A comprehensive, systems level understanding of cell signaling networks requires methods to efficiently assay multiple signaling species, at the level of single cells, responding to a variety of stimulation protocols. Here we describe a microfluidic device that enables quantitative interrogation of signaling networks in thousands of individual cells using immunofluorescence-based readouts. The device is especially useful for measuring the signaling activity of kinases, transcription factors, and/or target genes in a high throughput, high content manner. We demonstrate how the device may be used to measure detailed time courses of signaling responses to one or more soluble stimuli and/or chemical inhibitors as well as responses to a complex temporal pattern of multiple stimuli. Furthermore we show how the throughput and resolution of the device may be exploited in investigating the differences, if any, of signaling at the level of a single cell versus at the level of the population. In particular, we show that NF-κB activity dynamics in individual cells are not asynchronous and instead resemble the dynamics of the population average in contrast to studies of cells overexpressing p65-EGFP. Molecular & Cellular Proteomics 8: 433–442, 2009.

Specific intracellular signaling responses are the result of precisely regulated activation of multiple proteins and protein complexes occurring dynamically in space and time. A detailed understanding of the outcome of signaling responses commonly requires multiple experimental perturbations of the underlying biomolecular network by using receptor ligands followed by measurements of the activities of key kinases and transcription factors (1–4). Traditionally inferences about the network structure and function are made based on cell population analyses, e.g. through measurements of protein amount and activity status by immunoblotting, but the results might be misleading because of averaging out and masking of distinct responses by individual cells (5–8). Thus, single cell-based assays are becoming more common, including tracking of live cell probes or analysis of fixed and stained cells by flow cytometry and high content cell screening (HCCS)1.

Flow cytometry is a high throughput alternative technique that does not require genetic manipulation associated with using fluorescent protein tags. There are many examples of its use in signal transduction research, including network reconstruction in lymphocytes (4), phosphoprotein profiling of hematopoietic cancers (15), and identification of pathway-selective inhibitors (16). Unfortunately the method is not well suited for adherent cells because cell detachment by mechanical force or chemical agents, such as trypsin, often destroys cell integrity and may unintentionally trigger signaling pathways (17, 18). Critically flow cytometry lacks subcellular resolution and cannot, for example, measure nuclear translocation of a transcription factor that is a key event in many signaling pathways.

Immunocytochemistry is a technique that can report on molecular localization in many adherent wild type cells with subcellular detail and is therefore well suited for studies of signaling on a single cell level. The throughput of this technique can be increased through automatic imaging afforded by HCCS, which has allowed investigation of the effects of small molecule inhibitors and RNA interference on cell morphology, cell cycle transit, and other cellular phenotypes (19–22). An important consideration for the multiwell assays used in HCCS is ensuring uniform treatment of each well; this is

1 The abbreviations used are: HCCS, high content cell screening; EGFP, enhanced green fluorescent protein; NF-κB, nuclear factor-κB; PDMS, polydimethylsiloxane; TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; CV, coefficient of variation; IKK, IκB kinase; JNK, c-Jun NH2-terminal kinase; Erk, extracellular signal-regulated kinase.
often precluded by variations in the volume of liquid dispensed into each well (23). The resulting variability in the concentration of applied reagents hinders fair and quantitative comparisons and limits the ability of HCCS to resolve small differences in cell signaling responses. This issue is exacerbated in more complex protocols, such as sequential exposure of cells to different media, because of errors that accumulate when changing media. Moreover repeated media aspirations might unintentionally remove cells from the wells (24). Because these assays are also difficult to miniaturize (25), HCCS experiments may consume large quantities of expensive or valuable cells and reagents.

In principle, the precise chemical delivery capabilities of a microfluidic device can overcome some of the limitations associated with conventional HCCS. Laminar flow inside microfluidic channels allows one fluid to be completely displaced by another, allowing much more precise and consistent sample treatment compared with that achieved in multiwell plates (26), leading to more uniform cell responses (27). Shear forces can be minimized by modulating device geometry and flow rates, thus preventing cells from being stressed or accidentally removed when exchanging liquids. Fluid volumes are on the order of nanoliters so that an entire microfluidic assay can be performed with less reagent volume than might be needed for a single well experiment. Additionally, microfluidic assays can be easily automated, leading to increased throughput.

To date, microfluidic devices have been used to culture a variety of adherent mammalian cell types, such as neurons (28), chemotactic neutrophils (29), hepatocytes (30), and myoblasts (31). Additionally, high throughput microfluidic assays have been described for studying cell proliferation (32), real-time gene expression (33), and stem cell differentiation (34). However, there is surprisingly little work combining high content cell screening and microfluidics, especially in a way advantageous for studying signaling pathways and dynamics (35).

In this report, we describe a device that harnesses the advantages of microfluidics to enable precise, high-throughput immunofluorescence analysis of signaling dynamics in single cells. We demonstrate how the device can be used to obtain high content measurements of various kinases and transcription factors in response to perturbations by cytokines, growth factors, and small molecule inhibitors. As an example of one application of the device, we also show how the device may be used to investigate single cell versus population level responses, such as in the nuclear factor-κB (NF-κB) signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Device Fabrication**—The dual layer monolithic polydimethylsiloxane (PDMS) device was fabricated using standard soft photolithography protocols. Briefly, the upper valve layer mold was fabricated by spin coating SU8-2025 (Microchem, Newton, MA) onto a 3-inch-diameter silicon wafer (Montco Silicon Technologies, Spring City, PA) at 1500 rpm for 70 s. The wafer was then soft baked at 95 °C for 2 h, exposed to ~1.1 J/cm² broadband UV light using a high resolution transparency mask (In Tandem Design, Towson, MD), hard baked at 95 °C for 1 h, developed in SU-8 developer (Microchem), and finally baked at 200 °C for 30 min. The lower fluidic layer mold was fabricated by spin coating SPR 220-7.0 (Shipley, Marlborough, MA) at 730 rpm for 30 s onto a 3-inch-diameter silicon wafer precoated with hexamethyldisilazane (Sigma), then soft baked at 115 °C for 8 min, exposed to ~0.6 J/cm² broadband UV light using a high resolution transparency mask (In Tandem Design), developed in AZ400K developer (Clariant, Charlotte, NC) diluted 4-fold in distilled water, and then rounded by baking at 125 °C for 15 min. Both molds were coated with trimethylchlorosilane vapor for 30 min, then 10:0.5 g parts A:B of RTV615 (GE Silicones, Wilton, CT) was spin-coated onto the fluidic layer mold at 1600 rpm for 60 s, and 35 g:7 g RTV615 was poured onto the valve layer mold. The molds were baked at 80 °C for 45 min, and then the control layer mold was cut, hole-punched, cleaned, and aligned onto the fluidic layer mold. The layers were cured to each other at 95 °C for at least 90 min, and the final device was cut, hole-punched, cleaned, and reversibly bonded to clean number 1 coverglasses (Fisher) by baking at 80 °C overnight. Note that the control layer mask was enlarged by 1.6% to account for thermal shrinkage of the PDMS due to cooling after the initial bake, a common adjustment in the fabrication of multilayer PDMS devices. The final chambers were approximately parabolic in cross-section with 30-μm height and 250-μm width.

**Cell Culture**—NIH-3T3 cells (a gift from Dr. Jin Zhang, Department of Pharmacology, The Johns Hopkins University) were maintained in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% calf bovine serum (ATCC, Manassas, VA), 100 units/ml penicillin (Invitrogen), and 100 units/ml streptomycin (Invitrogen). A-549 cells (ATCC) were maintained in Ham’s F-12 medium (Invitrogen) with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were serum-starved in low serum medium (0.1% fetal bovine serum) for at least 16 h before each experiment to induce quiescence. Cells were stimulated with tumor necrosis factor (TNF) (Roche Applied Science) or platelet-derived growth factor (PDGF)-BB (Sigma) at the indicated concentrations in low serum medium.

**Device Operation**—Devices were flushed with 0.1% gelatin for 30 min to coat the glass substrate, and then the gelatin was flushed away with low serum medium. Serum-starved cells were seeded at a concentration of 9 × 10^4 cells/ml and then allowed to attach for at least 4 h. Control channels were slowly filled with water at 5 p.s.i. to prevent air from being forced from the control channel into the test chambers when valves are actuated at higher pressure during normal operation. Valve actuation was controlled by a set of miniature three-way solenoid valves (The Lee Co., Westbrook, CT) that toggled between 0 and 20 p.s.i. pressure sources. The sequence of valve actuation was controlled by custom C++ programs connected to the valves via a digital input/output computer card (PCI-6517, National Instruments, Austin, TX). Experiments were typically run unattended.

**Multiwell Assay**—A glass bottom 96-well plate (Mattec Corp., Ashland, MA) was coated with 0.1% gelatin for 30 min and then rinsed with low serum medium. Serum-starved NIH-3T3 cells were seeded in each well and allowed to attach for 4 h. Culture medium was manually aspirated and replaced with TNF-containing medium for the indicated concentration and times. Cell fixation and immunocytochemistry followed the same protocol as for microfluidic devices as described below.

**Antibodies**—Primary antibodies against p65, c-Rel, phospho-ATF-2, phospho-c-Jun, c-Fos, Fra-1, phospho-Akt1/2, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against phospho-Erk1/2 and phospho-Akt1/2 were from Upstate
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(Charsottesville, VA). Secondary antibodies for immunocytochemistry and flow cytometry were Alexa Fluor 488 or 594 conjugated to highly cross-absorbed goat anti-rabbit IgG or goat anti-mouse IgG (Invitrogen). Secondary antibodies for Western blot were Alexa Fluor 680-conjugated goat anti-rabbit IgG (Invitrogen) and IR Dye B00-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA).

Immunocytochemistry—Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min, permeabilized in 0.1% Triton X-100 (Sigma) for 5 min, blocked in 10% goat serum (Invitrogen) for 60 min, stained with primary antibody solution (1:100 antibody + 2 μg/ml Hoechst 33258 (Sigma) in 10% goat serum) for 60 min, and then stained with secondary antibody solution (1:200 secondary antibody + 1:40 Texas Red-conjugated phalloidin (Invitrogen)) for 60 min with PBS washes in between each step. All solutions were made in PBS buffer. For multiwell assays, fluid exchange was performed by manual aspiration, and for microfluidic devices, positive or negative hydrostatic pressure was used to push or suck staining reagents into the device within 1 min. Multiwell plates and devices were imaged under non-saturating conditions on a motorized Zeiss Axiovert 200M epifluorescence microscope using Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO).

Western Blot—Nuclear extracts were made using the NE-PER reagent according to the manufacturer’s recommendations (Pierce). Protein concentrations were measured by BCA assay (Pierce), and equal amounts of denatured protein from each sample were loaded onto a 4–20% polyacrylamide gradient gel (Pierce). Proteins were transferred to a nitrocellulose membrane, blocked with 1:1 Odyssey blocking buffer:PBS for 1 h, stained with primary and secondary antibodies for 1 h each, and then visualized on an Odyssey infrared scanner (Licor, Lincoln, NE).

Flow Cytometry—NIH-3T3 cells were starved in 0.1% serum medium overnight and then stimulated as indicated in the figure legends. Cells were then trypsinized, spun down, resuspended in 2% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, and then kept in storage solution (1% BSA in PBS) until staining. Cells were washed at least once with PBS in between each step. On the day of flow cytometry (FACSDiv, BD Biosciences), cells were incubated in the appropriate primary and secondary staining solution for 30 min each with washes in between and then resuspended in storage solution at 1 × 10^6 cells/ml.

Image Analysis—Custom MATLAB (Mathworks, Natick, MA) programs were written to process and analyze images. Images were flat field- and dark field-corrected (36) and then stitched together to form a composite image used for all subsequent analysis. Nuclear regions were segmented by thresholding a Hoechst image using Otsu’s method, and candidate nuclei were filtered for size (for example, to eliminate overlapping cells). The pixel-averaged immunocytochemical signal in each segmented nucleus was then computed. In all time courses shown, the median is plotted to lessen the effects of outliers due to possible image artifacts. Error bars are the approximate S.E., specifically the 68% confidence interval of the median. For spatial analysis, the location of each cell was taken as the centroid of the nuclear region. Copies of programs are available upon request.

RESULTS

The microfluidic device is a compact monolithic two-layer PDMS chip reversibly sealed to a glass coverslip (Fig. 1A). The bottom layer contains an array of chambers for cell experimentation connected by a distribution tree to inlets that provide access to desired reagents (Fig. 1B). Fluid flow in the device is gated by pushdown valves in the top layer (37). Some valves control fluid flow entering through a single inlet or leaving through an outlet, whereas other valves are arranged in a multiplexed pattern (38), allowing multiple subsets of chambers to be addressed using relatively few valves. At the beginning of a typical experiment, the glass coverslip substrate is coated with gelatin (or other matrix of choice, including that composed of various extracellular matrix proteins), and cells are seeded into the chambers and allowed to attach and spread. Next cell media containing stimulation agents are delivered in various temporal patterns as defined by valves actuated by a computer-controlled pressure source. Following stimulation, cells are fixed, permeabilized, and stained in situ by introducing the appropriate fluids in sequence, and then the device is imaged on an automated, motorized microscope and analyzed using custom designed software (see “Experimental Procedures”). The glass coverslip with affixed cells can be removed and preserved, and the device can be cleaned and reused. For brevity, henceforth we shall refer to the device as Imstain, a shorthand for automated in-chip immunostaining.

Imstain can be operated in various basic modes that can be activated in sequence so that its single device design suffices for a multitude of concurrent or sequential experiments. In the first mode, a specific fluid can be selected by closing the valves for all other inlets and delivering to a particular chamber by actuating a subset of the multiplexed valves (Fig. 1C, top panel). In the second mode, all fluid flow is stopped by closing valves for all inlets and valves at both ends of every experimental chamber to incubate cells in the newly introduced medium in the absence of flow and shear stress (Fig. 1C, second panel). A time course of the response to a single reagent (e.g., as in Fig. 2B; discussed below) can be performed by using mode 1 to introduce the reagent to all test chambers one by one with intermediate pauses in mode 2 for the desired time interval. In the third mode of operation, the dead volume between the inlets and test chambers can be flushed through “sacrificial” chambers to bring a new fluid to the entrance of the test chambers in preparation for delivery (Fig. 1C, third panel). A time course of the response to multiple reagents (e.g., as in Fig. 4B; discussed below) can be performed by repeated cycling between modes 3, 1, and 2 using modes 3 and 1 to flush and deliver a specific fluid to a particular test chamber with intervening pauses in mode 2 for desired time intervals. As an initial test, we performed such cyclic switching between modes to alternately deliver dye and water to a single test chamber. We found that this led to a complete and reproducible exchange of the fluid inside the test chamber (Fig. 1D). In the fourth mode, a subset of valves can be actuated to divide the chambers into four fluidically isolated groups, each with its own inlet and outlet (Fig. 1, B and C, bottom panel). By also actuating a subset of multiplexed valves to address multiple test chambers at the same time, this mode can be used to determine the time course of one signaling readout responding to up to four different stimuli in parallel (e.g., as in Fig. 3E; discussed below). Alternately multiple signaling readouts responding to the same stimulus...
can be measured by performing four identical time courses in parallel and then staining each group with a different antibody (e.g. as in Fig. 3A; discussed below).

There are several important features that are crucial to the proper function of this device. The sacrificial chambers that flank the top and bottom of each group of test chambers not only allow dead volume to be flushed through the device but also ensure an even distribution of cells to the test chambers by diverting the liquid close to the walls of the inlet from which cells are commonly excluded (39). Another important feature is a valve at the end of each test chamber that prevents cells from being exposed to stimuli that might otherwise diffuse backward into the chamber. Finally fluidic resistances between inlets and chambers are low and equalized, allowing for
uniform fluid exchange within seconds in each test chamber (Fig. 1E). The brief but rapid fluid flow allows multiple complete exchanges of fluid over cells in the test chamber without exposing them to prolonged and potentially deleterious episodes of shear stress, and we found no morphological signs of stress or loss of cells even after multiple fluid exchanges (supplemental Fig. 1).

These features are designed to increase the consistency of responses from single cells uniformly treated with a signaling stimulus. To demonstrate this, we contrasted the use of Imstain versus a 96-well glass bottom plate to stimulate mouse NIH-3T3 fibroblasts with 10 ng/ml TNFα for 0, 4, 8, or 12 min. The cells were then stained for an NF-κB subunit, p65, which translocates to the nucleus following TNFα stimulation (40). Nuclear p65 concentrations were quantified in each cell and then averaged for each time point. Each time point was repeated eight times in the same device or plate from which a coefficient of variation (CV; standard deviation of the replicates divided by their mean) was computed. We found that CV values for experiments performed in Imstain were 5% or less, which was 2–5 times lower than in wells (Fig. 1F). Furthermore examining distributions of nuclear p65 obtained in

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**Fig. 2. Detailed signaling time course measured in a single device.** A, composite image and zoomed views of a single device used to measure the time course in B. Each row is a separate test chamber used to obtain data for a stimulation time point. Stains for p65 (antibody; green), actin (phalloidin; red), and DNA (Hoechst 33258; blue) are shown. Scale bars are 500, 50, and 20 μm from top to bottom. B, a detailed time course of nuclear NF-κB activity in response to 10 ng/ml TNFα in NIH-3T3 mouse fibroblasts showing biphasic response dynamics. The plot shows median activity with error bars indicating the S.E. Note the non-linear timescale. C, Western blot of nuclear p65 levels in response to 10 ng/ml TNFα in NIH-3T3 cells with p53 nuclear loading control; the graph shows the quantified band intensities.
Imstain revealed no systematic differences in measurements taken from different chambers of the device (supplemental Fig. 2). We also found that unintentional cell aspiration in the wells, which is common in procedures with multiple washing steps, resulted in cell density variations that were more than 60% larger than in Imstain (Fig. 1G). Note that the comparison was performed on the same day using the same source of cells seeded at identical confluency, stimulated and stained with the same reagent mixtures, imaged on the same day on the same microscope, and background-corrected and analyzed in identical ways to minimize contributions to the CV by these aspects. Thus, this experiment shows that measurements performed in Imstain are significantly more consistent than in a traditional multiwell assay.

To illustrate that Imstain can be used to monitor signaling events at arbitrary temporal resolution following stimulation onset, we measured the response of mouse NIH-3T3 fibroblasts exposed to 10 ng/ml TNFα sampled at unevenly spaced time points up to 6 h. The resulting data set is shown in the composite image in Fig. 3A and contains about 200 cells per time point for a total of over 6,600 cells. Nuclear concentrations of p65 were quantified in each cell, and the average single cell response (Fig. 2B) showed the expected biphasic behavior with a peak at ~30 min, trough at ~60 min, and partial recovery thereafter (41, 42). The automatic data analysis allowed one to re-examine individual cells that might possess distinctive properties one may find of interest, e.g. to inspect cells with unusually high p65 activity. We re-examined cells whose p65 activity was three or more standard deviations above the mean, corresponding to 1% of the total population. Some of these cells displayed apoptotic morphology with artifically bright fluorescence, and the other cells did not have any obvious distinguishing morphological features suggesting that their high p65 activities are part of normal variation about the mean. The response on the cell population level was consistent with the results of the Western blot of p65 in the nuclear extracts (Fig. 3C). We could not perform the equivalent flow cytometry experiment as it could not resolve the nuclear translocation of p65.
we performed an experiment in which the activity of four TNFα-responsive transcription factors (46) (Fig. 3D) was monitored in response to TNFα in the presence or absence of SC-514, a putative selective IKK inhibitor (47), and then monitored the dynamic expression and localization of the NF-κB subunits p65 and c-Rel and the AP-1 transcription factor subunits ATF-2 and c-Jun. This experiment required performing eight time courses for the four transcription factors with or without SC-514. To perform this simultaneously in one device, we divided the chambers into four fluidically isolated groups (mode 4; as defined above). In two of the four groups cells were stimulated with TNFα alone, and in the other two groups cells were preincubated with 100 μM SC-514 and then stimulated with TNFα in the continued presence of SC-514. Each group of chambers was then stained either for p65 and ATF-2 simultaneously or c-Rel and c-Jun simultaneously using species-specific secondary antibodies. Throughout the time course, we found that nuclear translocation of NF-κB subunits directly activated by IKK (Fig. 3D) was incompletely inhibited in the presence of SC-514 (Fig. 3E). This correlates with a previous report that, at individual time points, SC-514 partially reduces p65 translocation induced by interleukin-1β and incompletely blocks NF-κB-mediated transcription (47). The AP-1 subunits, which are believed to respond to TNFα in an IKK-independent manner, were also partially suppressed by SC-514 (Fig. 3E). This finding suggests that SC-514 may cross-inhibit JNK in vivo, that IKK might cross-activate AP-1 through an as yet uncharacterized JNK-independent mechanism (48), or that depressed NF-κB activity leads to decreased TNFα production and paracrine signaling and hence lower TNFα-dependent AP-1 activity. More generally, this experiment shows how Imstain may be used to investigate the temporal response of multiple signaling proteins in response to multiple stimuli in pairwise combinations.

Moreover, Imstain may be used to study signaling responses to multiple stimuli presented sequentially to cells. To demonstrate this, we used Imstain to expose cells to four 10-min TNFα pulses repeated every 100 min with intervening washout periods and measured NF-κB dynamics every 12 min for over 6 h. In this experiment, assigning time points to the test chambers in chronological order would cause conflicts in which two chambers that cannot be addressed simultaneously need to be flushed with the same liquid at the same time, but such conflicts can be easily avoided by assigning the time points in a different order (Fig. 4A). We found that NF-κB activity returned nearly to baseline after each pulse of TNFα (Fig. 4B). Interestingly NF-κB responded rapidly to every pulse of TNFα but to a lesser degree each time (Fig. 4B); this may reflect poststimulus down-regulation of the TNF receptor (49). Notably this multiple stimulus experiment would be cumbersome to perform by hand in a multwell format as it would require dozens of rapid washes and fluid manipulations, and complete removal of each stimulus would be difficult to ensure. In Imstain, however, the experiment is automated and can be run unattended, and microfluidic flow ensures complete fluid exchange.

Finally to demonstrate the utility of the single cell resolution provided by our device, we addressed the still unresolved question of whether the kinetics of NF-κB activation in single cells are similar to those of the population average (12). Experiments in cells overexpressing p65-EGFP have suggested that p65 activity spikes periodically over time with the timing and frequency varying from cell to cell (50); this is distinctly different from the biphase response of the population average (42). As Imstain can measure distributions of single cell responses at specific time points, we considered whether the distribution of nuclear p65 levels observed in Imstain would be consistent with cells displaying desynchronized spiky p65 activity. In p65-EGFP tracking experiments, cells show a stereotypical initial spike of p65 activity, peaking about 30 min after TNFα stimulation (50). Likewise we observed that the distributions of p65 activity at 0 and 30 min were essentially different from the biphasic response of the population average (42). As Imstain can measure distributions of single cell responses at specific time points, we considered whether the distribution of nuclear p65 levels observed in Imstain would be consistent with cells displaying desynchronized spiky p65 activity. In p65-EGFP tracking experiments, cells show a stereotypical initial spike of p65 activity, peaking about 30 min after TNFα stimulation (50). Likewise we observed that the distributions of p65 activity at 0 and 30 min were essentially different from the biphasic response of the population average (42). As Imstain can measure distributions of single cell responses at specific time points, we considered whether the distribution of nuclear p65 levels observed in Imstain would be consistent with cells displaying desynchronized spiky p65 activity. In p65-EGFP tracking experiments, cells show a stereotypical initial spike of p65 activity, peaking about 30 min after TNFα stimulation (50).
secondary spikes have the same amplitude as the initial spike, then the p65 activity distributions at these later time points should be at least as dispersed as at 30 min of stimulation, but we found experimentally that the distributions were less disperse (Fig. 5A). If instead secondary spikes have a lower amplitude (30% of the initial spike; as reported in Ref. 50), then it might be possible to reconcile the observed dispersions with the model of desynchronized p65 spikes. Using the reported relationship between the initial and secondary p65 spikes, we predicted the expected distributions resulting from prolonged TNFα stimulation as a weighted average of the distributions from 0 to 60 min (see supplemental methods for the mathematical derivation). We found that the observed and expected distributions were significantly different (Fig. 5B; $p < 10^{-323}$ by Kolmogorov-Smirnov test, $n = 2687$), indicating that nuclear translocation of p65 in individual wild type cells does not display asynchronous spikes. Instead distributions of p65 activity normalized as a Z-score (the activity minus the population mean divided by the standard deviation) were unimodal and superimposable at every time point (Fig. 5A, inset), suggesting a single population of cells responding in relative synchrony. This example illustrates how data obtained from wild type cells using Imstain may be used to evaluate the significance of heterogeneous responses observed in cells with genetically encoded reporters.

**DISCUSSION**

In this report we describe Imstain, a microfluidic device capable of performing a variety of high content experiments to characterize cell signaling dynamics. The device has the single cell resolution characteristic of live cell imaging but allows one to visualize dynamic signaling events in cells free of exogenous genetic perturbations. Furthermore the device can be used to analyze thousands of cells, a number similar to that used in flow cytometry experiments, but provides subcellular detail and preserves the spatial distribution of the cells analyzed. Additionally a broad variety of cell lines can be studied in Imstain. In addition to mouse fibroblasts and human lung epithelia used in the above experiments, we have cultured a dozen different cell lines in Imstain devices, including delicate primary cells (supplemental Fig. 3). Imstain offers superior accuracy and uniformity in reagent delivery compared with traditional multiwell assays and is also compatible with existing HCCS antibodies and microscopic imaging tools. The miniaturized format of Imstain leads to a dramatic drop in material usage, enabling routine seeding of a device using a cell solution containing less than 90,000 cells, and an entire device can be stained with less than 100 ng of each antibody. We also automated Imstain experiments with simple off-the-shelf parts (e.g. tubes, solenoid valves, and digital output card), whereas automating HCCS experiments might require access to expensive robot systems. In short, our microfluidic device substantially extends the capabilities of HCCS and, more generally, can help reduce the barriers to using HCCS as a routine, quantitative, high throughput tool for studying signaling dynamics in single cells (35).

We demonstrated the utility of Imstain for high content cell screening by stimulating cells with different ligands and/or small molecular inhibitors and measuring the response of various kinases, transcription factors, and target genes. We showed that population-averaged signaling dynamics obtained in this way are comparable to that obtained by traditional methods albeit with increased throughput and spatial and temporal resolution. We also considered signaling dynamics at the level of single cells, specifically in the TNFα-NF-κB signaling pathway. Distributions of NF-κB activity in wild type cells measured in Imstain were not consistent with individual cells displaying the desynchronized spiky NF-κB oscillations seen in live cell experiments. Instead our results suggest that it is much more likely that individual cells respond with dynamics close to that of the population average. Similar analyses can be useful in inferring the difference, if any, of the behavior of individual cells versus the population average in other signaling pathways. Likewise the analysis
can be used to quantitatively evaluate individualized cell responses predicted, for example, by mathematical models.

The throughput of the device can be extended in a number of straightforward ways. For example, the device can be easily scaled up n-fold by duplicating the fluidic pattern n times. The device will not become substantially more complicated because the number of required multiplexed valves scales logarithmically with the number of test chambers. The throughput can be further enhanced by staining for multiple targets simultaneously. We demonstrated dual staining, and more targets could be resolved if primary antibodies are conjugated to distinguishable fluorophores similar to polychromatic flow cytometry (51). Alternately, cells could be repeatedly stained and bleached as in multiple epitope ligand cartography (52). Additionally, modules can be added upstream to enhance functionality, such as a mixer to automatically generate a range of stimulus concentrations or to combine several stimuli in a combinatorial fashion.

The primary advantage of using microfluidic devices is in the exquisite control over the cellular microenvironment, which might allow investigation of the effects of complex, more biologically realistic spatiotemporal cell stimulation patterns. Imstain provides a powerful example of the utility of tight control of extracellular conditions coupled to high content cell screening. We anticipate that devices like Imstain will allow rapid accumulation of data related to cell signaling and cell-cell communication in cell lines and primary cells subjected to complex changes in their extracellular milieu.

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