

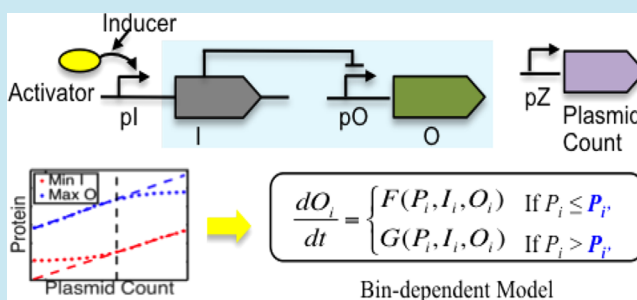
Modeling Genetic Circuit Behavior in Transiently Transfected Mammalian Cells

Junmin Wang,^{*,†} Samuel A. Isaacson,[‡] and Calin Belta[†][†]The Bioinformatics Graduate Program, Boston University, Boston, Massachusetts 02215, United States[‡]Department of Mathematics, Boston University, Boston, Massachusetts 02215, United States**S** Supporting Information

ABSTRACT: Binning cells by plasmid copy number is a common practice for analyzing transient transfection data. In many kinetic models of transfected cells, protein production rates are assumed to be proportional to plasmid copy number. The validity of this assumption in transiently transfected mammalian cells is not clear; models based on this assumption appear unable to reproduce experimental flow cytometry data robustly. We hypothesize that protein saturation at high plasmid copy number is a reason previous models break down and validate our hypothesis by comparing experimental data and a stochastic chemical kinetics model.

The model demonstrates that there are multiple distinct physical mechanisms that can cause saturation. On the basis of these observations, we develop a novel minimal bin-dependent ODE model that assumes different parameters for protein production in cells with low *versus* high numbers of plasmids. Compared to a traditional Hill-function-based model, the bin-dependent model requires only one additional parameter, but fits flow cytometry input–output data for individual modules up to twice as accurately. By composing together models of individually fit modules, we use the bin-dependent model to predict the behavior of six cascades and three feed-forward circuits. The bin-dependent models are shown to provide more accurate predictions on average than corresponding (composed) Hill-function-based models and predictions of comparable accuracy to EQuIP, while still providing a minimal ODE-based model that should be easy to integrate as a subcomponent within larger differential equation circuit models. Our analysis also demonstrates that accounting for batch effects is important in developing accurate composed models.

KEYWORDS: synthetic biology, modeling, transient transfection



In synthetic biology, there has been an increased use of transfection systems in mammalian cells in recent years. One reason for this increase is that transfection enables the production of important biomedical-related proteins, which can only become biologically active within mammalian cells.^{1–4} Transient transfection is a common method for the delivery of foreign genetic materials into mammalian cells.^{5–7} The transfected genetic materials utilize the cells' innate transcriptional and translational machineries to get expressed. Transiently transfected genes are only expressed temporarily, and do not become integrated into the host's genome. Compared with stable transfection, transient transfection offers faster expression of transfected genes, with higher expression levels. It also has lower cytotoxicity and induces no mutagenesis.^{3,8,9} It has been shown to be an effective technique for speeding up the screening of novel synthetic designs.¹⁰ These properties have motivated the investigation of transient transfection in mammalian synthetic biology.^{3,11}

Modern synthetic biology is inseparable from the computational models that guide the construction of synthetic networks.¹² One challenge in building such models for mammalian cells arises from the need for a more

comprehensive understanding of the cellular mechanisms underlying the transfection system.^{12,13} Another challenge is predicting the behavior of genetic circuits based on the behavior of the building blocks of the circuits, also known as modules.^{14–20} Chemical kinetic models have proven capable of describing circuit behavior in prokaryotic cells, which replicate foreign plasmids,^{21,22} and in stably transfected eukaryotic cells in which plasmids are genome-integrated.²³ Plasmid copy number is assumed fixed in both of these scenarios. For transiently transfected mammalian cells (TTMC), there is a large variation in plasmid copy numbers across a population.^{24,25} Binning cells by plasmid copy number is a common practice for analyzing flow cytometry data in this context (Figure 1(b)).^{26–28} Subpopulations of cells with similar plasmid counts can then be studied in groups (Figure 1(c)). Developing a modeling approach that is compatible with binning is a prerequisite to building predictive models for complex circuits in TTMC. Davidsohn *et al.* developed a traditional Hill-function-based model for TTMC,²⁶ where the

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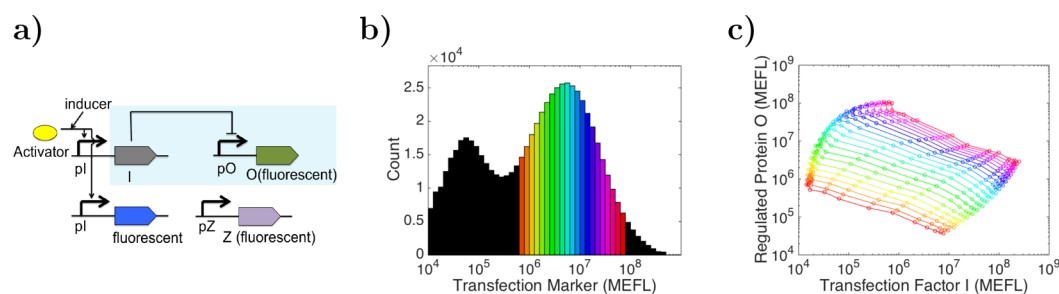


Figure 1. (a) Abstraction of a system comprised of a transfection marker and a module (in blue) encoding a transcriptional regulatory switch. See Figure S1(a) and ref 26 for more detailed illustrations. The induced (input) gene *I*, activated by an inducer, regulates the expression of *O*, the regulated (output) gene. *Z*, the transfection marker, is used to estimate plasmid copy number. (b) Distribution of the transfection marker. The black bins are ignored because they represent untransfected cells (data from ref 26). (c) Dose–response curves obtained from an experiment (data from ref 26). Averaged measurements binned by the expression level of *Z* are shown by color. Cells are separated into bins of width 0.1 on a log scale. Each curve corresponds to a different bin. The first bin, represented by the curve at the bottom, contains cells with the lowest plasmid counts. Each dot represents the average concentrations of the induced protein and the regulated protein within a bin at a certain inducer level. Concentrations of the induced and the regulated proteins have units of MEFL. Details about data generation and binning can be found in Supporting Information Section I.1.

rate of protein production is assumed to be proportional to the average plasmid copy number in each bin. Unfortunately, as they demonstrated, this model does not fit their flow cytometry data well.

In this work, we hypothesize that high plasmid copy number may cause saturation in the levels of expressed proteins, leading to the breakdown of traditional Hill-function-based models in this context. To validate our hypothesis, we study detailed two-stage gene expression models of a transient cotransfection system *via* the Gillespie algorithm,^{29,30} bin the simulated data by plasmid counts, and calculate the average protein concentrations within each bin. The agreement between the simulated results and the experimental data suggests that when physical gene expression parameters lie within a particular range, saturation of the rate of either transcription or translation can give rise to the observed saturated protein concentrations in experiments. These results suggest that the precise mechanism leading to the saturation of protein levels cannot be distinguished from just single-time flow cytometry measurements. To facilitate predictive modeling of circuits, we next develop a bin-dependent ordinary differential equation (ODE) model that splits flow cytometry data into two subsets based on plasmid copy number. This coarse-grained model can more accurately account for saturation in protein levels compared to standard Hill-function models, but avoids the need to specify a precise biological mechanism giving rise to saturation. For each plasmid copy number subset we fit separate kinetic parameters to the model, motivated by observations from the detailed stochastic model simulations. The resulting bin-dependent model is shown to outperform a traditional Hill-function-based model in reproducing input–output relationships for individual modules, yet requires only one additional parameter. By composing models fit to these *individual modules*, the bin-dependent model is also shown to predict the behavior of circuits composed of multiple modules more accurately than Hill-function-based models, while offering comparable accuracy to the EQuIP method of ref 26. As the bin-dependent model is itself described by standard chemical-kinetics type ODEs for chemical concentrations, it can be easily integrated as a subcomponent within other differential equation circuit models, and easily extended to include more biological details or features for any given system. Note, in the remainder,

species are denoted by Roman text, and concentrations by italicized text.

RESULTS AND DISCUSSION

Experimental Data. The first step in building our circuit model is to examine experimental data. In this paper we adopt a bottom-up approach to making circuits *via* the assembly of individual modules, where a module is defined as a single transcriptional regulatory switch, consisting of a transcription factor, the downstream regulated promoter and its gene. As an example of the types of modules we will use, consider a module comprising a fluorescent-reporter system involving three fluorescent genes: the induced (input) gene, the regulated (output) gene and the transfection marker (Figure 1(a)). The expression levels of the fluorescent genes are measured *via* flow cytometry, with the fluorescence intensities used as proxies for the concentrations of the fluorescent proteins. The induced gene is regulated by a constitutive activator protein, and an external inducer whose concentration can be controlled. The product of the induced gene serves as a transcription factor for the regulated gene, controlling the latter's expression of a fluorescent reporter. The induced gene's product is not fluorescent, but is measured by coexpressing a fluorescent reporter gene of a different color from a promoter that has the same sequence but is encoded on a different plasmid.³¹ The expression of the induced gene can be modulated by changing the amount of the inducer. Expression of the induced gene and the regulated gene at various inducer levels forms a dose–response curve (Figure 1(c)). In TTMC, expression levels are largely determined by the numbers of plasmids transfected in individual cells,^{25,26} which cannot be controlled and are highly variable across a population. It is, therefore, necessary to estimate the plasmid copy numbers so that the effect of variation in copy numbers on gene expression can be captured. This is often achieved by cotransfecting another constitutively expressed fluorescent protein, which serves as the transfection marker (Figure 1(a)). The induced gene, the regulated gene, and the transfection marker can be encoded on either one plasmid or separate plasmids. The former ensures that there is a one-to-one correspondence among the genes. In comparison, the latter is often preferred as separate plasmids can be absorbed by cells more readily due to smaller sizes, interference among the transcriptional units is minimized,

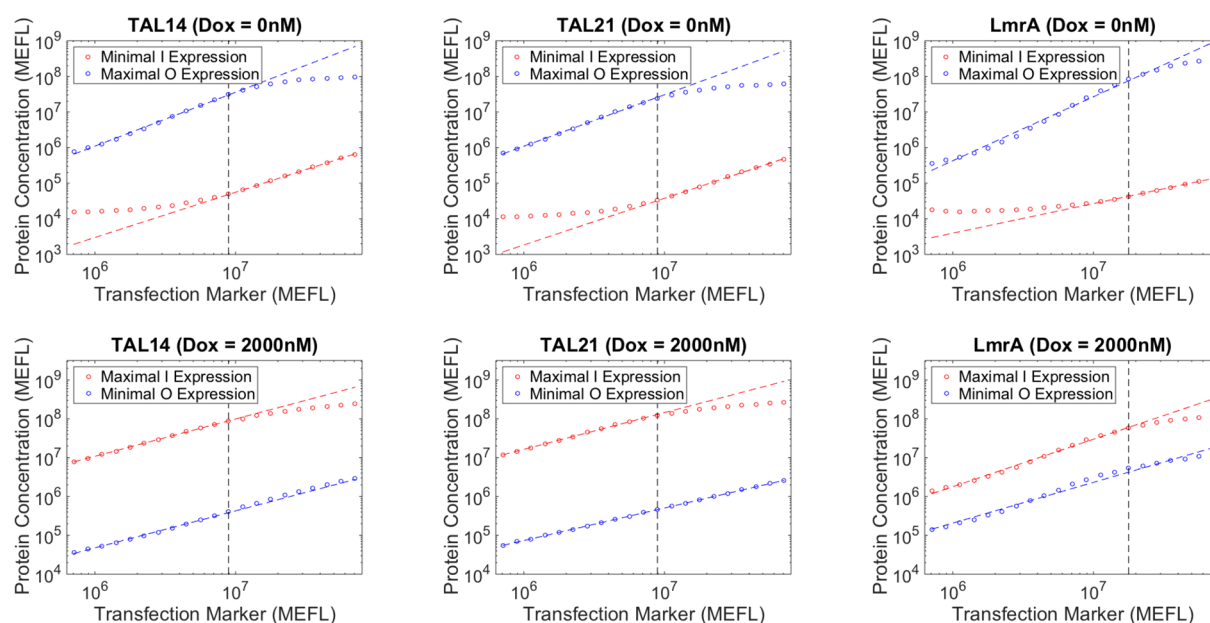


Figure 2. Maximal and minimal expressions of the induced gene I and the regulated gene O for TAL14, TAL21, and LmrA. In the figures, the x-axis corresponds to the concentration of the transfection marker, and the y-axis to the concentration of the input and the output proteins (here concentrations are in units of MEFL). Shown in red is the induced gene I, and in blue the regulated gene O. Each dot is the average protein concentration of cells from one bin. On the top row the circuit is induced at 0 nM; on the bottom row, 2000 nM. On the top row, least-squares regression lines are fit to red dots from 10^7 to $10^{7.9}$ MEFL (TAL14 and TAL21) or from $10^{7.3}$ to $10^{7.9}$ MEFL (LmrA), and to blue dots from $10^{5.8}$ to 10^7 MEFL (TAL14 and TAL21) or from $10^{5.8}$ to $10^{7.3}$ MEFL (LmrA). On the bottom row, least-squares regression lines are fit to red dots from $10^{5.8}$ to 10^7 MEFL (TAL14 and TAL21) or from $10^{5.8}$ to $10^{7.3}$ MEFL (LmrA), and to blue dots from $10^{5.8}$ to $10^{7.9}$ MEFL. The dots are calculated from the flow cytometry data of ref 26.

and the concentrations of individual proteins can be adjusted more easily.^{32,33} In what follows, we assume the transfection marker has been encoded on a separate plasmid for all models and experiments. We also assume the induced gene serves as an inhibitor of the regulated gene.

Fluorescence readings from flow cytometers can be converted to standard units of molecules of equivalent fluorescein (MEFL) via TASBE control.^{26,34,35} Standardized data are segmented into bins by plasmid counts so that subpopulations of cells with similar plasmid counts can be studied in groups (Figure 1(c)).^{26,27,36} Since flow cytometry measurements are typically log-normal distributed or a mixture of two log-normal distributions,^{37,38} binning is performed on a log scale to ensure that each bin contains relatively equal numbers of cells. The width of bins is selected depending on the resolution at which analysis is to be conducted. An example of binning can be found in Supporting Information Section 1.1. In this paper, we will focus on the average temporal behavior within each bin, with the goal of developing ODE models that can be directly parametrized from binned flow cytometry data.

Protein Concentration vs Plasmid Copy Number. Hill functions are commonly used to model transcriptional regulation in ODE models (Figure 1(a)) (see Supporting Information Section 3 for a mathematical definition of a Hill function). Davidsohn *et al.* developed a traditional Hill-function-based model to describe the time evolution of the induced and the regulated proteins in TTMC (Figure 1(a))^{26,39} (see Supporting Information Section 3). A key assumption of their model is that the log of the maximal production rate of the regulated protein is a linear function of the log of the transfection marker. This assumption is supported by findings of several other studies in different biological contexts.^{25,40} However, this assumption is only

partially supported by the experimental data in ref 26, shown here in Figure 2. When the induced gene is minimally induced (0 nM of inducer), *i.e.*, the regulated protein expressed without repressor, the log of the regulated protein's concentration grows proportionally to the log of the transfection marker between $10^{5.8}$ and 10^7 MEFL for TAL14 and TAL21 or between $10^{5.8}$ and $10^{7.3}$ MEFL for LmrA. When the induced gene is fully induced (2000 nM of inducer), the log of the induced protein's concentration also grows linearly in the log of the transfection marker between $10^{5.8}$ and 10^7 MEFL for TAL14 and TAL21 or between $10^{5.8}$ and $10^{7.3}$ MEFL for LmrA. Figure 2 also suggests that when either the induced gene or the regulated gene is maximally expressed, the concentrations of both the induced and the regulated proteins saturate starting from $10^{7.1}$ MEFL for TAL14 and TAL21 or $10^{7.4}$ MEFL for LmrA.

Furthermore, Figure 2 and the data in ref 26 suggest that when the induced gene is induced at 0 nM, the log of the induced protein's concentration is near-constant for low plasmid copy numbers.²⁶ When the induced gene is fully induced, *i.e.*, the regulated protein fully repressed, the log of the regulated protein's concentration grows linearly across all bins.

We now develop a detailed stochastic model of the plasmid system, similar to the one Davidsohn *et al.* constructed experimentally.²⁶ This model will enable us to explore possible mechanisms contributing to the observed saturation of protein concentrations at high plasmid copy number, as well as the near constant protein concentrations at low plasmid copy number. We do not attempt to fit this model to the single-time flow cytometry data directly as it is too complex to fit accurately without the incorporation of additional experimental measurements. Instead, *our purpose here is to use the stochastic model to gain a qualitative understanding of which*

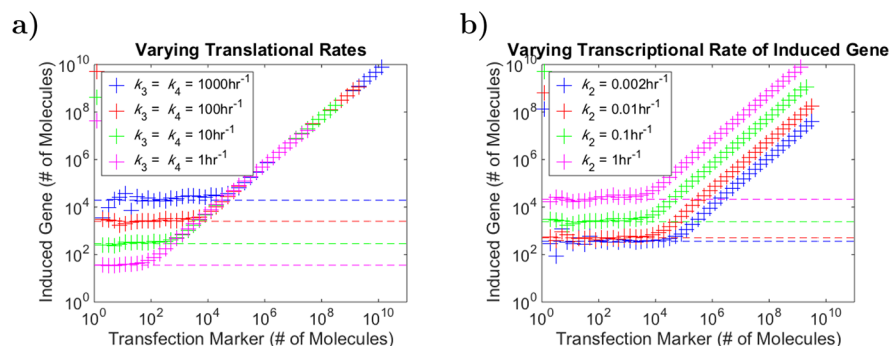
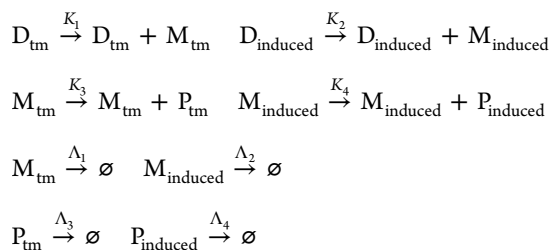


Figure 3. Simulations of our stochastic model suggest that either increasing translation rates (a) or decreasing transcriptional rates (b) can extend the near-constant induced gene levels at low copy plasmid numbers. The x -axis and y -axis stand for number of molecules of the transfection marker and the induced protein in each bin. Best fit horizontal lines are drawn for reference. (a) Comparison of models in which the translational rates decrease in order from 1000 to 1 molecule per mRNA per hour. (b) Comparison of models in which the transcriptional rate of D_{induced} increases from 0.002 to 1 molecule per plasmid per hour.

biological hypotheses, and what ranges of physical gene expression parameters, may contribute to the observed saturation effect. Our ultimate goal is to develop a simple model that qualitatively describes our limited set of data, avoiding further time-intensive experimental assays. Therefore, in the next subsection, we develop a more simplified ODE model that can be parametrized from just the limited flow cytometry data, building from the qualitative understanding of the two-plasmid system our stochastic model provides.

In our stochastic model, cells are cotransfected by a mixture of induced gene plasmids and transfection marker plasmids. We focus on the dynamics of the transfection marker and the induced gene, which are integrated on separate plasmids. The total initial number of plasmids transfected in a given cell is assumed to follow a log-normal distribution.^{26,37} This assumption is because the shape of the protein distribution is known to reflect the shape of the underlying plasmid distribution,⁴¹ and the protein distribution is often observed to be approximately log-normal.^{37,38} The conditional distribution of the number of each of the two types of plasmids, given the total number of plasmids, is assumed to be binomial.²⁶ This is because the plasmids we consider are assumed to be well-mixed, of relatively small and similar sizes, and hence indistinguishable for purposes of cotransfection.²⁶ In the remainder, we choose values for kinetic parameters such that they span the parameter distributions calculated from transcriptomics and proteomics data given in ref 42. We select parametric values for the initial plasmid distributions based on the polymerase chain reaction (PCR) findings of refs 24, 40, 43. The biochemical reactions in our model are shown below:



where D , M , and P stand for plasmid, mRNA, and protein. Subscript “tm” stands for the transfection marker, and “induced” for the induced gene that is cotransfected. Λ_i ($i = 1-4$) are first order degradation rate constants. Depending on the hypothesis underlying each model, K_i ($i = 1-4$) are defined either as normal first-order rate constants, where $K_1 = k_1 \cdot D_{\text{tm}}$,

and K_2 , K_3 , and K_4 are defined similarly, or as Michaelis–Menten (MM) equations, where a saturated K_1 is defined as $K_{1,\text{max}} \cdot \frac{D_{\text{tm}}}{D_{\text{tm}} + K_{D_{\text{tm}}}}$, and saturated K_2 , K_3 , and K_4 are defined similarly. $K_{1,\text{max}}$ represents the maximal value of K_1 , and $K_{D_{\text{tm}}}$ the half saturation constant. Further details of the models, including plasmid dilution mechanism and length of the simulation, can be found in Supporting Information Section 2.1. Using StochKit and GillesPy, for each fixed set of parameters we simulate this model using the Gillespie method 400 000 times.^{29,30,44,45} This is comparable to the number of experimental samples generated in ref 26. After simulation, we divide the simulated data based on the transfection marker into bins of width 0.2, which is comparable to values that are typically chosen in flow cytometry experiments.^{26,27,36} We then calculate the geometric mean of the induced protein’s concentrations for each bin.

To examine the mechanisms that contribute to the near-constant induced reporter concentrations at low plasmid copy number, and the saturating induced reporter concentrations at high plasmid copy number, we systematically vary individual or pairs of parameters while holding the remaining parameters constant. We begin by examining possible mechanisms that lead to near-constant induced reporter concentrations at low plasmid numbers, creating two cohorts of models. In each cohort we assume that K_i are normal first-order rate expressions, *i.e.*, $K_1 = k_1 D_{\text{tm}}$ with K_2 , K_3 , and K_4 defined similarly. The first cohort varies only the translational rate constants k_3 and k_4 , while the second cohort varies only the induced gene’s transcriptional rate, k_2 . Simulations of the stochastic model demonstrate that either increasing translation rates, or decreasing transcription rates, can lead to the observed constant induced reporter levels at low plasmid copy numbers (Figure 3).

We next investigate mechanisms that may cause protein concentrations to saturate at high plasmid copy numbers. Though the physical mechanism has not been proven, several experimental studies conclude that some steps of the transcription process may saturate in cells expressing large amounts of mRNA.^{46,47} It has also been suggested that the cationic liposomes used in transfection inhibit the process of transcription.⁴⁸ Hence, it is possible that a high concentration of liposomes (associated with high plasmid copy numbers) is also a mechanism that induces saturation in transcription rates. Motivated by these possible mechanisms, we modify our

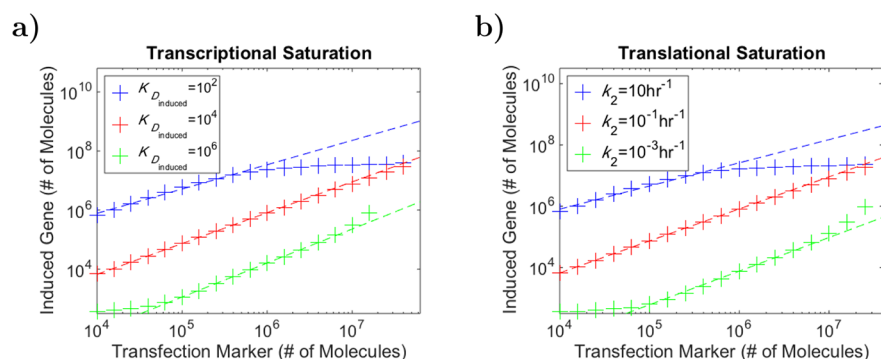


Figure 4. Simulations of our stochastic model suggest that either saturation of transcriptional kinetics (a) or saturation of translation kinetics (b) can lead to regimes where the induced gene reporter level saturates at high plasmid copy numbers. The x -axis and y -axis stand for number of molecules of the transfection marker and the induced protein in each bin. Least squares regression lines are drawn for reference. (a) Comparison of models built under the hypothesis of transcriptional saturation. The half saturation constant $K_{D_{\text{induced}}}$ increases in order from 10^2 to 10^6 molecules, and $K_{D_{\text{tm}}}$ is held fixed at 10^4 molecules. (b) Comparison of models built under the hypothesis of translational saturation. The transcriptional rate of the induced gene decreases in order from 10 to 10^{-3} molecule per plasmid per hour, and the transfection marker transcribes at a constant rate of 10^{-1} molecule per plasmid per hour.

stochastic model to incorporate saturation of transcriptional kinetics. We now take the transcription rates, K_1 and K_2 , to be given by saturating MM approximations with MM constants, $K_{D_{\text{tm}}}$ and $K_{D_{\text{induced}}}$ (see Supporting Information Section 2.1). Here smaller K_D values correspond to saturation beginning at lower plasmid copy numbers. By systematically varying both K_D values (see Supporting Information Section 2.1), we observe that transcriptional saturation may induce protein saturation when $K_{D_{\text{induced}}} \ll K_{D_{\text{tm}}}$ (see Figure 4(a)). That is, protein levels as a function of the amount of plasmid may saturate if the transcriptional rate of the induced reporter saturates at a lower level of plasmid than that at which the transcriptional rate of the transfection marker saturates.

Finally, we now investigate whether translational saturation can also induce saturation in protein levels at high plasmid copy numbers. Tachibana *et al.* presented experimental evidence which suggests that protein synthesis saturates when a large amount of mRNA is present.²⁴ Motivated by this study, we now consider a version of our stochastic model where the transcriptional rates $K_1 = k_1 D_{\text{tm}}$ and $K_2 = k_2 D_{\text{induced}}$ are nonsaturating first order reactions as in our first model, but the translation rates K_3 and K_4 are saturating MM approximations. Since the induced gene and the transfection marker are homologous fluorescent genes, we use the same maximal translation rates and same MM constants in K_3 and K_4 (see Supporting Information Section 2.1). This final version of our model suggests that under the hypothesis of translational saturation, protein reporter saturation can be observed if $k_2 \gg k_1$, *i.e.*, if the induced gene transcribes faster than the transfection marker's gene (see Figure 4(b)).

In summary, we have demonstrated two different physical mechanisms that may induce a near-constant level of the induced gene reporter at low plasmid copy numbers (high translation rates or low transcription rates). We thank a reviewer for pointing out another possible mechanism; that flow cytometry measurements at low plasmid copy numbers are susceptible to experimental noise such as autofluorescence, and instrumental limitations. In the absence of experimental noise, but under our modeling assumptions, our stochastic models demonstrate that even with linear production rates a near-constant level of the induced gene reporter will be

observed at low plasmid copy number. This arises as the normalized histograms of the plasmid encoding the transfection marker within each of the leftmost bins had relatively constant modes (see Figure S7 and Supporting Information Section 2.3 for more details).

Our models also demonstrate two different physical mechanisms that may induce a saturating level of induced gene reporter for high plasmid copy numbers (having the induced gene transcription kinetics saturate at lower plasmid levels than needed for saturation of the transfection marker gene transcription kinetics, or having translational saturation with the induced gene transcribing faster than the transfection marker's gene). Note that the results we have derived do not depend on the precise choice of bin width (see Figure S2 in Supporting Information). In Supporting Information Section 2.2 we show that these results persist when considering an alternative model for the initial plasmid distributions within cells. In Supporting Information Section 1.2 we explain why the observed saturation region at high plasmid copy number within the flow cytometry data is unlikely to be due to experimental noise.

Our analysis poses a challenge to the characterization of circuit behavior in TTMC. The stochastic models demonstrate there are multiple (physical) mechanisms that can explain the observed saturation (constant levels) of the induced gene reporter at high (low) plasmid copy numbers. Due to the complexity of these models it seems unlikely one could fit them, or even select which is most appropriate, from just single-time-point flow cytometry data.

Bin-Dependent ODE Model. Though mechanistic details cannot be disentangled from single-time flow cytometry measurements, characterization of modules remains a critical problem to be addressed. This is needed to enable the development of models that can predict the dynamics of circuits/pathways with more components, and which exhibit more complicated behaviors. To further this goal, we now develop a simple, phenomenological ODE model that can accurately describe single-time transient transfection flow cytometry data. While development of a more physically detailed model would be ideal, as shown in the last subsection it would require additional experimental data to be uniquely determined.

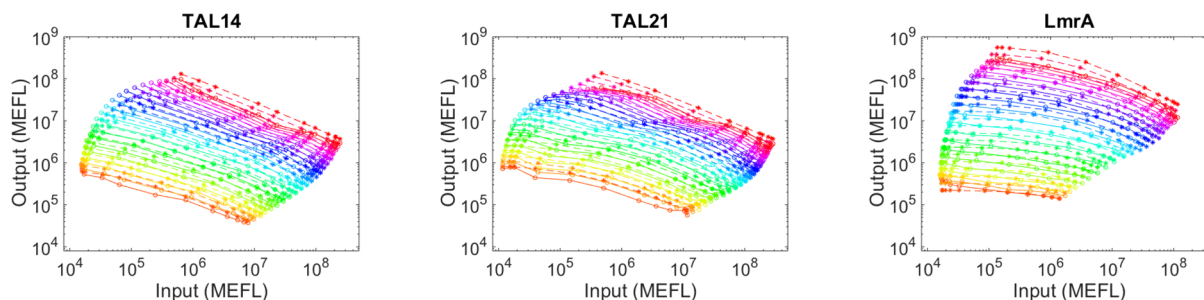


Figure 5. Comparison between experimental data and the traditional Hill-function-based TAL14, TAL21, and LmrA models. Plasmid copy number is shown by color. Solid lines are experimental data, and dashed lines are model fits. The experimental data in the plots are from ref 26.

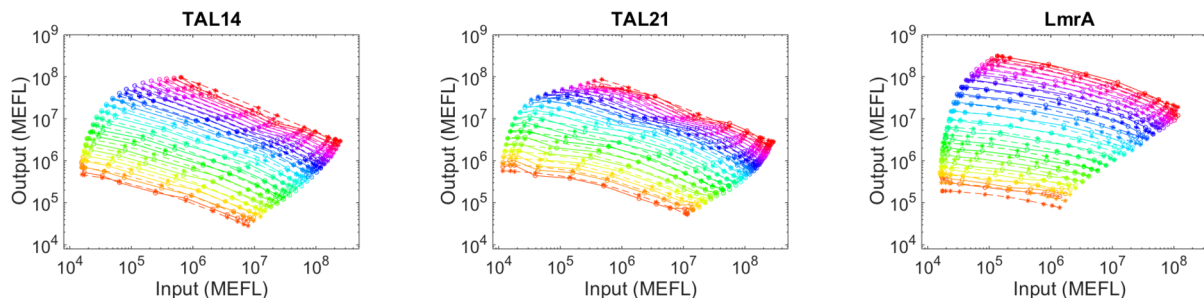


Figure 6. Comparison between experimental data and the bin-dependent TAL14, TAL21, and LmrA models. Plasmid copy number is shown by color. Solid lines are experimental data, and dashed lines are model fits. The experimental data in the plots are from ref 26.

To account for the observed saturation in protein concentration, we propose replacing the traditional Hill-function-based model (see Supporting Information Section 3) with a bin-dependent model. The bin-dependent model divides flow cytometry data into two subsets based on plasmid copy number, *i.e.*, one with and one without saturation.

$$\frac{dI_i}{dt} = \alpha_i \cdot \phi(t) - \lambda \cdot I_i \quad \phi(t) = \left(\frac{1}{2}\right)^{\lfloor t/T \rfloor}$$

$$\frac{dO_i}{dt} = \begin{cases} \beta \cdot \phi(t) \cdot \left(\frac{P_i}{P_1}\right)^f \cdot \left(\frac{1 - \gamma}{1 + \left(\frac{I_i}{d}\right)^h} + \gamma\right) - \lambda \cdot O_i & \text{if } P_i < P_{i'} \\ \beta \cdot \phi(t) \cdot \left(\frac{P_{i'}}{P_1}\right)^f \cdot \left(\frac{P_i}{P_{i'}}\right)^g \cdot \frac{1 - \gamma}{1 + \left(\frac{I_i}{d}\right)^h} \\ \quad + \beta \cdot \phi(t) \cdot \left(\frac{P_i}{P_1}\right)^f \cdot \gamma - \lambda \cdot O_i & \text{if } P_i \geq P_{i'} \end{cases} \quad (1)$$

where I_i and O_i are the concentrations of the input and the output in the i -th bin, and i' is the separating bin. The separating bin is chosen to be the bin at which average concentrations of the cotransfected protein switch from linear growth to saturating growth. α_i , the production rate of the induced protein in the i -th bin, is assumed time-invariant because I_i is induced by a constant concentration of inducer. We do not explicitly characterize the functional form of how α_i depends on the plasmid level as we simply fit a different value of α_i for each bin. $\phi(t)$ captures that the population-average plasmid counts decrease due to cell division over time.²⁶ T is length of the cell cycle; λ_I and λ_O are dilution/degradation rates of I and O . β is the maximal average production rate of

the regulated protein for cells in the first bin, *i.e.*, cells that have minimal plasmid counts P_1 . P_i is the midpoint of the i -th plasmid count bin. f and g capture the relationship between the concentrations of the transfection marker and the maximal production rates of the output protein for low and high copy numbers, respectively. The bin-dependent model only requires one additional parameter than a standard Hill-function-based model (see Supporting Information Section 3).

We fit the traditional Hill-function-based model (see Supporting Information Section 3) and the bin-dependent model (eq 1) to the TAL14, TAL21, and LmrA data sets from ref 26 for validation (TAL14, TAL21, and LmrA are names of the repressors).²⁶ Both models are simulated for 46 h since an average delay of 25 h in plasmid expression is expected.²⁶ Protein loss is assumed to arise purely from dilution, as both the input and output proteins are very stable on the time scale of the experiments.²⁶ We therefore take $\lambda_I = \lambda_O = \lambda$, and calculate them based on the length of the cell cycle, which spans approximately 20 h.²⁶ Davidsohn *et al.* constructed the circuits using the rtTA and GAL4/UAS system: the input (repressor) is activated by a constitutive rtTA protein and doxycycline, and expression of the output (EYFP), which is inhibited by the input, is driven by a constitutive Gal4 protein.²⁶ A detailed representation of the circuit structure can be found in Figure S1. rtTA and Gal4, which are indispensable for protein activation, are both constitutively expressed and are not considered as limiting factors for the production of the input and the output. Omitting rtTA and Gal4 leads to an abstraction of the circuit structure that can be studied by our models, as is shown in Figure 1(a). For the bin-dependent model, the bin that separates flow cytometry data into subsets of fast and slow protein production is chosen to be $10^{7.1}$ MEFL for TAL14 and TAL21, and $10^{7.4}$ MEFL for LmrA since in the data set, saturation in protein production is observed to the right of 10^7 MEFL and $10^{7.3}$ MEFL, respectively (Figure 2). Model fitting is implemented *via* minimizing the mean-squared

errors (MSE) between the log of observed and predicted concentrations of the regulated proteins (details of model fitting can be found in [Supporting Information Section 4](#)). We log-transform the concentrations to reduce the absolute errors that are often associated with measurements of large protein concentrations on a linear scale.⁴⁹ For our specific implementation, we use Matlab's GlobalSearch algorithm to locate the set of parameter values that produce the global minimum error.⁵⁰ The optimal parameter fits and the errors in the fit models are shown in [Tables S2 and S3 in Supporting Information Section 5](#), and the fit model values *versus* the experimental values of the fluorescent reporters are shown in [Figures 5 and 6](#). Our results suggest that the bin-dependent model fits the data well for all plasmid copy numbers despite having only one more parameter compared to the Hill-function-based model ([Table 1](#)).

Table 1. MSE of the Models

repressor	goodness of fit	
	Hill-function-based	bin-dependent
TAL14	0.013	0.004
TAL21	0.015	0.005
LmrA	0.020	0.009

We further compare the Hill-function-based model and the bin-dependent model *via* cross-validation. We conduct a 12-fold cross-validation by randomly dividing the flow cytometry data into 12 subsets of the same size, fitting the models separately on each combination of 11 subsets, and then testing the models on the single subsets that were left out.⁵¹ The fitting errors and the testing errors are then averaged over the 12 combinations of subsets. Our results suggest that both the fitting errors and the testing errors of the bin-dependent models are 1.5–2 times better than those of the Hill-function-based models ([Tables 2 and 3](#)). The bin-dependent model

Table 2. Averaged Fitting Errors of the Models within the 12-Fold Cross-Validation

repressor	fitting errors ^a	
	Hill-function-based	bin-dependent
TAL14	0.013	0.006
TAL21	0.017	0.009
LmrA	0.018	0.013

^aSee [Supporting Information Section 4](#) for the definition of fitting errors.

Table 3. Averaged Testing Errors of the Models within the 12-Fold Cross-Validation

repressor	testing errors ^b	
	Hill-function-based	bin-dependent
TAL14	0.014	0.007
TAL21	0.017	0.008
LmrA	0.019	0.013

^bSee [Supporting Information Section 4](#) for the definition of testing errors.

shows a less significant improvement for LmrA than for TAL14 and TAL21. A possible explanation is that for LmrA, the saturation effect is observed in six bins to the right of $10^{7.3}$ MEFL rather than in nine bins to the right of 10^7 MEFL. For

each repressor, we choose the model that produces the least testing error among 12 cross-validated models to be the best model. We evaluate the best models for each plasmid copy number. The results indicate that the bin-dependent models produce not only lower but also more consistent errors across all bins ([Figure 7](#)). The errors of the Hill-function-based models get large near 10^7 MEFL and $10^{7.8}$ MEFL for all repressors. This signals that there are patterns in the data that are not explained by the Hill-function-based models.⁵² The bin-dependent model produces larger errors for LmrA than for TALER repressors because there are slight indications of a near-constant region at low plasmid numbers for LmrA ([Figure 2](#)). In summary, we find that the bin-dependent model consistently provides significantly better fits to the experimental data than the Hill-function-based model.

Note, for high-plasmid-count subsets, our bin-dependent model assumes the log of the maximal protein production rate is approximated as a linear function of the log of the transfection marker. Although the relationship is arguably better fit by other functions, our assumption leads to a model with a good fit across the entire data set, while only requiring one additional parameter.

Modular Composition. To validate the predictive power of the bin-dependent model, we develop models for the six two-repressor cascades and three of the feed-forward circuits shown in [ref 26](#) (for which we were given the experimental data from [ref 26](#)). The exact structure of the cascades and the feed-forward circuits can be found in [Figures 3\(A\) and 5\(A\) of ref 26](#) or [Supporting Information Figures S1\(b\) and S1\(c\)](#), with [Figures 8\(a\) and 10\(a\)](#) providing abstractions that highlight the key parts of the circuits. A two-repressor cascade can be decoupled into two modules, with the output of the first module acting as the input of the second module ([Figure 8\(a\)](#)). Similarly, a feed-forward circuit can be decoupled into three modules ([Figure 10\(a\)](#)). The bin-dependent models for cascades and feed-forward circuits are constructed, and their agreement with experimental measurements are compared with that of the Hill-function-based and the EQUiP models developed in [ref 26](#). Specifically, we compare simulations of the circuit models to experimental data by measuring the differences between simulated and observed concentrations of EYFP 72 h post transfection (experimental data from [ref 26](#)). Full details of the experimental protocol can be found in [ref 26](#). The equations and parameters for the bin-dependent models can be found in [Supporting Information Section 6 and 8](#). The bin-dependent circuit models are developed by composing together the individual module models that were *individually fit* in the previous section. We *do not refit* the equations for each model to data for the complete two-module cascades or three-module feed-forward circuits. In this way we can assess how well models fit to individual modules can predict circuit behavior when composed together. To offer a comparable study to [ref 26](#), we use the parameters Davidsohn *et al.* fit for the Hill-function-based models for cascades.²⁶ Hill-function-based models for feed-forward circuits were not studied in [ref 26](#). We therefore construct Hill-function models of feed-forward circuits by composing the parametrized Hill-function models of individual modules developed in the previous section.

Like most biological data, calibrated flow cytometry is subject to batch effects. Parameters in the models of the modules need to be rescaled so that they are brought to the same scale before the models are connected into a circuit.

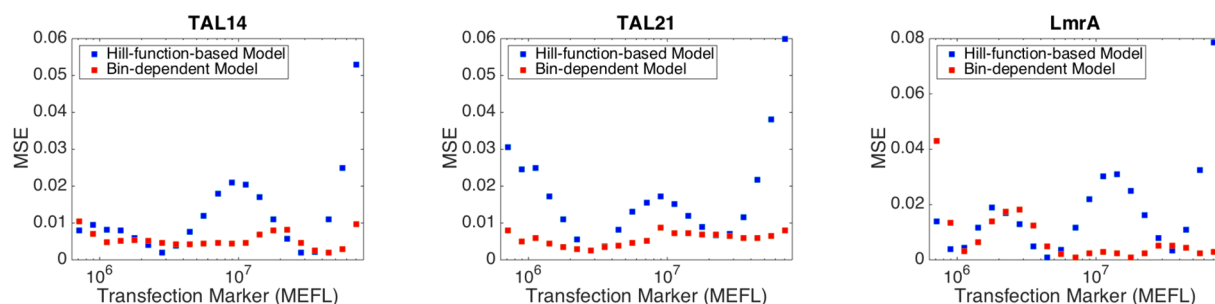


Figure 7. Testing errors of the best cross-validated models within each bin.

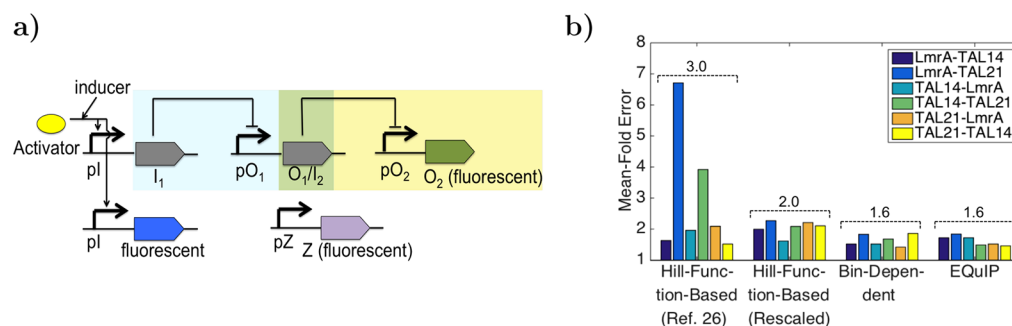


Figure 8. (a) Abstraction of the structure of the cascade. I_1 inhibits I_2/O_1 , which further inhibits O_2 . The expression of I_2/O_1 and O_2 is driven by a constitutive Gal4 protein and is omitted from the plot. The overlapping component of the modules is shown in the blended color. (b) Comparison of the mean-fold errors of the Hill-function-based models, with and without rescaling,²⁶ the bin-dependent models, and EQUIP²⁶ for each cascade. The experimental data the models are validated against are from ref 26. Numbers on top of the dotted lines represent the average mean-fold errors of six cascades.

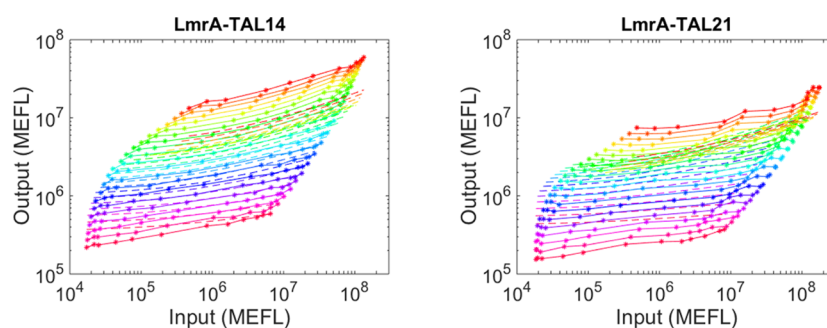


Figure 9. Comparison between experimental data and predictions made by the bin-dependent models for LmrA-TAL14 and LmrA-TAL21 cascades. Plasmid copy number is shown by color. Solid lines are experimental data, and dashed lines are model fits. Experimental data in the plots are from ref 26.

Rescaling is a two-step process, where systematic variation between modules is first removed to facilitate modular composition, and then reincorporated in the model to enable a fair comparison between model predictions and experimental data. The rescaling method we used can be found in Supporting Information Section 6. Davidsohn *et al.* determined the scaling factors among batches directly from experimental data by comparing the means and the tightness of the data of different batches for all the modules and cascades (note this calculation does not rely on EQUIP).²⁶ Values of the scaling factors for each input protein I , output protein O , and transfection marker can be found in Section 12 of the Supporting Information of ref 26. We use these scaling factors to rescale the parameters of the bin-dependent models since rescaling in our context is first-order linear compensation,²⁶ *i.e.*, there is no difference between rescaling the parameters and fitting the parameters to rescaled data. To understand the effect of cross-batch compensation on model predictions, we

also rescale the parameters of the Hill-function models provided in ref 26 by the same scaling factors. Since scaling factors for both modules and cascades are provided in ref 26, we perform both steps of rescaling amid construction of cascade models. The equations and parameters for the bin-dependent model and the rescaled Hill-function-based models for the cascades can be found in Supporting Information Section 6. For feed-forward circuits, we did not have experimental data with which to calculate scaling factors, and so we only performed parameter rescaling at the modular level. The equations and parameters for the feed-forward circuits can be found in Supporting Information Section 8.

The agreement between experimental measurements and model predictions for the six cascades is illustrated in Figure 9 and the figures of Supporting Information Section 7. For all six cascades, the bin-dependent model is able to capture the positive association between the input and the output (Figure 9). It also captures the buffer-like behavior of the cascades; *i.e.*,

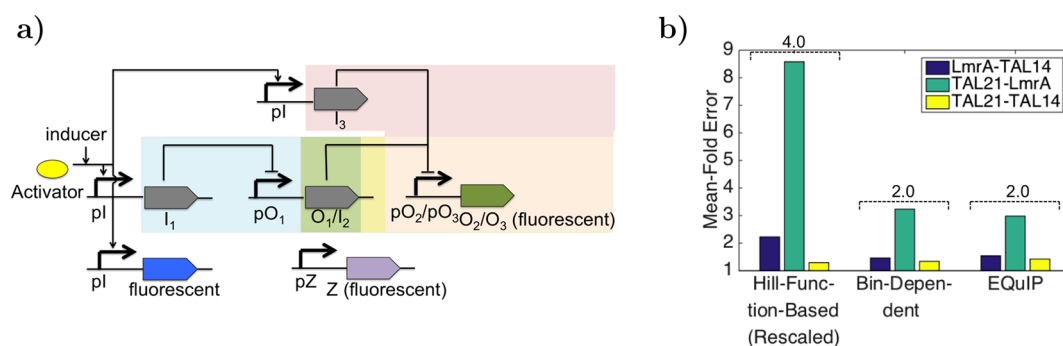


Figure 10. (a) Abstraction of the structure of the feed-forward circuit. I_1 inhibits I_2/O_1 , which further inhibits O_2 . I_3 inhibits O_3 . The expression of I_2/O_1 and O_2/O_3 is driven by a constitutive Gal4 protein and is omitted from the plot. The overlapping components of the modules are shown in the blended colors. (b) Comparison of the mean-fold errors of the rescaled Hill-function-based models, the bin-dependent models, and EQuIP²⁶ for each feed-forward circuit. The experimental data the models are validated against are from ref 26. Numbers on top of the dotted lines represent the average mean-fold errors of three feed-forward circuits.

the dynamic range of the output is narrower compared to that of the input due to low cooperativity of the regulatory modules (Figure 9 and Supporting Information Section 7).⁵³

To further investigate how well our composed circuit models fit the experimental data, we examined the average mean fold error, defined as the average over all six cascades of the mean-fold errors over all induction levels of each individual cascade (see Supporting Information Section 4 for details and formulas). The rescaled bin-dependent model is found to outperform the Hill-function-based model presented in ref 26, with an average mean-fold error of 1.6 fold for the former *vs* 3.0 fold for the latter. The 1.6 fold average error of the bin-dependent model also outperforms the average error of the Hill-function model with rescaling, which was found to be 2.0 fold (Figure 8(b)). This indicates that inconsistent scales due to batch effects contribute significantly to the magnitude and the inconsistency of the errors. In addition, the rescaled bin-dependent model also produces smaller mean-fold errors than the rescaled Hill-function model for all individual cascades (Figure 8(b)). In ref 54, we examined a different rescaled Hill-function model, based on composing the Hill-function models we parametrized for individual modules in the preceding subsection. For this rescaled Hill-function model we observed an average mean fold error of 1.8.⁵⁴ As we fit the Hill-function model using a different optimization routine than used in ref 26, this illustrates that the parameter estimation procedure can also influence the relative accuracy of different models. Finally, we note that the accuracy of the bin-dependent model varies relative to EQuIP, achieving a smaller mean-fold error for some cascades and larger error others (see Figure 8(b)). The average over all six cascades is the same as EQuIP (1.6), which is considered high accuracy based on results reported in the literature.^{26,27,34,55,56}

Besides cascades, the bin-dependent model also facilitates relatively accurate predictions for feed-forward circuits. The agreement between experimental measurements and model predictions for the three feed-forward circuits is illustrated in the figures of Supporting Information Section 9. The average error over all three feed-forward circuits is the same as EQuIP (2.0) and is much lower than the rescaled Hill-function-based model (4.0) (Figure 10(b)). The bin-dependent model captures the qualitative behavior of the circuit—the output is weakly affected by a change in the input at low inducer levels due to two opposing regulations: $I_1 \dashv O_1 \dashv O_2$ and $I_3 \dashv O_3$, and negatively associated with the input at high inducer levels

as inhibition becomes the dominant force. The relatively large error for the TAL21-LmrA circuit is likely to be batch-specific, as measurements of the output are below 10^4 MEFL at low plasmid copy number (see Figure S10). Note, such low levels of MEFL are not observed in any of the other circuit data sets.

Despite the relatively high accuracy of the bin-dependent model, we note that the simplicity of its representation of the gene expression process may in some contexts sacrifice accuracy. Figure 9 shows an under-prediction for two out of six cascades, the cause for which may be attributed to the non-negligible amount of time over which transcription and translation take place. The time lag between expression of I_2/O_1 and O_2 may be better captured by delay differential equations (DDE).

CONCLUSIONS

We have developed a bin-dependent ODE model that describes regulatory mechanisms *via* the use of standard Hill function type terms, while offering comparable accuracy to the EQuIP model of ref 26. Parameterized, bin-dependent models of individual modules should be relatively straightforward to integrate as subcomponents within larger existing ODE and DDE models of circuits. Moreover, it should also be relatively straightforward to modify a parametrized bin-dependent model to incorporate *additional*, previously characterized regulatory components (*i.e.*, for studying promoters coregulated by multiple transcription factors). In this way we expect that bin-dependent models for individual modules should be able to be composed with a variety of existing, well-characterized differential equation models that describe components of synthetic and systems biology networks.

Another benefit to the bin-dependent-model-based approach is that it is fairly robust to sampling noise in experimental data. The input–output data sets, which the ODE models are fit to, comprise the geometric means of measured protein concentrations within each bin. These data points may not be well separated, and hence appear noisy, when using sparse flow cytometry data sets. The model fitting step helps overcome this sampling noise by using deterministic ODEs based on widely used biochemical relationships (such as Hill-functions).

The bin-dependent model presented here establishes a framework for characterizing fundamental synthetic constructs and predicting circuit behaviors quantitatively in TTMC. As we demonstrated with the stochastic model, there are different mechanisms that may contribute to saturation in protein

production, a common phenomenon in TTMC. The value of the bin-dependent model lies in both its easy integrability with other differential equation models, and in its ability to describe the saturation effect in flow cytometry data accurately without specifying precise mechanistic details for how saturation occurs. The method presented here should be applicable to similar flow cytometry data sets, allowing the possibility to construct a well-characterized library of *in silico* models for regulatory switches. The quantitative parameters of such regulatory switches could then be used in constructing new predictive models for the behaviors of more complicated circuits. Our work represents one more step toward building a systematic workflow that can guide circuit design in TTMC.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00166.

Experimental details, two-stage gene expression models, Hill-function-based models, details of model fitting, optimal parameter fits of ODE models, models for cascades, cascade predictions, models for feed-forward circuits, and feed-forward circuit predictions (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: dawang@bu.edu.

ORCID

Junmin Wang: 0000-0003-3413-2004

Author Contributions

J.W. analyzed data, developed and conducted computational analysis, and wrote the manuscript. S.A.I. and C.B. helped develop the computation framework and models, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TTMC, transiently transfected mammalian cells; ODE, ordinary differential equations; PCR, polymerase chain reaction; MEFL, molecules of equivalent fluorescein; MM, Michaelis–Menten; DDE, delay differential equations.

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