High-throughput microfluidic single-cell analysis pipeline for studies of signaling dynamics

Ryan A Kellogg[1], Rafael Gómez-Sjöberg[2,4], Anne A Leyrat[3,4] & Savaş Tay[1]

[1]Department of Biosystems Science and Engineering, ETH Zürich, Zurich, Switzerland. [2]Department of Bioengineering, Stanford University, Stanford, California, USA. [3]Department of Bioengineering, Stanford University, Stanford, California, USA. [4]Present addresses: Quanticel Pharmaceuticals, San Francisco, California, USA (R.G.-S.); Fluidigm Corporation, South San Francisco, California, USA (A.A.L.). Correspondence should be addressed to S.T. (savas.tay@bsse.ethz.ch).

Published online 26 June 2014; doi:10.1038/nprot.2014.120

Time-dependent analysis of dynamic processes in single live cells is a revolutionary technique for the quantitative studies of signaling networks. Here we describe an experimental pipeline and associated protocol that incorporate microfluidic cell culture, precise stimulation of cells with signaling molecules or drugs, live-cell microscopy, computerized cell tracking, on-chip staining of key proteins and subsequent retrieval of cells for high-throughput gene expression analysis using microfluidic quantitative PCR (qPCR). Compared with traditional culture dish approaches, this pipeline enhances experimental precision and throughput by orders of magnitude and introduces much-desired new capabilities in cell and fluid handling, thus representing a major step forward in dynamic single-cell analysis. A combination of microfluidic membrane valves, automation and a streamlined protocol now enables a single researcher to generate 1 million data points on single-cell protein localization within 1 week, in various cell types and densities, under 48 predesigned experimental conditions selected from different signaling molecules or drugs, their doses, timings and combinations.

INTRODUCTION

The increasing spatial and temporal resolution of the imaging of dynamic cellular processes[1], such as protein translocation[2,3], protein interactions[4], post-translational modification[4], diffusion[5] and local concentration[6], the transcription of single mRNA molecules[7–9] and organelle function[10,11], has opened the door to many new biological questions[12]. Single-cell longitudinal imaging is the gold standard in cell and systems biology, owing to the ability of this technique to capture dynamics and variability of individual cells. Live-cell imaging and single-cell analysis of dynamic signal processing have greatly enhanced our understanding of basic immune responses[13], development[14], biological noise[15] and drug responses[16]. However, experiments to measure single-cell dynamics using manual pipetting and conventional (dish- or flask-based) cell culture methods have limited throughput and reproducibility, and they confer on researchers only a partial ability to manipulate the cellular environment precisely in real time. Furthermore, the large medium volumes used around the cells contribute to background fluorescence, inhomogeneity in concentration owing to convective mixing and weakened cell-to-cell paracrine signaling owing to the dilution of secreted molecules.

Microfluidics overcomes difficulties in traditional live imaging by miniaturizing fluid control to size scales that match those of single cells[17]. Microfluidic cell culture enables precise and automated specimen manipulation during live-cell experiments in nanoliter-volume chambers that allow physiological diffusive signaling between cells. Multiple patterns of complex temporal stimuli can be delivered in parallel to culture microchambers in a single experiment, resulting in marked increases in throughput, particularly when combined with automated image analysis routines. Transient increasing or decreasing stimuli, as well as rapid pulse trains of chemicals, can be seamlessly delivered to cells when membrane valve–based microfluidic systems are used. Moreover, precise spatial manipulation is possible through pairing with technologies such as optogenetics[18,19] or optical tweezers[20]. Finally, the use of microfluidics facilitates closed-loop control with continuous experimental manipulation based on real-time imaging feedback[21]. When combined with a robust image analysis pipeline, a single researcher can generate and analyze rich multiparameter data sets on the timescale of weeks rather than years.

Previously, we developed an automated microfluidic cell culture system[22] and used it to study immune signaling dynamics[2], cell migration[23] and stem cell differentiation[22], all while accounting for natural variability between individual cells. Precision fluidic control and high throughput achieved by this system enabled us to discover digital, all-or-none activation of the NF-κB immune pathway after inflammatory signaling inputs[2], and made possible the development of a widely applicable computational model of this pathway[2] and of coordinated cell migration in a population context[23]. Here we describe an accessible end-to-end pipeline for microfluidics-based single-cell analysis, including the production of microfluidics chips, hardware and software setup for controlling and automating chip operation, parallelized single-cell dynamic imaging experiments, automated image and data analysis, and cell retrieval from the chip for gene expression analysis using the Fluidigm BioMark microfluidics-based qPCR system[17,18,24] (**Fig. 1**).

Applications of the protocol

Although this protocol focuses on cell signaling applications, the method described herein is useful for many applications involving dynamic monitoring of cells under variable stimulation type and timing, and it is equally well suited to labeling studies in fixed cells across many experimental conditions or time points. In the field of stem cell biology, questions concerning how cell fate depends on the combination, concentration and duration of soluble input signals are widely interesting and are among those that the present approach was designed to answer[22]. Stem cells have been maintained in the chip described herein for 2 weeks without signs of differentiation or loss of renewal potential[22]. Owing to the ability to program arbitrary stimulation procedures, complex and

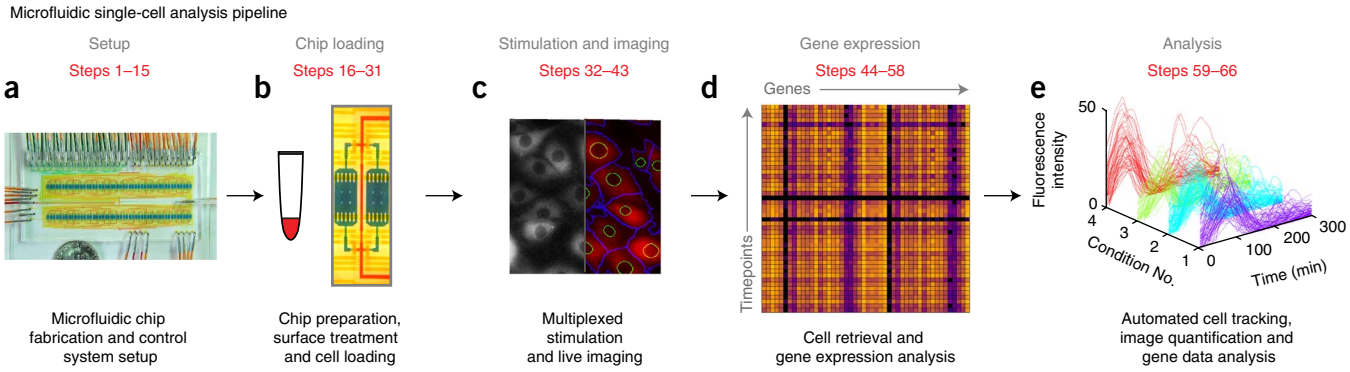

Figure 1 | Single-cell live-imaging and gene expression analysis pipeline. (**a**) PDMS-based microfluidic chips are produced from silicon molds. The chip described (called the Cell Culture Chip[22]) contains 96 independent ~1 mm × 1 mm × 35 µm (35-nl) chambers. Computer-controlled hardware for actuating on-chip elastomeric valves is set up. (**b**) Chips are mounted to the stage of a live-imaging microscope, and chamber surfaces are treated with cell adhesion molecules (typically fibronectin) to facilitate cell attachment. Cells prepared in suspension are loaded into each chamber and allowed to grow to the desired confluency. Typically, 30% of chamber volume is exchanged with fresh medium at 2-h intervals to prompt rapid cell growth. (**c**) Programmable chip control enables the delivery of unique stimulation patterns for each chamber in parallel with live-cell imaging. Imaging cells with fluorescent reporters facilitates readout of dynamic cell signaling events, such as translocation from the cytoplasm to the nucleus. (**d**) After in-chip stimulation, cells are trypsinized, and they readily detach from the chamber surface (typically in <1 min); their flow is directed through the microbore tubing off-chip to a 96-well plate that is to be subjected to immediate lysis and one-step reverse transcription and specific-target amplification for gene expression analysis. The use of the Fluidigm microfluidic qPCR system enables performing up to (96 × 96 =) 9,216 simultaneous qPCR measurements. (**e**) For live microscopy, image analysis automatically tracks cells and quantifies responses to produce single-cell trajectories of signaling dynamics. For images generated during microfluidic qPCR, fluorescence for each reaction over time is quantified to determine relative abundance of each gene template sequence. Images in **a** and **b** are adapted with permission from Gómez-Sjöberg *et al.*[22]. Copyright 2013 American Chemical Society.

otherwise labor-intensive protocols for protein or RNA labeling can be programmed and performed unattended for up to 96 experimental conditions. Moreover, compared with wide-field imaging in a 96-well plate, imaging in a microfluidic system with a chamber height of ~35 µm leads to vastly improved image signal-to-noise because of the reduction in medium volume and background fluorescence.

Comparison with other approaches

A number of microfluidic technologies have been described for cell culture applications, including automated antibody labeling for measuring signal transduction across multiple time points in fixed cells[25–27]; the study of single-cell dynamics under microfluidic gradients[28]; and microfluidic perfusion of cell culture arrays[29], single-cell trap arrays[30,31] and single- or few-cell isolation in microwells[32]. Overall, these methods lack the throughput capacity of the present protocol in terms of the number of parallel experimental conditions that can be set up. The method described herein involves the use of a chamber whose geometry allows natural diffusive signaling and physical contact between cells. Depending on the biological question being addressed, it may be suitable to choose an alternative chip design that, for example, traps and isolates single cells. The present protocol, including chip fabrication and computer control, surface treatment, cell loading and maintaining long-term viability, along with retrieval from the chip for downstream analysis, is applicable to mammalian cell culture in other multilayer polydimethylsiloxane (PDMS) chip designs.

Experimental design

Microfluidic chip design. Multilayer soft lithography[33] enables the fabrication of microfluidic devices with integrated membrane valves that allow highly complex, parallelized fluid manipulation that have led to paradigm shifts in single-cell genomic[34,35] and transcriptomic[36,37] analysis. Chip fabrication begins with the design and preparation of molding masters on silicon wafers. The chip is typically designed using CAD software, and this design is printed to produce photomasks, which are, in turn, used to produce molds using standard UV photolithography in a clean-room facility[38,39]. In multilayer microfluidics, a positive photoresist is typically used to generate channels with rounded cross-sections, and a negative photoresist is used to generate channels with rectangular cross-sections[40]. For mold-making protocol steps, see Gómez-Sjöberg *et al.*[22]. The transparent, biocompatible and gas-permeable PDMS is cured on the molds to create microscale features in the molds. By carefully aligning and bonding multiple PDMS layers on top of each other, it is possible to generate on-chip valves, and these valves can create a dense fluidic functionality that is analogous to the electronic functionality on a computer chip[33].

The microfluidic cell culture chip[22] provides a broadly applicable platform that overcomes the limitations of traditional live cell experiments with respect to automation, precision and throughput. The chip contains 96 individually controllable chambers with a surface area of ~1 × 1 mm^2 and a height of 35 µm, for a volume of 35 nl. Each chamber can support the culture of 1–1,000 mammalian cells, depending on cell size. Vials containing cell medium and other reagents are connected to the chip through thin tubing. Up to 16 input reagent vials can be connected to the chip at a time. Flows from one or multiple inputs (which are combined by an on-chip mixer) are directed to selected chambers. An on-chip peristaltic pump enables highly precise delivery of predetermined volumes of reagents into chambers. The chip is constructed from two PDMS layers: the 'flow' layer, which contains cells and reagents, is positioned above a 'control' layer that creates microfluidic valves[41]. Overall, the chip functions as

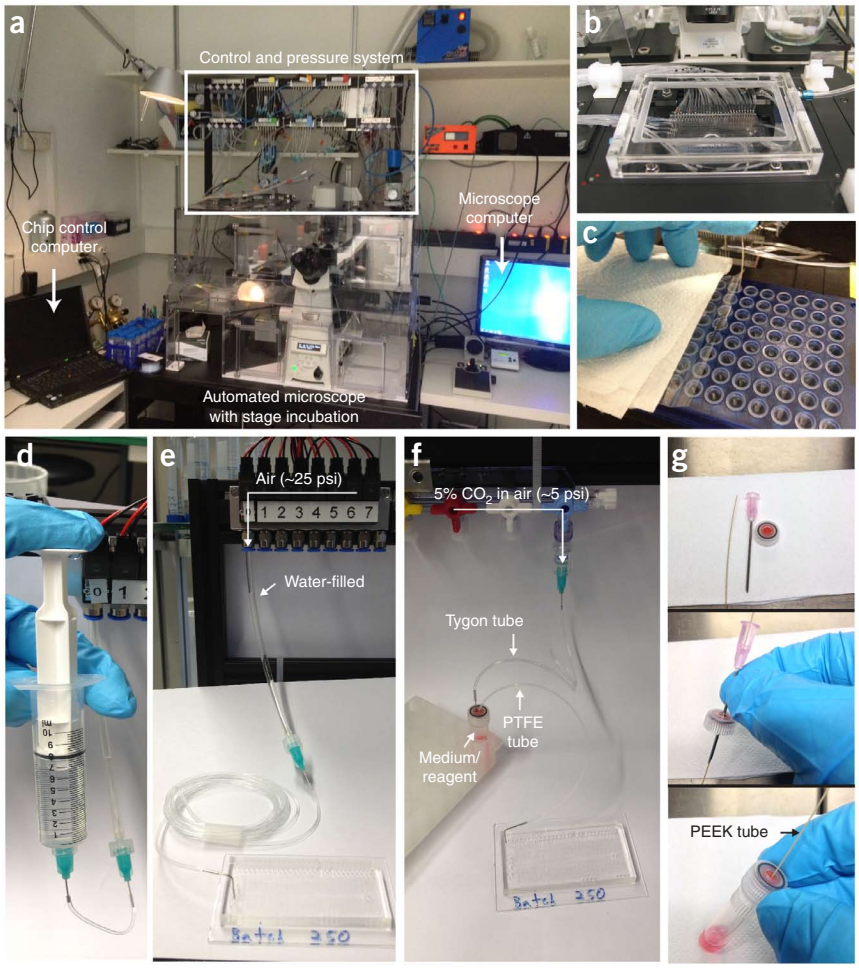

Figure 2 | Photos of the microfluidic cell culture system setup. (**a**) System overview. Solenoid valves that pressurize on-chip valves are controlled by a USB controller box operated by a laptop computer. An air pressure source is connected to the solenoid valves (required pressure ~20–30 psi). The valve control system and computer are portable for use on different microscopes. A separate computer controls a fully motorized inverted fluorescence microscope (pictured: Nikon Ti-Eclipse). The microscope contains an incubation system to control temperature, humidity and CO_2. (**b**) The chip is secured tightly to the microscope stage using a stage plate with a viewing area that is sufficiently large to see the chambers and surrounding fluidic circuitry on the chip. Slots in the stage cover allow tubing connections to the chip, and tubing is secured using plastic stage clips. (**c**) Cell retrieval from the chip. An ~10-cm-length section of PEEK tubing is connected to either the upper or lower waste outlet on the chip. Trypsin is delivered to chambers and cells are washed out serially through the tubing. A PBS wash step prevents cross-contamination between chambers. The cells are deposited into a 96-well plate containing 5 µl of lysis buffer for qPCR processing. Each well should be sealed to prevent evaporation during cell retrieval. (**d**) A syringe can be used to fill control lines with water. (**e**) Control lines connect the chip to a computer-controlled pneumatic valve manifold supplied with air, typically at 20–30 psi. Tygon tubing of sufficient length to reach the chip on the microscope then connects to the corresponding valve on the chip. (**f**) To connect pressurized reagent inputs to the chip, Tygon tube connects a port on a manual manifold to a vial containing the desired reagent or medium. The connection from the vial to the chip is made with PTFE tubing. (**g**) Process for making vials to be pressurized for flowing reagents or cells into chip. Although PTFE tubing is used for medium and reagents, PEEK tube is used for loading cells. A 20-gauge needle is inserted through a rubber membrane screw cap, and 0.02-inch PEEK tubing is inserted through the needle. The needle is then removed.

a miniaturized 96-well plate in which manipulation of nanoliter volumes for each chamber can be independently controlled and fully automated (**Fig. 2**).

Controlling and imaging the chip. Obtaining on-chip valves that control chip functions requires hardware to deliver pressure to specified valves and software to implement different sequences of valve opening and closing. An air pressure source is connected to computer-controlled solenoid arrays that direct pressure to specified valves on the chip. Scripting in MATLAB or LabVIEW enables the automation of chip functions, including chamber surface treatment, cell loading, cell feeding and stimulation, cell staining, cell retrieval from the chip and chip cleaning. Input reagents, such as cell medium, are pressurized with 5% CO_2 in an air-gas mixture to equilibrate the vial contents with the appropriate gas composition. The chip is mounted securely on the stage of an inverted automated fluorescence microscope with environmental control to maintain temperature (37 °C), gas composition (5% CO_2 in air) and humidity (>95%) around the chip. Manufacturer-provided or open-source microscope control software such as µManager (https://micro-manager.org) is used to carry out time-lapse imaging of cells in chambers on the chip. The chip can be used with either wide-field or confocal microscopes. Although compared with wide-field systems confocal microscopes may provide improved image quality, wide-field systems have the advantage of higher speed of image collection and lower phototoxicity, which are important when studying signaling dynamics and when fine time resolution needs to be maintained while acquiring images across many chip locations.

Maintaining cell viability in microfluidic chips. PDMS provides excellent permeability to oxygen and CO_2, which facilitates cell culture in closed PDMS devices[42]. As with any live-imaging experiment, cell health is negatively affected by phototoxicity, and thus illumination time and intensity should be minimized. Toxicity from un-cross-linked PDMS can be eliminated by fully curing PDMS chips, by autoclaving them or by using chemical extraction methods[43]. The small volume of microfluidic chambers means that cells rapidly consume nutrients and require feeding at frequent intervals. Cell feeding in the cell culture chip can be performed using the on-chip pump to gently exchange a portion of the chamber solution with fresh medium. A default 2-h feeding

interval with 30% medium replacement is suitable for cultures of 3T3 fibroblasts, RAW264.7 macrophages and probably other cell types. As a consequence of the small chamber volume, the concentration of ligands introduced into the chambers decays exponentially as the ligands degrade and as cells process and internalize them. This concentration variation may be used to generate periodic input signals to cells. To achieve constant ligand concentration, the medium containing the ligand can be continually perfused using pressure-driven flow or the on-chip peristaltic pump. Please note that a no-ligand feeding condition should be included in stimulation experiments as a control to determine the possible influence of the feeding method on cell response.

Cell tracking and data analysis. Owing to the volume of image data generated by the present protocol, automated processing and quantification is essential. Incorporating fluorescent reporters that label cellular compartments, such as the nucleus, can greatly aid computerized cell tracking and data extraction. Such labels may be genetically encoded in the cells' DNA, or small-molecule fluorophores may be added to cells before imaging. A number of software tools are available for image quantification. The MATLAB Image Processing Toolbox provides a full set of functions for customized implementation of essentially any image analysis task. We use MATLAB routines for the analysis of dynamic NF-κB nuclear translocation and oscillation in cells[2]. Open-source packages such as CellProfiler provide a broad range of image analysis functions that can be customized without programming. For large or complex image-processing sets, it may be necessary to parallelize the tasks and to carry out the analysis on a high-performance computer cluster. After cell tracking and image quantification, individual cell trajectories are assessed (typically in MATLAB or Excel) to draw conclusions about single-cell and population responses to each input condition.

Limitations. Microfluidic cell culture experiments are inherently more complex and require longer setup times than experiments conducted with traditional methods, although the labor and time investment is recovered many-fold by gains in throughput, automation, reproducibility and image quality. However, the biggest payoff is in experiments that deliver very high information density (large number of data points per chamber or per cell, collected over the course of the culture). Simple experiments with a single-endpoint readout might not be suited to the microfluidic platform.

The chip design described here is such that the chip has limited ability to generate chemical gradients, and it also requires medium to flow over cells to deliver new stimuli, a process that disturbs the paracrine signaling field in the chamber. Other chip designs enable gradient formation and diffusive feeding, which preserve the paracrine field[28,44]. The chip design described in the present protocol is not optimized for generating smoothly varying (i.e., sinusoidal) inputs to cells. Gene expression analysis is currently limited to analysis of all cells within a chamber rather than single cells, although isolation and analysis of a cell of interest may be achieved in the current chip design using optical trapping methods[45].

MATERIALS

REAGENTS

- PDMS and curing agent (Momentive, cat. nos. RTV-615A and RTV-615B)
- Trimethylchlorosilane (TMCS) (Sigma-Aldrich, cat. no. 92360)
  ! CAUTION TMCS is highly toxic and should be handled only in a fume hood; wear proper personal protective equipment.
- Pluronic F-127 (Sigma-Aldrich, cat. no. P6867)
- Fibronectin from human plasma (Millipore, cat. no. 341635-1MG)
- Cells. Cells should be adherent or semiadherent on fibronectin or other ECM proteins. Cells used in the PROCEDURE as an example application of the protocol are NIH3T3 mouse fibroblasts and RAW264.7 mouse macrophages genetically modified to express NF-κB (p65) fluorescent fusion proteins obtained as per relevant literature[2,46]
- Signaling molecules for cell stimulation. Our laboratory has used tumor necrosis factor alpha (TNF) (Life Technologies, cat. no. PMC3014) and lipopolysaccharide (LPS) (Sigma-Aldrich, cat. no. L4524)
- Hydrogen peroxide (H_2O_2), 30% (vol/vol) (Sigma-Aldrich, cat. no. 95313-1L)
  ! CAUTION H_2O_2 is corrosive; wear proper personal protective equipment.
- CellsDirect one-step qRT-PCR kit containing resuspension buffer, lysis enhancer and SuperScript III/Platinum Taq mix (Life Technologies, cat. no. 11753-100)
- Assay loading reagent (Fluidigm, cat. no. 85000736)
- Sample loading reagent (Fluidigm, cat. no. 85000735)
- TaqMan gene expression assays (Applied Biosystems, cat. no. 4331182)
- TaqMan universal PCR master mix (Applied Biosystems, cat. no. 4304437)
- TE buffer (Ambion, cat. no. AM9849)
- TaqMan primers (Applied Biosystems)
- Double-distilled water (ddH_2O)
- Dulbecco's PBS (Life Technologies, cat. no. 14190250)
- DMEM (Life Technologies, cat. no. 32430-027)
- FBS (Sigma-Aldrich, cat. no. F2442-500ML)
- Trypsin-EDTA, 0.05% (wt/vol) (1×) (Trypsin, Life Technologies, cat. no. 25300-054)

EQUIPMENT

- Automated fluorescence inverted microscope with environmental control chamber. We have implemented this protocol on Leica DMI6000B and Nikon Ti-E microscopes
- Pneumatic solenoid valves and controller boxes. Parts and assembly instructions are available at http://www.microfluidics.ethz.ch/chip_culture_protocol
- Silicon master molds. The silicon cell culture chip molds were obtained from the Stanford Microfluidics Foundry (http://www.stanford.edu/group/foundry/)
- BioMark HD system with integrated fluidic circuit (IFC) controller MX (Fluidigm)
- 48.48 Dynamic Array IFC (Fluidigm)
- Sonicator (SonoSwiss, cat. no. SW 6H)
- Plasma machine (Diener, FEMTO Version A)
- Spin coater (SPS-Europe, cat. no. SPIN150-NPP)
- Vacuum pump (Varian, cat. no. SH-110) and desiccator (Thermo Scientific Nalgene, cat. no. EW-06520-05)
- Punching press and 20-gauge round hole punch (Syneo, Accu-Punch MP)
- Laboratory oven (Thermo, Heratherm OSM60)
- Laminar flow hood (Thermo, Herasafe KS)
- Orbital mixer and 100-ml disposable cups (Thinky, cat. nos. ARE-250 and 250-100DSP)
- Precleaned glass slides, 75 × 50 mm (Fisher, cat. no. W56948)
- Stainless steel microbore tubes, 0.025-inch outer diameter (o.d.) × 0.013-inch inner diameter (i.d.) × 0.75 inches long (New England Small Tube)
- Luer manifolds five-port (Cole Palmer, cat. no. EW-30600-43)
- Disposable stainless steel dispensing needles (23 gauge, 0.5 inches long, type 304, i.d. 0.017 inches, o.d. 0.025 inches, McMaster-Carr)
- Pressure regulator 0–30 psi (Airtrol, cat. no. R-800-30-W/K)
- Polyetheretherketone (PEEK) tubing (510 μm o.d. × 65 μm i.d., IDEX no. 1543)

- Polytetrafluoroethylene (PTFE) tubing (0.022-inch i.d. × 0.042-inch o.d., Cole Parmer, cat. no. EW-06417-21)
- Tygon microbore tubing (0.02 inches × o.d. 0.06 inches, VWR, cat. no. S-54-HL)
- Microtubes, 2 ml (Sarstedt, cat. no. 72.694.006)
- Membrane screw caps (Sarstedt, cat. no. AG 65.3716)
- 10-ml syringe (BD Medical, cat. no. 14-826-13)
- 0.2-μm syringe filter (Thermo Fisher Nalgene, cat. no. 191-2020)
- Scotch tape (or equivalent clear tape)
- 5% CO_2 pressure source
- Compressed air source
- Valve control computer with Windows (Microsoft) and MATLAB (MathWorks, version 2006 or later) installation. A laptop computer allows the valve control system to be portable between different microscopes
- Analysis computer with Windows (Microsoft) and the following software: CellProfiler open-source package (http://www.cellprofiler.org) for image analysis, and MATLAB (MathWorks, version 2006 or later) and Fluidigm real-rime PCR analysis software (Fluidigm) for qPCR data analysis

REAGENT SETUP

6% (vol/vol) H_2O_2 solution (0.5 l) Add 100 ml of 30% (vol/vol) H_2O_2 to 400 ml of ddH_2O. Store the resulting solution at 4 °C; it will be stable for 1 month.

Medium for 3T3 fibroblasts and RAW264.7 macrophages (DMEM + 10% (vol/vol) FBS) Add 50 ml of FBS to 450 ml of DMEM. Store the resulting mixture at 4 °C; it will be stable for 1 month.

100 μg/ml fibronectin solution (1 ml) Add 100 μl of fibronectin (1 mg/ml) to 900 μl of PBS. Freshly prepare the solution and use it immediately.

0.2% (wt/vol) Pluronic solution Add 0.1 g of Pluronic powder to 50 ml of PBS. Store the resulting solution at 4 °C; it will be stable for 6 months.

Lysis buffer solution (300 μl) Thaw resuspension buffer on ice. Combine 250 μl of resuspension buffer and 25 μl of lysis enhancer (all part of the CellsDirect kit). Freshly prepare before use and keep it on ice.

EQUIPMENT SETUP

Chip mold setup Obtain flow and control layer cell culture chip molds on the basis of the AutoCAD DXF chip design file (**Supplementary Data**) by using one of several organizations that provide microfluidic fabrication services: Trianja (http://www.trianja.com), SIMTech Microfluidics Foundry or FlowJEM (http://www.flowjem.com). In addition, the Stanford Microfluidics Foundry provides a summer school for microfabrication techniques (http://stanford.edu/group/foundry).

The cell culture chip contains on-chip elastomeric valves that are actuated by an external pressure source. Gómez-Sjöberg *et al.*[22] developed a setup that uses three-way solenoid valves that are computer-controlled by a custom USB interface. This control system can be used generally for multilayer PDMS chips. Construct the control system by following the detailed instructions and by downloading the related software and drivers that are available at http://www.microfluidics.ethz.ch/chip_culture_protocol.

Computer setup Prepare the valve control computer and analysis computer by installing the software listed in the Equipment list.

PROCEDURE

Chip fabrication from existing molds ● TIMING 36 h

1| Place the flow and control mold wafers inside a closed wafer carrier box with a small beaker containing a few drops of TMCS. Incubate the wafers for 60 min and use them within 1 h of the incubation.

2| Make an aluminum foil container by molding foil around a 110-mm-diameter Petri dish. To make the flow layer, weigh 38 g of PDMS in a 100-ml disposable cup and add curing agent at a 1:10 weight-to-weight ratio (3.8 g) for each mold. Mix in an orbital mixer for 3 min at 2,000 r.p.m. and defoam for 5 min at 2,200 r.p.m. Pour 40 g of the resulting PDMS over the wafer.

3| Degas the PDMS flow layer by placing the aluminum foil container in a larger Petri dish in a vacuum desiccator. Make sure that the PDMS does not overflow and that inrushing air does not tip the wafer when the desiccator is refilled with air.

4| To cure the PDMS flow layer, cover and bake it for 1 h at 80 °C. Make sure that the substrate is level.

5| To make the control layer, weigh 22 g of PDMS in a 100-ml disposable cup and add curing agent at a 1:10 ratio (2.2 g) for each mold. Mix in an orbital mixer for 3 min at 2,000 r.p.m. and defoam for 5 min at 2,200 r.p.m.. Place the cup containing mixed PDMS in a vacuum desiccator until all air bubbles have been removed (~20 min). Pour PDMS over a wafer covering three-quarters of substrate and, using a spin coater, spin the control mold wafer for 12 s at 500 r.p.m., and then for 1 min at 2,500 r.p.m. to achieve a control layer thickness of ~30 μm. To cure the control layer, cover and bake it on a level surface for 1 h at 80 °C.

6| To coat glass slides with a thin PDMS layer, first clean 50 × 75-mm glass slides by 45-min sonication in ddH_2O. After sonication, dry the slides at 80 °C for ~45 min. Weigh and mix 1:10 PDMS (38 g of PDMS, 3.8 g of curing agent) as in Step 2. Use a spin coater to spin 1:10 PDMS onto 50 × 75-mm slides for 15 s at 500 r.p.m. and then for 60 s at 2,500 r.p.m. Bake the slides at 80 °C for 1.5 h to cure the PDMS.

7| Remove the PDMS from the flow layer by cutting out around the channels, and peel the PDMS carefully off the wafer. Align and punch the indicated holes with a 20-gauge round-hole punch. Capture all punched plugs on a piece of tape. Clean the flow layer after punching by coating it with tape and by pressing the tape into the mold.

8| Plasma-treat the control wafer and cut-out PDMS flow layer for 15 s at 45 W with the plasma machine. Position the flow layer cut-outs above the control layer (still on the mold) under low magnification. Make alignment marks on the two coincident layers, and bring the layers together. Irreversibly bind the flow to the control layer by baking it for 2 h at 80 °C.

9| Cut out the chip from around the channels and carefully peel it off the control wafer. Punch the remaining control layer holes. Clean and plasma-treat (15 s at 45 W) surfaces of the PDMS-coated slide and control-flow assembly. Position the chip on a PDMS-coated slide and bake it at 80 °C for a minimum of 36 h before use.
▲ **CRITICAL STEP** This final bake is essential to minimize the amount of un-cross-linked polymers remaining in the PDMS. After the chip-making procedure is mastered, the rate of chip failure owing to manufacturing defects is <10%.

Chip control system setup ● TIMING 1 d
10| Assemble a 48-valve control system (six manifolds with eight pneumatic solenoid valves each, operated by two-valve controller boxes) connected to a pressurized air source, and then install USB driver software (see Equipment Setup).

11| Mount pneumatic solenoid valve assembly and control boxes near the microscope. Please note that the use of a portable mounting frame is convenient for transporting the control system between multiple microscopes (see **Fig. 2** for an example mounting frame). Make sure that the water-filled Tygon microbore tubings from each pneumatic valve connect to the chip on the microscope stage and actuate control lines on the chip (**Fig. 2c**).

12| On the mounting frame, attach four five-port Luer manifolds that are end-to-end connected to a pressurized 5% CO_2 source through a 0–30-psi pressure regulator. Attach the Tygon microbore tubing to each manifold port of sufficient length to reach vials containing reagents for input into the chip. This tubing is used to pressurize the reagents for injection into the chip (**Fig. 2f**).

13| Set up the cell culture chip graphical user interface (GUI) software (available at the web link reported in the Equipment Setup).

14| (Optional) It is good practice to clean the control line tubing for every new chip. For this purpose, use a syringe or pressurized reservoir to fill each control line with 6% (vol/vol) H_2O_2. Elevate the tube ends to prevent them from being drained by gravity. Incubate the H_2O_2 in the control lines for 1 h.

15| Place the tube ends in a container and elevate the pressure within the system to expel the H_2O_2. Rinse each tube with ddH_2O.

Chip preparation on a microscope ● TIMING 1–2 h
16| Clean the residual PDMS from the bottom of the microscope slide using a razor blade. Apply Scotch tape to the glass surface to remove residual PDMS and dust particles. Inspect the chip under low magnification periodically until the bottom surface is visually free of dust or PDMS.

17| Position the chip on a microscope stage so that all chambers of the chip are visible. Tighten the stage screws to secure the position (**Fig. 2b**).

18| Use a syringe or a pressurized reservoir to fill each control line tube with ddH_2O (**Fig. 2d**).

19| Connect the control line tubes to the chip (**Figs. 2e**,**3**).

20| Turn the control line pressure to 0. Push the 'Close All' button on the GUI (**Fig. 4**). Gradually increase the control line pressure to 25 psi (1.72 bar). Inspect the chip to ensure that there is no delamination leading to leakage outside the chip or between the control and flow layers.
? TROUBLESHOOTING

21| Test that the control lines are connected correctly by closing the valves in the GUI software and ensuring that the appropriate valve is closed on the chip by inspection under low magnification.

22| Connect the waste vials to the Waste1Up and Waste1Dn flow outlets on the chip (**Fig. 3**).

23| Secure the tubes so that stage movement does not apply force on the chip (**Fig. 2b**).

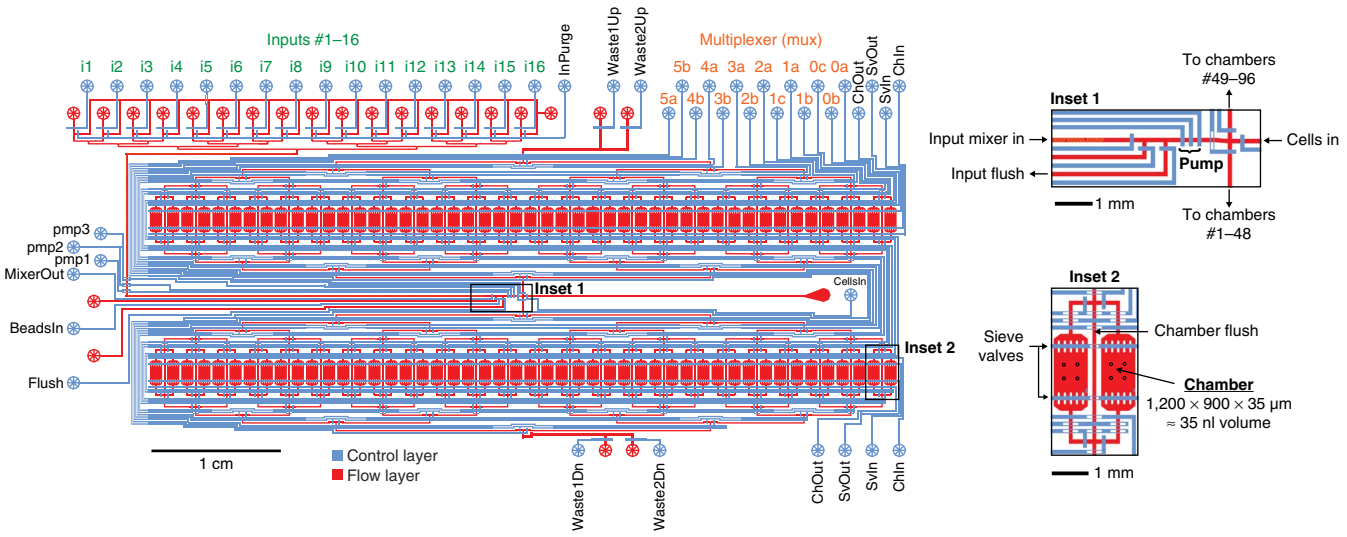

Figure 3 | Cell culture chip layout. The chip contains 96 individually addressable chambers, and it can be connected to up to 16 input reagent vessels. Cells, medium and reagents are contained in the red 'flow' layer, whereas membrane valves are created by the blue 'control' layer. The multiplexer (mux) valves control which chamber or chambers are selected. Cells can be loaded into chambers using the CellsIn port. Cell and reagent input vials are pressurized with 5% CO_2 to 3–10 psi (200–700 mbar) and delivered to the chip through microbore tubing by pressure-driven flow. Input lines converge and pass through a herringbone mixer, enabling the researcher to select reagents one at a time or to mix multiple reagents before they enter chambers. PBS connected to the InPurge line clears the input manifold and mixer when selecting a new input, and the waste flows out through the mixer flush outlet. Inset 1 shows an on-chip peristaltic pump that can generate precisely metered flow to chambers, which enables replacement of a specific fraction of the chamber volume. Pumping precision (1 pump cycle) is ~0.35 nl. Inset 2 shows an enlarged view of the microfluidic chambers, which are ~1 mm^2 in area and 35 µm in height for an ~35-nl volume. Typically, 100–200 large (i.e., 3T3 fibroblast) or 200–300 small (i.e., RAW264.7 macrophages) cells are grown in a chamber. Sieve valves at the entrance and exit of each chamber to enable trapping large cells and particles (>20 µm diameter). A chamber flush line enables the researcher to clear the flow path before directing new reagent to chambers.

Chip surface treatments ● TIMING 6 h–overnight

▲ CRITICAL To promote cell adhesion, chamber surfaces are to be treated with proteins such as fibronectin, collagen or gelatin. In our tests with mouse 3T3 fibroblast and RAW macrophage cell lines, fibronectin treatment resulted in rapid and reliable cell attachment, so treatment with fibronectin is covered as an example in this subsection of the PROCEDURE.

24| Prepare four input vials (**Fig. 2g**) containing 1 ml of Pluronic (0.2% (wt/vol) in PBS), 1 ml of fibronectin (100 µg/ml), 1 ml of medium and 1 ml of PBS, respectively. Set the inlet pressure to 7 psi (480 mBar).

25| Connect the Pluronic and fibronectin vials to input #1 and #2 on the chip, connect the medium vial to input #3 and connect the PBS vial to the InPurge flow inlets (**Fig. 3**).

26| Flush air from the tubes by opening the inlet valves for Pluronic, fibronectin, medium and PBS, one at a time, and direct the flow-through Input Flush (**Fig. 3**) until the air has been eliminated from the flow path for each input.

27| Remove all air from the chip: first, close all chip outlets (Waste1Up, Waste2Up, Waste1Dn and Waste2Dn). Second, open the multiplexer and chamber valves.

28| Remove any stray air bubbles by applying flow pressure to a closed channel to force air out through the PDMS matrix.

29| To block nonchamber surfaces with Pluronic to prevent cell attachment in these areas, first close all valves that allow flow to the chambers by selecting 'All Chambers' in the chamber dropdown box and activating 'Flush' and 'Ch In Closed' checkboxes in the software GUI (**Fig. 3**). Second, close all flow outlets on the chip by ensuring that Waste1Up, Waste2Up, Waste1Dn and Waste2Dn are all closed. Finally, open input #1 (Pluronic), MixerOut and Pump to allow Pluronic flow into the nonchamber areas of the chip. As Pluronic flows in, air in the nonchamber areas of the chip is pushed out through the PDMS. After all air has been removed from the chip (~15 min), continue incubation with Pluronic for 1 h.

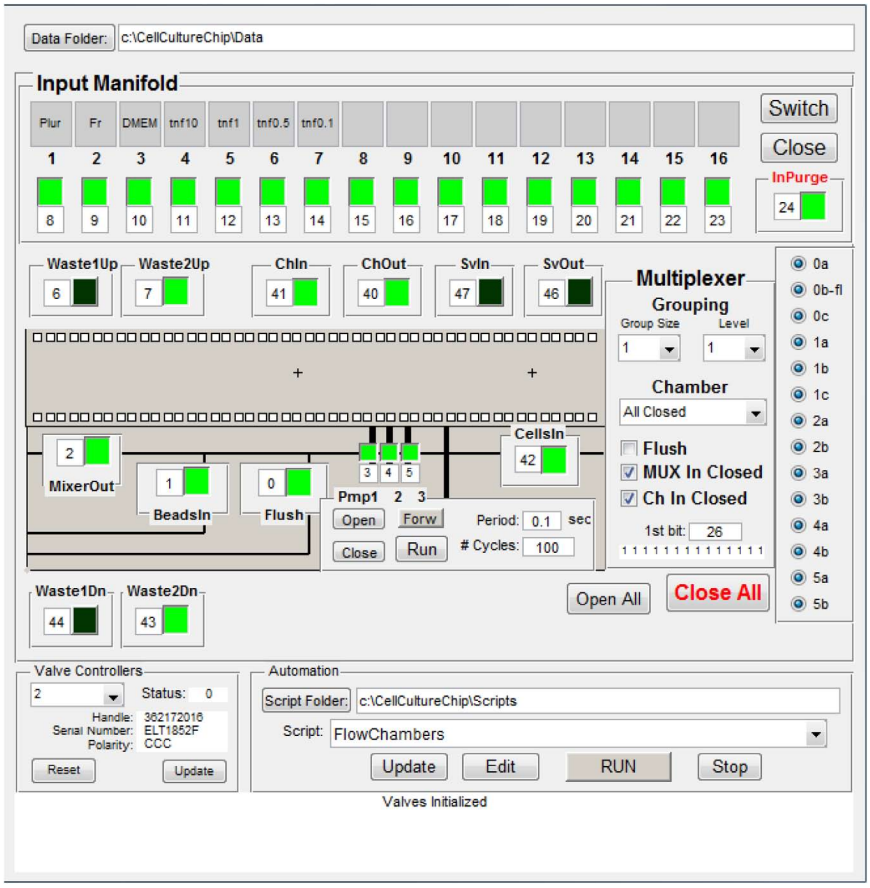

Figure 4 | Cell culture chip control GUI. The software enables the researcher to perform the manual actuation of on-chip valves and confers the ability to run automated scripts to carry out sequential valve operations that implement cell culture and stimulation functions.

30| Wash away the Pluronic from the chip by using PBS: first, close input #1 (Pluronic) and open InPurge (PBS). Open Waste1Up and Waste1Dn to allow PBS to flow through the chip. Continue to allow PBS flow across all nonchamber surfaces for 30 min.
▲ **CRITICAL STEP** Pluronic must be completely washed from the chip. Residual Pluronic may enter chambers and prevent cell adhesion.

31| Treat chamber surfaces with fibronectin: close InPurge (PBS) inlet and open Input #2 (fibronectin). Allow fibronectin to flow through the nonchamber areas of the chip, displacing the PBS, for 10 min. Finally, open the valves to allow flow to chambers by using the GUI to open ChIn and ChOut and by de-selecting 'Flush' and 'Ch In Closed' in the multiplexer panel. Again close the exits from the chip (Waste1Up and Waste1Dn). Now fibronectin will flow into the chambers, displacing the air in the chambers. Incubate the fibronectin in the chambers for 2 h–overnight.

Preparation of cell suspension and loading of cells onto the chip ● **TIMING 2–4 h**

32| Prepare a single-cell suspension of cells with a concentration ranging from 5×10^5 to 2×10^6 cells per ml. The typical volume consumed during cell loading into 96 chambers is 25–50 µl. Preparing suspensions toward the high end of the concentration range specified above enables faster loading owing to fewer seeding rounds. The use of microbore PEEK tubing helps prevent clumping of cells during loading. Connect the vial containing the cell suspension to the cell input channel on the chip and pressurize it with 5% CO_2 at 3–5 psi.
? TROUBLESHOOTING

33| Load the cells into the chambers automatically (option A) or manually (option B). Please note that either option is essentially equivalent.

(A) Automated cell loading using scripting

(i) Use a cell suspension containing 5×10^5 cells per ml to force the cells to flow into each chamber, and immediately acquire a 10× phase-contrast image of the chamber. Cells will appear as circular bright spots in the image, which can be detected by applying an intensity threshold to the image.

(ii) Count and make a record of the number of detected cells loaded in each chamber. Cells begin adhering to the fibronectin surface within minutes.

(iii) After completing one round of seeding, wait for 5 min, and repeat Step 33A(i,ii) above. Cells from the previous seeding round remain attached as additional cells are loaded. Acquire a second image and update the cell count for each chamber.

(iv) Repeat Steps 33A(i,ii) until the cell count for each chamber reaches a specified target (such as 150 fibroblasts or 200 macrophages). The target number of cells per chamber is chosen on the basis of the cell size and growth rate to achieve ~80% confluence during the experiment.
? TROUBLESHOOTING

(B) Manual cell loading

(i) Perform a similar procedure as for option A above, but use visual estimation of the cell count in each chamber.
(ii) Manually force the cells to flow to each chamber in turn.
(iii) After one seeding round, evaluate which chambers require additional cells and seed additional cells in the chamber after a few minutes have elapsed to allow cell adherence.
? TROUBLESHOOTING

Imaging and feeding cells ● TIMING 4 h–overnight

34| Set up time-lapse imaging in the microscope control software: store the stage positions for each imaging location and/or chamber of interest. Specify a desired time lapse between the images to be acquired in sequence. For imaging cell growth, a 30-min interval between image acquisitions is typically sufficient. 10× magnification phase-contrast imaging is sufficient to monitor cell growth in the chip.

35| Feed cells in the chip at predefined intervals as detailed below. Please note that the following numbers have been satisfactory for a variety of cell types, but it might be necessary to perform experiments to determine the optimal feeding volume and frequency required by different cell types. For one feeding round, use the on-chip pump to exchange 30% of the volume in each chamber containing cells. One pump cycle replaces ~1% of the chamber volume. Therefore, to replace 30% of the chamber volume, operate the pump for 30 cycles. The pump is composed of three microfluidic valves in series. The following valve control sequence performs one pump cycle: [0 0 1] – [1 0 1] – [1 0 0] – [1 1 0] – [1 1 1], where 0 indicates that the valve is open (depressurized) and 1 indicates that the valve is closed (pressurized). The time delay between each valve operation determines the flow rate. Maximum pump speed is limited by the response time of the pneumatic solenoid valves and driving electronics. We typically operate in the range of a 0.1–0.05-s delay between pump valve operations, for flow rates of 30–60 nl/min.

36| Repeat the feeding procedure detailed in Step 35 every 2 h. The feeding interval can be adjusted to 1 h for dense cultures or to every 3 h for sparse cultures. In our experience with 3T3 fibroblasts and RAW264.7 macrophages, 2-h feeding intervals with 30% chamber replacement generates optimal cell growth. Occasionally (perhaps 5% of experiments), debris may enter the chip, which blocks the flow.
? TROUBLESHOOTING

37| Acquire phase-contrast images at the previously specified locations during feeding to monitor cell health. Hardware focus technologies (such as Perfect Focus System, Nikon) help maintain focus at many locations across the large chip surface.
? TROUBLESHOOTING

Conducting a cell stimulation experiment ● TIMING variable; 2 h–2 weeks

38| Prepare solutions of the experimental input reagents in vials at the desired concentrations (e.g., cytokines at different dilutions in medium). For example, TNF dilutions may span from 0.01 to 100 ng/ml to test the NF-κB dose response. To test the role of stimulus timing, researchers may deliver pulses that have varied duration or frequency. Inputs may be combined using the on-chip mixer. Use PTFE tubing to connect the input vials to the chip.

39| Connect inputs to the chip, pressurize them with 5% CO_2 at 7 psi and prime each input directing the flow through the flush output on the chip until the air in the tubing and channel is replaced by fluid from the vial.

40| Program cell stimulation for each chamber: for each input time, record the chambers receiving the input, the mode of input (pumping to replace a fraction of chamber volume or pressure-driven flow to fully replace volume) and which inputs to select. For example, for 10-min TNF input pulses spaced at 1-h intervals, TNF is provided at the 0-min, 60-min and 120-min (etc.) time points, and cell medium is provided at the 10-min, 70-min and 130-min (etc.) time points.
▲ **CRITICAL STEP** Log the timing of each stimulus delivery, as well as the times of image acquisitions, so that image data can be properly aligned with stimulation timing.

41| Fine-tune imaging positions and focus using the microscope software before starting the experiment. Specify imaging settings for fluorescence acquisition: for imaging nuclear-cytoplasm shuttling dynamics, we acquire GFP and dsRed channels using a 20× long-distance objective. Perform at least one round of imaging before starting the experiment, and then start a stimulation script to begin the experiment. Perform imaging and chip operations as independent processes. Continue imaging as the on-chip stimulation experiment proceeds.

42| Wait for completion of the experiment.

43| After the stimulation experiment is complete, either fix and stain the cells to relate the dynamic signaling response to additional protein endpoint measurements or collect the live cells from the chip for gene expression analyses.

Retrieving cells from the chip for gene expression analysis ● TIMING 1 h

44| Prepare a 96-well plate in which 5 µl of lysis buffer solution is added to one well for each chamber of cells to be retrieved from the chip. For example, an experiment to measure gene expression at 30-min intervals over 24 h uses half the plate (48 chambers, one per time point).

45| Connect a vial containing 1 ml of trypsin to the chip input.

46| Use a length of 10 cm of 0.02-inch (o.d.) PEEK tubing to connect the chip output to the waste (**Fig. 2c**).

47| Deliver trypsin to each chamber containing cells. Allow 1–3 min for cell detachment. Please note that efficiency of cell retrieval by this method is typically ~90%.

48| For each chamber containing detached cells for collection, position the unconnected end of PEEK tubing into a well containing lysis buffer on a 96-well plate; force 1–3 µl of cell medium to flow through each chamber containing detached cells and collect the outflow through PEEK tubing into a 96-well plate containing 5 µl of lysis buffer (**Fig. 3c**); flush 3 µl of PBS through the tubing to clear them for the contents of the next chamber (collect the outflow on a Kimwipe).

? TROUBLESHOOTING

High-throughput qPCR using the Fluidigm BioMark system ● TIMING 4 h

49| Thaw 2× CellsDirect reaction mix on ice, as well as the 20× TaqMan gene expression assays (18 µM TaqMan primer pairs) of interest. For studies of inflammatory signaling, relevant assays might include NF-κB–regulated genes.

▲ CRITICAL STEP Make sure the 2× CellsDirect reaction mix is completely thawed, and mix this reagent thoroughly before use.

50| Prepare, in a 1.5-ml tube, a primer pool containing each TaqMan assay (initial concentration 20×) diluted 100 times in TE buffer to a final concentration of 0.2×. In this example, if the final volume of the assay mix is 150 µl and there are 48 TaqMan genes, add 1.5 µl of each TaqMan assay and complete with 78 µl of TE buffer.

51| Prepare a reaction mix having the following components in a 1.5-ml tube:

Component	Volume to add per reaction (µl)	Final concentration
2× CellsDirect reaction mix	5	1×
TE buffer	1.5	—
Primer pool (0.2× or 180 nM)	2.5	45 nM
SuperScript III/Platinum Taq mix (from CellsDirect kit)	1	—

52| Distribute the 10 µl of the reaction mix in 48 wells of a 96-well plate.

■ PAUSE POINT The plate can be stored at –20 °C for 3 months.

53| Load 1 µl of cell lysate prepared in Step 48 into each well.

54| Perform one-step reverse transcription (RT) and specific target amplification (STA) reaction by using the thermal cycling protocol described in the in-text table below:

Cycle number	RT	Taq activation	STA – 14 cycles	
			Denaturation	Annealing/extension
1	15 min at 50 °C			
2		2 min at 95 °C		
3–16			15 s at 95 °C	4 min at 60 °C
Hold				4 °C

55| Dilute the RT-STA product obtained in Step 54 by one-third by adding 22 µl of TE buffer to each reaction mixture. Please note that dilutions between one-half and one-fifth are also possible at this stage.

56| Split the 96-well plate into two halves, left and right. To each of the left-hand 48 wells of the plate, add 3.75 µl of Fluidigm 2× assay loading reagent and 3.75 µl of each individual TaqMan assay (20×).

57| To each of the right-hand 48 wells of the plate, add 3.4 µl of the diluted cDNA solution obtained in Step 52, 3.75 µl of the TaqMan master mix and 0.4 µl of 20× gene sample loading reagent.

58| Follow the instructions in the Fluidigm real-time PCR User Guide (http://www.fluidigm.com/home/fluidigm/Support/UG/Real-Time-PCR_Analysis_ug_68000088.pdf) to prime the microfluidic qPCR chip (48.48 Dynamic Array IFC) with assays and samples, perform the qPCR and use the Fluidigm real-time PCR analysis software to calculate cycle threshold (Ct) values for each qPCR.

59| Export the Ct values calculated in Step 58 from the Fluidigm software using the *File* → *Export* menu command and by selecting 'Heat Map Results (*.csv)' under 'Save as type'. This selection generates a comma-separated value (.csv) file containing Ct values in table format with genes (assays) in columns and conditions (samples) in rows.

60| Import the Ct data from the file generated in Step 59 into MATLAB using the 'csvread' function. In MATLAB, Ct and relative expression can be readily explored and plotted. Relative gene expression can be calculated as $2^{(Ct\_reference - Ct\_gene)}$.

Automated analysis of microscopy images ● TIMING variable

61| Transfer images from the microscope computer to the analysis computer, in which CellProfiler is installed (see Equipment Setup).

62| In CellProfiler, use the 'IdentifyPrimaryObjects' module to segment nuclei in images with a nuclear label (i.e., H2B-GFP fluorescent fusion protein), which automatically calculates and applies a threshold to the image so that only bright objects (nuclei) remain.

63| In CellProfiler, use the 'IdentifySecondaryObjects' module to segment cytoplasm in images with cytoplasmic fluorescence (such as p65-dsRed) using the nuclei segmented in Step 62 as a seed for each cytoplasm segmentation.

64| Use the 'MeasureObjectIntensity' module in CellProfiler to quantify the total and mean fluorescence for the nucleus and cytoplasm in each image.

65| Use the 'ExportToSpreadsheet' module in CellProfiler to save cell locations and fluorescence measurements in an Excel document.

66| Use the 'TrackObject' module in CellProfiler to connect objects between images and to produce single-cell trajectories of nuclear and cytoplasmic fluorescence over time. Alternatively, use a tracking algorithm implemented in MATLAB to extract trajectories (such as http://physics.georgetown.edu/matlab/code.html).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
20	Chip delamination	Poor binding between layers	Use plasma treatment to increase the bond strength
32	Cell clumping during loading	Cells are left in suspension for too long	Agitate the cell vial by gentle vortexing or tapping Trim the ends of the tubing
33A(iv), 33B(iii)	Poor cell attachment	Poor surface treatment	Ensure that Pluronic is washed away thoroughly before fibronectin treatment
	Poor cell health in the chip	Incomplete PDMS curing	Bake the chip for an additional 24–48 h
		Cell medium evaporation from the vial	Ensure that the cell medium input vials are tightly sealed to prevent evaporation
		Cell medium evaporation from the chip	Ensure 95% humidity around the chip and seal the tubing slots on the stage cover with tape
36	Debris is causing the chip to clog	Dust particles are present in the tubing	Flush the inlet tubing briefly with cell medium or PBS before connecting it to chip
		Cellular aggregates	Ensure that cells are fully dissociated by trituration before loading
		Particulates in medium and/or serum	Pass cell medium and/or serum and reagents through a 0.2-μm syringe filter before preparing input vials
37	Microscope is losing focus over time	Chip is moving on the stage during the experiment	Secure the tubing tightly to the stage with tape or stage clips to ensure that there is no force on the chip during stage movement
48	Inconsistent volume in the 96-well plate after cell retrieval	Evaporation from wells during the retrieval procedure	Cover each well individually with tape immediately after cell retrieval

● **TIMING**

Steps 1–9, chip fabrication from existing molds: 36 h
Steps 10–15, chip control system setup: 1 d
Steps 16–23, chip preparation on a microscope: 1–2 h
Steps 24–31, applying chip surface treatments: 6 h–overnight
Steps 32–33, preparation of cell suspension and loading of cells into the chip: 2–4 h
Steps 34–37, imaging and feeding cells: 4 h–overnight
Steps 38–43, conducting a cell stimulation experiment: variable; 2 h–2 weeks
Steps 44–48, retrieving cells from the chip for gene expression analysis: 1 h
Steps 49–60, high-throughput qPCR using the Fluidigm BioMark system: 4 h
Steps 61–66, automated analysis of microscopy images: variable

ANTICIPATED RESULTS

This protocol enables researchers to perform multiplexed stimulation and imaging of cells in a high-throughput manner. The correct application of the protocol enables the generation of image data that convey information about single-cell dynamics under parallel input conditions extracted using automated methods (**Fig. 5a**,**b**). When combined with high-throughput gene expression technologies, such as the BioMark system, the use of the cell culture chip enables the analysis of up to 96 genes in cells under complex stimulation patterns sampled finely in time (30 min between gene expression time points over a 12-h total duration in the example detailed here) (**Fig. 5c**). Overall, this microfluidic single-cell analysis pipeline provides a new paradigm for live-cell studies, which overcomes problems with cell manipulation, precision and automation in order to unravel complex questions around dynamic responses and their heterogeneity at the single-cell level.

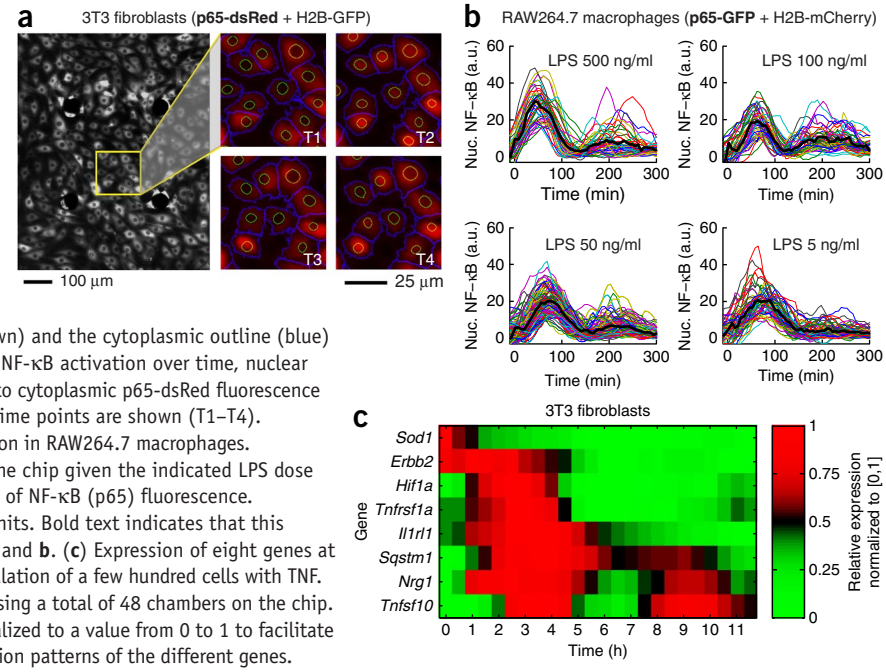

Figure 5 | Anticipated results. (**a**) Live fluorescence imaging of 3T3 fibroblast cells under TNF stimulation expressing p65-dsRed and H2B-GFP fusion proteins and automated image analysis. The left grayscale image is composed of two stitched vertical adjacent images in the chamber and shows p65-dsRed fluorescence. The four dark spots arranged in a rectangular pattern are PDMS pillars that are included in the chip design to prevent chamber collapse. In the right panel of four images, red fluorescence is due to p65-dsRed, nuclear outline (yellow) is determined from H2B-GFP image (not shown) and the cytoplasmic outline (blue) is determined from the p65-dsRed image. To quantify NF-κB activation over time, nuclear p65-dsRed fluorescence intensity is measured relative to cytoplasmic p65-dsRed fluorescence intensity. Images corresponding to four consecutive time points are shown (T1–T4). (**b**) Single-cell responses to changes in LPS concentration in RAW264.7 macrophages. Graphs correspond to cells in different chambers on the chip given the indicated LPS dose at time $T = 0$. The y axes show mean nuclear intensity of NF-κB (p65) fluorescence. Colored lines indicate different cells. a.u., arbitrary units. Bold text indicates that this fluorescence channel is displayed and quantified in **a** and **b**. (**c**) Expression of eight genes at 30-min time points after on-chip stimulation of a population of a few hundred cells with TNF. Two chambers comprised each of the 24 time points, using a total of 48 chambers on the chip. Values are relative expression ($2^{Ct\_gapdh - Ct\_gene}$)-normalized to a value from 0 to 1 to facilitate a heat map display for comparing the temporal expression patterns of the different genes.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS We acknowledge S. Quake for supervising earlier stages of this work. We thank M. Covert and his group for useful discussions and for making an earlier version of the image analysis software available, as well as for the gift of 3T3 cells with the p65-DsRed fusion protein. This work was supported by a European Research Council (ERC) starting grant and a Swiss National Science Foundation grant to S. Tay.

AUTHOR CONTRIBUTIONS R.A.K. optimized the protocol and developed methods for cell retrieval and gene expression analysis, and wrote the manuscript. R.G-.S. and A.A.L. are the original developers of the microfluidic cell culture chip and software. S.T. optimized the protocol for cell signaling studies and supervised the signaling project. All authors edited the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

1. Fernandez-Suarez, M. & Ting, A.Y. Fluorescent probes for super-resolution imaging in living cells. *Nat. Rev. Mol. Cell Biol.* **9**, 929–943 (2008).
2. Tay, S. *et al.* Single-cell NF-κB dynamics reveal digital activation and analogue information processing. *Nature* **466**, 267–271 (2010).
3. Albeck, J.G., Mills, G.B. & Brugge, J.S. Frequency-modulated pulses of ERK activity transmit quantitative proliferation signals. *Mol. Cell* **49**, 249–261 (2013).
4. You, X. *et al.* Intracellular protein interaction mapping with FRET hybrids. *Proc. Natl. Acad. Sci. USA* **103**, 18458–18463 (2006).
5. Reits, E.A. & Neefjes, J.J. From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell Biol.* **3**, E145–147 (2001).
6. Kim, S.A., Heinze, K.G. & Schwille, P. Fluorescence correlation spectroscopy in living cells. *Nat. Methods* **4**, 963–973 (2007).
7. Muramoto, T. *et al.* Live imaging of nascent RNA dynamics reveals distinct types of transcriptional pulse regulation. *Proc. Natl. Acad. Sci. USA* **109**, 7350–7355 (2012).
8. Delebecque, C.J., Lindner, A.B., Silver, P.A. & Aldaye, F.A. Organization of intracellular reactions with rationally designed RNA assemblies. *Science* **333**, 470–474 (2011).
9. Yunger, S., Rosenfeld, L., Garini, Y. & Shav-Tal, Y. Quantifying the transcriptional output of single alleles in single living mammalian cells. *Nat. Protoc.* **8**, 393–408 (2013).
10. Puchner, E.M., Walter, J.M., Kasper, R., Huang, B. & Lim, W.A. Counting molecules in single organelles with superresolution microscopy allows tracking of the endosome maturation trajectory. *Proc. Natl. Acad. Sci. USA* **110**, 16015–16020 (2013).
11. Shim, S.H. *et al.* Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. *Proc. Natl. Acad. Sci. USA* **109**, 13978–13983 (2012).
12. Spiller, D.G., Wood, C.D., Rand, D.A. & White, M.R. Measurement of single-cell dynamics. *Nature* **465**, 736–745 (2010).
13. Germain, R.N., Robey, E.A. & Cahalan, M.D. A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* **336**, 1676–1681 (2012).
14. Kulesa, P.M. & Fraser, S.E. Cell dynamics during somite boundary formation revealed by time-lapse analysis. *Science* **298**, 991–995 (2002).
15. Cai, L., Dalal, C.K. & Elowitz, M.B. Frequency-modulated nuclear localization bursts coordinate gene regulation. *Nature* **455**, 485–490 (2008).
16. Neumann, B. *et al.* High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods* **3**, 385–390 (2006).
17. Junkin, M. & Tay, S. Microfluidic single-cell analysis for systems immunology. *Lab. Chip* **14**, 1246–1260 (2014).
18. Levskaya, A., Weiner, O.D., Lim, W.A. & Voigt, C.A. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature* **461**, 997–1001 (2009).
19. Bugaj, L.J., Choksi, A.T., Mesuda, C.K., Kane, R.S. & Schaffer, D.V. Optogenetic protein clustering and signaling activation in mammalian cells. *Nat. Methods* **10**, 249–252 (2013).
20. Grier, D.G. A revolution in optical manipulation. *Nature* **424**, 810–816 (2003).
21. Milias-Argeitis, A. *et al. In silico* feedback for *in vivo* regulation of a gene expression circuit. *Nat. Biotechnol.* **29**, 1114–1116 (2011).
22. Gómez-Sjöberg, R., Leyrat, A.A., Pirone, D.M., Chen, C.S. & Quake, S.R. Versatile, fully automated, microfluidic cell culture system. *Anal. Chem.* **79**, 8557–8563 (2007).
23. Vedel, S., Tay, S., Johnston, D.M., Bruus, H. & Quake, S.R. Migration of cells in a social context. *Proc. Natl. Acad. Sci. USA* **110**, 129–134 (2013).
24. Sanchez-Freire, V. *et al.* Microfluidic single-cell real-time PCR for comparative analysis of gene expression patterns. *Nat. Protoc.* **7**, 829–838 (2012).
25. Cheong, R., Wang, C.J. & Levchenko, A. High-content cell screening in a microfluidic device. *Mol. Cell. Proteom.* **8**, 433–442 (2009).

26. Cheong, R., Wang, C.J. & Levchenko, A. Using a microfluidic device for high-content analysis of cell signaling. *Sci. Signal.* **2**, pl2 (2009).
27. Cheong, R., Rhee, A., Wang, C.J., Nemenman, I. & Levchenko, A. Information transduction capacity of noisy biochemical signaling networks. *Science* **334**, 354–358 (2011).
28. Frank, T. & Tay, S. Flow-switching allows independently programmable, extremely stable, high-throughput diffusion-based gradients. *Lab. Chip* **13**, 1273–1281 (2013).
29. Hung, P.J., Lee, P.J., Sabounchi, P., Lin, R. & Lee, L.P. Continuous perfusion microfluidic cell culture array for high-throughput cell-based assays. *Biotechnol. Bioeng.* **89**, 1–8 (2005).
30. Chung, K., Rivet, C.A., Kemp, M.L. & Lu, H. Imaging single-cell signaling dynamics with a deterministic high-density single-cell trap array. *Anal. Chem.* **83**, 7044–7052 (2011).
31. Faley, S.L. *et al.* Microfluidic single cell arrays to interrogate signalling dynamics of individual, patient-derived hematopoietic stem cells. *Lab. Chip* **9**, 2659–2664 (2009).
32. Roach, K.L. *et al.* High-throughput single-cell bioinformatics. *Biotechnol. Prog.* **25**, 1772–1779 (2009).
33. Melin, J. & Quake, S.R. Microfluidic large-scale integration: the evolution of design rules for biological automation. *Ann. Rev. Biophys. Biomol. Str.* **36**, 213–231 (2006).
34. Vollmers, C., Sit, R.V., Weinstein, J.A., Dekker, C.L. & Quake, S.R. Genetic measurement of memory B-cell recall using antibody repertoire sequencing. *Proc. Natl. Acad. Sci. USA* **110**, 13463–13468 (2013).
35. Kalisky, T. & Quake, S.R. Single-cell genomics. *Nat. Methods* **8**, 311–314 (2011).
36. Ottesen, E.A., Hong, J.W., Quake, S.R. & Leadbetter, J.R. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science* **314**, 1464–1467 (2006).
37. Warren, L., Bryder, D., Weissman, I.L. & Quake, S.R. Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. *Proc. Natl. Acad. Sci. USA* **103**, 17807–17812 (2006).
38. Qin, D., Xia, Y. & Whitesides, G.M. Soft lithography for micro- and nanoscale patterning. *Nat. Protoc.* **5**, 491–502 (2010).
39. Lecault, V. *et al.* High-throughput analysis of single hematopoietic stem cell proliferation in microfluidic cell culture arrays. *Nat. Methods* **8**, 581–586 (2011).
40. Unger, M.A., Chou, H.P., Thorsen, T., Scherer, A. & Quake, S.R. Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* **288**, 113–116 (2000).
41. Thorsen, T. Microfluidic large-scale integration. *Science* **298**, 580–584 (2002).
42. Berthier, E., Young, E.W. & Beebe, D. Engineers are from PDMS-land, biologists are from Polystyrenia. *Lab. Chip* **12**, 1224–1237 (2012).
43. Millet, L.J., Stewart, M.E., Sweedler, J.V., Nuzzo, R.G. & Gillette, M.U. Microfluidic devices for culturing primary mammalian neurons at low densities. *Lab. Chip* **7**, 987–994 (2007).
44. Kolnik, M., Tsimring, L.S. & Hasty, J. Vacuum-assisted cell loading enables shear-free mammalian microfluidic culture. *Lab. Chip* **12**, 4732–4737 (2012).
45. Landenberger, B., Hofemann, H., Wadle, S. & Rohrbach, A. Microfluidic sorting of arbitrary cells with dynamic optical tweezers. *Lab. Chip* **12**, 3177–3183 (2012).
46. Wall, E.A. *et al.* Suppression of LPS-induced TNF-α production in macrophages by cAMP is mediated by PKA-AKAP95-p105. *Sci. Signal* **2**, ra28 (2009).