OFF state of the switch consists of a double-stranded (ds) DNA template ("D'') with a partially single-stranded (ss) (and thus incomplete) T7 RNA polymerase (RNAP) promoter region. The switch is turned ON by the binding of ssDNA or ssRNA activator ("A'') that completes the RNAP promoter region. The resulting template ("DA'') has a nicked promoter but still transcribes well [13]. (Experimental characterization of genelets used DNA activator [13]; RNA activator would likely be less efficient than its DNA counterpart [17].) To provide a sharp threshold of activation, an inhibitor strand (ssDNA or ssRNA, "I'') can bind to a complementary free-floating activator A, resulting in a functionally inert activator-inhibitor complex "AI.'' (this reaction is not explicitly implemented for the circuit presented in this paper.) Using these design motifs for switches and signals, networks with arbitrary connectivity can be constructed modularly, in principle, as continuous-time analog neural networks [18]. In a typical reaction network, RNA outputs will be produced by RNAP from upstream switches using NTP as fuel, and these outputs will serve as inputs for downstream switches. At the same time, the degradation of RNA signals by *Escherichia coli* ribonuclease H (RNase H) and/or ribonuclease R (RNase R) removes RNA signal, thereby undoing the regulatory effect of the RNA signals.

The synthetic *in vitro* transcriptional circuits ('genelets') have been used to create bistable circuits, oscillators, and drive other molecular machines using modular architecture with programmable connectivity [13], [19], [20]. Thus, it would be possible to experimentally understand and characterize elementary reactions and system dynamics for our proposed circuit in the near future. A notable difference from the models of Barkai and Leibler is that, for our proposed biochemical circuit, the RNA signals act directly as the activator and repressor of the system, obviating the need for translation machinery. The deterministic dynamics of the model is given by the set of reaction rate equations below.

$$\begin{aligned}
\dot{D}_A^{OFF} &= -k_{ON}AD_A^{OFF} + k_{OFF}D_A^{ON} \\
\dot{D}_R^{OFF} &= -k_{ON}AD_R^{OFF} + k_{OFF}D_R^{ON} \\
\dot{A} &= k_{p,A}D_A^{ON} + \alpha k_{p,A}D_A^{OFF} - k_{ON}AD_A^{OFF} \\
&\quad -k_{ON}AD_R^{OFF} - k_{ANN}AR - \delta_A A \\
\dot{R} &= k_{p,R}D_R^{ON} + \alpha k_{p,R}D_R^{OFF} - k_{ANN}AR \\
&\quad +\delta_A C - \delta_R R \\
\dot{C} &= k_{ANN}AR - \delta_A C
\end{aligned} \tag{1}$$

where D_A and D_R are synthetic DNA templates coding RNA signals A and R, while C is the complex of A and R. The templates D_A and D_R have incomplete promoter regions for RNAP such that the transcription rates are slow. When the RNA signal A binds and completes the incomplete promoter region, the transcription rate increases. Therefore, D_A^{ON} is the ON-state switch, i.e., D_AA complex, while D_A^{OFF} is simply D_A.

To effectively achieve time-scale separation for the dynamics of A and R, $k_{p,A}$ and δ_A should be much larger than $k_{p,R}$ and δ_R, respectively. To adjust production rates, one possibility is tuning the catalytic rate of RNAP for templates by changing the consensus sequence right after the promoter region; a simpler solution would be using high concentration of template D_A coding for A. To adjust degradation rates, we can utilize the property of RNase R that can by itself degrade single-stranded RNAs with extensive secondary structure provided that a single-stranded 3′ overhang more than 4-base long is present [21]. By incorporating extensive secondary structures for 3′ end of R while leaving 3′ end of A structure-free, we can achieve the desired differential degradation for A and R. We observed oscillation with experimentally plausible parameter values (Figure 2).

To gain insight into the essential elements required for oscillations, we simplified the deterministic rate equations by making quasi-steady-state assumptions for templates D_A, D_R, and activator A. The resulting two-variable system with the repressor R and the complex C as the two slow variables

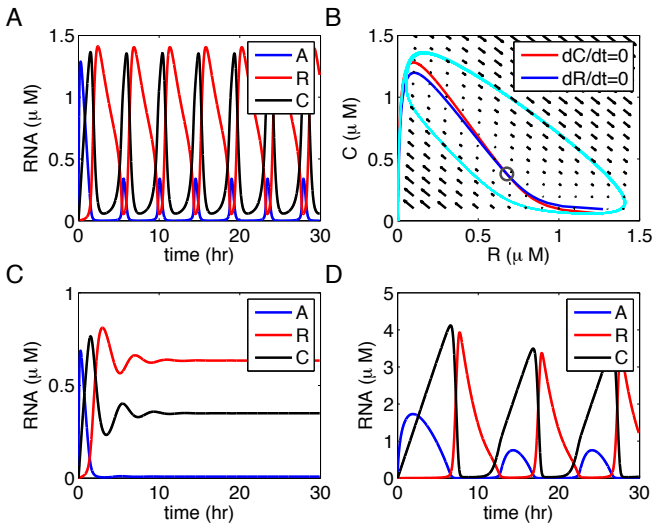

Fig. 2. Dynamics of the synthetic oscillator model. a, b) Time-courses and phase portrait of the first-order model (1), where $k_{on} = 10^5/M/s$, $k_{off} = 0.003/s$, $k_{ANN} = 2 \times 10^5/M/s$, $D_A^{tot} = 200\ nM$, $D_R^{tot} = 100\ nM$, $\delta_A = 0.003/s$, $\delta_R = 10^{-4}/s$, $k_{p,A} = 0.03/s$, $k_{p,R} = 0.003/s$, $\alpha = 0.01$. c) Time-courses of the Michaelis–Menten RNAP model (2) assuming first-order RNase R reactions, where $k_{cat,A,ON} = 0.2/s$, $k_{cat,R,ON} = 0.02/s$, $k_{cat,A,OFF} = 0.02/s$, $k_{cat,R,OFF} = 0.002/s$, $K_{M,A,ON} = K_{M,R,ON} = 300\ nM$, $K_{M,A,OFF} = K_{M,R,OFF} = 3\ \mu M$, $E^{tot} = 55\ nM$. d) Time-courses of the Michaelis–Menten RNAP and RNase R model (2), where $k_{d,cat,A} = 0.2/s$, $K_{d,M,A} = 500\ nM$, $k_{d,cat,R} = 0.066/s$, $K_{d,M,R} = 5\ \mu M$, $E_d^{tot} = 15\ nM$,

is written as follows:

$$\begin{aligned} \dot{R} &= k_{p,R} D_R^{tot} \frac{\alpha k_{off} + k_{on}\tilde{A}(R)}{k_{off} + k_{on}\tilde{A}(R)} \\ &\quad -k_{ANN}\tilde{A}(R)R + \delta_A C - \delta_R R \\ \dot{C} &= k_{ANN}\tilde{A}(R)R - \delta_A C \end{aligned}$$

where D_R^{tot} is the sum of the ON- and OFF-state templates, and $\tilde{A}(R)$ is the quasi-equilibrium value of A by solving $\dot{A} = 0$. The trajectories of the full model with respect to the solution $\tilde{A}(R) = f(R)$ result in small differences because the time-scale separation is not perfect. We note that the two-dimensional model would remain a good approximation of the full model for parameter choices where the dynamics of A become faster with respect to the dynamics of R.

We demonstrate the following properties of the simplified oscillator model:

1) *The system is bounded:* We can prove that $R < \frac{k_{p,A} D_A^{tot}}{k_{ANN}\tilde{A}(R)}$, and thus, $\dot{C} < k_{p,A} D_A^{tot} - \delta_A C$. Consequently, C is bounded. Using this upper bound on C, we conclude that $\dot{R} < k_{p,R} D_R^{tot} + k_{p,A} D_A^{tot} - \delta_R R$.

$$R < \frac{k_{p,R} D_R^{tot} + k_{p,A} D_A^{tot}}{\delta_R} \quad C < \frac{k_{p,A} D_A^{tot}}{\delta_A}$$

2) *There is a single equilibrium point:* Let

$$P(\tilde{A}) = \frac{\alpha k_{off} + k_{on}\tilde{A}(R)}{k_{off} + k_{on}\tilde{A}(R)},$$

$P(\tilde{A})$ is a monotonic increasing function of $\tilde{A}$ and bounded by α and 1. Solving $\dot{R} = 0$ results in

$$C = \frac{k_{ANN}\tilde{A}(R)R + \delta_R R - k_{p,R} D_R^{tot} P(\tilde{A})}{\delta_A};$$

solving $\dot{C} = 0$ results in $C = \frac{k_{ANN}\tilde{A}(R)R}{\delta_A}$. The two nullclines coincide when $g(R) = \delta_R R - k_{p,R} D_R^{tot} P(\tilde{A}) = 0$. We can show that if

$$k_{p,a} D_A^{tot} > k_{off}(D_A^{tot} + D_R^{tot}), \text{ then } \frac{\partial \tilde{A}(R)}{\partial R} < 0,$$

which holds true for the choice of parameters. Since $g(0) < 0$, $g(\frac{k_{p,R} D_R^{tot}}{\delta_R}) > 0$, and $\frac{\partial g(R)}{\partial R} = \delta_R - k_{p,R} D_R^{tot} \frac{\partial P(\tilde{A})}{\partial \tilde{A}} \frac{\partial \tilde{A}(R)}{\partial R} > 0$, we conclude that there is a unique positive solution for $g(R) = 0$.

3) *The trace of J determines the existence of a limit cycle:* Notice that $det(J) = \delta_A(\delta_R - k_{p,R} D_R^{tot} \frac{\partial P(\tilde{A})}{\partial \tilde{A}} \frac{\partial \tilde{A}(R)}{\partial R}) > 0$, thus the sign of tr(J) determines stability of the unique equilibrium point $tr(J) = -k_{ANN}(\tilde{A} + R\frac{\partial \tilde{A}}{\partial R}) - \delta_A - \delta_R + k_{p,R} D_R^{tot} \frac{\partial P(\tilde{A})}{\partial \tilde{A}} \frac{\partial \tilde{A}(R)}{\partial R}$.

Combining these observations with 1) and 2), we can apply the Poincare-Bendixson theorem to conclude that system (1) has a limit cycle whenever $tr(J) > 0$.

B. In Vitro Barkai-Leibler Oscillator with Crosstalk

We now consider a more realistic model, taking into account that both the activator and repressor switches use a common enzyme, RNAP, to transcribe their RNA signals, while the activator and repressor RNA signals are degraded by a common enzyme, RNase R. (To simplify presentation, we do not explicitly model the enzyme reactions of RNase H.) The limited load capacity of enzymatic processes, known as Michaelis-Menten kinetics, introduces nonlinear aspects on the system dynamics such as zero-order ultrasensitivity [22]. Following typical Michaelis-Menten assumptions, i.e. low enzyme concentration and quasi-steady-state approximation for enzyme-substrate complexes, we replace the first-order reaction rates with enzyme-mediated reactions with enzyme concentrations (E^{tot} or E_d^{tot}), catalytic rates (k_{cat} or κ_{cat}), and Michaelis constants (K_M or κ_M). The reaction rate equations for D_A and D_R are unchanged.

$$\begin{aligned} \dot{A} &= \frac{E^{tot}\left(\frac{k_{cat,A,ON}}{K_{M,A,ON}} D_A^{ON} + \frac{k_{cat,A,OFF}}{K_{M,A,OFF}} D_A^{OFF}\right)}{1 + \frac{D_A^{ON}}{K_{M,A,ON}} + \frac{D_A^{OFF}}{K_{M,A,OFF}} + \frac{D_R^{ON}}{K_{M,R,ON}} + \frac{D_R^{OFF}}{k_{M,R,OFF}}} \\ &\quad -k_{ON} A D_A - k_{ON} A D_R - k_{ANN} A R \\ &\quad -E_d^{tot} \frac{\frac{\kappa_{cat,A}}{\kappa_{M,A}} A}{1 + \frac{A}{\kappa_{M,A}} + \frac{R}{\kappa_{M,R}} + \frac{C}{\kappa_{M,A}}} \qquad (2) \\ \dot{R} &= \frac{E^{tot}\left(\frac{k_{cat,R,ON}}{K_{M,R,ON}} D_R^{ON} + \frac{k_{cat,R,OFF}}{K_{M,R,OFF}} D_R^{OFF}\right)}{1 + \frac{D_A^{ON}}{K_{M,A,ON}} + \frac{D_A^{OFF}}{K_{M,A,OFF}} + \frac{D_R^{ON}}{K_{M,R,ON}} + \frac{D_R^{OFF}}{K_{M,R,OFF}}} \\ &\quad -k_{ANN} A R + E_d^{tot} \frac{\frac{\kappa_{cat,A}}{\kappa_{M,A}} C - \frac{\kappa_{cat,R}}{\kappa_{M,R}} R}{1 + \frac{A}{\kappa_{M,A}} + \frac{R}{\kappa_{M,R}} + \frac{C}{\kappa_{M,A}}} \\ \dot{C} &= k_{ANN} A R - E_d^{tot} \frac{\frac{\kappa_{cat,A}}{\kappa_{M,A}} C}{1 + \frac{A}{\kappa_{M,A}} + \frac{R}{\kappa_{M,R}} + \frac{C}{\kappa_{M,A}}} \end{aligned}$$

To compare the above model and the linear system (1) in a mathematically controlled way [23], we require that there is no extra degree of freedom for the Michaelis–Menten enzyme reactions. Thus, we set the production rates for both first-order model and Michaelis–Menten enzyme

model equivalent at the unique fixed point of the system. For example, $k_{p,A}$ and α constrain the RNAP parameters as follows.

$$\begin{aligned} k_{p,A} &= \frac{\frac{k_{cat,A,ON}}{k_{M,A,ON}} E^{tot}}{1+\frac{\bar{D}_A^{ON}}{k_{M,A,ON}}+\frac{\bar{D}_A^{OFF}}{k_{M,A,OFF}}+\frac{\bar{D}_R^{ON}}{K_{M,R,ON}}+\frac{\bar{D}_R^{OFF}}{K_{M,R,OFF}}} \\ \alpha &= \frac{k_{cat,A,OFF}}{K_{M,A,OFF}} / \frac{k_{cat,A,ON}}{K_{M,A,ON}} \end{aligned}$$

For the Michaelis–Menten RNAP reaction equations, we can follow the model reduction procedure for the first-order model because A is still a function of R only. On the other hand, the Michaelis–Menten RNase R reaction equations are functions of A, R, and C, making the model reduction approach difficult. We focused our attention to numerically probing system behavior as the saturation levels for RNAP and RNase change. Using the above constraints, the location of equilibrium point and molecular flux (production and degradation rates) at the equilibrium point remain identical for different enzyme parameter choices.

We note that the moderate saturation of RNAP significantly deteriorated the oscillation (Figure 2C), while the moderate saturation of RNase R increased the period of oscillations — at high levels of saturation the oscillations effectively disappear. (Figure 2D). Thus, including the crosstalk effects of substrates competing for free production enzyme effectively destroy the ability of the system to oscillate while increased competition for degradation enzyme seemed to qualitatively alter but not immediately destroy oscillations.. Towards understanding these effects, the next section develops a theoretical framework for quantifying crosstalk.

III. Towards Quantifying Crosstalk

Crosstalk arises from species competing for commonly shared enzymes. We will focus on two types of enzyme-mediated reactions in biochemical systems, reactions involving production and degradation. We suppose all other reactions do not involve enzymes and mechanistically depend on binding among non-enzyme components (thus operating on a slower time scale) — we call these reactions 'interaction' reactions. We decompose the dynamics of a system accordingly:

$$\dot{x} = f_c(x) = P_c(x) + I(x) - D_c(x), \;\; x(0) = x_0 \qquad (3)$$

where $x \in \mathbb{R}^n$, $P_c(x)$ ($D_c(x)$) describes the rates of production (degradation) from enzyme-mediated reactions with crosstalk effects and $I(x)$ describes the dynamics of interaction reactions. We will suppose that $P_c(x), D_c(x)$ in (3) satisfy the Michaelis Menten assumption. If x_i is not produced (degraded) by an enzyme-mediated reaction, then the corresponding entry in $P_{c,i}(x)$ ($D_{c,i}(x)$) is 0. For notational convenience, we will suppose that there are n_s substrate species indexed as $x_1, ..., x_{n_s}$ that combine with enzymes to produce n_p products: $x_{\alpha(1)}, ..., x_{\alpha(n_p)}$. For example, if $x_{\alpha(i)}$ is produced by substrates $x_1, ..., x_{n_k}$ in competition with other enzyme-sharing substrates $x_1, ..., x_{n_c}$, we will write $P_{c,\alpha(i)}(x)$ as

$$\begin{aligned} P_{c,\alpha(i)}(x) &= E^{tot} \sum_{j=1}^{n_k} k_{cat,j} g_j(x_1, ..., x_{n_c}) \qquad (4) \\ &\equiv E^{tot} \sum_{j=1}^{n_k} k_{cat,j} \frac{\frac{x_j}{k_{M,j}}}{1+\sum_{l=1}^{n_c} \frac{x_l}{k_{M,l}}} \end{aligned}$$

Similarly, we let $x_{d(1)}, ..., x_{d(n_d)}$ denote the list of system states that are degraded commonly by one or more enzymes and suppose that for a given x_i degraded by one or more enzymes shared by competing substrates $x_{d(1)}, ..., x_{d(n_c)}$, $D_{c,i}(x)$ can be written as

$$\begin{aligned} D_{c,i}(x) &= E_d^{tot} \kappa_{cat,i} u_i(x_{d(1)}, ..., x_{d(n_c)}) \\ &\equiv E_d^{tot} \kappa_{cat,i} \frac{\frac{x_i}{\kappa_{M,i}}}{1+\sum_{l=d(1)}^{d(n_c)} \frac{x_l}{\kappa_{M,l}}} \qquad (5) \end{aligned}$$

The processes of production and degradation are fundamental to system function: without production reactions, a system cannot synthesize the various components required to achieve intended dynamics and without degradation, a system quickly saturates and is potentially unstable. However, any production or degradation reaction involving competing substrates will elicit crosstalk effects. Thus, crosstalk arising from enyme-mediated reactions is a fundamental feature of any sufficiently complex biochemical system.

To quantify crosstalk in such systems, we need to compare the dynamics of system (3) against the dynamics of a reference system that is free of crosstalk. We note that the dynamics of such a reference system will still retain any crosstalk-free dynamics. This may include terms modeling nonlinear saturated production of a metabolite via a single dedicated enzyme (which is sometimes the mode of production in large metabolic networks). However, in the reference system, we suppose there are additional dedicated enzymes for each substrate (originally competing for a shared enzyme in system (3)). Such a system, that reflects the complexity and function of the original system may be impossible to synthesize physically. However, there are (simpler) scenarios where it is possible to design a system with dedicated enzymes for each substrate. Though such systems do not scale nicely, they provide a motivation for defining the following *conceptual* crosstalk-free system:

Definition 1: [Alternative System] Given the system (3) with equilibrium point x_e, define the alternative production function $P(x) : \mathbb{R}^n \to \mathbb{R}^n$, where for each nonzero entry $P_{\alpha(i)}(x), i = 1, ..., n_p$, if $x_{\alpha(i)}$ is produced from substrates $x_1, ..., x_{n_k} \in \{x_1, ..., x_{n_s}\}$ in system (3), then $P_{\alpha(i)}(x)$ is defined as

$$\begin{aligned} P_{\alpha(i)}(x) &\equiv E^{tot} \sum_{j=1}^{n_k} k_{cat,j} h_j(x_j) \qquad (6) \\ &\equiv E^{tot} \sum_{j=1}^{n_k} k_{cat,j} \frac{\frac{x_j}{K_{M,j}}}{1+\frac{x_j}{K_{M,j}}}. \qquad (7) \end{aligned}$$

Implicitly, we posit the presence of $E_1, ..., E_{n_s}$ enzymes that combine respectively with $x_1, ..., x_{n_s}$, with identical concentration totals $E_1^{tot} = \ldots = E_{n_s}^{tot} = E^{tot}$ and

identical Michaelis Menten constants. Similarly, we define $D(x) : \mathbb{R}^n \to \mathbb{R}^n$ to be a vector of functions describing the degradation dynamics for n_d species each produced by n_d different enzymes. If $x_{d(1)}, ..., x_{d(n_c)}$ are substrates competing for degradation enzyme E_d then we write the degradation rate of $x_{d(i)}$ as

$$D_{c,d(i)}(x) \equiv E_d^{tot}\kappa_{cat,i}v_i(x_{d(i)}) \quad (8)$$

$$\equiv E_d^{tot}\kappa_{cat,i}\frac{\frac{x_i}{\kappa_{M,i}}}{1+\frac{x_i}{\kappa_{M,i}}} \quad (9)$$

We then define the *alternative system* to be

$$\dot{x} = f_a(x) = P(x) + I(x) - D(x),\ x(0) = x_0 \quad (10)$$

Remark 1: The alternative system retains the same set of interaction reactions as the original system and thus, sequestration, activation and inhibition, or branch displacement reactions are all conserved. Wherever production or degradation of a component of the system is concerned, however, the dynamics of the alternative system are modified to remove competition effects between substrates. Note that the effects of nonlinear saturation are still retained through the use of Michaelis Menten functions.

Remark 2: The alternative system is a conceptual reference system to compare against the crosstalk system (3). For the comparison to be fair, it is important that (10) satisfy *internal equivalence* [23], namely that we hold fixed any dynamics or parameters unassociated with crosstalk, e.g. $I(x)$ or $x(0)$. The remaining degrees of freedom are the functional forms of $P(x)$ and $D(x)$ and their respective parameters. Following the process in [23], we impose constraints on the external behavior to achieve *external equivalence* of the alternative system: 1) the enzymes satisfy the Michaelis Menten assumption and 2) in the absence of competing species, the production (degradation) rates of $P_{c,\alpha(i)}(x)$ and $P_{\alpha(i)}(x)$ ($D_{c,d(i)}(x)$ and $D_{d(i)}(x)$) coincide. We can think of $P_{\alpha(i)}(x)$ as the limit of $P_{c,\alpha(i)(x)}$ when exactly one of $x_1, ..., x_s$ are nonzero. These constraints imply that the Michaelis constants, catalytic constants, and enzyme concentrations for $P_{\alpha(i)}(x)$ and $P_{c,\alpha(i)}(x)$ are identical. Thus, as we compare the behavior of both systems, any differences in production or degradation rates are purely due to effects of crosstalk.

With the definition of an alternative system in place, we can now quantify the amount of crosstalk that occurs between a pair of species in system (3).

Definition 2 (Pairwise Crosstalk): Let x_i and x_j be two distinct species in the biochemical system (3). Writing $P_{c,i}(x)$ as in equation (4), we define the *directed pairwise production crosstalk* of x_j on x_i as

$$c_p(x_i, x_j) = \begin{cases} P_i(x) - P_{c,i}(x), & x_j \in \{x_1, ..., x_{n_c}\} \\ 0, & \text{otherwise.} \end{cases} \quad (11)$$

Writing $D_{c,i}(x)$ as in equation (5), we define the *directed pairwise degradation crosstalk* of x_j on x_i as

$$c_d(x_i, x_j) = \begin{cases} D_i(x) - D_{c,i}(x), & x_j \in \{x_1, ..., x_{n_c}\} \\ 0, & \text{otherwise.} \end{cases} \quad (12)$$

We define the *undirected pairwise production crosstalk* between x_i and x_j as $C_p(x_i, x_j) \equiv c_p(x_i, x_j) + c_p(x_j, x_i)$ and the *undirected pairwise degradation crosstalk* as $C_d(x_i, x_j) \equiv c_d(x_i, x_j) + c_d(x_j, x_i)$. We refer to $C(x_i, x_j) \equiv C_p(x_i, x_j) + C_d(x_i, x_j)$ as the *pairwise competitive crosstalk* between x_i and x_j.

Remark 3: By construction, $C_p(x_i, x_j) \geq 0$ and $C_p(x_i, x_j) = C_p(x_j, x_i)$. C_p does not, in general, define a metric. However, if we restrict the domain of C_p to a *$\mathcal{P}$-class*, defined as a set consisting of a single product $x_{\alpha(i)}$ and substrates $x_1, ..., x_{n_c}$ competing for the enzyme that produces $x_{\alpha(i)}$, then it follows immediately from the definitions that C_p is a pseudometric.

Remark 4: Similarly, if we consider any set of species $\mathcal{D}$ degraded by a single enzyme, $C_d(x_i, x_j)$ then defines a metric on the associated discrete state space $\mathcal{D} = V_s$ of degradable species. In particular, the 2-tuple $(\mathcal{D}, C_d)$ is a metric space and if f is some contraction mapping on $(\mathcal{X}, C_d)$, then we can apply the contraction mapping theorem.

Remark 5: If K is the number of distinct enzymes in the system and x_1 competes for enzyme E_1, x_2 for E_2,..., x_K for E_K, then by Definition 2, defining $\Delta_p(x) = P_c(x) - P(x)$ and $\Delta_d(x) = D_c(x) - D(x)$ we get

$$\begin{aligned} f_c(x) - f_a(x) &= P_c(x) - D_c(x) - P(x) + D(x) \\ &= \Delta_p(x) - \Delta_d(x) \\ &= \sum_{k=1}^{K} -c_p(x, x_k) + c_d(x, x_k), \end{aligned}$$

where $c_p(x, x_k), c_d(x, x_k)$ is the vector of associated pairwise crosstalk terms. This result shows that the production crosstalk and degradation crosstalk vectors can be recovered through an appropriate collection of states that representatively span the set of enzymes for which competitive crosstalk occurs.

Definition 3 (System Crosstalk): The *total system crosstalk* for system (3), denoted $\mathcal{F}$ is defined as $C_{\mathcal{F}} \equiv \sum_{i<j} C(x_i, x_j)$

IV. Crosstalk and Robust Design

Since crosstalk is fundamental to system function, it is important to understand how to design a system in a way that reduces crosstalk. Intuitively, if we want production of a particular state to be reliable, we should impose the constraint that the populations of all other competing species are relatively small in comparison with the population of the state we wish to produce. On the other hand, if we want a state to follow a reference trajectory, it is important that we understand how the relative (dynamic) populations of each species drive the crosstalk that our target state experiences. In this section, we present results that can be viewed as two types of design criteria: 1) criteria that impose constraints on the dynamics of the system in order to keep the effects of crosstalk small or bounded 2) criteria for attenuating crosstalk by altering specific system parameters.

Our first result shows that by effectively bounding competing species, we can make the dynamics of a particular state robust.

Proposition 1 (Designing robust $x_{\alpha(i)}$): Suppose $x_{\alpha(i)}$ is produced by a single substrate x_i in competition with $x_1, ..., x_{i-1}, x_{i+1}, ..., x_{n_c}$ for enzyme E. Further, suppose $x_{\alpha(i)}$ is degraded by enzyme E_d, in competition with $x_{d(1)}, ..., x_{d(n_d)}$. If for a given x_0, there exists $\rho > 0$ and $\delta > 0$ such that the solution of the crosstalk system (3), $x_c(t)$, lies in both the set

$$S^i_\rho = \{x \in \mathbb{R}^n_{>0} | \sum_{j=1, j\neq\alpha(i)}^{n_c} \frac{x_j}{k_{M,j}} \approx \rho\} \tag{13}$$

and the set

$$S^i_\delta = \{x \in \mathbb{R}^n_{>0} | \sum_{j=d(n_1), j\neq\alpha(i)}^{d(n_d)} \frac{x_j}{\kappa_{M,j}} \approx \delta\} \tag{14}$$

and $x_i \gg \rho, \delta$ for all $t \geq 0$, then the magnitude of the crosstalk

$$|C_p(x_{\alpha(i)}, x_l) + C_d(x_{\alpha(i)}, x_q)| \approx O(\frac{1}{x_i}),$$

for any $l \neq i$ and $q \neq i$, and for any interval of time $[t, t+\tau]$

$$|x_{\alpha(i),c} - x_{\alpha(i),a}| \approx O(\frac{\tau}{x_{\alpha(i),a}})$$

Proof: We write $P_{c,\alpha(i)}(x)$ and $P_{\alpha(i)}(x)$ as before. The first result follows directly from computing the magnitude of the difference and the second from integrating over the time interval. ■

In practice it may be difficult to force the trajectories of all other competing states associated with a single enzyme to be small. Likewise, it may be difficult to increase the Michaelis constants of all other competing species while maintaining the same Michaelis constant for $x_{\alpha(i)}$. Such a constraint may be difficult to satisfy with an oscillator featuring two or three states coupled by the same production enzyme. In order for oscillations to occur, one state must be presumably abundant or another state relatively depleted. When competing for the same enzyme, this presents a difficulty for the depleted state as it returns from the minimum in its oscillatory trajectory to its peak, since it must outcompete the more abundant state for production enzyme. Analogous complications arise from a shared degradation enzyme. Conceptually, one solution to avoid this unwanted crosstalk coupling is to provide two or three independent enzymes that facilitate the production and degradation of oscillatory states, but the number of enzymes are limited so this approach is not scalable.

Next, we take a different approach. Rather than considering the dynamical constraints that must be imposed for the crosstalk system to match the alternative system, we consider effectively tuning certain aspects of the system so that the crosstalk can be rendered negligible.

While it may be difficult to introduce n independent enzymes to drive and regulate the trajectories of n different states, it is certainly possible to incorporate extra substrates that reliably maintain a steady state and exhibit population sizes that wash out the fluctuations of native system species. We emphasize this result, for its conceptual importance, but more especially for its practical significance.

Proposition 2: Let $x_1, ..., x_s$ be the set of system functional substrates and let $x_{s+1}, ..., x_{s+b}$ be a set of extra substrates that compete for the same enzyme as $x_1, ..., x_s$. Similarly, let $x_{d(n_d)+1}, ..., x_{d(n_d)+w}$ be a set of extra substrates that compete for degradation enzyme. Suppose these extra substrates maintain steady state. If the trajectory of $x_c(t)$ satisfies

$$\sum_{j=1}^{s} \frac{x_j}{k_{M,j}} \ll \sum_{j=s+1}^{s+b} \frac{x_j}{k_{M,j}}$$

and

$$\sum_{j=1}^{d(n_d)} \frac{x_j}{\kappa_{M,j}} \ll \sum_{j=d(n_d)+1}^{d(n_d)+w} \frac{x_j}{\kappa_{M,j}}$$

then $f_c(x)$ is robust to crosstalk and can be approximated with linear production and degradation functions $P_L(x), D_L(x)$, scaled to reflect the dominating flux of the junk substrates.

Proof: The result follows immediately from approximating the denominators in each entry of $P_c(x)$ and $D_c(x)$ with the dominant steady state terms of the extra substrates. ■

Since the denominators in the production and degradation functions are approximately constant, production and degradation become linear processes. Again, since the extra substrates are at steady state, this is effectively the same as increasing the Michaelis constants of competing substrates. It is these Michaelis constants that dictate the effects of production and degradation crosstalk in system (3).

The next theorem reveals a second parameter that is key to attenuating crosstalk: the total production and degradation enzyme concentrations. Intuitively, if we want to reduce competition among the species for enzyme, then by increasing the amount of enzyme, each substrate will be degraded or produced as if degradation or production were a first order process — the availability of substrate dictates the progress of the reaction.

Theorem 1: Suppose x_q are bounded for $q = 1, ..., n_s$ and x_l are bounded for $l = d(1), ..., d(n_d))$. Then as E^{tot} and $E_d^{tot} \to \infty$ then $C_p(x_i, x_j), C_d(x_i, x_j) \to 0$ for all $i \neq j$ and taking $E^{tot} \to \infty, E_d^{tot} \to \infty$ simultaneously, then the total system crosstalk $C_{\mathcal{F}} \to 0$.

Proof: First, we consider a single production enzyme, E, as the argument is identical from enzyme to enzyme. Suppose x_i is produced by E in combination with substrates $x_1, ..., x_k$ that form complexes $(x_l : E), l = 1, ..., k$ complex in competition with $x_1, ..., x_{n_c}$ and E^{tot} is large, we instead write $x_l = x_l^{free} + (x_l : E)$ since there will be a nontrivial amount of enzyme-substrate complex formed unlike the usual limiting enzyme assumption in the Michaelis-Menten formulation. Suppose $x_j \in \{x_1, ..., x_{n_c}\}$, we will show

$$\lim_{E^{tot}\to\infty} c_p(x_i, x_j) = 0,$$

(otherwise, $c(x_i, x_j) = 0$ already). First, in the crosstalk system (3), detailed balance yields

$$(x_i : E) = \frac{x_i^{free} E^{free}}{K_{M,i}}$$

and by conservation of mass, we get

$$\begin{aligned} E^{free} &= E^{tot} - \sum_{l=1}^{n_c}(x_l : E) \\ &= E^{tot} - \sum_{l=1}^{n_c}(x_l - x_l^{free}) \\ &\geq E^{tot} - \sum_{l=1}^{n_c} x_l \end{aligned}$$

Following the derivation in [19], the modified production rates can then be written as

$$P_{c,i} = \sum_{q=1}^{k} k_{cat,q} \frac{x_q^{free} E^{free}}{K_{M,q}}$$

and substituting for $x_q^{free} = \frac{x_q}{1+\frac{E^{free}}{K_{M,q}}}$ we get

$$P_{c,i} = \sum_{q=1}^{k} k_{cat,q} \frac{x_q E^{free}}{K_{M,q} + E^{free}} \tag{15}$$

Similarly, for the alternative system, we can derive

$$P_i = \sum_{q=1}^{k} k_{cat,q} \frac{x_q E_q^{free}}{K_{M,q} + E_q^{free}}.$$

By definition of the alternative system, $E_q^{tot} = E^{tot}$ for $q = 1, ..., k$. For each q, we have

$$E_q^{free} + E_q^{free} \frac{x_q}{K_{M,q} + E_q^{free}} = E^{free} + E^{free} \sum_{l=1}^{n_c} \frac{x_l}{K_{M,l} + E^{free}}.$$

and

$$\begin{aligned} c_p(x_i, x_j) &= \sum_{q=1}^{k} k_{cat,q} \left(\frac{x_q E_q^{free}}{K_{M,q} + E_q^{free}} - \frac{x_q E^{free}}{K_{M,q} + E^{free}} \right) \\ &= \sum_{q=1}^{k} k_{cat,q} \left(E^{free} - E_q^{free} + \sum_{l \neq q} \frac{E^{free} x_l}{K_{M,l} + E^{free}} \right) \end{aligned}$$

and as E^{tot} approaches infinity, since x_l are bounded, all free x_l are sequestered by enzyme, so that the free enzyme can be expressed as $E^{free} = E^{tot} - \sum_{l=1}^{n_c} x_l$. Similarly, $E_q^{free} = E^{tot} - x_q, q = 1..., k$. Thus, by direct substitution $\lim_{E^{tot} \to \infty} c(x_i, x_j) =$

$$\lim_{E^{tot} \to \infty} \sum_{q=1}^{k} k_{cat,q} \left(-\sum_{l=1}^{n_c} x_l + x_q + \sum_{l \neq q} \frac{E^{free} x_l}{K_{M,l} + E^{free}} \right)$$

$$= 0.$$

Since x_i and x_j were arbitrary, then the undirected crosstalk $C_p(x_i, x_j)$ must also approach 0 for all i and j as E^{tot} increases.

A similar proof shows the degradation rates satisfy

$$\lim_{E_d^{tot} \to \infty} C_d(x_i, x_j) \to 0$$

Thus, by the definition of $C_{\mathcal{F}}$ and the algebraic properties of limits,

$$\begin{aligned} \lim_{E^{tot}, E_d^{tot} \to \infty} C_{\mathcal{F}} &= \lim_{E^{tot}, E_d^{tot} \to \infty} \sum_{i,ji<j} C(x_i, x_j) \\ &= \sum_{i,j,i<j} \lim_{E^{tot} \to \infty} C_p(x_i, x_j) \\ &+ \sum_{i,j,i<j} \lim_{E_d^{tot} \to \infty} C_d(x_i, x_j) \\ &= 0 \end{aligned}$$

■

This theorem suggests that by increasing the total production and degradation enzyme, the production and degradation crosstalk correspondingly decrease. Indeed, this is made clear by examining the alternate expressions for production and degradation in equation (15) and substituting the expression for $E^{free} = E^{tot} - \sum_{l=1}^{n_c} x_l$. As we increase the total amount of enzyme, both the alternative and crosstalk production rates become approximately linear, with the alternative system approaching slightly faster than the crosstalk system. This suggests a mode of adjusting system performance by simply attenuating the production or degradation crosstalk. Again, this theorem is practically significant in *in vitro* systems where total enzyme concentrations can be tuned with high precision. As a final example, we illustrate how tuning enzyme concentrations and the effective Michaelis constants in the Barkai-Leibler system attenuate crosstalk to recover oscillatory dynamics.

Example 1 (Tuning the Barkai-Leibler System): In the Barkai-Leibler system (2), when RNAP is saturated, the templates D_A and D_R vie for the common RNAP, resulting in crosstalk: they effectively down-regulate each other's transcription rates. This crosstalk interferes with A promoting its own production through D_A. Because the production and degradation of A must be on a fast time-scale to achieve oscillation, we see that RNAP-mediated crosstalk deteriorates oscillation (Figure 2c).

On the other hand, RNA signals A, R, and C experience degradation crosstalk as they each compete for saturated RNase R, i.e. through crosstalk the three RNA signals become mutually activating. Except for R activating A (which is weak when $\kappa_{M,R} > \kappa_{M,A}$), this mutual activation would fortify existing activating reaction cascades — A promotes creation of R and formation of C, R promotes formation of C, and C returns to R. Therefore, qualitatively, the saturation of RNase R helps the circuit oscillate (Figure 2d). In Figure 3, we see that by increasing RNAP and introducing background template, we effectively reduce the production crosstalk and we recover oscillations in the Barkai-Leibler system (2). We note that by simultaneously increasing all enzyme concentrations, the first-order production degradation model (1) and subsequent analysis becomes valid again, but over a different set of parameters. Thus models with linear production and degradation are not only instrumental in characterizing simplified system dynamics but represent the limit for large enzyme concentrations or small substrate concentrations.

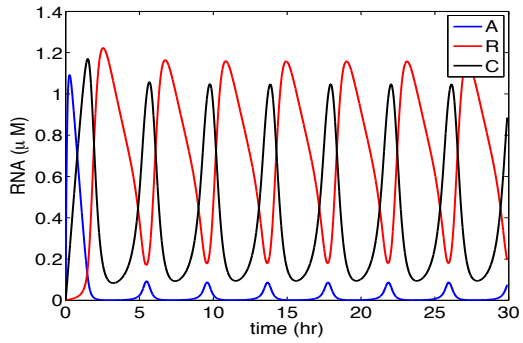

Fig. 3. A simulation of the Barkai-Leibler system using identical parameters as Figure 2c), but with four times the amount of RNAP ($220nM$) and 1.08 μM of background template.

V. Future Work & Conclusions

In this work, we introduced signaling crosstalk as a fundamental phenomenon of enzyme-mediated reactions in biochemical systems. As a motivating example, we introduced an *in vitro* version of the Barkai-Leibler oscillator and demonstrated that under simplifying assumptions, the system would oscillate. We posed a more realistic model that introduced crosstalk in the production functions and consequently, the system did not oscillate. We then posed a theoretical framework for quantifying crosstalk and used it to derive several dynamical constraints and theoretical results that suggest design strategies for attenuating crosstalk. Finally, we showed that by effectively changing the Michaelis constants or by changing the total enzyme concentrations, the Barkai-Leibler system (1) was able to recover oscillatory behavior.

We note that in this work we did not model degradation products. Because the chemical identity of degradation products for a single enzyme-substrate pair can vary from one instance of a degradation reaction to another, the resulting number of states required to describe all potential degradation products can be staggering. Furthermore, degradation products can result in crosstalk in the interaction terms — - a type of crosstalk we did not explore. Future research will address crosstalk from degradation products, hidden regulatory interactions from background gene expression, and develop a framework for quantifying crosstalk *in vivo*.

Additionally, crosstalk from interference with degradation products will have nearly negligible effects upon initialization of an in vitro system but will dramatically increase as the system runs for an extended period of time. These degradation products interfere on a faster time scale than enzyme-mediated reactions and can partially or completely sequester vital components of the system, thus changing the effective binding energies between reactants and ultimately the future reaction rates. Finally, the interactions are uncertain as the populations of the each of the potential degradation products is uncertain and highly dependent on the activity of the enzyme. Thus, crosstalk from degradation interference is fundamentally different than crosstalk from production or degradation. Future research will address crosstalk from degradation products, hidden regulatory interactions from background gene expression, and develop a framework for quantifying crosstalk for *in vivo* systems.

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