Electronic Supplementary Material (ESI) for Lab on a Chip.

SUPPLEMENTARY INFORMATION

SI 1: Cell size measurement using impedance cytometry

Impedance cytometry is a useful non-invasive technology that allows to monitor different cell properties, such as cell size, stiffness and deformability[1,2].

Measurements were performed at the Centre for Hybrid Biodevices, Electronics and Computer Sciences, University of Southampton, using the setup described elsewhere[3]. The flow was set to 10 µl/min, and 5 µm and 7 µm polystyrene calibration beads were used to build the calibration curve. The module of the electric impedance is proportional to the volume of the cell, hence its cubic root is proportional to the cell diameter; the proportionality factor was determined from the calibration beads, that have known sizes.

Figure 1 shows the histograms of the diameters of the two cell populations used in the experiments: B3Z, T cells, and K89, antigen presenting cells. The graphs were obtained from 3 samples for each cell line, and fitted with a Gaussian curve on GraphPad Prism. Average diameters calibrated against polystyrene beads were found to be 12.84 ± 1.53 µm for B3Z (3 samples, 254 cells) and 12.37 ± 1.18 µm for K89 (3 samples, 725 cells).

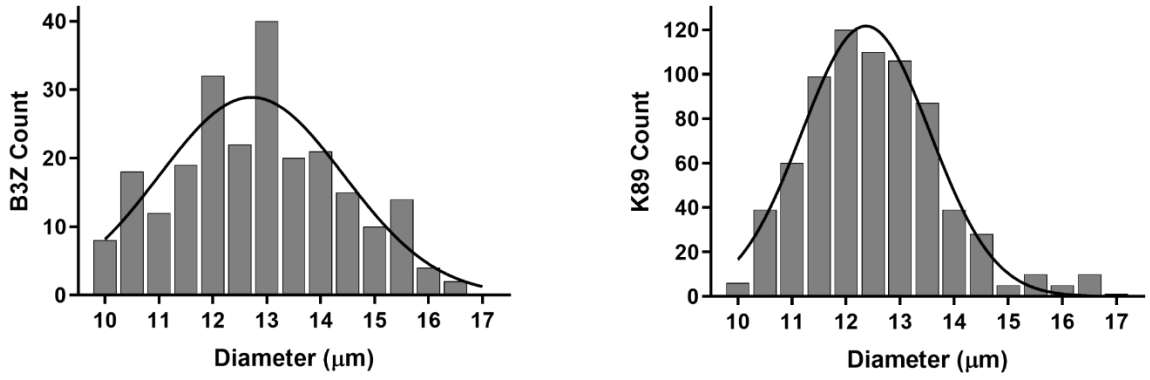

Figure 1 Cells size characterization using single cell microfluidic impedance cytometry. Cells diameters are proportional to the cubic root of the impedance module, and can be calibrated with commercial beads of known sizes. B3Z sizes (254 cells, 3 samples) were fitted with a Gaussian of amplitude 28.87, mean 12.72 µm and SD 1.709. K89 sizes (725 cells, 3 samples) were fitted with a Gaussian of amplitude 121.7, mean 12.37 µm and SD 1.185.

SI 2: Dilution subcloning and sensitivity assay

Initially, sensitive subpopulations of the T cell line were selected using dilution subcloning, and periodically B3Z sensitivity to the SIINFEKL peptide was measured. B3Z expresses the Escherichia coli lacZ reporter gene for β-galactosidase under control of the IL-2 promoter. Therefore activation via the T cell receptor in response to low concentrations of SL8 (<1 pM) bound to the H-2K^b complexes of K89 cells[4] can be measured using a chromogenic substrate (CPRG) of β-galactosidase.

B3Z subcloning was carried out by diluting cells to a statistical concentration of 5 cells/ml and adding 200 µl of the suspension to each of the 96-wells in two flat bottomed

cell culture plates (1 cell/ well). Wells positive for B3Z cell growth were harvested at day 7 of culture to test for sensitivity to SL8.

To confirm specificity of the subclones, 10^5 B3Z cells from each subclone were added to two wells containing 10^5 K89 in a total volume of 200 µl of growth media. SL8 peptide was added to one of the two wells at a final concentration of 5 nM. Cells were co-incubated overnight at 37°C, 5 % CO_2. Cells were then pelleted (290 g, 5 min, room temperature) and the supernatant was removed and replaced with 100 µl of a mixture of CPRG (CPRG; 91 mg CPRG, Roche) ,1.25 ml Nonidet-p40 (Sigma) and 9 ml 1 M MgCl2 (Sigma) per 1 litre phosphate buffered saline (PBS) for each well. The plates were left to react at room temperature, and the colour change was quantified after 6 hours using a BioRad 680 microplate reader. Readings were taken at a wavelength of 595 nm and at 695 nm as a reference. Results are depicted in Figure 2, illustrating that the wells positive to cell growth were all specific to the presentation of SL8 at the K89 surface, showing lacZ activity only in the presence of peptide.

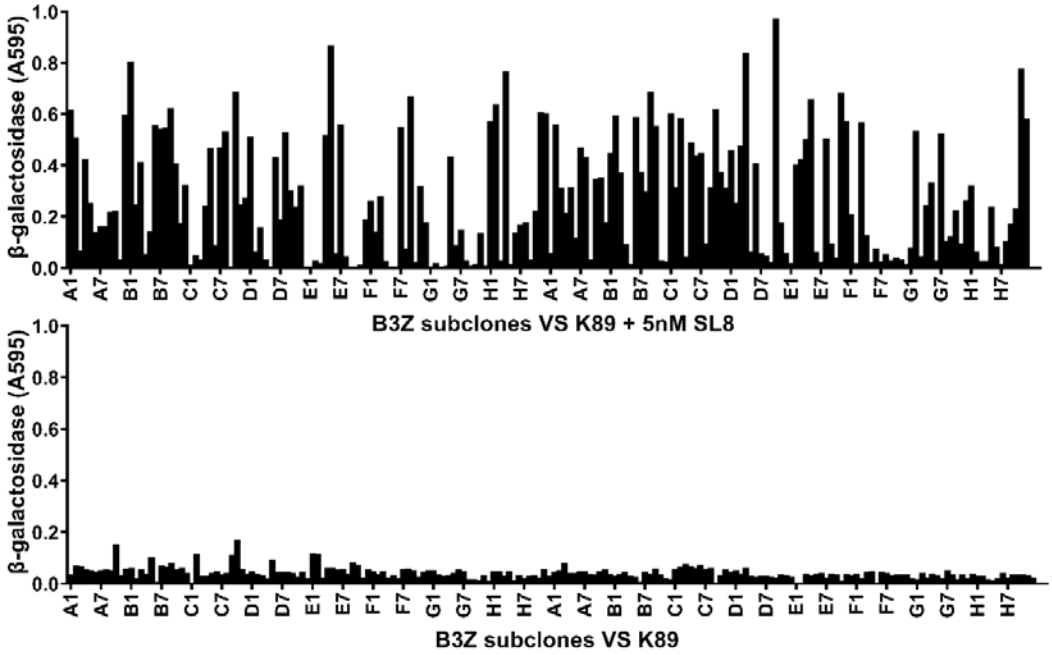

Figure 2 Results from a specificity test ran on two 96-wells plates of the B3Z subclones. Each subpopulation from a single well is depicted by a histogram column in the top diagram and the correspondent column in the bottom graph. Absorbance was measured with a microplate reader at a wavelength of 595 nm. The top graphs represent the lacZ activity of subclones in response to K89 loaded with SL8 peptide, while the bottom graphs represent the lacZ activity of subclones in response to K89 alone (control).

A selection of 5 subclones was expanded for 9 days, and a new sensitivity assay was performed. In this case, subclones were exposed to K89 (1:1 cells ratio) co-incubated with a serial dilution of peptide concentrations (10 pM to 625 nM). No peptide was added to the last well, as a control for the specificity of the readings. The same protocol previously described was followed, and results are depicted in Figure 3. As a control, an aliquot from the original B3Z population (cultured in parallel throughout the selective subcloning) was tested, showing that the selection for sensitive subclones was successful.

Dilution subcloning and sensitivity assays were periodically performed to guarantee the reliability of the cell model throughout the project.

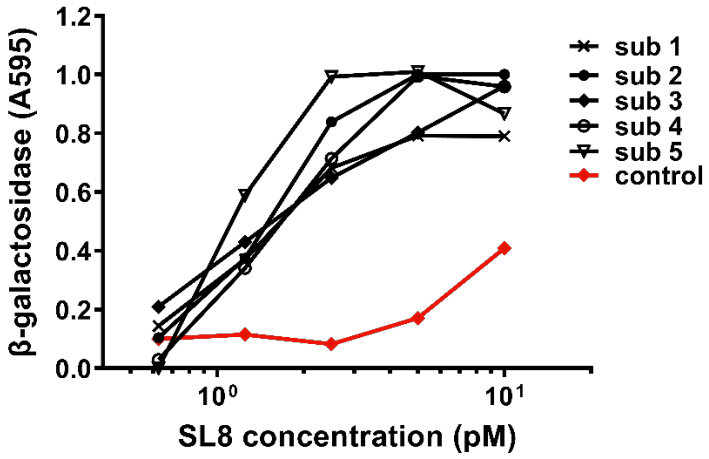

Figure 3 Sensitivity assay on five B3Z subclones. LacZ activity of the subclones is quantified in response to various peptide concentrations loaded on the K89 APCs. The control represents the starting population from which subclones were selected. It is shown how the dilution cloning method allowed to improve the sensitivity of the model system.

SI 3: Peptide presentation chase experiment

To optimize the SL8 peptide loading protocol of K89 cell line, an antibody specific for the SIINFEKL:H-2K^b complex was used (25D1.16) in flow cytometry experiments. Two sets of experiments were carried out aimed to identify the minimum incubation time for peptide loading, and the stability of the SIINFEKL:H-2K^b complex on peptide-loaded K89.

The minimum incubation time was evaluated by pulsing samples of the cell line with SIINFEKL for different times (20-40-60-80 min) before washing off the unbound epitope and tagging the H-2K^b/SL8 complexes.

The chase experiment was performed by pulsing K89 for 1 hour with SIINFEKL, washing off the unbound peptide and staining the samples at different times after the wash (0-30-60-90-120 min).

Results from both experiments are depicted in Figure 4, and showed that the minimum incubation time for optimal loading was 20 minutes, while 120 minutes after washing off unbound peptide, the exposed SL8 was already reduced by nearly 30% from the initial loading. A secondary increase that peaked 1 hour after removing the unbound peptide was noted, and is thought to arise from the loading of internalised SIINFEKL onto H-2K^b in the ER as previously demonstrated[5].

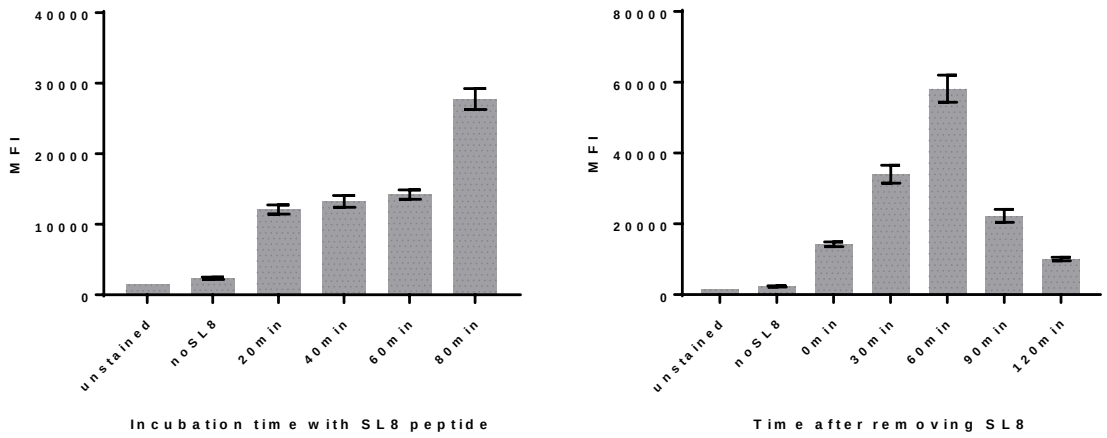

Figure 4 Antigen presentation on K89 surface over time. MFI was obtained after 25D1.16 staining, and 95% CI was calculated for each sample. In the left graph, the samples were incubated for different times (20-40-60-80 minutes) with SL8 before staining; in the right graph, all samples were incubated with the epitope for 1 hour, and signal was read at different times after washing off the unbound peptide (0-30-60-90-120 minutes). Controls: unstained K89 and stained K89 in absence of SL8 (first two columns of each graph).

SI 4: Flow cytometry B3Z stimulation assays

Flow cytometry was used to measure the bulk response of the cell population. Serial dilution experiments were performed to define the appropriate concentrations of soluble stimulants (antiCD3 and ionomycin) and antigen concentrations for the single cells experiments. Stimulation and recording protocols are reported in Methods.

As reported in literature[6], low concentrations of ionomycin (100 nM = 74.7 ng/ml) primarily act by discharging the Endoplasmic Reticulum (ER) calcium stores, while high concentrations (10 µM = 7470 ng/ml) dissipate the ion gradient across the plasma membrane. As such, the experiments were run spanning across a similar range (from 50 ng/ml to roughly 10 µg/ml). This is consistent with the concentrations tested from other groups[7–10]. The normalized percentage of fluorescence increase was determined as (MFI-MFI_0)/MFI_0*100; this was considered a better indicator compared to absolute values, to dissociate from the basal fluorescence decrease observed between samples over time. Results are shown in Figure 5a; as expected, higher concentration of ionomycin induce a greater normalized increase in fluorescence, that corresponds to a higher level of intracellular calcium. As suggested by the fit (red line), the increase seems to reach a plateau at higher ionomycin doses, as likely the intracellular calcium level tends to balance the extracellular concentration. All experiments were run in RPMI-1640 Media (Sigma-Aldrich, nominal calcium concentration of 0.42mM).

For antiCD3 stimulations, the serial dilution ranged between 100 µg/ml and 2 µg/ml (see Figure 5b). AntiCD3 soluble antibodies elicited a calcium increase with a usual delay of roughly 30 seconds. This pattern can be observed in Figure 5c, and it is likely linked to the different way antiCD3 stimulates the cells: in this case a receptor-ligand interaction needs to occur at the cell surface to initiate the activation cascade. For this reason, the peak value of the MFI after stimulation (MFI_{peak}) was selected to analyse the data.

B3Z were stimulated with K89 pulsed with different concentrations of SL8 peptide, as described in Methods and Results. An example of flow cytometry results of B3Z stimulated with K89 in presence and absence of the epitope is reported in Figure 5d, that also illustrates the data processing pipeline.

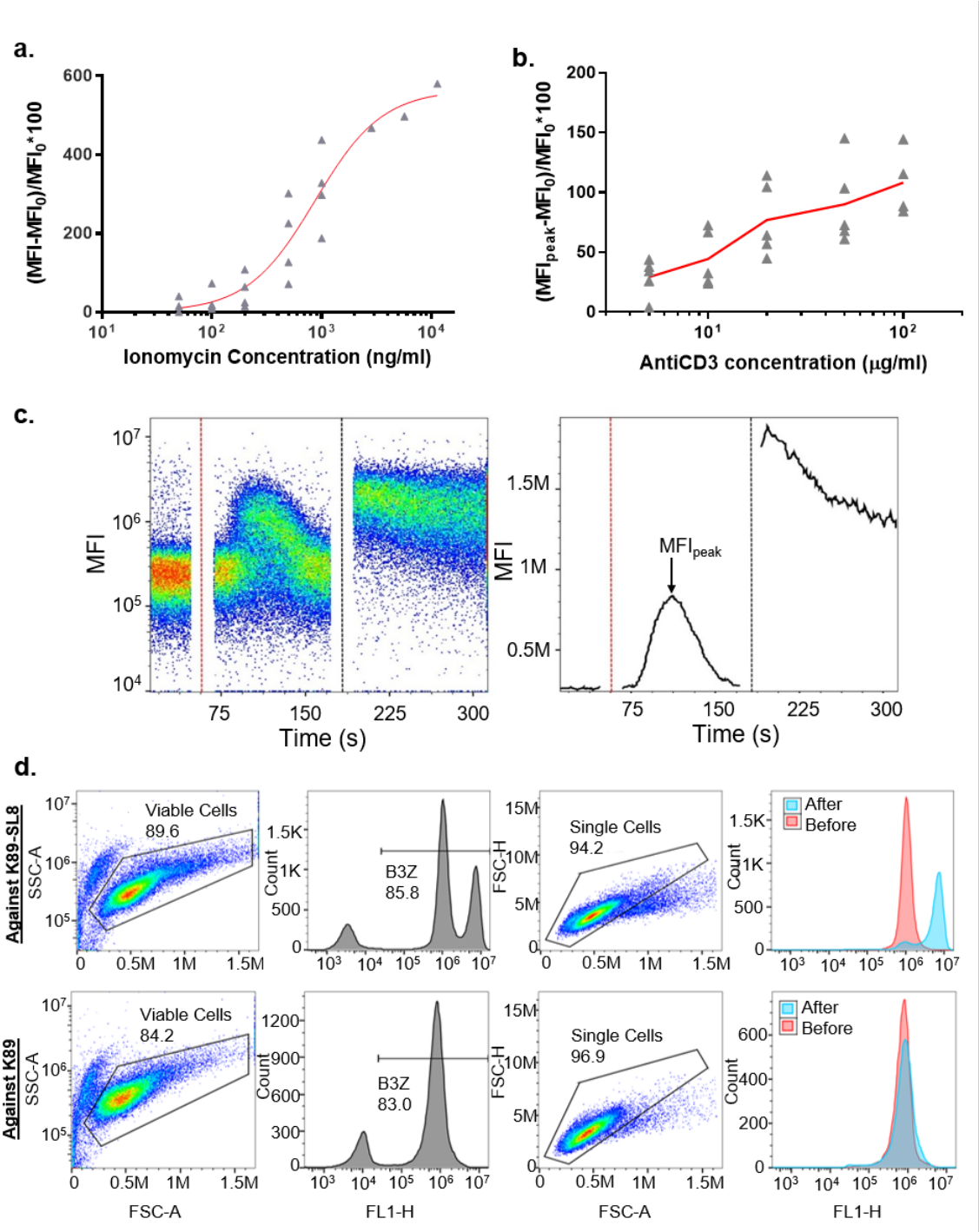

Figure 5 a) Ionomycin titration results. Each triangle indicates a tested sample; samples are merged from different experiments, comprising both technical and biological replicas. The fluorescent increase after ionomycin stimulation was normalized on the basal level of the sample before ionophore addition. The dose-response curve was fitted using the symmetrical sigmoidal function from GraphPad Prism ($R^2 = 0.90$), assuming that at high concentrations of ionomycin, the

intracellular calcium will tend to balance the ionic concentration in the buffer. b) Soluble antiCD3 titration. Each triangle indicates a tested sample; samples are merged from different experiments, comprising both technical and biological replicas. The peak of the fluorescent increase after antiCD3 stimulation was normalized on the mean basal level of fluorescence of the sample. The red line interpolates the mean values between the replicas. c) Typical time response of a B3Z population stimulated initially with antiCD3 and following with ionomycin. On the left side, the scatter plot of the population over time; on the right side, the Mean Fluorescence Intensity of the same population is represented over time. The vertical red-dotted lines mark the time of addition of 50 ng/ml soluble antiCD3; the vertical black-dotted lines highlight the instant of 1 µM (747 ng/ml) ionomycin addition. These graphs are from a single population, while being representative of the usual results obtained in several experiments. d) Representative data of B3Z biological activations obtained by spinning down the population with SL8-pulsed K89 and recording the MFI shift in flow cytometry. The top 4 graphs show the gating process on a B3Z population stimulated with K89 loaded with 10µM SL8; the bottom 4 graphs on the left show the same gates on a B3Z population exposed to K89 not loaded with peptide. In both cases, the fourth graph shows the FL1 histogram of the B3Z before (light blue) and after (red) mixing with K89.

SI 5: Master fabrication

Wafers were initially rinsed with acetone and isopropanol, dried under a nitrogen gun and left overnight to dehydrate at 200°C. TI prime (adhesion promoter, MicroChemicals GmbH) was spincoated onto the wafer at 500 rpm for 5 s/ 3000 rpm for 30 s/ 100 rpm for 5 s, followed by a 2 minutes bake at 120°C. SU8-3025 negative photoresist (MicroChem Ltd) was spincoated at 500 rpm for 5 s/ 2100 rpm for 30 s/ 500 rpm for 5 s, followed by a soft bake at 65°C for 5 minutes and at 95°C for 15 minutes. The wafer was then exposed to UV light to give a 150 mJ/cm^2 dose, followed by a post-exposure bake at 65°C for 5 minutes and at 95°C for 10 minutes. Master features were developed rinsing in EC solvent (Microposit™) for 4.5 minutes, followed by a final wash in isopropanol for 1 minute. Feature profiles were checked at the profilometer, and the master was eventually hard-baked for 2 min at 150°C.

SI 6: Optimization of wells occupancy

Wells height was optimized in order to trap high numbers of single B3Z. Masters with various thicknesses of SU8 were developed, allowing to cast wells of different heights. B3Z single occupancies were quantified in each case, and results are shown in Figure 6a. Wells ranging between 17 and 27 µm gave satisfactory results.

Seeding cell concentration was also assessed by incubating different cell numbers on top of several agarose plates (27 µm wells). All incubations lasted 30 minutes, and 2 plates were tested for each concentration. For each plate, images of 3 different arrays were taken and processed using ImageJ. The counts were run each on one entire spot of 4200 wells. Results are reported in Figure 6b. Good occupancies were observed starting from 5:1 cell:well ratio. Using cell concentrations of 10:1 or above, 90% occupancies were achieved. An excessive increase in the cell numbers resulted in greater chances of double occupancies (despite the limit given by the exclusion geometry), and washing out all the cells that didn't settle inside the wells resulted more difficult.

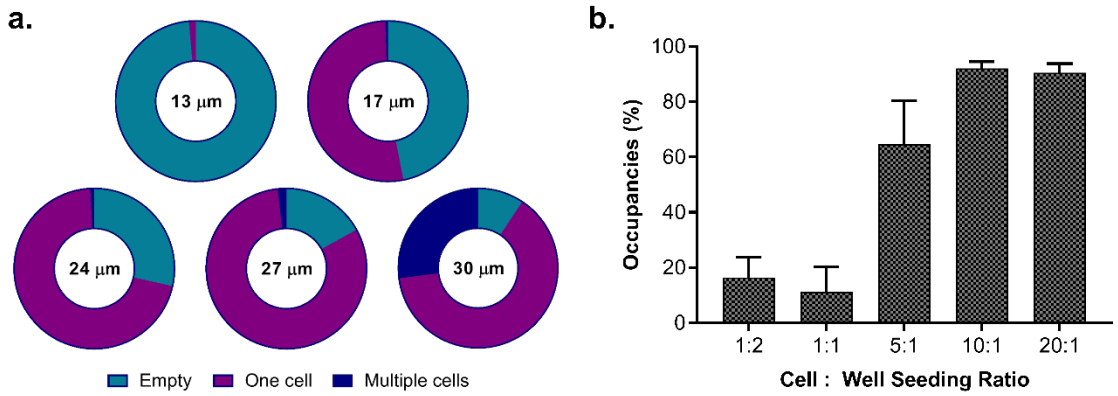

Figure 6 a) Occupancies of microwell arrays of various heights. Empty wells and single/multiple occupancies are shown for 13-17-24-27-30 µm wells; data reported good occupancies for traps ranging from 17 to 27 µm. Shallow wells cause cells loss in the washes, while deep wells show increased percentages of double occupancies. b) B3Z occupancies of 27 µm wells using different cell:well seeding ratios (2 experiments, 3 arrays measured from each experiment/concentration).

SI 7: ROIs definition

An example of automatic location of the cells within a trapping plate is presented in Figure 7. ImageJ uses a time frame from the recorded video; by the personalized protocol, it is possible to build ROIs around each single cell in a simple and effective way. To prove the correct functioning of the "gating" process, the ROIs are overlaid in yellow on top of the second image that depicts the same plate in a composite image. In this case, cells were stained in red using a fluorescent membrane dye. A strong correlation between automatically selected ROIs and well position is evident.

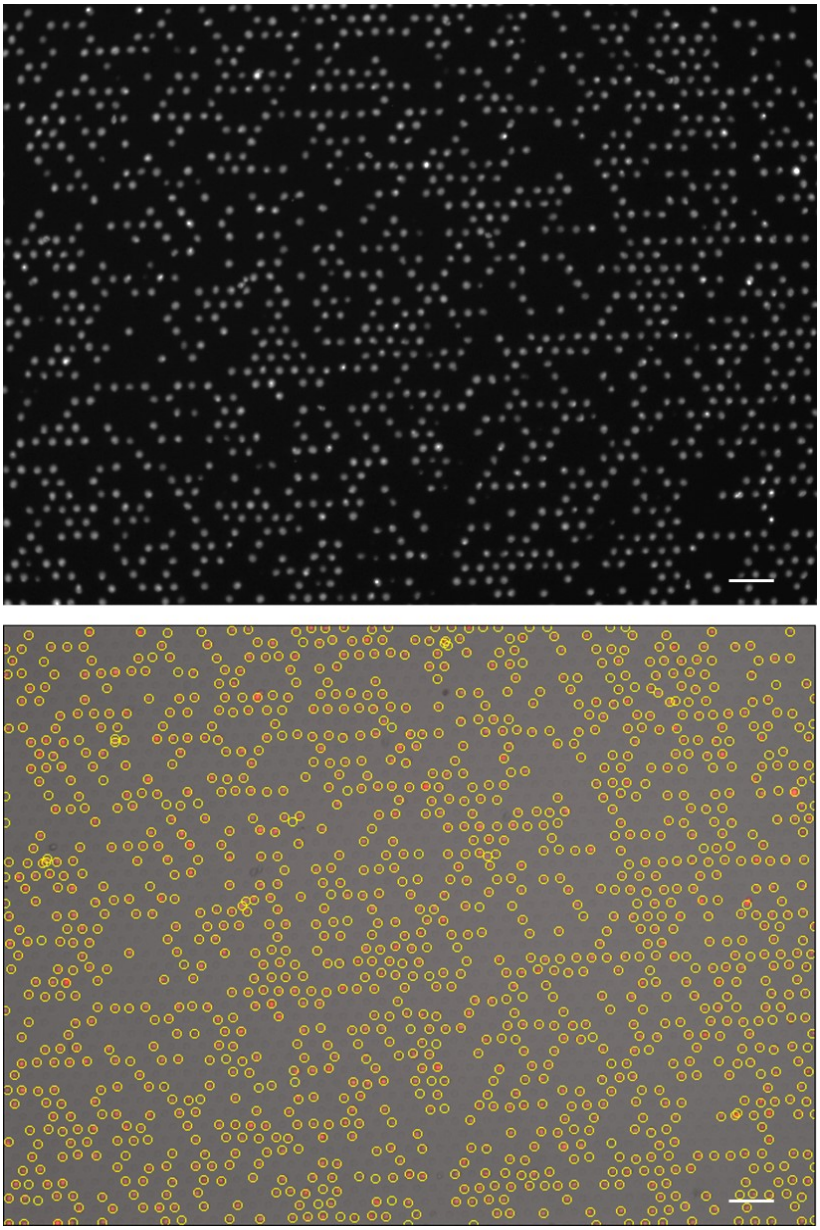

Figure 7 Automatic cell location and labelling. The same image is reported above: on the top, the greyscale image taken from the fluorescent channel, used to find the maxima and define the ROIs; on the bottom, the composite image (fluorescent channel and brightfield) is used to prove the correct ROI gating: the yellow circles are the ROIs, each of them is labelled with a number and the X-Y coordinates of its centre. Scale bar 100 µm.

SI 8: T cell activation using centrifugation to approximate T cells and APC

In flow cytometry experiments, T cell – APCs contact was induced by mixing the two populations at a 1:1 cell ratio and centrifuging the sample. Different centrifugation speeds and durations resulted in different percentages of T cell activations, suggesting that the contact force plays a role in T cell activation.

After dosing B3Z with calcium-chelating fluorescent dye and co-incubation of K89 with 10 µM SL8 for 1 hour, the two populations were aliquoted in different tubes. The basal fluorescence signal of each B3Z sample was recorded before K89 addition. B3Z:K89 were mixed at a 1:1 cell numbers ratio, and K89 were resuspended in a 50 µl volume

of media just before addition to the 200 µl sample of B3Z. Each sample containing the cell mixture was then spun down with different time-speed settings, before being resuspended with a pipette and put back on the rack to record the B3Z calcium signals for other 2.5 minutes.

Centrifuge times were varied between 1 and 3 minutes, while the speeds ranged between 59 and 835 rcf (800-1400-2000-3000rpm on a 5415D Eppendorf centrifuge combined with rotor F-45-12-11). Results are reported in Figure 8a, and indicate the percentages of activated B3Z gated on the FL-1 histogram of the single B3Z population after K89 addition. The number of activated cells increases by both prolonging the centrifugation time and increasing the spinning speed, suggesting that the force applied on the APC/T cells during cell-cell contact has a role in the signalling cascade. Number of doublets within each sample didn't correlate with the number of activators (Figure 8b), hence the pellet resuspension using a pipette was sufficient to break the T cell/APC bonds. This result confirms that conducting the analysis on the single cell gate doesn't bias the readings. A similar set of experiments has been reported previously[11], which focused on T cell-APC contact duration rather than the force influence.

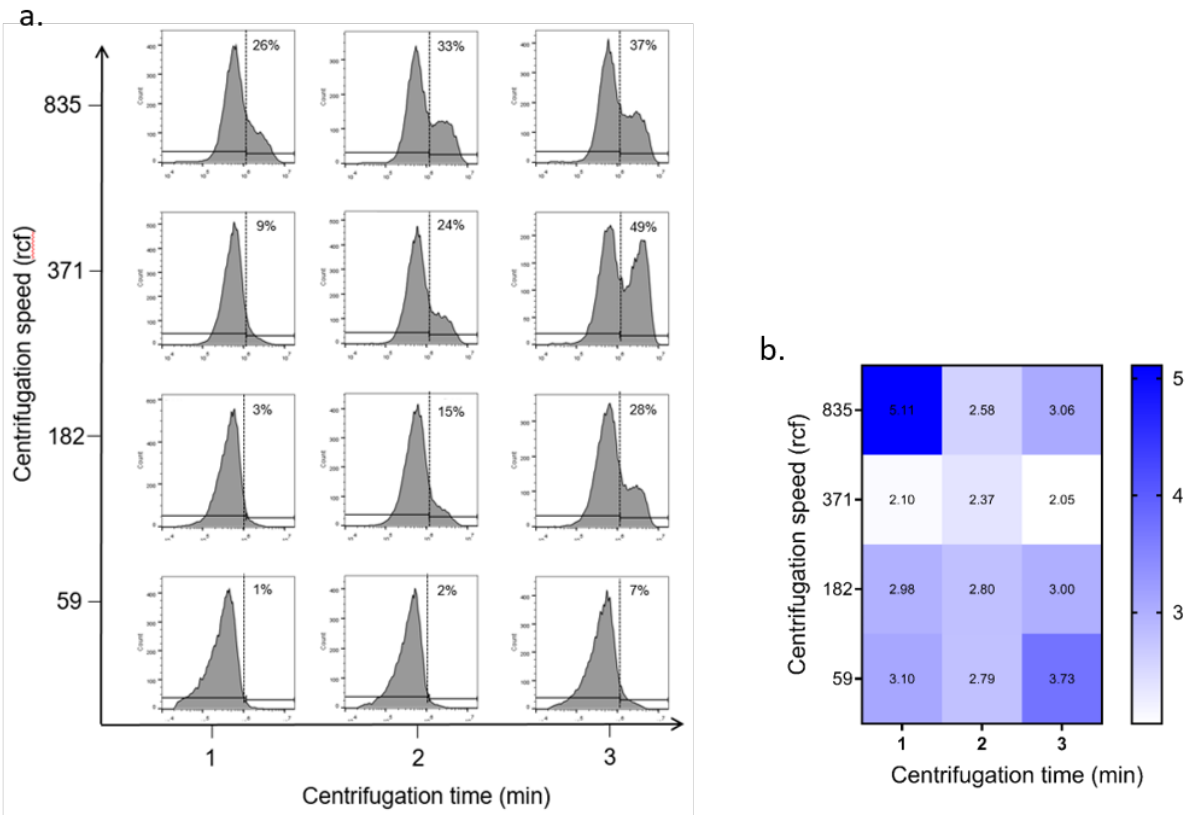

Figure 8 a) FL-1 histograms of single B3Z after 1:1 addition of 10µM SL8 loaded K89 and spinning of the mixed population at different centrifugation speeds and times. Percentages of activators among the samples are indicated in the graphs. b) Doublets percentages after B3Z:K89 centrifugation and pipette disruption of the pellet, gated on the FSC-A:FSC-H graph. No correlation between the spinning settings and the cell couples were observed. The random fluctuations could be related to variances in the manual pipetting.

SI 9: Controls for synchronised T cell activation

Control recordings are shown in Figure 9. They include: B3Z signalling when the trapping plate is not exposed to any stimulation (Figure 9a), B3Z signalling after capping with the

PDMS-covered lid in absence of the K89 layer (Figure 9b) and a comparison of the top 100 T cells traces (sorted on the mean MFI of each recording) after pairing with a confluent layer of K89 in presence (Figure 9c-d-e) and absence (Figure 9f-g-h) of SIINFEKL peptide.

No significant calcium fluctuations were recorded in response to the mechanical operations involved in the device. Importantly, T cells did not respond to monolayers of K89 APC grown on the lid in absence of the relevant epitope.

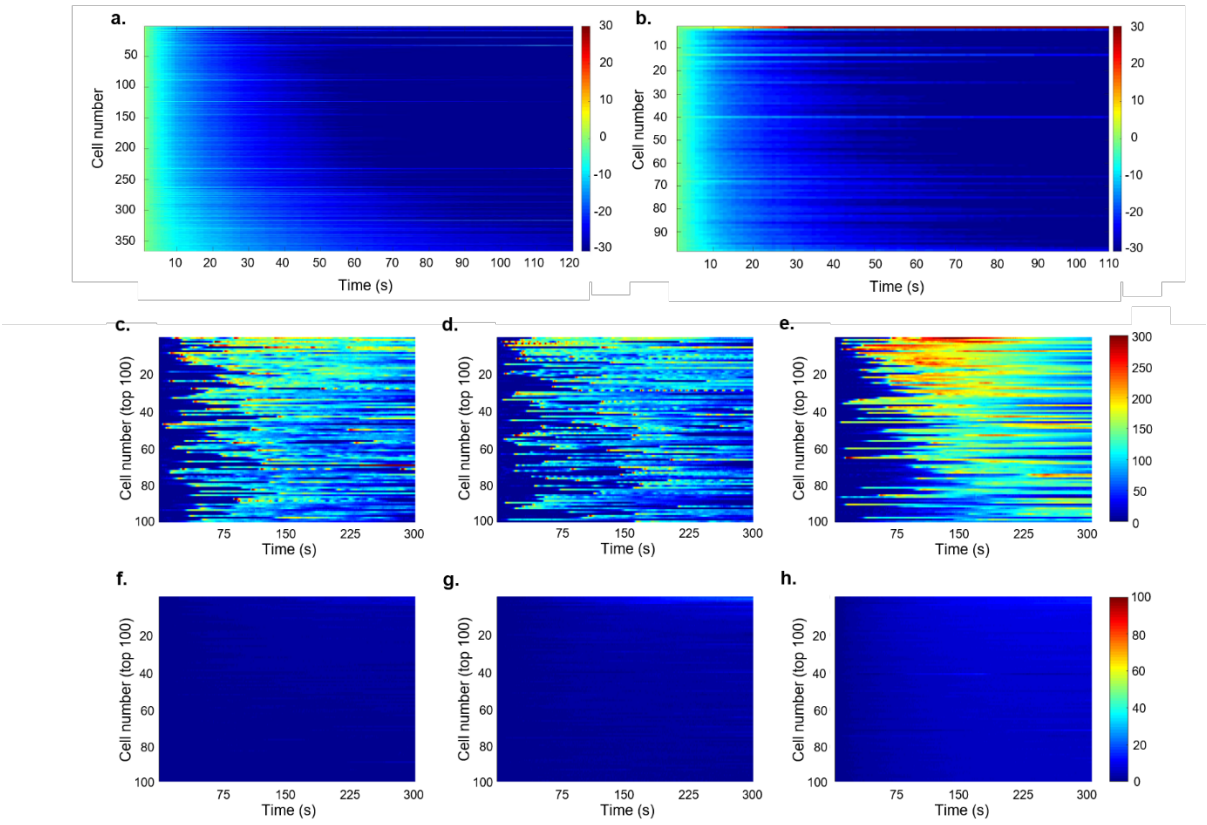

Figure 9 Controls. a) single T cells signalling in absence of any stimulation (being in contact with the agarose trapping device only). b) single T cells responses when pushed against the PDMS membrane, in absence of K89. c-d-e) top 100 T cells signals (sorted on mean MFI over the recording) after being paired to K89 pulsed with 1 µM SIINFEKL, 3 experiments. f-g-h) top 100 T cells signals (sorted on mean MFI over the recording) after being paired to K89, in absence of peptide, 3 experiments. All traces were normalized on the first frame and expressed as a percentage of the initial value.

SI 10: Co-visualization of T cell responders and APCs to prove pairing

To confirm that activation only occurred in cells that were in contact with APCs, we used APCs labelled with the red membrane dye PKH26, to enable co-visualisation with the T cells (recorded in the FITC channel, see overlay in Figure 11a). An array of B3Z was selected and recorded over time using FITC filter to capture the signalling of lymphocytes. The same spot was subsequently recorded using Rhodamine filter to gate on K89 time traces. APCs Rhodamine recording proved that the membrane dye

signal was constant over time (Figure 11b), and did not induce artefacts in the FITC detection channel.

For the analysis, ROIs were created around each individual K89 cell (Rhodamine channel) to filter the FITC time series (Figure 11c), proving that T cells sharing the well with antigen presenting cells (FITC signal recorded in the APCs ROIs) showed the usual activation profiles. According to this analysis, 328 activated T cells could be identified. Similarly, after analysing calcium oscillations of lymphocytes (from the FITC time series, see example of activations in Figure 11d) and determining the responders (n=366), we superimposed the activators ROIs on the PKH26-labelled image (Figure 11e), showing that such positions were shared with K89 cells.

The discrepancy between the detected activators might easily be due to a slight misalignment of the cells sharing the same well, and subsequent signal loss in the first analysis.

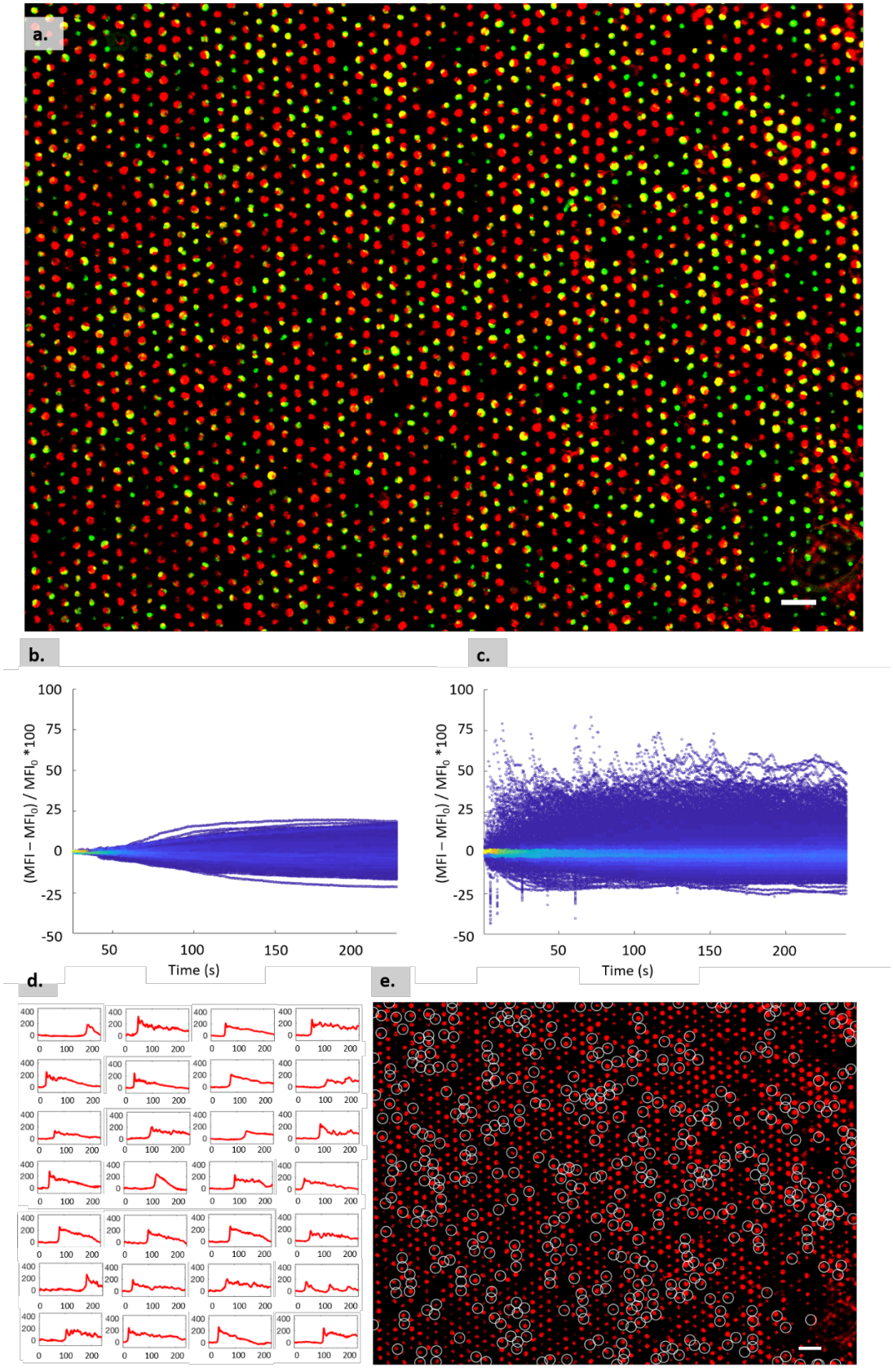

Figure 10 Combinatorial study of B3Z and K89 ROIs. a) Fluo-8AM stained B3Z (green) and membrane-stained K89 (red) were paired using the microwell array-lid system. b) Red membrane-stained K89 signals in the green channel over time: no fluctuations could be detected. c) K89 ROIs were superimposed to the FITC recording of B3Z calcium signalling: 328 B3Z activations could be highlighted using K89 positions. B3Z responders were detected from the FITC recording, see examples of single cell activations in d), and their position was highlighted with white circles on a red channel image: results match the finding pictured in c). Scale bar 100 µm.

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