

Cell Stimulus and Lysis in a Microfluidic Device with Segmented Gas–Liquid Flow

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We describe a microfluidic device with rapid stimulus and lysis of mammalian cells for resolving fast transient responses in cell signaling networks. The device uses segmented gas–liquid flow to enhance mixing and has integrated thermoelectric heaters and coolers to control the temperature during cell stimulus and lysis. Potential negative effects of segmented flow on cell responses are investigated in three different cell types, with no morphological changes and no activation of the cell stress-sensitive mitogen activated protein kinases observed. Jurkat E6-1 cells are stimulated in the device using α -CD3, and the resulting activations of ERK and JNK are presented for different time points. Stimulation of cells performed on chip results in pathway activation identical to that of conventionally treated cells under the same conditions.

The identities of many proteins involved in cell signaling in mammalian cells are known, as are the topologies of the signal transduction networks in which the proteins operate.^{1–6} However, quantitative data on the dynamics of signaling induced by different stimuli and in different cell types are scarce. Large sets of self-consistent and dynamic measures of protein activities, concentrations, and states of modification are essential for the construction of accurate models of biological networks. One reason for the dearth of such data sets is the cumbersome and complex nature of the experiments that are required. A typical process flow in these experiments starts with the addition of stimuli to cells (cytokines or growth factors) under controlled conditions of concentration, time, and temperature, followed at various intervals by cell lysis and the preparation of extracts. The lysis step is usually performed on ice using buffers that contain a variety of inhibitors so that further biomolecular activities are minimized during and after cell lysing. Analysis using quantitative immunoblotting,⁷ protein arrays,⁸ mass spectrometry,^{9,10} or other analytic

techniques then follows. Our overall aim is to achieve this process flow in microdevices and thereby expand greatly the number and reliability of experiments designed to collect information on signaling pathways. Here we describe approaches to the problem of stimulating and mixing cells in a microdevice. This microdevice has been designed so that it can be integrated in the future with microfabricated separators and detectors to miniaturize complete experimental protocols.

Some of the signaling events exhibit fast transient responses (15–45 s),¹¹ and analysis of these requires very short mixing times and well-controlled and reproducible stimulus conditions. These pathways can be difficult to reproducibly probe with conventional laboratory techniques, as even small fluctuations in the manual handling become significant at short times. Microfluidic systems offer the potential for reproducible and automated analysis with good control over experimental parameters like volume, concentration, and temperature. However, one of the challenges in microfluidic systems is to achieve sufficiently short mixing times. Mixing under the laminar flow conditions typically found in microfluidic devices occurs by diffusion, a relatively slow process for biological material and biochemical reactants. Another issue is the dispersion or residence time distribution (RTD) associated with parabolic flow profiles in laminar flow. Depending on position in the flow channel, differences exist in the retention time of the material flowing through the device, adding further uncertainty to the duration of stimulus in microfluidic devices.

Considerable effort has gone into improving mixing in microfluidic systems, including enhancing the diffusive mixing by lamination of liquid streams,^{12–14} introducing chaotic advection through patterned channel walls,^{15,16} or mixing through three-

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dimensional channel networks.¹⁷ The fabrication of these mixing schemes generally requires additional process steps, and working with whole cells ($\sim 10\text{--}20\ \mu\text{m}$ in diameter) limits the smallest dimension of the laminates that can be used. The challenges with mixing in microfluidic systems have also influenced the approach to cell lysis in microdevices. Where the typical biochemistry protocols involve chemical lysis agents, in microsystems a variety of lysis methods have been studied, including electrical fields,^{18,19} pure mechanical lysis,^{20,21} and a few devices using chemical lysis relying on diffusive mixing.²² In some devices relying on chemical lysis, mixing is enhanced by focusing the sample stream into a narrow stream flanked on both sides by lysis buffer, with the width of the streams determined by the flow ratio of sample to lysis buffer.²³ However, the need for large lysis buffer flow rates compared to sample flow rate results in significant dilution of the cell lysate, which is undesirable for many applications.

Air segmentation of liquid streams has long been used in continuous analysis devices to help separate samples, enhance mixing, and minimize dispersion.^{24–27} Recently, flow segmentation has also been used to achieve rapid mixing in microfluidic-based devices. Recirculation within the liquid segments drives mixing. Both immiscible liquid–liquid^{28,29} and gas–liquid³⁰ segmented flow devices have been developed. In addition to mixing enhancement, the reduced dispersion in segmented flow offers improved RTD³¹ compared to laminar flow and therefore improves exposure time control. However, none of the existing microfluidic-based devices are fully biocompatible due to the use of surfactants, which lowers the surface tension, or use of ethanol-based solvents.

In this paper, we present a microfluidic device that uses the enhanced convective mixing observed in segmented gas–liquid flow to obtain short mixing times and narrow RTD, making analysis of fast transient responses of cell signal pathways possible. By not relying on any surfactants to aid formation of the segmented flow, the device is completely biocompatible. It works directly with cell culture media and an inert gas. The device also utilizes a well-established chemical lysis protocol. Furthermore, the mixing of agents into the cell stream is done with a high ratio of cell stream to stimulus and lysis buffers, minimizing dilution

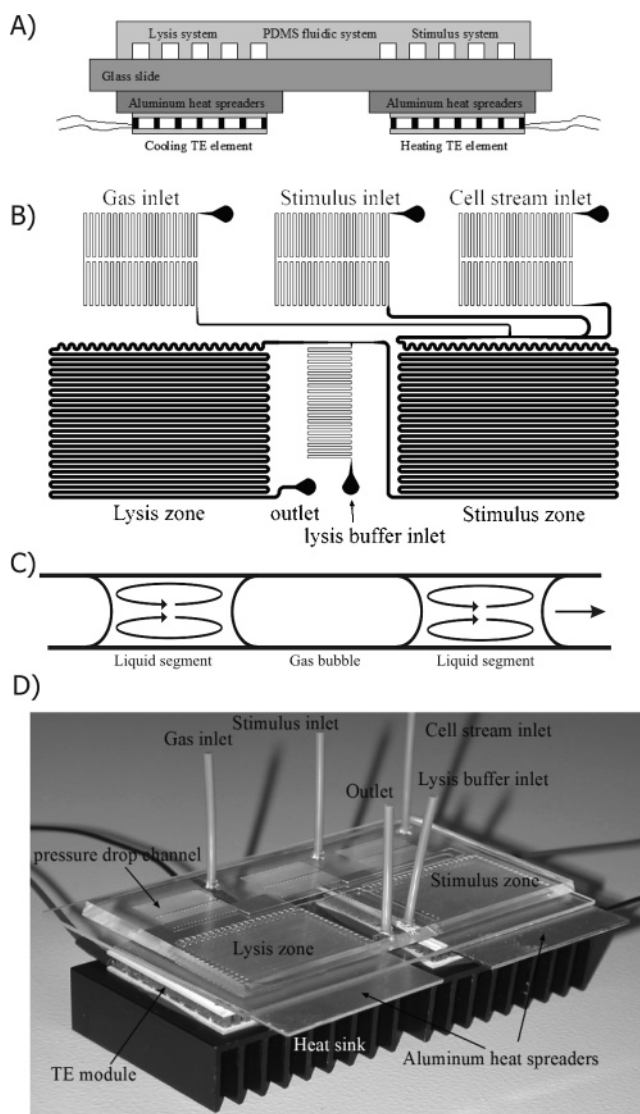


Figure 1. (A) Schematic of device with thermal control setup consisting of thermoelectric elements attached to the backside through aluminum heat spreaders. (B) Schematic of the fluidic system layout, with two main zones, one for cell stimulus and one for cell lysis. (C) Schematic of the recirculation in segmented flow that creates the enhanced convective mixing. (D) Photograph of device placed in the temperature control setup.

of the final cell lysate. Finally, the device integrates a thermal control setup for optimal performance during cell stimulus and lysis.

MATERIALS AND METHODS

Device Design. The device consists of a simple fluidic system defined in a poly(dimethylsiloxane) (PDMS) layer that is bonded to a glass slide. Schematics of the device and of the fluidic system design are shown in Figure 1A and B. All fluidic features are $300\ \mu\text{m}$ deep. There are three primary inlet ports, one for the cell stream, one for the stimulus, and a gas inlet for formation of the segmented gas–liquid flow. Pressure drop channels at each inlet help create sufficient pressure drops to minimize the periodic fluctuations associated with the creation of bubbles and thus ensure a stable formation of the flow segments. The pressure drop channels are $50\ \mu\text{m}$ wide, while the geometry where the gas inlet

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meets the cell and stimulus streams is 300 μm expanding gradually to 400 μm , the width used in the main channel system. The expansion helps in the formation of uniform and short slugs required for effective mixing by recirculation in the liquid segments (Figure 1C). The main channel system consists of two connected zones. Cell stimulus takes place in the first zone, and cell lysis is initiated in the second zone by introducing a lysis buffer via a secondary inlet located between the two zones. Each of the zones has a length of ~ 800 mm. The residence time of the sample in each zone can be adjusted by changing the flow rates, so that a single device design can be used for analyzing multiple stimulation time conditions. At typical slug velocities between 5 and 20 mm/s, the residence time in each of the zones can be adjusted from 40 to 160 s. Since both stimulus and lysis buffer are introduced only from one side of the channels, each of the zones starts with a meander structure that breaks the flow symmetry in the slugs so that mixing will progress across the centerline.^{28,30} The stimulus and lysis zones are spatially separated on the device so that independent temperature regulation of the two zones is possible. Temperature regulation for the two zones is achieved by using a thermal control setup consisting of a thermoelectric (TE) heater at 37 °C to mimic physiological conditions during stimulation and a TE cooler at 4 °C to inhibit further signaling during lysis (Figure 1A). Because of the low thermal mass and high surface-to-volume ratio in microfluidic systems, rapid sample cooling from stimulus temperature to lysis temperature is possible, resulting in increased experimental control and enhanced reproducibility.

Device Fabrication. The device was fabricated using soft lithographic techniques,³² by molding PDMS (Dow Corning Sylgard brand 184 silicone elastomer, Essex-Brownell Inc.) on masters fabricated from silicon wafers with SU8 photoresist (SU8-2050, Microchem Corp.). Two layers of 150- μm -thick SU-8 were spun onto 100-mm silicon wafers and baked. Photolithography was then used to define a negative image of the channel system in the resist. After a cross-linking bake, the wafers were developed using SU-8 developer (Microchem Corp.). PDMS was molded on the SU-8 master for 2 h at 70 °C, after which the device was peeled from the mold and cut to size, and inlet and outlet ports were punched into the material ($1/16$ -in. o.d.). The device was sealed by bonding the PDMS to precleaned glass slides (25 \times 75 mm, Corning) using an oxygen plasma (PDC-32G, Harrick Scientific) to activate both surfaces. Fluidic connections were achieved using PEEK tubing ($1/16$ -in. o.d., 250- μm i.d., Upchurch Scientific) inserted into the inlet and outlet ports and glued in place with 5-min epoxy (Devcon). Due to the simple fabrication scheme, the devices could be made as single-use devices that are disposed of after use in a biological assay.

Experimental Setup. The device was mounted into a thermal control setup to achieve the desired temperature regulation of the stimulus and lysis zones (Figure 1D). TE heaters and coolers (30 \times 30 mm, TE-127-1.0-2.5, TE Technology Inc.) were attached to the backside of the glass slide through aluminum heat spreaders (40 \times 32 \times 2 mm). Heat sinks were attached to the backsides of the TE modules. Temperature was regulated using proportional integral controllers (TC-24-10, TE Technology Inc.)

with temperature-sensing thermistors attached to the aluminum heat spreaders providing the temperature feedback for the controllers. Liquid delivery of cell sample, stimulus, and lysis buffer was done using syringe pumps (Cole Parmer 74900 series). Gas was delivered by a pressure-regulated nitrogen gas cylinder. The cell sample was always delivered in a ratio of 4:1 to the stimulus; thus, stimulus was delivered in a 5 \times concentration. Similarly, lysis buffer was delivered in a ratio of 1:4 to the total flow rate of cell stream plus stimulus and, thus, also had to be in a 5 \times concentration. Typical cell sample flow rates were from 25 to 100 $\mu\text{L}/\text{min}$. The input cell density in all experiments was $\sim 2 \times 10^6$ cells/mL corresponding to ~ 200 cells/slug, on average. A typical experiment typically collected 300–400 μL of sample, resulting in at least 6×10^5 cells collected for analysis per sample. Including flow stabilization time (3–5 min), each sample could be collected within 15 min of start of the experiments.

Fluidic Characterization. Characterization of slug length and slug velocity was performed using an inverted microscope setup (Axiovert 200, Zeiss) equipped with a high-speed camera (MF-046C, Allied Vision Technology GmbH). Slug length and slug speed were analyzed by capturing a series of images at specific time intervals. The known channel width was used to calibrate pixel count with slug length and velocity.

Mixing in the segmented flow was quantified in time-resolved measurements using a laser scanning confocal microscope (Zeiss LSM 510) with a 10 \times air objective, where the 488-nm argon line was used as the excitation wavelength. The extent of mixing was determined by tracking the spatial distribution of a tracer dye (Rhodamine B). The dye was introduced at one side of the microchannel, and its distribution was observed at different distances downstream of the injection point. Details on the measurement and data processing procedure are described elsewhere³⁰

Numerical Modeling. Numerical simulation using CFD-ACE (CFDRC Corp.) was used to aid the design of the device and estimate performance. 3D steady-state simulations of the thermal control setup were performed to make sure that the temperature distribution in the stimulus and lysis zones was as expected. Furthermore, 2D simulations of the segmented flow using volume of fluid (VOF), simulations combined with convective and diffusive species transport, were used to estimate mixing performance of the device. The effect of slug length and velocity on the mixing characteristics was modeled.

Cell Cultures and Treatments. Jurkat E6-1 human T-cell lymphoma, SKW 6.4 human B-cell lymphoma, and U937 human monocytes (all from ATCC) were used in this study. All cells were grown in RPMI medium 1640 with 25 mM HEPES (Catalog No. 224000089, InVitrogen) supplemented with 10% certified heat inactivated fetal bovine serum (Catalog No. 10082139, InVitrogen), penicillin (100 units/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and L-glutamine (2 mM) at 37 °C in a humidified 5% CO₂ incubator.

For cell signaling experiments, Jurkat E6-1 cells were treated with anti-CD3 (Catalog No. 555336, BD Bioscience) at a final concentration of 2 $\mu\text{g}/\text{mL}$. SKW 6.4 and U937 cell were treated with a combination of phorbol 12-myristate 13-acetate (PMA; Catalog No. 19-144, Upstate) and ionomycin (Sigma-Aldrich) at final concentrations of 25 ng/mL and 0.5 $\mu\text{g}/\text{mL}$, respectively.

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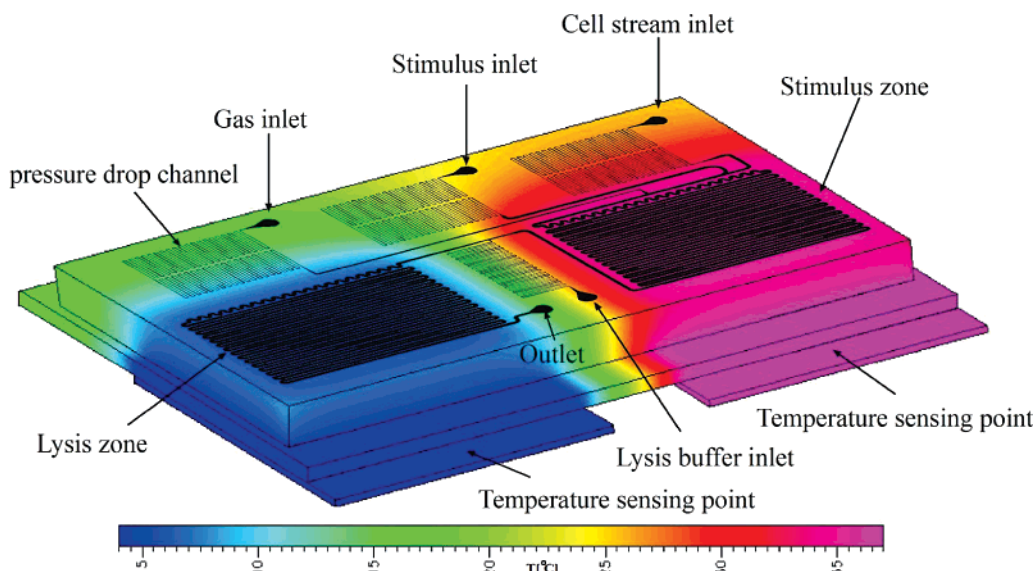


Figure 2. Three-dimensional finite element simulation of the temperature distribution in the device, showing good temperature uniformity in both stimulus and lysis zones.

Cell Viability Staining. Cell viability was determined using acridine orange (A-1301, Molecular Probes) as viable dye and ethidium bromide (E-1305, Molecular Probes) as a stain for dead cells. The dyes were used at a final concentration of 1 $\mu\text{g}/\text{mL}$ each. Analysis was performed using an inverted fluorescence microscope (Axiovert 200, Zeiss). The number of nonviable cells after a treatment was adjusted for the naturally occurring nonviable cells from the original cell culture by using eq 1.

$$\% \text{ nonviable cells} = \frac{\% \text{ nonviable}_{\text{treated}} - \% \text{ nonviable}_{\text{control}}}{100 - \% \text{ nonviable}_{\text{control}}} \times 100 \quad (1)$$

Cell Morphology Analysis. Cell fragmentation was analyzed by forward scatter signaling in a flow cytometer (FACSCalibur, BD Biosciences). Cells for flow cytometry analysis were fixed after treatment in 1% paraformaldehyde at 37 $^{\circ}\text{C}$ for 10 min and washed twice in wash buffer consisting of 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Positive control populations of cells with increasing degree of fragmentation were prepared by passing cell suspensions 5, 10, and 20 times through a 60-mm long needle with an inner diameter of 150 μm .

MAPK Signaling. Analysis of MAPK signaling was done using flow cytometry (FACSCalibur, BD Biosciences). Cells for flow cytometry analysis were fixed after treatment in 1% paraformaldehyde at 37 $^{\circ}\text{C}$ for 10 min and washed twice in wash buffer consisting of 0.1% BSA in PBS. The cells were permeabilized in 100% methanol and stored at -20 $^{\circ}\text{C}$ before further analysis. Analysis was performed using $\sim 10^5$ cells/assay. The cells were washed twice in the wash buffer before antibody staining. Rabbit polyclonal anti-phospho-ERK (Catalog No. 9101, Cell Signaling Technology), anti-phospho-JNK (Catalog No. 9251, Cell Signaling Technology), and anti-phospho-p38 (Catalog No. 9211, Cell Signaling Technology) were used as primary antibodies in final dilutions of 1:200, 1:25, and 1:25, respectively. The cells were incubated in 25 μL of wash buffer with the desired dilution of primary antibody

for 1 h. After incubation with the primary antibody, the cells were washed twice (0.05% Tween-20 was added to the wash buffer in the case of anti-phospho-ERK), then resuspended in 25 μL of 1:200 dilution of secondary antibody in wash buffer (Alexa 488-conjugated goat anti-rabbit IgG, Catalog No. A11008, Molecular Probes), and incubated for 1 h at room temperature. Finally, the cells were washed twice, resuspended in wash buffer, and transferred to flow cytometry tubes for analysis.

Immunoblots. Quantitative immunoblot assays of ERK and JNK activation were performed using cell lysates. The lysis buffer consisted (at final concentration) of the following: 1% Triton X-100, 150 mM NaCl, 10 mM β -glycerophosphate, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 10 mM NaF, 1 mM Na_3VO_4 , 10 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ pepstatin, and 10 $\mu\text{g}/\text{mL}$ chymostatin. The insoluble (pellet) and soluble (supernatant) fractions were isolated by centrifugation (10 min at 20000g). Immunoblots of phospho-ERK and phospho-JNK were performed using the same primary antibodies as for flow cytometry, but this time at 1:1000 and 1:500 dilutions, respectively. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. After blocking (30–60 min at room temperature), blots were probed overnight at 4 $^{\circ}\text{C}$ in primary antibody, washed 3×5 min in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20), incubated 1 h at room temperature in secondary antibody (1:5000 IRDye800-conjugated donkey anti-rabbit IgG, Rockland Immunochemicals), and finally washed 3×5 min in TBS-T. Blots were scanned on an Odyssey imaging system and quantitated using Odyssey software (Li-Cor Biosciences)

RESULTS AND DISCUSSION

Characterization of Flow and Thermal Properties. To ensure that the device met the necessary requirements for reproducible analysis of rapid cell signaling pathways, we performed modeling and device characterization. Temperature during both stimulus and lysis has to be controlled accurately. Figure 2 shows a three-dimensional (3D) thermal simulation of the temperature distribution in the device in which the layout of the fluidic system has been superimposed. There is good temperature

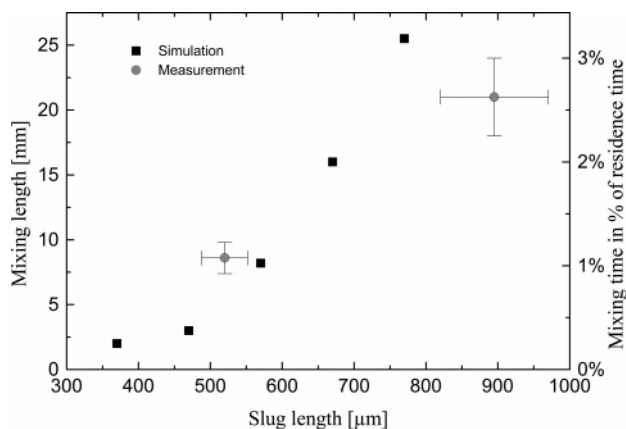


Figure 3. 2D VOF simulation of mixing in segmented flow showing the effect of the slug length on mixing efficiency. Included in the figure are experimental data for mixing in 520- and 950- μm -long slugs. The error bars denote one standard deviation.

uniformity in both the stimulus and lysis zones, and the temperatures at the points where thermistors provide feedback to the temperature controllers correspond well to the temperature in the respective zones. Heating and cooling both require ~ 0.4 W, which is easily obtained using standard single-stage TE elements. During experiments, the temperature in the stimulus zone typically fluctuated 0.1 – 0.2 $^{\circ}\text{C}$ around the set point (37 $^{\circ}\text{C}$), while the temperature in the lysis zone showed slightly higher fluctuations of 0.4 – 0.5 $^{\circ}\text{C}$ around the set point (4 $^{\circ}\text{C}$). Thus, adequate temperature control was achieved.

To achieve good time control in the system, it is necessary to have short mixing times compared to the total stimulus duration, which in our case is given by the residence time in the stimulus zone. Figure 3 shows a 2D VOF simulation of the effect of slug length on mixing length. The right y-axis in the figure gives the mixing time in percentage of the stimulus time that the mixing lengths correspond to for an 800-mm-long stimulus zone. Experimental mixing data are included for approximately 520- and 900- μm -long slugs. The experimental data were obtained by tracking the spatial distribution of an injected tracer dye (Rhodamine B) at different distances downstream of the injection point. Both simulation and experiments show that the mixing length increases rapidly with slug length. It is therefore important to produce short liquid slugs in order to achieve effective mixing. At the longer slug length, the experimental data show slightly faster mixing than predicted by simulation, which is to be expected since the simulations do not include mixing enhancements from small flow modifications arising from surface roughness and breakage of the thin liquid film (meniscus) at the side walls.³⁰ Using a gas pressure of 82.7kPa with total volumetric flow rates (sum of cell stream and stimulus volumetric flow rates) varying from 30 to 120 $\mu\text{L}/\text{min}$, the current design produces slug lengths from ~ 675 μm in the low-flow-rate range up to 800 μm for the high-flow rates. Therefore, we operated in a regime with mixing lengths well below 30 mm, resulting in mixing times that were less than $\sim 3\%$ of the total stimulus time. The slug velocity varies almost linearly from 5 mm/s to just over 20 mm/s in the given flow range corresponding to residence time in the stimulus zones varying from approximately 40 to 160s. Increasing the liquid flow rate and the gas pressure allows even higher slug velocities to be obtained,

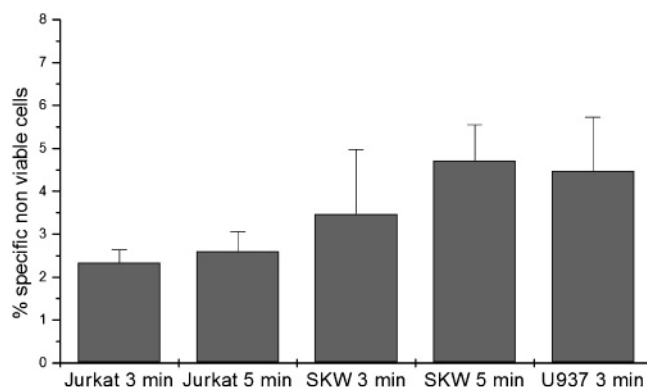


Figure 4. Viability tests for Jurkat E6-1, SKW 6.4, and U937 cells after segmented flow through device with either 3- or 5-min residence time. The error bars denote one standard deviation.

but it is probably better to achieve shorter residence times by decreasing the length of the zones.

Viability and Cell Stress Analysis. We were concerned that unstimulated cells flowing through the device would activate cell stress pathways, interfering with bioassays of interest. Our goal was to leave unstimulated cells unperturbed and stimulated cells as close as possible to cells undergoing conventional treatment for signaling pathway analysis. A series of viability and morphology tests were conducted, and special attention was devoted to activation of MAPK pathways as these are known to be influenced by cellular stresses.⁶ In normal operation of our device, cells are lysed after flow through the stimulus zone. However, to assay the potential negative effects of segmented flow on cell viability, morphology, and activation of stress-induced pathways, cells were flowed through the entire device (both stimulus zone and lysis zones) in the absence of stimulus and lysis buffers. The results therefore represent a worst case scenario of influence from segmented flow through the device on the behavior of the cells and also include the effect of all the cell handling, including loading of cells into syringes, flow through connection tubes, and collection of the cells at the outlet.

For viability testing, the cell stream was flowed through the device in the presence of acridine orange (viable cell stain) and ethidium bromide (nonviable cell stain) in tissue culture medium. No lysis buffer was introduced. Two flow conditions were chosen, resulting in 3- and 5-min total residence time in the system, corresponding to slug velocities of approximately 9 and 5 mm/s. Figure 4 shows the results for three different cell types, Jurkat E6-1, SKW 6.4, and U937. There was a slight increase in the number of ethidium bromide-positive cells after segmented flow through the system, but for all cell types and flow conditions, less than 5% of cells were affected.

Even though the viability testing did not show any significant increase of the number of dead cells, cell fragmentation could have occurred without being detected by viability stains. However, cell fragmentation can be detected by forward scatter signal in flow cytometry. Cells for flow cytometry were fixed in paraformaldehyde. To maximize the effect of shear stress on the cells, they were fixed at the outlet instead of in the device itself (by adding paraformaldehyde through the lysis buffer inlet). Typical forward scatter histograms for flow conditions with 5-min total residence time in the system are shown for all three cell types under

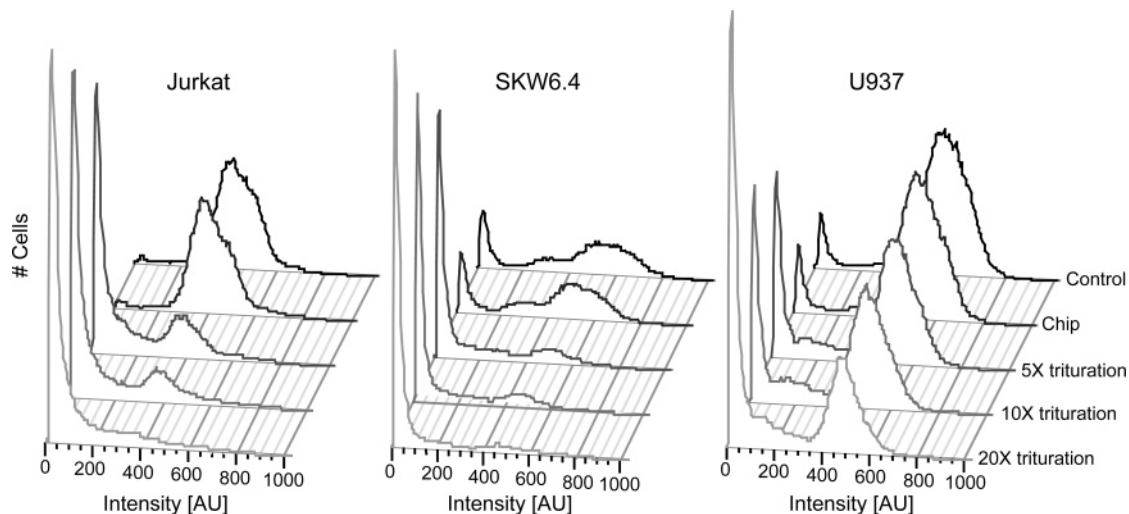


Figure 5. Forward scatter histograms for morphology analysis of Jurkat E6-1, SKW 6.4, and U937 cells. Included are histograms for cells subjected to segmented flow through the entire device (chip), histograms from control cell populations (control) and histograms for cells triturated by passing them 5, 10, and 20 times through a 60-mm-long 150- μ m-inner diameter needle to induce increasing degrees of cell fragmentation.

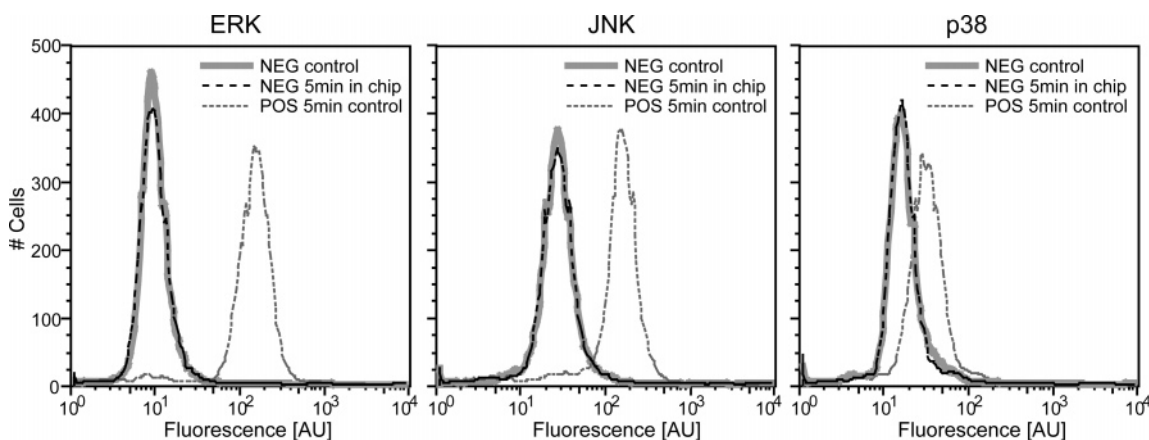


Figure 6. Histograms from flow cytometry analysis of ERK, JNK, and p38 pathway activation in Jurkat E6-1 cells subjected to either segmented flow (NEG 5-min chip), stimulation with α -CD3 (POS 5-min control), or no treatment (NEG control).

investigation in Figure 5. No stimulus was applied to the cells in these experiments. Included are histograms for negative control cell population fixed directly from the cell cultures as well as histograms from cell populations with increasing degrees of fragmentation due to trituration of the cells by passing them (5, 10 and 20 times) through a needle. The histograms within each cell type are virtually identical for the control population and the cell population flowed through the device, while the triturated populations show a decrease in the whole cell population and an increase of cell fragments (increased low-intensity scattering). This was also the case for cells flowed through the device with 3-min total residence time (data not shown). From these data we concluded that there are no detectable morphological changes in any of the cell types for the flow conditions tested here.

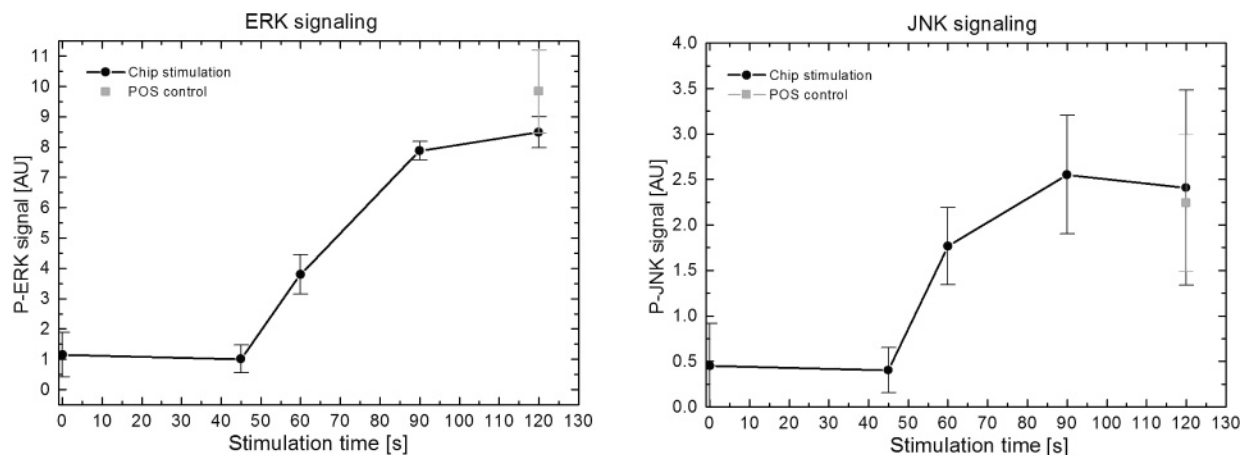
Next, we wanted to demonstrate that flowing cells through the device did not in itself trigger activation of signaling pathways in cells, an important consideration for the use of the device in the analysis of signaling events. We focused on the three main signaling pathways of MAP kinases, assaying ERK, JNK, and p38 phosphorylation. Both JNK and p38 are known to respond to cellular stresses; JNK is also known as the stress-activated protein kinase.⁶ Cells were flowed through the entire device before being

fixed at the outlet after 3- and 5-min residence time in the system and then stained for the phosphorylated forms of ERK, JNK, and p38. Figure 6 shows typical flow cytometry histograms of untreated Jurkat E6-1 (human T-cell lymphoma) from a control population as well as cells after 5 min in the segmented flow device. These two populations show nearly identical distribution profiles. As a positive control for cell activation, cells were treated with anti-CD3 in a conventional cell culture dish, a stimulus of the T-cell receptor pathway that induces activation of all three MAP kinases in Jurkat E6-1 cells. Anti-CD3-induced JNK, p38, and ERK activation was clearly distinguishable from the unstimulated state of untreated cells or cells flowed through our device. Similar results were obtained in SKW 6.4 (human B lymphocytes) and U937 (human monocytes), as summarized in Table 1. In these last two cases, a mix of PMA and ionomycin was used as a stimulus to generate the positive control cells. In all three cases, there was no significant activation of the three MAP kinase pathways after segmented flow through the device under fast-flow (3-min residence time) or slow-flow (5-min residence time) conditions (Table 1). Finally, anti-CD3 antibodies were introduced through the stimulus inlet to stimulate Jurkat E6-1 cells in the device, and the cells were collected and fixed after 5-min residence

Table 1. Geometric Means of Fluorescence Intensities from Flow Cytometry Analysis of ERK, JNK, and p38 Activation in Jurkat E6-1, SKW 6.4, and U937 Cells Subjected to Various Treatments^a

	Jurkat E6-1			SKW 6.4			U937		
	ERK	JNK	P38	ERK	JNK	P38	ERK	JNK	P38
5-min unstimul chip	9.7 ± 0.6	26.5 ± 0.6	18.2 ± 1.4	12.0 ± 1.1	24.7 ± 2.1	20.4 ± 0.6	7.5 ± 0.2	13.2 ± 0.1	14.0 ± 0.9
3-min unstimul chip	9.6 ± 0.5	28.0 ± 1.2	18.2 ± 1.6	12.9 ± 0.2	26.5 ± 0.9	20.2 ± 1.4	7.2 ^b	14.0 ^b	13.1 ^b
negative control	8.9 ± 0.6	26.0 ± 2.8	17.9 ± 2.9	10.9 ± 0.8	22.6 ± 0.7	17.8 ± 1.2	7.3 ± 0.7	11.7 ± 0.5	13.5 ± 0.3
5-min anti-CD3 chip	120 ± 19	119 ± 17	30.9 ± 4.7						
5-min positive control	129 ± 8	124 ± 10	28.5 ± 2.9	98.3 ± 6.4	110 ± 4.6	49.5 ± 2.4	81.8 ± 2.4	75.1 ± 2.3	23.3 ± 1.1

^a All data are the result of triplicate measurements unless otherwise noted. ^b Single measurement.

**Figure 7.** ERK and JNK signaling in Jurkat E6-1 cells stimulated with α -CD3 for different times. Stimulation and cell lysis was performed with the microfluidic device (chip stimulation) and with conventional methods (POS control). The error bars denote one standard deviation.

time. The flow cytometry profiles for phospho-ERK, phospho-JNK, and phospho-p38 were similar to those of cells treated for 5 min in a conventional cell culture dish (geometric means of the distributions in fluorescence intensities are shown in Table 1). This similarity demonstrates that results of signaling pathway analysis obtained using this device are comparable to those obtained by conventional methods.

Rapid Stimulation and Lysis of Jurkat Cells. Having established that segmented flow through devices does not appear to activate signaling pathways artificially, short stimulations of Jurkat E6-1 cells were performed within the devices followed by integrated cell lysis. The stimulation zone was kept at 37 °C while the lysis zone was kept at 4 °C using the thermal control setup. Cells were stimulated with anti-CD3 in the device using flow conditions that gave 45-, 60-, 90-, or 120-s residence time in the stimulus zone. Cells were lysed by introducing a lysis buffer that contained a variety of inhibitors to minimize further signaling following lysis. Quantitative immunoblotting was used to analyze the ERK and JNK signaling. Figure 7 shows the results from triplicate experiments at each time point. The phospho-ERK signal starts to increase after 45 s of stimulation and begins to level off at ~8-fold activation after ~90 s. A similar time delay in Ca²⁺ response after T-cell receptor activation has been observed previously.³³ The fast ERK response is resolved with relatively small variations between experiments performed with the same stimulation conditions showing the reproducibility of the stimulation and effectiveness of the integrated lysis in the microfluidic

device. This level of time control can only be achieved with rapid lysing of the cells so the inhibitors can quickly stop the signaling events. Viability dye analysis showed that there were no remaining viable cells, when cells were mixed with lysis buffer in the device (data not shown). As a further test of the effectiveness of the cell lysis, the insoluble fractions of the lysates were analyzed separately. Triton-insoluble fractions generally include most of the nuclear materials, while the cytoplasmic signaling proteins should be soluble. Less than 5% of the activated ERK was found in the insoluble fraction in each of the lysates, showing that lysis was complete (data not shown). The phospho-JNK signaling shows the same trend as the phospho-ERK signal, leveling off at ~5-fold activation after 90 s, albeit with larger variations between the triplicate experiments. This was due in part to the distortion of the electrophoretic separation of JNK from the large amount of BSA the cell lysate and in part because the phospho-JNK signal was weaker, i.e., a feature of the immunoblot assay, not the device. Changing to serum-free media would significantly reduce the amount of BSA although it might also change the response of the cells. Another alternative is to perform analysis using protein arrays⁸ instead of immunoblots as these should be less sensitive to the presence of BSA in the lysates. Figure 7 also includes the results of conventional stimulation and lysis of Jurkat cells with 120-s stimulation time. In our opinion, it is not possible to achieve reproducible stimulation times of <120 s using conventional methods. As with the flow cytometry experiments, we see comparable activation of ERK and JNK for stimulations performed in the device and those performed using conventional methods under the same conditions.

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CONCLUSIONS

We have presented results for a new microfluidic device that uses enhanced convective mixing in segmented gas–liquid flow to achieve sufficiently short mixing times to resolve fast transient responses in cell signaling pathways. A thermal control setup for optimal performance during stimulus and lysis was integrated with the device. We found no effects on cell behavior from the use of segmented flow, including no cellular stress-related activation of the ERK, JNK, and p38 pathways in three different cell types. Stimulation of cells performed in the device resulted in pathway activation comparable to that using conventional methods, and the good time control in the device resulted in reproducible analysis of fast transient responses in the cell signaling pathways. One interesting biological conclusion from these studies is that a time delay of ~45 s appears to exist between T-cell receptor

activation and activation of both ERK and JNK kinases. Presumably this reflects the minimum time required to assemble signaling complexes.

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