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Temporal gradients in microfluidic systems to probe cellular dynamics: A review

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Abstract

Microfluidic devices have found a unique place in cellular studies due to the ease of fabrication, their ability to provide long-term culture, or the seamless integration of downstream measurements into the devices. The accurate and precise control of fluid flows also allows unique stimulant profiles to be applied to cells that have been difficult to perform with conventional devices. In this review, we describe and provide examples of microfluidic systems that have been used to generate temporal gradients of stimulants, such as waveforms or pulses, and how these profiles have been used to produce biological insights into mammalian cells that are not typically revealed under static concentration gradients. We also discuss the inherent analytical challenges associated with producing and maintaining temporal gradients in these devices.

Keywords

attenuation; pulsing; frequency response; waveforms; eukaryote

The *in vivo* environment that cells experience is complicated due to the number of signals that cells are exposed to, as well as the spatiotemporal profile of these signals. The temporal profile of many compounds *in vivo* is oscillatory, for example hormone secretion, for reasons that are only now beginning to be understood or investigated [1–3]. These dynamic signals may be used to increase the signal-to-noise ratio of the compound over the thousands of proteins found in serum [4] or reduce receptor desensitization [5]. *In vitro*, the time profile of stimulant delivery can affect the response of the system under investigation, highlighting the need for accurate reproduction by *in vitro* systems of the temporal patterns found *in vivo*. In some cases, delivery of temporally varying stimulus patterns allow observation and determination of intracellular dynamics or population behavior, both of which are difficult to observe under a non-changing stimulus.

Advances in understanding the temporal dynamics of cellular systems are dependent on the analytical devices used to deliver the stimulant waveforms. The ability to accurately and precisely control fluid flows affords microfluidic systems new avenues to study cellular dynamics that have not been possible using conventional macroscale devices. Numerous

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microfluidic devices have been applied in the field of diagnostics [6], cell biology [7], system biology [8], and synthetic biology [9]. Over the last decade, the popularity of using microfluidic devices shifted the paradigm from not only analyzing cellular targets, to now also controlling the cellular microenvironments. For example, a growing field in microfluidic research is the generation of spatial molecular gradients for studying cellular chemotaxis [10], morphogenesis [11], or electrotaxis [12]. Several comprehensive reviews have been written on spatial gradients and their applications in biology [11–14]. More pertinent to the topic of temporal gradients, was a 2008 review article by Jovic *et al.* that summarized how macro-systems have been used to generate temporal gradients and how the advent of microfluidics provided a more effective platform to probe cellular dynamics [15]. The authors emphasized the need for the development of next generation microfluidic platforms for studying cellular dynamics.

This review article is focused on advancements in the field of microfluidic systems since 2009 that use or generate temporal gradients to study cellular dynamics. The applications in this review were restricted to mammalian cells, with a recent review demonstrating systems for investigating the dynamics of yeast and bacterial cells [16]. Finally, this review article is not meant to be comprehensive, only a few representative examples have been highlighted to bring the importance of dynamic stimulations and the use of microfluidic systems to generate these profiles to the attention of the readers.

1. Temporal gradient generation

In recent years, there have been many microfluidic devices capable of generating temporal gradients that have been developed and used for cellular studies. As in most microfluidic systems, the devices have a characteristic length scale that is in the micrometer range where the fluid dynamics are dominated by viscous rather than inertial forces. At these scales, the flow is laminar where parallel streams of fluid mix only by diffusion at their boundary. A mixture of glass and plastic devices have been described with the majority of the ones mentioned in this review being either poly(dimethyl siloxane) (PDMS) or a combination of PDMS and glass.

1.1 Microfluidic devices used to generate temporal gradients

A typical microfluidic system used to generate temporal gradients delivers two or more analytes to a mixing channel where they mix to homogeneity prior to delivery to the cells under study [17]. To produce time varying patterns of reagents, the ratio of the two reagents are varied in time while maintaining a constant volumetric flow rate. The output concentration waveforms can be in the form of pulses, square waves, or sinusoidal waves depending on the application as will be discussed in the following sections. In Section 1.2, more information will be given for producing the correct waveform by optimizing the time the reagents spend in the mixing channel because too little time may not mix the reagents to homogeneity, while too much time may allow dispersion to have a detrimental effect on the shape of the waveform.

More elaborate methods to produce temporal waveforms have also been developed. For example, pulse code modulation (PCM) has been used to produce and deliver stimulant waveforms to ganglia of *Aplysia californica* [18] and also to murine islets of Langerhans [19–22]. In PCM, discrete pulses of analyte are introduced into a flowing stream of buffer where they broaden and mix due to dispersion, producing a homogeneous output concentration that is proportional to the temporal density of the analyte pulses [23, 24]. For the device described in [20–22], two on-chip diaphragm pumps [25] were used to deliver pulses of glucose while also driving buffer through the microfluidic system. By varying the temporal density of the pulses, sine waves of fluorescein with various amplitudes (Figure 1)

and a frequency range from 0.026 – 0.061 Hz have been produced. As will be discussed in Section 1.2, the maximum frequency that could be produced with this system was limited by the dispersion of the waveforms in the mixing channel to ~ 0.061 Hz (16.4 s period) [21]. In a similar report, the ability to deliver more than one stimulant waveform to cells was demonstrated by including more pumping systems [19].

To decrease the time spent in the mixing channel, which is important for decreasing the amount of dispersion, active mixing can be utilized. There are multiple examples of systems with active mixing. One system utilized an acoustically-activated air bubble trapped in a microfluidic channel for enhancing mixing [26]. The system had piezoelectric transducers adjacent to a horseshoe structure (HSS) in a microfluidic channel (Figure 2). The transducers generated low intensity acoustic waves that produced acoustic microstreaming in the HSS. In this way, an air bubble trapped in the HSS vibrated due to the acoustic waves, which produced frictional forces between the boundary of the bubble and the surrounding medium. These forces then mix reagents rapidly in the double-ring recirculation regions. This system has been used to produce square wave pulses of color dyes [27]. Square waves with durations of 2.5–7.5 s and duty cycles of 0.5 were produced, although the authors did not specify if lower durations or duty cycles were possible. While this system was not utilized for cellular studies, the system should be useful for such applications.

The systems described above are suitable for a small number of cells, but other methods have been developed for controlling and delivering spatial and temporal signals to large cell culture areas. In several systems, polycarbonate nanoporous membranes have been integrated into microfluidic devices [28–31]. Cells are cultured on the membrane, which also serves as a partition from the fluid channel underneath (Figure 3). This configuration allows analytes from the underlying channel to diffuse rapidly through the thin membrane into the cell culture region, reducing the time to establish a gradient, both spatial and temporal, from hours to minutes. The Spence group has used this methodology for quantifying the amount of nitric oxide released from erythrocytes using a plate reader for high throughput detection [29, 30]. VanDersarl *et al.* [31] produced temporal gradients underneath a 24 μm thick polycarbonate membrane with 5% porosity where Chinese hamster ovary cells were cultured. Finite element simulations were used to investigate the concentration profiles above the membrane when 5 min pulses at 0.5 duty cycle were delivered to the underlying channels. A pulse of the fluorescent DNA-labeling dye, DAPI, was delivered to Chinese hamster ovary cells and the time for cell staining was consistent with their model (Figure 3). The cells started to respond to a change in dye within 45 s and reached a stable fluorescence level in less than 6 min. An added benefit to these types of systems is that because the bulk flow velocity is very low in the cell chamber, the amount of shear force is minimal, limiting the perturbations on the cells under study.

In the above microfluidic devices, all systems required an external device to manipulate the fluid flow and generate temporal patterns. Recently, Mosadegh *et al.* [32] developed a microfluidic circuit that used an embedded flow switching mechanism to produce an oscillatory output concentration from two constant input flows. The microfluidic device was made of PDMS with passive auto-regulating components analogous to electronic circuit components, such as a diode and transistor. Although a given set of fabricated components produced one oscillation frequency, different device parameters can be used to vary the oscillation frequency. In an extended work from the same group [33], the authors demonstrated a flow switching valve capable of adjusting oscillation period in the range of 0.0175 – 0.00278 Hz with duty cycles from 0.2 – 0.5. Similar work has also been reported by others [34–36] to control flows and reduce the bulky external hardware that is typically used with microfluidic systems. These microfluidic architectures provide a simple and

scalable system that, while not yet applied to cellular studies, could easily be implemented to study cellular dynamics.

1.2 Gradient dispersion

In all applications where dynamic gradients of concentrations are delivered to cells, the gradients will undergo dispersion while traveling through the microfluidic device. The effect of dispersion is particularly significant in pressure driven flows where the fluid has a parabolic flow profile, with lower flow velocities near the channel walls and higher velocities in the channel center [37, 38]. The dispersion broadens the waveforms and lowers the amplitude, potentially affecting the cellular response. Because the amount of dispersion is proportional to the concentration gradient, there is a larger effect on high frequency waveforms compared to low frequency waves, where the designation between high and low frequencies is characterized by a cutoff frequency (f_c) [24]:

$$f_c \approx \frac{1}{2\pi} \left(\frac{U^3}{DL} \right)^{1/2} \quad (1)$$

where U is mean flow velocity in the channel, D is the effective Taylor dispersion-adjusted diffusion coefficient, and L is the length of the channel. As can be seen from this equation, a large linear velocity and short channel length will lead to a large value of f_c because the waveform will quickly pass through the channel, reducing the amount of broadening that can occur.

In this way, any channel can be thought of as a low pass filter where the amplitude of high frequency waves are greatly attenuated and the amplitude of low frequency waves are not. The attenuation of the waveform amplitude as a function of input frequency and channel length can be estimated by:

$$\left\| \frac{C_{out}(f, L)}{C_{in}(f, 0)} \right\| \approx \exp \left(-\frac{f^2}{f_c^2} \right) \quad (2)$$

where $C_{out}(f, L)$ is the amplitude of the output waveform and $C_{in}(f, 0)$ is the amplitude of the input waveform. The ability to predict the amount of dispersion that will occur using a given channel geometry is critical to applying the correct waveforms of stimulant to the biological system under investigation.

2. Applications of dynamic stimulations to cells

2.1 Neurons

Neurons are highly complex cells that are connected via synapses, where information flows through the rapid release of neurotransmitters. The chemicals released from vesicles into the synapse bind to postsynaptic receptors and may initiate or terminate various cellular functions. The disruption of synaptic transmission, for example, failure to activate postsynaptic receptors such as ligand-gated ion channels, can result in various neurological and psychiatric disorders. It is also known that different temporal patterns of neurotransmission can cause different responses from neurons, which are particularly important for processes such as learning and memory [39]. Understanding the mechanisms involved in neuronal signaling cascades would help in a number of different diseases.

Recently, a microfluidic local perfusion (μ LP) chamber has been developed to study signaling between synapses using dissociated postnatal rat hippocampal neurons [40]. The entire device was made from PDMS and had two cell chambers 900 μ m apart that contained

separate populations of neurons. The two chambers were connected by 150 parallel “grooves”, each 7.5 μm wide $\times$ 3 μm high. A perfusion channel ran perpendicular to the grooves close to the postsynaptic compartment. The perfusion channel was 50 μm in width and 100 μm in height. The floor of the device was made of glass, which allowed visualization of the cells. Dendrites from one cell population would reach into the grooves a short distance but still form synapses with axons growing from the other cell population. The perfusion system was located close to the postsynaptic compartment to ensure stimulant was delivered over the synaptic regions within the microgrooves (Figure 4). Three parallel flows were delivered to the perfusion channel with glutamate used as the stimulant in the center flow, while buffer was used as the outer flows. This approach allowed diffusion of glutamate to the side flows, but not to the microgrooves creating a very focused perfusion area. The end of the flow channel was connected to a syringe pump that withdrew the solution at a flow rate of 25 $\mu\text{L}/\text{h}$. Using this system, glutamate was delivered to dendrites in one of two temporal profiles. The first profile consisted of three pulses of glutamate, 1 min duration with a 5 min rest period between pulses, and the second profile was a 3 min constant stimulation with glutamate. The pulsatile profile was generated by adding or removing 30–40 μL in the center perfusion well. When the stimulant was absent in the inlet well, the buffers in the outer flows completely covered the perfusion area. A higher level of the phosphorylated transcription factor, cAMP response element-binding (CREB) protein, was observed using the pulsed delivery of glutamate as opposed to the constant delivery, suggesting the activation of new gene expression that paralleled formation of long-term memory in whole organisms (Figure 4).

In another report, a microfluidic system was developed for studying kinetic properties of ligand-gated ion channels (LGIC) [41]. Upon binding a neurotransmitter agonist, LGIC on post-synaptic cells open, allowing particular ions to enter the cell which can then either initiate or inhibit an action potential. Typically the kinetics of these receptors are faster than metabotropic receptors which rely on downstream cascades to initiate their effects. Botzolkis *et al.* developed a PDMS microfluidic device to generate rapid pulses ($\sim 400 \mu\text{s}$) of gamma-aminobutyric acid (GABA), similar to neurotransmitter exposure times found *in vivo*. Upon binding GABA, Cl^- enters the cell, hyperpolarizing it, inhibiting action potentials. To recreate the dynamics of *in vivo* signaling, the authors wanted to develop a device to produce rapid pulses of GABA while measuring ion current through a membrane patch of HEK293T cells expressing $\alpha 1\beta 3\gamma 2\text{S}$ receptors, a receptor isoform expressed in adult hippocampal synapses. The device consisted of three fluidic channels that were 3 cm long and 100 μm wide with 50 μm spacing between the channels. The middle channel tapered to a final width of 10 μm . The outer channels contained the buffer and the middle channel contained 1 mM GABA. The entire device was submerged in a bath solution and the patch pipette containing excised outside-out membrane patches was placed directly at the outlet of one of the buffer channels. To generate a brief pulse of GABA, the PDMS device, situated on an acrylic platform, was moved orthogonally via a stepper motor so that the patch pipette was now positioned in front of the middle channel (Figure 5A). To remove the stimulus, the device was moved so that the pipette was at the outlet of a buffer channel. In this way, solution exchange times of 100 μs could be achieved with pulses of 1 mM GABA at either 400 μs (synaptic) or 10 ms (conventional) duration applied to the membrane patches. Currents evoked by the synaptic pulses were smaller compared to conventional pulses ($169.9 \pm 55.3 \text{ pA}$ vs. $225.4 \pm 56.4 \text{ pA}$) and the authors suggested that these values resembled the hippocampal inhibitory post-synaptic currents. Also, they found that the longer pulse times decreased the sensitivity of GABA receptors at high stimulation frequency (20 Hz) compared to low stimulation frequency (1 Hz) (Figure 5B). Because this pulse of GABA did not require mixing to achieve the final concentration, the effect of dispersion was minimized. The microfluidic device used in this study generated one of the fastest solution exchange times and pulse durations used in cellular studies.

2.2 Islets of Langerhans

Islets of Langerhans are micro-organs present in the pancreas that play a vital role in maintaining blood glucose homeostasis by releasing a number of peptides into the bloodstream, including insulin and glucagon. Unregulated secretion of these peptides can result in various metabolic diseases like type 2 diabetes mellitus [42] and as such, it is of great interest to understand islet physiology.

Glucose-stimulated release of insulin from β -cells involves coupling of metabolism and ionic signals, culminating in increases in intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) and insulin secretion that oscillate with a period of ~ 5 min. Because *in vivo* insulin secretion is also oscillatory with a similar period, the islets in the pancreas must be synchronized [43]. One hypothesis of how synchronization occurs is via entrainment of all islets to oscillatory glucose levels. Entrainment is defined here as the control and regulation of the oscillatory nature of islets to an external forcing signal [44]. To test this hypothesis, Zhang *et al.* used a 3-layer glass-PDMS microfluidic system for generation of glucose waveforms by the PCM method described in Section 1.1. These waveforms were then delivered to single islets of Langerhans held in a 200 nL chamber on the device where $[\text{Ca}^{2+}]_i$ was monitored by a fluorescent dye [21, 22]. Using this device, single islets were stimulated with a constant 11 mM glucose followed by a sinusoidal glucose profile with an amplitude of 1 mM and a period of 5 min. After switching to the oscillatory concentration, the $[\text{Ca}^{2+}]_i$ oscillations phase-locked to the glucose waves, remaining locked even when the glucose wave was shifted in the middle of the experiment 180° (Figure 6A).

This microfluidic device was further extended to test if multiple islets can synchronize by entraining the entire population to one glucose waveform – similar to what has been hypothesized to occur to the large numbers of islets in the pancreas [22]. To ensure that all of the approximately 20 islets in the 1 mm diameter chamber would experience the same glucose wave, the flow was split after the mixing channel and delivered via four inlets to the cell chamber. This approach maintained the glucose waveform in the chamber and enabled all islets to be entrained to the same glucose waveform, which resulted in a synchronized output from the islet population (Figure 6B) [22]. These results support the hypothesis that islets can be entrained to oscillatory glucose levels.

In a study by Lo *et al.* [45], a multimodal microfluidic system was developed to study the effect of hypoxia on islets. Hypoxia is a state when cells do not receive adequate oxygen leading to cell death. In islets, hypoxia leads to changes in glucose metabolism, decreasing overall ATP yield, and ultimately lowering insulin release. Quantifying the effect of hypoxia is important for islet transplant therapy where islets are injected into the hepatic portal system where the islets experience lower oxygen levels as compared to physiological levels, which has been suggested to impair glucose-insulin coupling. The authors also tested if preconditioning islets to intermittent low O_2 levels can improve insulin release observed under hypoxic conditions. The multilayered PDMS microfluidic device had a channel where O_2 gas was flowed, and above this channel, 10 islets were held in an 8 mm diameter chamber where glucose could be delivered (Figure 7A). Glucose was changed from 3 to 14 mM at hypoxic (5% O_2) and normoxic (21% O_2) O_2 levels while changes in $[\text{Ca}^{2+}]_i$, mitochondrial potential, and insulin secretion were monitored. $[\text{Ca}^{2+}]_i$ and mitochondrial potential were monitored using Fura-2 and Rhodamine 123, respectively, whereas insulin was collected at 1 min intervals and measured off-chip by an ELISA. Hypoxic conditions inhibited oscillations of $[\text{Ca}^{2+}]_i$ induced by 14 mM glucose, and lowered the average $[\text{Ca}^{2+}]_i$ level as compared to islets that were exposed to normoxic conditions (21% O_2) (Figure 7B). During the hypoxic state, insulin secretion was suppressed approximately 70% which the authors' state may have been due to decreased ATP production from the mitochondria, as evidenced by a low Rh123 fluorescence. When islets were pre-conditioned with alternating

1 min pulses of 5% and 21% O₂ (totaling 60 min), improved insulin secretion and dynamics were observed as compared to without the pulses. The authors suggested that intermittent hypoxia preconditioning improved islet function via a mitochondrial K_{ATP} channel mechanism.

2.3 Other mammalian cells

As mentioned above, [Ca²⁺]_i plays a pivotal role in cell signaling. The overall [Ca²⁺]_i dynamics observed are due to the kinetics of extracellular Ca²⁺ entry, and to the kinetics of [Ca²⁺]_i clearance via multiple cellular pumps and ion channels. Understanding how extracellular signals are converted and processed into overall [Ca²⁺]_i changes can provide insights into both of these kinetic features.

Recently, microfluidic devices have been used to generate chemical waveforms to resolve temporal dynamics of single cells. Jovic *et al.*, [46] presented a microfluidic device to study the Ca²⁺-pathway of the G-protein M3 muscarinic receptor system in single HEK293 cells. The PDMS device had two inlet reservoirs, one loaded with carbachol, a muscarinic receptor agonist, dissolved in imaging media and the other reservoir with imaging media alone. The channels from these two reservoirs merged into a single outlet channel where the cells were located. The fluids were delivered through the device by pumping via an external pressure source using Braille pins [47]. A series of square waves of carbachol were produced by sequentially valving the fluids from the two reservoirs. The authors varied the stimulant concentration (C), stimulant duration (D), and rest period (R) between pulses of carbachol, and observed the [Ca²⁺]_i dynamics resulting from this periodic stimulation and compared their experimental results to results from multiple mathematical models. It was observed that with long R-values, the cells phase locked to the carbachol pulses, but when R was decreased from 64 to 8 s with constant D (24 s) and C (10 nM), the cells did not remain phase locked. The two main mathematical models that were tested gave similar results as experimentally observed when a constant carbachol concentration was used, but neither of the models could accurately reflect all the various [Ca²⁺]_i dynamics that were experimentally observed using frequency-dependent stimulations, such as small “sub threshold” spikes (Figure 8). The authors stated that phase-locking analysis was able to examine the differences in recovery and activation properties between the models.

In a later paper, these authors explored how cell-to-cell variability affects the conversion of periodic extracellular signals into periodic intracellular signals [48]. They also investigated the temporal parameters of the extracellular signal that could synchronize a large number of cells even in the presence of cellular variability. A similar device as described above was used to produce pulses of carbachol and deliver these to HEK293 cells. Various ranges of the parameters concentration (C), duration (D) and rest period (R), were then investigated while monitoring [Ca²⁺]_i responses. A response fidelity was determined which represented the percentage of cells in a population that synchronized to the carbachol pulses. The tested values of the parameters were: C = 10, 17.5 and 25 nM, D = 16, 24 and 32 s, and R = 8, 24 and 64 s. It was found that increasing C from 10 to 25 nM, while both D and R were fixed at the mid-value of 24 s increased the response fidelity from 40 to 90%. Similarly, increasing D to 32 s or R to 64 s with C fixed at 10 nM and R or D at 24 s, respectively, increased the fidelity to ~70%. Using pharmacological agents, several examples of the importance of protein expression levels on the response fidelity were also provided. The authors predict that their methodology may be broadly applicable to other oscillatory signaling systems.

Kuczenski *et al.* [49] developed a microfluidic “chemical signal generator” to generate dynamic gradients of ionomycin for delivery to NIH-3T3 fibroblasts while monitoring [Ca²⁺]_i levels. Ionomycin is a selective Ca²⁺ ionophore that transports extracellular Ca²⁺ into the cell. The device was made of PDMS with channels 50 μm high and 300 μm wide

and had two inlets and an outlet. The stimulus (1 μM ionomycin) and blank solutions were loaded into the two inlet channels and merged into the outlet channel where NIH-3T3 fibroblasts loaded with the Ca^{2+} -sensitive dye, Fluo-4, were located. The position of the interface of the two fluids in the outlet channel was dependent on the relative velocity ratios of the two solutions. Using feedback-regulated pressure reservoirs to control the pressure of each inlet solution, the interface was shifted laterally across the cells in time producing pulses and waves of ionomycin. Because repetitive stimulation with ionomycin in the presence of low extracellular $[\text{Ca}^{2+}]$ (1 μM), produced decreasing pulses of Fluo-4 fluorescence, the authors suggested that intracellular Ca^{2+} stores were being depleted instead of ionophore-mediated transport of extracellular Ca^{2+} . The authors suggested that the chemical signal generator could be applicable to studying other calcium regulatory mechanisms and also to investigate frequency-regulated pathways.

In work by Sun *et al.* [50], a hydrodynamic gating method was developed for studying ATP-induced calcium signaling via P2Y receptors in HeLa cells. In this work, a PDMS microfluidic device with a t-shaped intersection was used that had a sample inlet (S), sample waste outlet (SW), buffer inlet (B), and buffer waste outlet (BW). By applying a negative pressure on either the SW or BW reservoir, either B or S was delivered to the cells (Figure 9A). By switching between the two reservoirs where the negative pressure was applied, pulses of S could be delivered to the cell. Rise times as short as 20 ms were produced and 100 ms pulse durations were demonstrated with duty cycles of 0.5 with only slight dispersion-induced alterations of the waveforms. They used their device to deliver 20 s pulses of 20 μM ATP at 100 s intervals to Fluo-3 loaded HeLa cells (Figure 9B). In some cells, the authors noted that the $[\text{Ca}^{2+}]_i$ spikes induced by the ATP pulses gradually decreased in amplitude upon repetitive stimulation, which they believed was due to receptor desensitization. The authors concluded by stating that they anticipate their method could be useful in studying cellular toxicology and pharmacology.

2.4 Cellular toxicity

Cytotoxicity is important in the field of drug discovery and environmental risk assessment. In the process of drug development, several potential compounds are evaluated for their *in vitro* actions on cells to limit animal experimentation [51, 52]. In conventional studies, a constant drug concentration is applied to cells and cytotoxicity measurements are evaluated at the end of the experiment. This end-point approach limits the understanding of how the spatial and temporal kinetics of drug delivery affects the pharmacokinetic response, dictating a need for the development of new tools that allow real-time kinetic analysis of drug-induced cytotoxicity and apoptosis.

Recently Atencia *et al.* developed a microfluidic device to study cell toxicity by examining intracellular protein synthesis and degradation [53]. The device consisted of a top fluidic channel, $320\text{ }\mu\text{m} \times 1.5\text{ mm}$ (height $\times$ width) with two inlets and one outlet (Figure 10A). Four pairs of holes (vias) were placed along this outlet channel, which led to four “buried channels” underneath that ran perpendicular to the top channel. The buried channel dimensions were $600\text{ }\mu\text{m}$ wide and 3 mm long and because the inlet and outlet of each of these buried channels was at the same axial position along the main channel, there was no pressure differential in the buried channel and therefore no bulk flow. The device was fabricated using layers of double-sided tape enabling facile fabrication. Vero cells transfected with green fluorescence protein (GFP) were used as a reporter of intracellular protein synthesis and loaded in the bottom four channels of the device and then sealed with the top section. Either cell media or cycloheximide (CHX) was flowed from one of the inlets in the top channels while buffer was flowed from the other, producing two laminar streams in the top channel, and a spatially smooth gradient in the bottom channels. Cycloheximide is a compound that inhibits eukaryotic protein translation by interfering with RNA machinery

[54]. For cells not exposed to CHX in the bottom channel, the GFP fluorescence increased linearly in time demonstrating normal protein synthesis. Cycloheximide was then delivered to cells for durations of 12, 3, and 7 h within a 43 h total time span. Upon exposing the cells to this compound, GFP fluorescence decreased indicating protein degradation, but could be regenerated when the cycloheximide was removed (Figure 10B). It was found that the degradation rate of GFP during CHX exposure, and GFP accumulation during cell recovery, was independent of CHX pulse duration. The authors hypothesized that short cycles of toxin could be used to infer cell growth and cell fate in a faster manner than conventional assays, although they state that further tests with wider ranges of toxin concentration would be required to validate this hypothesis.

In another study by Shin *et al.* [55], a microfluidic device was developed to assess the toxicity of cadmium (II) chloride (CdCl_2) to a mouse embryonic fibroblast cell line, BALB/3T3. The microfluidic device consisted of three modules for different types of Cd^{2+} exposure. One set of cells was exposed to a constant concentration of Cd^{2+} , a second set of cells were exposed to a time-varying concentration of Cd^{2+} , and a third set of cells were exposed to a blank solution. To produce temporal gradients, a “concentration generation zone” was used where the flow rates from the four inlets were varied and the solution mixed along a zigzag channel prior to delivery to the cells located downstream. The temporal system also had a fifth channel connected to the main channel for introducing a washing medium after cell treatment and an additional loading port for loading cells. The temporal profile was similar to an exponential decay curve, where the maximum Cd^{2+} that the cells were exposed was 135 μM , and the concentration decreased to 30 μM over 60 min. To maintain an equivalent dose, a 51 μM Cd^{2+} solution was delivered in the constant exposure conditions. The amounts of reactive oxygen species (ROS) within the cells were then compared using a fluorescent probe (dichlorofluorescein diacetate). It was revealed that no significant difference in ROS levels was present between perfusion of constant and temporal delivery of Cd^{2+} . The system demonstrated the possibility of performing multiple experimental set up on a single microfluidic device and that the system could be easily adopted for studying other compounds.

3. Conclusion

In this review, several examples were provided how time-varying stimulations can induce a different cellular response than what would be observed when using static stimulations. Due to the ability to accurately and precisely manipulate fluid flows, microfluidic devices are ideal platforms to perform experiments using time-varying signals. While temporal gradients can reveal new directions for investigating and understanding cellular behavior, the effect of dispersion on the shapes of the gradients must be taken into account to ensure an accurate waveform is being delivered to the cells under investigation. With further desires to go to faster waveforms, newer or more refined microfluidic devices will be required. On the other hand, it is important to maintain a simple fabrication and implementation strategy so non-microfluidic researchers can use them in their own applications. It is our hope that the applications and devices discussed in this review show the readers that the use of temporal gradients is an exciting field that has a myriad of opportunities for understanding cellular dynamics.

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Highlights

- Review article covering 2009 – present
- Topics include microfluidic devices capable of producing gradients with a focus on mammalian cells
- Also included are selected examples of these waveforms on cell dynamics

Figure 1. Microfluidic perfusion system for generating glucose gradients

A. Top view of a microfluidic design that utilized PCM to produce waveforms of glucose before delivering to the cell chamber. The valving channels are shown in gray and fluidic channels in black. **B.** Using PCM, a continuous gradient of sine waves were generated at 0.00245 Hz with a median value of 50 μM and amplitudes of 10, 25, 35, and 40 μM . The 5 dashed horizontal lines indicate the fluorescence intensities of standard solutions of 0, 25, 50, 75, and 100 μM fluorescein. Reprinted with permission from reference [20]. Copyright 2008 American Chemical Society.

Figure 2. Schematic of a chemical waveform generator using acoustically activated bubbles

The left image shows the experimental setup of a piezoelectric transducer that generated low intensity acoustic waves adjacent to a microfluidic channel. The image on the right shows the bubble trapped in a HSS. In the absence of acoustic waves (top image), the two liquids entering the HSS were not mixed, as evidenced by the laminar flow of the two solutions. When the piezoelectric transducer was turned on, the two liquids quickly mixed as shown by the homogeneous fluorescence in the channel. Reprinted with permission from reference [26]. Copyright 2009 Royal Society of Chemistry.

Figure 3. Generation of temporal gradients in large cell culture systems

A. Cross-section view of a large cell culture device where the cells are located on top of a polycarbonate membrane that resides above a flow channel. As shown, alternating buffer and fluorescent plugs produces temporally varying patterns in the cell culture region above the channel. **B.** Square wave pulses of DAPI (red line), 5 min in duration with 0.5 duty cycle, were generated in the fluidic channel and compared to results produced from a simulation of the signal above the membrane (blue line). Reprinted with permission from reference [31]. Copyright 2011 Royal Society of Chemistry.

Figure 4. A multi-inlet microfluidic perfusion device to study synapse-to-nucleus signaling

A. Schematic of a microfluidic local perfusion chamber where the flows from three inlets are delivered to synapses formed in the microgrooves. On the sides of the microgrooves, four wells provide access to the two neuronal chambers for maintaining cell growth. The right image depicts a zoomed-in view of the laminar flow from the 3-inlets going across the microgrooves. **B.** Merged fluorescence micrograph of Alexa Fluor 488 hydrazide (green), Alexa Fluor 568 hydrazide (red), and Alexa Fluor 633 hydrazide (blue) added to the middle perfusate channel, postsynaptic compartment, and presynaptic compartment, respectively. Scale bar = 100 μm. Note the minimal diffusion out of the perfusate channel into the microgrooves. On the right is an image of a MAP2-immunolabeled neuron (green) indicating no morphological changes growing across the perfusion channel (white lines) in the microgrooves. Scale bar = 50 μm. **C.** Fluorescence images of neurons when dendrites were exposed to 3 min pulses of glutamate (left image) or a constant glutamate

concentration (right image). The temporal profile of the glutamate concentration is shown on top of each image. Scale bar = 20 μm . Reprinted with permission from reference [40]. Copyright 2010 Elsevier.

Figure 5. Synaptically relevant pulses produced by a microfluidic device

A. Schematic of the PDMS microfluidic system attached to an acrylic platform. The glass electrode was held stationary while the platform was moved, via the stepper motor, producing ultrashort pulse durations. **B.** Pulses of 1 mM GABA were delivered at either 1 Hz (top panels) or 20 Hz (bottom panels) with durations of 400 μ s (left panels) or 10 ms (right panels) while monitoring receptor current. Reprinted with permission from reference [41]. Copyright 2009 Elsevier.

Figure 6. Islet $[Ca^{2+}]_i$ response to glucose waveform

A. Using a microfluidic system similar to that shown in Figure 2, the $[Ca^{2+}]_i$ concentration within a single islet was measured using Indo-1 fluorescence (black line) upon exposure to an oscillatory glucose concentration (red line). The $[Ca^{2+}]_i$ phase locked to the glucose oscillations even during the presence of a 180° shift in the glucose concentration at ~42 min. Reprinted with permission from reference [21]. Copyright 2010 American Chemical Society. **B)** The $[Ca^{2+}]_i$ oscillations from 21 islets, as measured by Fura-2 fluorescence, synchronized after exposure to oscillatory glucose levels (dashed line). On bottom is the average Fura-2 signal from the 21 islets showing coherent oscillations from the population during oscillatory glucose exposure. Reprinted with permission from reference [22]. Copyright 2011 American Physiological Society.

Figure 7. A multimodal microfluidic device to study islet hypoxia

A. Cross-sectional view of the device developed to vary the O₂ concentration in the perfusate. O₂ was flowed through the bottom channel and could pass through the thin PDMS sheet into the islet chamber. Islets held in the islet chamber were perfused with Krebs Ringer (KR) buffer (pink) under different glucose concentrations. **B.** The [Ca²⁺]_i response, as measured by the change in Fura-2 fluorescence, under different O₂ tensions are shown. The blue line is the average response under normoxic conditions (21% O₂), the red line is the average response under hypoxic conditions (5% O₂), and the green line is the response when islets were preconditioned by intermittent hypoxic (IH) pulses. As seen, the islets exposed to IH pulses responded similarly to islets under normoxic conditions. Reprinted with permission from reference [45]. Copyright 2012 American Chemical Society.

Figure 8. Single cell response to a periodic stimulation using a microfluidic platform

HEK293 cells were stimulated with carbachol using the waveform shown at the top. The $[Ca^{2+}]_i$ response to these carbachol pulses, as monitored by a FRET-based intracellular sensor, from a single cell is shown. The smaller peaks, known as sub threshold peaks, were experimentally observed, but not predicted by mathematical models. Reprinted from reference [46], copyright [46].

Figure 9. ATP-induced Ca^{2+} changes in single HeLa cells

A. Schematic diagram of experimental setup to generate pulses of ATP using negative pressure applied to the BW and SW reservoirs. Also shown is the instrumentation for optical detection of $[\text{Ca}^{2+}]_i$ changes in single HeLa cells. **B.** $[\text{Ca}^{2+}]_i$ changes induced by pulses of ATP are shown. Ten consecutive pulses of $20 \mu\text{M}$ ATP were delivered, each 20 s in duration. Reprinted with permission from reference [50]. Copyright 2011 Springer.

Figure 10. Diffusion-based gradient generator for examining cell toxicity

A. On the left is the top layer of the microfluidic device where reagents are delivered to the two inlets. The two vias allow fluidic contact to the channels on the bottom layer (shown on the right) where the cells are located. **B.** A population of cells along the bottom channel were exposed to cycles of CHX for the durations of time shown at the top of the plot. The GFP fluorescence as a function of time is shown for cells exposed to different concentrations of CHX. During the periods of CHX exposure, GFP fluorescence decreased indicating protein degradation, while during the periods without CHX, protein synthesis was reinitiated as indicated by increased GFP fluorescence. Reprinted with permission from reference [53]. Copyright 2012 Royal Society of Chemistry.