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Biological signal generators: integrating synthetic biology tools and *in silico* control

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Abstract

Biological networks sense extracellular stimuli and generate appropriate outputs within the cell that determine cellular response. Biological signal generators are becoming an important tool for understanding how information is transmitted in these networks and controlling network behavior. Signal generators produce well-defined, dynamic, intracellular signals of important network components, such as kinase activity or the concentration of a specific transcription factor. Synthetic biology tools coupled with *in silico* control have enabled the construction of these sophisticated biological signal generators. Here we review recent advances in biological signal generator construction and their use in systems biology studies. Challenges for constructing signal generators for a wider range of biological networks and generalizing their use are discussed.

Graphical Abstract

Keywords

biological signal generators; control; optogenetics; microfluidics; signaling dynamics; biological networks

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Introduction

Biological networks consist of interacting components that regulate one another through post-translational modifications, regulation of transcription, and other mechanisms (Figure 1). Advances in reporting and imaging technologies have allowed us to see multiple steps in the transmission of information through the cell as external stimuli are processed into intracellular responses. It is now apparent that biological components encode information in many ways, including in both the amplitude and the dynamics of their activity [1, 2, 3, 4, 5]. This is akin to the processing of information by electrical circuits, which take input signals and convert them to output signals to achieve a desired outcome and where information is encoded in both the frequency and amplitude of the signal. Electrical engineers use signal generators, which create programmable signals such as voltage waves, to understand the importance of specific electrical signals and unravel the function of components and subcircuits. Until recently, biologists have had no equivalent tool for probing biological networks.

The importance of biological signal generators

Standard static perturbations of biological networks (such as gene knockouts or knockdowns) yield information about the identity of components in a biological network but lack kinetic information. Step-shocks such as rapid overexpression, have been theoretically and experimentally shown to be inferior to time-varying inputs, such as oscillations, for identifying network components, protein interactions, and kinetic properties [6, 7]. Additionally, step responses can be insufficient for characterizing networks containing feedback or feed-forward loops [8, 9, 10]. There has been extensive research in engineering and systems biology devoted to the design of optimal stimuli for discrimination between models of complex systems [7, 11]. However, without a biological signal generator (Box 1) capable of producing complex time-varying signals the application of these tools to unraveling biological networks is limited. In synthetic biology applications, biological signal generators have the potential to dramatically improve the design process by allowing the behavior of individual biological components or genetic circuits to be well characterized over a range of inputs [12, 13]. Additionally, fine-tuning and control of biological networks for applications in biotechnology or biomedicine requires the ability to dynamically program component activity in the network. Recent advances in synthetic biology and *in silico* control have started to allow the creation of the necessary biological signal generators for these and other applications.

Synthetic biology tools for biological signal generators

Conditional systems for modulating protein function are common research tools in the biological sciences [14]. Often these systems work by controlling protein concentration, as in systems where a ligand-responsive transcriptional activator or repressor acts on a specific promoter to control the expression and subsequent production of a protein of interest. Biological signal generators of protein function can be built around these conditional systems by using appropriate instrumentation and controllers to drive the ligand-effector signal that generates output from the conditional system, or biological **plant**. Control

strategies often require development of predictive mathematical models to describe how the conditional system responds to the effector.

Many conditional systems employ promoters naturally regulated by nutrients or stress. One of the first examples of a closed-loop biological signal generator utilized a stress-responsive promoter and osmotic stress to drive gene expression in yeast [15]. Similar controllers have been built utilizing control of nutrient-responsive promoters [16, 17]. However, nutrients and stress regulate many processes within the cell, making them less-than-ideal effectors. Significant effort in synthetic biology has focused on designing chemically-inducible “orthogonal” conditional systems that interact minimally with existing cellular regulation and machinery [18, 19, 20, 21]. These orthogonal induction systems are well suited for constructing signal generators. Fracassi *et al* used an orthogonal Tet-responsive promoter to drive an intracellular protein concentration signal in mammalian cells [22]. As the toolkit of orthogonal conditional systems continues to expand new possibilities are emerging for designing biological signal generators. For example, recent development of modular CRISPRi technologies have enabled the activation or repression of multiple orthogonal promoters simultaneously [23, 24]. Systems that regulate protein function rather than protein concentration are also possible. The O’Shea group created a biological signal generator using an analog-sensitive version of protein kinase A (PKA). By controlling PKA-activity and therefore downstream signals of TF activity they were able to determine how the amplitude, frequency, and duration of TF activity encoded differential gene expression [5].

Biological signal generators built around chemically-regulated biological plants have used microfluidic technology to control the effector signal [5, 25, 17, 22]. Microfluidic devices allow for fast, time-varying effector signals due to the small volumes of media in play. However, there are limitations. Most microfluidic devices allow for only small amounts of cell culture and cell material is difficult to recover from the device, often limiting downstream assays to single-cell imaging of fluorescent reporters (though see Lane, *et al* [26]). An alternative to chemical effectors is to drive the plant using light effectors (optogenetics). Light presents some advantages over chemical effectors because its wavelength, intensity, temporal duration, and spatial dimension can be precisely controlled in cultures of cells. Synthetic biology advances have created optogenetic tools effective for controlling a variety of cellular processes, from gene regulation to protein localization [27, 28, 29, 30]. The optogenetic toolkit continues to expand and efforts are in progress to make technology for building light-responsive biological plants and the necessary light-control hardware more accessible [31, 32]. Optogenetics has been used as an effector in large culture vessels because light can be more quickly added and removed than a chemical [33, 34, 13]. One of the first demonstrations of *in silico* feedback control to create a biological signal generator of protein concentration used a light-responsive promoter to drive gene expression in cultures of yeast [35]. Subsequent powerful examples of optogenetic signal generators include a regulator for maintaining protein concentration in chemostat cultures of microbes [34], a biological signal generator for characterizing a synthetic inverter gene circuit in *E. coli* [12], a high-throughput kinase activity generator in mammalian cell cultures [4], and single-cell level control of gene expression in a population of *E. coli* [36].

***In silico* controllers for biological signal generators**

In some of the simplest biological signal generators, the output of the biological plant is assumed to directly follow this effector signal—the effector signal is turned on when output is desired and turned off when it is not. For systems in which the plant responds rapidly and faithfully to the effector signal, this can be an effective approach [4, 3, 25, 5]. However, many biological plants are affected by interactions with the cellular milieu, including feedback due to stress and resource limitation [37, 38]. Additionally, delays and waveform differences between the effector signal and plant output often need to be considered [34]. In these cases, a more sophisticated controller is required to design an appropriate effector signal.

Open-loop control strategies use a model of the biological plant to design effector signals that lead to the desired output [13, 17]. Characterization of an appropriate model requires measuring the relationship between effector input and plant output (the **input/output relationship**) and fitting a model that can describe this behavior over a wide-enough range of conditions [12]. An alternative to open-loop control is closed-loop control, where the error between the actual and desired output of the plant is used to determine the effector signal. Unlike open-loop controllers, closed-loop controllers can compensate for unexpected disturbances (such as those caused by environmental fluctuations) in a process known as **disturbance rejection**. The majority of biological signal generators that have been implemented to date and that are capable of generating complex signals such as ramps and oscillations utilize closed-loop control [17, 16, 22, 35, 39, 40, 41, 42]. However, utilizing a predictive photoconversion model of the optogenetic biological plants, Olson and colleagues were able to generate sophisticated signals in *E. coli* gene expression using open-loop control [43].

One of the simplest forms of closed-loop control is bang-bang control. In bang-bang control, the effector signal is turned on when the actual output falls below the desired output and turned off when the actual output rises above the desired output. This kind of controller is easy to implement and has successfully been used to create biological signal generators around slowly changing biological plants [34]. However, bang-bang controllers tend to oscillate around the desired output, particularly when there is a lag between the effector signal and the output response. Proportional-integral-derivative (PID) controllers are widely used in industrial control systems and have been successfully implemented in biological signal generators [17, 35]. PID controllers apply correction based on the proportional, integral, and derivative terms of the error signal. Compared to bang-bang controllers they take account of much more information, including changes in the error with time. More sophisticated controllers are also possible. For example, model predictive control (MPC) relies on a dynamic model of the underlying biological plant, often an empirical model obtained from **system identification** techniques, to design an effector signal. Model predictive control has been used to build biological protein signal generators and control processes such as cell growth [17, 22, 15, 35]. MPC can anticipate future events using the underlying model and thus usually provide more accurate signal generator output as well as better disturbance rejection, at the cost of additional computational expense.

Insights gained from biological signal generators

Biological signal generators can be used to input complex intracellular signals into a network of interest (Figure 1) to gain biological insight. An important aspect of biological networks is how they transmit and transform intracellular signals. Dynamic intracellular signals, such as pulses in kinase and transcription factor activity, are known to carry information and generate distinct downstream responses [44, 45, 5, 3]. These dynamic signals can be transformed by subnetworks within the cell with important consequences for cellular response. For example, linear enzymatic cascades often function as low pass filters, integrating high frequency signals and transmitting lower frequency signals with high fidelity [25, 46].

The Ras-ERK pathway regulates important cellular decisions such as proliferation and is known to function as a low-pass filter of Ras-activity with a specific bandwidth [46]. Recently, Bugaj, *et al* [4] utilized a biological signal generator of Ras activity to examine how cancer mutations disrupt this property in the Ras-Erk pathway (Figure 2A). They use the Opto-SOS optogenetic system [46, 29] to drive different frequency signals of Ras activity and observe the filtering properties of the Ras-ERK cascade. They found that filtering activity is altered in a lung cancer cell line (H1395) leading to low-fidelity activation in cancer cells at frequencies where normal cells transmit the signal faithfully. This led to abnormally high Erk output and aberrant cell-cycle entry in response to otherwise non-proliferative pulsatile inputs. This example shows that biological signal generators can give valuable insight into how alteration of signaling dynamics drives disease.

Harrigan, *et al* [40] recently used a biological signal generator to determine the requirements for dynamic feedback regulation in the *S. cerevisiae* mating pathway. Signaling cascades are tightly regulated by factors which prevent the pathway from over- or under-activating. In response to mating pheromone, a MAPK cascade in haploid yeast drives expression of genes needed for mating as well as repressors of upstream signaling to downregulate the pathway. Harrigan, *et al* used a biological signal generator with an optogenetically controlled biological plant driving protein concentration of three regulators of the *S. cerevisiae* mating pathway: *SST2*, *MSG5*, or *GPA1*. Their closed-loop optogenetic control (CLOC) system continuously sampled and measured mating pathway output (using a fluorescent reporter and flow cytometry) and tuned output from the biological signal generator (i.e. regulator concentration) to achieve the expected wild-type mating pathway dynamics. Using this scheme they were able to determine the necessary dynamics of the three critical regulators. They found that while static induction of *SST2* can rescue the wild-type phenotype, dynamic control of *MSG5* and *GPA1* is required to rescue the defect in mating pathway regulation caused by knocking out these genes. This result, which could not be obtained using classical over- or under-expression experiments, highlights the utility of a biological signal generator for studying inherently dynamic cellular processes.

The signals generated by biological signal generators can themselves be used to control additional biological processes. Metabolic engineering is one area where there is significant potential for this kind of control. Organisms can be engineered with exogenous enzymes to

produce valuable products such as drugs and fuels. However, the pathways engineered to synthesize these products divert resources and sometimes generate toxic intermediates, causing a burden on cell growth. Recently, Zhao, *et al* [33] used an optogenetic biological signal generator to control the concentration of metabolic enzymes in yeast engineered to produce isobutanol. By using the signal generator to regulate the concentration of enzymes shunting pyruvate through the natural metabolic pathway versus the engineered pathway they were able to design a signal that maximized isobutanol production by optimizing the trade-off between cell growth and production. Thus, the biological signal generator allowed researchers to balance the metabolic needs of the cells with production of a desirable product.

Another exciting aspect of biological signal generators is that they can be used to design dynamic control schemes that force cells to access cellular regimes that are not normally possible. Lugagne *et al* [39] used a biological signal generator to control the concentration of two repressors (lacI, tetR) involved in a bistable genetic toggle switch engineered in *E. coli* (Figure 2B). By designing an appropriate output from the biological signal generator they were able to maintain the bistable genetic toggle switch near its unstable equilibrium, a state it could normally never maintain due to biological noise. This ability could find relevant applications in driving cellular decision-making, controlling cell fate, and understanding differentiation dynamics.

Conclusions and Perspectives

Recent advances show the potential of biological signal generator technology for elucidating and controlling biological networks. Though powerful, current limitations prevent this technology from being widely adopted. Most biological signal generators have been implemented in yeast, *E. coli*, or common mammalian cell lines, systems which have received the vast amount of synthetic biology development and for which many biological “plants” already exist. Further development of orthogonal conditional systems is necessary to allow more biological signal generators to be developed. Additionally, most biological plants used in biological signal generators manipulate protein concentration. Tools that allow protein activity and localization to be controlled would allow more varied intracellular signals to be generated. The measurement technology needed for closed-loop control also limits biological signal generator implementation. Sensors that allow real-time assessment of diverse cellular processes, such as gene expression and metabolism, could allow more sophisticated signal generators to be constructed.

Future work should also focus on democratizing biological signal generators. Biological signal generator construction has thus far required expertise in synthetic biology, control theory, and laboratory automation. This presents a barrier for many systems biologists. Open-source microfluidics or optogenetics hardware combined with controllers designed to allow biologists to implement control algorithms around their biological plant could allow many more laboratories to adopt this technology.

Ultimately, systems biology will be well-served by having the equivalent of an electrical engineer's signal generator. It remains to be seen what biological discoveries and applications will be enabled by this technology.

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Glossary

Input/Output Relationship (I/O):

An input-output relationship describes the output of a circuit or biological network in response to specific inputs. I/O relationships are critical for developing models that can be used to design controllers.

Open-loop control:

In open-loop control, also called non-feedback control, the control action or effector signal from the controller is independent of the actual output of the plant that is being controlled.

Closed-loop control:

In closed-loop control, also called feedback control, at least part of the effector signal is determined by the output of the plant. Closed-loop systems are designed to automatically achieve and maintain the desired output by comparing the actual output with the desired output to create an error signal that is used by the controller to adjust the effector signal.

Disturbance rejection:

Disturbance refers to unwanted inputs that affect the control system's output. Disturbance rejection is the (partial) elimination of the effects of these disturbances through an appropriate control scheme. Disturbance rejection is a possible feature of closed-loop control strategies.

System identification:

The process of determining an appropriate model for the system under control based on experimental data.

Plant:

The system under control is known as the plant. For example, the thermostat in your home controls the furnace (**plant**) such that a desired temperature is maintained.

Effector:

In this article, we define an effector to be the component that regulates the biological plant. In a biological signal generator, the effector signal is determined by the controller and appropriate hardware and instrumentation is used to deliver the effector signal to the biological plant.

Bandwidth:

Bandwidth determines how much information can be transmitted through a signaling pathway per unit time. The larger the bandwidth of a biological network, the more faithfully it can follow a rapidly varying input signal

Reference Signal:

The reference signal is the user-defined setpoint for the system under control (*i.e.* the **plant**). The controller attempts to drive the plant such that the system output matches the reference signal, which may vary in time.

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Box 1: Components of a Biological Signal Generator

An electronic signal generator generates electronic signals specified by a user. In much the same way, a biological signal generator should produce a desired intracellular signal (such as kinase activity or transcription factor (TF) concentration) that is specified by the user (see **A**). The basic components of a biological signal generator are shown in **B**. Some, or all, of these components might be needed in order to get the desired range of signals from the biological signal generator.

A critical component of a biological signal generator is the biological **plant**, which is controlled to produce the desired output signal. For example, the plant might be a chemically-inducible promoter that drives expression of a TF, whose concentration is the desired output. Some early biological signal generators consisted of nothing more than the plant and offered only limited precision or control. In our example, chemical inducer might be added to induce the promoter and dramatically overexpress the desired TF.

More sophisticated signals can be generated by developing a control scheme for the plant. A controller can send an effector signal (u) to the plant (in our example, u is the amount of chemical inducer) to achieve a desired biological signal, namely, the user-defined **reference signal**. If the output of the biological signal generator has no influence on the effector signal, this is called **open-loop** control. Open-loop control schemes *can* be very effective [13, 43] when coupled with accurate models of the biological plant that allow appropriate effector signals to be designed. However, because the output of the biological signal generator is not fed-back to control the effector signal these types of controllers cannot compensate for changes in the plant with time nor reject unexpected disturbances. **Closed-loop**, or feedback, control schemes can compensate for these biological changes by using the output of the biological signal generator to determine the effector signal. In a closed-loop control scheme the output is measured by a sensor and compared to a user-defined desired output to create an error signal, which is fed into a controller that determines the effector signal that will best drive the plant towards producing the desired output signal.

In principle, a biological signal generator can be implemented entirely within a cell. For example, regulators (signal generators that keep their output at a constant level) have been implemented using entirely *intracellular* components [38, 47, 48]. However, biological signal generators capable of producing more complicated output signals commonly consist of an intracellular biological plant with *extracellular in silico* implementation of the controller and sensing as shown in **B**.

Figure 1:

Biological networks, such as the cartoon network shown here, sense extracellular stimuli and convert that stimuli into appropriate intracellular responses. The network transforms a signal and information is encoded in the dynamics and amplitude of component activity (*e.g.* red signal). A biological signal generator can bypass the upstream network to produce controllable levels of component activity (*e.g.* blue signal) making it possible to dissect how information is transmitted or to control specific desirable responses.

Figure 2:

Signal generators can be used to gain insight into biological systems. In Bugaj, *et al* (A) a biological signal generator was used to drive waves of Ras activity and measure how normal and diseased Ras-ERK cascades transmitted that activity. Diseased networks transmitted the signal with less fidelity, leading to hyperactivity of ERK and incorrect interpretation of normally non-proliferative signals. (B) Signal generators can also be used to maintain biological systems in a desirable state. Lugagne, *et al* used a biological signal generator to control the concentration of two repressors in a bistable toggle switch. By pulsing the concentration of the repressors the authors were able to maintain the toggle switch near its unstable state (orange), a state that could not be obtained without dynamic control.