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Long-term model predictive control of gene expression at the population and single-cell levels

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Gene expression plays a central role in the orchestration of cellular processes. The use of inducible promoters to change the expression level of a gene from its physiological level has significantly contributed to the understanding of the functioning of regulatory networks. However, from a quantitative point of view, their use is limited to short-term, population-scale studies to average out cell-to-cell variability and gene expression noise and limit the nonpredictable effects of internal feedback loops that may antagonize the inducer action. Here, we show that, by implementing an external feedback loop, one can tightly control the expression of a gene over many cell generations with quantitative accuracy. To reach this goal, we developed a platform for real-time, closed-loop control of gene expression in yeast that integrates microscopy for monitoring gene expression at the cell level, microfluidics to manipulate the cells' environment, and original software for automated imaging, quantification, and model predictive control. By using an endogenous osmoresponsive promoter and playing with the osmolarity of the cells' environment, we show that long-term control can, indeed, be achieved for both time-constant and time-varying target profiles at the population and even the single-cell levels. Importantly, we provide evidence that real-time control can dynamically limit the effects of gene expression stochasticity. We anticipate that our method will be useful to quantitatively probe the dynamic properties of cellular processes and drive complex, synthetically engineered networks.

model based control | computational biology |
high osmolarity glycerol pathway | quantitative systems biology

Understanding the information processing abilities of biological systems is a central problem for systems and synthetic biology (1–6). The properties of a living system are often inferred from the observation of its response to static perturbations. Time-varying perturbations have the potential to be much more informative regarding the dynamics of cellular functions (7–12). Currently, it is not possible to precisely perturb protein levels in an analogous manner, even though this perturbation would be instrumental in our understanding of gene regulatory networks. Indeed, despite the development of novel regulatory systems, including various RNA-based solutions (13), transcriptional control by means of inducible promoters is still the preferred method for manipulating protein levels (14, 15). Unfortunately, inducible promoters have several generic limitations. First, there is a significant delay between gene expression activation and effective protein synthesis. Second, many cellular processes can interfere with gene expression through internal feedback loops whose effects are hard to predict. Third, the process of gene expression shows significant levels of noise (16–18). Given these limitations, novel experimental strategies are required to gain quantitative, real-time control of gene expression *in vivo*.

Here, we see the problem of manipulating gene expression to obtain given temporal profiles of protein levels as a model-based control problem. More precisely, we investigate the effectiveness of computerized closed-loop control strategies to control gene expression *in vivo*. In model-based closed-loop control, a model of the

system is used to constantly update the control strategy based on real-time observations. We propose an experimental platform that implements such an *in silico* closed loop in the budding yeast *Saccharomyces cerevisiae*. We show that gene expression can be controlled by repeatedly stimulating a native endogenous promoter over many cell generations (>15 h) for both time-constant and time-varying target profiles and at both the population and single-cell levels. Recently, Miliadis-Argeitis et al. (19) also proposed an approach for feedback control of gene expression in yeast. In contrast to their work, we propose a method that is effective at the single-cell level, for time-varying target profiles, and robust despite the presence of strong internal feedback loops. We start by describing the gene induction system and the experimental platform before discussing its efficiency.

Results and Discussion

Controlled System. We based our approach on the well-known response of yeast to an osmotic shock, which is mediated by the high osmolarity glycerol (HOG) signaling cascade. Its activation leads to the phosphorylation of the protein Hog1 (Fig. 1A), which orchestrates cell adaptation through glycerol accumulation. Phosphorylated Hog1 promotes glycerol production by activating gene expression in the nucleus as well as stimulating glycerol-producing enzymes in the cytoplasm. After they are adapted, the cells do not sense the hyperosmotic environment anymore, the HOG cascade is turned off, and the transcriptional response stops (20–22). In control terms, yeast cells implement several short-term (non transcriptional) and long-term (transcriptional) negative feedback loops that ensure perfect adaptation to the osmotic stress (10, 23). Because of these adaptation mechanisms, it is a priori challenging to control gene expression induced by osmotic stress. It is, thus, an excellent system to show that one can robustly control protein levels, even in the presence of internal negative feedback loops. Several genes are up-regulated in response to a hyperosmotic stress. These genes include the nonessential gene *STL1*, which codes for a glycerol proton symporter (24, 25). We decided to use its native promoter (pSTL1) to drive the expression of yECitrine, a fluorescent reporter. Applying an osmotic stress transiently activated the HOG cascade (Fig. 1B), and yECitrine levels reached modest values (600 fluorescence units) (Fig. 1B). Importantly, when short but repeated stresses were applied, pSTL1 could be repeatedly activated, and much higher levels could be reached (Fig. 1C).

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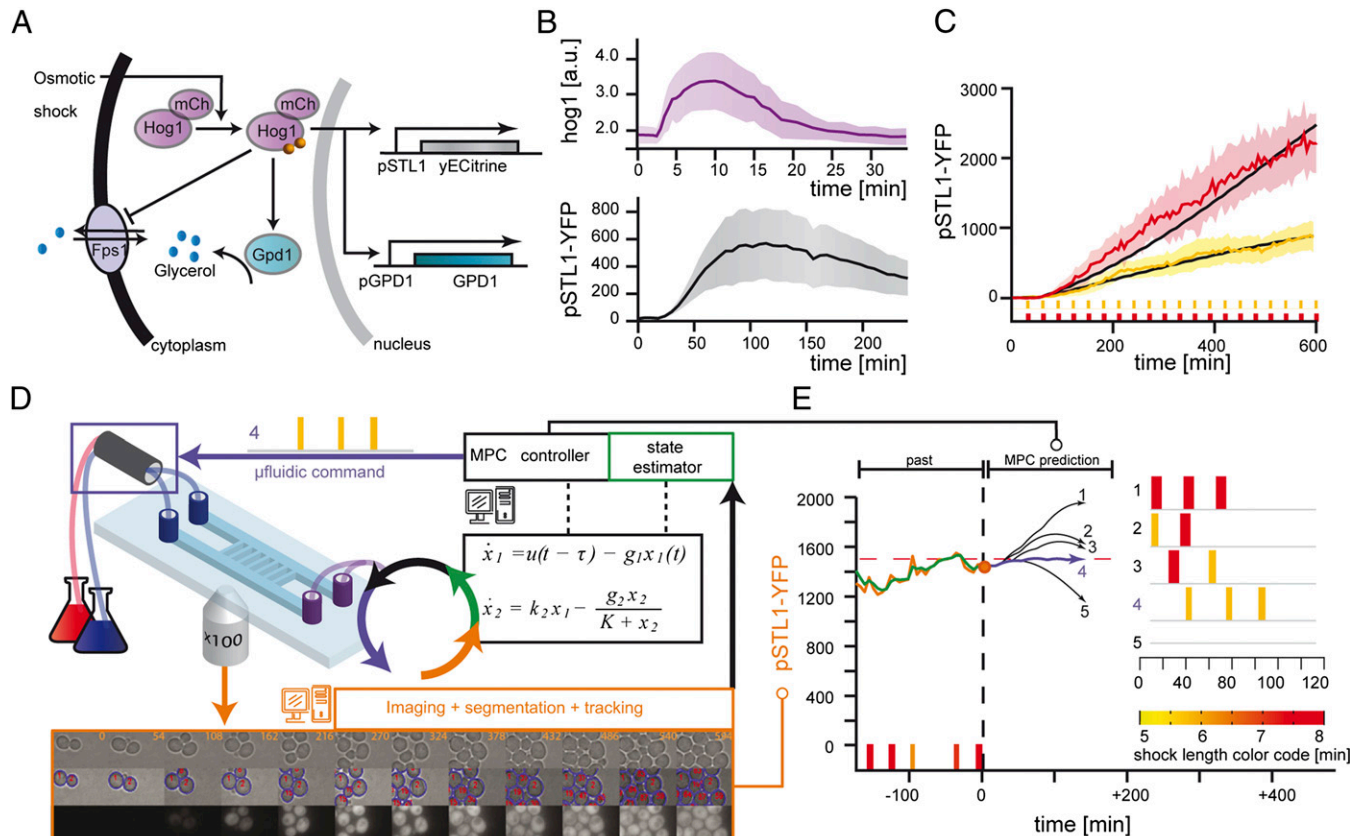


Fig. 1. A platform for real-time control of gene expression in yeast. (A) A hyperosmotic stress triggers the activation and nuclear translocation of Hog1. Short-term adaptation is mainly implemented by cytoplasmic activation of the glycerol-producing enzyme Gpd1 and closure of the aqua-glyceroporin channel Fps1. Long-term adaptation occurs primarily through the production of Gpd1. (B) When maintained in a hyperosmotic environment (1 M sorbitol), the HOG cascade was quickly activated, which is seen by Hog1 nuclear enrichment. This transient signaling response lasted typically <20 min. The expression level of pSTL1-yECitrine (YFP) increased after an ~20-min delay, peaked around 600 fluorescence units after 100 min, and then decayed. (C) In contrast, the fluorescence level showed a continuous increase when stimulated periodically (T = 30 min). The increase rate was larger for longer pulses (red, 8 min; yellow, 5 min). Black curves are the expected behaviors based on our model of the pSTL1 induction. Solid lines and their envelopes are the experimental means and SDs of the cells' fluorescence. (D) Yeast cells grew as a monolayer in a microfluidic device that was used to rapidly change the cells' osmotic environment (blue frame) and image their response. Segmentation and cell tracking were done using a Hough transform (orange frame). The measured yECitrine fluorescence, either of a single cell or of the mean of all cells, was then sent to a state estimator connected to an MPC controller. A model (black frame) of pSTL1 induction was used to find the best possible series of osmotic pulses to apply in the future so that the predicted yECitrine level follows a target profile. (E) At the present time point (orange circle), the system state is estimated (green), and the MPC searches for the best input (pulse duration and number of pulses) (see text and [SI Materials and Methods](#)), which minimizes the distance of the MPC predictions (black curves) to the target profile (red dashed line) for the next 2 h. Here, the osmotic series of pulses that corresponds to the blue curve (4) was selected and sent to the microfluidic command. This control loop is iterated every 6 min.

A closed-loop control of the pSTL1 activity requires the acquisition and analysis of live cell images, the computation of the input (i.e., osmolarity) to be applied in the near future, and the ability to change the cells osmotic environment accordingly (Fig. 1 *D* and *E*).

Experimental Platform. To observe the cells and control their environment, we designed a versatile platform made of standard microscopy and microfluidic parts. The microfluidic device contained several 3.1- μm -high chambers that were connected by both ends to large channels through which liquid media could be perfused (Fig. 1D). Because the typical diameter of an *S. cerevisiae* cell is 4–5 μm , the cells were trapped in the chamber and grew as a monolayer. Their motion was limited to slow lateral displacement due to cell growth (Fig. S1). This design allowed for long-term cell tracking (>15 h) and relatively rapid media exchanges (~2 min). The HOG pathway was activated by switching between normal and sorbitol-enriched (1 M) media.

Model of pSTL1 Induction. To decide what osmotic stress to apply at a given time, we used an elementary model of pSTL1 induction. Many models have been proposed for the hyperosmotic

stress response in yeast (10, 26–30). We used a generic model of gene expression written as a two-variable delay differential equation system, where the first variable denotes the recent osmotic stress felt by the cell and the second variable is the protein fluorescence level (Fig. 1D, *Materials and Methods*, Table S1, and *SI Materials and Methods*). Because our goal was to show robust control, despite the presence of unmodeled feedback loops, the adaptation mechanisms described above were purposefully neglected. The choice of this model was also motivated by the tradeoff between its ability to quantitatively predict the system’s behavior (favors complexity) and the ease of solving state estimation problems (favors simplicity). Despite its simplicity, we found a fair agreement between model predictions and calibration data corresponding to fluorescence profiles obtained by applying either isolated or repeated osmotic shocks of various durations (Fig. 1C and Fig. S2).

Closing the Loop. The fluorescence intensity of a single cell arbitrarily chosen at the start of the experiment, or the average fluorescence intensity of the cell population, was sent to a state estimator (extended Kalman filter discussed in [SI Materials and Methods](#)) connected to a model predictive controller (31). Model Predictive Control (MPC) is an efficient framework well-adapted

Closed-Loop Population Control Experiments. Our first goals were to maintain the average fluorescence level of a cell population at a given constant value (set-point experiment) and force it to follow a time-varying profile (tracking experiment). Both types of experiments lasted at least 15 h, starting with a few cells and ending with 100–300 cells in the field of view (Fig. S3). The control objective was to minimize the mean square deviations (MSDs) between the mean fluorescence of the population of cells and the target profile. We succeeded in maintaining the average fluorescence level at a given constant value or forcing it to follow several given time-varying profiles (Fig. 2 A–D, Figs. S3, S4, and S5, and Movies S1, S2, and S3). Admissible time-varying target profiles were obviously constrained by the intrinsic timescales of the system, such as the maximal protein production and degradation rates. However, within these constraints, graded responses could be obtained. In Fig. 2C, for example, the trapeze slope is less steep than what maximal pSTL1 induction can deliver (Fig. 2 A and B). Note that our control strategy opened the possibility to reach higher fluorescence levels than what full induction with a step shock would allow (compare with Fig. 1B). Indeed, because of cell perfect adaptation to hyperosmotic stresses, a sustained 1 M sorbitol shock triggers only a transient gene expression and fluorescence peaks at moderate levels (Fig. 1B). By using repeated, well-separated pulses,

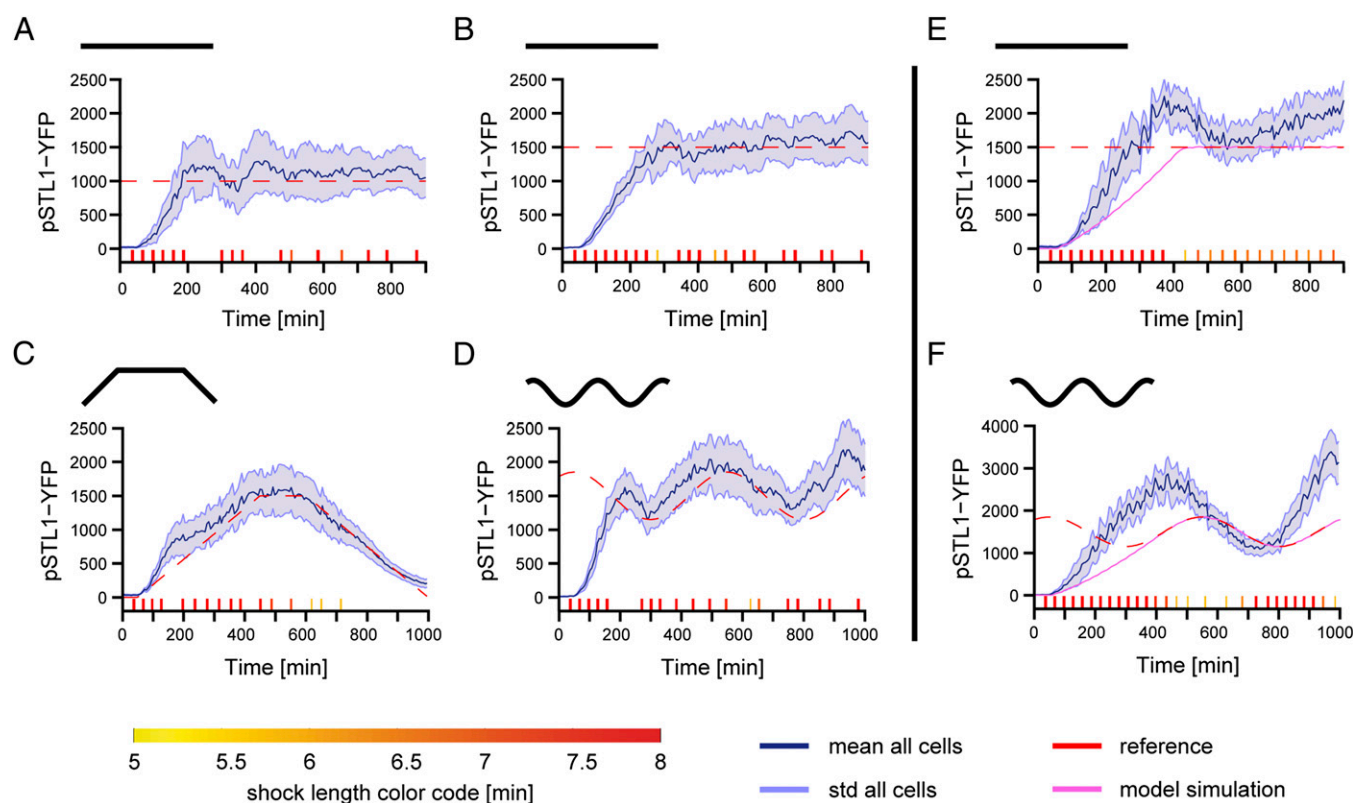
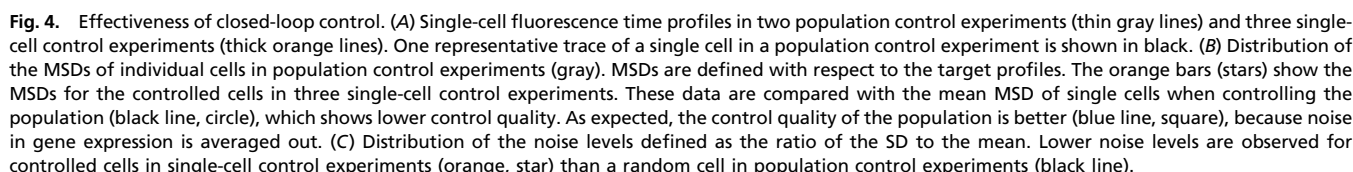


Fig. 2. Real-time control of gene expression can be achieved at the population level. (A and B) Set-point control experiments with target values 1,000 and 1,500 fluorescence units (f.u.; red dashed line). This unit is the same across all graphs (no renormalization). To avoid desensitizing the HOG pathway, the controller repeatedly applied short osmotic pulses (durations between 5 and 8 min). The timeline of osmotic events is shown at the bottom of each graph (color code along the bottom). Shock starting times and durations were computed in real time. The measured mean cell fluorescence is shown as solid blue lines. The envelopes indicate SD of the fluorescence distribution across the yeast population. (C and D) Tracking control experiments. In C, the target has a trapezoidal shape (maximum at 1,500 f.u.). In D, the target is sinusoidal (average value at 1,500 f.u.). In both cases, the mean level of fluorescence successfully follows the time-varying target profile. (E and F) Open-loop control experiments. Two examples of open-loop control (the osmotic inputs were computed using our model before starting the experiments) showing poor control quality. Errors accumulate over time. The simulated behavior of the system is represented in violet.

Closed-Loop Single-Cell Control Experiments. In a second set of experiments, we focused on the real-time control of gene expression at the single-cell level. We tracked one single cell over at least 15 h and used its fluorescence to feed the MPC controller. As shown

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Microscopy and Experimental Setup. We used an automated inverted microscope (IX81; Olympus) equipped with an X-Cite 120PC fluorescent illumination system (EXFO) and a QuantEM 512 SC camera (Roper Scientific). The YFP filters used were HQ500/20× (excitation filter; Chroma), Q515LP (dichroic; Chroma), and HQ535/30M (emission; Chroma). All these components were driven by the open-source software μ Manager (40), a plug-in of ImageJ (41), which we interfaced with Matlab using in-house-developed code. The temperature of the microscope chamber, which also contained the media reservoirs, was constantly held at a temperature of 30 °C by a temperature control

system (Life Imaging Services). Images were taken with a 100× objective (PlanApo 1.4 NA; Olympus). The fluorescence exposure time was 200 ms, with fluorescence intensity set to 50% of maximal power. The fluorescence exposure time was chosen such that the fluorescent illumination did not cause noticeable effects on cellular growth over extended periods of time. Importantly, illumination, exposure time, and camera gain were not changed between experiments, and no data renormalization was done. Therefore, the fluorescence intensities can be directly compared across experiments.

Image Analysis. The cellular boundaries were identified on the bright-field image using a circular Hough transform implemented in Matlab (42). For tracking, we compared the current image with the previous one, defined a cell-to-cell distance matrix, and used linear optimization to match pairs of cells. The tracking process was made more robust by also considering the last but one image if a gap was detected (caused by rare segmentation errors). The YFP fluorescence level in each cell was defined as the mean fluorescence level taken over the cell area after subtraction of the background fluorescent level. The signaling activity of the Hog1 cascade can be estimated by measuring the Hog1 nuclear enrichment. We defined the nuclear enrichment of Hog1::mCherry as the difference between the minimal and maximal fluorescence intensities within a cell. Maximal and minimal Hog1::mCherry intensities were computed by averaging the fluorescence of the 15 brightest and 15 dimmest pixels, respectively.

Modeling. The controller used a two dimensional ordinary differential equation (ODE) model to predict the behavior of the system:

$$\dot{x}_1 = u(t - \tau) - g_1 x_1$$

and

$$\dot{x}_2 = k_2 x_1 - \frac{x_2}{K + x_2},$$

where x_1 denotes the recent osmotic stress and x_2 denotes the protein fluorescence level. The osmotic input (u) is shifted by $\tau = 20$ min to account for the observed delay in the system. The remaining parameters have been estimated based on several calibration experiments: $g_1 = 4.02 \times 10^{-3}$, $k_2 = 0.58$, $g_2 = 37.5$, $K = 750$, and $\tau = 20$ (SI Materials and Methods, Table S1, and Fig. S2).

State Estimation. We implemented an extended Kalman filter, which estimates the system state based on fluorescent observations and the model of the system. The parameters of the filter (measurement noise R and process noise Q) were set to $R = 2,500$ and $Q = \text{diag}(0.37, 925)$.

Model Predictive Control. The controller searches for osmolarity profiles that minimize the squared deviations between model output and target profile within the next 120 min, while fulfilling the input constraints (pulse duration of 5 to 8 min separated by at least 20 min). In practice, this problem is recast into a parameter search problem, in which parameters are used for encoding stress starting times and shock durations and solved using the global optimization tool CMAES. Because image analysis and parameter search may take up to 3 min, the input to be applied is not immediately available at the time of the measurement. Consequently, we apply at time t the input that was computed at time $t - 3$ min.

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- Bhalla US, Ram PT, Iyengar R (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science* 297:1018–1023.
- Hooshangi S, Thiberge S, Weiss R (2005) Ultrasensitivity and noise propagation in a synthetic transcriptional cascade. *Proc Natl Acad Sci USA* 102:3581–3586.
- Cai L, Dalal CK, Elowitz MB (2008) Frequency-modulated nuclear localization bursts coordinate gene regulation. *Nature* 455:485–490.
- Celani A, Vergassola M (2010) Bacterial strategies for chemotaxis response. *Proc Natl Acad Sci USA* 107:1391–1396.
- Baumgartner BL, et al. (2011) Antagonistic gene transcripts regulate adaptation to new growth environments. *Proc Natl Acad Sci USA* 108:21087–21092.
- O'Shaughnessy EC, Palani S, Collins JJ, Sarkar CA (2011) Tunable signal processing in synthetic MAP kinase cascades. *Cell* 144:119–131.
- Walter E, Pronzato L (1997) *Identification of Parametric Models from Experimental Data* (Springer, Berlin).
- Bennett MR, et al. (2008) Metabolic gene regulation in a dynamically changing environment. *Nature* 454:1119–1122.
- Hersen P, McClean MN, Mahadevan L, Ramanathan S (2008) Signal processing by the HOG MAP kinase pathway. *Proc Natl Acad Sci USA* 105:7165–7170.
- Muzzey D, Gómez-Urbe CA, Mettetal JT, van Oudenaarden A (2009) A systems-level analysis of perfect adaptation in yeast osmoregulation. *Cell* 138:160–171.
- Shimizu TS, Tu Y, Berg HC (2010) A modular gradient-sensing network for chemotaxis in *Escherichia coli* revealed by responses to time-varying stimuli. *Mol Syst Biol* 6:382.
- Pelet S, et al. (2011) Transient activation of the HOG MAPK pathway regulates bimodal gene expression. *Science* 332:732–735.
- Rao CV (2012) Expanding the synthetic biology toolbox: Engineering orthogonal regulators of gene expression. *Curr Opin Biotechnol*, 10.1016/j.copbio.2011.12.015.
- Voigt CA (2006) Genetic parts to program bacteria. *Curr Opin Biotechnol* 17:548–557.
- Khalil AS, Collins JJ (2010) Synthetic biology: Applications come of age. *Nat Rev Genet* 11:367–379.
- Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. *Science* 297:1183–1186.
- Balázsi G, van Oudenaarden A, Collins JJ (2011) Cellular decision making and biological noise: From microbes to mammals. *Cell* 144:910–925.
- Munsky B, Neuert G, van Oudenaarden A (2012) Using gene expression noise to understand gene regulation. *Science* 336:183–187.
- Miliadis-Argeitis A, et al. (2011) In silico feedback for in vivo regulation of a gene expression circuit. *Nat Biotechnol* 29:1114–1116.
- de Nadal E, Alepuz PM, Posas F (2002) Dealing with osmotic stress through MAP kinase activation. *EMBO Rep* 3:735–740.
- Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 66:300–372.
- Miermont A, Uhlenendorf J, McClean M, Hersen P (2011) The dynamical systems properties of the HOG signaling cascade. *J Signal Transduct* 2011:930940.
- Yi TM, Huang Y, Simon MI, Doyle J (2000) Robust perfect adaptation in bacterial chemotaxis through integral feedback control. *Proc Natl Acad Sci USA* 97:4649–4653.
- Ferreira C, et al. (2005) A member of the sugar transporter family, Stl1p is the glycerol/H⁺ symporter in *Saccharomyces cerevisiae*. *Mol Biol Cell* 16:2068–2076.
- O'Rourke SM, Herskowitz I (2004) Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. *Mol Biol Cell* 15:532–542.
- Klipp E, Nordlander B, Krüger R, Gennemark P, Hohmann S (2005) Integrative model of the response of yeast to osmotic shock. *Nat Biotechnol* 23:975–982.
- Hao N, et al. (2007) A systems-biology analysis of feedback inhibition in the Sho1 osmotic-stress-response pathway. *Curr Biol* 17:659–667.
- Mettetal JT, Muzzey D, Gómez-Urbe C, van Oudenaarden A (2008) The frequency dependence of osmo-adaptation in *Saccharomyces cerevisiae*. *Science* 319:482–484.
- Zi Z, Liebermeister W, Klipp E (2010) A quantitative study of the Hog1 MAPK response to fluctuating osmotic stress in *Saccharomyces cerevisiae*. *PLoS ONE* 5:e9522.
- Zechner C, et al. (2012) Moment-based inference predicts bimodality in transient gene expression. *Proc Natl Acad Sci USA* 109:8340–8345.
- Findeisen R, Imsland L, Allgower F, Foss BA (2003) State and output feedback nonlinear model predictive control: An overview. *Eur J Control* 9:179–195.
- Tamás MJ, et al. (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol Microbiol* 31:1087–1104.
- Csete ME, Doyle JC (2002) Reverse engineering of biological complexity. *Science* 295:1664–1669.
- Iglesias PA, Ingalls BP (2009) *Control Theory and Systems Biology* (MIT Press, Cambridge, MA).
- Uhlenendorf J, Bottani S, Fages F, Hersen P, Batt G (2011) Towards real-time control of gene expression: Controlling the hog signaling cascade. *Pac Symp Biocomput* 2011:338–349.
- Menolascina F, di Bernardo M, di Bernardo D (2011) Analysis, design and implementation of a novel scheme for in-vivo control of synthetic gene regulatory network. *Automatica* 47:1265–1270.
- Toettcher JE, Gong D, Lim WA, Weiner OD (2011) Light-based feedback for controlling intracellular signaling dynamics. *Nat Methods* 8:837–839.
- Regot S, et al. (2011) Distributed biological computation with multicellular engineered networks. *Nature* 469:207–211.
- Sprinzak D, et al. (2010) Cis-interactions between Notch and Delta generate mutually exclusive signalling states. *Nature* 465:86–90.
- Edelstein A, Amodaj N, Hoover K, Vale R, Stuurman N (2010) Computer control of microscopes using µManager. *Curr Protoc Mol Biol*, 10.1002/0471142727.mb1420s92.
- Rasband WS (1997–2012) *ImageJ* (US National Institutes of Health, Bethesda, MD). Available at <http://imagej.nih.gov/ij/>. Accessed July 29, 2012.
- Ballard DH (1981) Generalizing the Hough transform to detect arbitrary shapes. *Pattern Recognit* 13(2):111–122.