Supplementary Appendix for

Droplet encapsulation improves accuracy of immune cell cytokine capture assays

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Dropmaker design (separate file): "Yuan et al. SI Dropmaker design AutoCAD file"
Droplet generation (separate file): "Yuan et al. SI Droplet generation video file"

Supplementary Information Text:
Materials and Methods

Microfluidic chip design and fabrication

Microfluidic chips are fabricated from polydimethylsiloxane (PDMS) polymer (Dow Corning, Sylgard 184, USA) according to published methods[30] with minor modifications. We spin coat photoresist SU8-3025 (Microchem, USA) to a thickness of 37 μm. After we cut PDMS slab from the mold and punch (Biopsy punch, 1 mm diameter; Ted Pella, Harris Uni-Core, cat. no. 15110-10) tubing holes, we remove the hole-punch debris by transparent adhesive tape followed by sonication (30 sec) in an isopropanol sonication bath. We then use compressed air to further clean the punch holes and remove isopropanol. We then completely dry the PDMS slab overnight in a 65 °C oven prior to plasma bonding to a glass slide. To make the PDMS chip hydrophobic for generating aqueous droplets, the chip channels are treated with Aquapel (Aquapel, cat. no. 47100) for 30min and device is dried in a 65 °C oven before use.

Cell lines

Both NK-92 MI cell line (ATCC® CRL-2408™) and K562 cell line (ATCC® CCL-243™) are purchased from ATCC.

Cell culture

For NK-92 MI cells, the base medium is Alpha Minimum Essential medium (ThermoFisher Scientific, cat. no. 12561-049) without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. To make the complete growth medium, the base medium is supplemented to obtain final concentrations: 0.2 mM inositol; 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, cat. no. M6250-100ML); 0.02 mM folic acid (Sigma-Aldrich, cat. no. F8758-25G); 12.5% horse serum and 12.5% FBS (Sigma, cat. no. F2442). For K562 cells, the base medium is DMEM supplemented with 10% FBS; and 1% penstrep (Hyclone, cat. no. SV30010). Both NK-92 MI cells and K562 cells are cultured with their complete growth medium in 37 $^{\circ}$C, 5% CO_2 incubator.

We stimulate cells by incubating 0.5 ml cells (at ~5×10^6 cells/ml) with 1 µl Cell Stimulation Cocktail (eBiosciences™, cat. no. 00-4970-93) for 5 h. Stimulation reagent was removed by washing twice with 1 ml 1X PBS. Immediately prior to encapsulation we remove dead cells using a dead cell removal kit (Miltenyi Biotech, cat. no. 130-090-101) according to manufacturer's instructions.

Cell staining

Live cells are stained with CellTracker™ Green CMFDA Dye (ThermoFisher, cat. no. C2925). Briefly, we centrifuge cells at 600 g for 5 min and remove all supernatant. We then resuspend the cells with 1 ml pre-warmed cell CellTracker™ Green CMFDA Dye solution (1 μM in PBS). We incubate for 30min in a 37 $^{\circ}$C cell culture incubator and then wash cells 3 times with 1 ml PBS.

Cell coating

IFN-γ Secretion Assay-Detection Kit (APC) is purchased from Miltenyi Biotech (cat. no. 130-090-762). To coat capture antibody onto the surface of NK-92 MI cells, 2×10^6 cells are transferred to 1.7 ml Eppendorf tube and collected by centrifugation at 600 g for 5 min. Then cells are resuspended with 180 μl ice-cold PBS and 20 μl IFN-γ capture antibody is added to the cell suspension. Cells are incubated on ice for 30 min and then washed with 1 ml PBS for 3 times to remove unbound capture antibody.

Droplet generation

We prepare two cell suspensions, one containing NK-92 MI cells coated with IFN-γ capture antibody (2×10^6 cells/100 μl in complete NK-92 MI medium) and the second containing K562 cells stained with CellTracker™ green (8×10^6/100 µl in complete NK-92 MI medium). We use syringe pumps to flow these two cell suspensions into two separate inlets of a co-flow microfluidic chip (chip design shown in *SI Appendix* Fig. S1 and AutoCAD file included as SI File 1). These streams merge within the chip and the merged aqueous stream is cut into droplets by a stream carrying the continuous phase, HFE7500 fluorocarbon oil (Novec™, 3M, USA) containing 2% (w/v) surfactant (008-FluoroSurfactant, Ran Biotechnologies, USA). We use flow rates of 100 µl/h for each aqueous stream and 1000 µl/h for the continuous phase to generate droplets with diameter of 40 µm. Video of droplet making is included as supplementary file *SI Droplet generation video*.

Droplet incubation and microscope imaging

After generation, droplets are incubated in 37 °C, 5% CO_2 incubator for desired time. We pipette ~2 µl droplet emulsion into a disposable hemocytometer (Bulldog Bio, cat. no. DHC-N420) counting chamber pre-filled with ~5 µl HFE7500 oil (containing 2%) surfactant as spacer oil to allow droplets to float into a monolayer. Droplets are analyzed with a 10X air objective on TCS-SP5 confocal microscope (Leica Microsystems Inc., IL, USA). Lasers of 488 nm and 640 nm are used to excite the CellTracker green and APC fluorescent dyes, respectively. Each image is taken at scanning rate of 8000 Hz with line average of 6 to ensure imaging quality.

Release cells from droplets and test viability

We release encapsulated cells by destabilizing droplets and letting them merge with 1 ml PBS. By releasing droplet contents into this relatively large volume of PBS, we ensure that any unbound secreted IFN-γ is greatly diluted and cannot be captured at significant levels by non-secreting cells. Briefly, we carefully remove HFE7500 oil with 2% surfactant from underneath the droplet emulsion and add 1ml PBS on the top of the emulsion. We then add 400 µl HFE7500 containing 20% (v/v) PFO (1H,1H,2H,2H-Perfluorododecyltrichlorosilane, Sigma-Aldrich, cat. no. 729965) and immediately mix the solution gently. After 5 min, the PBS (cell containing) and the HFE7500 phases separate into upper and lower layers, respectively. We transfer the upper PBS layer into a fresh 1.7 ml Eppendorf tube, pellet the cells at 600 g for 5 min and remove the supernatant. We wash the cells twice with 1 ml PBS and re-suspend the final cell pellet with 200 µl PBS. To test viability, we stain a fraction of these cells with 0.4% Trypan Blue (ThermoFisher, cat. no. 15250061) to label dead cells and analyze by microscopy. We confirm these results by treating another fraction of these cells with DAPI (Sigma-

Aldrich, MO, USA; final concentration of 1 μg/ml), which stains the nuclei of dead cells, and analyzing by flow cytometry.

Cell sorting by flow cytometry

Flow cytometry FACS Aria (Becton-Dickenson, USA) is used to perform cell sorting. We use a 100 μm nozzle. We use forward scatter (FSC) and side scatter (SSC), set to 250 V and 350 V, respectively, to identify the population of single live cells. We set the FITC fluorescence parameter to 300 V, which separates Cell Tracker green-stained K562 cells and NK-92 MI cells (not stained with Cell Tracker) into distinct populations. We set the APC fluorescence parameter to 500 V to identify NK-92 MI cells that secrete IFN-γ.

Software, image processing and statistical analysis

The AutoCad (AutoDesk, USA) software is used to design the mask for lithography. Image processing and analysis are done using Image J (National Institutes of Health, USA). Flowjo is used for analyzing FACS results. Origin (OriginLab, USA) is used for plotting graphs. Each experiment is performed independently at least in duplicate.

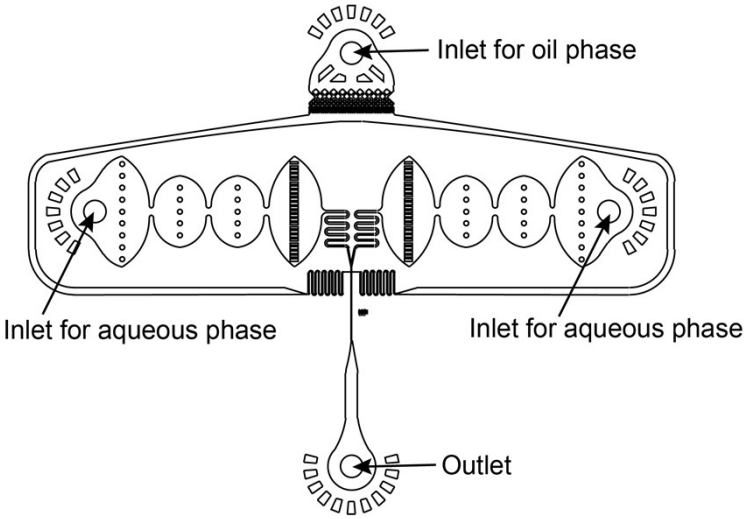

Figure S1: Co-flow device.

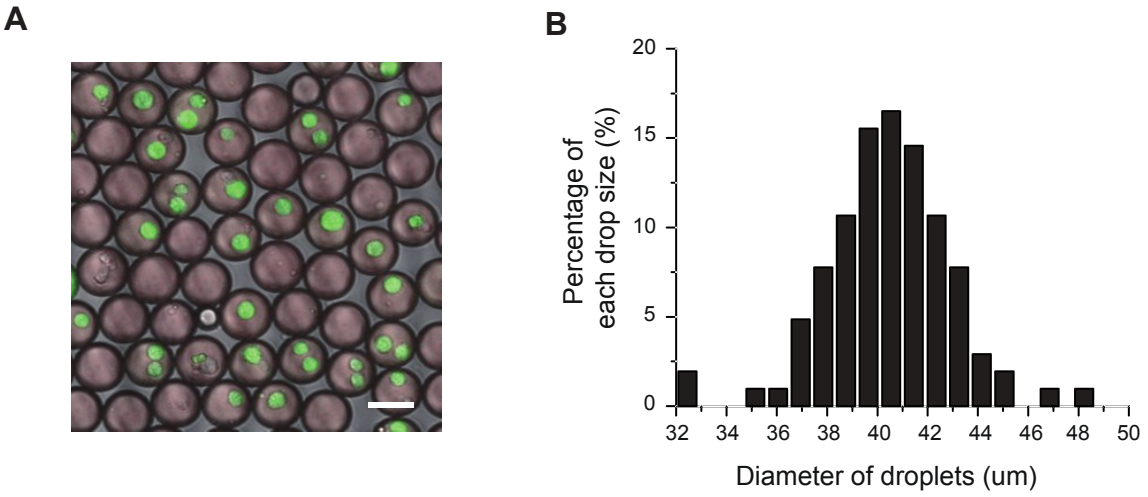

Figure S2: Droplet size distribution. A) Droplets containing cells are imaged under fluorescence microscopy. Scale bar represents 40μm. B) Size distribution of droplets. We use an Image J size analysis tool to determine the size of ~300 imaged droplets and plot these results as a size vs frequency histogram.

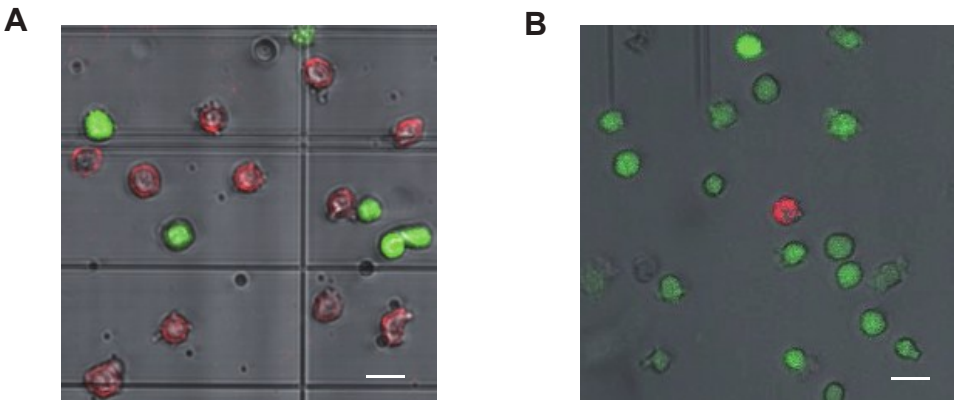

Figure S3: Cells released from the droplet mixtures. Droplets with either stimulated NK-92 MI cells or non-stimulated NK-92 MI cells are generated independently and then mixed together. After 12h incubation, cells are released from droplet mixtures. **A)** Cells released from the 1:1 droplet mixture. **B)** Cells released from the 1:100 droplet mixture. Unstained NK-92 MI cells treated with stimulus cocktail are activated and show APC fluorescence on the cell surface in droplets. Green-stained NK-92 MI cells, which are not treated with stimulus cocktail, do not show any APC fluorescence. Scale bar=20 μm.

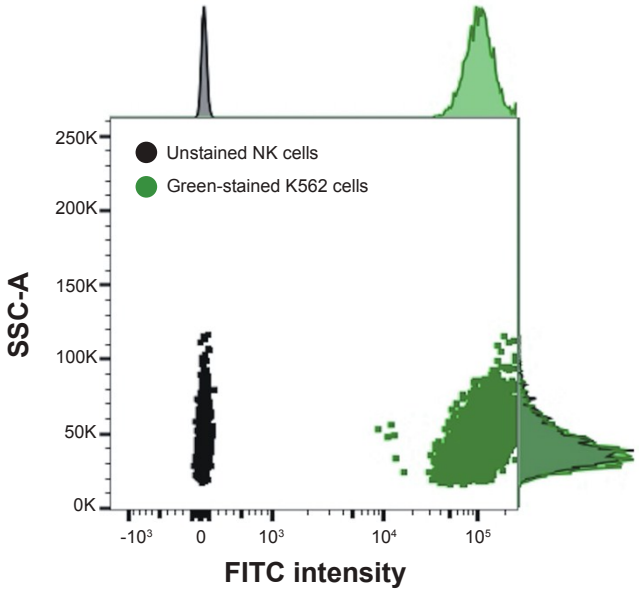

Figure S4: Cell Tracker green stained K562 cells are easily distinguished from unstained NK-92 MI cells. Unstained NK-92 MI cells and K562 cells stained with Cell Tracker Green are analyzed by flow cytometry separately and the results are plotted on the same axes. When FITC fluorescence intensity is plotted against side-scatter, the K562 cell population is distinct and well-separated from the unstained NK-92 MI cell population, represented by green dots and black dots, respectively.

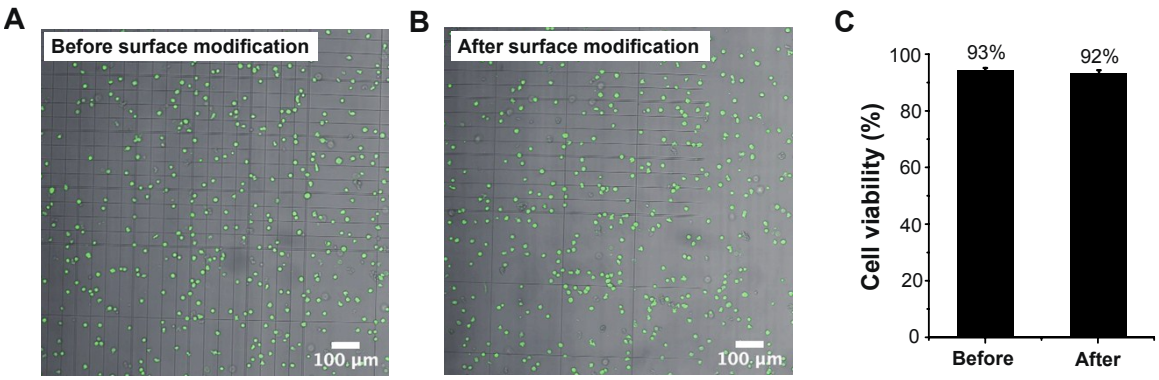

Figure S5: Cell viability before and after surface modification with capture reagent. (A) NK-92 MI cells without treatment of IFN-γ capture reagent are stained with Calcein AM. (B) NK-92 MI cells coated with IFN-γ capture reagent are stained with Calcein AM. (C) Analysis of cell viability before and after cell surface modification. Error bar represents two independent experiments.

Table S1: Summary of false positive and false negative rates observed for cells incubated in bulk or in droplets.

	False positive rate (% IFN-γ^+ in non-stimulated cells)	False negative rate (% IFN-γ^- in stimulated cells)
1:1 cell mix without droplets	98% (+/- 3%)	0.4% (+/- 0.5%)
1:100 cell mix without droplets	2% (+/- 4%)	94% (+/- 0.1%)
1:1 cell mix with droplets	1% (+/- 1%)	0.1% (+/- 0.1%)
1:100 cell mix with droplets	0.2% (+/- 0.2%)	2% (+/- 2%)