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proTeOn and proTeOff, New Protein Devices That Inducibly Activate Bacterial Gene Expression

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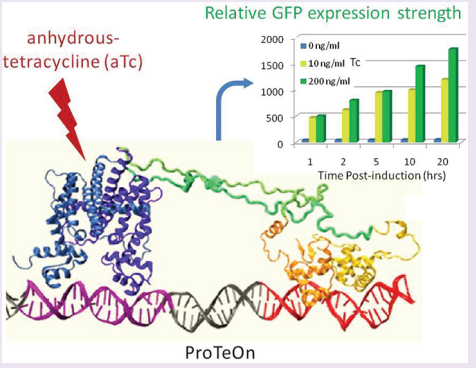
Supporting Information

ABSTRACT: Using an original workflow, we have modeled, constructed, and characterized two new molecular devices that inducibly activate gene expression in *Escherichia coli*. The devices, prokaryotic-TetOn and prokaryotic-TetOff, were built by fusing an inducible DNA-binding protein domain to a transcription activation domain and constructing a complementary synthetic promoter sequence through which they could control downstream gene expression. In particular, the transactivators were built using variants of the tetracycline repressor, TetR, and the transactivating domain of the LuxR activator. The complementary promoter sequence included TetR's operator, *tetO*, and elements of the *lux* promoter. These specific protein domains and their operator sites were chosen as they have been thoroughly studied and well characterized. First, our methodology began with optimizing the geometry of the molecular components using molecular modeling. We did so to achieve an unprecedented combination of controllable and transactivating function in bacterial organisms. The devices were then built to activate the expression of green fluorescent protein. Their unique function was found to be robustly tight and activating many-fold increases of expressed gene levels, as measured by flow cytometry experiments. The devices were further characterized with stochastic kinetic models. The new devices presented herein may become useful additions to the molecular toolboxes used by biologists to control bacterial gene expression. The methodology used may also be a foundation for the design, development, and characterization of a library of such devices and more complex gene regulatory networks.



Controllable gene regulatory systems are the subject of continued, intense investigations. In addition to being critically important in explaining how phenotypes emerge from genotypes in living organisms, their components are rapidly becoming integral in efforts toward engineered gene expression control.[1−10] Two well-studied example regulatory systems are the tetracycline (*tet*) and luminescence (*lux*) operons. The tetracycline repressor protein, TetR, and numerous TetR derivatives afford a remarkably robust function of inducible repression and derepression of gene expression. Thus, they have been employed in numerous synthetic biology and bioengineering applications.[11−14] Switching gene expression on and off by TetR and its derivatives simply depends on the presence or absence of the antibiotic tetracycline (Tc). The *lux* operon's transcription activator protein, LuxR, is another attractive controllable molecular component for engineering applications. When induced, it activates gene overexpression by recruiting transcriptional machinery to the promoter. In addition, a number of variant activators have been identified that upregulate transcription over a range of strengths with varied dependence on inducer molecules.[15−18]

Herein, we present two novel synthetic molecular devices that inducibly upregulate bacterial gene expression. We have designed, built, and characterized these devices using a unique methodology that is based on both computational and experimental efforts. Both devices are composed of an inducible DNA-binding domain and a transcription activation domain. In particular, TetR and the reverse-TetR (rTetR) derivative were chosen for the inducible domains while the transactivating domain of LuxR was incorporated for transcription upregulation.[19−22] TetR protein and its derivative, rTetR, dissociate from and bind to the *tetO* operator sequence in response to Tc, respectively.[19−21] This behavior made them attractive candidates for controlling our devices. In addition, LuxRΔN(2−162) (LuxRΔN) is the C-terminal, constitutive transactivating domain of the full length LuxR activator. Strong, constitutive transcription activation is achieved with this variant lacking the N-terminal residues 2−162 of the full protein.[22] These specific protein components were selected as they have been thoroughly studied and well characterized. Specifically, the protein:DNA-operator crystal structures for both domains as well as the kinetic parameters that govern their protein:protein and protein:DNA interactions were available in the literature.

We designed, constructed, and characterized the two new devices using an original workflow that integrates experimental synthetic biology, molecular modeling, and stochastic reaction kinetic simulations. This methodology may be directly implemented for the

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development of other biological devices and larger regulatory networks. The devices we present here, prokaryotic-TetOn and prokaryotic-TetOff (proTeOn and proTeOff), function in an Tc-dependent manner: the proTeOn synthetic protein (PROTEON) activates gene expression in the presence of Tc, and the proTeOff synthetic protein (PROTEOFF) activates expression in the absence of Tc. Orders of magnitude higher expression levels are observed in the activating states compared to the basal expression levels of both systems. With these new devices, there is now a dial of activated expression to complement those of basal and repressed gene expression.

While the proTeOn and proTeOff systems are functionally unique, their designs are conceptually inspired by the widely used TetOn and TetOff systems, which function in eukaryotic systems.[23,24] Gossen and co-workers fused the TetR protein to a mammalian transactivating domain, building molecular devices that activate eukaryotic gene expression in a Tc-dependent manner. To our knowledge, there were no prokaryotic transcription factors homologous to TetOn or TetOff prior to the construction of proTeOn and proTeOff. Additionally, our kind of engineering approach to synthetic biology, using molecular dynamic simulations to guide system design and stochastic simulations to enhance system characterization, has not been previously reported in the literature.

The proposed behaviors of proTeOn and proTeOff are illustrated in Figure 1. As shown, anhydrotetracycline (aTc), a Tc derivative, was used as the inducer in our experiments. For proTeOn, in the absence of aTc, the inducible DNA binding domain, rTetR, does not bind the *tetO* operator and the PROTEON protein does not upregulate the target gene, green fluorescence protein (*gfp*).[21] Upon activation with aTc, rTetR binds the inducer, undergoes a conformational change and binds *tetO*.[21] This binding brings LuxRΔNnear its operator site, allowing it to bind *luxbox* and upregulate *gfp* transcription through RNA polymerase (RNApol) recruitment to the promoter.[25−27] For proTeOff, in the absence of aTc, its inducible DNA binding domain, TetR, binds *tetO*.[2,20,28,29] This binding brings LuxRΔNclose to its operator site, permitting it to bind *luxbox*, and upregulate *gfp* transcription.[25−27] After the addition of aTc, TetR binds the small molecule, undergoes a conformational change and releases *tetO*.[2,20,28,29] Upon dissociation of TetR:*tetO*, the LuxRΔN:*luxbox* interaction is destabilized and transcription upregulation is terminated.

To achieve these phenotypes, we first used molecular modeling to design the geometry of the synthetic transactivator/promoter pairs of each system. The important feature of proTeOn and proTeOff on which we focused was the optimized interaction between the inducible synthetic transactivator protein that upregulates gene expression and its complementary synthetic promoter. Next, we built and characterized both systems experimentally. Last, implementing stochastic simulations, we explored the system dynamics and quantified unknown kinetic parameters of key interactions. The workflow is detailed in the Methods section. In the Results and Discussion section, we begin by presenting elements of the model-driven design of the devices.

ProTeOn and proTeOff can be applied to robustly manage prokaryotic gene expression. Variant systems and more complex inducible gene regulatory networks can also be designed and constructed using this workflow and the proTeOn and proTeOff systems as a foundation.

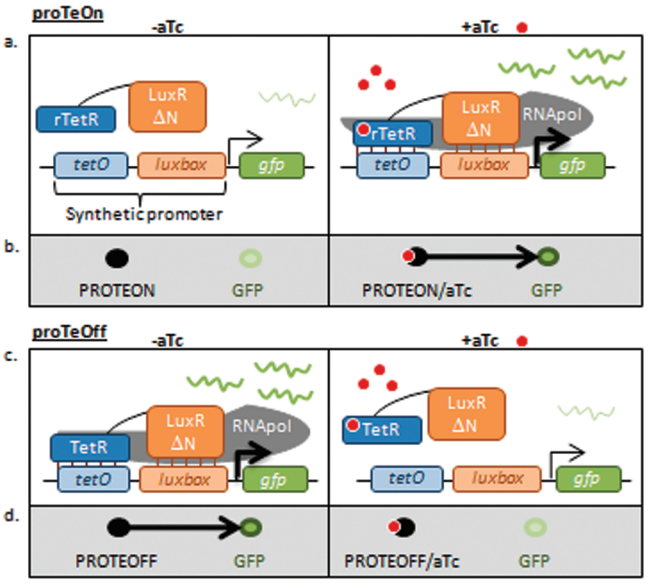

Figure 1. Function of proTeOn and proTeOff. (a) proTeOn behavior. In the absence of aTc, rTetR does not bind *tetO* and PROTEON does not upregulate *gfp*. Upon activation with aTc, rTetR binds the inducer, undergoes a conformational change, and binds *tetO*, bringing LuxRΔNnear its operator site to bind *luxbox* and upregulate *gfp* transcription through RNApol recruitment to the promoter. (b) proTeOn logic. In the absence of aTc, PROTEON does not control GFP expression. Upon induction with aTc, PROTEON upregulates GFP. (c) proTeOff behavior. In the absence of aTc, TetR binds to *tetO*, bringing LuxRΔNnear its operator site to bind *luxbox* and upregulate *gfp* transcription through RNApol recruitment. After the addition of aTc, TetR binds to the small molecule, undergoes a conformational change, and releases *tetO*. Upon dissociation of TetR:*tetO*, the LuxRΔN:*luxbox* interaction is destabilized, and upregulation by RNApol recruitment is terminated. (d) proTeOff logic. In the absence of aTc, PROTEOFF upregulates GFP expression. Upon the addition of aTc, PROTEOFF's control on GFP expression is terminated.

RESULTS AND DISCUSSION

proTeOn and proTeOff Designs. For proTeOn and proTeOff to perform efficiently, both protein domains (TetR/rTetR and LuxRΔN) must readily bind their operator sequences upon induction, LuxRΔNmust recruit RNApol to the promoter, and then RNApol must bind to the promoter and begin transcription. Numerous length and space requirements are associated with each of these steps, and we systematically accommodated each of them by designing the devices prior to construction. To satisfy the above geometric constraints, we built molecular models of each system using MOE[30] and NAMD.[31] With the chosen protein components and DNA operator sites as starting points, the entire DNA promoter sequence and the linker amino acid sequence were designed in tandem.

First, the synthetic promoter for both systems was designed, and the optimized sequence is annotated in Figure 2a. The major aim of the promoter design was to minimize the distance between the two DNA-bound protein domains while maintaining favorable binding between the domains and their operators. The interactions between RNApol and the specific residues of the promoter to which it binds (the UP element and the −10 region) had to be accommodated as well. To meet these aims, the operator sites were integrated into the promoter such that the two domains bound along the same face of the DNA (Figure 2b,

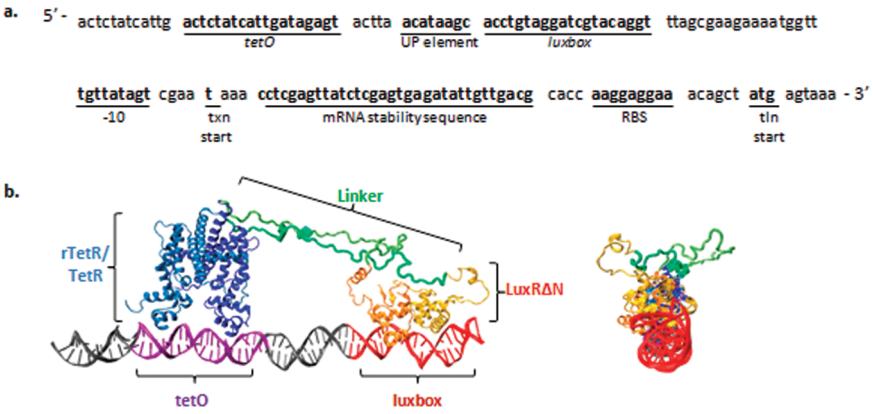

Figure 2. proTeOn and proTeOff systems' design. (a) proTeOn and proTeOff synthetic promoter sequence. Both PROTEON and PROTEOFF bind and recruit RNApol to this synthetic promoter sequence. Moving from 5′ to 3′: the rTetR/TetR protein domain binds *tetO*, RNApol binds the UP element and the −10 region, LuxRΔNbinds the *luxbox*, the mRNA stability sequence stabilizes the mRNA transcript, and ribosomes bind the RBS of this resulting mRNA message. (b) proTeOn molecular model. Both proTeOn and proTeOff are designed to assemble as shown. The inducible DNA binding domain (rTetR/TetR, blue) binds the *tetO* operator (purple), and the transcription activator domain (LuxRΔN, orange) binds the *luxbox* (red). The two domains bind their operators along the same face of the DNA double helix and are connected (TetR/rTetR's C-terminus to LuxRΔN's N-terminus) by a linker peptide (green).

side view) while maintaining a minimum distance between the two operator sites. The final design was scrutinized to ensure that neither DNA-bound protein domain was encroaching the residues of the UP element or −10 region as this would inhibit efficient RNApol recruitment.

Next, we optimized the peptide linker required to connect the two DNA-bound protein domains (TetR/rTetR's C-terminus to LuxRΔN's N-terminus). We did this by assuming it resembles a polymer in good solution and choosing a length that would minimize the entropic elastic tension effects. The optimal linker length was determined to be 150 Å, and the final sequence is the product of 5 repeats of a 9-amino acid subunit, ARTQYSESM. The individual subunits are connected by single glycine residues, and this 49-amino acid peptide is flanked by 3 glycine residues on each side. These were chosen to ensure linker flexibility and promote correct protein domain folding. The final optimized proTeOn system, meeting all geometric requirements, is illustrated in Figure 2b.

Characterization of proTeOn and proTetOff Behavior. To investigate the behavior of both systems and determine the optimal conditions and applications of each device, we performed two sets of experiments. First, cells expressing the transactivators were induced with a range of aTc concentrations. Second, cultures were primed with these aTc amounts prior to transactivator expression. In both experiments we monitored the resulting differential expression of the target gene, *gfp*, over time. Induction experiments were repeated for all inducer concentrations and time points. The reported trends were observed across the replicates.

proTeOn. Upon administration of aTc, proTeOn upregulates target genes by 1 h post-treatment and achieves 15-fold upregulation through long times. With a low (10 ng/mL) aTc concentration, proTeOn can achieve steady state expression of targets 10-fold over untreated cultures just 5 h post-treatment. With a high (200 ng/mL) aTc concentration, target gene expression can reach steady state by 10 h post-treatment at levels 15-fold over untreated cultures. These behaviors are displayed as both expression means and population distributions in Figure 3a. Basal GFP expression levels are observed from the synthetic promoter in the absence of aTc. There is no repressed state for the proTeOn system as presented here.

PROTEON expressed in the presence of aTc gives target gene upregulation within 2 h. Steady state upregulation is achieved by 5 h after the transactivator's initial expression, with GFP expression 10-fold over uninduced controls. PROTEON is thus rapidly transcribed, translated, and folded and becomes functional when under the control of a free, nonrepressed, promoter. This is shown in Figure 3b. The mean behaviors taken across biological replicates are discussed in the Supporting Information and shown in Supporting Figure 3.

Applications of proTeOn would be appropriate in systems when quick bursts or long-term gene upregulation is desired. Such behavior is governed by rTetR's responses to induction with aTc. This control is achievable with inducer levels as low as 10 ng/mL aTc. aTc concentrations outside of this range, or scaled throughout an experiment, may be useful depending upon the desired level of activation. Notably, long-term target gene upregulation increases with aTc up to 200 ng/mL. Thus, inducing with a range of aTc concentrations, proTeOn can be tuned to robustly upregulate gene expression for both acute and long time scales.

proTeOff. In the absence of aTc, proTeOff activates target gene expression. Upon administration of aTc, upregulation can be reduced by half just 1 h post-treatment. Steady state target gene expression is only one-half and one-fifth that of untreated samples with low and high aTc concentrations, respectively. This is observed as early as 5 h and remains through long times. While activation by proTeOff is significantly reduced with low aTc concentrations, a subset of activator proteins still appears to bind the promoter and upregulate transcription. With GFP levels at only one-fifth of the untreated controls, we consider the low GFP expression observed with 200 ng/mL aTc to be the synthetic promoter's basal expression level. These low basal expression levels are observed through long times. These behaviors are presented also as expression means and population distributions in Figure 4a. Please note that there is also no repressed state for the proTeOff system as presented here.

PROTEOFF expressed in the absence of aTc leads to target gene upregulation within 1 h and maintains it through long times. PROTEOFF is therefore quickly transcribed, translated, and

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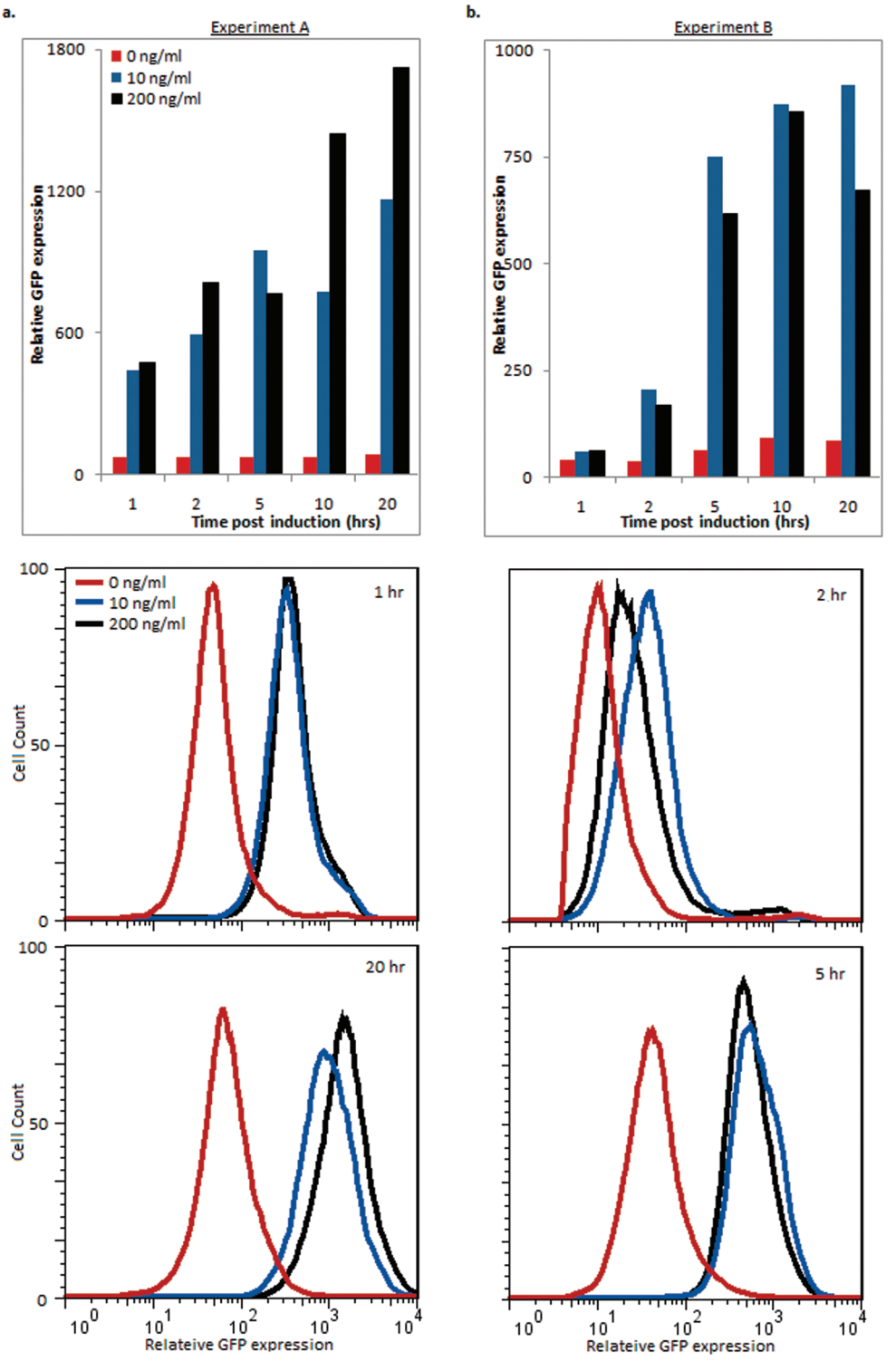

Figure 3. proTeOn system phenotype analysis by flow cytometry. Mean GFP expression and expression distribution were analyzed by flow cytometry 1, 2, 5, 10, and 20 h post-treatment for both experimental set-ups as described. Induction experiments were repeated for all inducer concentrations and time points, and the reported trends were observed across replicates. (a) Cells expressing PROTEON were induced with 0, 10, and 200 ng/mL aTc. By 1 h after aTc treatment, proTeOn upregulates GFP expression. Steady state expression is reached by 5 and 10 h with low and high aTc concentrations (respectively) and maintained through 20 h. Maximum overexpression is 10- and 15-fold above uninduced controls with low and high aTc levels, respectively. (b) PROTEON was expressed in cells precultured with 0, 10, and 200 ng/mL aTc. In low and high aTc, significant proTeOn activity is observed 2 h after PROTEON expression is induced. Steady state activity is achieved by 5 h and maintained through 20 h. Maximum upregulation is 10-fold above uninduced controls with both aTc concentrations.

folded and is functional when under the control of a free, nonrepressed promoter. In the presence of low and high aTc concentrations, the device activity is maintained at less than one-fifth of untreated cultures 1 h after the PROTEOFF's initial

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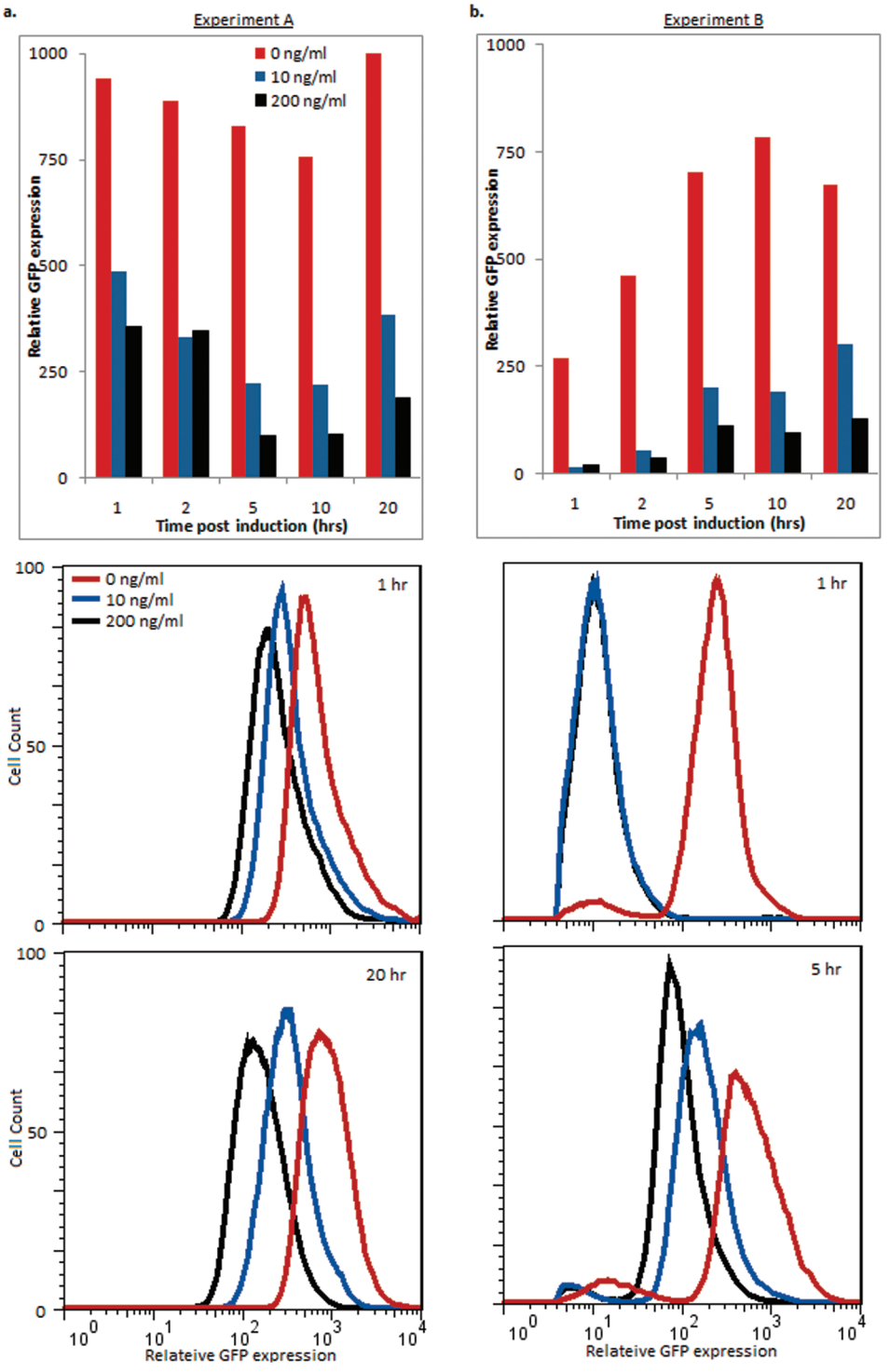

Figure 4. proTeOff system phenotype analysis by flow cytometry. Mean GFP expression and expression distribution were analyzed by flow cytometry 1, 2, 5, 10, and 20 h post-treatment for both experimental set-ups as described. Induction experiments were repeated for all inducer concentrations and time points, and the reported trends were observed across replicates. (a) With 0 ng/mL, proTeOff upregulates GFP expression. With 10 and 200 ng/mL aTc, expression is reduced to half that of untreated samples by 1 h. Steady state expression is reached by 5 h and maintained through 20 h post-treatment. Minimum expression is one-half and one-fifth that of untreated samples with low and high aTc, respectively. (b) PROTEOFF was expressed in cells precultured with 0, 10, and 200 ng/mL aTc. In 0 ng/mL aTc, proTeOff activity is observed (*via* GFP upregulation) 1 h after PROTEOFF expression is induced. In low and high aTc, reduced proTeOff activity is observed across all times and steady state activity is achieved by 5 h after initial PROTEOFF expression. High aTc levels maintain the reduced device activity and thus basal GFP expression. Low concentrations permit GFP levels to rise to one-third that of untreated cultures. Both of these behaviors are observed through 20 h.

expression. An aTc concentration of 200 ng/mL maintains this very low basal activity, thus basal gene expression, through long times. Treatment with low aTc allows the target's expression to rise to one-third of untreated cultures by 5 h after the

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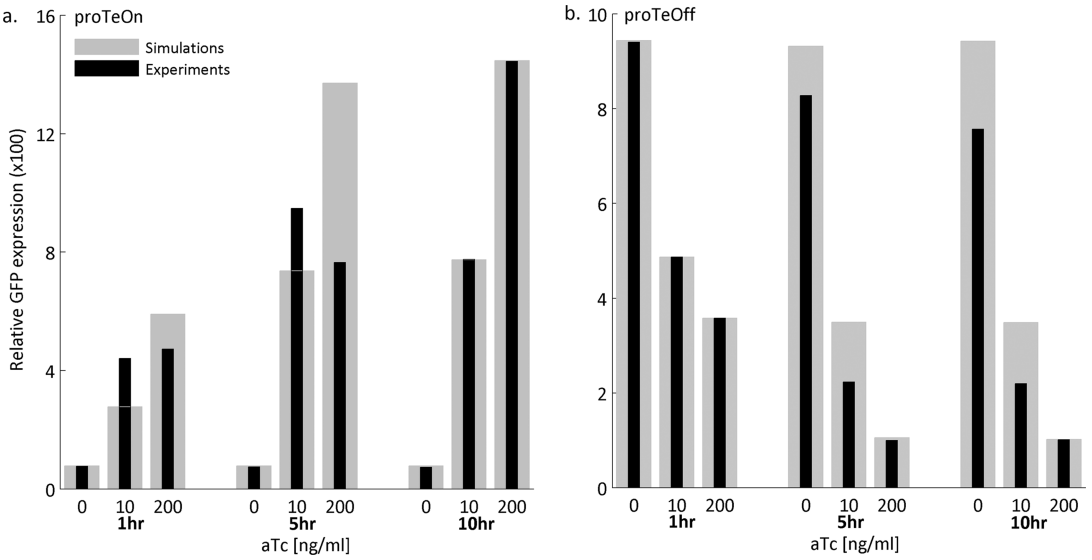

Figure 5. proTeOn and proTeOff average GFP expression by stochastic simulations. Simulation and experimental results of the average GFP expression at 1, 5, and 10 h, when 0, 10, and 200 ng/mL aTc is administered. Overall, a good agreement between experimental and computational results is observed. (a) proTeOn average culture GFP. (b) proTeOff average culture GFP.

transactivator's initial expression, where it remains through long times. This elevation is due to a subset of free PROTEOFFs binding to the promoter and recruiting RNApol. These data are shown in Figure 4b. The mean behaviors taken across biological replicates are discussed in the Supporting Information and shown in Supporting Figure 3. proTeOff can be applied to upregulate gene expression for both short and long times. Continuous long-term upregulation as well as upregulation with intermittent periods of low expression can be achieved. These behaviors are governed by TetR's response to aTc. aTc concentrations as low as 10 ng/mL effectively achieve periods of low expression. Target gene expression drops with increasing aTc concentrations of 10−200 ng/mL. aTc concentrations outside of this range or scaled throughout an experiment may also achieve a desired phenotype. Thus, proTeOff can be tuned to upregulate gene expression for short, long-continuous, and long-intermittent time scales using a range of aTc concentrations.

Characterization of proTeOn and proTeOff kinetics. In addition to experimentally testing proTeOn and proTeOff, we have assessed the kinetics of both systems by conducting stochastic simulations. This was done to characterize the systems at a finer resolution than can be achieved in the lab alone. We aimed to quantify the strength of the interactions between the device components by developing a stochastic model that captures the time profiles of measured GFP probability distributions. We modeled the transcription, translation, regulatory, and degradation events with stochastic kinetics. We built the models and conducted the simulations as described before[32−41] and discussed in the Supporting Information. Stochastic model parameters that did not exist in the literature were fit to match the untreated experimental phenotypes (0 ng/mL) and the behaviors upon induction with both low and high aTc concentrations (10 and 200 ng/mL). The mean GFP expression is captured by the model for both proTeOn and proTeOff at short times post-aTc induction (1 and 5 h) as well as at steady state (10 h), as shown in Figure 5. Overall, the mean GFP levels achieved by the simulation trajectories agree well with the experimental observations.

A discrepancy is observed between the simulation and experimental results at 5 h with 200 ng/mL aTc. This may be attributed to high aTc levels retarding cellular processes, such as protein overexpression.[42] By 10 h, this effect is no longer experimentally significant, and a good match is therefore observed between theoretical and experimental results. Due to their stochastic nature, the models can also capture the GFP distributions observed experimentally at short and long times. The GFP distributions at 10 h for proTeOn and 1 h for proTeOff, when 0, 10, and 200 ng/mL aTc are administered, are presented in Figure 6. For both systems, and across all aTc concentrations, the distributions generated *in silico* match those observed *in vivo*.

The protein:DNA binding strengths in the proTeOn and proTeOff systems are characterized by four kinetic parameters. These values, extracted from the models, are given in Table 1. Binding of aTc to PROTEON increases the affinity of the latter for the promoter 10,000-fold. Binding of PROTEOFF to aTc leads to a decrease in the affinity of PROTEOFF to *tetO* by 10^8 times. Both PROTEON and PROTEOFF enhance *gfp* expression significantly when bound to *tetO* by recruiting RNApol to the promoter. In our simulations, this is realized by increasing the binding strength of RNApol to DNA. Conforming to the simulation results, binding of PROTEON to *tetO* increases the binding strength of RNApol to DNA approximately 22 times, whereas binding of PROTEOFF to *tetO* increases the binding strength around 14 times.

Conclusions. The engineered proTeOn and proTeOff systems can all be applied to tightly control gene expression with aTc in prokaryotes. Incorporating appropriate tags in the target genes' transcripts may render proTeOn and proTeOff powerful tools for cytosolic, membrane-associated, and secreted protein overexpression.

More broadly, however, the workflow presented here can be implemented in the design, construction, and testing of a library of variant devices and regulatory networks. Such devices include those that respond to sugars, proteins, amino acids, metabolites, toxins, and other small molecules. With these, one may efficiently engineer gene expression responses as applications demand.

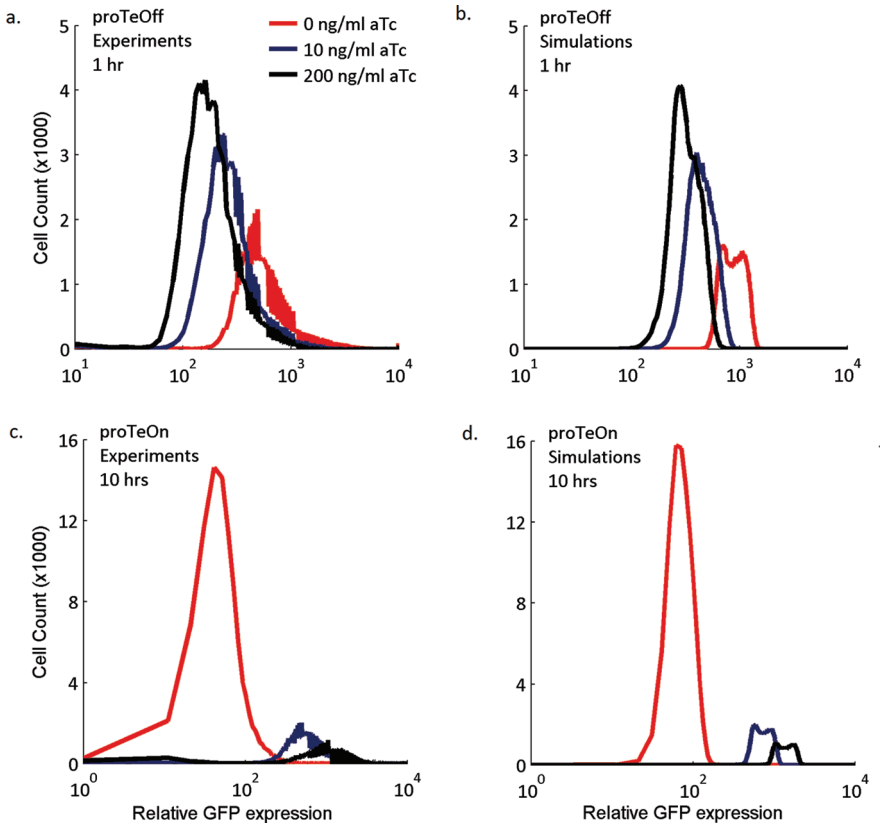

Figure 6. proTeOn and proTeOff GFP distribution by stochastic simulations. Simulation (b,d) and experimental (a,c) results of the distribution of GFP expression throughout the cell population when 0, 10, and 200 ng/mL aTc are administered. The simulation results largely agree with the experimental phenotypes. (a,b) proTeOff GFP distribution at 1 h. (c,d) proTeOn GFP distribution at 10 h.

Table 1. Dissociation Constants of the Key Biomolecular Interactions Underlying proTeOn and proTeOff[a]

	binding affinity	
biomolecular interactions	proTeOn	proTeOff
PROTET + *tetO* ↔ PROTET:*tetO*	2.5×10^{-5}	2.5×10^{-10}
PROTET:aTc2 + *tetO* ↔ PROTET:*tetO*:aTc2	2.5×10^{-10}	2.5×10^{-2}
RNApol + pro + *tetO* ↔ RNApol:pro:*tetO*	3.7×10^{-9}	3.7×10^{-9}
RNApol + pro + PROTET:*tetO* ↔ RNAp:pro:*tetO*:PROTET	1.67×10^{-10}	2.56×10^{-10}

[a] The dissociation constants are estimates from our modeling efforts. The units for the second order reactions (reactions 1 and 2) are M, whereas for the third order reactions (reactions 3 and 4) they are M^2.

As the collection of well characterized synthetic devices grows, we may also be able to combine them, along with naturally occurring parts, into larger gene regulatory networks to achieve more complex desired phenotypes. Feedback loops, feedforward loops, and AND and OR logic gates are a few possible networks. These devices and networks may then be used either as tools for controlling the expression of a single gene or as interoperable parts of larger regulatory networks that control multiple genes independently. The current proTeOn and proTeOff devices and the kinetic and structural details that have been identified for each are a firm stepping stone from which this work can expand. Using our workflow, integrating experimental synthetic biology, molecular modeling, and stochastic reaction kinetic simulations, the required effort and expense that scale with system complexity will also be reduced. Molecular devices that can tunably regulate the expression of a single or a handful of genes in response to sugar, protein, amino acid, metabolite, toxin, and other small molecule levels may provide synthetic biology research and the bioengineering industry the tools they need for efficient, diverse gene expression control.

METHODS

PROTEON and PROTEOFF Parts. PROTEON and PROTEOFF are composed of an inducible DNA binding domain and a DNA binding transcription activator, connected by a linker peptide. The reverse tetracycline repressor (rTetR) is the N-terminal, inducible DNA binding domain in PROTEON, while the tetracycline repressor (TetR) is in PROTEOFF, rendering both systems responsive to anhydrotetracycline (aTc).[2,19−21,28,29] LuxRΔN(2−162) (LuxRΔN) is the DNA-binding activator at the C-terminus of both synthetic proteins.[22] LuxRΔNis the C-terminal domain of the full length LuxR transactivator. It lacks N-terminal residues 2−162 of the full length LuxR and possesses strong constitutive transactivator activity.[22] In both systems, the two domains are connected by a 150 Å, 55 amino acid peptide linker (its design is discussed below).

proTeOn and proTeOff Synthetic Promoter Parts. The proTeOn and proTeOff synthetic promoter is composed of sequences from the *tet* and *lux* operons' promoters. Operator sites for TetR/rTetR[2,20,29]

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and LuxRΔN,[25,26,29] RNApolymerase (RNApol) binding sequences (the UP element and −10 region),[27] transcription and translation start sites, an mRNA stabilizing sequence, and a ribosomal binding site[43] are included. These sequences are annotated in Figure 2a. Green fluorescence protein mutant 3 (GFP) is the reporter molecule used to monitor the systems' behavior and is under the control of the synthetic promoter.[44] A second synthetic promoter, containing the RBS of the *lux* promoter and lacking the mRNA stability sequence was also tested. This promoter provided *gfp* transcript increase upon induction. However, a parallel increase in GFP protein was not observed.

Molecular Modeling. We designed the proTeOn and proTeOff systems using molecular modeling with MOE[30] and with NAMD.[31] We built models of the systems utilizing the known structure of the inducible DNA binding domain (rTetR or TetR) bound to the *tetO* operator (PDB code 1QPI)[19] and the transcription activator domain (LuxRΔN) bound to the *luxbox* (PDB code 1H0M).[45] The linker size was determined by assuming the scaling of polymer's end-to-end vector distance in a good solvent.[46] The specific sequence was then determined to minimize proteolysis in bacteria. We thus designed a 55-amino acid linker peptide, which is expected to be linear, flexible, and hydrophilic.[47]

System Construction. Both synthetic activator genes were synthesized by GENEART using standard molecular biology techniques, cloned into the expression vector pT7-FLAG1 (p1118 Sigma), at KPN1 restriction sites, using Top10 *Escherichia coli* (*E. coli*) cells (C404010 Invitrogen). The final constructs are illustrated in Supporting Figure 1a. The synthetic promoter and *gfp*[44] were synthesized by GENEART, on pMK, a pUC19 derived expression vector that is compatible in *E. coli*, high copy, and kanamycin-resistant. This construct is illustrated in Supporting Figure 1b. proTeOn and proTeOff are contained on these two plasmids and were transformed into chemically competent BL21(DE3)-T1 *E. coli* cells (B2935 Sigma) by heat shock for characterization.

Gene Expression Control by Tetracycline in Bacterial Cultures. BL21(DE3)-T1 cells containing the proTeOn and proTeOff systems were cultured in selective LB media, at 30 °C to facilitate temperature-sensitive folding of LuxRΔN, agitating at 200 rpm. Cultures were maintained in midlog growth. We completed initial experiments to first confirm the solubility and stability of PROTEON and PROTEOFF and then to establish promoter specific gene regulation by aTc (rather than a general soluble protein upregulation).

Basal, low, medium, and high levels of the synthetic activators were maintained with 0, 0.25, 0.75, and 1 mM IPTG, respectively. Each system was induced over a range of aTc concentrations, 1, 10, and 200 ng/mL. The total soluble protein was isolated from cultures with CelLyticB reagent (B7435 Sigma), separated by size on a 10% polyacrylamide gel, and transferred to PVDF membrane. PROTEON was detected by primary mouse monoclonal anti-FLAG M2 antibody (F3165 Sigma), GFP by mouse monoclonal anti-GFP antibody [LGB-1] (ab291 Abcam), and loading control RNApol by mouse monoclonal anti-RNApol sigma 70 antibody [2G10] (ab12088). Biotinylated, polyclonal sheep antimouse secondary antibody, VECTASTAIN ABC kit (PK-4002 Vector Laboratories), and Amersham ECL Western blotting detection reagent (RPN 2109) were used to complete the specific detection of each protein. Relative quantification of each protein was performed using ImageJ software, publicly available at http://rsb.info.nih.gov, taking the ratio of protein of interest to RNApol density. PROTEON and PROTEOFF are soluble and stable at high intracellular concentrations, and gene regulation by aTc is specific to genes under the control of the synthetic promoter. This is discussed in Supporting Information and illustrated in Supporting Figure 2.

We characterized each system maintaining PROTEON and PROTEOFF expression with 0.75 mM IPTG and controlling the activity of each device with 0, 10, and 200 ng/mL aTc. Two sets of experiments were performed. In experiment setup A, PROTEON and PROTEOFF production was induced overnight with 0.75 mM IPTG and 0 ng/mL aTc; at t = 0 h cultures were treated with 0, 10, and 200 ng/mL aTc. In experiment setup B cultures were treated overnight with 0, 10, and 200 ng/mL aTc and 0 mM IPTG; at t = 0 PROTEON and PROTEOFF expression was induced with 0.75 mM IPTG. Experiments A investigated the proTeOn and proTeOff system dynamics upon induction with aTc, whereas experiments B provided insight on the PROTEON and PROTEOFF protein production and maturation dynamics.

IPTG and aTc levels were maintained in the cultures at all $t > 0$ h. Induction experiments were repeated for all inducer concentrations and time points, and reported trends were observed across replicates.

Cell samples were collected for analysis by flow cytometry at t = 1, 2, 5, 10, and 20 h, fixed with 4% paraformaldehyde for 30 min at RT, washed with 1x phosphate buffered saline (PBS), and stored in 1x PBS at 4 °C. Individual cells' GFP expression was measured by flow cytometry using a FACScalibur (BD Biosciences). A total of 100,000 cells was investigated per sample with excitation at λ_{ex} = 488 nm and subsequent fluorescence detection at λ_{em} = 530 ± 30 nm. The cytometry data was collected using CellQuest (BD Biosciences) and analyzed using FlowJo (Tree Star) software. Each sample's healthy cell population was selected by first removing erroneous events (due to electronic noise) that fell below a minimum emission at λ_{em} = 530 ± 30 nm and then removing events that fell outside of the characteristic side-scatter and forward-scatter range for single *E. coli* cells. The differential GFP expression of the selected cells was analyzed and compared across samples.

Mean Gene Expression Data Analysis. All cytometry data was collected as described above. Prior to evaluating data across replicates, all GFP expression values were normalized to their corresponding (same system and time point) 0 ng/mL aTc samples. Basal expression (achieved with 0 ng/mL) from the proTeOn system is denoted with a value of 1. In contrast, maximal expression (also achieved with 0 ng/mL) from the proTeOff system is denoted with a value of 1. This basis is consistent for both experimental setups and through all times. Normalized GFP expression averages and standard errors were then calculated for replicates.

Stochastic modeling. We built computer models of proTeOn and proTeOff to further characterize the experimental behavior of each system. A hybrid stochastic-discrete and stochastic-continuous algorithm called Hy3S was used.[38,39] Hy3S couples chemical Langevin equations with discrete kinetic Monte Carlo, modeling a system's behavior at the resolution of biomolecular interactions in individual cells. Characteristics of numerous natural and synthetic biological systems have previously been described using this approach.[32,37,38,40,41] The simulations were carried out under a number of key assumptions and parameters that are discussed in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information. Detailed description of the stochastic kinetic reaction models of protein devices and further details on experimental characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Foster, T. J., Davis, M. A., Roberts, D. E., Takeshita, K., and Kleckner, N. (1981) Genetic organization of transposon Tn10. *Cell 23*, 201–213.

(2) Hillen, W., and Berens, C. (1994) Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu. Rev. Microbiol. 48*, 345–369.

(3) Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., and Meier, I. (1983) Control of expression of the Tn10-encoded tetracycline resistance genes. Equilibrium and kinetic investigation of the regulatory reactions. *J. Mol. Biol. 169*, 707–721.

(4) Engebrecht, J., Nealson, K., and Silverman, M. (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell 32*, 773–781.

(5) Kaplan, H. B., and Greenberg, E. P. (1985) Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol. 163*, 1210–1214.

(6) Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., Jr., and Greenberg, E. P. (1996) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. U.S.A. 93*, 9505–9509.

(7) Kritzer, J. A. (2010) Grand challenge commentary: Beyond discovery: probes that see, grab and poke. *Nat. Chem. Biol. 6*, 868–870.

(8) Vickers, C. E., Blank, L. M., and Kromer, J. O. (2010) Grand challenge commentary: Chassis cells for industrial biochemical production. *Nat. Chem. Biol. 6*, 875–877.

(9) Callura, J. M., Dwyer, D. J., Isaacs, F. J., Cantor, C. R., and Collins, J. J. (2010) Tracking, tuning, and terminating microbial physiology using synthetic riboregulators. *Proc. Natl. Acad. Sci. U.S.A. 107*, 15898–15903.

(10) Isaacs, F. J., and Collins, J. J. (2005) Plug-and-play with RNA. *Nat. Biotechnol. 23*, 306–307.

(11) Elowitz, M. B., and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature 403*, 335–338.

(12) Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature 403* 339–342.

(13) Hasty, J., McMillen, D., and Collins, J. J. (2002) Engineered gene circuits. *Nature 420*, 224–230.

(14) Ramalingam, K, T., J., Maynard, J, and Kaznessis, Y. (2009) Forward engineering of synthetic bio-logical AND gates. *Biochem. Eng. J.* 38–47.

(15) Egland, K. A., and Greenberg, E. P. (2001) Quorum sensing in *Vibrio fischeri*: analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol. 183*, 382–386.

(16) Fuqua, C., Winans, S. C., and Greenberg, E. P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol. 50*, 727–751.

(17) Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol. 176*, 269–275.

(18) Sitnikov, D. M., Schineller, J. B., and Baldwin, T. O. (1995) Transcriptional regulation of bioluminesence genes from *Vibrio fischeri*. *Mol. Microbiol. 17*, 801–812.

(19) Orth, P., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nat. Struct. Biol. 7* 215–219.

(20) Saenger, W., Orth, P., Kisker, C., Hillen, W., and Hinrichs, W. (2000) The tetracycline repressor-a paradigm for a biological switch. *Angew. Chem., Int. Ed. 39*, 2042–2052.

(21) Scholz, O., Henssler, E. M., Bail, J., Schubert, P., Bogdanska-Urbaniak, J., Sopp, S., Reich, M., Wisshak, S., Kostner, M., Bertram, R., and Hillen, W. (2004) Activity reversal of Tet repressor caused by single amino acid exchanges. *Mol. Microbiol. 53*, 777–789.

(22) Choi, S. H., and Greenberg, E. P. (1991) The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent lux gene activating domain. *Proc. Natl. Acad. Sci. U.S.A. 88*, 11115–11119.

(23) Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5547–5551.

(24) Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science 268*, 1766–1769.

(25) Devine, J. H., Shadel, G. S., and Baldwin, T. O. (1989) Identification of the operator of the lux regulon from the *Vibrio fischeri* strain ATCC7744. *Proc. Natl. Acad. Sci. U.S.A. 86*, 5688–5692.

(26) Egland, K. A., and Greenberg, E. P. (1999) Quorum sensing in *Vibrio fischeri*: elements of the luxI promoter. *Mol. Microbiol. 31* 1197–1204.

(27) Finney, A. H., Blick, R. J., Murakami, K., Ishihama, A., and Stevens, A. M. (2002) Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the lux operon during quorum sensing. *J. Bacteriol. 184*, 4520–4528.

(28) Berens, C., and Hillen, W. (2003) Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *Eur. J. Biochem. 270*, 3109–3121.

(29) Ramos, J. L., Martinez-Bueno, M., Molina-Henares, A. J., Teran, W., Watanabe, K., Zhang, X., Gallegos, M. T., Brennan, R., and Tobes, R. (2005) The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev. 69*, 326–356.

(30) *MOE: Molecular Operating Environment* (2005) Chemical Computing Group, Montreal.

(31) Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. *J. Comput. Chem. 26*, 1781–1802.

(32) Biliouris, K., Daoutidis, P., and Kaznessis, Y. N. (2011) Stochastic simulations of the tetracycline operon. *BMC Syst. Biol. 5*, 9.

(33) Hill, A. D., Tomshine, J. R., Weeding, E. M., Sotiropoulos, V., and Kaznessis, Y. N. (2008) SynBioSS: the synthetic biology modeling suite. *Bioinformatics 24*, 2551–2553.

(34) Salis, H., and Kaznessis, Y. N (2005) Numerical simulation of stochastic gene circuits. *Comput. Chem. Eng. 29*, 577–588.

(35) Kaznessis, Y. N. (2009) Computational methods in synthetic biology. *Biotechnol. J. 4*, 1392–1405.

(36) Kaznessis, Y. N. (2011) Mathematical models in biology: from molecules to life. *Wiley Interdiscip. Rev.: Syst. Biol. Med. 3*, 314–22.

(37) Weeding, E., Houle, J., and Kaznessis, Y. N. (2010) SynBioSS designer: a web-based tool for the automated generation of kinetic models for synthetic biological constructs. *Brief Bioinform. 11*, 394–402.

(38) Salis, H., and Kaznessis, Y. (2005) Accurate hybrid stochastic simulation of a system of coupled chemical or biochemical reactions. *J. Chem. Phys. 122*, 54103.

(39) Salis, H., Sotiropoulos, V., and Kaznessis, Y. N. (2006) Multi-scale Hy3S: hybrid stochastic simulation for supercomputers. *BMC Bioinf. 7*, 93.

(40) Sotiropoulos, V., and Kaznessis, Y. N. (2007) Synthetic tetracycline-inducible regulatory networks: computer-aided design of dynamic phenotypes. *BMC Syst. Biol. 1*, 7.

(41) Tuttle, L. M., Salis, H., Tomshine, J., and Kaznessis, Y. N. (2005) Model-driven designs of an oscillating gene network. *Biophys. J. 89*, 3873–3883.

(42) Golan, D.E.; A. H. T., Armstrong, E.J.; Armstrong, A.W. (2005) *Principles of Pharmacology: the Pathophysiologic Basis of Drug Therapy* Lippincott Williams and Wilkins, Philadelphia.

(43) Meynial-Salles, I., Cervin, M. A., and Soucaille, P. (2005) New tool for metabolic pathway engineering in *Escherichia coli*: One-step method to modulate expression of chromosomal genes. *Appl. Environ. Microbiol. 71*, 2140–2144.

(44) Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene 173* 33–38.

(45) Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P., and Marco, S. D. (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *EMBO J. 21*, 4393–4401.

(46) Doi, M., and Edwards, S. F. (1986) *The Theory of Polymer Dynamics*, Clarendon Press, Oxford.

(47) George, R. A., and Heringa, J. (2002) An analysis of protein domain linkers: their classification and role in protein folding. *Protein Eng. 15*, 871–879.