Pulsing cells: how fast is too fast?

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(Received 24 July 2008; published online 26 August 2008)

Signal transduction pathways are complex coupled sets of biochemical reactions evolved to transmit and process information about the state of the immediate cell environment. Can we design experiments that would inform us about the properties and limitations of signal processing? Recent studies suggest that this indeed can be achieved by exciting a cell with carefully designed oscillatory stimuli. Although this analysis has its caveats, complex temporal stimulation of signal transduction networks can serve to rapidly advance our understanding of these information channels and ultimately create intelligent ways of controlling them. [DOI: 10.2976/1.2969901]

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The complexity of biological signaling networks is reflected in the scores of genes and proteins that can interact with each other in the form of linear and branched cascades or feedback and feedforward loops, giving rise to robustly regulated responses to the myriad environmental stimuli and stresses. Understanding the dynamical aspects of this complexity has been aided by the use of mathematical modeling (Asthagiri and Lauffenburger, 2000; Kholodenko, 2006) and quantitative high-throughput experimental techniques, the hallmark of modern day systems biology. In particular, relatively recently, devices allowing precise dynamic handling of the cell media, including those operating on the microscale (Whitesides *et al.*, 2001; Melin and Quake, 2007), have dramatically expanded the range of stimuli used to interrogate cell behavior.

On the conceptual level, the functional purpose of a signal transduction network inside a cell—transforming a range of inputs from the external environment into the desired output—is remarkably similar to the functions of a circuit board in an electrical device [Figs. 1(A) and (B)] (Lok, 2002; Hasty *et al.*, 2002). Although the components of living cells and electronic devices and their modes of operation are clearly vastly different, the apparent similarity of the functional needs suggests that similar tools of analysis might be employed and perhaps used to reveal common control and regulation principles. Fortunately, many of the tools developed by electrical engineers were prompted by the increased complexity of the systems they designed, systems complex to such an extent that sometimes they had unpredictable functionality—precisely the situation we might be dealing with in cell biology. It is therefore tempting to think that one can use an analog of various electronic testgears, and more importantly, the pertinent analytical techniques to better understand the wiring of living cells. Some examples of such approaches have already emerged, e.g., in the analysis of chemotaxing cells (Levchenko and Iglesias, 2002; Yi *et al.*, 2000).

Two recent studies have explicitly set out to use precise and complex cell stimulation and dynamical systems theory to unravel the complexity of the high osmolarity glycerol (HOG) pathway in budding yeast, thereby furthering our understanding of its regulation (Hersen *et al.*, 2008; Mettetal *et al.*, 2008). In their paper, Hersen *et al.* demonstrate the value of a technique used in circuit theory for characterizing a linear system: measuring the output response to a train of sinusoidal or nonsinusoidal pulses oscillating at different frequencies. Drawing inspiration from the concept of bandwidth in communication systems, they characterized the dynamical properties of the HOG pathway by calculating its "bandwidth" (see below for details) and showed that this pathway can act as a low-pass filter (LPF). Additionally, they were able to place bounds on the kinetic rate constants of the pathway. The concurrent and conceptually similar study by Mettetal *et*

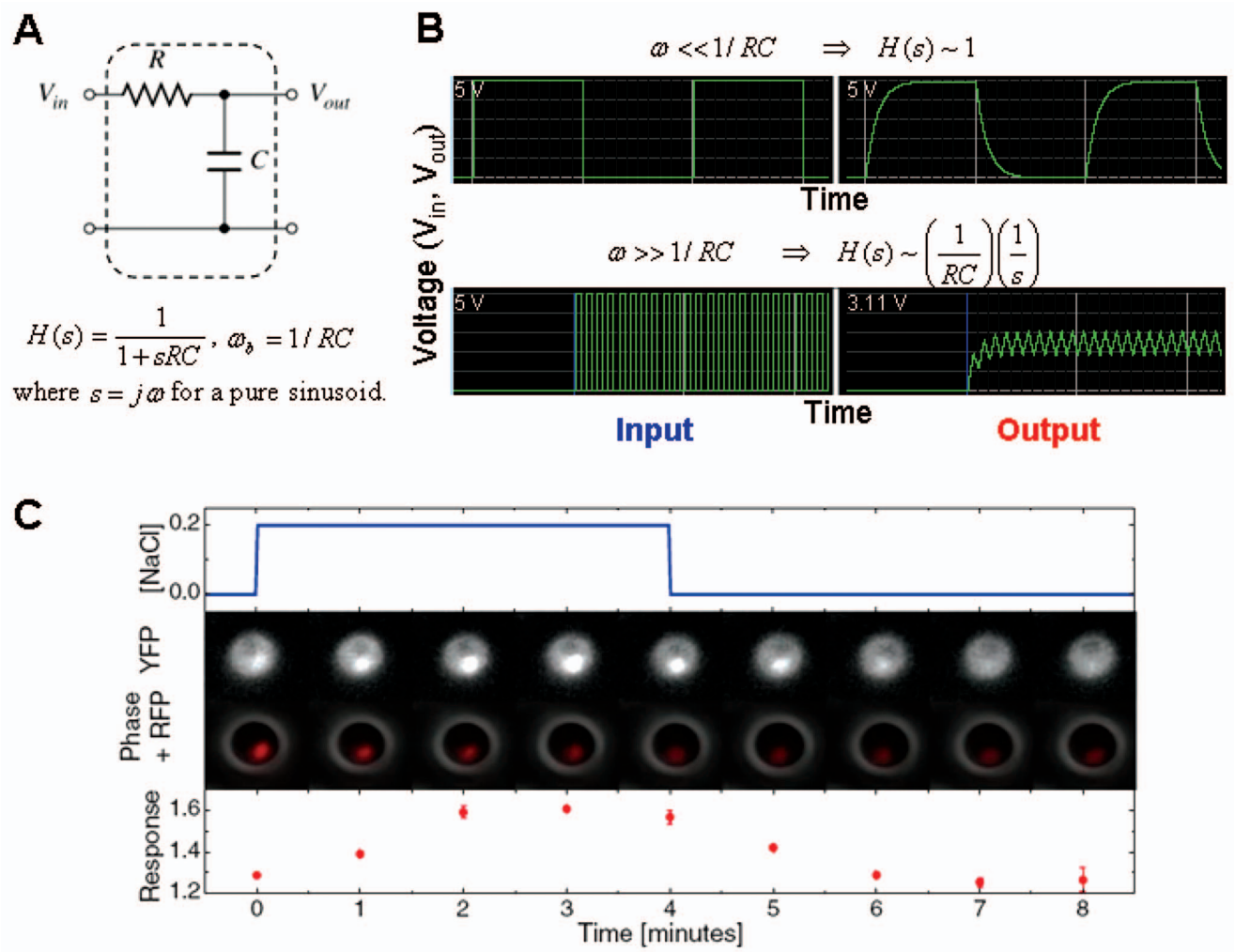

Figure 1. The HOG pathway for osmoadaptation behaves like a low-pass filter (LPF) in response to an oscillatory square wave input, in a manner analogous to an electronic LPF consisting of a resistor *R* and a capacitor *C*, as shown in (A). The transfer function ($H(s)$) and filter bandwidth (ω_b) relations of the *RC* circuit are shown below the circuit. (B) At low input frequencies of the square wave $\omega \ll 1/RC$ (top left panel), the circuit acts like a unity gain system (as seen from the amplitudes of the input and output waves), and the output (top right) closely follows the input, except for the time delay involved in charging and discharging the capacitor. When $\omega \gg 1/RC$ (bottom left), the LPF acts like an integrator, which has a transfer function of $(1/s)$. This can be seen from the overall envelope of the response (bottom right), which resembles the charging cycle of an *RC* circuit following a step input. An LPF attenuates high frequencies, as seen from the decreased amplitude of the individual charge and discharge cycles of the capacitor (bottom right), a consequence of the fast changing input pulse train, which does not allow complete charging and discharging of the capacitor. (C) The HOG pathway response to a step input of high osmolarity involves activation of the Hog1 MAPK, which then translocates inside the nucleus, as shown by Hog1-yellow fluorescent protein (Hog1-YFP) protein localization in the middle panel. Following a return to iso-osmolar environment (low cycle of square wave), the pathway deactivates resulting in translocation of Hog1-YFP out of the nucleus. The nucleus is identified by a nuclear marker Nrd1-red fluorescent protein. The average translocation response of the population (red circles in the bottom panel) are defined as the ratio of average nuclear YFP fluorescence to the average whole-cell YFP fluorescence. Figure 1(C) is reprinted from Mettetal *et al.* (2008), *Science*, **319**(5862), 482–484, with permission from the American Association for the Advancement of Science.

al. also characterized the frequency response of the same pathway, using it to develop a simplified yet predictive model of the osmoadaptation response and highlighting the importance of fast acting feedbacks, not involving gene expression, in regulating this response. Their study highlighted the fact that osmoadaptation is dominated by a fast acting Hog1-dependent negative feedback that does not depend on protein synthesis and to a lesser extent by a Hog1-independent negative feedback that controls the transmembrane protein Fps1. Negative feedback through Hog1 mediated gene expression was needed only over longer time scales and more intense osmolar shocks.

Measuring the frequency response of a linear system can be relatively simple and informative. (Linear systems have the simple property that changes in output are proportional to changes in input, and the output to a combination of inputs is

the same as the combined sum of outputs to the individual inputs.) First, in response to a sinusoidal input, a linear system output is a scaled and possibly phase-shifted sinusoid of the same frequency. Measuring the output amplitude and phase shift over a range of driving frequencies allows for the complete characterization of the input–output response in the frequency domain, i.e., the so-called transfer function. Since any periodic time-domain signal can be decomposed into a Fourier series, i.e., a sum of sinusoids; once the frequency response is known, the response of the linear system to virtually any input can be predicted (Oppenheim *et al.*, 2002). Although most networks in cell signaling are nonlinear and can even sometimes have two or more distinct responses to the same input, a linear model is a good approximation when the perturbed network is not far away from a resting state (Khalil, 2002). Second, the potentially complex differential equations and convolution operations required for modeling the system in the time domain become much simpler algebraic operations in the frequency domain, a scarce mathematical convenience that is hard to dismiss in a sea of biological complexity.

Careful selection of input stimuli can lead to a better understanding of the dynamical properties of a signaling pathway, by highlighting the possible dominant regulatory mechanisms. For example, if a system is given a step input and shows robust perfect adaptation to it, it can be shown to have an integral feedback connection in the pathway (Yi *et al.*, 2000). Alternately, near perfect adaptation may be the result of a feedforward loop or a negative feedback loop (Levchenko and Iglesias, 2002; Behar *et al.*, 2007). Indeed, most biological experiments are restricted to step inputs of different heights or short pulses of variable height and width. These have proved to be informative stimulus conditions, both from the point of view of simplicity of analysis (e.g., a step input only has a single parameter of dependence, the level of the input) as well as the ease of experimental implementation. However, theoretical analysis shows considerable advantages of oscillatory inputs for analyzing linear stochastic gene regulation and signaling networks (Lipan and Wong, 2005). In particular, such measurements can improve signal-to-noise ratios by observing the response over multiple input cycles of the oscillatory input and allow variations of several different input parameters, including the amplitude, the time period, and the slopes of the increasing or decreasing edges of the pulse.

Osmoregulation, which involves active mechanisms allowing a cell to monitor and regulate the osmotic pressure, and consequently the cell shape and relative water content, is a type of homeostatic mechanism that is critical to continual cellular well-being. It is especially important for yeast cells, which live in widely varying osmotic environments, and being nonmotile need to develop an appropriate response to the vagaries of the environment. In the HOG pathway, *S. cerevisiase* monitor osmotic changes through the plasma membrane-localized sensor histidine kinase Sln1, which under normal ambient conditions is active and inhibits mitogen activated protein kinase (MAPK) signaling by phosphorylating the kinase Ssk1. Following loss of turgor pressure, the Sln1 phosphorelay system is inactivated, leading to dephosphorylation of Ssk1, which activates mitogen activated protein kinase kinase kinases (MAPKKKs) Ssk2 and Ssk22, which in turn phosphorylate the MAPKK Pbs2. The pathway is also activated through another route, the Sho1 branch, which includes many proteins common to the pseudohyphal and pheromone pathway, and activates Pbs2 through the MAPKKK Ste11. Active Pbs2 then phosphorylates Hog1, which translocates to the nucleus and triggers a transcriptional response. This includes genes that increase the production of glycerol, thereby increasing the internal osmolarity of the cell. In addition to gene transcription mediated production of glycerol, several Hog1-dependent and independent mechanisms are involved in osmoadaptation, including the crucial regulation of the aquaglyceroporin Fps1. Following osmoadaptation and an increased turgor pressure or following the return to an iso-osmolar environment, the pathway activation is turned off, and phosphatases further inactivate the pathway, leading to Hog1 MAPK translocation out of the nucleus (Klipp *et al.*, 2005). The HOG pathway has been extensively studied and has been reviewed elsewhere (Hohmann, 2002; Hohmann *et al.*, 2007; Saito and Tatebayashi, 2004).

Hersen *et al.* stimulated individual yeast cells using an oscillatory square-wave excitation pattern by alternately exposing the cells to a hyperosmolar sorbitol solution or an iso-osmolar solution for defined periods of time [similar to Fig. 1(C)]. The investigators then tracked the translocation of Hog1-green fluorescent protein into and out of the nucleus in individual cells. They found that for a slowly varying pulse train, i.e., a square wave of low frequency, the Hog1 nuclear localization follows the input closely. However, as the input oscillation frequency increases beyond a certain range, the output oscillations die out and are replaced by the typical HOG pathway response to a step input, i.e., a transient increase in Hog1 nuclear translocation followed by a return to basal nuclear levels. It is as if the pathway is "blind" to the oscillations if they happen fast enough. Strikingly, the response levels for the high frequencies match the response to a step input of half the amplitude of the input square wave, thus suggesting that the pathway is averaging the input square wave, or in other words, integrating it. When the amplitudes of the oscillations of Hog1 nuclear translocation are plotted against the frequency of the input wave, the response resembles that of a LPF [Figs. 1(A) and (B)], thereby allowing the investigators to calculate the bandwidth of the signaling pathway.

The bandwidth of a signal transduction pathway can be an obscure term. One way to understand it is in relation to its definition for a communication network, where it corre-

sponds to the information carrying capacity of the network. Thus, the larger the bandwidth of a signaling pathway, the shorter its response time to a rapidly varying signal, and the more information it can carry about the input signal. Hersen *et al.* measured the bandwidth for a number of readouts in addition to Hog1 activity, including a mechanical readout in the form of the cell size as well as gene expression readouts. All these readouts demonstrated an LPF response, though interestingly, the mechanical response in the form of cell size was shown to be nearly an order of magnitude faster than the signaling response. The researchers used their bandwidth measurements on different mutant strains to place bounds on the activation and deactivation rates of the Sln1 and Sho1 branches of the pathway. They found that the Sln1 branch dominated the activation dynamics of the HOG pathway and allowed cells to integrate fast fluctuating inputs from the environment. This observation is interesting when contrasted with the fact that while yeast has two branches that feed into the osmoadaptation pathway, it is the faster Sln1 branch, and not the Sho1 branch, that is conserved in fungi, indicating the importance of a fast response to osmolar changes in the environment (Furukawa *et al.*, 2005; Krantz *et al.*, 2006). Their results also suggest that the HOG pathway bandwidth is likely to be limited by the deactivation rates of components at or downstream of Pbs2, thereby highlighting the role of pathway phosphatases as an avenue for future investigation.

Though the use of oscillatory stimuli promises valuable insights (Lipan and Wong, 2005), the difficulty in implementing a generalized experimental technique has thus far curbed its widespread use in biology laboratories. The key to the success of Hersen *et al.* has been the leverage of microfluidics as a means for reliably generating an oscillatory external environment. Microfluidics refers to an integrated system of miniaturized components handling small fluidic volumes on the nanoliter scale and below (Whitesides *et al.*, 2001). One of the prominent applications of microfluidics in the field of biology has been the generation of spatially graded stimuli for the study of gradient sensing (Keenan and Folch, 2008; Paliwal *et al.*, 2007). The capability of microfluidics to accurately control the temporal dimension of the extracellular environments has been less explored. Hersen *et al.* utilized one of the classic simple designs in microfluidics, a Y-junction, to generate square-wave inputs with different frequencies. One of the caveats of this design is that it is most applicable for cell types that adhere to the glass substrate and are relatively insensitive to shear stress from direct fluid flow over them. However, other published designs have already addressed these issues (Groisman *et al.*, 2005; Wang *et al.*, 2008; Paliwal *et al.*, 2007; Irimia *et al.*, 2007; Sabounchi *et al.*, 2006) and can be easily incorporated with the flow scheme deployed in this work for a wider application [Figs. 2(A) and (B)].

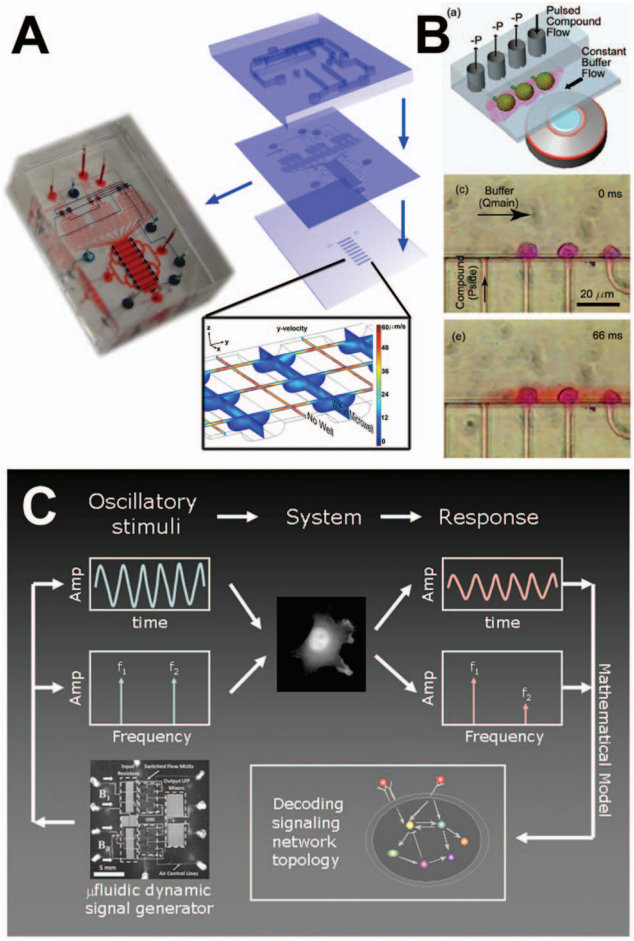

Figure 2. Microfluidics-based approaches for exposing various cell types to novel stimulation profiles for the reverse engineering of cell signaling networks. (A) Shear sensitive cell types can be exposed to ligand gradients by employing techniques that reduce or prevent flow induced shear stress. Wang *et al.* (2008) present a design where neuronal cells are plated on wells etched on a glass cover-slip (bottom layer of the multilayer microfluidic device). Graded (spatially varying) or oscillatory (temporally varying) stimuli are presented to the cells through media flowing in the fluidic layer (middle layer), and media flow is regulated by a control layer of valves (top layer of the multilayer device). Cells are not directly exposed to the media flow but through diffusion of the media down the wells. (B) A biochemical pulse generator integrated with a cell trapping mechanism as designed by Sabounchi *et al.* (2006). Cells, including those that do not adhere to a substrate, can be captured using a negative pressure generated in the side channels while pulsing through a solenoid valve sends requisite stimulation pulses in the top layer. (C) An iterative process consisting of a careful design of microfluidics-based experimental platforms, quantitative experimentation, and analysis of cellular response in the time or frequency domain, as well as mathematical modeling of signaling pathways can lead to a deeper understanding of the structure and regulatory mechanisms of signal transduction pathways. (A) from Wang *et al.* (2008), *Lab Chip* **8**(2), 227–237 and the photograph of the microfluidic dynamic signal generator in (C) from Azizi and Mastrangelo, 2008, *Lab Chip* **8**(6), 907–912, are reproduced by permission of The Royal Society of Chemistry. (B) from Sabounchi *et al.* (2006), *Appl. Phys. Lett.* **88**(18), 183901, is reprinted with permission from American Institute of Physics, copyright 2008.

The work by Hersen *et al.* may provide a model for similar studies in other signal transduction pathways, but there are some important concerns that might limit the generality of the oscillatory input based method. As mentioned above, the concept of frequency domain analysis using a transfer function applies to linear time-invariant systems. However, most biological systems are highly nonlinear. Furthermore, the assumption that the input perturbations are small enough, which allows the nonlinear system to be represented in a linearized form, may not be valid, depending on the pathway being studied or the applied stimulus amplitudes. In addition, the frequency domain transfer function approach can be difficult to apply for multiple-input–multiple-output systems. Biological systems are often multiple-input, possibly with multiple output variables too, making them difficult to interpret as pathways with a particular bandwidth, etc. The scalable and high-throughput nature of microfluidics could come to the rescue in addressing this problem (Meyvantsson and Beebe, 2008). Presence of multiple experimental regions, each devoted to a single input and output, is easy to achieve on the same microfluidic chip, thereby allowing the calculation of transfer functions between each input–output pair. Another potentially limiting factor of the analysis is implicit reliance on fast perfect adaptation of the Hog1 response, allowing rapid restimulation of the pathway. However, perfect adaptation might be a rather special property limited to very few biochemical systems, thus leading to non-standard oscillatory or even non-oscillatory responses. The technique would also be inapplicable to systems that can show progressively declining oscillatory output, i.e., due to gradual downregulation of pathway components. The analysis would also have to be interpreted differently if used on systems that are inherently oscillatory with an internal frequency, e.g., even in response to a step stimulus. The analysis would have to rely on taking into account potential resonance frequencies, etc. Thus, it remains to be seen whether the application of the analysis tools described by Hersen *et al.* would extend to many other signaling systems or remain confined to rather special, though important cases.

In spite of the above caveats, the studies by Hersen *et al.* and Mettetal *et al.* clearly illustrate the power of seeking "simplicity in complexity," i.e., developing simplified mathematical models or conclusions for signaling networks, based on their input–output characteristics and thus developing intuition about their structure [Fig. 2(C)]. This can be contrasted with the equally popular and successfully detailed mathematical modeling approach, where most biochemical reactions in the network are modeled using previously experimentally derived or fitted values for the large numbers of kinetic rate constants and concentrations (Schoeberl *et al.*, 2002; Chen *et al.*, 2004; Shao *et al.*, 2006). Detailed models have also been successfully used for modeling the yeast HOG pathway (Klipp *et al.*, 2005), and a combination of both approaches promises a better understanding of this signaling network. Importantly, these studies provide an approach that can be tailored toward finding bounds on the kinetic rate constants and unraveling the regulatory mechanisms of signaling pathways in general.

ACKNOWLEDGMENTS

S.P. and C.J.W. contributed equally to this article, which was written and edited by S.P., C.J.W., and A.L.

During the editorial review of the commentary, a study of metabolic gene regulation in yeast was published; wherein again, a microfluidic device generated oscillatory stimulation profile was used to characterize the response (Bennet *et al.*, 2008, *Nature*, July 30 [Epub ahead of print] PMID:18668041).

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