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Biomolecular resource utilization in elementary cell-free gene circuits

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***Abstract*— We present a detailed dynamical model of *in vitro* behavior of transcriptional circuits that explicitly takes into account the contributions of essential molecular resources and that demonstrates (1) how resources are utilized in circuits with multiple components, and (2) the consequences of limited resource availability. The model is validated using a recently developed and well-characterized *in vitro* environment—a cell-free biochemical 'toolbox'—and a number of simple test circuits that allowed us to confirm the existence of biomolecular 'crosstalk' and isolate its individual sources. The implications of crosstalk for biomolecular circuit design and function are also discussed.**

I. INTRODUCTION

The past several decades have witnessed significant advances in the biological sciences driven by the introduction of techniques from historically separate research areas such as mathematics, physics, computer science, and engineering. The rapid growth and a number of research successes has led to the emergence of whole new disciplines, chief among them 'synthetic biology' [1], promising significant insights into biological circuit function and a wide array of technological benefits. An important aspect of synthetic biology is the use of models to guide circuit construction and understand behaviors that, even in seemingly simple systems, regularly defy intuition [2], [3]. 'Toy circuits'—those without any specific biotechnological application or direct natural analog—are also regularly used to explore fundamental biological mechanisms and design principles [4]; among their argued benefits are a relatively well-controlled construction process and a certain degree of confidence with the necessary scope of any associated mathematical model.

A. A simple two-gene circuit

As an example of a toy circuit, we consider a simple system consisting of two genes under the control of constitutive promoters, x and y, which code for a generic protein X and fluorescent reporter Y, respectively. A 'naive' description of the system is as follows: x is transcribed into mRNA x_m which is then translated into X, while in parallel the reporter gene is transcribed to produce an mRNA y_m, which is then translated into an 'immature' (i.e., dark) reporter protein $\mathrm{Y_d}$ that matures into the visible Y. This simple description is shown schematically in Fig. 1 and summarized by the following set of chemical reactions:

$$\begin{aligned} x &\to x + x_m & y &\to y + y_m \\ x_m &\to \varnothing & y_m &\to \varnothing \\ x_m &\to x_m + \mathrm{X} & y_m &\to y_m + \mathrm{Y_d} \\ & & \mathrm{Y_d} &\to \mathrm{Y}\ . \end{aligned}$$

In constructing this reaction set we have assumed that the proteins are stable against degradation, although mRNA transcripts are readily degraded by ribonucleases. We further assume that the reactions take place in large volumes (so that stochasticity in the expression of individual molecules does not affect the overall dynamics [5]) and that their dynamics can be suitably approximated using mass-action kinetics, and rewrite them as a set of ordinary differential equations (ODEs):

$$\frac{\mathrm{d}[x_m]}{\mathrm{d}t} = k_{x,TX}[x] - k_d[x_m] \tag{1a}$$
$$\frac{\mathrm{d}[\mathrm{X}]}{\mathrm{d}t} = k_{x,TL}[x_m] \tag{1b}$$
$$\frac{\mathrm{d}[y_m]}{\mathrm{d}t} = k_{y,TX}[y] - k_d[y_m] \tag{1c}$$
$$\frac{\mathrm{d}[\mathrm{Y_d}]}{\mathrm{d}t} = k_{y,TL}[y_m] - k_{mat}[\mathrm{Y_d}] \tag{1d}$$
$$\frac{\mathrm{d}[\mathrm{Y}]}{\mathrm{d}t} = k_{mat}[\mathrm{Y_d}] \tag{1e}$$

where $k_{y,TX}, k_d, k_{y,TL}, k_{mat}, k_{x,TX}$, and $k_{x,TL}$ are the various reaction rates of the circuit. This model has a structure common to many other ODE models of gene regulatory circuits: for each gene there is one equation for transcription and one for translation, the degradation of mRNAs is not regulated by any other species in the circuit, and that the production and degradation terms are linear [6], [7]. According to this simple model, the expected behavior of the output signal when all background reactions reach steady state is

$$\frac{\mathrm{d}[\mathrm{Y}]}{\mathrm{d}t}\Big|_{SS} = \frac{k_{y,TL}\ k_{y,TX}}{k_d}[y]\ , \tag{2}$$

that is, the rate of increase in fluorescent signal is related only to the fixed concentration of y and constant reaction rates.

An assumption implicit to this model is that the molecular machinery fundamental to all gene regulatory circuits—for example, transcription initiation factors, RNA polymerase, and ribosomes—exist in sufficiently high concentrations and that their utilization by one component has no noticeable effect on others in the same reaction volume. It is thus not unexpected that our simple model shows x to have no effect on $\mathrm{d}[\mathrm{Y}]/\mathrm{d}t\big|_{SS}$. Recently, however, significant evidence has supported the existence of indirect coupling ('crosstalk')

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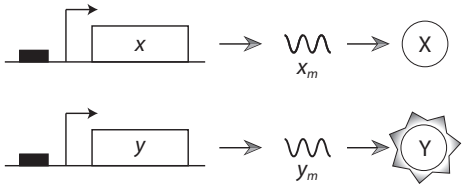

Fig. 1. A 'naive' picture of a circuit containing two genes x and y driven by constitutive promoters (represented by filled rectangles). Genes are transcribed (in the direction indicated by the right-angle arrows) into mRNAs x_m and y_m, which are then translated into a generic protein X and immature fluorescent reporter $\mathrm{Y_d}$, respectively. The reporter then matures into the visible Y. The proteins are stable against degradation but the mRNAs are readily degraded (not shown). The two genes do not directly interact.

between expressing genes across a range of biological networks, including those involved in transcriptional regulation [8] and post-translational processing [9]. It has been suggested that this crosstalk arises via the forced sharing of the molecular resources needed for circuit function [10], [11]. And while theoretical frameworks for the analysis of crosstalk are being developed (e.g., [12]); models that may be used to explore the effects of crosstalk introduced via resource utilization are still lacking.

We thus set out to develop a more detailed mathematical model for the simple *in vitro* gene circuit shown in Fig. 1, but with a level of complexity sufficient to capture and predict crosstalk arising from the sharing of molecular resources. An important model design criterion was that the model have a general form that could be easily expanded to more complex circuits and that the individual sources of crosstalk and their relative contributions to the total could be identified with a small number of simple experiments.

B. A cell-free biochemical 'toolbox'

The value of any biological model lies in its ability to accurately capture and predict true biological phenomena; however, given the complexity of biological systems and the context-dependence of circuit components (which often have unpredicted interactions with the host; see, e.g., [13], [14]), experimental verification of biomolecular circuit models can be challenging. As a result there has been considerable interest in developing relatively simple *in vitro* platforms that can be used for circuit development, characterization, and model verification [15]–[17]. Important steps towards this goal have been made in recent years by V. Noireaux and colleagues at the University of Minnesota with the development of a cell-free 'toolbox': an *in vitro* system that allows transcription and translation (TX-TL) processes to take place using molecular machinery extracted from *E. coli* [18], [19]. Endogenous DNA and mRNA from the cells is eliminated during extract preparation, so that synthetic gene circuits of interest may be studied in isolation with no other genetic material present in the reaction. In contrast with other *in vitro* systems that use non-native RNA polymerases and/or have a high cost per use, the toolbox enables rapid and inexpensive testing of natural circuits that may be later implemented *in vivo* with little or no modification. The toolbox also allows for control over nutrients, reaction conditions, and the concentration of circuit components—control which is difficult to achieve *in vivo*. It is thus an ideal environment that may be used to establish the validity of biological circuit models and in particular confirm the existence of crosstalk in even simple genetic circuits.

In Section II we give our detailed model of the two-gene system described above with the roles played by essential molecular resources made explicit. In Sections III and IV, the simulated and cell-free experimental results are shown for a number of simple test circuits that allowed us to confirm the existence of biomolecular 'crosstalk' and isolate its individual sources. A discussion of these results and the implications for circuit design is given in Section V.

II. DETAILED MODEL FOR CONSTITUTIVE EXPRESSION OF TWO GENES IN VITRO

A more detailed chemical reaction network model for the two-gene system described in Section I-A that makes explicit the role of TX-TL machinery is as follows:

$$
\begin{aligned}
\mathrm{E} + \mathrm{S1} &\rightleftharpoons \mathrm{ES1} & \mathrm{R} + x_m &\rightleftharpoons x_m{:}\mathrm{R}\\
\mathrm{ES1} + x &\rightleftharpoons x{:}\mathrm{ES1} & \mathrm{R} + y_m &\rightleftharpoons y_m{:}\mathrm{R}\\
\mathrm{ES1} + y &\rightleftharpoons y{:}\mathrm{ES1} & x_m{:}\mathrm{R} &\to x_m{:}\mathrm{R} + \mathrm{X}\\
x{:}\mathrm{ES1} &\to x{:}\mathrm{ES1} + x_m & y_m{:}\mathrm{R} &\to y_m{:}\mathrm{R} + \mathrm{Y_d}\\
y{:}\mathrm{ES1} &\to y{:}\mathrm{ES1} + y_m & \mathrm{Y_d} &\to \mathrm{Y}\ .\\
x_m &\to \varnothing\\
y_m &\to \varnothing
\end{aligned}
$$

where E, R, S1, and ES1 represent free core RNA polymerase (RNAP), free ribosome, the primary 'housekeeping' sigma factor (necessary for transcription initiation), and the sigma factor-RNAP holoenzyme, respectively. In addition, (:) represents RNAP holoenzymes bound to DNA and ribosomes bound to mRNA transcripts. The ODEs for this expanded model are:

$$\frac{\mathrm{d}[x_m]}{\mathrm{d}t} = k_{x,TX}[x{:}\mathrm{ES1}] - k_d[x_m] - \frac{\mathrm{d}[x_m{:}\mathrm{R}]}{\mathrm{d}t} \tag{3}$$

$$\frac{\mathrm{d}[y_m]}{\mathrm{d}t} = k_{y,TX}[y{:}\mathrm{ES1}] - k_d[y_m] - \frac{\mathrm{d}[y_m{:}\mathrm{R}]}{\mathrm{d}t} \tag{4}$$

$$\frac{\mathrm{d}[x_m{:}\mathrm{R}]}{\mathrm{d}t} = k_{\mathrm{X}+}[\mathrm{R}][x_m] - k_{\mathrm{X}-}[x_m{:}\mathrm{R}] \tag{5}$$

$$\frac{\mathrm{d}[y_m{:}\mathrm{R}]}{\mathrm{d}t} = k_{\mathrm{Y}+}[\mathrm{R}][y_m] - k_{\mathrm{Y}-}[y_m{:}\mathrm{R}] \tag{6}$$

$$\frac{\mathrm{d}[\mathrm{X}]}{\mathrm{d}t} = k_{x,TL}[x_m{:}\mathrm{R}] \tag{7}$$

$$\frac{\mathrm{d}[\mathrm{Y_d}]}{\mathrm{d}t} = k_{y,TL}[y_m{:}\mathrm{R}] - k_{mat}[\mathrm{Y_d}] \tag{8}$$

$$\frac{\mathrm{d}[\mathrm{Y}]}{\mathrm{d}t} = k_{mat}[\mathrm{Y_d}] \tag{9}$$

$$\begin{aligned}\frac{\mathrm{d}[\mathrm{ES1}]}{\mathrm{d}t} &= k_{\mathrm{ES1}+}[\mathrm{E}]\big([\mathrm{S1}]_{\mathrm{tot}} - [\mathrm{ES1}]\big)\\ &\quad - k_{\mathrm{ES1}-}[\mathrm{ES1}] - \frac{\mathrm{d}[x{:}\mathrm{ES1}]}{\mathrm{d}t} - \frac{\mathrm{d}[y{:}\mathrm{ES1}]}{\mathrm{d}t}\end{aligned} \tag{10}$$

$$\begin{aligned}\frac{\mathrm{d}[x{:}\mathrm{ES1}]}{\mathrm{d}t} &= k_{x+}[\mathrm{ES1}]\big([x]_{\mathrm{tot}} - [x{:}\mathrm{ES1}]\big)\\ &\quad - k_{x-}[x{:}\mathrm{ES1}]\end{aligned} \tag{11}$$

$$\begin{aligned}\frac{\mathrm{d}[y{:}\mathrm{ES1}]}{\mathrm{d}t} &= k_{y+}[\mathrm{ES1}]\big([y]_{\mathrm{tot}} - [y{:}\mathrm{ES1}]\big)\\ &\quad - k_{y-}[y{:}\mathrm{ES1}]\end{aligned} \tag{12}$$

with the following conservation relations:

$$[\mathrm{E}] = [\mathrm{E}]_{\mathrm{tot}} - [x{:}\mathrm{ES1}](1 + k_{x,TX}\frac{L_x}{V_{TX}}) - [y{:}\mathrm{ES1}](1 + k_{y,TX}\frac{L_y}{V_{TX}}) - [\mathrm{ES1}] \quad (13)$$

$$[\mathrm{R}] = [\mathrm{R}]_{\mathrm{tot}} - [x_m{:}\mathrm{R}](1 + k_{x,TL}\frac{L_x}{V_{TL}}) - [y_m{:}\mathrm{R}](1 + k_{y,TL}\frac{L_y}{V_{TL}}) \; . \quad (14)$$

$[\mathrm{E}]_{\mathrm{tot}}$, $[\mathrm{R}]_{\mathrm{tot}}$, $[\mathrm{S1}]_{\mathrm{tot}}$, $[y]_{\mathrm{tot}}$, and $[x]_{\mathrm{tot}}$ represent the fixed total concentrations of (free and complexed) RNAP, ribosome, primary sigma factor, and genes in the reaction volume, and the factors of the form $(1 + k\frac{L_i}{V})$ account for multiple loading of holoenzymes on the mRNA templates [20]. L_i is the length (in bp) of gene i and V_{TX} and V_{TL} represent the rates of progression (in nucleotides per second) of RNAP along the DNA and ribosome along the mRNA, respectively.

III. SIMULATED AND EXPERIMENTAL RESULTS

Of the molecular species in our model, we might expect the RNAP (E) and ribosomes (R) to contribute a crosstalk-type effect to the output signal; for example, since E (in the form of the holoenzyme ES1) binds to both x and y, an increase in the concentration of x could result in a sequestration of ES1 away from y and thus a decrease in the output Y. Similarly, an increase in the amount of x_m could decrease the amount of free R available to translate y_m. These predictions were tested computationally and experimentally with two circuits specifically designed to distinguish between the possible sources of crosstalk: in one case, x encodes a small untranslated RNA to which there there is no ribosomal binding (Fig. 2A), and in the other, it encodes a 'dummy' protein with no direct interactions (Fig. 2B).

A. Fluorescent reporter only

As a preliminary test of the model and to provide a baseline for performance in the absence of a second gene, we set $[x]_{\mathrm{tot}} = 0$ and solve expression dynamics using parameters drawn from the literature (Table I). Simulated fluorescence ($\propto [\mathrm{Y}]$) is plotted as a function of time in Fig. 3A, along with experimental results. Both simulation and experiment show a linear increase in output after a short 'ramp up' phase and are in good agreement.

B. Untranslated RNA

To determine the contribution of RNAP holoenzyme alone to crosstalk in the circuit, we use a gene that is transcribed into an RNA molecule that is not bound by ribosomes and thus not translated (Fig. 2A) at two low but biologically-relevant concentrations, $[x]_{\mathrm{tot}} = 0.1$ nM and $[x]_{\mathrm{tot}} = 1$ nM. In simulation, the identity of this gene is fixed by setting the rate of association of R to x_m, $k_{\mathrm{X}+}$, equal to zero. We find that neither concentration had any discernible effect on the rate of production of Y as compared with the single-plasmid control; both simulated functions are linear and exactly overlaid and highly consistent with experiment (Fig. 3B). We can thus conclude that when additional genes are present in low concentrations, and when using biological parts with their native binding affinities and reaction rates, holoenzyme does not appear to contribute to any crosstalk between those additional genes and the circuit output.

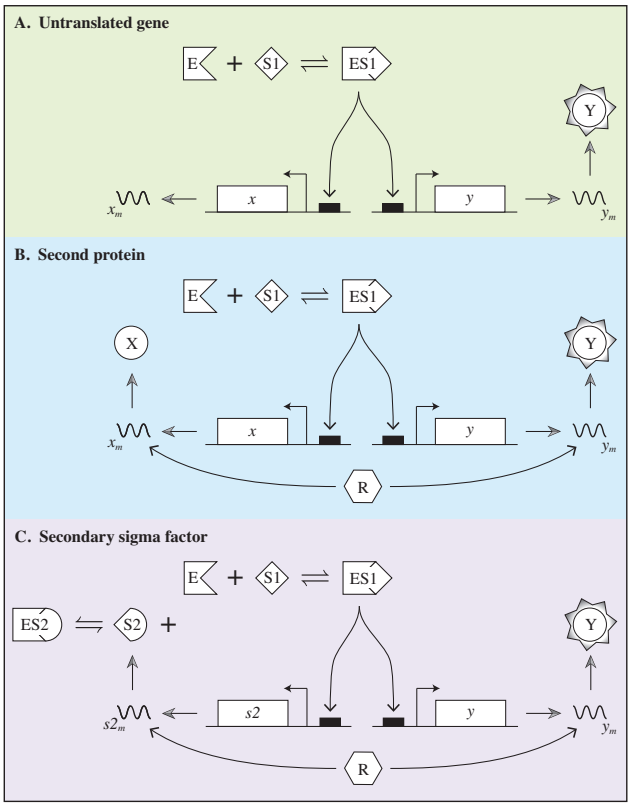

Fig. 2. Two-gene circuits tested in the cell-free toolbox environment. The difference between circuits is in the second gene, which encodes either (A) an untranslated RNA, (B) a 'dummy' protein with no direct interactions, or (C) a secondary sigma factor. Symbols are as in Fig. 1, with additional arrows representing complex formation and the regulatory roles of various molecular species.

C. Additional protein-coding gene

We now consider the effect of ribosome sequestration on circuit output, using a second protein-coding gene whose final product has no direct interactions with any other model components, i.e., a 'dummy' protein (Fig. 2B). We use the full model of Section II with all rates and concentrations positive. Similar to the single-plasmid control and untranslated RNA circuit, simulated and experimental data both show a linear increase in output after a short 'ramp up' phase; however, the model predicts a slope of d[Y]/dt that is different for $[x]_{\mathrm{tot}} = 0.1$ nM and $[x]_{\mathrm{tot}} = 1$ nM (Fig. 3C), and, though preliminary and with substantial variance, the data is consistent with this predicted behavior. This suggests that unlike holoenzymes, ribosomes may be a limiting resource and that even low levels of auxiliary ribosome targets can lead to a reduction in the circuit output.

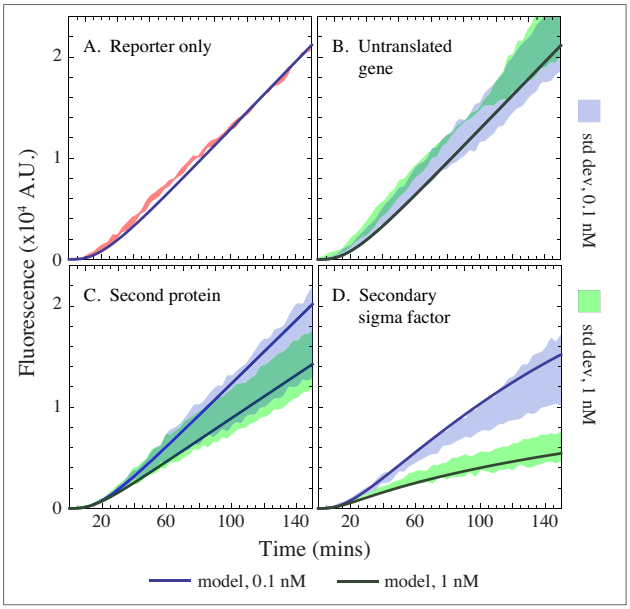

Fig. 3. Modeling and experimental results for the fluorescent reporter alone (A) and for implementations of the circuits schematized in Fig. 2, in which the second gene encodes a small untranslated RNA (B), a second protein (C), and a secondary sigma factor (D). Blue and light green shaded areas indicate the standard deviation of measurements (n=2) made with $[x]_{\text{tot}} = 0.1$ nM and $[x]_{\text{tot}} = 1$ nM, respectively. Total reporter concentration ($[y]_{\text{tot}}$) is fixed at 2 nM in all simulations and experiments. DNA components used in experiments were derived from *E. coli*; see Appendix for details.

IV. SPECIAL CASE: ALTERNATIVE SIGMA FACTORS

In the previous section we showed simulated results and experimental data suggesting that a generic protein which does not interact directly (for example, as a transcription factor) with a constitutively expressing fluorescent reporter may still affect its expression via indirect sequestration of ribosomes. While this particular resource loading effect is common to all protein-coding genes, certain classes of proteins with dynamically changing concentrations may introduce additional undesirable effects; for example, alternative sigma factors that can compete for access to free core RNAP and in doing so reduce the concentration of specific holoenzymes, which in turn reduces activity from sigma factor-specific promoters. Experimental evidence supporting sigma factor sequestration has been found *in vivo* [21] and using purified sigma factor subunits [22]. Alternative sigma factors hold significant potential for the design of complex genetic circuits: the substantial promoter selectivity that sigma factors confer to RNAP [23] can lead to a significant increase in the variety of available transcriptional control elements, beyond the standard library of repressors and activators that are now commonly used.

We set out to determine if our model formalism predicts additional resource-loading-type effects when a secondary constitutively-expressed sigma factor is introduced to the system. We thus add the following equation to our model:

$$\frac{\mathrm{d}[\mathrm{ES2}]}{\mathrm{d}t} = k_{\mathrm{ES2}+}[\mathrm{E}][\mathrm{S2}] - k_{\mathrm{ES2}-}[\mathrm{ES2}] \ , \qquad (15)$$

and modify Eqs. (7) and (13) to be

$$\frac{\mathrm{d}[\mathrm{S2}]}{\mathrm{d}t} = k_{s2,TL}[s2_m{:}\mathrm{R}] - \frac{\mathrm{d}[\mathrm{ES2}]}{\mathrm{d}t} \qquad (16)$$

$$\begin{aligned}[\mathrm{E}] = [\mathrm{E}]_{\text{tot}} &- [s2{:}\mathrm{ES1}](1 + k_{s2,TX}\frac{L_{s2}}{V_{TX}}) \\ &- [y{:}\mathrm{ES1}](1 + k_{y,TX}\frac{L_y}{V_{TX}}) \\ &- [\mathrm{ES1}] - [\mathrm{ES2}] \ . \end{aligned} \qquad (17)$$

(Notationally, references to 'x' and 'X' have been replaced with '$s2$' and 'S2' to emphasize that these equations represent specific properties of sigma factors.) The results of our simulation are shown along with experimental data for $[s2]_{\text{tot}} = 0.1$ nM and $[s2]_{\text{tot}} = 1$ nM in Fig. 3D. We note that (1) core RNAP sequestration by a secondary sigma factor has a more pronounced effect on the circuit output than does the ribosome loading, and particularly at higher gene concentrations, and (2) unlike the untranslated RNA and 'dummy' protein cases, the rates of fluorescence increase are sublinear for the 2.5 hours of the experiment.

V. DISCUSSION

We have presented a detailed model for a simple two-gene regulatory circuit *in vitro* that makes explicit the important functional roles played by RNA polymerase, sigma factors, and ribosomes and that allows us to understand and predict precisely how these resources are shared between components in multi-gene circuits. In particular, the model predicts that, in circuits composed of naturally occurring biological parts with realistic binding affinities, even a single noninteracting protein-coding gene added at a low concentration to a circuit introduces significant crosstalk through ribosomal loading. The model also shows a significant decrease in the output signal when a constitutively expressed secondary sigma factor is added to the circuit. These predictions are supported by our experimental results and further suggest the validity of the cell-free toolbox as an experimental platform for characterizing novel synthetic biocircuits.

With confidence so established, we may use the model to answer additional questions about the system; for example, without modifying any circuit component, can we determine at what level of additional genes does the RNAP holoenzyme become a limiting resource, and below what concentration does ribosomal loading not lead to any significant crosstalk? In Fig. 4 we see how the production rate of the output (d[Y]/dt) is affected by the concentration of a second gene over 6 orders of magnitude. As before, we use the simulated untranslated-RNA gene (with $k_{\mathrm{X}+} = 0$) to isolate and predict the effect of holoenzyme utilization. We find that crosstalk arising from limited holoenzyme availability begins to appear when the concentration of additional genes is ∼30 nM (Fig. 4A), or 15X the reporter concentration ($[y]_{\text{tot}}$) of 2 nM. On the other hand, ribosome-related crosstalk (as determined by the model) begins to manifest itself at concentrations as low as 1% of $[y]_{\text{tot}}$, or ∼20 pM (Fig. 4B). This latter result in particular suggests that ribosome utilization effects may be difficult to avoid in any natural circuit of even minimal

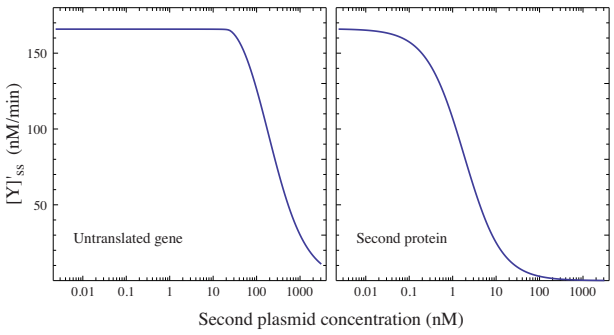

Fig. 4. Rate of increase of fluorescence at system 'steady state' as a function of the concentrations of the secondary gene when it codes for (A) an untranslated RNA or (B) a typical protein.

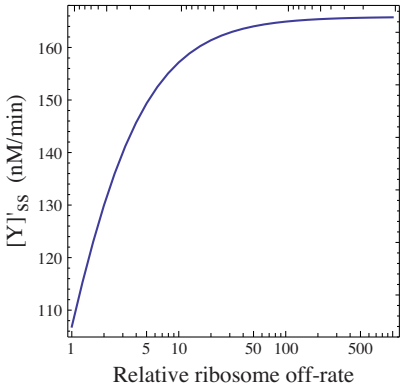

Fig. 5. Rate of increase of fluorescence at system 'steady state' as a function of the strength of the second gene's ribosomal binding site relative to the fluorescent reporter's.

complexity. Experimental efforts aimed at confirming these results are ongoing.

However, we are not limited to naturally-occurring parts when constructing new biomolecular circuits; synthetic biological tools allow us to adjust many properties of a given circuit component, including degradation rates and holoenzyme and ribosomal binding affinities. This model may then be used as a circuit design aid, to predict, for example, how much the ribosomal binding off-rate must be reduced in order to reduce ribosomal crosstalk. According to the model, with $[x]_{\text{tot}} = 1$ nM and all other parameters held fixed, a 50-fold decrease in $k_{\text{X}-}$ is needed for a complete elimination of ribosome loading effects (Fig. 5). The effects of variation in other circuit parameters may be similarly tested.

The model proposed here is a foundational one that may be easily expanded to include any number of genes. We have made no assumptions as to the timescales of various reactions or the relative concentrations of reacting species—assumptions that are commonly made in the application of Michaelis-Menten kinetics to biocircuit analyses. Furthermore, although we have used a mass-action deterministic approach in the construction of our model ODEs, the chemical reaction network formalism is easily adopted to stochastic simulation should the size of the reaction volumes of interest be decreased as to require it.

APPENDIX

A. Methods

Preparation of the cell-free TX-TL system was described previously [18], [19]. We used the reporter gene deGFP, a variant of eGFP more translatable in the cell-free toolbox. The transcriptional repressor *tetR* was expressed from a $\text{P}_{\text{LlacO}-1}$ regulatory part, composed of a strong promoter specific to σ^{70} flanked with two *lac* operators. Sigma factor F (σ^{28}), the secondary sigma factor used in the circuits, was expressed from a OR2-OR1-Pr regulatory part, a strong promoter specific to σ^{70} flanked with two lambda Cl operators. The untranslated RNA gene used is an RNA-based transcriptional regulator expressed off plasmid pAPA1256 from [24]. Data were collected over two separate experimental runs using 384-well plates and a Victor X3 plate reader set at 29°C. Simulations were done using Mathematica.

B. Model parameters

Values for reaction rates and other model parameters are listed in Table I. References are listed when available. When only a dissociation constant K_d ($= k_-/k_+$) could be found or measured, the on-rate (k_+) is taken to be 1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$, with the off-rate ($k_-$) set to $K_d \times k_+$. We note that in cell-free systems, the speeds of RNAP and ribosomes are slower than what has been measured *in vivo*.

TABLE I
MODEL PARAMETERS

Param.	Value
$k_{y,TX}$	0.05 s^{-1}
k_d	0.0012 s^{-1} [18]
$k_{y,TL}$	0.05 s^{-1}
$k_{x,TX}$	0.05 s^{-1}
$k_{x,TL}$	0.05 s^{-1}
$k_{\text{ES1}+}$	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
$k_{\text{ES1}-}$	4.3×10^{-4} s^{-1} [22]
$k_{\text{ES2}+}$	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
$k_{\text{ES2}-}$	1.2×10^{-3} s^{-1} [22]
$k_{\text{X}+}$	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
$k_{\text{X}-}$	350 s^{-1}
$k_{\text{Y}+}$	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
$k_{\text{Y}-}$	500 s^{-1}

Param.	Value
k_{mat}	0.003 s^{-1} [18]
k_{y+}	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
k_{y-}	0.017 s^{-1}
k_{x+}	1.7×10^6 $\text{M}^{-1}\text{s}^{-1}$
k_{x-}	0.017 s^{-1}
L_y	800 bp
L_x	800 bp
V_{TX}	3 bp·s^{-1} [25]
V_{TL}	2 bp·s^{-1} [25]
$[\text{R}]_{\text{tot}}$	1500 nM [26]
$[\text{E}]_{\text{tot}}$	100 nM [19]
$[\text{S1}]_{\text{tot}}$	30 nM [19]
$[y]_{\text{tot}}$	2 nM

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