Supplementary Information

Integrated platform for cell culture and dynamic quantification of cell secretion

Alicia J. Kaestli, Michael Junkin, Savaş Tay

Supplementary materials and methods:

Device fabrication

The device design and fabrication protocol was previously described in detail (Kellogg et al., 2014). In brief, the chip was fabricated using standard multilayer soft lithography (Unger et al., 2000). The chips were designed in AutoCAD (Autodesk Inc., USA), and printed at 40,000 dpi onto transparencies (Fine Line Imaging, Minneapolis, USA). Photolithography was used to make the control and flow molds on 4-inch silicon wafers (Figure 2a). The control mold was made with SU-8 3025 (MicroChem, USA) spun at 3000 rpm to a height of 25 μm. The flow mold was made with AZ 50XT (MicroChem, USA) spun at 4500 rpm and then reflowed, and with SU8-3010 (MicroChem, USA) spun at 3000 rpm. The final height of the AZ 50XT and SU-8 3010 on the flow mold was 15 μm and 10 μm, respectively. The reflow process consisted of heating the wafer up to 200°C and maintaining the temperature for a total of 13 hours to hard bake the resist after reflow. This process caused the reflowing of AZ 50XT into rounded channels (Unger et al., 2000).

To produce polydimethylsiloxane (PDMS) chips from the silicon molds, the molds were first coated with chlorotrimethylsilane (92360, Sigma-Aldrich Chemie GmbH, Switzerland) for 15 minutes in a fume hood. This coating renders the molds non-stick to PDMS. Then, 69 g of a 10:1 mixture of PDMS (RTV-615, Momentive Specialty Chemicals Inc., USA) was cast onto the control layer and degassed. The flow mold was spun with 10:1 PDMS at 2800 rpm for 1 minute. Both layers were then baked at 80°C for 45 minutes. The control layer was pealed off the silicon mold, punched with a 710 μm inner diameter biopsy punch (CR0350255N20R4, Syneoco, USA), aligned, bonded to the flow layer via plasma treatment for 15 seconds at 45W (Femto #112296, Diener electronic GmbH + Co. KG, Germany), and baked for 2 hours at 80°C. The input and output holes on the aligned PDMS chip was then punched using the same 710 μm inner diameter biopsy punch. A glass slide (1100020, Biosystems Switzerland AG, Switzerland) was cleaned via a 5 minute sonication with ultra pure water. The aligned PDMS layers were bonded to glass via plasma treatment, and baked overnight at 80°C to strengthen bonding.

Antibody spot patterning

The antibody spot patterning protocol was fully automated. No operator input was needed after the reagents were connected to the device. The script run for the antibody patterning of the device is described below.

To begin patterning, biotinylated BSA (29130, Thermo Fisher Scientific) at a concentration of 2 mg/mL in distilled water was flowed through the antibody spot chambers (Figure 3bii). All button valves were opened, and the cell chambers were closed. Every 10 minutes, biotinylated BSA was flowed into all antibody spot chambers for 5 minutes. Next, flow was stopped, and the solution was incubated in the chambers for 5 minutes. This process was continued for a total of 90 minutes. All button valves were

closed, and the antibody spot chambers were then washed with 0.05% PBS Tween (P7949, Sigma-Aldrich Chemie GmbH, Switzerland) for 20 minutes. Then, the same protocol was repeated with 1 mg/mL neutravidin (3100, Thermo Fisher Scientific) dissolved in PBS (Figure 3biii).

Next, the button valves were closed, and the surrounding neutravidin was passivated by flowing 2 mg/mL biotinylated BSA through the antibody spot chambers in the chip (Figure 3biv). Every 10 minutes, biotinylated BSA was flowed into all antibody spot chambers for 5 minutes. Next flow was stopped, and the solution was incubated in the chambers for 5 minutes. This process was continued for a total of 90 minutes. Afterwards, the antibody spot chambers were then washed with 0.05% PBS Tween for 20 minutes. After this step, there are now neutravidin spots specifically underneath the button valves. Next, the same protocol was repeated with starting block buffer (37578, Thermo Fisher Scientific, USA).

Subsequently, the antibodies were patterned onto the neutravidin spots (Figure 3bv). Here, it is possible to either stop the script, and connect a cold and freshly prepared antibody solution to the chip, or continue running the script with the antibody solution having been plugged into the chip at the beginning of the experiment. No difference was found in connecting freshly prepared solution or running the script antibody plugged into the chip from the start of the patterning protocol. Button valves were opened, and biotinylated anti-TNF (T9160-14, US Biological, USA) at a concentration of 7.5 µg/mL in PBS was flowed through the antibody spot chambers in the chip. Every 10 minutes, biotinylated-BSA was flowed into all antibody spot chambers for 5 minutes. Next, flow was stopped, and the solution was incubated in the chambers for 5 minutes. This process was continued for a total of 120 minutes. Then, all button valves were closed, and the antibody spot chambers were washed with 0.05% PBS Tween for 20 minutes. The antibody spots are now patterned underneath the button valves (Figure 3bvi).

As a final step, the antibody spots are blocked using starting block buffer. Specifically, starting block buffer was flowed through the antibody spot chambers in the chip. All button valves were opened, and the cell chambers were closed. Every 10 minutes, buffer was flowed into all antibody spot chambers for 5 minutes. Next, flow was stopped, and the solution was incubated in the chambers for 5 minutes. This process was continued for a total of 90 minutes. All button valves were closed, and the antibody spot chambers were then washed with 0.05% PBS Tween for 20 minutes.

At the end of the antibody patterning protocol, the antibody chambers were additionally washed with PBS for 20 minutes. To prevent pressure buildup in the antibody spotting chamber from water diffusion through the PDMS (Garcia-Cordero et al., 2013), the valves adjacent to the antibody spotting chamber and waste port were opened every 90 minutes to relieve the built up pressure.

Step	Reagent	Product Information	Button valve opened or closed	Total time in antibody spot chamber
1	2 mg/mL Biotinylated BSA	29130, Thermo Fisher Scientific, USA	Open	90 minutes
2	1 mg/mL Neutravidin	3100, Thermo Fisher Scientific, USA	Open	90 minutes
3	2 mg/mL Biotinylated BSA	29130, Thermo Fisher Scientific, USA	Closed	90 minutes
4	Starting block buffer	37578, Thermo Fisher Scientific, USA	Closed	90 minutes
5	7.5 μg/mL Biotinylated anti-TNF	T9160-14, US Biological, USA	Open	120 minutes
6	Starting block buffer	37578, Thermo Fisher Scientific, USA	Open	90 minutes

Table S1: Summary of antibody patterning protocol. There are 6 steps in the antibody spotting protocol. For each step, the reagent and product information are noted. Additionally, it is marked if the button valves were opened or closed during each step of the protocol. Finally, it is indicated for how long each reagent remained in the antibody spot chamber. During this time, flow of 5 minutes, and an incubation of 5 minutes were repeated for each reagent for the time indicated in the last column of the table.

Antibody spot validation

To validate the neutravidin spot underneath button valves (Fig 3ci), steps 1 through 4 in Table 1 were implemented. Then, 2 μg/mL of atto 565-biotin (92636, Sigma-Aldrich Chemie GmbH, Switzerland) in PBS was incubated in the antibody spot chamber for 90 minutes. After incubation, all button valves were closed, and the antibody spot chambers were then washed with 0.05% PBS Tween for 20 minutes.

To validate the antibody spot underneath button valves (Fig 3cii), a fluorescently labeled antibody specific to the patterned anti-TNF antibody was added to the chamber. This allowed visualization of the location of the anti-TNF antibody spots. To accomplish this, steps 1 through 5 of Table 1 were first implemented. Next, to test if the biotinylated anti-TNF antibody was successfully patterned underneath the button valves, a fluorescent antibody against the patterned antibody was added to the antibody spot chambers. Specifically, 5 μg/mL of Alexa 488 Goat Anti-Rat IgG (H+L) (A-11006, Thermo Fischer Scientific, USA) in PBS was flowed into the antibody spot chambers for 5 minutes, and then mixed over a revealed antibody spot for 90 minutes. After the incubation, all button valves were closed, and the antibody spot chambers were then washed with 0.05% PBS Tween for 20 minutes. The fluorescence of the spots was now imaged to quantify the consistency of the anti-TNF antibody patterning.

To validate the binding of an antigen to an antibody (Fig 3ciii), steps 1 through 4 in Table 1 were implemented. Then, step 5 was completed using 2 μg/mL of biotinylated anti-GFP antibody (ab6658, Abcam, USA) in PBS. Next, 16 μg/mL of enhanced green fluorescent protein (eGFP) (4999-100, BioVision, USA) in PBS was incubated over the antibody spots, via 5 minutes flow followed by 5 minutes incubation, repeated for a total of 90 minutes. After, all button valves were closed, and the antibody spot chambers were washed with 0.05% PBS Tween for 20 minutes. The fluorescence of the spots was now imaged to quantify antigen binding to the pattered antibody spots.

Converting fluorescence intensity to TNF molecules

To convert the fluorescence intensity measured to a number of TNF molecules, the maximum likelihood function and variability for each fluorescent value was calculated (Fig S4). First, a normalized probability function was calculated using the mean and standard deviation of data points in the calibration curve (Fig. S4a). Next, the maximum likelihood and standard error for each fluorescent value in the normalized probability function was computed (Fig. S4b). Additionally, the limit of detection of the assay was calculated as the sum of the mean fluorescence intensity of media and three times the standard deviation of the mean. If a measured fluorescence intensity value is below the limit of detection, this intensity represents zero TNF molecules with the standard error of the fluorescence intensity. In contrast, if a measure fluorescence intensity is above the limit of detection, the intensity represents the maximum likelihood number of TNF molecules and standard error for that intensity.

Cell culture

Cell culture of the RAW 264.7 cells was previously described (Junkin et al., 2016). No further changes to this protocol were made. The number of cells seeded into a cell culture chamber is controlled by (1) the concentration of cells flowed into the device, (2) the amount of time cells are flowed into the device, and (3) the presence of a cell trap. The cell trap has the same design as the trap used in our previous paper (Junkin et al., 2016). With the trap, and certain loading densities, we could achieve single cell resolution in our chip (Figure S7). The secretion of the single cell reported is within an order of magnitude of single cell secretion measured in our previously published device (Junkin et al., 2016). Specifically, to seed cells into the chip, a solution of 10^5 cell mL^{-1} was prepared. After flowing fresh media through the cell chambers to wash out unbound fibronectin, the cell solution was flowed into the cell chambers. A video showing the flow of cell through the cell chamber, and eventual trapping of two cells is shown in Movie S3. Cells were flowed into the chip for around 10 minutes or until the desired confluency of cells in each cell chamber was achieved. Then, flow was stopped, and cells outside of the cell chamber were washed away with media.

Throughout experiments, cell viability was tracked by looking at membrane integrity by imaging the cells every 5 minutes. From these videos, we could determine the viability of cells as cells having intact membranes. We identified a cell as dead if it either (1) no longer had an intact membrane or (2) had no change in shape for at least 30 minutes. An example image of cells at the beginning and end of the experiments is shown in Figure S7.

Supplementary figures:

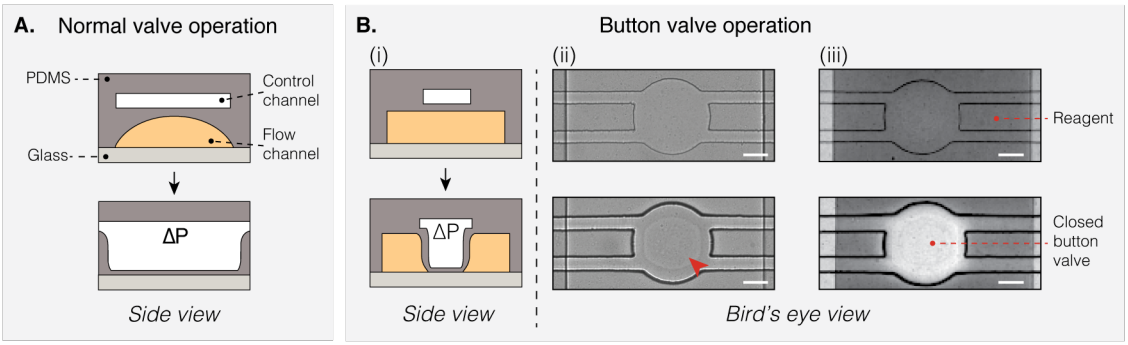

Fig. S1 Differences in conventional and button PDMS valve operation. (A) In a traditional pneumatic PDMS valve, the control channel is at least the same width as the flow channel. When the control line is pressurized, the thin PDMS membrane between the control and flow layers is defected to the bottom of the flow channel. Thus, reagent flow through the flow channel is stopped. (B) (i) In button valve operation, the control channel is narrower than the flow channel (Garcia-Cordero and Maerkl, 2013; 2014; Garcia-Cordero et al., 2013; Maerkl and Quake, 2007). Thus, when the control line is pressurized, only a small surface of the flow channel is blocked from the reagent. (ii) An image of an open (top) and closed (bottom) microfluidic button valve. The surface blocked by the valve is indicated with an arrow. In these images, the flow channel is filled with a transparent reagent (PBS). (iii) The flow channel is filled with food dye (dark regions in image). When the button valve is open (top) food dye can flow below the valve. In contrast, when the button valve is closed, the food dye is pushed away, and the surface below the valve is absent of food dye. Thus, the light patch shows where the button valve is closed. Scale bar is 50 μm.

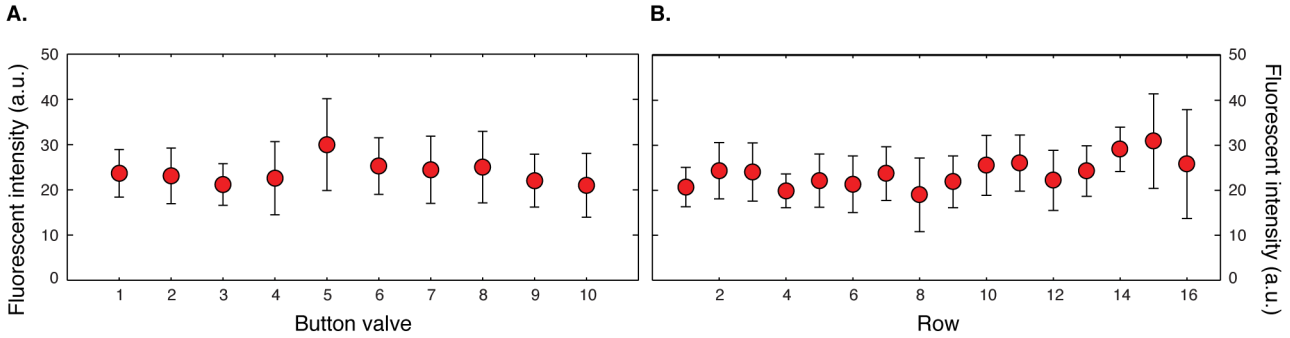

Fig. S2 Variation in capture antibody density on antibody spots. Capture antibodies were patterned on the chip. After patterning, a fluorescent antibody against the capture antibody was added to visualize how much capture antibody was present on each spot. No significant difference in capture antibody density was observed between button valves or rows. (A) The variation in capture antibody density across the 10 button valve control lines. Button valve 1 refers to the antibody spots closest to the cell chamber (left most spots Figure 2a), whereas antibody spot 10 refers to the spots closest to the waste outlet (right most spots Figure 2a). (B) The variation in capture antibody density across the 16 rows in the chip. Row 1 refers to the top row close to high density of control line inlets (top of chip in Figure 2a), whereas row 16 refers to the row closest to the input inlets (bottom of chip in Figure 2a). Error bars indicate standard deviation.

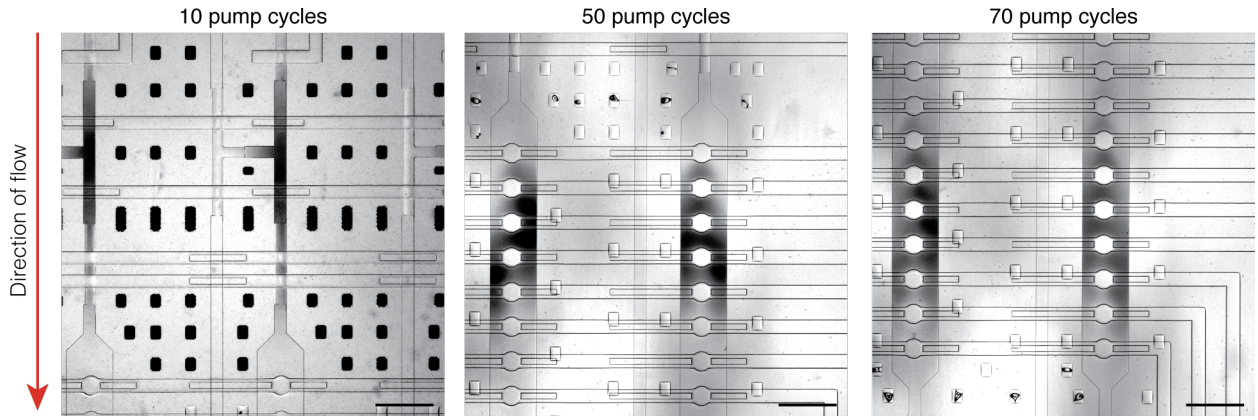

Fig. S3 Variation in peristaltic media transfer pump parameters. Steps 1 and 2 of the fluid transfer steps shown in Figure 4a were tested using different parameters for the media transfer pumping. Cytokine media was represented with food dye (dark fluid in channels). Fresh media was represented with PBS (clear fluid in channels). A pump cycle is defined as the actuation of one 120° pattern, as defined in Unger et al (2000). In brief, a pattern of 6 valve configurations was used over 3 valves. Specifically, the pattern 010, 011, 001, 101, 100, 110 was used. Here, 0 refers to a closed valve, and 1 refers to an open valve. The total pattern (one pump cycle) in the left-most picture was repeated 10 times. An image was taken at the end of each pumping script with the specified number of pump cycles above each photo. The pumping parameter used for cell and calibration experiments was 50 pump cycles. Scale bar is 500 μm.

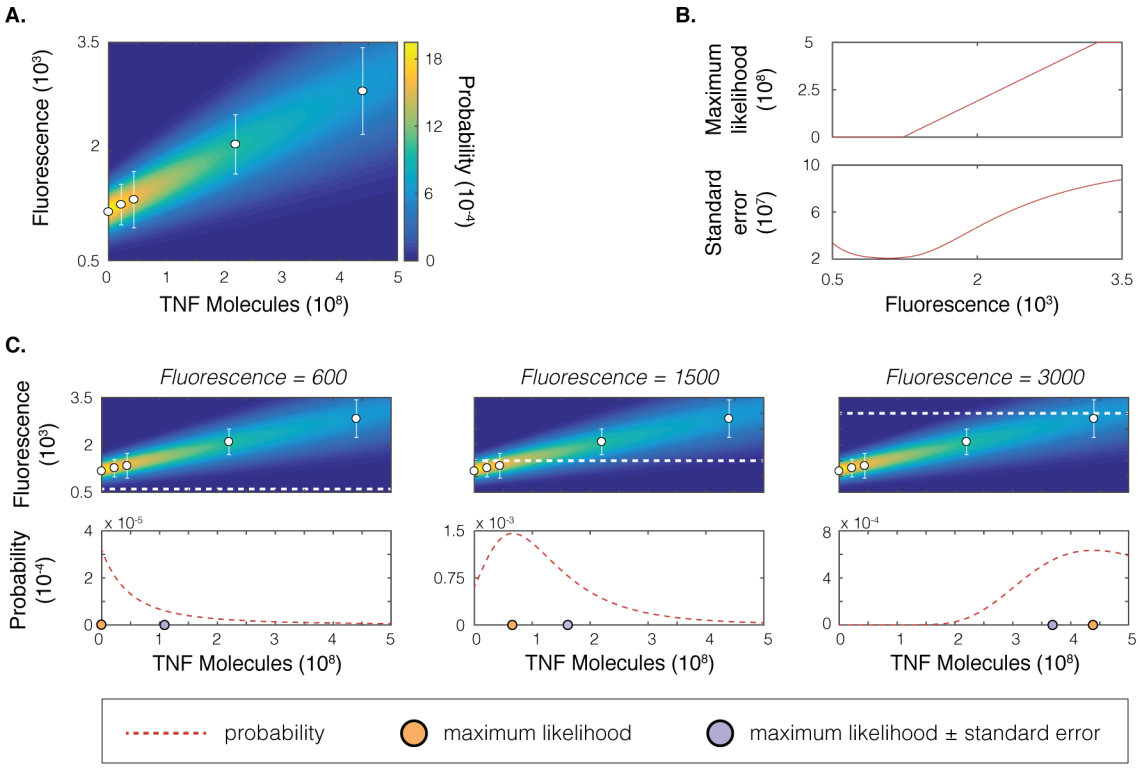

Fig. S4 Quantification of TNF secretion. (A) The normalized probability function is plotted as a heat map. The function is derived from the mean and standard deviation of the calibration curve. The calibration curve is plotted as white circles. Error bars represent standard deviation. (B) The maximum likelihood and standard error for fluorescence values are calculated. (C) For three different fluorescence values, the probability is plotted. These examples show how the maximum likelihood and standard error values in (B) were calculated. The maximum likelihood was calculated as the number of TNF molecules with the maximum probability, for a given fluorescence value.

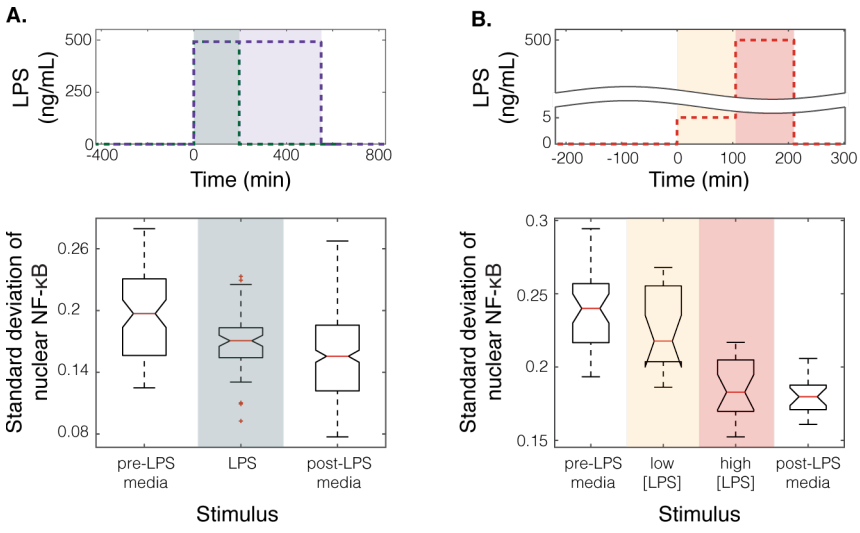

Fig. S5 Variability in macrophage cellular NF-κB response. The top plots show the stimulus conditions the cells were exposed to. The graph plots the standard deviation of nuclear activity of all single cells during each indicated simulation condition. A high standard deviation indicates higher cell-to-cell variability. Specifically, a higher standard deviation indicates higher variability around the average cellular NF-κB response plotted in Figures 6a and 6b. The notches in the bar plots represent the 95% confidence interval the standard deviation of the cellular NF-κB response during each stimulus. (A) The standard deviation of the cellular NF-κB response due to a single LPS pulse. This stimulus mimics a chronic inflammatory response. The cellular variability of cells exposed to a 3 hour (green dashed line) and 9 hour (purple dashed line) were studied as a single group. Cellular variability was highest in during the first media stimulation, and decreased during the first LPS stimulation. (B) The standard deviation of the cellular NF-κB response due to LPS ramp (orange dashed line). This stimulus mimics and increasing inflammatory condition. Variability was highest during the first media stimulation, and decreased during the stimulation with a high concentration of LPS.

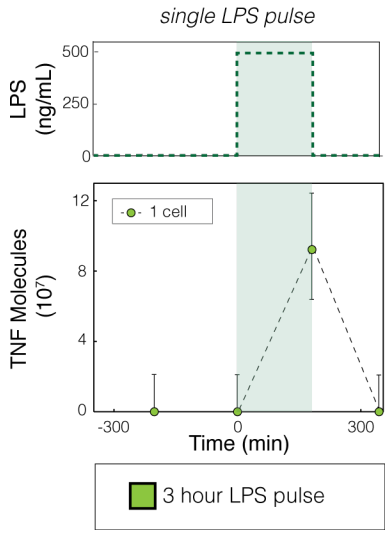

Fig. S6 The TNF secretion response of a single RAW 264.7 cell to a 3 hour LPS pulse. The white and green background indicates when the cell was exposed to media and 500 ng/mL LPS, respectively.

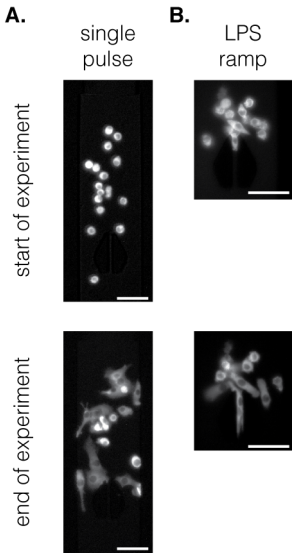

Fig. S7 Morphology of RAW 264.7 cells at the start and end of an experiment. (A) The morphology of RAW 264.7 cells at the start of a single pulse 500 ng/mL LPS experiment. The cellular NF-κB response is shown in Figure 5d. (B) The morphology of RAW 264.7 cells at the start of an LPS ramp experiment. The cellular NF-κB response is shown in Figure 5e. The scale bar is 50 μm.

Movie S1 Mixing of food dye in an antibody spot chamber. This movie illustrates step 3 of Figure 4a. Here, the antibody spot chamber is filled with cytokine filled media, represented by food dye (dark liquid) and PBS. The mixing pump is used to mix the contents of the antibody spot chamber. A button valve is open, and thus the cytokine media is in contact with an exposed antibody spot. Scale bar is 500 μm.

Movie S2 Time needed to introduce a new reagent in the cell chamber. The movie shows the time needed to introduce a new stimulus into the cell chamber. The cell chambers were filled with water, and food dye was flowed into the chip from an inlet port. It took ~3.5 seconds for the food dye to travel from the input port to the cell chamber. Scale bar is 500 μm.

Movie S3 Cells being seeded into the cell chamber. Cells are flowed into the cell chamber until the desired confluency is reached. Cells are trapped by a cell trap. The valves surrounding the cell trap were closed at time 20.6 seconds. The scale bar is 20 μm.

References:

Garcia-Cordero, J.L., Nembrini, C., Stano, A., Hubbell, J.A., and Maerkl, S.J. (2013). A high-throughput nanoimmunoassay chip applied to large-scale vaccine adjuvant screening. Integr Biol (Camb) *5*, 650–658.

Junkin, M., Kaestli, A.J., Cheng, Z., Jordi, C., Albayrak, C., Hoffmann, A., and Tay, S. (2016). High-Content Quantification of Single-Cell Immune Dynamics. Cell Rep *15*, 411–422.

Kellogg, R.A., Gómez-Sjöberg, R., Leyrat, A.A., and Tay, S. (2014). High-throughput microfluidic single-cell analysis pipeline for studies of signaling dynamics. Nat Protoc *9*, 1713–1726.

Unger, M.A., Chou, H.P., Thorsen, T., Scherer, A., and Quake, S.R. (2000). Monolithic microfabricated valves and pumps by multilayer soft lithography. Science *288*, 113–116.