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Optogenetic characterization methods overcome key challenges in synthetic and systems biology

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Systems biologists aim to understand how organism-level processes, such as differentiation and multicellular development, are encoded in DNA. Conversely, synthetic biologists aim to program systems-level biological processes, such as engineered tissue growth, by writing artificial DNA sequences. To achieve their goals, these groups have adapted a hierarchical electrical engineering framework that can be applied in the forward direction to design complex biological systems or in the reverse direction to analyze evolved networks. Despite much progress, this framework has been limited by an inability to directly and dynamically characterize biological components in the varied contexts of living cells. Recently, two optogenetic methods for programming custom gene expression and protein localization signals have been developed and used to reveal fundamentally new information about biological components that respond to those signals. This basic dynamic characterization approach will be a major enabling technology in synthetic and systems biology.

In electrical engineering, systems of astonishing complexity are constructed using a hierarchical design and analysis framework that intentionally avoids physical descriptions of the materials. Basic components such as resistors, capacitors and transistors (**Fig. 1**) are designed to transform electrical input signals into outputs that can be relayed to other components[1]. After fabrication, proper component function is validated using two standard devices: an electronic function generator and an oscilloscope. Function generators are used to create programmable inputs, such as voltage sine waves of fixed amplitude and variable frequency, which are sent into components, and oscilloscopes are used to measure the resulting component outputs (see **Box 1** for a glossary of engineering terms). Component signal processing properties, such as the relative amplitude (gain) and time delay of output relative to input for various signals, are captured using phenomenological, or 'black box' mathematical models. The models are used to reliably assemble components into circuits with more advanced capabilities, such as band-pass filters or amplifiers, and eventually into complete systems, such as computers or radios[1] (**Fig. 1**). The electrical systems engineering framework can also be applied in reverse to divide and conquer systems that are too complex to be understood intuitively. When presented with an unknown or broken system, the input-output (I/O) relationships of subcircuits and subcomponents can be measured individually to build up an understanding of overall system function or to debug failure modes.

Beginning in the late 1990s, researchers began to apply the basic principles of the electrical systems engineering framework to forward-engineer synthetic biological systems[2–4] and reverse-engineer natural networks[5,6]. In the biological systems engineering framework, a transcriptional repressor and its cognate promoter might be considered a single component, called a transcriptional inverter, because it converts a high input signal (concentration of the repressor) into a low output signal (transcription from the repressible promoter) and vice versa. In two of the papers that inspired synthetic biology, a transcriptional toggle switch[2] and oscillator[3] were assembled from two and three *Escherichia coli* inverters consisting of non–cross-reacting (orthogonal) repressor and promoter pairs. A wide range of synthetic gene circuits have since been designed and built for engineering purposes[7–26] and as highly tractable models to study the basic principles of biological regulation[27–40]. In addition, multiple components such as sensors, circuits and output genes (actuators) have been combined to build complete synthetic biological systems such as biosensing[41–44], tumor-invading[45,46], pattern forming[47–49] and edge-detecting[50] bacteria (**Fig. 1**) as well as synthetic microbial ecologies[51,52]. However, the gains of synthetic biology have been hard fought. The performance of most biological system designs cannot be predicted from the known properties of the components, and most published systems required years of design and debugging to complete[53–56].

To understand why synthetic biology lags so far behind electrical engineering, consider that both electronic and biological components perform 'ideally' (i.e., exhibit intended or desired performance) under certain operating conditions and nonideally under others. Because electronic components are designed to exacting size, shape and material property specifications, their nonidealities can readily be 'engineered away', for most operating conditions. For example, modern resistors exhibit ideal impedance for input signals (e.g., voltage oscillations) with frequencies varying over many orders of magnitude. However, when signals reach the gigahertz range, modern resistors perform unpredictably (**Fig. 2**). Electrical components can still be used under nonideal conditions, but in these regimes, characterization of their performance features becomes indispensable.

Though biomolecular function is much more difficult to design than electronic component function, synthetic biologists have begun to engineer away biological component nonidealities as well. For example, in bacteria, the translation initiation rate of a protein coding sequence is, to a first approximation, determined by the binding affinity of the 16S ribosomal RNA for the Shine-Dalgarno sequence in the ribosome binding site (RBS) of the mRNA. However, downstream gene sequences can form secondary and tertiary structure with the RBS, which can change the translation rate in unpredictable ways. To reduce RBS context dependence, a bicistronic design was recently developed such that a leader RBS drives translation of a 16-amino-acid peptide whose coding sequence contains a second RBS[57]. During translation of the leader peptide, the ribosome

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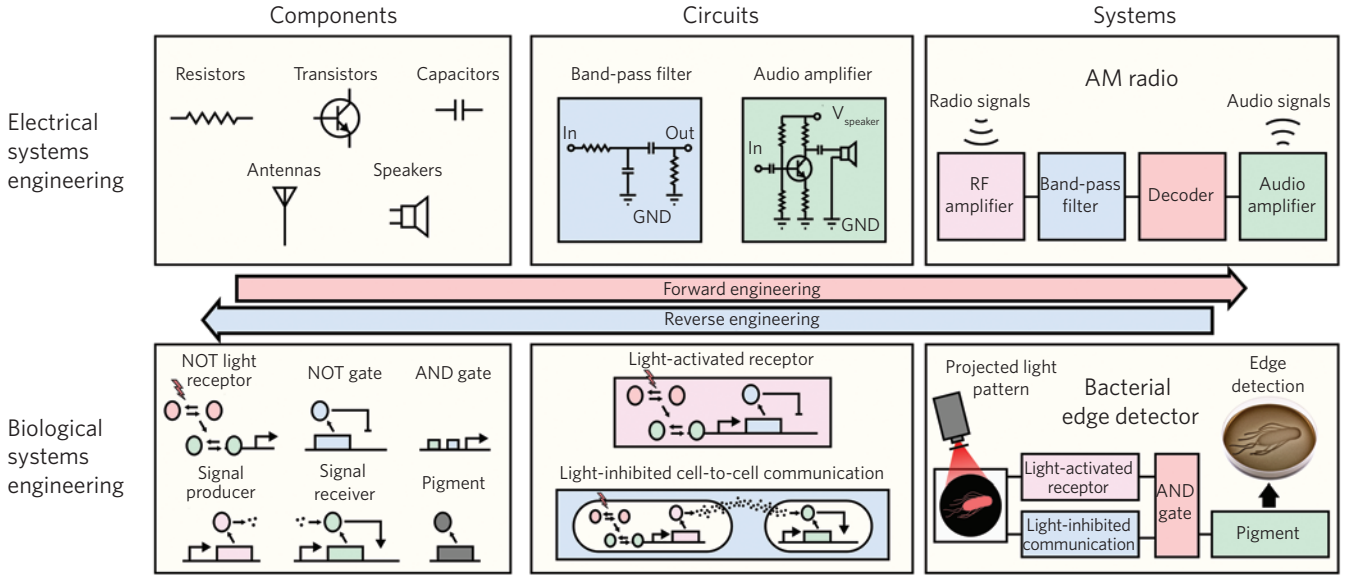

Figure 1 | **Parallels between electrical and biological systems engineering.** Electrical and biological systems exhibit similarities in their scaling of complexity, from components to circuits, and from circuits to systems. For example, an AM radio can be constructed from resistor, capacitor, transistor, antenna and speaker components, and circuits built from assemblies of these components. A light-sensing bacterial edge detector can be constructed from biological components including a red light–deactivated receptor, transcriptional NOT and AND gates, a protein producer and sensor of a diffusible signaling molecule and a pigment-producing enzyme, and circuits built from these components. The process of specifying a desired system and assembling it from components and circuits is termed 'forward engineering', and the process of disassembling and characterizing an existing system to understand it is termed 'reverse engineering'. RF, radio frequency.

helicase unwinds structure[58] at the second RBS. The result is that for a given leader RBS, the free energy of base pairing between the second Shine-Dalgarno sequence and 16S rRNA largely determines the translation initiation rate from bicistronic designs, regardless of the downstream gene[57]. Bicistronic designs should accelerate the design and debugging of synthetic biological systems, which often depend sensitively on the expression levels of the component genes. Similar efforts have also been made to insulate promoters from the impact of downstream mRNA sequences[59,60] and adjacent gene expression cassettes[61,62], and cistrons on a multigene mRNA[60] from one another. Though nucleic acid component function is being optimized with some success, protein design remains a major challenge[63,64]. Without models and theory for designing protein structure at the level of primary sequence, most biological components will continue to operate in a manner that changes with input signals, and genomic, cellular and environmental context. The ultimate success of synthetic and systems biology therefore hinges on our ability to characterize biological component function in relevant cellular contexts.

Characterization challenges

We identify five characterization challenges currently facing synthetic and systems biology (**Fig. 3**). First, many chemical effectors that are commonly used to probe biological processes via activation of a particular receptor or pathway (in particular, sugar, antibiotic or growth factor effectors) may also affect unknown or poorly characterized pathways. Alternative pathway activation can feed back to alter the perfor-

Box 1 | Glossary of engineering terms

Amplifier: a circuit that increases the amplitude of a signal.

Bandwidth: (i) a measure of the flow of information contained in a signal, often expressed in bits per second; (ii) the width of a frequency range.

Biological component: a genetically encoded molecule such as a promoter, noncoding RNA, transcription factor or enzyme that accepts an identifiable input and produces an identifiable output.

Black box: a circuit abstraction in which the inner workings of a circuit are ignored such that the performance of the device can be specified solely by its input-output signal transformation.

Feedback control: an approach to stabilize system dynamics wherein observations of the output signal of a system are periodically or continuously used to adjust the input.

Filter: a circuit which passes only a specified set of frequencies contained in a signal; types include low-pass and high-pass filters, which respectively pass frequencies below and above a specified cutoff, as well as band-pass filters, which pass frequencies contained within a specified bandwidth.

Fourier transform: a reversible mathematical transformation that decomposes a time-varying signal into a distribution of sinusoids with a range of frequencies.

Frequency analysis: an approach for characterizing the signal transformation of a black-box circuit in which sinusoids with a range of frequencies are used as circuit inputs and the effects of the signal transformation are extracted from the characteristics (e.g., amplitudes and phase shifts) of the resulting responses.

Function generator: a tool that produces desired signals or waveforms and is often used to characterize or debug circuits and systems.

Oscilloscope: a tool that connects to a specific point or points of a circuit and displays the dynamics of the signal passing through.

White noise: a signal that is composed of a flat frequency spectrum.

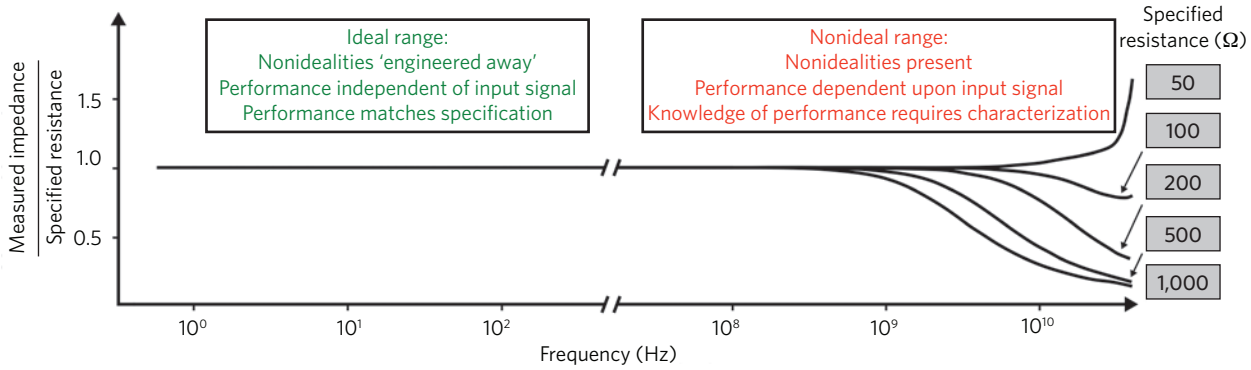

Figure 2 | Nonidealities in electronic components. Resistors are electrical components that inhibit the flow of current, an effect quantified as the impedance. The resistors shown here have been engineered to have a specific impedance over voltage input signals with a wide range of frequencies. This ideal behavior enables circuits to be designed without specific knowledge of the physical construction of each resistor. However, for very high frequencies (gigahertz range), idealized performance breaks down in a manner dependent on the physical construction of the resistor. It is therefore inadvisable to use these resistors in circuits that operate at these frequencies. However, if no other choice were available, the component could still be used by noting the impedance at the operating frequency as identified by the characterization data shown here. Biological components are akin to electrical components operating outside of their ideal ranges, because it is difficult to engineer away nonidealities at the molecular level. Thus, good characterization is paramount to engineering with biological components. Adapted from a figure provided courtesy of Vishay Intertechnology, Inc., from Technical Note 60107.

mance of a component of interest in unknown ways, thus confounding analysis. Extracellular effectors that activate only a single pathway should therefore be identified. Second, many biological components respond dynamically to intracellular signals, such as transcription factor concentration or kinase activity, as opposed to extracellular signals. Therefore, well-defined intracellular signals, rather than extracellular signals, should be generated in order to directly probe biological components. The third challenge is to understand how biological components transform many different types of dynamical input signals, such as pulses or sine waves of different amplitudes or periods, into outputs in an otherwise fixed cellular context. Fourth, different components can directly or indirectly influence one another when present in the same cell, and such 'composition effects' must be understood. For example, a synthetic circuit consisting of an inducible LacI repressor driving green fluorescent protein (GFP) expression from a LacI-repressible promoter exhibits modified output dynamics, including delayed repression and accelerated derepression timescales when additional LacI operator sites, such as those that would be present in the promoter of a new downstream component, are present in the cell[65]. Additionally, mRNAs from a component can compete for the cellular ribosome pool, both reducing overall levels and increasing cell-to-cell variability in expression of other proteins[66]. In another example, high-level expression of proteins tagged for degradation can saturate proteasomes, altering the dynamics of otherwise unlinked components that make use of proteolysis[67,68]. Finally, the metabolic[69] and physiological state of the cell[70] and the extracellular growth environment[71] can alter the performance of components in ways that are only beginning to be explored. To reliably forward-engineer or reverse-engineer biological systems, the impact of host cell or environmental variables on component performance must be systematically investigated[72].

The biological function generator approach

Recently, several groups have used time-varying light and chemical signals to program custom gene expression and protein localization dynamics in bacteria, yeast and mammalian cells[73–77]. Because biological rather than effector signals are being controlled, we refer

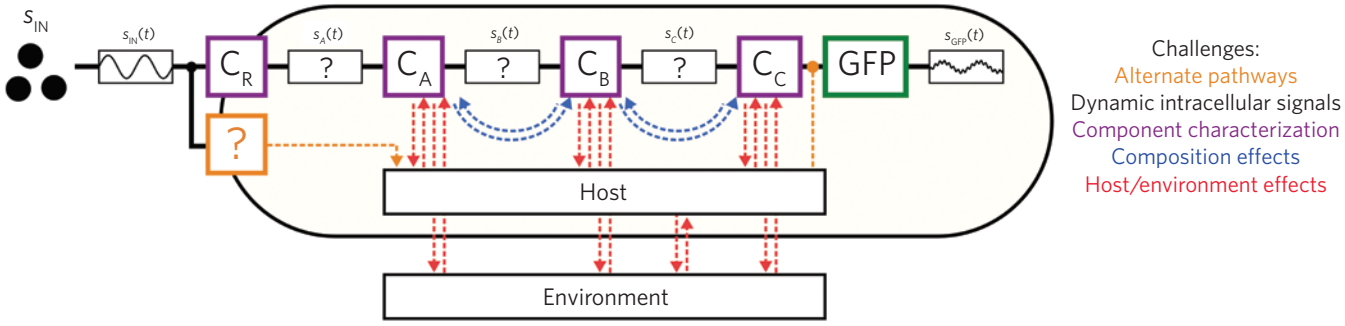

Figure 3 | Challenges in characterizing biological components, circuits and systems. First, an extracellular signal can alter the performance of components indirectly and via poorly understood mechanisms. Signals should be chosen that avoid stimulating alternative pathways. Second, the activities of biological components (e.g., receptor component; C_R) are dynamic and should be probed dynamically by creating time-varying input signals (e.g., $s_{IN}(t)$) and measuring time-varying output signals (e.g., $s_A(t)$). Third, components should be well-characterized in isolation. Fourth, the performance of components may change when they are connected to other components, and these composition effects should be measured. Fifth, components can interact with the host cell and environment dynamically and vice versa. The interactions between the components, cellular host and environment should be probed and understood. C_R and GFP can also result in composition or host effects, but these are not shown for clarity.

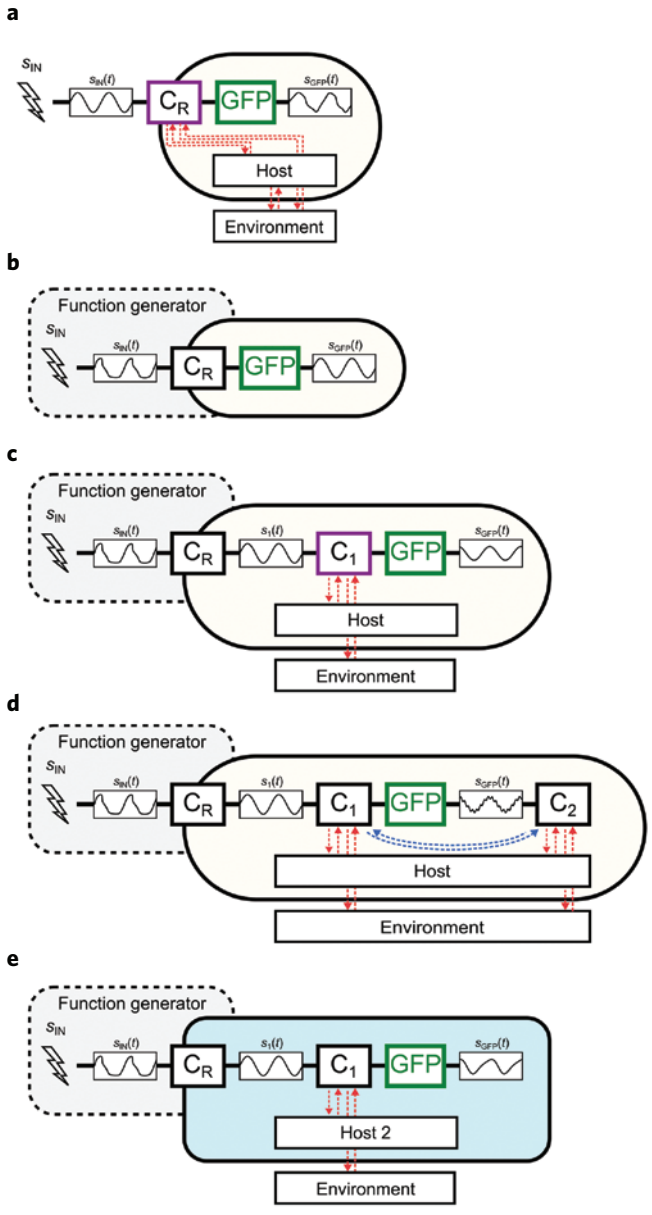

Figure 4 | Developing and using biological function generators to characterize biological components, circuits and systems. (**a**) Receptor characterization. The dynamic I/O of a chosen receptor component (C_R) is first characterized. Here a light-sensitive receptor is used to regulate the expression of intracellular GFP. By measuring the I/O for this system over a wide range of input signals, a model of the signal transformation can be constructed for C_R (which incorporates the effects of C_R on the host and environment if necessary). (**b**) Receptor control. Using the model developed in **a**, the intracellular output of C_R can be programmed to be $s_{GFP}(t)$ by computing the necessary light input signal $s_{IN}(t)$. (**c**) Component characterization. For component characterization, the biological function generator is used to produce a range of input signals $s_1(t)$ for the component or circuit C_1. By directly measuring the signal transformations of C_1, a model of its signal-processing properties can be characterized. (**d**) Composition effect characterization. To characterize composition effects, a system is constructed in which C_R drives C_1, and C_1 drives both C_2 and GFP. The signal-transformation properties of C_1 in isolation were characterized previously, so differences in I/O in this arrangement are due to composition effects with C_2. (**e**) Host-environment effect characterization. Using the biological function generator, a component can be characterized in a new host or environmental condition. For example, the differences in the signal transformation behavior of C_1 in host 2 relative to its behavior in the host shown in **c** can be attributed to host effects.

to these methods as 'biological function generators'[77]. The common first step is to identify a receptor or pathway, and define its input and output. Chemical receptors, such as nutrient or metabolite-inducible promoter systems, are well established. However, because many are linked to complex regulatory and metabolic networks, it can be difficult to identify those whose inputs are not recognized by alternative pathways, or whose outputs are not subject to feedback or other complicating regulation. For example, in the one chemical-based biological function generator approach, gene expression output is controlled from the *Saccharomyces cerevisiae* high osmolarity glycerol (HOG) stress-response system using microfluidically generated sorbitol input signals[75]. However, because the HOG system adapts to changes in osmolarity, a 20-min interval is required between sorbitol pulses that themselves can be no more than 8 min long. Despite the use of online feedback control to adjust the input signal in response to unexpected deviations in the output, the difference between the desired and observed gene expression signals was relatively large in that study.

Genetically encoded light receptors (optogenetic tools)[78] provide several advantages over chemical receptors. After the initial repurposing of microbial channelrhodopsin for the optical control of neural activity[79], light-switchable proteins including light-oxygen-voltage (LOV) domains, phytochromes and cryptochromes have been used to control gene expression, protein localization and other intracellular processes[80,81]. Because light receptors typically respond to lower light intensities than what is typically used for fluorescent protein imaging, and many model organisms do not exhibit sensitivity to light, light receptors tend to have simple I/O mappings, thus satisfying the first challenge (**Fig. 3**). Furthermore, phytochrome-family proteins can be rapidly converted between active and inactive conformations by different light wavelengths, whereas LOV domains and cryptochromes revert to a dark-adapted ground state in seconds to minutes after removal of activating blue light. Rapid reversibility allows better quantitative and temporal control of receptor output than is expected for many chemical systems, which can be limited by slow ligand unbinding or chemical transport delays across the cell membrane. Finally, a simple microcontroller and several light-emitting diodes are sufficient to create highly reproducible, multiplexed light signals at microsecond timescales. By contrast, it is challenging to generate multiplexed and subsecond chemical signals using microfluidics.

The common second step in the biological function generator studies is to characterize the dynamical I/O properties of the selected receptor using a fluorescent reporter gene[73–77] (**Fig. 4a**). Given sufficient measurements, one should be able to formulate a mathematical model that accurately predicts receptor outputs for input signals that have not been tested[77]. Because the only purpose of the model is to predict receptor I/O, a black-box model will typically be preferred over a more complicated version reflecting the receptor molecular biology. Once it is parameterized with experimental data, the model can be used to simulate the receptor response to a wide range of different inputs *in silico*, thus enabling the computational design of effector signals capable of driving biological signals with desired quantitative and temporal features (**Fig. 4b**) and satisfying challenge two. Additionally, if receptor outputs can be measured using fluorescent proteins in real time, an *in silico* feedback controller can adjust the designed input signal to account for any deviations from the model prediction[73–75].

Biological components that can accept the receptor output as an input can now be characterized using receptor-generated functions (**Fig. 4c**), thus addressing the third challenge. For example, if the receptor output is an inducible promoter, and the component of interest is a transcriptional inverter composed of LacI and its target promoter P_{tac}, LacI can be expressed from the receptor output promoter, and GFP can be expressed from the *tac* promoter. Then, a wide range of time-varying LacI protein signals can be generated, and the corresponding transcription rate from the P_{tac} promoter can

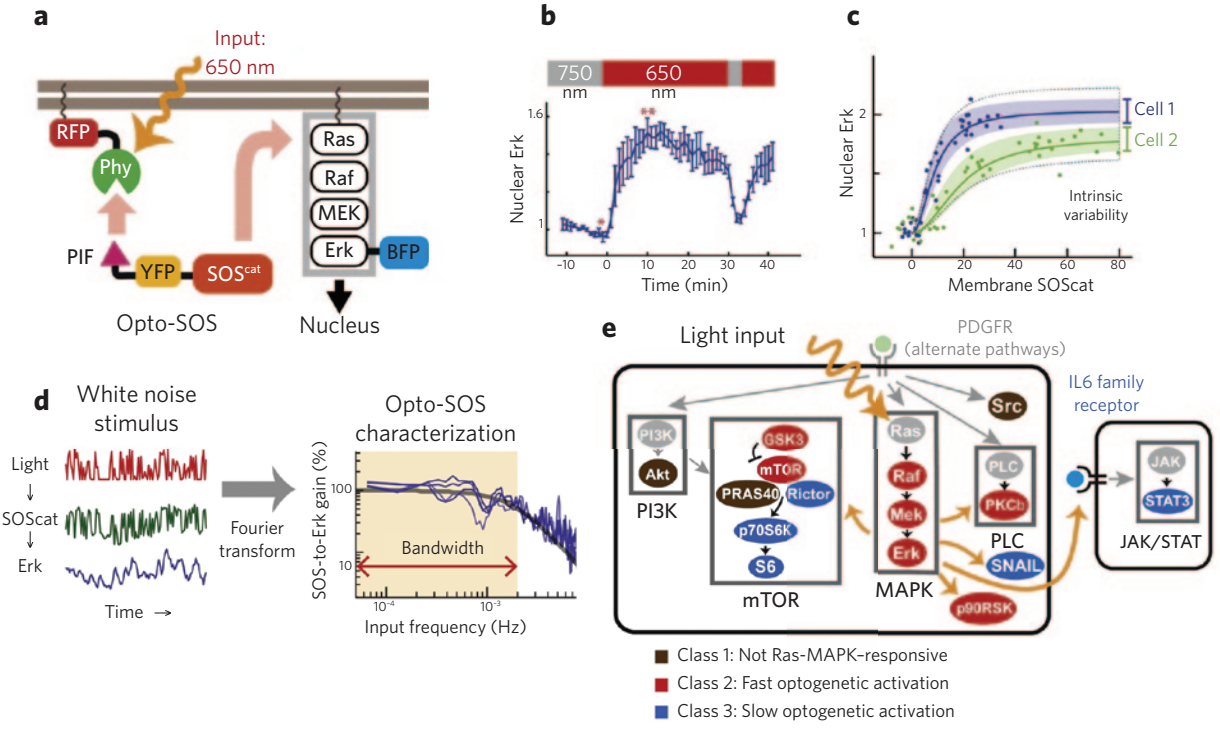

Figure 5 | Optogenetic reverse engineering of Ras-Erk signaling. (**a**) The opto-SOS system for receptor characterization. (**b**) Light activation and deactivation dynamics of the system output. (**c**) Opto-SOS enables single-cell dose-response measurements to characterize the steady-state relationship between the membrane-localized SOScat input and the nuclear-localized Erk output signals. (**d**) Measurement of the frequency response of Ras-Erk using a white noise light signal. Ras-Erk effectively passes signals with periods greater than ~5 min but attenuates faster signals. (**e**) Mapping of the extended Ras-Erk circuit as revealed by opto-SOS, PDGF and proteomics analysis. Adapted from ref. 76 with permission from Elsevier.

be determined based on GFP output. By comparing input (LacI protein concentration) and output (P_{tac} promoter transcription rate), the dynamical inverter I/O properties can be directly characterized. After a component is characterized in isolation, it can be combined with other components in the same cell and its I/O properties can be remeasured. By comparing the two conditions, any composition effects could be determined (**Fig. 4d**, challenge four). Finally, the fifth challenge can be addressed by repeating I/O measurements for a single component in different host cells or growth environments (temperature, medium, cell density, etc.; **Fig. 4e**).

Two of the biological function generator studies used light-driven biological signals to characterize genetic circuits. In the first, the authors used their previously engineered light-reversible phytochrome-based protein-protein heterodimerization system[82] to dynamically control signaling from a guanine nucleotide exchange factor at mammalian cell membranes in order to characterize downstream circuit responses[76]. In the second study, we used our previously engineered phytochrome-family bacterial two-component systems[83,84] (TCSs) to program custom protein expression signals in *E. coli* and used those signals to characterize the dynamical I/O of a synthetic transcriptional inverter circuit[77]. In those studies, advanced dynamics such as sine waves of programmable frequencies and a complex white noise signal were used to reveal new and unexpected information about the biological circuits being studied.

Optogenetic reverse engineering of Ras-Erk signaling

The opto-SOS biological function generator. Mammalian cells make fate decisions based on extracellular information detected by membrane-bound receptors. Many of these receptors transmit information intracellularly through the Ras-Erk cascade, a module that has been demonstrated to drive critical cellular decisions such as proliferation, differentiation and arrest[85]. The pathway functions by regulating the activity of the serine/threonine kinase Erk, which, upon phosphorylation, is translocated to the nucleus, where it regulates the activity of several downstream gene expression pathways. Previous approaches for studying Ras-Erk signaling have relied on upstream chemical inducers of Ras such as platelet-derived growth factor (PDGF) and epidermal growth factor in NIH-3T3 cells or nerve growth factor in PC12 cells. However, these chemical inducers also affect several alternate signaling pathways, obfuscating the outcomes of signaling from Ras-Erk[76].

To gain direct control of Ras-Erk, the opto-SOS system was developed[76], which utilizes the red light–activated and far-red light–deactivated phytochrome B–phytochrome interacting factor 6 (PhyB-PIF6) protein heterodimerization system[82]. Opto-SOS enables light-switchable colocalization of membrane-bound Ras and the catalytic domain of the guanine nucleotide exchange factor Son of Sevenless (SOS), termed SOScat, an upstream activator of Ras (**Fig. 5a**). Using live-cell fluorescence microscopy, a fluorescent protein fusion to the activator is used to directly monitor the activity of the input signal, which is read out by localization of the reporter to the plasma membrane. A different fluorescent protein reporter fusion to Erk enables simultaneous measurement of the biological output signal via nuclear translocation. SOScat localization and disassociation from the membrane occur in under 1 min, and nuclear Erk levels stabilize 6 min after a change in the light control signal (**Fig. 5b**).

Characterization of the Ras-Erk module. The opto-SOS system was used to determine the precision with which the Ras-Erk module transmits steady-state signals[76]. That is, the researchers wished to identify if the module can be used to detect only coarse-grained 'high' and 'low' signals or if it can discriminate small differences in input signal. Projecting and holding different ratios of red and far-red light onto populations of opto-SOS–containing cells resulted in

a population-averaged dose-response curve with substantial noise. However, single-cell measurements demonstrated that individual cells exhibit much greater ability to transmit precise signals (**Fig. 5c**).

To examine the dynamic signal processing of Ras-Erk, the researchers performed a frequency analysis[76], a well-established approach that has been adapted from electrical systems engineering[86] (**Fig. 5d**). In frequency analysis, one measures how a component transforms both the phase and amplitude of sinusoidal input signals of different frequencies into outputs. Rather than characterizing the response to different frequencies independently, the researchers used a higher-throughput approach[76] in which the input is a white noise signal composed of variable periods from 1 min to 2 h. The resulting input (SOScat membrane localization) and output (Erk nuclear localization) measurement data were Fourier-transformed, allowing the system gain (i.e., output amplitude divided by input amplitude) to be evaluated as a function of input frequency. For inputs with periods faster than 4 min, the output was dramatically attenuated. In the parlance of electrical engineering, Ras-Erk is therefore a low-pass filter with high bandwidth (**Fig. 5d**). From a biological perspective, this result shows that Ras-Erk can respond to relatively transient input signals but suggests the circuit may have evolved to filter stochastic fluctuations in input signal that result from biochemical noise such that cell-fate decisions are not made in response to false stimuli.

Characterization of downstream pathways. The researchers next used reverse-phase protein arrays, a high-throughput proteomic tool, to measure both the total abundance and phosphorylation levels of downstream proteins (outputs) in response to three different inputs including a 20-min pulse of red light, sustained red light

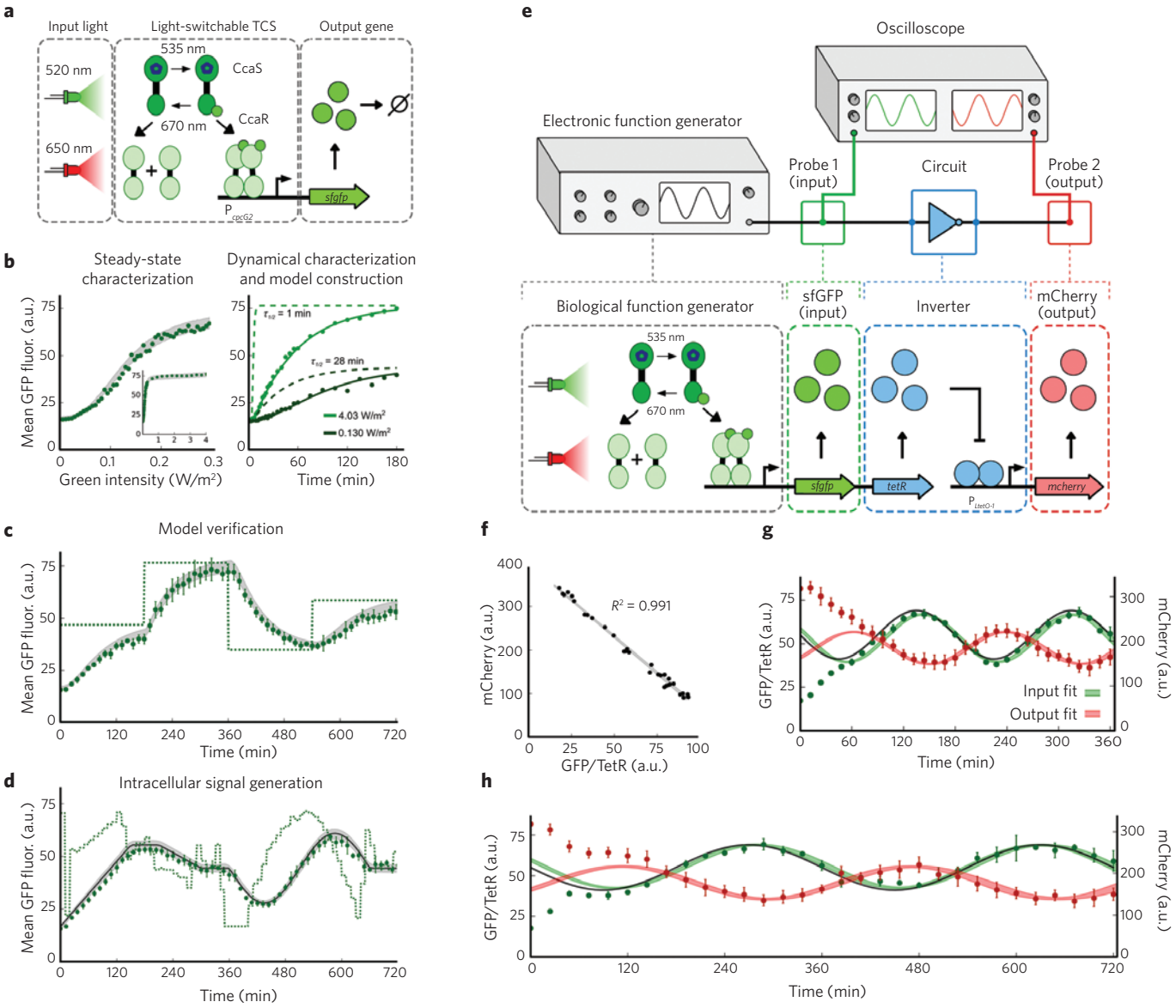

Figure 6 | Forward engineering biological systems by optogenetic component characterization. (**a**) The green and red light-switchable CcaS-CcaR TCS was used to control the expression of sfGFP. (**b**) Steady-state and dynamical characterization experiments were performed with the CcaS-CcaR system with light as an input and sfGFP as an output. (**c**) A model was constructed that enables the accurate prediction of sfGFP output as a function of input light signals. (**d**) Using the model, input signals can be designed to generate desired intracellular dynamics of sfGFP. (**e**) Using the CcaS-CcaR function generator to perform a simple characterization of the TetR-$P_{LtetO-1}$ inverter. (**f**) The steady-state I/O response of the TetR-$P_{LtetO-1}$ inverter. (**g**,**h**) The dynamic I/O response also exhibited a linear response, as the 180-min (**g**) and 360-min (**h**) period sinusoidal TetR-sfGFP input signals are linearly transformed into inverted sinusoidal outputs with the same period and a delay of ~7 min. Adapted from ref. 77.

and a PDGF control[76] (**Fig. 5e**). Of the 180 proteins screened, 27 incurred significant changes in response to PDGF. These proteins were grouped into three classes based upon their light responses. Eight class I proteins were identified that respond to neither of the light inputs, 12 class II proteins were found that respond to both light inputs, and seven class III proteins were shown to respond only to the sustained light input. Thus, Ras-Erk signaling is insufficient to activate class I proteins, which indicates that these proteins are possibly used to drive alternative or combinatorial responses for cell-fate decisions. By contrast, Ras-Erk signaling is sufficient to elicit a rapid response for class II proteins. PKCβ was shown to be a class II protein despite previous research indicating that it was solely active upstream of Ras-Erk. Finally, class III proteins demonstrate a dependence on the dynamics of the Ras-Erk signal. One of these proteins, STAT3, only responds after more than 1 h of sustained red light.

The researchers devoted further study to the slow-timescale link between Erk and STAT3 (ref. 76), which could not be explained by prior studies of any signaling pathway. STAT3 is known to be downstream of two pathways, one of which contains a class I protein and thus cannot provide the link to Ras-Erk. The other, a Janus kinase pathway activated by the interleukin-6 (IL-6) family of cytokines (small extracellular signaling proteins), was hypothesized to be the link. To determine whether cytokine signaling was being used, a coculture experiment using opto-SOS–containing cells and wild-type NIH-3T3 cells was devised. Immunofluorescence assays of STAT3 activity revealed that the wild-type cells were indeed activated in an Erk-dependent manner by nearby opto-SOS–containing cells (**Fig. 5e**). The coculture experiments also showed that the opto-SOS cells did not have increased STAT3 activity. Thus, it appears that Erk activates the STAT3 response of neighboring cells via a cell-cell communication signal, while remaining insensitive to the signal. Finally, the researchers investigated the slow response of the Erk-STAT3 link. It may appear that the response is slow because the cells need to both produce and respond to the cytokine and then finally activate STAT3, incurring time delays in the process. However, when the co-culture experiments were performed with two 1-h pulses of red light separated by 1 h of far-red light rather than a 2-h pulse of red light, the STAT3 response was very limited. Thus the researchers hypothesized that there may be cellular circuits in place that internally measure the duration of Erk activity and only release cytokines when a sufficient amount of time has elapsed.

Optogenetic characterization of a synthetic transcriptional inverter

Light-switchable two-component systems. Our recent biological function generator approach enables simultaneous generation and observation of protein expression signals in exponentially growing *E. coli* populations[77]. Extracellular light signals are converted into intracellular protein expression signals via light-switchable bacterial TCSs. In particular, we used our previously engineered CcaS-CcaR system, which is activated by green light and deactivated by red light[84] (**Fig. 6a**), and Cph8-OmpR system, which is activated in the dark or by far-red light and deactivated by red light[83]. Each TCS consists of a sensor histidine kinase with a phytochrome-family photosensory domain and bifunctional kinase/phosphatase domain (CcaS or Cph8) and a cognate response regulator (CcaR or OmpR), which when phosphorylated by the sensor histidine kinase activates transcription from an output promoter P_{cpcG2} or P_{ompC}.

Light-switchable TCS characterization. By expressing superfolder GFP (sfGFP) from each output promoter, we systematically characterized the steady-state TCS I/Os to different ratios of green:red light (**Fig. 6b**) or different intensities of red light. Then, we demonstrated that the gene expression response to a step change in light input consists of three distinct time-scales: a pure delay during which transcription rate does not change, a time window during which transcription transitions from the initial to final rate and a fixed time required for the output protein (sfGFP) to reach the new steady state after an instantaneous change in its transcription rate. The pure delay was present only in the CcaS-CcaR system, whereas the kinetics of the transcription-rate transition were dependent on the intensity of light when switching both systems away from the ground state but constant when switching in the other direction. Finally, the timescale of sfGFP transitions matched the growth rate of the cells, an expected result for stable proteins. Simple ordinary differential equation models consisting only of the delay and two sequential exponential transitions were sufficient to fit the observed dynamics. By parameterizing the models with experimental measurements of the response to a variety of step changes, we quantitatively predicted the response of the TCSs to multiple non–experimentally tested step changes linked in series (**Fig. 6c**).

Light-switchable TCS control. By combining model simulations with an *in silico* optimization algorithm, we designed light signals to drive gene expression dynamics to quantitatively follow user-defined reference time courses with low error (**Fig. 6d**). For example, we designed light signals to drive sfGFP to transition both linearly and biphasically between defined start and end points over 180 min, the latter of which was ~80% faster than a single step change in light input. We then generated sine waves with programmable amplitudes and periods as well as a complex waveform combining linear ramps, holds and sinusoids over 12-h experiments with equally high predictability (**Fig. 6d**).

Component characterization using CcaS-CcaR. Using our function-generator approach, we characterized how a synthetic transcriptional inverter composed of the commonly used Tet repressor (TetR) and $P_{LtetO-1}$ promoter transformed signals (**Fig. 6e**). By coexpressing sfGFP and TetR from the P_{cpcG2} promoter and mCherry from $P_{LtetO-1}$, we could directly observe both the input and output of $P_{LtetO-1}$. At steady state, the inverter showed a highly linear I/O over the range of TetR levels produced by CcaS-CcaR (**Fig. 6f**). We then programmed TetR sine waves and linear ramps and measured the relationship between sfGFP and mCherry signals (**Fig. 6g,h**). These measurements revealed that the inverter was not only linear at steady state, but also dynamically linear. The output signals were well fit by inverted sine waves with an ~7-min delay relative to the input.

Discussion of results

The reverse-engineering approach used to characterize the Ras-Erk module led to several conclusions regarding its single-cell steady-state and dynamic I/O properties. Furthermore, direct control of SOS activity enabled the effects of Ras-Erk to be decoupled from those of alternate pathways that respond to the widely used chemical activator PDGF. The biological function-generator method revealed unexpected responses to Ras-Erk signaling, a lack of responses previously attributed to Ras-Erk and new downstream connections that depend on Ras-Erk dynamics.

Our forward-engineering study yielded several promising results for synthetic biology. First, we demonstrated that the nonlinear dynamics of two different multiprotein signal transduction pathways could be accurately modeled using simple step-response light input and gene expression output measurements. This result suggests that step-like changes in protein abundance, created using our function generator, could similarly be used to characterize biological components that respond to protein inputs. Such an approach could greatly simplify the characterization and modeling of synthetic biological components that lack convenient extracellular effectors. Second, the CcaS-CcaR I/O was remarkably robust to several genetic and environmental perturbations. These include

the addition of *tetR* to mRNA encoding sfGFP, the presence of an additional plasmid carrying $P_{LtetO-1}$–*mCherry* and its corresponding antibiotic, and the extra TetR and mCherry expression load. Any performance differences arising from these context and environmental changes were accounted for by remeasuring a single parameter, the cell growth rate, and adjusting it in the model. Finally, that the inverter is highly linear over the range of TetR levels used suggests that other transcriptional components can be engineered to operate within linear ranges as well. Linear transcriptional components are appealing because they are simple to characterize and model. Although linear components do not exhibit the same richness of signal transformation capabilities as nonlinear components, they can nonetheless be used to perform simple signal transformations, such as amplification and attenuation, which are useful when connecting components with different I/O signal strength requirements, a common scenario in synthetic biology. Libraries of linear components could therefore accelerate predictive design and scale-up in gene circuit complexity, both of which are major goals of synthetic biology.

Looking forward

Development of improved biological tools. The existing function generator systems can be used to directly characterize the I/O of other biological components. However, additional developments could improve throughput of data collection and reduce other external variables that confound measurements. In the case of the bacterial[77] and yeast[73] light-switchable systems, continuous-culture instruments containing both light-emitting diode inputs and fluorescence detectors would permit uninterrupted, high-temporal-resolution I/O measurements for longer experimental timescales and improved control of growth phase, culture density and media conditions. In the case of our systems, online fluorescence monitoring would permit the introduction of *in silico* feedback control, which could make the generated biological input signals even more robust to perturbations arising from composition effects, component-host interactions or environmental conditions.

It should also be possible to replace fluorescent probes with high-throughput sequencing approaches to increase the number of outputs that can be measured. For example, mRNA barcoding and RNA sequencing methods could be used to simultaneously measure the mRNA input and output from each node in a much larger synthetic or natural transcriptional circuit in order to avoid challenges of spectral overlap in highly multiplexed fluorescent proteins[87]. The dynamics of the input and output proteins could then be inferred using a standard model of translation[88].

When combined with the recent clustered regularly interspaced short palindromic repeats (CRISPR) interference method, function generators with transcriptional outputs could be used to dynamically perturb the expression of virtually any gene in almost any laboratory organism by controlling the expression of a short guide RNA designed to base pair with a genomic promoter[89,90]. Then, by combining custom short guide RNA dynamics with high-throughput sequencing analysis, one could analyze how signals flow through known and unknown downstream promoters, and discover promoters that respond to specific dynamical signals in a manner akin to the reverse-phase protein arrays analysis in the opto-SOS study. For example, the cellular response to programmed dynamics of the mammalian tumor suppressor p53 (ref. 91), the *S. cerevisiae* HOG pathway[92], *Bacillus subtilis* competence regulators ComS and ComK[93], or the *E. coli* SOS DNA damage response regulator LexA[94] could all be investigated. It seems likely that the ability to generate custom intracellular signals will increasingly reveal that dynamical signal processing is a widespread paradigm in biology.

Single-cell biological function generator methods could also be used to study the role of gene expression noise in cellular decision making and population dynamics. To generate single-cell functions, the authors of the Ras-Erk study[76] used a digital micromirror device adapted to project spatially controlled patterns of light onto a microscope stage[74]. This approach should be amenable to use with our light-switchable TCSs as well. The length-scale limit of digital micromirror device–based microscope systems is as low as ~500 nm, which could theoretically allow single bacteria in a dense microcolony to be controlled with light. Finally, it should be possible to multiplex function generators using orthogonal input signals and receptors. Multiple independent intracellular signals could then be used to analyze multi-input promoters[16,95] or logic gates[20], or multiple nodes in the same or different circuits simultaneously.

Managing the combinatorial explosion in characterization experiments. Although complete component characterizations may be desirable (**Fig. 4**), they will often be impractical when the number of components or contexts is large—a common scenario in biology. More reasonably, a limited set of characterization measurements may be performed and used to parameterize a more general model of component I/O and context dependencies. In an initial characterization of a new class of components (e.g., transcriptional activators), data sets may need to be large in order to construct models that accurately capture all relevant performance characteristics. However, additional components from the same class should then be well fit by the same model architecture with a different parameterization. Those components that are not well fit would likely be subject to different molecular interactions or modes of regulation and should be placed in a different class. The accuracy of newly constructed models can be verified by comparing the simulated responses of components to previously untested inputs to experimental measurements of those same input signals. Model development and data collection will reinforce one another, with better models increasing the amount of biological understanding achieved through measurements, such that fewer measurements can be made to achieve yet more understanding. In the end, improved understanding achieved through biological function generator measurements should accelerate progress toward the major goals of systems and synthetic biology.

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Competing financial interests

The authors declare no competing financial interests.

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