Microfluidic Temporal Cell Stimulation

by

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Abstract

This thesis presents a novel microfluidic platform for investigating cellular signaling networks, combining a programmable system for generating arbitrarily complex temporal input stimulant concentration profiles with real-time fluorogenic monitoring of cell physiological response. While cellular assay automation has been demonstrated in microfluidic devices, applying microfluidics to more difficult, multi-parametric systems biology problems, like the analysis of signal transduction pathways, requires a higher level of automation to rigorously control environmental parameters and detect output signals. Addressing this challenge, I developed a JAVA-based programmable microvalve system for automated control of reagent delivery enabling the end user to generate complex chemical waveforms and perform multiple assays on individually addressable cell chambers on chip.

Typically, signaling behavior has been inferred from cellular responses to step inputs or slow square waves [1-3]. Generation of arbitrary waveforms in these experiments involved either complex programmable syringe pump systems or physically moving a sample through a spatial gradient to create temporal changes [4, 5]. My device used computer-controlled elastomer valves to dynamically generate arbitrary concentration profiles with waveform periodicities as short as 10 seconds. This kind of complex signal input to cellular networks has not previously been reported.

To demonstrate this platform, experiments were performed monitoring the change of intracellular free calcium in response to the application of histamine. Cells were treated with a dye that fluoresces only when bound to calcium within the cytosolic compartment. Live cell imaging was used to observe both spontaneous oscillations in response to constant stimulant concentrations as well as response peaks driven by applied waveforms generated on-chip. This system allowed the following of the response of individual cells to changes in applied stimulant. Using this platform, scientists can create new, complex input signals and observe output responses, opening new avenues of experimentation in systems biology.

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Introduction

A platform for temporal cell assays

Cells live in an environment in constant flux. There are periodic changes on a vast order
of time scales, from circadian rhythms on a 24-hour cycle, to neurons firing in the
millisecond range. They react to these changes in incredibly complex ways that are only
somewhat understood. Microfluidics offers us a framework for replicating these
environments, and casting light on their mechanisms, in contrast to typical in vitro
investigations that involve a single step increase of concentration of the chemical of
interest. The difficulty of changing the concentration dynamically in a culture dish
precludes more complex experiments. The rate at which additions and dilutions can be
performed, and the accuracy achieved by this method leaves much to be desired in the
range of possible experiments.

Many researchers have looked to microfluidics to address these shortcomings. With
characteristic lengths and volumes much closer to those of the cells themselves, these
new technologies can create environments with more precision and speed than traditional
methods. Generating time varying chemical concentrations is one of the new techniques
being used to probe deeper into network responses. To date, microfluidic waveform
generation has focused on low frequency step changes in concentration [2, 3]. The work
described in this thesis was focused on generating intermediate concentrations from
inputs, applying those concentrations to cells on chip, and observing the response.

We developed a platform to investigate cellular response to complex temporal inputs. By providing precise control of the culture environment, experiments that would be impossible with traditional laboratory equipment can now be done inside PDMS microfluidic chip. Specifically, rapid, cyclical changes of a reagent of interest in the fluid surrounding the cells can be accomplished. Take for example, the creation of a two minute period sinusoidal concentration waveform in a micro-well. To achieve this with 5% resolution would take 20 cycles of volumes mixing, evacuation, and replacement per minute, or pipetting at about 1 Hz. Using our platform, this can be accomplished on-chip, without prohibitively difficult pipetting operations.

The first major piece of the platform is the complex input generation. Though time varying concentrations have been produced in microfluidic devices before, none provided the flexibility and control we were seeking. Generation of arbitrary waveforms was previously very difficult, requiring, for example, complex computer controlled syringe pumps systems, or physically moving a sample through a spatial gradient to create temporal changes [5, 6]. By using a pulse width modulation approach, we were able to generate arbitrary time varying concentrations on chip.

The second piece of the platform was integrated cell culture on chip. This allowed us to control the fluid environment of the cells precisely and quickly by having them downstream of the concentration generator. Because the volumes and dimensions were so
small (10 nl, 20μm), the fluid surrounding the cells was easily displaced by incoming concentration waveforms. Building on the work of other researchers in our lab, cell sieves were incorporated into the devices, downstream of the concentration generator [7, 8]. These sieves trapped about 10 cells each during cell seeding, providing consistent numbers and locations of cells within the device.

**Figure 1:** The letters MIT written in fluorescein concentration by generating the appropriate waveform on chip

An example waveform is shown above in Figure 1, with the orange showing the fraction of an input reagent (fluorescein in this case). In seven minutes, a concentration profile that resembles the letters MIT was generated on a chip to demonstrate the capability of the device to create complex arbitrary concentration profiles. When creating arbitrary intermediate concentrations, the settling time to reach a desired concentration was about 30 seconds. If only step changes were required, the settling time was as low as 5 seconds.
Lower flow rates to ensure good mixing during intermediate concentration generation precluded faster switching speeds.

These time varying concentrations can tell us much more about cellular response than steady state concentrations, which typically involved a single step of concentration change. For example, the signaling network being used may act as a low, high, or bandpass filter, an amplifier or dampener. These properties are either difficult or impossible to elucidate with a single time invariant bolus [2, 9, 10]. Being able to mimic \textit{in vivo} environment changes such as circadian rhythms on the scale of hours, to hyperosmolar signaling pathways on the scale of minutes, to cell migratory responses on the scale of seconds [10, 11], enables experiments that more closely represent biological conditions. Part of our approach was to treat the cells as a “black box”, much in the same way that circuits can be probed by observing response to pulses. Models for responses can be derived based on these responses, without delving into the incredible complexity of cellular networks [3]. Instead of modeling square waves as sinusoids for Fourier analysis, we can actually generate accurate sinusoidal inputs. A researcher can quickly specify the type of waveform to be generated, and immediately test it on the cells on chip. This flexibility and programmability makes this platform especially well suited to this sort of study.

There has been much work in microfluidics to create different kinds of gradients. The linear gradients created by combinatorial mixers, for example, have been widely used [12]. Spatial gradients have also been used in screening applications to deliver a broad
range of doses to a series of cell populations [13]. These static gradients did not offer the
temporal variability sought for this work.

Other temporal work doesn’t have the ability to combine cell culture and waveform
generation, typically focusing on one or the other of these aspects. One reported
technique used a very complex system with a submicron accuracy manipulator, and up to
16 pre-mixed inputs [5]. While this device is capable of fast, complex waveforms, it can
only assess one cell at time as opposed to entire populations in our chip, and requires
very elaborate experimental equipment when compared to standard microfluidic
experimental setups.

More recently, a microfluidic platform was developed that uses a pulse modulation
approach, switching between states and using fluid mixing to create intermediate
concentrations. The multi-layer PDMS devices used in the study are much more complex
than ours, and have only had waveform generation demonstrated, not combined culture or
cell stimulation [14].

Another reported technique to create temporal concentration changes involves moving
the interface of a co-laminar flow. Using pressure to drive multiple fluid input sources,
control of gradient position within 2% of the output channel, and response time <0.1
second has been achieved. This system in general, however, is quite complex, involving
computer-controlled feedback regulated pressure sources. While enabling rapid step
changes at defined points within a microchannel, the ability to generate general
spatiotemporal waveforms is limited [4]. The Toner group (MIT-Harvard HST/ MGH) applied this co-laminar technique to the temporal stimulation of adherent mammalian cell lines [3]. The position of the flow interface could be swept across a parallel set of channels containing cells by regulating input pressure. Controlling which channels were receiving stimulus as a function of time, many temporal profiles were tested simultaneously. While this approach has the ability to assay entire populations of cells, like our device, it only allows for step changes in concentration between two states as opposed to intermediate concentrations. Likewise, the Oudenaarden group at MIT has done work dealing with temporal stimulus of yeast cells, but the time scales involved were long, tens of minutes to hours, as well as being only step changes [2].

To illustrate the utility and capabilities of multilayer PDMS microfluidic devices as tools to stimulate cell populations with complex temporal waveforms, we selected calcium response to histamine in HeLa cells as a model system. The cells take up a calcium sensitive dye, then, when stimulated with histamine, release calcium into the cytoplasm, causing the dye to fluoresce under a FITC filter set. Stimulating the cells with periodic concentrations of histamine shows different behavior than is observed from simple step inputs.
Discussion and Results

Overview of platform

We developed a platform for investigating cellular response to complex time varying concentrations. The major components of the platform are:

- Microfluidics
  - Concentration Waveform generation
  - Integrated cell culture on chip

- Hardware
  - Inverted fluorescence microscope (Olympus IX71)
  - Cooled CCD camera (Apogee U2000)
  - Shutter (Sutter Lambda SC),
  - Microscope stage environment control (WeatherStation),
  - Motorized stage (Prior)

- Software
  - In house developed JAVA software
  - MicroCCD
  - ImageJ
**Design Development**

To create time varying concentrations from a reagent and a diluent, a pulse width modulation-type approach was attempted. The basic idea was to interleave injections of each input in a set ratio, and update the desired ratio frequently. Smaller resolutions allow faster updates, but less precision, where larger ratios allow more exact concentration mixing, but are updated less frequently. Using a resolution of 20 mixing steps was sufficient in most cases, providing ±2.5% concentration accuracy, and updates about every second.

![Diagram](image)

**Figure 2:** First waveform generator chip, with valves for peristaltic metering (red) on the input channels (blue) and a long outflow channel for diffusive mixing.
The early chips used peristaltic metering to control injected volume, and a series of herringbone structures to mix the fluids once injected [15]. In the schematic shown in (and other subsequent schematics) the red lines represent control channels, the blue represents rounded channels, capable of being valved, and the yellow and green are rectangular channels. The asterisks are ports to interface the device to the world. This chip design used the sets of three valves for peristaltic metering on two input channels to pump either the upper or lower fluid into the mixing channel. A sequence of images in Figure 3 shows the mixing of fluorescein and water.

Figure 3: Fluorescein and water mixing at 1 frame per second. Sequential injections of water and fluorescein creating intermediate concentrations.

In this sequence, the bright green fluid is the fluorescein input, and the dark fluid is water. An injection of fluorescein is almost to the mixing chamber in the first frame, and followed by water in the second and third. Frames four and five are pure fluorescein, followed by another water injection in frame six. As these fluids flow through the
channel, mixing eliminates small local gradients, producing smooth concentration outputs. Valve actuation was operated at 20 Hz, injecting fluid every 150 ms. Imaging was performed at one frame per second under FITC with a Sony XCD-X710CR color camera.

This design was effective at generating waveforms on a several minute scale, 180 second period sinusoids, for example, shown in Figure 4. To generate this waveform, valve actuation was again set to 20 Hz. The output concentration was measured fluorometrically using a Sony XCD-V50 black and white camera, imaging at one frame per second. Signal intensity was measured in ImageJ.
Figure 4: Sinusoidal concentration waveform generated on chip (blue diamonds) by metering fluorescein and water as a demonstration of device capability. The output concentration matches very well with the prescribed concentration (orange).

With such a large outlet channel and low flow rates, dispersion and diffusion prevent shorter periods. To increase waveform speed, and to begin to culture cells on chip, the devices show in Figure 5 were designed.
Figure 5: Initial versions of integrated waveform and cell culture, with peristaltic metering on each assay input, and an additional input for cell loading. One chip has serpentine diffusive mixer, and the other a direct input to the cell chamber.

These devices, like the preceding generation, have a pair of inputs with metering valves. Another input port was added, for the initial loading of cells. Two versions were made, one with a long inlet to ensure mixing, and one with a shorter channel to allow for quicker changes of concentration. Culture chambers with cell sieves were used to help ensure uniform cell counts and seeding conditions. Two types of chambers were used, with 400 μm (not shown) and 800 μm diameters.

Fluorescein was again used to quantify the ability of the chip to produce specified gradients. Shown in Figure 6 is an example waveform, with three different concentrations flowing through the serpentine channel. Each was generated for one minute, with 5 Hz valve actuation. The first is mostly past the exit, the middle, lower concentration entirely in the mixer, and the last still being generated by the metering valves.
Figure 6: Fluorescein waveform in progress, traveling through the serpentine channel from left to right. Each concentration was generated for one minute, showing the flexibility in making intermediate concentrations.

More fluorometric characterization experiments were done with this chip, showing the types of gradients possible. In Figure 7, a series of step concentration changes were defined in the software, as show in Table 1. Each concentration was generated for one minute, with valves operating at 6 Hz, and output fluorescence measured at one frame per second under FITC with a Sony XCD-X710CR color camera.

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
<th>360</th>
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</thead>
<tbody>
<tr>
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<td>0.00</td>
<td>0.10</td>
<td>0.15</td>
<td>0.20</td>
<td>0.50</td>
<td>0.80</td>
<td>1.00</td>
<td>0.95</td>
<td>0.25</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 1: Defined waveform for on chip mixing.
Intermediate Concentrations

Figure 7: Dynamic creation of intermediate concentrations of analyte in the central microchannel generated from a table defined in the software source

Once used in experimentation, some areas for improvement of the design for gradient generation and cell assaying arose. (1) There was no way to purge the inputs to ensure the desired fluid had completely rinsed out the previous fluid. (2) The input used to load cells tended to become fouled with debris and cells after seeding, rendering it unsuitable for other use. (3) Though this chip has dedicated cell input lines, the total number of inputs is low, making complex assays more difficult. (4) If any input fluids are changed, there is a risk of introducing bubbles, as well as the need to flush the previous fluid out of the input well. To perform the desired assays, at least three reagents are required, necessitating changing inputs with this chip design. (5) The herringbone mixers were shown empirically to be unimportant for mixing, as the interspersed fluids mixed well through dispersion and diffusion as they flowed through the chip.
Figure 8: Version two (V.2) of the combined culture and assay device with an added fluid input as well as a bypass for purging input lines.

The next generation device (V.2) (Figure 8) used the same base SU-8 masks (orange) for the cell culture area, but changed the surrounding chip architecture. The number of inputs was increased to four, and a bypass around the cell culture area added. This allows the input fluids to be purged to ensure the desired fluid flowing through the chamber later. Throughout these design changes, cell culture on chip remained a challenge. Through these tests, the 800μm cell traps provided approximately four times as many cells per chamber and more robust fabrication. These were used in all future chip designs.

Two more designs were created, one to allow multiple experiments per chip (V.4), and another with more limited functionality but easier fabrication and setup (V.3). The simple
chip (V.3) required only eight control inputs, and being smaller, could easily be fabricated in large numbers. These chips were useful for developing protocols, as many procedures could be tested with less time spent on setup. Both versions had dedicated peristaltic metering valves on the exit, and used the 800μm cell traps. With these chips, cells were cultured reliably on chip, due to protocol refinements as described in the Culture Development section.
Figure 9: Cell culture devices versions three and four featuring peristaltic metering on the exit that can be applied to any input, and a bypass for purging input fluids.

The four-chamber device (V.4) featured four inputs, facilitating seeding as well as assays. The chambers were individually addressable, allowing different experiments to be run in each chamber. By exposing cells cultured under identical condition to different stimuli, the number of variables per experiment was reduced. This also reduced set up time, as
four unique experiments could be conducted each time a chip was prepared. V.4 satisfied our requirements of multiple experiments per chip, reliable cell culture, and waveform generation, and was the final realized design in the development of the platform. Hundreds of these devices were cast and used in developing the culture and assay protocols.
**Culture Development**

As previously mentioned, there are many challenges to reliable robust cell culture on chip. The base protocol was adapted from Wang et al. [8]. The following protocol steps were the most important for reliable repeatable culture; without them culture viability was 10% at best. Using an off-chip peristaltic roller pump while loading cells provided a constant flow rate. By eliminating the variations caused by manually operating a 50µl glass syringe, the cells were not exposed to rapid pressure fluctuations. Another change was to eliminate the 0.2% gelatin as specified in [8]. We did find that a pretreatment of Dulbecco's Phosphate-Buffered Saline (DPBS) in the chip for at least two hours to be necessary, though, to pre-hydrate the chip.

Pipette tips (10µl, no barrier) filled with media were placed in the inputs to provide continuous perfusion while the chips were incubated, detached from the computer controlled valves. These tips were filled with ~100µl of media, almost to the point of overflowing. Before using the tips, lengths of tygon tubing filled with media were used. The tips improved the culture conditions by providing a larger media reservoir and reducing the capillary effect that would stop gravity driven flow in the small-bore tubing.

To solve the problems that occasionally arose with bubbles forming at the tip-chip interface, media was pre-equilibrated to internal incubator conditions. When this was not sufficient to prevent their formation, they could typically be removed by pushing fluid out of the ports in question. In the event that not all the air was removed in this way,
small bubbles were forced into the bulk PDMS by applying several psi of pressure to the input tube of interest using a regulated compressed air supply.

The images in Figure 10 show the cells immediately after seeding (A), mostly confined to the cell sieves. In the next image (B), six hours later, the cells have spread and attached to the glass floor of the chamber. This is a typical density found after seeding, similar to those used in experimentation.
Figure 10: A) HeLa cells in sieves immediately after seeding, mostly in the cell sieves. B) Six hours after seeding, spread and attached. This seeding density was typical, and similar to that used in assay experiments. The round culture chambers are 800 μm in diameter and 25 μm high.
**System Control**

The next major piece of the platform is control and automation of chip operations and data collection. To accomplish this, several capabilities had to be added to our in-house, open source JAVA platform for microfluidics [16].

The waveforms were defined in the source JAVA software files by a function that mapped desired concentration to elapsed time. Functions can be piecewise defined or continuous. The piecewise method was used to define a series of steps or pulses, whereas the continuous functions were better suited smooth shapes such as sinusoids or ramps.

To control the motorized stage, the open source library “RXTX” [17] was incorporated into our JAVA, to communicate with the stage over a serial interface. The stage can be sent to specific locations as defined in the software quickly and easily, allowing data to be collected unattended from different parts of the chip.

To operate the Sutter Lambda SC shutter, the jd2xx library [18] was added to our JAVA libraries to communicate over USB with FTDI device [19] inside Sutter shutter. This allows the JAVA full control over shutter operations. The shutter was later connected directly to the camera in a slave mode, for more precise timing of the exposure and shutter operation.

To operate an Apogee U2000 cooled CCD camera [20], a Visual Basic script was used to call MicroCCD operations. This script passes imaging parameters (exposure, binning,
temp, etc) written to operate the camera. Metadata such as the location on the chip, mixed concentration, and timestamp can be saved into the filename or other metadata locations.

These adaptations to existing code allowed complex experiments to be run easily, all from a simple GUI front end.
Proof-of-Concept Platform Experiments in Cell Signal Transduction

Cell signaling is complex, and in many cases, poorly understood. This platform was developed with the hope that it would help to elucidate some of the underlying mechanisms of cellular response, specifically network response to time varying signals. These pathways are central to cellular activity, and have roles in nearly every aspect of cellular function [21].

The idea is to use a “black-box” approach to determining behavior: Observe the reactions to a variety of inputs, and try to derive a model for the behavior. To take the first steps towards that goal, we chose to investigate the calcium response to histamine in HeLa cells. Details of the culture conditions and assay procedures can be found in the appendices. Cellular calcium response to histamine exhibits complex oscillatory behavior [22].

This calcium response has many interesting consequences [21], but is a fairly easy system to investigate using fluorometric assays (Invitrogen kit number F36205). Cells maintain calcium levels in the cytoplasm much lower than their environment, with resting levels at ~100nM. To maintain this low level, ATPase pumps actively push calcium ions into the endoplasmic reticulum or out of the cell entirely. Upon receipt of an appropriate trigger, be it an extra- or intracellular stimulus, calcium ions flow rapidly from the stores into the cytoplasm, activating a variety of downstream responses [21]. This calcium in the
cytoplasm activates a fluorescent dye (Fluo 4), which can be measured by fluorescence microscopy. This made it an ideal choice for proving the utility of this platform. With a platform capable of stimulating cells with time varying concentrations of histamine, investigations into these response phenomena are available in greater depth.

The investigations into temporal stimulation of HeLa cells exposed several interesting results. Most noticeable is the significant heterogeneity within cell populations. In Figure 11, a kymograph of responses from several cells in a population is shown.
Figure 11: Kymograph of responses, each vertical striped column represents a cell. Initially, the cells are oscillating asynchronously in constant histamine, but when the histamine is instead pulsed (beginning at the arrows) the calcium release of the cells largely synchronizes.

Each banded column in Figure 11 represents a cell oscillating in response to applied histamine. Time is the vertical axis, increasing downward. A low level of histamine is initially applied continuously for three minutes (top of Figure 11). Responses to this are largely asynchronous, with each cells responding differently. After this initial period, pulses of stronger histamine are applied at intervals (starting at the arrows), returning to
low levels in between. The cells largely synchronize to these pulses, as shown on the figure, in contrast to the initial responses.

Another interesting behavior is the apparent adaptation response to histamine. For a given concentration, oscillation frequency decreases over time. This would seem to indicate that the cells are responding to histamine by lowering their sensitivity to it.

**Figure 12:** Frequency decrease in calcium response to histamine. Cells were stimulated with square wave pulses of histamine, and the number of peaks per stimulus decreases on subsequent histamine pulses.

In Figure 12, the response of a single member of a cell population to a series waves of three minutes of histamine, three minutes of assay buffer (from the Invitrogen kit). The
first response shows rapid oscillations, but the number of peaks per histamine bolus drops in subsequent pulses.

As a demonstration of platform’s ability to generate complex waveforms, a ramp of histamine concentration was generated from 0-100 μM over 10 minutes. An example cell response is shown in Figure 13.

Figure 13: Ramped histamine concentration and a single cellular response. The ability to generate stimuli such as these, and observe single cell responses shows the strength of this platform, and the complexity of the pathways and responses under investigation.
This response of a single cell to a complex input shows the versatility and power of this platform, as well as the complexity of the biological response, highlighting the need for such a platform.
Conclusions

A platform for investigating cellular response to time varying signals was developed. A temporal gradient generator was developed. Combining this with on chip culture, and requisite hardware, the platform is operational. A researcher can culture cells on chip, specify up to four unique experiments to be run on individually on the cell sieves, and have them all run unattended. The design can be scaled to larger numbers of individually addressable cell populations if desired.

Future experiments could investigate the complexities and heterogeneities of calcium oscillations in cells, or any number of other cellular responses of interest. Also, the downstream consequences of such stimuli would be quite interesting to investigate as well. Calcium signaling is known to be involved in cancer metastasis [23], axon growth [24], and calcium oscillations have been shown to “increase efficiency and specificity of gene expression” [1]. So prevalent is calcium signaling that it is “almost be easier to list the cell processes that are not controlled by Ca2+ than those that are controlled by it” [25]. Better tools to investigate this broad field, such as this platform, will have profound effects on our knowledge of signaling and its effects, from fertilization at conception to apoptosis and death.
References


[18] https://jd2xx.dev.java.net/.


Appendices

Methods

Cell Culture protocol

A human derived immortal cell line, HeLa, was used for all experiments. Cells were cultured in 25 cm² flasks in DMEM + 10% FBS + P/S at 37 °C and 5% CO2. After much trial and error, a protocol was established to culture cells on chip. Prior to plasma bonding, chips were baked “face up” at 80 °C for at least 3 hours. Both glass slides and chips were rinsed briefly with acetone, the ethanol, and blown dry with air. Chips and slides were then plasma treated, bonded, and baked at 80°C for >5 minutes to ensure a good bond.

Once bonding was complete, the chips were placed in a vacuum chamber, and held at ~600 mTorr for several minutes. Immediately upon release of the vacuum, the chips were filled with DPBS, the vacuum accumulated in the chip removing any trapped air bubbles. The chips were then equilibrated with DPBS in the channels for ~2 hours. Media for use in the chips was equilibrated open in the incubator, to allow it to achieve correct gas ratio and pH.

To collect cells for seeding into the chips, media was aspirated out of a culture flask, then the flask rinsed with DPBS and aspirated. For a 25 cm² flask, 0.5 mL of Invitrogen TrypLE was added, and the flask returned to the incubator for 5 minutes. The cells were
then no longer adherent, and 2 mL of media added. This was then aliquoted into conicals, and centrifuged at 1000 RPM for 2 minutes. The cells were resuspended in a few hundred microliters of media, depending on initial number of cells. At this point, the chip should have been equilibrating with the DPBS for several hours. A droplet of media is then placed over the inputs, to ensure no air is entrained during the seeding process. A port to load cells is chosen, and the others closed. The dense mixture of cells is loaded into a 10μL pipette tip, which is then inserted into the desired port. Using an off chip pump, suction is applied at the exit, at 0.5 μL/minute. The cells are pulled into the chip, and caught in the cells traps. Once an appropriate seeding density is established, pre-equilibrated media is loaded into similar tips, and inserted into the other ports. Then the cell port is closed, and the others opened, pulling fresh media over the cells. The tip with cells is replaced with media, and all the ports opened. Fresh media is allowed to flow over the cells for a few more minutes. Then the tips are filled up completely, the chip disconnected from the control lines, and placed in the incubator. The filled tips provide a slow gravity driven perfusion keeping fresh media flowing over the cells for up to ~16 hours if necessary.

After cells have become adherent in the chip, as low as 6 hours, but usually 24 hours later, they are ready to be assayed. The microscope culture chamber is heated, humidified, and the pre-mixed CO2 blend bled into the chamber. The chip is reconnected to the control lines, and the assay reagent loaded into the clean inputs (i.e., those not used for cell loading).
The Assay

The assay is a kit from Invitrogen, *Fluo-4 NW Calcium Assay Kits (F36205)*. To perform this assay on the chip, the reagents (Dye, Histamine, Buffer) were prepared as per the kit protocol. Dye was perfused over the desired cells for 30 minutes at 37 °C. Once the dye had been taken up, the fluorescent source was turned on, and the cells brought into the focus of the camera. The desired stimulation profile is then dynamically generated and driven over the cells. Imaging is done under FITC with an Apogee U2000 16 bit CCD camera, cooled to 0 °C, binned 2x2, giving a final resolution of 800x600 pixels. The camera drives a Sutter shutter, controlling fluorescent exposure. The light source is also passed through 6.25% neutral density filter to reduce negative photo effects on the cells and dye.

Device manufacture

The devices were made using multilayer soft lithography techniques [26]. Using top actuated valves, the fluid flow channels and thus cell culture areas were directly on glass.

Before casting, both molds were treated with trichloromethylsilane as a mold release agent.

The control mold was cast ~4mm thick with 5:1 base: hardener ratio PDMS (Sylgard 184). A thin layer of 20:1 PDMS was poured on the flow mold. Both molds were then
exposed to vacuum in a bell jar for about 5 minutes to degas the PDMS and remove bubbles that may have been trapped on the features. The flow mold was then spun at 2200 RPM to get an appropriate membrane thickness over the rounded channels. This mold was allowed to “rest” for ~30 minutes to allow non-uniformities from the spinning process to flatten out. Then control mold was then baked at 80°C for 15 minutes, and the flow for about 13 minutes (until just barely tacky). The control layer was then cut and demolded, and manually aligned with the flow layer. This was done for each chip on the mold, then the flow mold, now with control on top, was placed back in the 80°C oven for a minimum of 2 hours to allow the layers to bond and cure more fully. The devices were then removed from the mold and baked for several more hours. Port were punched with Harris Uni-core biopsy punches, 0.5mm for control lines and 0.75mm for flow lines. Cellophane tape was used to remove debris from devices, and which were then rinsed with acetone and ethanol sequentially, and blown dry. Glass slides were washed and dried in a similar manner. To bond the chips to the slides, a plasma etch treatment was used. The PDMS and glass slide were placed into the plasma chamber, with the surfaces to be bonded face up. The chamber was evacuated to ~600mTorr and then the plasma ignited for 30 seconds. Following this, and venting the chamber, the device was placed on the glass, and then baked again for at least 5 minutes to ensure a good bond. At this point devices were ready for experimental use.
**Mold Fabrication**

To create the molds for casting, photoresist was patterned on 75mm silicon wafers. High resolution transparency masks from CAD/Art Services [outputcity.com] were used to define the features. To create rounded resist suitable for valved channels, AZ-50XT was used. To create vertical sidewall channels, various SU-8 resists were used. Exact fabrication protocols are described below.

Wash the wafer with acetone, methanol, isopropanol, air dry.

Bake the wafer in 130C oven for ~5 minutes.

For rounded channels (able to be valved) taller than 10 μm, use AZ 50

For square channels, use appropriate SU-8 formulation (10 or 50, typically)

For AZ 50:

1. Wafer on coater

2. Place a few drops of adhesion promoter (HDMS) on wafer while spinning ~1k rpm.

Wait for it to dry. Stop coater.

3. Pour out a ~1" circle of resist on wafer.

4. Spin to get desired thickness (~45 seconds @ 2500 RPM for 30μm)

5. Hotplate @ 85C 2 minutes

6. Hotplate @ 115C for 10 minutes

7. Wait. 1 hr usually sufficient
8. Expose in 1 minute bursts on broadband, once for every ~7 microns in thickness, wait at least 10 seconds between exposures

9. Place in 1:3 diluted "400K" developer

10. When fully developed rinse with water

11. Place on plate at low temp, ramp slowly (one hour) to 120C to reflow.

12. If solvent resistance is required (subsequent su-8, eg) place in 130C oven (directly from plate) at least overnight.

13. Before going on to next step, test in non-feature area for solvent resistance with acetone on a q-tip

SU-8

1. Pour ~1" circle of resist on wafer.

2. Spin slowly ~500 rpm to spread resist

3. Spin at desired rpm for ~45 seconds (1500 rpm for 20μm)

4. Prebake as per specs

5. Expose ~1 minute/25 microns (can be continuous)

6. Postbake as per specs

7. Develop on coater with Proplyene Glycol Methyl Ether Acetate (PGMEA)

8. Rinse with isopropanol on coater while spinning at ~500 rpm

9. Inspect small features to ensure full development
These procedures were used serially to create molds with both rounded and square features. SU-8 was typically used for control molds, as it was more reliable and more robust.

**Data Analysis**

Data were collected as sequences of 800x600 (binned 2x2 from native resolution) 16 bit TIFFs from an Apogee U2000 cooled CCD camera. Imaging was typically performed though a 10x objective, with a 6.25% passage neutral density filter in place, and a FITC filter set.

Images were loaded into ImageJ [nih.gov], cells in the region of interested outlined, and brightness measured for each cell in each image.