Chapter 2

Negative Regulation Gene Circuits for Efflux Pump Control

Daniel A. Charlebois, Junchen Diao, Dmitry Nevozhay, and Gábor Balázsi

Abstract

Synthetic biologists aim to design biological systems for a variety of industrial and medical applications, ranging from biofuel to drug production. Synthetic gene circuits regulating efflux pump protein expression can achieve this by driving desired substrates such as biofuels, pharmaceuticals, or other chemicals out of the cell in a precisely controlled manner. However, efflux pumps may introduce implicit negative feedback by pumping out intracellular inducer molecules that control gene circuits, which then can alter gene circuit function. Therefore, synthetic gene circuits must be carefully designed and constructed for precise efflux control. Here, we provide protocols for quantitatively modeling and building synthetic gene constructs for efflux pump regulation.

Key words Synthetic gene circuit, Efflux pump, Feedback, Mathematical modeling

1 Introduction

Synthetic biology aims to rationally design biological devices for specific predefined purposes [1–5]. This includes building synthetic gene circuits that function as switches [6], oscillators [7], and logic gates [8, 9] to control the secretion of substrates such as drugs or biofuels for clinical or industrial applications [10]. This goal can be achieved by expressing efflux pump proteins across the cellular membrane [11]. However, the secreted substrates can be toxic to the cells, making it important to precisely control their production, secretion, and removal. This can be achieved with synthetic gene circuits that precisely control efflux pump expression. However, efflux pumps can also lower intracellular inducer concentrations by pumping inducer molecules out of the cell along with the intended substrates. This introduces an implicit negative feedback loop that may alter the function of gene circuits that control efflux pump expression.

Previously, we built and characterized synthetic gene circuits with negative regulation and negative feedback, aptly named the "negative regulation" (NR) and "negative feedback" (NF) gene

Jeffrey Carl Braman (ed.), *Synthetic Biology: Methods and Protocols*, Methods in Molecular Biology, vol. 1772,
https://doi.org/10.1007/978-1-4939-7795-6_2,

© Springer Science+Business Media, LLC, part of Springer Nature 2018

circuits [12]. Both gene circuits are TetR-based and inducible by tetracycline analogs. The NR gene circuit consists of a tetracycline repressor (TetR) driven by a P_{GAL1} promoter, where TetR inhibits transcription of a downstream $P_{GAL1-D12}$ promoter controlling reporter gene expression in an inducer-dependent manner (Fig. 1a). The NF gene circuit is similar to the NR gene circuit, except that TetR inhibits the transcription of its own gene as well as a reporter gene since both the genes are driven by identical $P_{GAL1-D12}$ promoters (Fig. 1b) [13, 14]. Importantly, the NF gene circuit reduces gene expression heterogeneity [15–17] over a wide range of inducer concentrations, and it linearizes the dose response prior to saturation compared to other gene circuits that lack auto-regulation (such as the NR circuit) [12, 18].

Considering the importance of precise efflux control, in a recent study [11] we thoroughly investigated the efficiency and precision of controlling ATP-binding cassette (ABC) efflux pumps with low and high precision, by modifying the NR and NF gene circuits, respectively. ABC multidrug resistance pumps are highly conserved across bacteria, fungi, and mammals, and cause resistance to certain antibiotic treatments and chemotherapies by pumping a wide range of compounds out of the cell [19–21]. To build the NRpump (Fig. 1c) and NFpump (Fig. 1d) gene circuits in the budding yeast *Saccharomyces cerevisiae*, we replaced the

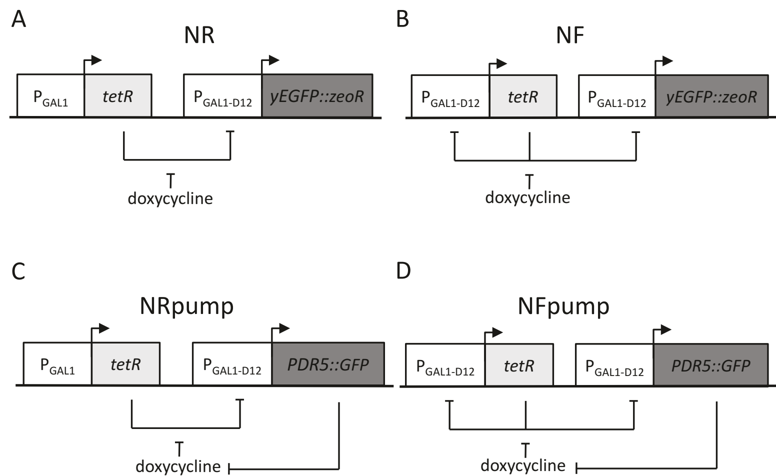


Fig. 1 Diagrams of the NR, NF, NRpump, and NFpump synthetic gene circuits. (**a**) Negative Regulation (NR) gene circuit. This gene circuit consists of a regulator gene (*tetR*) that represses target gene (*yEGFP::zeoR*) expression in an inducer (doxycycline) concentration-dependent manner. (**b**) NF gene circuit. This gene circuit differs from NR in a single aspect: tetR autorepression (negative feedback). (**c**) Negative Regulation pump (NRpump) gene circuit. This gene circuit is identical to NR, except for the target gene (*PDR5::GFP*). (**d**) NFpump gene circuit. This gene circuit differs from NRpump in a single aspect: TetR autorepression (negative feedback). Blunt arrows in each panel indicate repression

passive target gene (*yEGFP::zeoR*) in the previously characterized versions of the NR and NF gene circuits [12] with *PDR5::GFP*, a fusion of the yeast pleiotropic drug resistance (PDR) ABC pump and the GFP fluorescent reporter. We chromosomally integrated both of these modified NR and NF gene circuits into the *PDR5*-knockout cells to avoid potential confounding interactions with the native copy of the *PDR5* gene. Importantly, the Pdr5p::GFP fusion protein pumped out the inducer doxycycline from yeast cells, creating an implicit feedback loop. Consequently, Pdr5p altered the dose-responses of the original NR and NF gene circuits substantially in budding yeast. In addition to the expected reduction in inducer sensitivity from efflux pump function, we observed an unexpected increase of inducer sensitivity in the NRpump and NFpump strains at low inducer concentrations. Using a combined mathematical-experimental approach, we found that reduced TetR expression caused this additional change. Overall, these findings provide insight into how efflux pumps can alter gene network function and highlight the importance of mathematical modeling in elucidating and predicting unexpected consequences of gene circuit modifications.

Using inducible synthetic gene circuits to control efflux pump expression could mitigate the toxicity of secreted biochemicals while ensuring proper yield. However, while designing such systems, consideration should be given to the implicit feedback that results from the efflux pumps acting not only on their intended substrate, but also on the inducer. In this chapter, we describe step by step the protocols required to mathematically model and build synthetic gene circuits for efflux pump control. This should be useful for basic research (e.g., studies of efflux-pump-mediated drug resistance), as well as industrial and biomedical applications, and also for predicting and understanding the consequences of modifying natural or synthetic gene networks that regulate an efflux pump or other active target genes.

2 Materials

2.1 Strains

1. Haploid *Saccharomyces cerevisiae* strain YPH500 (α, *ura3-52*, *lys2-801*, *ade2-101*, *trp1Δ63*, *his3Δ200*, and *leu2Δ1*).
2. *Escherichia coli* XL-10 Gold strain.

2.2 Plasmids, Sequences, and Oligonucleotides Used in Plasmid Construction

1. The *PDR5* sequence was obtained from the *Saccharomyces* Genome Database (SGD) database. The full length is 4536 base pairs, including the start codon (ATG) and stop codon (TAA).
2. The plasmid containing the original *PDR5::GFP* fusion [22] was a gift from Dr. Won-Ki Huh (Seoul National University, Seoul, South Korea).

3. Primers used for constructing the *PDR5::GFP*-containing plasmid:

   PDR5-BamHI-f: 5′-gcgcggatcctattaaaATGCCCGAGGCCAAGCTTAAC-3′

   neGFP-XhoI-r: 5′-gcgcctcgagCTATTTGTATAGTTCATCCATGC-3′

4. Primers used for sequencing verification of the *PDR5::GFP*-containing synthetic gene circuits:

   PG-Seq1 –f: 5′-ACAGAACCGTATCAAGGGTGTC-3′

   PG-Seq2 –f: 5′-TTCTTCTCTGTTAGAAATCTTTTCG-3′

   PG-Seq3 –f: 5′-TATTTCACTGGAGAAACCTTTGTTACG-3′

   PG-Seq4 –f: 5′-GAAAGGTTCGATAACTGCAGCTG-3′

   PG-Seq5 –f: 5′-TCTACGTTTATGTTGGTTCTATGG-3′

   PG-Seq6 –f: 5′-GGAAACATTCTTGGACACAAATTGG-3′

   PG-Seq7 –r: 5′-CATCTCTCACTGTAGAAAGAATTG-3′

   PG-Seq8 –r: 5′-TCAGCTGCAGTTATCGAACCTTTC-3′

   PG-Seq9 –r: 5′-CTTCGTAACAAAGGTTTCTCCAGTG-3′

   PG-SeqA –r: 5′-AAAAGATTTCTAACAGAGAAGAAAATGC-3′

   PG-SeqB –r: 5′-CTATCGACACCCTTGATACGGTTCTG-3′

   PG-SeqC –f: 5′-ACTCATGGTTTTGATCTTGGTGCAGATAC-3′

   PG-SeqD –f: 5′-TGTACTAACTGAAAAGAATGCAAATGACC-3′

   PG-SeqE –f: 5′-ATGGTGCTCATAAATGCCCTGCTGACG-3′

   Backbone-r: 5′-CGCGTTGGCCGATTCATTAATGC-3′

   GAL1AgeI-f: 5′-CTTCACCGGTCGCGTTCCTGAAACGCAG-3′

5. Primers used for the verification of the *pet56-his3Δ200* locus:

   TRP-Seq-f: 5′-GGTGAAAACCTCTGACACATGCAGCTCC-3′

   HIS-Begin-r: 5′-ACGCTTTACTAGGGCTTTCTGCTCTGTC-3′

6. Primers used for the verification of the orientation of insertion into the pRS403 (*his* marker) plasmid (together with Backbone-r from above):

   Insert-f: 5′-AATTGGAGCGACCTCATGCTATACCTG-3′

2.3 Yeast Cell Transformation

1. G418, 100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL 500 μg/mL.
2. PEG, 50% w/v (molecular weight 3350).
3. Lithium Acetate, 1.0 M and 100 mM.
4. Salmon sperm DNA solution, 2 mg/mL, stored at −20 °C.
5. TE Buffer (PH 8.0): Tris–HCl (PH 8.0), 10 mM; EDTA, 1 mM.
6. Nexcelom cell counter.
7. Sterilized glass beads or plate spreader.

2.4 Medium

1. YPD medium: 5 g of yeast extract, 10 g of peptone, 19 mg of adenine, add deionized water to 450 mL and autoclave for 20 min. Add 50 mL of 20% glucose after autoclaving (*see* **Notes 1–3**).
2. Synthetic complete medium (per liter): 6.7 g of nitrogen base without amino acids, 2 g of drop-out mix with complete amino acids, 19 mg adenine, add deionized water to 900 mL, autoclave, then add 100 mL 20% glucose or galactose.
3. Synthetic dropout medium without certain amino acid(s) (per liter): 6.7 g of nitrogen base without amino acids, 2 g drop-out mix without certain amino acid, 19 mg adenine, add deionized water to 900 mL, autoclave, then add 100 mL 20% glucose or galactose.

2.5 Endogenous PDR5 Knockout

1. Same reagents as in yeast cell transformation (Subheading 2.3).
2. Primers used for *PDR5* knockout:

   PDR5-deletion-UP45: 5′-TTAAGTTTTCGTATCCGCTCGTCGAAAGACTTTAGACAAAAATG-3′

   PDR5-deletion-DN45: 5′-CATCTTGGTAAGTTTCTTTTCTTAACCAAATTCAAAATTCTATTA-3′

   PDR5-deletion A: 5′-TTGAACGTAATCTGAGCAATACAAA-3′

   PDR5-deletion B: 5′-TACCTAAAACGACTAGCAATTCA-3′.

   PDR5-deletion C: 5′-GCCTCTTTGTTGTTTACAATGTC-3′

   PDR5-deletion D: 5′-TCACACTAAATGCTGATGCCTAT-3′

   Kan B: 5′-CTGCAGCGAGGAGCCGTAAT-3′

   Kan C: 5′-TGATTTTGATGACGAGCGTAAT-3′

2.6 Site Directed Mutagenesis

1. QuickChange II XL site-directed mutagenesis kit.
2. Plasmid constructed with the *PDR5::GFP* gene.
3. 14 mL BD Falcon polypropylene round-bottom tubes.
4. 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 80 μg/mL).
5. Isopropyl-1-thio-beta-D-galactopyranoside (IPTG, 20 μM).
6. pH 7.5 NYZ+ Broth (per liter): 10 g of NZ amine, 5 g of yeast extract, 5 g of NaCl, add deionized H_2O to 1 L, NaOH (for pH adjustment).
7. LB Agar (per liter): 1 g of NaCl, 10 g of trypotone, 3 g of yeast extract, 20 g of agar.
8. Primers used for site-directed mutagenesis:

   PDR5-S558Y-f: 5′-CTATTTCCGTGGTTATGCTAT GTTTTTTGCAATTCTATTCAATGC-3′

   PDR5-S558Y-r: 5′-CATAGCATAACCACGGAAATA GAATGTAGAAGTATCACC-3′

   PDR5-G312A-f: 5′-GTTTCCGGTGCTGAAAGGAA GCGTGTCTCCATTGCTGAAGTCTCC-3′

   PDR5-G312A-r: 5′-CGCTTCCTTTCAGCACCG GAAACACCTCTGACGATGTCGTTACC-3′
9. Sequencing primers used to confirm *PDR5* mutations:

   Insert-f from Subheading 2.2 and GALSeqA-f (anneals to pRS4* plasmids in the DNA region immediately upstream of the *pGAL10*-controlled gene):

   5′-CAAACCTCTGGCGAAGAATTG-3′

2.7 Stochastic Simulation and Mathematical Modeling

A software environment such as Matlab (The Mathworks, Inc., https://www.mathworks.com) with the SimBiology, Symbolic Math, and Statistics toolboxes can be used for stochastic simulations, calculations, and statistics. Alternatively, non-commercial, freely available software such as Octave [23], Dizzy [24], or Python (Python Software Foundation, https://www.python.org) can be used for the same purpose.

3 Methods

3.1 Construction of the Reporter Plasmid with PDR5::GFP

Here, we describe the construction of the reporter plasmid with the *PDR5::GFP* gene [22] under the control of the TetR-repressible $P_{GAL1\text{-}D12}$ promoter [14]. It also has the *HIS3* gene as the auxotrophic marker for *S. cerevisiae* and the extended *pet56-his3Δ200* locus [25] for chromosomal integration. Construction consists of two steps: insertion of the *PDR5::GFP* gene into the plasmid (Protocol A) and addition of the *pet56-his3Δ200* locus

(Protocol B). The original *PDR5::GFP* fusion gene (5274 bp) has two internal *BamH*I sites; therefore, we have to divide it into two parts to make fragments that we can insert between the *BamH*I and *Xho*I sites in the parental pDN-G1GZmbh plasmid [12, 26].

3.1.1 Protocol A

1. Amplify the cassette containing the *PDR5::GFP* gene by PCR (35× cycles: 10 s at 98 °C; 10 s at 54 °C; 120 s at 72 °C; Phire polymerase) using the pair of primers PDR5-BamHI-f, neGFP-XhoI-r and the original PDR5::GFP plasmid [22] as a template. Purify the final PCR product (~5300 bp) using an agarose gel electrophoresis and gel extraction kit of your choice (*see* **Note 4**).
2. Digest the PCR product from **step 1** with the *Afl*II and *Xho*I restriction enzymes. This will result in two fragments, small and large (~400 bp and ~4900 bp, respectively). Run the reaction products in agarose gel electrophoresis and extract both the fragments using a gel extraction kit of your choice.
3. Perform a second digestion reaction of the small fragment from **step 2** using the *BamH*I enzyme and clear it up using a kit of your choice.
4. Digest the pDN-G1GZmbh [12, 26] plasmid with the *BamH*I and *Xho*I restriction enzymes. Separate the reaction products using agarose gel electrophoresis and extract the large fragment (backbone) using a gel extraction kit of your choice.
5. Perform a sticky-end ligation reaction using the backbone fragment from **step 4** and both the small and large digested PCR fragments from **steps 2–3** to produce a plasmid with the *PDR5::GFP* gene inserted into the backbone. Run the ligation reaction overnight at room temperature to increase efficiency.
6. The following day, transform competent *E. coli* cells with the product from the overnight ligation and seed bacteria onto a selective agar plate with added ampicillin to select for transformed cells. Incubate the plate in a 37 °C incubator overnight.
7. Pick several colonies from the plate from **step 6** and grow them separately overnight in the LB medium with ampicillin added. Purify the plasmids using a miniprep kit of your choice.
8. Sequence the plasmid samples with the primers for *PDR5::GFP* verification (Materials and Methods, Subheading 2.2, **item 4**) to confirm proper insertion of the gene into the plasmid. If all the steps are successful, this protocol will result in the intermediate plasmid pDN-G1PGbh.

3.1.2 Protocol B

1. Digest both pDN-G1PGbh from Protocol A and pDN-T2dPGxh [25] plasmids with the *Ahd*I and *Avr*II restriction enzymes. Separate reaction products using agarose gel electro-

phoresis and extract the large fragment of pDN-G1PGbh and the small fragment of pDN-T2dPGxh using a gel extraction kit of your choice.

2. Perform a sticky-end ligation reaction using both the extracted fragments from **step 1** to produce a plasmid with the *pet56-his3Δ200* locus inserted into the backbone. For better efficiency, run the ligation reaction overnight at room temperature.
3. Follow the *E. coli* transformation and DNA preparation procedures as described in **steps 6–7** of Protocol A.
4. Sequence the plasmid samples with the primers for *pet56-his3Δ200* locus verification (Materials and Methods, Subheading 2.2, **item 5**) to confirm proper insertion of the sequence into the plasmid. If all the steps are successful, this protocol will result in the pDN-G1PGlbh plasmid, which will be further used for yeast transformation (*see* **Note 5** with regards to homology locus for yeast transformation).

3.2 Yeast Cell Transformation

1. Pick up a single colony from a yeast cell plate and inoculate into 5 mL synthetic complete (SC) medium. Depending on the yeast strain, the SC medium can be substituted by synthetic drop-out (SD) medium (e.g., SD medium without histidine or trypsin). Shake the cell culture in an incubator at 300 rpm and 30 °C overnight.
2. Measure cell density of the overnight culture on a Nexcelom cell counter, calculate the cell culture volume that contains 2.5×10^8 cells, inoculate the calculated volume of cell culture into 50 mL fresh SC (or SD) medium to reach a cell density of 5×10^6/mL.
3. Shake the cell culture in an incubator at 300 rpm and 30 °C for 3–5 h, until the cell density reaches 2×10^7/mL.
4. Harvest the cells in sterile 50 mL centrifuge tubes at 8000 × *g* for 4 min.
5. Pour off the supernatant carefully, resuspend the cell pellets with 25 mL sterile H_2O, and centrifuge again.
6. Repeat **step 5** one time.
7. Pour off the supernatant carefully, and resuspend the cells with 1 mL Lithium Acetate (100 mM) thoroughly, and transfer the suspension to a sterile 1.5 mL tube.
8. Centrifuge the suspension at 13,000 × *g* for 5 s, then remove the supernatant with a pipette.
9. Resuspend the cell pellet with 500 μL Lithium Acetate (100 mM), mix thoroughly by pipetting and vertexing.

10. Boil 100 μL (50 μL for control, 50 μL for experimental sample) salmon sperm DNA for 5 min, and quickly chill on ice or in ice water. Keep on ice before using for transformation (*see* **Note 6**).
11. Vortex the cell suspension and pipette 50 μL samples into a pre-labeled 1.5 mL tube (sterile); pellet the cells and remove the Lithium Acetate supernatant with a pipette.
12. Make transformation mix containing the following ingredients, carefully add them into the cell pellets in the order listed below:

    240 μL of PEG 3350.

    36 μL of Lithium Acetate (1 M).

    50 μL of salmon sperm DNA.

    34 μL of linearized plasmid DNA (0.1–10 μg) or sterile H_2O for control (*see* **Note 7**).
13. Vortex each tube vigorously until the cell pellet has been completely mixed. This usually takes at least 1 min.
14. Incubate the mixture at 30 °C for 30 min. Shake the tubes, turning them upside down repeatedly or use a thermomixer at 600 rpm.
15. Heat shock for 20 min at 42 °C, and then chill on ice for 2 min.
16. Centrifuge the cells at 8000 × *g* for 30 s, and then remove the supernatant with a pipette.
17. Pipette 1 mL sterile H_2O into the 1.5 mL tube, and resuspend the cell pellets gently.
18. Take 100 μL cell culture, dilute it 10 times and 100 times separately.
19. Plate 100 μL aliquots of the original transformation mix, 10 times dilution and 100 times dilution onto 3 selective plates separately.

3.3 Construction of PDR5-Containing Synthetic Gene Circuits

The construction of *PDR5* containing gene circuits follows a similar method as other gene circuits described before [12]. Each synthetic gene circuit is created by integrating two separate plasmids—reporter and regulator. The reporter plasmid, as described in Subheading 3.1, contained *PDR5::GFP* gene [22] under the control of TetR-repressible PGAL1-D12 promoter [13]. The regulatory plasmid contained the gene encoding the TetR repressor as the regulator [11, 12]. We developed all regulatory circuits earlier [12] and described them in detail in a previous book chapter [26]. We created a total of 12 PDR5-containing synthetic gene circuits, which are single-color and double-color versions of the following (including 4 *PDR5* mutants for control purposes): NRpump, NFpump, NRpump558, NFpump558, NRpump312, and NFpump312.

3.4 Strains and Media

All the PDR5-containing gene circuits were transformed into haploid *S. cerevisiae* strain YPH500 (*α*, *ura3-52*, *lys2-801*, *ade2-101*, *trp1Δ63*, *his3Δ200*, and *leu2Δ1*) (Stratagene) to create strains containing these synthetic gene circuits, which had a chromosomally integrated *pet56-his3Δ200* locus in their genome as described previously [25]. In the NRpump558/312 and NFpump558/312 versions, a single nucleotide mutation was introduced into the *PDR5* gene before following the same procedure for yeast genomic integration. In the S558Y mutant, the C base of the *PDR5* gene was changed to A at position 1673, while the G base was changed to C at position 935 in the G312A mutant. These mutants lack pump function and can serve as controls. Additionally, since *PDR5*-controling gene circuit integration can alter regulator expression, we also constructed 2-color versions of each gene circuit. In these 2-color gene circuits, the regulator gene *tetR* was replaced with the *tetR::mCherry* fusion gene in the regulator plasmid. Other procedures were the same for genomic integration. Finally, colonies with single integration were selected by PCR and flow cytometry as described before [12] . All cell cultures were grown in the YPD medium, synthetic complete medium, or synthetic drop-out medium with appropriate selection markers and 2% galactose or glucose.

3.5 Endogenous PDR5 Knockout

To make a kill curve to optimize the concentration of the selection agent, here we investigated the response to G418. The concentration must distinguish G418 resistance strains from non-resistance strains. Use any KanMX4 cassette containing strain from the same genetic background as positive control, since they are resistant to G418 (*see* **Note 8**).

1. Prepare YPD plates containing 400 μg/mL G418. For knocking out PDR5, we replace the endogenous PDR5 gene with a G418-resistant gene.
2. Primer design: Following the yeast deletion project website (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html). 10 primers need to be designed. UPtag, DNtag, UPstream 45, DNsteam 45, Confirmation primer A, B, C, D, KanB and KanC. 8 of them can be found on the yeast deletion project website with the reference of ORF name of *PDR5* (http://www-sequence.stanford.edu/group/yeast_deletion_project/Deletion_primers_PCR_sizes.txt). The two remaining primers, KanB and KanC, are universal for all KanMX4 cassette gene knockouts. Primer KanB (5′-CTGCAGCGAGGAGCCGTAAT-3′), primer KanC (5′-TGATTTTGATGACGAGCGTAAT-3′).
3. PCR: Amplify the linear oligo sequence containing the KanMX4 cassette with homologous sequence of *PDR5* by PCR. Follow the protocol on yeast deletion webpage for the

two rounds of PCR (www-sequence.stanford.edu/group/yeast_deletion_project) and then calculate the amount of DNA yield. Do additional rounds of PCR if more DNA is needed for transformation. Prepare a KanMX4 plasmid by growing the corresponding frozen *E. coli* strain. Make mini prep, midi prep, or maxi prep to obtain the KanMX4 plasmid from *E. coli*.

4. Transformation: follow the protocol in Subheading 3.2 for transformation. We used 0.3 μg of PCR products for transformation. Remember to include your negative control (no DNA in transformation) in the experiment.
5. Selection of knockout clones by confirmation PCR. Follow the protocol on the yeast deletion webpage (www-sequence.stanford.edu/group/yeast_deletion_project). Successful deletion can be confirmed by two PCR products with primer A and KanB, and primer D and KanC, while no product with primer A and B, or primer C and D. Otherwise, the knockout has failed and the procedure should be repeated.

3.6 Site-Directed Mutagenesis

There are three main steps in the mutagenesis process as listed below, each step has detailed instruction provided in the Agilent QuickChange II XL site-directed mutagenesis kit. Make sure to include the control reaction provided in the kit.

1. Run a PCR reaction with the designed primes containing the specific site mutation to amplify the entire plasmid.
2. Digest the template plasmid using DpnI restriction enzyme (NEB).
3. Transform competent *E. coli* cells provided in the kit (*see* **Note 9**).

3.7 Identification of Optimal Cell Culture Time for each Strain

This experiment determines the time for each strain to reach saturation, because cells need to grow in exponential phase to maintain stable growth conditions for the experiments. The goal of this measurement is to determine the starting cell density and the time for each strain to reach saturation without an inducer.

1. Pick a single colony from the yeast plate, inoculate into 1 mL SD medium with 2% glucose, incubate at 30 °C, shaking at 300 rpm overnight.
2. Measure cell density with a Nexcelom cell counter, and inoculate 5×10^5 cells into 1 mL SD medium with 2% galactose and appropriate selection marker. Then, incubate at 30 °C, while shaking at 300 rpm.
3. Measure cell density every 2 h for 24 h or until cell growth reaches stationary phase, meaning no cell density change in two consecutive measurements.

4. Plot the growth curves and estimate the time it takes for each strain to reach the stationary phase.

3.8 Measurement of Growth Rate

1. Streak cells from frozen stock on a selection plate with 2% glucose, incubate at 30 °C for 2 days.
2. Inoculate a single colony into SD medium with a selection marker and 2% glucose, incubate in the 30 °C incubator shaking at 300 rpm overnight.
3. Measure cell density with the Nexcelom cell counter, and inoculate 5×10^5 cells into 1 mL SD medium with 2% galactose, selection marker, and increasing concentrations of inducer (doxycycline). Incubate at 30 °C, and shaking at 300 rpm.
4. Measure cell density every 12 h and resuspend cells in fresh medium with starting cell density of 5×10^5 cells/mL. Measure cell density during the entire dose-response experiment.

3.9 PDR5 Functionality Measurement by Calcein-AM Red

1. Grow cells as described in Subheading 2.6 for 3 days until PDR5 expression becomes stable as measured by flow cytometry, meaning no significant change of GFP fluorescence distribution over two consecutive days.
2. Transfer cell culture from culture tube to sterilized 1.5 mL Eppendorf tube, centrifuge at 8000 × *g* for 3 min.
3. Remove the supernatant by pipetting, add 500 μL ice cold PBS into the cell pellet, resuspend the cells and mix well by vortexing, centrifuge at 8000 × *g* for 3 min.
4. Repeat **step 3**.
5. Measure cell density with Nexcelom cell counter, resuspend cells into 100 μL fresh SD medium with a cell density of 5×10^5 cells/mL.
6. Dissolve Calcein-AM red dry powder into DMSO, and prepare stock solution with concentration at 50 μg/mL.
7. Add 10 μL of 50 μg/mL Calcein-AM red to the cell culture, reaching a concentration of 5 μg/mL. No *PDR5* gene strain is used as a negative control.

3.10 Measurement of Reporter Gene Expression

Flow cytometry is performed every 24 h for normal dose-response characterization. For strains with only GFP fluorescence, a unit with a single-excitation laser (such as a Becton-Dickinson FACSCan) is sufficient, while a unit with two excitation lasers (such as a Becton-Dickinson FACSAria II) is necessary for taking dose-responses of 2-color strains and Calcein-AM red dye measurements to test PDR5 functionality. Flow cytometry data is collected to monitor the stabilization of target gene expression in the cell population. Once the target gene expression becomes stable, flow cytometry data is used to analyze dose-responses.

3.11 Data Analysis

Single-laser flow cytometer (FASCScan) data analysis:

1. Extract FSC, SSC, and FL1 (green fluorescence intensity) using the FACScan data package in Matlab (*see* **Note 10**).
2. To reduce extrinsic noise, apply a gate based on FSC and SSC, and select the gated area to include the highest cell density in each sample.
3. Read and process FL1 data based on the gating.
4. Calculate the arithmetic mean and standard deviation; exclude outliers, that is, any data point outside of three times the standard deviation, from the data set.
5. Subtract the FL1 value of YPH500 strain from that of each sample (*see* **Note 11**).
6. Normalize the mean of each sample to the sample in its corresponding strain that was induced at the highest doxycycline concentration.
7. Calculate arithmetic mean and CV of processed FL1 value.
8. Plot arithmetic mean and CV against doxycycline concentrations.

Two-laser flow cytometer (FACSAria II) data analysis:

1. Extract FSC, SSC, FL1 (green fluorescence intensity) and FL3 (red fluorescence intensity) using .fcs file reader scripts (such as fcsread) in Matlab (*see* **Note 10**).
2. The following steps are identical to the single-laser data analysis above, except that the mean and CV are calculated for both FL1 and FL3, and then plotted against doxycycline concentrations separately for each 2-color strain.

3.12 Mathematical and Computational Modeling

1. Develop a set of statements (facts from the known biology and reasonable assumptions) about the system. For example, for the NRpump gene circuit (Fig. 1c), the following statements provide a basis for the model (for a modeling example based on the NF gene circuit, along with the corresponding Dizzy software code, *see* ref. 24)
   (a) TetR represses a downstream efflux pump-reporter gene.
   (b) TetR and efflux-pump reporter proteins are relatively stable relative to *S. cerevisiae* cell cycle time and therefore are diluted at a constant rate given by cell growth.
   (c) TetR binds irreversibly to doxycycline and is inactivated when bound.
   (d) For doxycycline to bind to TetR, it must diffuse into the cell from the extracellular environment.
   (e) The PDR5 protein is an efflux pump that transports doxycycline out of the cell.

These statements can be translated into a set of biochemical reactions and then a mathematical model. E.g., the set of ordinary differential equations (ODEs) for NRpump is:

$$\frac{dx}{dt} = a_x - bxy - dx$$
$$\frac{dy}{dt} = C - bxy - fy - \frac{ky^h z}{K^h + y^h}$$
$$\frac{dz}{dt} = l_z + a_z F_z(x) - dz$$

where the variables x, y, and z are the concentrations of TetR species unbound to doxycycline, intracellular doxycycline, and the pump-reporter protein, respectively. The parameters a_x and a_z represent maximal gene expression of TetR and the gene of interest, respectively, l_z describes leaky pump-reporter protein synthesis, b is the rate of doxycycline binding to TetR, C is doxycycline influx into the cell and is proportional to extracellular doxycycline concentration, d is the dilution rate, and f is the combined rate of doxycycline dilution and diffusion out of the cell. The parameters for the pump term (last term on the right-hand side of the dy/dt equation) are K which is the concentration at which the activation of the pump is half-maximal, the pump Hill coefficient h, and the rate of Pdr5p mediated inducer efflux k ($k = 0$ for the NR circuit, and $k > 0$ for the NRpump circuit). $F_z(x)$ is a repression function that can be approximated with a Hill function:

$$F_z(x) = \frac{\theta^n}{\theta^n + x^n}$$

where θ is the repression threshold and n the Hill coefficient.

2. Estimate parameters to describe the model system. To obtain the exact values for these parameters for our experiment we fit the following analytic solution for z as a function of the control parameter C available for the NR gene circuit [14] to the experimental data:

$$z(C) = \frac{l_z + a_z F_z(x(C))}{d}$$

where

$$x(C) = \frac{a_x b - df - bC + \sqrt{(a_x b - df - bC)^2 + 4bda_x f}}{2bd}$$

The analytic equation was fit to the experimental data for the NR strain using Matlab's [27] curve fitting application (fit options: Nonlinear Least Squares, algorithm: Trust-Region, $r^2 = 0.9991$) guided by biologically realistic upper and lower parameter bounds. Fitting for the NRpump strain was based on these parameters, tuning additional parameters for the pump term by hand to fit the experimental data. The computational results for the ODE model were obtained using Matlab's ode45 differential equation solver using the code described in Box 1 (*see* **Notes 12** and **13**).

3. Consider the biochemical reactions underlying the mathematical model:

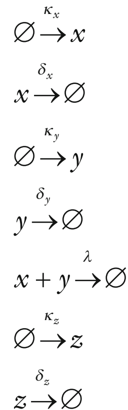
Box 1 Matlab "skeleton" code for the stochastic simulation of the NRpump gene circuit

```
%rates
ax = 43;          %maximal TetR production rate
az = 50;          %maximal Pdr5p::GFP production rate
lz = 10;          %leaky Pdr5p::GFP production rate
bprime = 10;      %doxycycline-TetR binding rate
d = 0.12;         %TetR and Pdr5p::GFP dilution rate
f = 1.2;          %doxycycline dilution-diffusion rate
n = 4;            %Hill coefficient for repression function
theta = 0.44;     %TetR-promoter dissociation constant
k = 200;          %rate of Pdr5p::GFP mediated efflux
K = 50;           %half-maximal pump activation parameter
h = 3.5;          %Hill coefficient for pump term
c = 10;           %doxycycline influx rate
dox = 0:15;       %extracellular doxycycline
t_end = 200;      %simulation end time
dox_cnt = 0;
for C = dox*c
    dox_cnt = dox_cnt+1;
%species
     x = 0; %unbound/active TetR protein
     y = 0; %intracellular doxycycline
     z = 0; %efflux pump-reporter protein
     %Gillespie SSA loop
     t = 0; %initialize simulation time
     while t < t_end
     %calculate reaction propensities
```

(continued)

Box 1 (continued)

```
      a = [ax (bprime*x*y+x*d) C ...
           (bprime*x*y+y*f+(k*y^h*z)/(K^h+y^h)) ...
           (lz+az*(theta^n/(theta^n+x^n))) z*d];
      a0 = sum(a); %combined reaction hazard
      %calculate time to next event
      r1 = rand;
      while r1 == 0
           r1 = rand;
      end
      t_next = ((1/a0)*(log(1/r1)));
      %update time
      t = t + t_next;
      %determine next reaction
      i = 1; mu = 0; amu = 0; r2 = rand;
      while amu < r2*a0
           mu = mu + 1;
           amu = amu + a(i);
           i = i + 1;
      end
      %reactions
      if mu == 1
           x = x + 1;
      elseif mu == 2
           x = x - 1;
      elseif mu == 3
           y = y + 1;
      elseif mu == 4
           y = y - 1;
      elseif mu == 5
           z = z + 1;
      elseif mu == 6
           z = z - 1;
      end
   end
   %save SSA data
   SSA_data(dox_cnt,1:3) = [x y z];
 end
 %figure
 plot(dox,SSA_data(:,3),'k.--')
```



where $k_x = a_x$, $\delta_x = d$, $k_y = C = doxc$, $\delta_y = f + ky^{-1}(y^h/(K^h + y^h))z$, $\lambda = b' = b/(V^*N_A)$ where V is the cell volume and N_A Avogadro's number, $k_z = l_z + a_zF_z$, and $\delta_z = d$ (*see* **Note 14**).

4 Notes

1. Please make sure to add adenine, otherwise the medium and plate turn red.
2. Sterilize glucose by filtering through a 0.22 μm filter.
3. Make sure to add glucose only after the sterilized liquid medium cools down.
4. You can use any polymerase of your choice for gene amplification but it is important to use a high-fidelity polymerase variant to minimize the chance of mutations introduced during the PCR step. Make sure to verify the sequence of the whole insert in your final plasmid in addition to flanking regions. *PDR5::GFP* is a relatively long gene and at least double coverage will be required for the entire coding region. Please use primers specified in Subheading 2.2, **item 4**.
5. We use the *pet56-his3Δ200* locus present in the YPH500 yeast strain (and derivatives) for integrating our plasmid. If you plan to use a different yeast strain for transformation, you might need to design and introduce your own homology region into the plasmid. Also, depending on the strain background, you might need to use a different auxotrophic marker.
6. Heat water to boiling temperature before taking salmon sperm DNA from the freezer, or use the PCR machine to boil the sample. If working with more than one sample, boil more salmon sperm DNA accordingly.
7. Use fresh PEG or less than 1 month old PEG (PEG concentration changes over time).

8. Most online protocols recommend using 200 μg/mL G418 for selection, but 400 μg/mL worked best for us.
9. Please make sure to use polypropylene round-bottom tubes, since tubes made of other materials can cause very low transformation rate.
10. Please contact us if you have questions about the Matlab code.
11. The YPH500 strain itself generates a low autofluorescence signal, which may need to be subtracted from the data.
12. The Matlab script is also available at: https://github.com/dacharle42/NRpump_SSA_MiMB.git.
13. Simulate the reaction equations using the Gillespie stochastic simulation algorithm [28] in Matlab (*see* Box 1) [27] or other software such as Dizzy [24]. Execute code over many independent runs to generate gene expression statistics for plotting and analysis. In practice, the number of runs is increased until the statistics do not change except within a small margin of error. To normalize the results, divide the mean reporter-pump protein number for each inducer concentration by the value obtained for the highest inducer concentration.
14. The reaction rate of inducer influx into the cell is given by *dox***c*, where *dox* is the concentration of extracellular doxycycline and *c* is the membrane permeability.

Acknowledgments

The authors would like to thank W.-K. Huh for kindly sharing the PDR5::GFP fusion, and M. Bennett, M. Lorenz, G. May, T. F. Cooper, G. Peng for helpful discussions, and Matthew Wu for editing the modeling section of the chapter. This research was supported by the NIH Director's New Innovator Award (1DP2 OD006481– 01) and an NIGMS Maximizing Investigators' Research Award (MIRA, 1R35GM122561) to G.B.; by an NSERC Postdoctoral Fellowship [Grant no: PDF-453977–2014] to D.C.; by the University of Texas Graduate School of Biomedical Sciences at Houston to J.D.; by Program # 1326 of the Ministry of Education and Science, Russian Federation to D.N.; and by the Laufer Center for Physical & Quantitative Biology. Daniel A. Charlebois and Junchen Diao contributed equally to this work.

References

1. Stephanopoulos G (2012) Synthetic biology and metabolic engineering. ACS Synth Biol 1:514–525
2. Nielsen J et al (2014) Engineering synergy in biotechnology. Nat Chem Biol 10:319–322
3. Khalil AS, Collins JJ (2010) Synthetic biology: applications come of age. Nat Rev Genet 11:367–379
4. Way JC et al (2014) Integrating biological redesign: where synthetic biology came from and where it needs to go. Cell 157:151–161
5. Purnick PEM, Weiss R (2009) The second wave of synthetic biology: from modules to systems. Nat Rev Mol Cell Biol 10:410–422
6. Collins JJ, Gardner TS, Cantor CR (2000) Construction of a genetic toggle switch in Escherichia coli. Nature 403(6767):339–342
7. Elowitz MB, Leibler S (2000) A synthetic oscillatory network of transcriptional regulators. Nature 403(6767):335–338
8. Anderson JC, Voigt CA, Arkin AP (2007) Environmental signal integration by a modular AND gate. Mol Syst Biol 3:133
9. Tamsir A, Tabor JJ, Voigt CA (2011) Robust multicellular computing using genetically encoded NOR gates and chemical 'wires'. Nature 469:212–215
10. Dunlop MJ et al (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487–487
11. Diao J et al (2016) Efflux pump control alters synthetic gene circuit function. ACS Synth Biol 5:619–631
12. Nevozhay D et al (2009) Negative autoregulation linearizes the dose-response and suppresses the heterogeneity of gene expression. Proc Natl Acad Sci U S A 106:5123–5128
13. Blake WJ et al (2006) Phenotypic consequences of promoter-mediated transcriptional noise. Mol Cell 24:853–865
14. Murphy KF, Balazsi G, Collins JJ (2007) Combinatorial promoter design for engineering noisy gene expression. Proc Natl Acad Sci U S A 104:12726–12731
15. Kaern M et al (2005) Stochasticity in gene expression: from theories to phenotypes. Nat Rev Genet 6(6):451–464
16. Charlebois DA, Kaern M (2012) What all the noise is about: the physical basis of cellular individuality. Can J Phys 90:919–923
17. Sanchez A, Golding I (2013) Genetic determinants and cellular constraints in noisy gene expression. Science 342:1188–1193
18. Nevozhay D, Zal T, Balázsi G (2013) Transferring a synthetic gene circuit from yeast to mammalian cells. Nat Commun 4:1451–1451
19. Charlebois DA, Balazsi G, Kaern M (2014) Coherent feedforward transcriptional regulatory motifs enhance drug resistance. Phys Rev E 89:052708
20. Lage H (2003) ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int J Antimicrob Agents 22:188–199
21. Balzi E, Goffeau A (1995) Yeast multidrug resistance: the PDR network. J Bioenerg Biomembr 27:71–76
22. Huh W-K et al (2003) Global analysis of protein localization in budding yeast. Nature 425:686–691
23. Eaton JW, Bateman D, Hauberg S (2008) GNU octave manual. Network Theory Ltd., Surrey
24. Ramsey S, Orrell D, Bolouri H (2005) Dizzy: stochastic simulation of large-scale genetic regulatory networks. J Bioinforma Comput Biol 3:415–436
25. Nevozhay D et al (2012) Mapping the environmental fitness landscape of a synthetic gene circuit. PLoS Comput Biol 8:e1002480
26. Nevozhay D, Adams R, Balázsi G (2011) Linearizer gene circuits with negative feedback regulation. Methods Mol Biol 734:81–100
27. MATLAB 2016 The MathWorks Inc.: Natick, MA
28. Gillespie DT (1977) Exact stochastic simulation of coupled chemical reactions. J Phys Chem 81:2340–2361