

Timing is everything: using fluidics to understand the role of temporal dynamics in cellular systems

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Abstract In biology, it is not only the magnitude of a chemical stimulus that determines cellular response; it is becoming increasingly clear that the timing of the stimulus is vastly important as well. Currently there is a paucity of data regarding cell behavior under dynamic stimulation conditions that are representative of what occurs in vivo. This is, at least in part, attributed to the lack of appropriate tools for generating time-varying stimulatory signals in highly diverse patterns. Fluidics on the macro and micro scale has provided a practical platform for dynamically stimulating cells in a highly controllable manner at physiological and supra-physiological time scales (seconds to a few hours). These fluidic systems have contributed substantially to our understanding of how cells process and react to dynamic stimulatory environments; while these setups provide the means to analyze and manipulate cellular behavior in these types of environments on the single-cell level and on a high-throughput level, improvements can be made to these platforms to enhance their utility for high-impact biological investigations of temporal dynamics.

Keywords Temporal dynamics · Stimulation patterns · Cellular systems · Fluidics · Microfluidics

1 Introduction

The chemical environment of a cell is highly dynamic, as manifested in hormonal regulation and neuronal signaling (Brabant et al. 1992; Goldbeter 1996; Laurent 2002). In order to survive and prosper in such an environment a cell must be able to appropriately interpret these chemical cues and react accordingly (Goldbeter 2008; Knobil 1981a). While the magnitudes of these chemical stimuli provide important information to a cell, the temporal patterns of the stimuli are equally if not more significant for the orchestration and coordination of cellular processes (Knobil 1981a; Li and Goldbeter 1989). Information is stored in the frequency of the chemical signals, and cells utilize their external and internal machinery to aptly decode the meaning of these stimuli (Brabant et al. 1992; Goldbeter 1996, 2008; Schofl et al. 1994).

Aside from several canonical hormone cycles, however, relatively little is known about cellular responses under diverse patterns of chemical stimulation on physiological timescales (Brabant et al. 1992). With recent advances in chemistry and genetic engineering, there is a relative abundance of real-time readouts for assessing cellular responses (Zhang et al. 2002) compared to the number of uses of such readouts in dynamic flow systems. A major limiting factor is the lack of technologies for generating well-controlled temporal patterns of chemical stimuli. Conventional techniques for assessing cellular responses under dynamic conditions are deficient in the diversity of chemical patterns that can be created, reproducibility, and potential for high-throughput. These techniques entail adding known amounts of chemicals to a culture of cells growing in culture dishes or on glass slides, effectively exposing cells only to a step increase in stimulant concentration (Paliwal et al. 2008); this setup is

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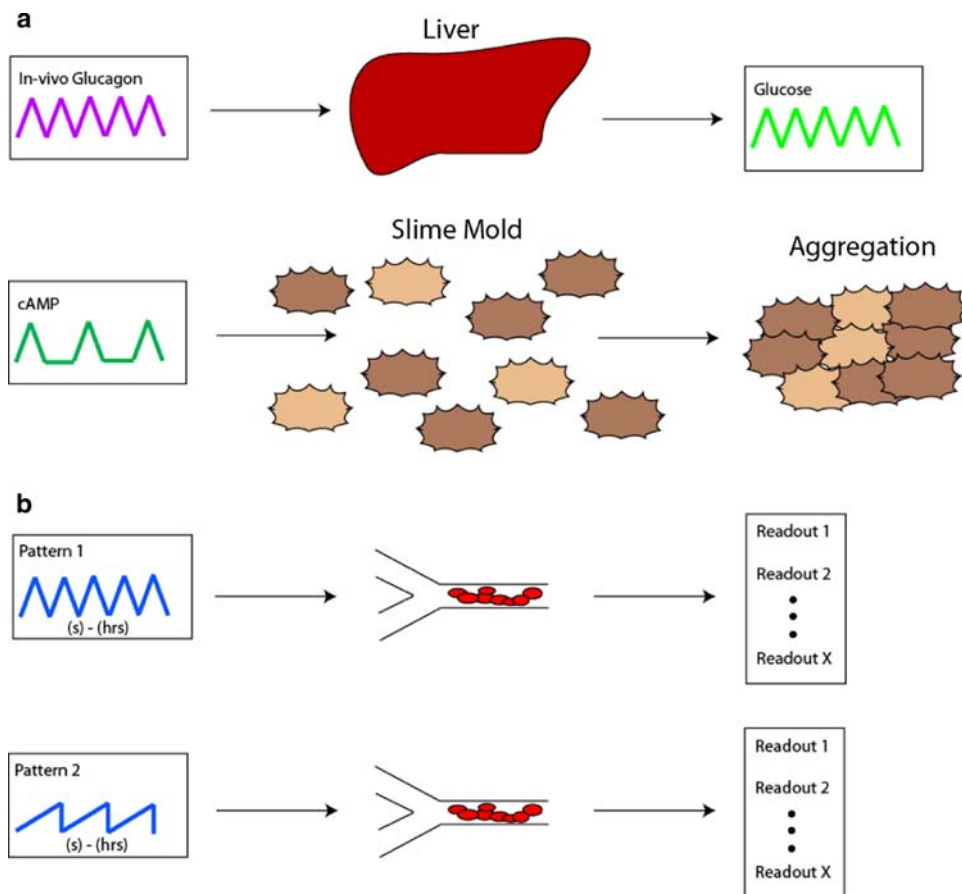
straightforward, but is labor-intensive and not amenable to quick and reproducible stimulus patterns. Perfusion chambers represent a step up in terms of reproducibility, but lack versatility and scalability (King et al. 2008). Herein lays the opportunity to utilize microfluidic technologies to provide versatile and precise temporal controls in a robust and user-friendly format to enhance our understanding and control of biochemical timing and rhythm (Fig. 1).

Early macrofluidic setups for analyzing cellular responses under dynamic patterns of stimuli were used to assess the physiological role that pulsatile chemical patterns played in certain cellular systems, such as liver cells (Schoffl et al. 1991, 1993; Weigle et al. 1984) and lens tissue (Brewitt and Clark 1988). Once certain differences in cellular response were observed between dynamic and constant chemical stimulatory conditions (such as the quantity of glucose released, the amplitude and frequency of calcium transients, the induction of transparency in lens tissue) and real-time readouts improved, the focus shifted from the physiological relevance of these chemical pulses to the mechanisms by which the external and internal cellular machinery interpret temporal patterns. While these were bulky systems that consumed large quantities of reagent, macrofluidics provided a platform

for making key observations of cellular and molecular responses [such as the levels of intracellular second messengers like calcium (Schoffl et al. 1993), cAMP (Schoffl et al. 1991), or chloride (Verkman et al. 1992)] under dynamic conditions and motivated the development of microfluidic technologies for similar types of studies. The findings enabled by the macrofluidic technology portrayed that there were substantial reasons for analyzing biological systems in this manner: there were clear differences between pulsatile chemical delivery compared to continuous delivery on the whole cell level [slime mold behavior (Robertson et al. 1972), bacteria movement (Block et al. 1982), glucose release (Weigle et al. 1984), lens tissue development (Brewitt and Clark 1988)] and on the intracellular level [calcium levels (Schoffl et al. 1993), cAMP levels (Schoffl et al. 1991)]. These observations indicate that pulsatile delivery is potentially very physiologically relevant or can be used to probe cellular processing properties.

The advent of microfluidics has provided a platform that not only minimizes reagent and cell consumption, but also harnesses the physical properties of fluids on the micro and nano scale for creating highly controllable, repeatable, and versatile chemical patterns for cellular stimulation (Beebe et al. 2002; Melin and Quake 2007; Shim et al. 2003;

Fig. 1 Temporal dynamics in nature and how microfluidics can be used to analyze this phenomenon. **a** Examples of naturally occurring time-varying chemical patterns in nature: pulsatile glucagon stimulates liver cells to produce higher levels of glucose compared to continuous stimulation (Weigle et al. 1984); slime mold naturally release the chemical cAMP in a pulsatile fashion when starved and aggregate as a result. Results have shown that the frequency of this chemical release is crucial to the aggregation process (Robertson et al. 1972). **b** Microfluidics provides an ideal platform for studying physiologically relevant temporal dynamics in cellular systems. Various methods have been developed to create time-varying (seconds to hours) chemical patterns for cellular stimulation, and there are a multitude of both real-time and endpoint readouts available to assess cellular responses



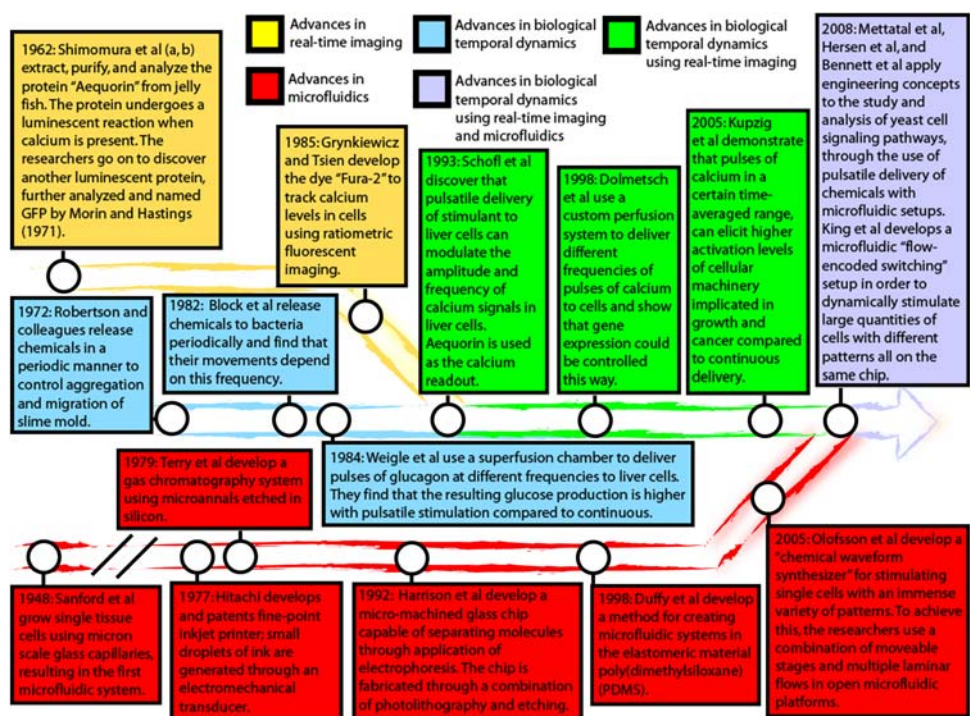
Whitesides et al. 2001); the review by Whitesides et al. (2001) provides a thorough overview of the application of soft-lithography for biology and biochemistry applications, as well as microfluidic device fabrication. These microfluidic setups, combined with mathematical and computer modeling, have resulted in key findings in terms of determining the architecture and dynamics of internal and external cellular machinery (Bennett et al. 2008; Hersen et al. 2008; Mettetal et al. 2008). In these most recent cases, yeast cells were used; platforms will need to be developed that can support more delicate and complex mammalian cells as well as real-time imaging and endpoint assessment of the state of their cellular machinery. In addition, microfluidic setups will need to be developed that have capabilities for high-throughput screening of the effects of versatile patterns of stimulation with frequencies over the range of seconds to hours, along with the ability to image in real-time. Ultimately, this is an exciting time for studying temporal dynamics on these time scales in cellular systems, because many readouts are readily available, and also because microfluidic technology provides an ideal platform for regulating the timing of stimulation of cells (Fig. 2). By providing an overview of the macrofluidic setups that led up to the development of current microfluidic setups (Fig. 2), we show the importance and the impact that research with these systems has had on our understanding of temporal dynamics in cell biology; we also point out the great opportunities available for next generation microfluidics in this field.

2 Macrofluidics in physiology

As early as 1972, researchers used fluidics to analyze temporal dynamics and control cellular behavior, providing the impetus for studying these crucial biological properties (Fig. 2). In this case the target system was slime mold (*Dictyostelium discoideum*) (Robertson et al. 1972); using well-controlled, external pulses of stimulant, Robertson and colleagues were able to control aggregation and migration of these cells. While this cellular system may sound unappealing and uninteresting, the discovery of its unique behavior in the face of rhythmic stimulation set off a wave of important theoretical analyses (Barkai and Leibler 2000; Li and Goldbeter 1992; Martiel and Goldbeter 1987; Novak and Tyson 1993; Tolic et al. 2000) and ushered further investigation into the rhythmic patterns of signaling in mammals (Brabant et al. 1992; Brewitt and Clark 1988; Dalkin et al. 1989; Knobil 1981b, Norstedt and Palmiter 1984; Steiner et al. 1982; Wildt et al. 1981). Just recently, a different form of slime mold was discovered to have “learning” capabilities when subjected to temporal patterns of temperature and humidity shocks (Saigusa et al. 2008).

In order to intricately study the effects of temporal patterns of chemical stimulation on human cellular systems, researchers required setups that could reliably and repeatedly generate pulsatile patterns of chemical stimulation, while also providing an appropriate environment for cells. The first prominent example of the application of

Fig. 2 Timeline, with select examples, of important advances in the areas of real-time imaging, microfluidics, and temporal dynamics in biology, as well as seminal research where these fields converged, resulting in key biological discoveries



macrofluidics for the study of cell biology on mammalian cells was by Weigle et al. (1984), who developed a system for analyzing the effect of pulsatile and continuous application of glucagon on glucose production in packed beds containing liver cells. The setup consisted of two 10-cc plastic syringes clamped vertically with a 105- μm membrane at the bottom. Polyacrylamide gel beads were then deposited and allowed to settle, followed by liver cells. A peristaltic pump was used to deliver glucagon to the respective columns and integrated glucose production was assessed over a 90-min period (continuous glucagon stimulation vs. six consecutive 3-min pulses). It was found that liver cells had a significantly higher glucose production rate under pulsatile stimulation conditions vs. continuous stimulation conditions over a physiological range of glucagon concentrations. This study illustrated early on that the timing of stimulation was very important in human cellular systems and demonstrated that fluidics coupled with endpoint readouts (glucose) could be used to assess the cellular responses in an important physiological system. Liver cells, in particular, need a very specific environment to grow; microfluidics can provide proper microenvironments for cells, and can be custom made for particular cellular systems so that temporal dynamics can be properly assessed. In a seminal study of lens development, Brewitt and Clark (1988) used a perfusion chamber to demonstrate that pulsed delivery of platelet derived growth factor (PDGF) led to the growth and transparency of lens tissue, whereas continuous administration of PDGF led to lens opacity, reduced protein content, and reduced weight.

Using a similar setup to that of Weigle et al. (1984), Schofl et al. (1993) created a superfusion chamber to study the temporal dynamics of liver cells, but on the molecular scale. The authors analyzed how square-wave pulses of stimulation at different frequencies affected cellular calcium levels in real-time, using aequorin as a readout. Since cellular calcium controls a number of important cellular functions, it is critical to assess how it is regulated through external chemical stimulation. This study revealed many significant properties regarding receptor control of the amplitude and frequency of calcium spikes. Applying this type of analysis also allowed the researchers to extract important kinetic parameters (such as time constants associated with calcium amplitude and response lag time), which are valuable for mathematical and computer models used to recapitulate molecular pathways. Ultimately, this demonstrates that a straightforward setup can be implemented to analyze the dynamics of internal and external cellular machinery as well as cellular behavior.

Other seminal studies were conducted with the use of simple, programmable fluidic setups such as the previous examples. Dolmetsch et al. (1998) discovered that the pattern of cellular calcium release controlled the expression

of certain genes in cells. In this study, cells were exposed to different frequencies of square-wave pulses of calcium; the patterns were generated through the use of a computer-controlled solenoid valve that selectively pumped from two reservoirs (one containing calcium, the other a calcium chelator; Fig. 3). In order to assess gene expression, cells were collected and analyzed separately, in an endpoint manner. A slightly modified version of this setup was utilized to assess the effect of calcium patterns on internal machinery implicated in cellular growth and cancer (Kupzig et al. 2005). Again, in this case, pulses of calcium were applied to cells and endpoint measurements were taken of the activation state of the internal cellular machinery. It was found that over a particular time-average calcium concentration, pulses of stimulation were more effective than continuous stimulation in inducing cellular machinery activation. This was yet another example of a biological study in which a simple fluidic premise was employed to make a substantial biological finding.

These setups, while providing key biological findings, are a bit difficult to setup and are bulky, meaning that they

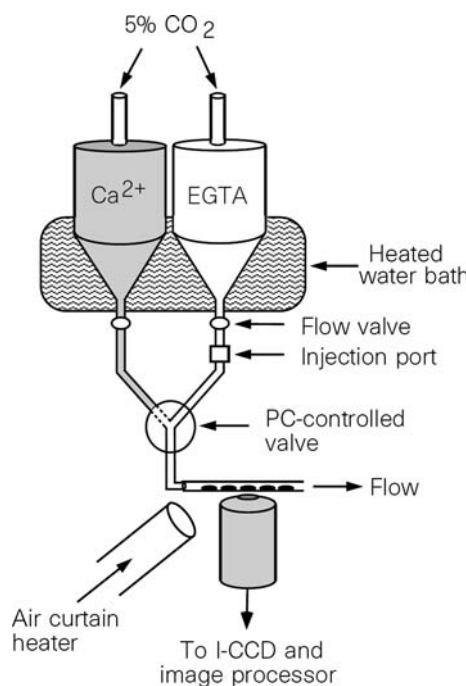


Fig. 3 The calcium clamp setup was developed in order to control calcium levels inside cells, in order to analyze the effect that the frequency of the calcium signals had on gene expression, and activation of cellular machinery implicated in growth and cancer. A computer-controlled solenoid valve was used to switch between two reservoirs (one containing calcium, the other containing EGTA, a calcium chelator). Real-time imaging was used to monitor the calcium levels, while endpoint readouts were used to quantify gene expression and cellular machinery activation. Reprinted by permission from Macmillan Publishers Ltd.: Nature 392:933–936, © (1998)

expand a great deal of reagents. For example, studies using exposure to expensive growth factors and cytokines in a flowing system could cost thousands of dollars or more per experiment if not scaled down. Researchers would benefit from the development of microfluidic systems that could perform similar functions with less volume, are easy to setup, but also generate more elaborate patterns to provide further insight into cellular and molecular behaviors.

3 Microfluidic setups

Recently developed microfluidic setups have overcome the limitations of the aforementioned macrofluidic setups by not only reducing the level of reagent consumption, but also by enhancing the breadth of stimulation patterns possible for probing of temporal dynamics in cells. To this end, Olofsson et al. (2005) developed a “chemical waveform synthesizer” (Fig. 4). In this microfluidic system, single cells are held fixed above an open microfluidic chamber possessing multiple laminar flow streams containing various cellular stimulants. The individual cells are then dipped into the laminar flow streams, and a moveable x - y stage is then used to precisely and rapidly expose the cell to a new flow stream or to another area of the same flow stream (where the concentration of the stimulant in that flow stream is different). The result is one of the most versatile setups for stimulant pattern generation. Swift switching between streams and precise positioning result in exquisite temporal and spatial resolution for cellular studies. However, the method currently is limited to electrical activity readouts through the patch-clamping technique, which might preclude any real-time imaging capabilities. In addition, the patch-clamp technique is very difficult to setup and the method is amenable currently to the study of single cells, and in that regard is not high-throughput (Chiu and Orwar 2004). It may be possible, however, to combine this setup with that developed by Estes et al. (2008) and others (Fertig et al. 2002; Huang et al. 2006; Schmidt et al. 2000) to facilitate patch-clamp analysis of a greater number of cells.

An elegant solution to the throughput limitations of the “chemical waveform synthesizer” was realized with the “flow-encoded switching” setup developed by King et al. (2008; Fig. 5). This microfluidic system consisted of two inlets and columns of “experimental” channels, where cells were cultured and stimulated, and “gap” channels, which were positioned in between “experimental” channels in order to properly control the position of flow streams. Manipulation and variation of just one fluidic parameter (either ratio of inlet flow rates, or inlet pressure) resulted in generation of dynamic temporal patterns. This setup is very powerful not only in its fluidic simplicity, but

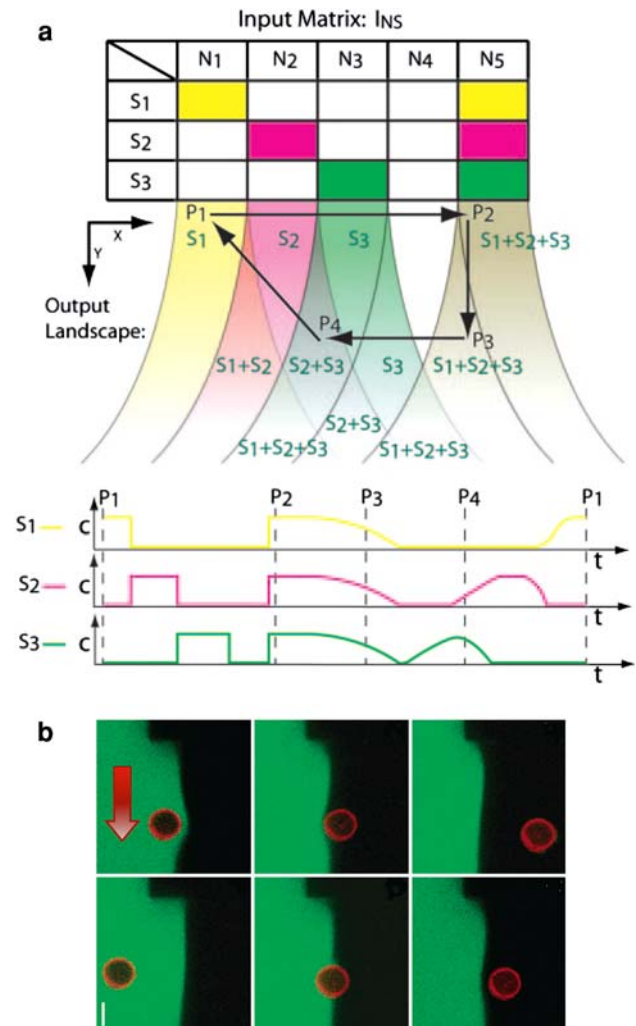


Fig. 4 The setup of the “chemical waveform synthesizer.” **a** For this example, there are five laminar flow streams (N1–N5) containing three different stimulants S1–S3. The stage is moved along the paths P1–P4 depicted below the “Input Matrix,” creating the stimulation patterns portrayed in the graphs. It can be seen that three different stimulation patterns with the three different stimulants are obtained during one run. Reprinted from Olofsson et al. Proc Natl Acad Sci USA 102:8097–8102 © (2005) National Academy of Sciences, USA. **b** A cell (in red) is moved between two laminar flow streams (green and black) to expose it to varying chemical environments. The red arrow indicates the direction of flow. The scale bar represents 10 μm . Reprinted with permission from Anal Chem 76:4968–4976, © (2004) American Chemical Society (Colour figure online)

also because it permits a user to test and observe multiple cellular stimulation conditions during one experimental run, on a single chip. These latter properties overcome some of the issues of throughput with microfluidic systems. In addition, the authors show how the setup can be multiplexed, to achieve control of more than one pattern generation parameter (duration, frequency, concentration, etc.). The “flow-encoded switching” cannot create stimulation patterns as diverse as the “chemical waveform

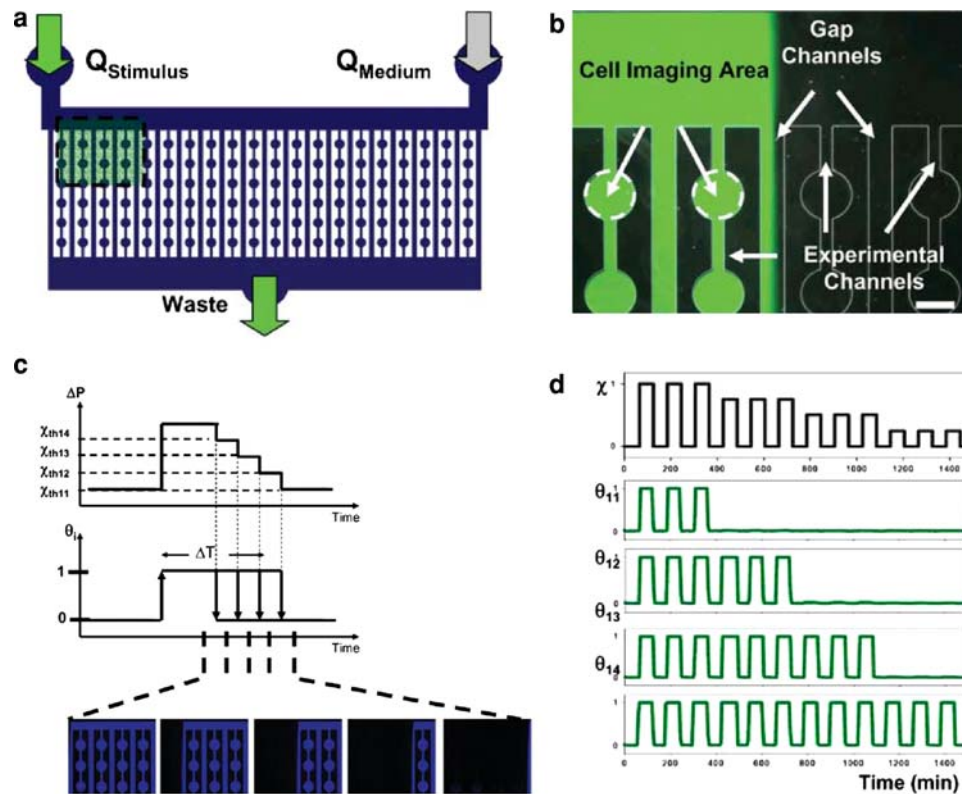


Fig. 5 The “flow-encoded switching” setup. **a** Schematic of the microfluidic device, with two inlet flow inputs, Q_{stimulus} and Q_{medium} , controlling the patterns of stimulation addressing the experimental channels. **b** The device is composed of “gap” channels and “experimental” channels. The “experimental channels” contain cells and convey the desired stimulation patterns to the cells; the “gap” channels provide a means of properly positioning the flow streams between “experimental channels.” **c** The duration of

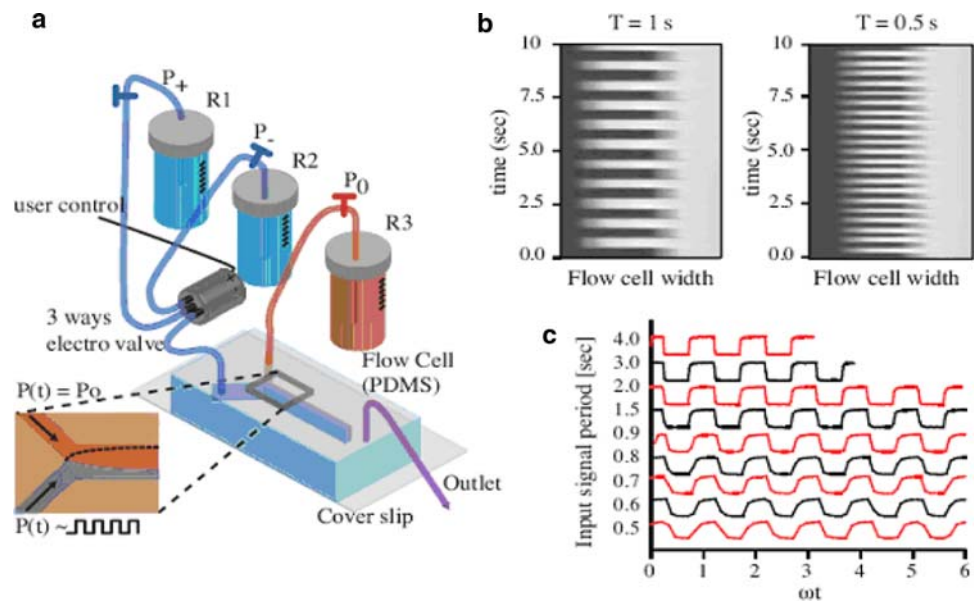
stimulation of individual “experimental” channels can be controlled by modulating one fluidic parameter, the inlet flow ratio in this case. As depicted this ratio is initially increased and then progressively reduced, resulting in different time spans of stimulation for the depicted “experimental” channels. **d** Fluorescence measurement of stimulation profile where pulse train duration is varied across several “experimental” channels. Reprinted from King et al. (2008)—reproduced by permission of The Royal Society of Chemistry

synthesizer” and experimental channels cannot be individually addressed. The setup is currently not optimized for rapid switching either; this issue cannot be simply settled by increasing inlet flow rates, because high shear stress levels can harm or detach cells (Lu et al. 2004) and can elicit certain cellular signals, like calcium (Ando et al. 1990); the previously mentioned microfluidic and macrofluidic examples did not take into consideration shear stress levels induced by fluid flow, despite its vast pertinence to cell viability and signaling. Evaporation can pose a tremendous problem for microfluidic setups especially for long-term studies on cells because of harmful osmolality shifts, but the implementation of special membranes can ameliorate this (Heo et al. 2006). Cell viability can be further compromised by the presence of bubbles, which can also adversely affect flow streams in microfluidic setups (Kang et al. 2007; Walker et al. 2004). Another potentially confounding factor is that cells secrete their own chemicals which can be taken up by their neighbors and influence their behavior; in the “flow-encoded switching” system,

experimental channels have cells arranged in series, such that cells downstream can sense chemicals released by cells upstream, which may potentially result in different behaviors. In the study by King et al. (2008), the rat hepatoma cell line H35 was used, which is known to release the growth-inducing factor transferrin (Shapiro and Wagner 1989). Ultimately, this latter setup demonstrates great potential and portrays how microfluidic designs can be easily parallelized to enable more efficient investigation of the effects of dynamic chemical stimulation on cells. It also highlights some of the challenges and constraints encountered with developing such microfluidic platforms.

While these aforementioned microfluidic platforms exhibit great potential for biological investigations, the studies were mainly device design oriented. A trio of seminal studies emerged recently in which yeast cells were exposed to dynamic chemical stimulation patterns using microfluidic technology and the resulting behavior of the internal cellular machinery was compared with existing mathematical models, in order to elucidate the mechanisms

Fig. 6 Rapid pulse generation setup used to study frequency response in yeast cells.
a Schematic of microfluidic setup, where inlet pressures are manipulated to generate pulses of different periods as depicted in **b** and **c**. When reservoir R2 is chosen, through the user-controlled three-way valve, then the contents of R3 fill the microchannel; when reservoir R1 is chosen, its contents fill the microchannel. Reprinted from Hersen et al. Proc Natl Acad Sci USA 105:7165–7170, © (2008) National Academy of Sciences, USA



of cellular machinery regulation and to gather important kinetic parameters for these components.

Hersen et al. (2008) developed a straightforward microfluidic setup for generating a fast-switching microfluidic system for delivering osmotic shocks to yeast cells (Fig. 6). Reminiscent of the previously mentioned macrofluidic setups, this system consisted of reservoirs with stimulant and neutral media; one could address cells with particular square-wave patterns of various frequencies in a programmable manner. Using this setup, the authors monitored the fidelity of internal cellular signals to the frequency of square-wave osmotic shocks; it was found that the internal machinery responsible for controlling the osmoadaptation response acted as a low-pass filter for this type of stimulation, since the real-time readouts used in this study showed an integrated response and vastly reduced oscillatory amplitude once the frequency of the shocks became too high. In this manner, the bandwidth of this cellular signaling system was deciphered, along with ranges for important kinetic parameters, such as the rate constants for phosphorylation, nuclear translocation, and deactivation of several HOG1 MAPK pathway components controlling cellular osmotic shock responses. The determination of such parameters serves to enhance mathematical and computer models of the behavior of internal cellular machinery. For this study, mathematical modeling was used to test the accuracy of the postulated network of cellular machinery architecture and make predictions about system behavior under certain altered conditions. This same setup and experimental procedure are potentially amenable to the analysis of other cellular machinery components as well.

The same signaling pathway was analyzed by Mettetal et al. (2008); in this study, a simple abstract model was

developed based upon the experimental data and was then compared to what was previously known about the molecular machinery. In doing so, the work highlighted the important roles of negative feedback loops in the yeast osmoadaptation response over short and long time scales. Bennett et al. (2008) used a slightly more sophisticated setup to study yeast cell metabolic gene regulation under dynamic stimulation conditions (Fig. 7). Applying mathematical modeling and microfluidics, these researchers discovered previously unreported properties of the internal cellular machinery that regulates metabolic inputs in yeast. This discovery was made upon the observation that their mathematical model of the molecular machinery dynamics did not correspond with their experimental data. Further characterizations of the molecular machinery were made by using a mutant strain of yeast and comparing its behavior to that of normal yeast; interestingly, it was found that under dynamically changing conditions, these cells responded similarly, despite their differing responses under static conditions. This finding implied that the molecular machinery in yeast had evolved “universally” to adapt to fluctuating environments.

These three studies exemplify an important, emerging niche for biological analysis, involving the union of microfluidics, real-time imaging of biochemical cellular responses, and mathematical and computer modeling. The essence of this experimental approach is captured through precise, reproducible delivery of well-controlled patterns of stimulation to cells, real-time imaging of cellular responses, and effectively recapitulating these stimulation patterns in a computer model to see how the *in vivo* and *in silico* models compare. Discrepancies reveal missing components in the computer model architecture or might

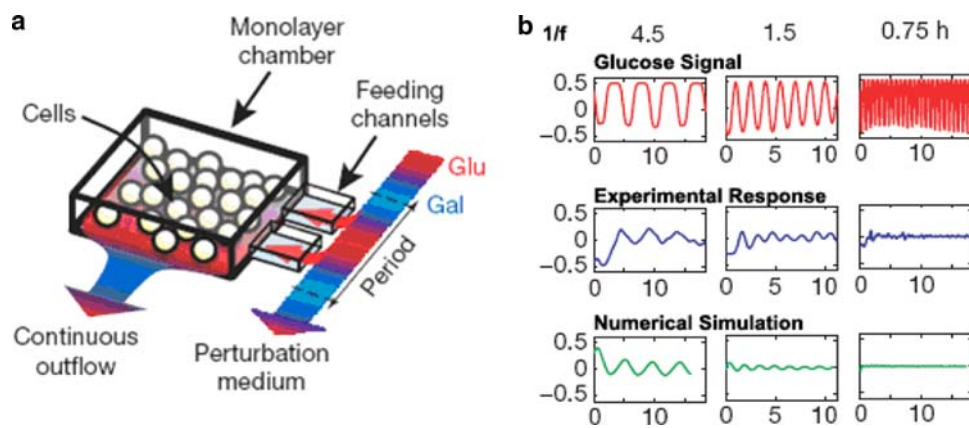


Fig. 7 Microfluidic setup used for analysis of metabolic gene regulation in yeast cells. **a** Yeast cells grown in a monolayer are addressed with different stimulation patterns of glucose which are created by a waveform generator and conveyed to the cells through the feeding channels. **b** Cellular responses to different frequencies of glucose stimulation were measured using real-time readouts, and mathematical models were developed in order to test the postulated

signal pathway architectures. As depicted in the graphs below, as the period of the glucose pulses diminished, the cellular response attenuated and eventually did not respond, which was predicted by numerical simulations; this indicated that this was a low-pass filter system. Reprinted by permission from Macmillan Publishers Ltd.: Nature 454:1119–1122, © (2008)

be indicative of suspect parameters or mechanisms, all of which can be potentially resolved with microfluidic technology through dynamic probing with different patterns of stimulation (Ingolia and Weissman 2008). The next generation of microfluidic setups for this type of analysis and other applications will need to be optimized for mammalian cells, high-throughput capabilities, and fast-switching, versatile stimulation patterns.

The three aforementioned examples used yeast cells as their model system; these cells are less complex in terms of their signaling pathways and upkeep. Several microfluidic setups have been developed that have taken advantage of this. With their microfluidic setup, Groisman et al. (2005) demonstrated that bacterial and yeast cells could be grown to high densities under chemostatic and thermostatic conditions from single cells, a feat that is very challenging to achieve using conventional techniques. The authors monitored colony growth from a single cell and analyzed the effect of the addition of an autoinducer upon the growth response. Repeated transient exposure to exogenous chemical signals was possible with the microfluidic device, however, these temporal dynamics were not explored in this work. Using a similar microfluidic setup, Balaban et al. (2004) used the system to identify two types of bacterial “persister” types upon transient exposure to antibiotics, a seminal finding and one that would have been difficult to achieve otherwise since single-cell analysis is not amenable with conventional laboratory techniques as opposed to microfluidics. While these two microfluidic setups had a highly flexible design allowing for easy control of colony growth and development, they were optimized for yeast and bacterial cell culture and not mammalian cell culture. Mammalian cells are generally more delicate and

challenging to culture compared to yeast and bacteria. Use of non-standard culture surfaces such as PDMS in microfluidic devices as well as changes in volume and flow can significantly add to these challenges, especially cell seeding and long-term culture (Mehta et al. 2007; Walker et al. 2004). To this end, Gomez-Sjoberg et al. (2007) developed a PDMS-based high-throughput, automated microfluidic cell culture system capable of long-term growth and analysis of mammalian cells (up to several weeks). In this work, transient stimulation exposures (hours to days) were applied in order to observe the effect on cell growth, differentiation, and motility; using human primary mesenchymal stem cells (hMSCs), the authors demonstrated that 96 h of stimulation with differentiation media was the minimum amount of time needed for cells to differentiate to an osteogenic lineage with their setup; in addition, it was shown that hMSCs stimulated continuously for 9 days with differentiation media exhibited reduced motility compared to previously unstimulated cells when the two groups were incubated with differentiation media after more than 18 h. Despite the many attractive features this microfluidic setup has for analysis of temporal dynamics in cellular systems, such as full automation of stimulation schedules, high-throughput capabilities, and time-lapse microscopic imaging, there exist several key drawbacks. One major limitation is that it is extremely difficult to fabricate and setup, compared for instance to the “flow-encoded switching” system. While the combination of the fluidic inputs and a multiplexer conveniently facilitated the formulation of individual media compositions that could individually address each of the 96 cell culture chambers, unlike the “flow-encoded switching” setup, the scheme is not very user-friendly or portable. With the flow rate used

in this study, the shear stress level was noted to be an order of magnitude smaller than the minimum level required to perturb differentiation or proliferation for the cells used in their study (Kreke and Goldstein 2004; Riddle et al. 2006), comparable to the shear stress levels used in the “flow-encoded switching” setup; as suggested by King et al. (2008), to achieve faster media switching through increased flow rates without significantly augmenting shear stress levels, cells could be placed in recessed wells. Even with this design improvement to the setup, the versatility of stimulation patterns that can be generated with the system developed by Gomez-Sjoberg et al. (2007) is not at the level of the “chemical waveform synthesizer.” This setup however portrays that long-term mammalian cell culture is achievable in microfluidic devices in an automated, high-throughput manner. Other setups which have achieved long-term mammalian cell culture include the portable, handheld recirculation system developed by Futai et al. (2006), the “airway epithelia on a chip” by Huh et al. (2007), “differentiation on a chip” by Tourovskaia et al. (2005), and the continuous perfusion setup developed by Hung et al. (2005).

There exists a great opportunity to analyze mammalian cells under dynamic stimulation so that cellular processing mechanisms can be better understood on a signal pathway level and eventually cellular behavior can be better understood and controlled on a systems or organ level. The caveat is that mammalian cellular processing is generally more complex than that of yeast and this means that more intricate techniques for analysis will be necessitated for analysis (Ingolia and Weissman 2008; Paliwal et al. 2008). This potentially means that more complex patterns of stimulation will be necessitated, compared to the sinusoidal or square-wave patterns used in the aforementioned yeast cell studies. In a physiological context, it has already been demonstrated that when certain rhythmic chemical patterns are disrupted in the body, it leads to pathological conditions (Brabant et al. 1992; Gambacciani et al. 1987; Van Couter and Refetoff 1985). This provides further motivation for investigating temporal dynamics with mammalian cells, in addition to getting a better grasp of how more complex pathways process dynamic chemical information.

4 Imaging

In order to assess temporal dynamics in cellular systems, appropriate readouts are needed. Compared to the degree to which they are utilized in microfluidic systems, there is a relative abundance of real-time fluorescent readouts that exist for tracking the localization, translocation, appearance, or degradation of target proteins. This development is a consequence of the ability to fuse green fluorescent

protein (GFP; or variants of GFP) to proteins of interest and genetically encode these in cells (Zhang et al. 2002). These types of readouts were implemented in the works by Bennett et al. (2008), Hersen et al. (2008), and Mettetal et al. (2008), and represent passive applications of these fluorescent constructs. In their microfluidic setup, King et al. (2007) created a high-throughput “gene expression living cell array,” which employed fluorescently tagged transcription reporters to capture levels of gene expression in real-time under chemical stimulation. These indicator cells are useful for evaluating longer term responses of cells on the timescale of expression and degradation of fluorescent proteins (many hours to days). However, in order to advance our understanding of temporal dynamics in cells and capture key dynamic intracellular parameters on the seconds to hours scale, such as biochemical messenger concentrations (calcium and cAMP for example), protein activity levels, and protein–protein interactions, specialized probes are required. In the aforementioned study by Schoffl et al. (1993), the fluorescent protein aequorin was used to quantify calcium concentrations in liver cells; while this probe has been used successfully in characterizing intracellular calcium levels in real-time, it does not afford high-throughput analysis as a result of its labor-intensive introduction into cells, which involves individual microinjection. Genetically encoded probes are more effective in this manner because they can be introduced to a large pool of cells all at once through various transfection methods. Many fluorescent probes implement the phenomenon known as fluorescence resonance energy transfer (FRET) in order to convey dynamic intracellular information (Miyawaki 2003). FRET is the radiation-less transfer of energy from an excited donor (in this context, usually a GFP variant) to an acceptor (a different GFP variant) (Jares-Erijman and Jovin 2003). These exist either as bimolecular probes, where two different types of proteins are labeled with two different GFP variants and their subsequent interactions lead to FRET signals, or unimolecular probes, where two different GFP variants are fused to a single macromolecule and subsequent conformational changes of the macromolecule lead to FRET signals. Bimolecular probes have the advantage of being easier to construct, however, quantifications derived from these signals are much more difficult to assess compared to unimolecular biosensors (Jares-Erijman and Jovin 2003). Unimolecular FRET probes are more difficult to construct to ensure viability and functionality, but signal quantitation is much easier compared to bimolecular probes mostly because the donor and acceptor fluorophores are in equimolar quantities (Miyawaki 2003). Several prominent unimolecular FRET biosensors have been developed to probe intracellular pathway dynamics: Cameleon (for intracellular calcium) (Miyawaki et al. 1997), Raichu-Ras

(measures levels of activated Ras) (Mochizuki et al. 2001), Picchu (indirect measure of EGFR and Abl kinase phosphorylation) (Kurokawa et al. 2001; Ting et al. 2001), cGMP probe (Honda et al. 2001; Sato et al. 2000), PKA probe (Nagai et al. 2002; Zhang et al. 2001), cAMP probe (Nikolaev et al. 2004), and most recently Rab5 probe (Kitano et al. 2008).

While FRET probes have provided tremendous insight into spatial and temporal cellular signaling dynamics, there are always concerns about slow kinetics in the face of dynamically fluctuating signals, low dynamic ranges, and interference that they cause, as with any reporter system (Tay et al. 2007). In many cases FRET probes are composed of functional proteins, and their introduction may alter signal dynamics of a target pathway if the probe is expressed in exorbitant quantities (Miyawaki 2003). Similarly, certain probes are designed to capture intracellular components, but if these probes are highly expressed in cells, then they may act as harmful buffers and if the probes compete for intracellular substrates with endogenous proteins, signal levels can be attenuated (Miyawaki 2003). In a study of the FRET biosensor for calcium, Cameleon, a unimolecular and bimolecular version were analyzed (Miyawaki et al. 1999). The authors concluded that high concentrations of the endogenous protein greatly affected the sensitivity of the probe to intracellular calcium levels, while the unimolecular version was not affected. Nonetheless, these probes introduce their own “interpretations” of the signaling dynamics, and accordingly need to be deconvolved if precise quantification of the target signal is desired (Gunawardena 2008). To this end, Tay et al. (2007) probed the kinetics and reliability of a Troponin-C-based calcium sensor through deconvolution of the transfer function of the biosensor based upon the input signal, which was the fluorescent signal from the calcium dye Indo-1 in this case. The authors concluded that the FRET probe showed quantitative reproducibility, which meant that algorithms could be developed in the future that could overcome the slow kinetics inherent to many current FRET probes. Fluorescent dyes for intracellular signal measurement generally exhibit faster kinetics and higher dynamic ranges compared to genetically encoded probes; however, the dyes can leak out of cells and thereby do not afford long-term temporal dynamics characterizations.

To date, only one study has utilized FRET based probes to measure intracellular signal dynamics in a microfluidic chip (Sawano et al. 2002). In this study, cells loaded with various probes were locally stimulated through coupled laminar flow streams and the resulting spatial dynamics were captured through the resulting FRET signals; the authors discovered that the degree of propagation of stimulus across a cell was dependent upon the density of receptors expressed on its surface. This result has

tremendous implications for cancer biology, since certain receptors are over-expressed in many forms of cancer (Hynes and Lane 2005). As FRET based biosensors improve and diversify, there will be a tremendous opportunity to implement them with microfluidics so that effective assessments of temporal dynamics of pathway components can be made.

One caveat of microfluidics, however, is that continuous real-time imaging can be a challenge. Some actuation systems, such as the programmable Braille display (Gu et al. 2004), are not transparent and are difficult to access by a microscope. Even with actuation setups that are amenable to real-time imaging by microscope, we have observed that PDMS-based microfluidic devices can be suboptimal for phase imaging due to unevenness or inconsistencies in device thickness or shape; oil-immersion imaging can be problematic due to PDMS absorption of the oil. In addition, microfluidic actuation can cause vibrations and unwanted movements that can render long-term imaging of temporal dynamics challenging. In this regard, future microfluidic setups will need to take into consideration these requirements for improving real-time microscopy for studies of temporal dynamics in cellular systems.

5 Conclusions

For decades fluidics has played a vital role in elucidating the effect of temporal stimulation dynamics on cellular systems, from simple organisms such as slime molds (Robertson et al. 1972) and yeast (Bennett et al. 2008; Hersen et al. 2008; Mettetal et al. 2008) to more complex systems such as liver cells (Schoffl et al. 1991, 1993; Weigle et al. 1984) and lens tissue (Brewitt and Clark 1988). The ability to dynamically stimulate cells has provided a means of investigating how cells process and interpret information on the molecular level as well as on a multi-cellular level. Seminal studies have effectively demonstrated the importance that non-static stimulation of cellular systems has in our understanding of temporal dynamics and the timing involved in biological systems; in this manner, fluidics overcomes the limitations of conventional static techniques used in biology laboratories. Several prominent examples were provided where simple fluidic setups were employed to deliver well-controlled, reproducible pulses of chemical stimulant to cells; these simple studies resulted in some of the most important biological discoveries (Balaban et al. 2004; Bennett et al. 2008; Brewitt and Clark 1988; Dolmetsch et al. 1998; Hersen et al. 2008; Kupzig et al. 2005; Mettetal et al. 2008; Robertson et al. 1972; Weigle et al. 1984). Recent efforts have resulted in the development of microfluidic platforms

with rapid pattern switching for single-cell studies [“chemical waveform synthesizer” (Olofsson et al. 2005)] or slower pattern switching for higher throughput characterizations [“flow-encoded switching” (King et al. 2008)]; the setup developed by Gomez-Sjoberg et al. (2007) provided a means for long-term maintenance of mammalian cell cultures (up to several weeks) in an automated, high-throughput fashion. An emerging niche for microfluidics in biological investigations has been for testing cellular network architectures under dynamic stimulation conditions, providing an optimal platform for scrutinizing existing mathematical and computer models of cellular signaling pathways to a greater degree, compared to conventional methods (Bennett et al. 2008; Hersen et al. 2008; Mettetal et al. 2008). These latter examples again portray that the simple principle of being able to deliver well-timed pulses of stimulant in combination with mathematical modeling can lead to vital biological discoveries. It is important to note that these studies were conducted on yeast cells, organisms which are much simpler than mammalian cells. In addition, despite knowing many of the components of a cellular signaling pathway, the way that these components interact is very complex and needs elucidation (Brabant et al. 1992). This provides the motivation for the development of next generation microfluidic platforms for investigation of temporal dynamics, since dynamic stimulation patterns can be used to probe signaling mechanisms in ways that static systems cannot (Ingolia and Weissman 2008; Paliwal et al. 2008). The results of such studies hold enormous physiological and therapeutic importance, since many times disruption of the intrinsic rhythms of these systems leads to pathological conditions (Brabant et al. 1992; Gambacciani et al. 1987; Van Couter and Refetoff 1985) and knowing the signaling mechanisms can help with drug development (Persidis 1998). As readouts for the state of cellular machinery improve and expand, this will provide an exceptional opportunity for the development of the next generation of microfluidic devices. It will be necessary for these devices to be amenable to real-time imaging, operate at suitable shear stress levels, and be optimized for supporting cells with complex microenvironments. Ultimately, the microenvironment must be sufficiently recapitulated so that measurements have a proper physiological meaning (Griffith and Naughton 2002; Khetani and Bhatia 2006; Tsang and Bhatia 2006). In addition, future setups will need to combine the versatility of stimulation pattern generation (media switching on the order of seconds to hours) of the “chemical waveform synthesizer” with high-throughput characteristics (preferably 384 or more parallel assays) and the ability to test multiple conditions on a single chip; this latter property was demonstrated by the user-friendly “flow-encoded switching” setup and the fully automated setup developed

by Gomez-Sjoberg et al. (2007). Although a large variety of pumps and valves have been developed, even performing simple two-fluid switching with seconds to hours periods over 96 or larger cell culture wells/channels is still a huge challenge; commercially available microfluidic perfusion setups do exist for mammalian and cell culture, but these are generally not tailored for temporal dynamics studies. Other challenges of adapting microfluidics for characterizing temporal dynamics include difficulties with long-term real-time imaging, cell seeding, controlling shear stress levels that may effect cell signaling and viability, and lack of accessibility to non-engineers. Ultimately, the idea is to have a straightforward microfluidic platform that can be accessible by both engineers and biologists, fostering greater collaboration between these two groups. The timing is right for microfluidic engineers to make a larger impact on the field of temporal dynamics in biology.

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