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## A pipette-tip based method for seeding cells to droplet microfluidic platforms

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**TITLE:**

A Pipette-Tip Based Method for Seeding Cells to Droplet Microfluidic Platforms

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**KEYWORDS:**

Droplets, Microfluidics, Tip Loading, Poisson Distribution, Cell Encapsulation, Cell-Pairing,  
Cellular Interactions

**SUMMARY:**

This article presents a protocol for seeding scarce population of cells using pipette-tips to  
droplet microfluidic devices in order to provide higher encapsulation efficiency of cells in  
droplets.

**ABSTRACT:**

Amongst various microfluidic platform designs frequently used for cellular analysis, droplet-  
microfluidics provides a robust tool for isolating and analyzing cells at the single-cell level by

eliminating the influence of external factors on the cellular microenvironment. Encapsulation of cells in droplets is dictated by the Poisson distribution as a function of the number of cells present in each droplet and the average number of cells per volume of droplet. Primary cells, especially immune cells, or clinical specimens can be scarce and loss-less encapsulation of cells remains challenging. In this paper, we present a new methodology that uses pipette-tips to load cells to droplet-based microfluidic devices without the significant loss of cells. With various cell types, we demonstrate efficient cell encapsulation in droplets that closely corresponds to the encapsulation efficiency predicted by the Poisson distribution. Our method ensures loss-less loading of cells to microfluidic platforms and can be easily adapted for downstream single cell analysis, *e.g.*, to decode cellular interactions between different cell types.

## INTRODUCTION:

In recent years, the use of microfluidics as a robust and versatile platform for cellular analysis at the single cell level has rapidly increased<sup>1</sup>. These platforms provide high-throughput screening of single cells and biological molecules with high precision and sensitivity using very small sample volume<sup>2-4</sup>. Among different types of microfluidic designs, droplet-based platforms enable high-throughput analysis of single cells by isolating them in an aqueous phase droplet surrounded by an immiscible phase that allows precise and accurate control over the cellular microenvironment<sup>5,6</sup>. Droplet-based microfluidics gives the flexibility to isolate single or multiple-cells in, both, aqueous and hydrogel droplets and is valuable in probing complex cellular behavior, such as protein secretion or cellular interactions<sup>7-9</sup>. Signaling and cross-talk amongst immune cells can be influenced by interactions with other cells in the microenvironment<sup>10</sup>. Isolation of single cells in droplets provides an effective noise-free analytical laboratory, free from the influence of external environmental factors for more efficient and accurate results<sup>11,12</sup>. Modifying the design of a droplet-microfluidic platform with multiple inlets allows the encapsulation of multiple cell types to study cellular interactions *via* cell-pairing<sup>12,13</sup>.

The process of encapsulation of cells in droplets is random and the rate of encapsulation of cells can be statistically determined using the formula for the Poisson distribution<sup>14,15</sup>. This rate of encapsulation can be estimated by considering the average rate of the arrival of cells at the droplet junction and assuming that the arrival of each cell is independent from the arrival of other cells<sup>16</sup>. Even though independent cell arrival cannot be guaranteed, in cases of sparsely distributed cells, the assumption of independence can be considered and the probability of a droplet containing one or more cells can be predicted as a function of the number of cells present in each droplet and the average number of cells per droplet<sup>16,17</sup>. Since this estimation of cellular encapsulation in droplets is dependent on the number of cells present in each droplet, one can suggest that increasing the concentration of the cells at the inlet will increase the average number of cells present in each droplet<sup>16</sup>. Therefore, to ensure single cell encapsulation, the cell concentrations must be reduced but this often leads to a large number of empty droplets<sup>18</sup>.

Loss of cells during loading by either attachment, sedimentation, and/or clumping in the syringe, tubing, or production device is a common drawback responsible for the deviation of

actual encapsulation values from the predicted encapsulation values<sup>19</sup>. This problem gets further exaggerated when seeding rare immune cells or clinical specimens as they are already scarce in population and the encapsulation of only a few cells, much lower than expected, does not provide sufficient data for experimental analysis. Plasmacytoid dendritic cells (pDCs) are a rare subset of immune cells that only constitutes approximately 0.2 - 0.6 percent of the entire white blood cell population<sup>20</sup>. These cells secrete massive amounts of type I interferons upon activation and thereby play a critical role in immune responses<sup>21</sup>. When studying the cellular behavior of such rare cells in droplets, it is imperative to prevent cell loss during cell seeding and encapsulation<sup>22</sup>. There are several design related developments that have ensured the encapsulation of single cells in droplets using active encapsulation methods that utilize different physical forces such as acoustic or electrical forces for generation of droplets containing single-cells<sup>23,24</sup>. However, these methods have their own limitations in terms of droplet production<sup>16</sup>.

In this study, we established a robust and straightforward method that circumvents the shortcomings of traditional methods for loading single or multiple cells to microfluidic devices. Our method, inspired by Rho *et al.*, utilizes differently-sized pipette tips for seeding small volumes of rare immune cells to droplet microfluidic platforms without significant sample loss and yielded results that are coherent with theoretical predictions<sup>25</sup>. This methodology can be easily and successfully adapted for several applications involving droplet-based microfluidics and applied for a wide variety of cell types or even microparticles.

## **PROTOCOL:**

### **1. 3-Inlet Polydimethylsiloxane (PDMS) Device Fabrication**

1.1 Measure 40 g of PDMS base in a conditioning mixer cup and add 4 g of PDMS curing agent to the base reagent in the cup, carefully, using a dropper.

1.2 Place the cup in the holder of the conditioning mixer and measure the total weight of the cup with the holder. Set the value of the centrifuge balance weight on the conditioning mixer accordingly.

1.3 Mix the base and curing agent in the conditioning mixer at 2000 rpm for 2 min followed by de-foaming at 2000 rpm for 2 min.

1.4 Prepare an aluminum boat, with a diameter approximately the same size as that of a 100 mm silicon wafer. Place the silicon wafer, fabricated for the replica molding process, in the aluminum boat and put this setup in a Petri dish (diameter = 120 mm, height = 20 mm).

Note: The size of the Petri dish depends on the size of the silicon wafer.

1.5 Remove the cup from the holder and pour the pre-cured PDMS mixture (contents of the cup), carefully, on the silicon wafer.

1.6 Place the Petri dish, containing the silicon wafer with the pre-cured PDMS mixture, in a desiccator for about 20 min to remove all the air bubbles.

1.7 Remove the Petri dish after 20 min and check for any remaining air bubbles that can be removed.

1.8 Place the Petri dish in an oven, set at 65 °C, for at least 3 h.

1.9 Remove the Petri dish from the oven after 3 h and carefully peel the cured PDMS from the silicon wafer.

1.10 Cut PDMS devices along the cut lines, using a knife or a scalpel. Punch holes at the inlets and outlet of each device using a 1.2 mm puncher. Clean each PDMS device with scotch tape to remove any dust or residual pieces of PDMS.

1.10.1 Optionally, blow with nitrogen to remove residual PDMS pieces.

1.11 Prepare glass slides by cleaning them with soap-water, followed by isopropanol and dry with nitrogen.

1.12 Bond a clean PDMS device with a clean glass slide in a plasma asher to close the flow lines. Use the following settings: Power: 50 W, Time: 45 s, Bleed delay time: 2 s, Process gas: Gas 1 (Air), Vent: Both valves, Restricted vent time: 60 s, Pump spin down time: 10 s, Vent hold time: 0 s, Gas shutoff time: 1 s, Turbo pumping enabled: 0. Disconnect all the other gas lines.

Note: The settings used for the plasma asher can vary according to the brand of the plasma asher used.

1.13 Prepare the silane solution by adding 50 µL of silane (1H,1H,2H,2H-Perfluorooctyltriethoxysilane) to 950 µL of fluorinated oil.

Note: Silane is toxic. Please operate under fume hood.

1.14 Draw the prepared silane solution in a syringe, which is connected to a Teflon tubing.

1.15 Salinize the device by flushing the prepared silane solution through the outlet of the device.

1.16 Place the device in an oven, set at 65 °C, for 30 min.

1.17 Remove the salinized device from the oven and flush excess silane out of the device with fluorinated oil.

1.18 Place the device back in an oven, set at 65 °C, for at least 1 h to complete the bonding process.

Note: The Protocol can be paused here.

## **2. Loss-Less Cell Encapsulation**

### **2.1. Cell harvesting**

2.1.1. Re-suspend Jurkat T cells in Roswell Park Memorial Institute (RPMI) medium at concentrations of  $1.0 \times 10^6$  cells/mL,  $2.0 \times 10^6$  cells/mL,  $4.0 \times 10^6$  cells/mL, and  $8.0 \times 10^6$  cells/mL; pDCs in hematopoietic serum-free culture media (e.g., X-VIVO 15) at concentrations of  $1.3 \times 10^6$  cells/mL,  $3.0 \times 10^6$  cells/mL, and  $13.0 \times 10^6$  cells/mL; A549 cells in Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of  $1.0 \times 10^6$  cells/mL.

Note: The type of cells and concentration of cells can vary based on the experiment. Labelling of cells can also be done based on the experiment.

### **2.2. Tip-loading for aqueous droplet generation**

2.2.1. Prepare fluorinated oil with 3% biocompatible surfactant mixture by adding 30 mL of surfactant to 20 mL of fluorinated oil (oil phase).

Note: The concentration of the surfactant added to the fluorinated oil determines the stability of the emulsion for different incubation periods. The concentration of the surfactant varies depending on the media used for specific cell-types.

2.2.2. Draw the oil phase mixture in a syringe (1 mL). Remove air bubbles from the syringe and connect it to a Teflon tubing of appropriate length.

2.2.3. Prepare a sample syringe by drawing biocompatible mineral oil in a syringe. Remove air bubbles and connect the syringe to a Teflon tubing of appropriate length.

2.2.4. Punch a PDMS plug with a diameter of 5 mm from a cured PDMS slab.

Note: The cured PDMS slab can be prepared using steps 1.1 to 1.9. Use a plain silicon wafer instead of a fabricated silicon wafer.

2.2.5. Punch another hole in the center of the plug with a 1 mm puncher.

2.2.6. Insert the plug in a 200  $\mu$ L pipette tip, from the larger end, such that it fits tightly.

Note: Use a 1000  $\mu$ L pipette tip for larger sample volume and larger cells. For 1000  $\mu$ L pipette tip, plugs of diameter ranging between 5 mm and 7 mm can be used. With a plug of diameter 5

mm, a sample volume of around 400  $\mu\text{L}$  can be aspirated in the pipette tip. If a plug of larger diameter is used (7 mm), more sample volume can be aspirated (around 900  $\mu\text{L}$ ).

2.2.7. Insert the tubing, which is connected to the syringe, in the PDMS plug, which has been inserted in the pipette tip. Push the syringe plunger slowly to fill the connected pipette tip with mineral oil. Push out all the residual air from the pipette tip.

2.2.8. Lower the pipette tip, connected to the syringe, in the sample solution and aspirate about 150  $\mu\text{L}$  of sample in the tip.

2.2.9. Repeat steps 2.2.4 to 2.2.8 to prepare a second sample syringe.

2.2.10. Carefully place all the three prepared syringes on the syringe pump.

2.2.11. Insert both the pipette tips, containing the sample, in the two inner inlets of the PDMS chip. Insert the tube containing the oil phase mixture in the outer inlet.

2.2.12. Set the value of the flow rates on the syringe pump as follows: continuous phase solution: 600  $\mu\text{L}/\text{h}$ , cell samples: 100  $\mu\text{L}/\text{h}$ , each. Enter and set the dimensions of the syringe.

Note: The diameter settings will vary based on the type of syringe.

2.2.13. Start the pump to flush sample solution through the inner channels of the device and oil phase through the outer channel of the device.

2.2.14. Plug in a tubing of appropriate length to the outlet to start collecting the droplets when the droplet formation is stable. The time of collection varies based on the experiment.

2.2.15. Collect droplets in a lock tube. Add 200  $\mu\text{L}$  of RPMI medium (without serum) on top of the collected droplets and incubate the sample.

Note: Incubation time of the collected droplets varies based on the experiment. Droplets are collected in a lock tube when flow cytometry-based analysis or isolation is performed after retrieving the cells from droplets by breaking the emulsion. It is possible to collect the droplets in a glass chamber if the experiment requires in-droplet microscopic analysis.

### 2.3. Emulsion breaking and cell retrieval for flow cytometric analysis

2.3.1. Prepare 20% 1H,1H,2H,2H-Perfluoro-1-octanol (PFO) solution (v/v) in fluorinated oil by adding 2 mL of PFO in 10 mL of fluorinated oil.

2.3.2. Remove excess oil from the bottom of the collection tube, containing the droplets, using a syringe.

2.3.3. Add 100  $\mu$ L of 20% PFO solution to the emulsion to break the emulsion and release the encapsulated cells into the aqueous phase. Tap and mix briefly. Do not vortex at this point. Incubate for 1-2 min.

Note: The amount of PFO added depends on the quantity of droplets produced. Keep adding additional PFO until the oil layer is completely dissolved. Keep in mind that PFO is toxic for the cells and that too high PFO concentrations or too long incubation in PFO might lead to increased cell death.

2.3.4. Spin the solution shortly at the lowest possible rcf for 30 s.

2.3.5. Prepare 100 mL cold Phosphate-Buffered Saline (PBS) solution supplemented with 2% Fetal Calf Serum (FCS) (2 mL of FCS in 98 mL of PBS).

2.3.6. Pipette 550  $\mu$ L of the aqueous fraction, immediately after centrifugation and transfer it to a new lock tube containing 500  $\mu$ L of cold PBS solution supplemented with 2% FCS, as prepared in step 2.3.5. Let any residual oil sink to the bottom of the new lock tube.

2.3.7. Aspirate 950  $\mu$ L of the aqueous phase containing the cells from this lock tube, carefully, without aspirating any residual oil and transfer the solution to a new lock tube.

2.3.8. Spin down the cells in the new lock tube for 10 min.

2.3.9. Re-suspend the cells in 300  $\mu$ L of cold PBS solution supplemented with 2% FCS, as prepared in step 2.3.5.

Note: The cells can be also re-suspended in any other suitable solution such as media depending on the experiment. Stain the cells, based on the experiment, for analysis using flow cytometry.

### **3. Cell Pairing**

#### **3.1. Cell harvesting and staining**

3.1.1. Count Jurkat T cells, from culture flask, and spin down the cells at 1500 rpm for 5 min.

3.1.2. Remove the supernatant and re-suspend  $1.0 \times 10^6$  cells in 1 mL PBS to get a concentration of  $1.0 \times 10^6$  cells/mL. The amount of PBS added depends on the cell count.

3.1.3. Repeat steps 3.1.1 to 3.1.2 to prepare a second sample of Jurkat T-cells with the same cell concentration.

3.1.4. Wash both samples twice with 1 mL of PBS at 1500 rpm for 5 min.



3.1.5. Re-suspend one cell sample with 1.25  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE) dye and the other cell sample with 1.25  $\mu$ M far red dye or 1.25  $\mu$ M cell proliferation dye at a cell concentration of  $1.0 \times 10^6$  cells/mL. The total staining solution is 1 mL for  $1.0 \times 10^6$  cells.

Note: Cells can be labelled with different dyes depending on the filters available in the flow cytometer or in the fluorescence microscope.

3.1.6. Incubate the cell samples with dyes for 10 min at 37 °C.

3.1.7. Stop the staining reaction by adding 1 mL of ice cold FCS after 10 min.

3.1.8. Wash the cell samples twice with 1 mL of PBS at 1500 rpm for 5 min.

3.1.9. Re-suspend the cell samples in RPMI media at a concentration of  $10.0 \times 10^6$  cells/mL, for each color.

### **3.2. Tip-loading for production of agarose hydrogel droplets for cell pairing**

Note: For cell pairing using agarose hydrogel droplets, maintain the temperature of the system between 27 °C and 37 °C throughout the droplet generation and collection process to prevent gelling of the hydrogels and to warrant cellular viability<sup>9</sup>.

3.2.1. Dissolve ultra-low gelling temperature agarose by heating it up to 75 °C in PBS at a concentration of 4% (w/v) and stir the mixture for 20 min.

3.2.2. Mix the agarose solution with labelled Jurkat T cells to yield an agarose concentration of 2% (w/v). Repeat this for the other sample with differently labelled cells.

3.2.3. Prepare fluorinated oil with 2% surfactant mixture by adding 20 mL of surfactant to 30 mL of fluorinated oil (oil phase mixture).

3.2.4. Follow the steps 2.2.2 – 2.2.14.

Note: Because of the viscous nature of low melting agarose and to ensure stable droplet production, set the value of the flow rates on the syringe pumps as follows: oil phase mixture: 2000  $\mu$ L/h, cell samples: 200  $\mu$ L/h. Enter and set the dimensions of the syringe.

3.2.5. Collect the droplets in a lock tube and incubate the droplets at 4 °C for 60 min.

### **3.3. Emulsion breaking and agarose bead retrieval for FACS analysis**

3.3.1. After incubation of droplets for 60 min, remove excess oil from the lock tube, containing the droplets, using a syringe.

3.3.2. Add 200  $\mu$ L of PFO to remove the oil interphase from the droplets.

Note: The amount of PFO added to the tube depends on the quantity of droplets produced. Keep adding additional PFO until the oil layer is completely dissolved.

3.3.3. Wash the collected agarose beads twice with 1 mL of cold PBS to remove oil completely by centrifugation at 1500 rpm for 10 min.

3.3.4. Analyze collected agarose beads using flow cytometry.

Note: It is also possible to observe the beads under a fluorescence microscope.

## REPRESENTATIVE RESULTS

For our experiments, we used a 3-inlet PDMS based microfluidic device with the height of 25 microns (**Figure 1**). In this device setup, we used the outer inlet for flushing the oil with surfactant and the two inner inlets for flushing the aqueous phases with cell suspensions or media. After generation and collection, the droplets are incubated for a couple of hours off chip before downstream analysis using flow cytometry. During the incubation period, serum components present in the media can interact with the surfactant and cause droplets to become unstable and disintegrate. It is therefore important to add an optimized concentration of surfactant to the fluorinated oil. We tested the stability of the monodispersed droplets containing hematopoietic serum-free culture media supplemented with 2% human serum with different concentrations of surfactant in fluorinated oil. It can be inferred from **Figure 2** that these monodispersed droplets are highly stable for up to 24 hours when at least 3% surfactant is added to the oil phase. Similar results were obtained with RPMI media with and without the addition of 10% FCS (data not shown). Therefore, droplet stability is highly dependent on optimal surfactant concentrations when working with different sources of culture media and serum components.

To demonstrate the encapsulation efficiency of our approach we first seeded the cells using tubing connected to syringes, which is the most conventional approach for seeding cells (**Figure 3A**). We harvested Jurkat T cells at different concentrations of  $1.0 \times 10^6$  cells/mL,  $2.0 \times 10^6$  cells/mL, and  $4.0 \times 10^6$  cells/mL and obtained an encapsulation efficiency that was lower than predicted values (**Figure 3B**). At  $1.0 \times 10^6$  cells/mL, the fraction of droplets that contained a single cell was 2.5%, which did not increase even upon using higher cell concentrations. To increase the cell-loading efficiency, we modified our previous approach and mounted the tubing at half the length to an elevated tripod and loaded the cell suspension in the half that was attached to the PDMS device (**Figure 4A**). Using this approach, we encapsulated Jurkat T cells at different concentrations of  $1.0 \times 10^6$  cells/mL,  $2.0 \times 10^6$  cells/mL, and  $4.0 \times 10^6$  cells/mL, and also rare pDCs at different concentrations of  $1.0 \times 10^6$  cells/mL,  $2.0 \times 10^6$  cells/mL, and  $12.0 \times 10^6$  cells/mL. We expected improved encapsulation rates by preventing cell sedimentation with this method. However, at all the concentrations tested, the experimental results were much lower than the predicted Poisson values (**Figure 4B** and **Figure 4C**).

Using our tip-loading approach we optimized our cell encapsulation rates to obtain experimental results coherent with the statistically predicted values (**Figure 5A**). For different concentrations of Jurkat T cells, the obtained encapsulation efficiency matched our calculated values at all concentrations (**Figure 5B**). Remarkably, even with adherent cells like A549 tumor cells, which tend to clump, we observed a slightly improved encapsulation efficiency at a cellular concentration of  $1.0 \times 10^6$  cells/mL (**Figure 5C**). We also assessed the efficacy of our system to encapsulate less available and scarce pDCs at different cell concentrations of  $1.0 \times 10^6$  cells/mL,  $3.0 \times 10^6$  cells/mL, and  $13.0 \times 10^6$  cells/mL (**Figure 5D**). To facilitate the loading of possibly larger volumes exceeding 200  $\mu$ L, *e.g.*, when working with cell lines or more abundant primary immune cells, we also investigated the cell encapsulation efficiency by using 1000  $\mu$ L tips (blue). We demonstrated that these 1000  $\mu$ L tips gave a similar encapsulation efficiency in comparison to the 200  $\mu$ L tips (yellow) (**Figure 5E**).

Dependent on the chip design and research question at hand, our tip loading technique can be used to load cells through either one inlet, for probing into cellular heterogeneity, or multiple inlets in parallel, for decoding cellular interactions. We compared the loading of Jurkat T cells (at a concentration of  $10.0 \times 10^6$  cells/mL) from one inlet to two differently labelled populations of Jurkat T cells (at a combined concentration of  $10.0 \times 10^6$  cells/mL) from two inlets (**Figure 6A** and **Figure 6B**). During encapsulation, the droplets were generated using ultra-low gelling temperature agarose and gelled after production to form agarose hydrogel beads that allowed subsequent downstream analysis *via* microscopy and flow cytometry (**Figure 6C** and **Figure 6D**). Microscopic analysis revealed that cell pairing was achieved at different combinations indicating for high throughput cell pairing (**Figure 6C**). Furthermore, analysis of the same population of hydrogel beads by flow cytometry revealed that beads without cells could be separated from beads with cells based on the distinct forward (FSC, size) and sideward (SSC, granularity) scatter pattern (**Figure 6D**). Gating on the population of beads without cells confirmed a lack of cell encapsulation by the absence of fluorescent signals. Additionally, gating on the bead population with cells revealed the existence of multiple sub-populations indicative for the encapsulation of differently labeled Jurkat T cells. Our results demonstrate that efficient cell pairing can be achieved, based on both microscopic and flow cytometric analysis, and showed a slightly increased encapsulation efficiency compared to the Poisson prediction (**Figure 6E**).

#### FIGURE AND TABLE LEGENDS:

##### **Figure 1. PDMS based droplet microfluidic device with three inlets and one outlet.**

The device consists of three inlets for continuous oil phase, cell culture media, and cell suspension, respectively. The generated droplets are collected at the outlet. The samples flow laminarly to the flow-focusing junction where they are encapsulated in droplets. At the inlets, filter structures hold large particles like protein or cell aggregates back. The diameter of the gaps in the filter structure are indicated by blue lines. The diameter of the channels at the production nozzle are indicated by red lines. The channel height on the entire chip was 25  $\mu$ m.

**Figure 2. Droplet stability over 24 hours.** The graphs show the area of droplets containing hematopoietic serum-free culture media + 2% human serum, over time for three different concentrations of surfactant **A)** 0.5% **B)** 3% **C)** 5%.

**Figure 3. Tubing based cell loading approach.** Jurkat-T cells are loaded at different concentrations to the device using a syringe connected to tubing. **A)** The illustration shows the experimental setup **B)** The cell encapsulation rate as determined by light microscopy. Dots: experimentally determined values; Closed lines: Poisson distribution. Error bar represents standard error of mean.

**Figure 4. Encapsulation of various cell types at different concentrations using a vertical tube loading approach.** Jurkat T cells and pDCs (of different concentrations) were encapsulated to determine the efficiency of cell encapsulation. **A)** The illustration shows the experimental setup for the vertical tube loading approach. **B)** The graph shows the encapsulation efficiency of Jurkat T cells. **C)** The graph shows the encapsulation efficiency of pDCs. Dots: experimentally determined values; Closed lines: Poisson distribution. Error bar represents standard error of mean.

**Figure 5. Tip loading approach to encapsulate different cells types.** **A)** Schematic illustration of the tip loading technique. **B)** The graph shows the Encapsulation efficiency of Jurkat cells. **C)** The graph shows the encapsulation efficiency of A549 cells. **D)** The graph shows the encapsulation efficiency of pDCs. **E)** The graph shows the encapsulation efficiency of Jurkat T cells using 200  $\mu$ L pipette tips (yellow) and 1000  $\mu$ L pipette tips (blue). Dots: experimentally determined values; Closed lines: Poisson distribution. Error bar represents standard error of mean.

**Figure 6. Cell pairing in droplets.** **A)** Schematic illustration of the tip loading approach for pairing distinct cells from 2 inlets in droplets. **B)** The graph shows the encapsulation of Jurkat T cells using one inlet or two inlets in parallel. The cell concentration for one tip is  $2.0 \times 10^6$  cells/mL and the cell concentration for two tips both  $1.0 \times 10^6$  cells/mL. Dots: experimentally determined values; Closed lines: Poisson distribution. **C)** The Fluorescence microscopic overlays of hydrogel beads and labelled Jurkat T cells. **D)** The graph shows the flow cytometric analysis of paired cells in agarose hydrogel beads. The plot demonstrates both forward scatter and sideward scatter. **E)** Comparison of cell numbers in agarose hydrogel beads as detected by fluorescence microscopy and flow cytometry. Bars: mean value; Whiskers: standard error of mean,  $n \geq 4$ . Error bar represents standard error of mean.

## DISCUSSION:

In this protocol, we have demonstrated an efficient and straightforward technique to load and encapsulate cells in droplets for high-throughput, single-cell analysis and to perform controlled cell pairing for cellular interaction studies. Furthermore, we have compared several conventional approaches to load cells to microfluidic devices and showed that our tip loading approach is a more efficient technique in comparison to other methods.

Studying clinical specimens or rare cell types scarce in number by droplet-based microfluidics possess some inherent challenges. Like we have also demonstrated, cells tend to sediment in syringes and surface of the tubing, thereby, preventing cellular encapsulation to conform to the predicted values. To evade this problem, some groups use stirring bars in the syringes. However, when using rare and limited cell populations, the total cell volume is also limited, thereby, limiting the use of large syringes and stirring bars. Furthermore, we also replaced more commonly used tubing with Teflon coated tubing to prevent cell attachment but this method did not improve the results and if the tubing is too long, the problem of cell attachment aggravates (data not shown). Alternatively, we used a vertical tube loading approach where the cells were loaded in the tubing and not in the syringe to prevent the loss of cells in large syringe volumes. Using this technique, cells with small sample volume can be loaded, *e.g.*, pDCs which are rare and limited. Also, the sample from the tubing is loaded to the device vertically to prevent cell sedimentation. The tubing used for cell seeding has small dimensions and can be compared to microchannels. The flow in the tubing is pressure driven and follows a parabolic velocity profile<sup>26</sup>. This implies that the maximum flow velocity is at the center of the tubing and minimum velocity is at the edges of the tubing<sup>27</sup>. When flushing a population of cells through the tubing, the velocity gradient causes the cells to be pushed towards the edges where they settle down because the velocity at the boundary is close to zero. The sedimentation or settlement of cells in the tubing, thereby, reduces the encapsulation efficiency as shown in the representative results where the experimental data did not match with the predicted model.

Another commonly adapted solution used by scientists, working with droplet microfluidics, is to increase the density of the cell culture media by addition of density matching reagents such as Iodinaxol to prevent cell sedimentation in syringes<sup>19</sup>. However, density matching reagents can influence cellular behavior and adversely affect the cytokine secretion by cells (data not shown)<sup>28</sup>.

Even though several small and big modifications in conventional cell loading techniques showed slight improvements in encapsulation efficiencies, the obtained experimental results still did not match the theoretical calculations. However, with the tip loading approach we could the overcome the limitations of previous methods and encapsulation efficiency governed by Poisson statistics. This technique is not only advantageous for loading suspension cells but can also be applied for loading adherent cells, such as primary keratinocytes and A549 to microfluidic chips. When using abundant cell lines, for example A549, K562, *etc.*, larger sample volume can be used. Therefore, depending on the volume of the sample, different sized pipette-tips can also be used and this simple technique can be adapted for both single-cell encapsulation and multiple cell encapsulation.

While low cell concentration is required to ensure the encapsulation of single cells in droplets, higher cells concentrations are desired to increase the average number of cells encapsulated in each droplet for studies related to cell pairing. There are several single-cell methods that have been previously described to pair immune cells on microfluidic chips or microfabricated nanowells<sup>29–31</sup>. In droplet microfluidics, Poisson statistics dictates that 1:1 cell pairing for two different cell types can be achieved at optimal cell concentrations. Based on the Poisson

prediction, there is also a probability that droplets might contain other combinations. While 1:1 cell pairing can be desirable to study cellular interactions at single cell level and results in increased cellular understanding, multiple cell pairing also has major advantages. It allows to comprehend the influence of multiple cells of one cell type on the other cell type. Cross talk between different immune cells help to generate an effective immune response against several infections and pathogens and also adds robustness to our immune system<sup>32</sup>. As such, cellular communication can be interrogated with high precision in distinct contexts, *e.g.*, 1:1, 2:1, 1:2, 2:2, 3:1, *etc.* yielding increased understanding on how single or pairs of cells control the induction of immune responses. This is particularly interesting to study for example the capacity of natural killer cells or cytotoxic T cells to serially kill their respective target cells.

As discussed, for multiple encapsulation of cells in droplets, higher cell concentrations are desired. However, when loading cells from one inlet for cell encapsulation, higher concentrations of cell sample can cause cells to aggregate at the inlet. This results in lower encapsulation rates and higher deviation from the theoretical values. To evade this problem, the cells can be loaded from two separate inlets as well. Theoretically, it would be possible to develop other microfluidic devices with multiple inlets to achieve even higher levels of cell encapsulation where an average on *x* number of cells is warranted. In this study we investigated the encapsulation efficiency of Jurkat T cells when loaded from both one inlet and two inlets using the same total concentration and obtained similar encapsulation efficiency. This modification allows researchers to pair different cell types on chip.

While this method aids in loading cells to microfluidic devices without significant loss of cells, there are certain precautions that need to be kept in mind. When filling the syringes with mineral oil and aspirating the cell sample in pipette tips, incorporation of air bubbles should be avoided and the entire system should be air-free. It is also important to keep in mind that the mineral oil should not mix with the sample. Pipette tips, containing samples, should be inserted firmly in the inlets of the microfluidic device, with utmost precaution, to prevent leakage and further incorporation of air bubbles. To summarize, tip-loading is a straightforward, yet, robust technique that allows for high-throughput analysis of cellular behavior through cell encapsulation without significant loss of cells in a cost-effective manner. When used with optimal sample concentrations at the inlet, this approach of loading cells with pipette-tips is very flexible and can be adapted for different cell types, especially for rare primary immune cells, to obtain higher encapsulation efficiency, close to predicted models.

#### **ACKNOWLEDGMENTS:**

We thank the Eindhoven University of Technology for generous support.

#### **DISCLOSURES:**

We have nothing to disclose.

#### **REFERENCES:**

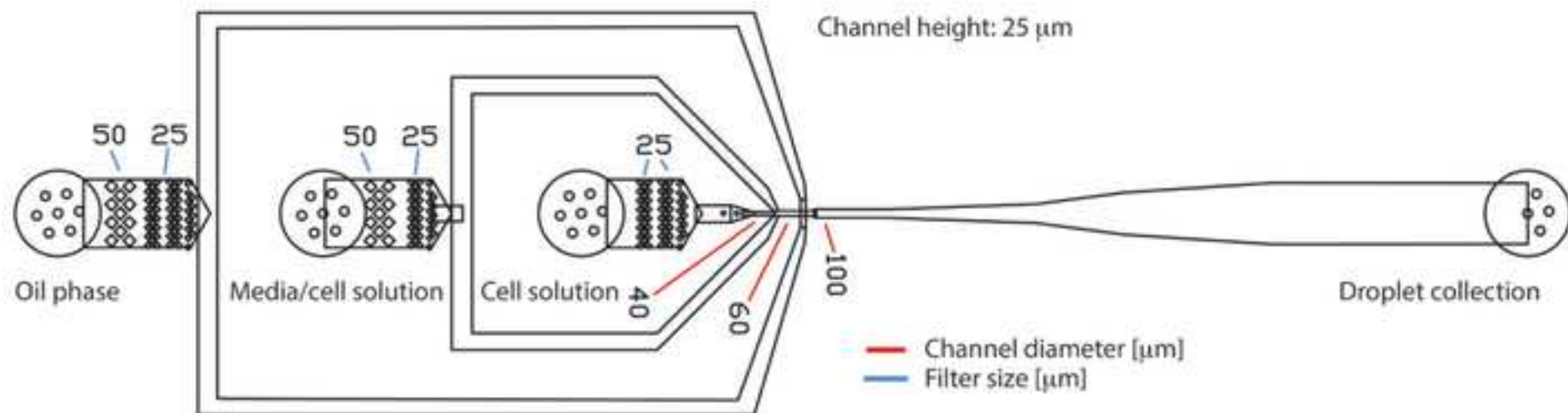
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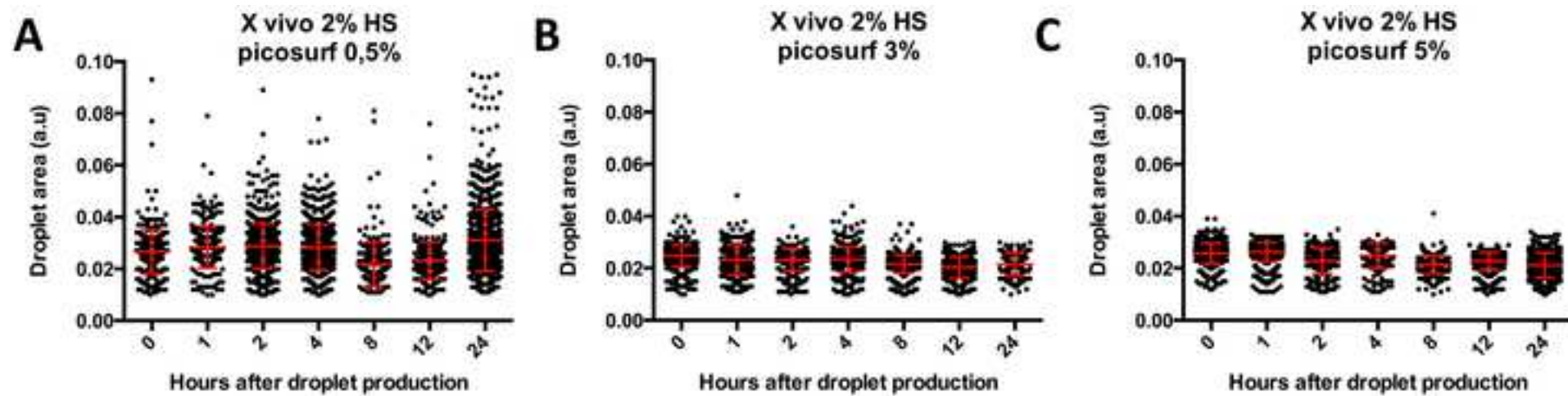
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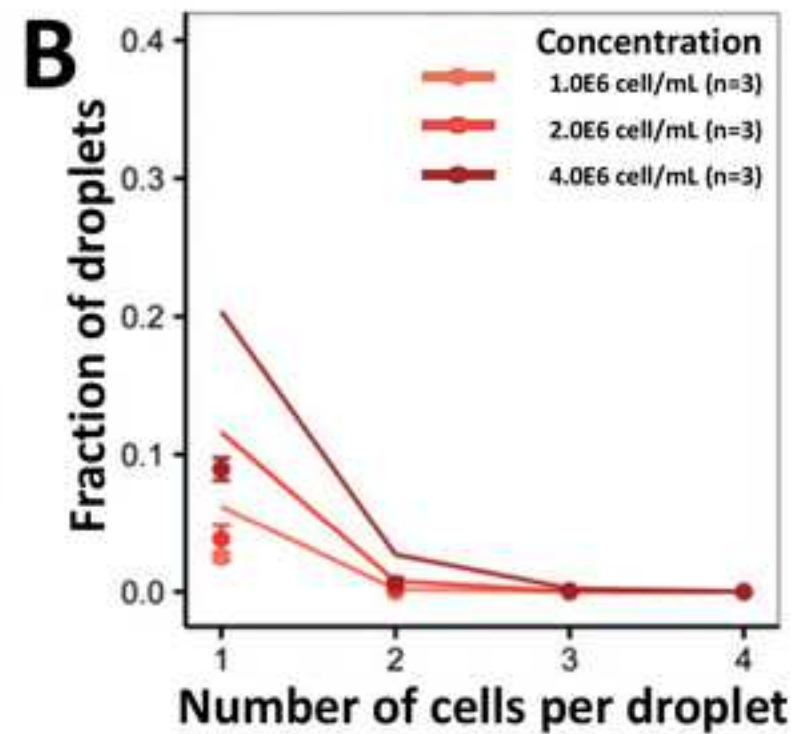
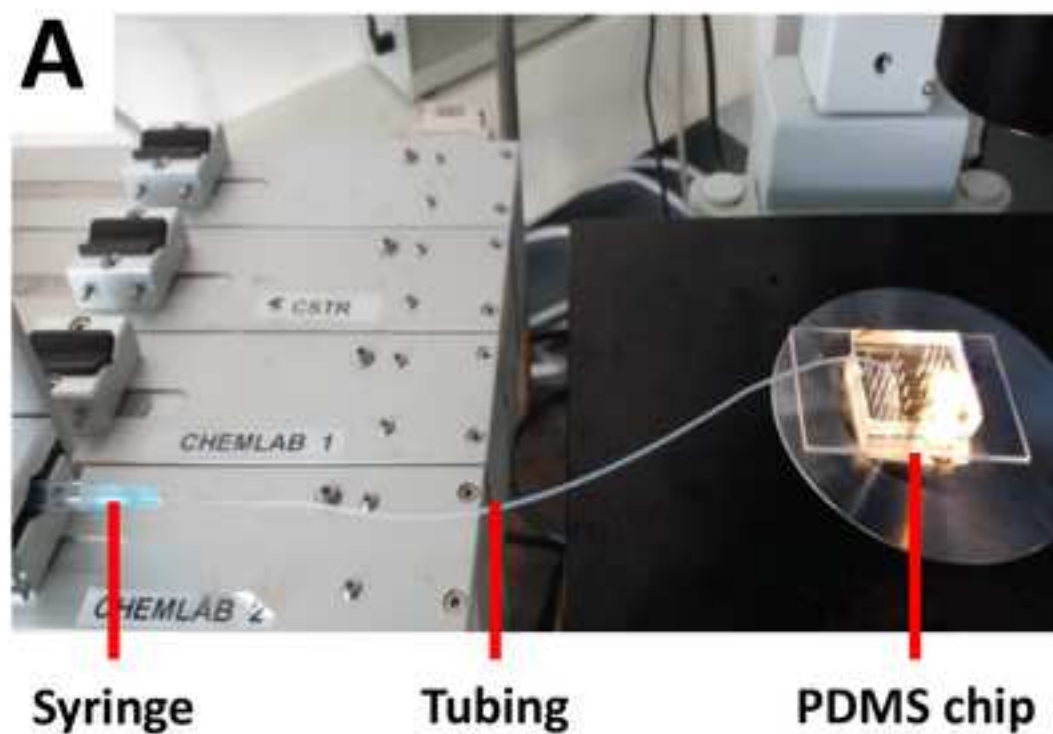
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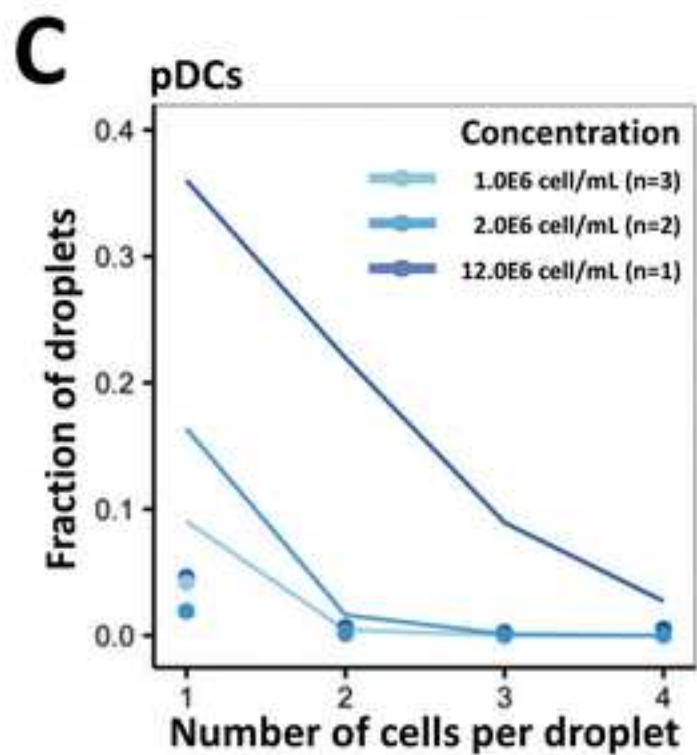
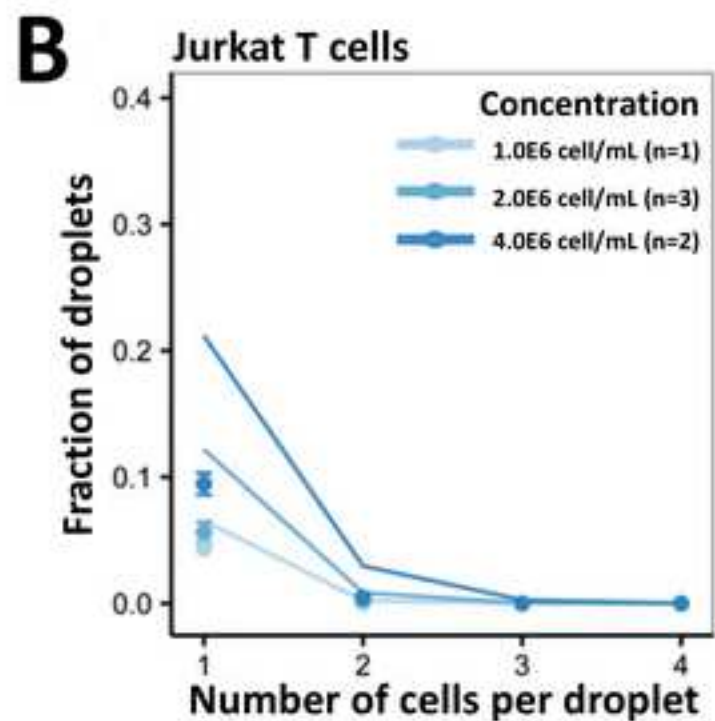
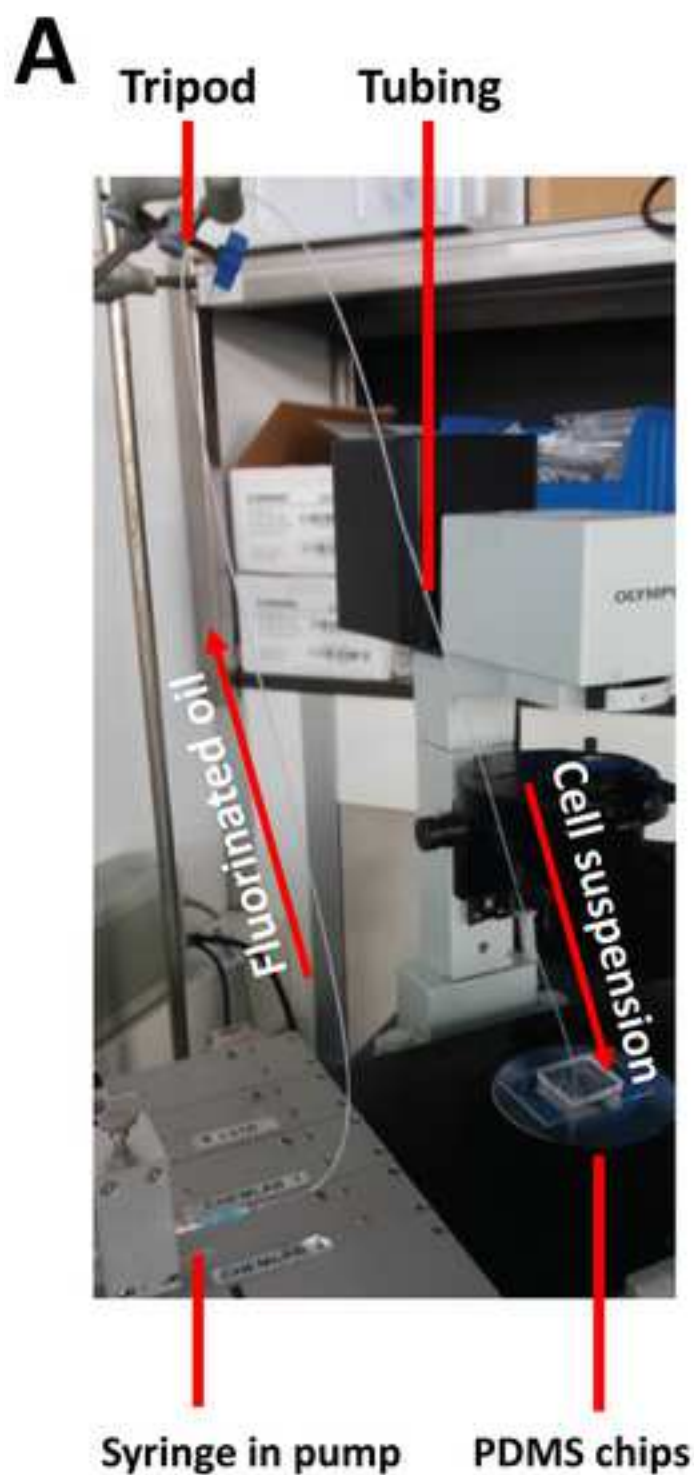


Figure 1









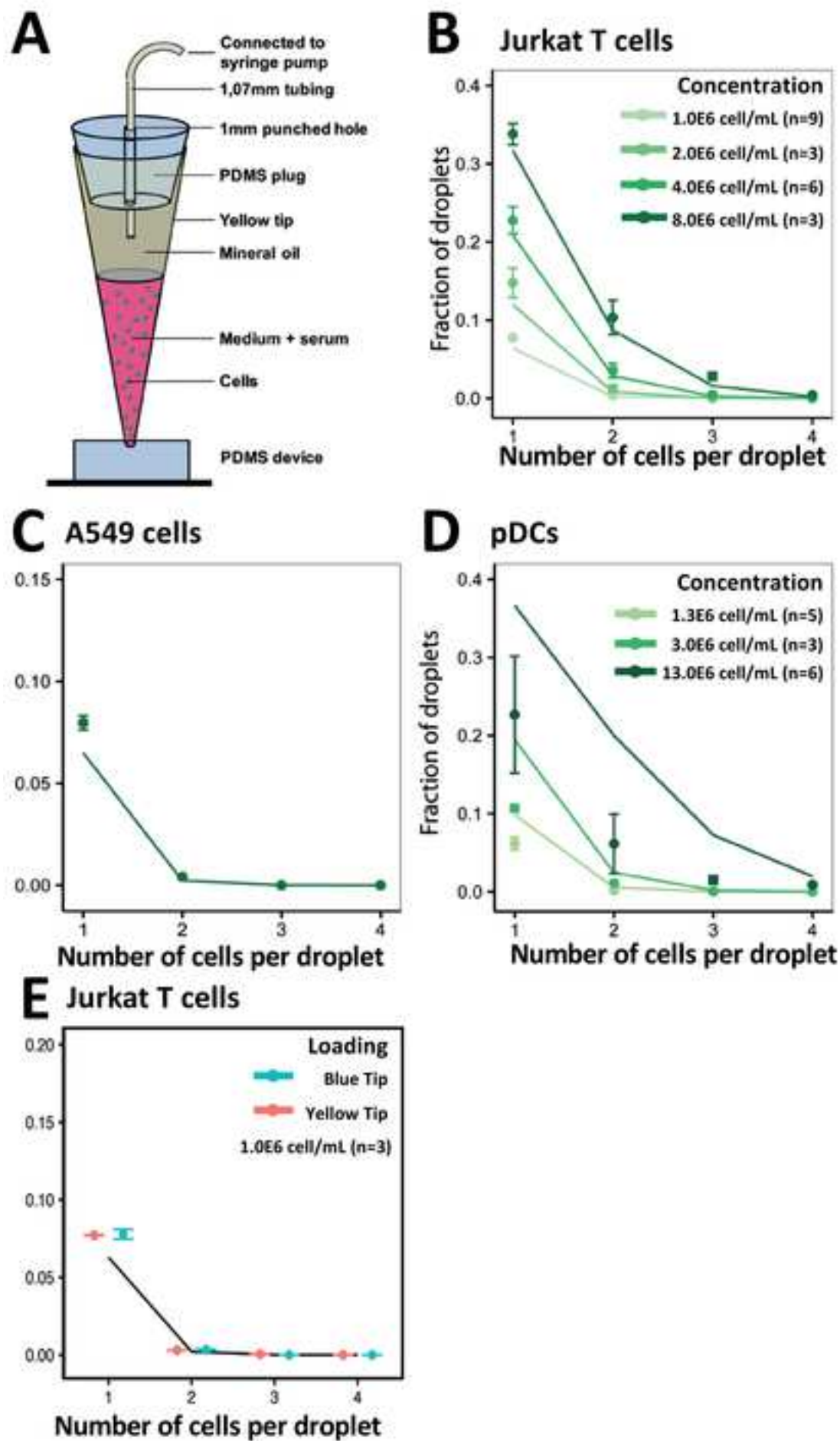
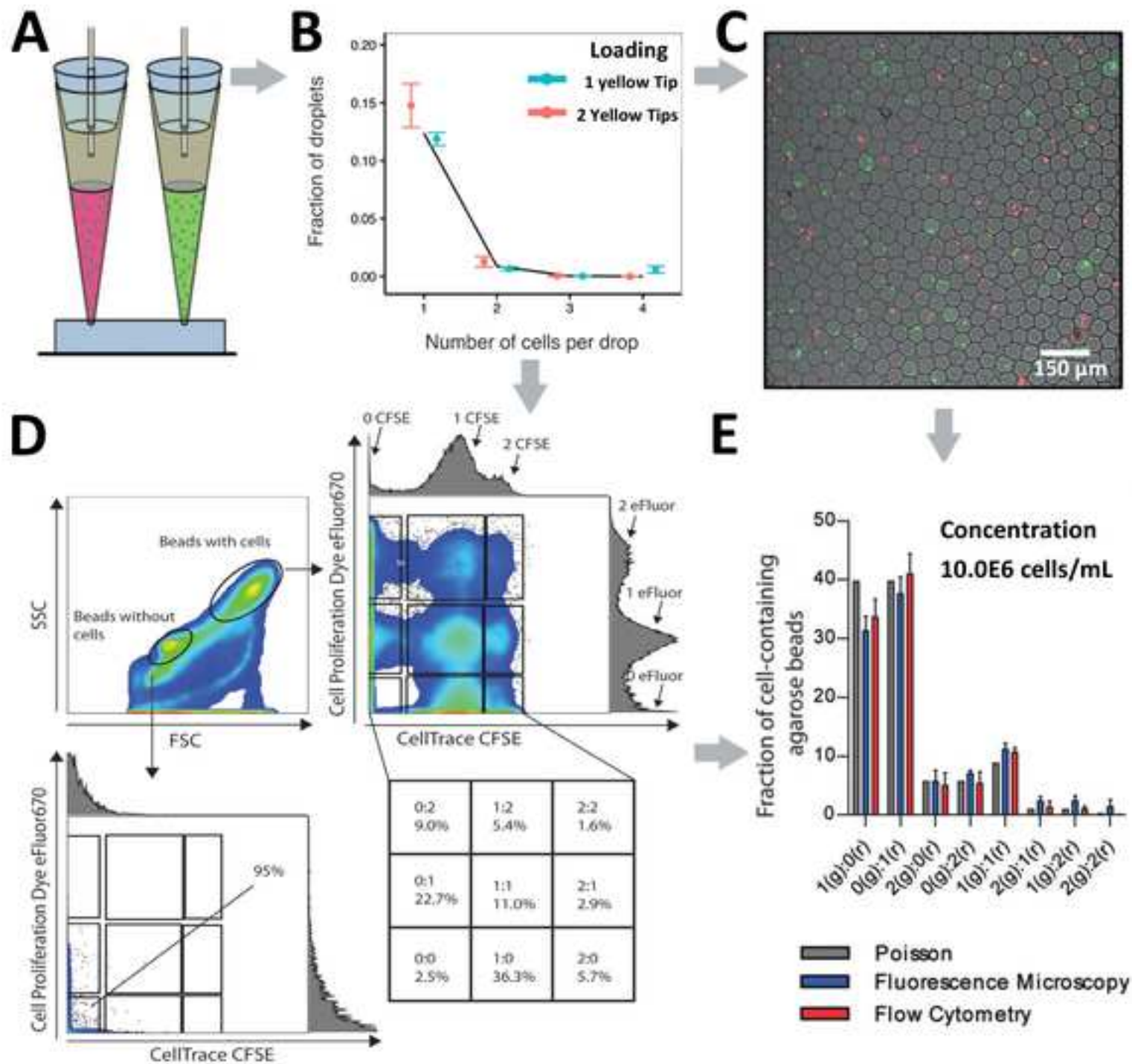




Figure 6



<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
1H,1H,2H,2H-Perfluoro-1-octanol	Sigma-Aldrich	171468-5G
1H,1H,2H,2H-Perfluorooctyltriethoxysilane	Fluorochem/UK	S13150
Agarose (Ultra-low Gelling Temperature)	Sigma-Aldrich	9012-36-6
BD Wegwerpspuiten met Luer-Lok-punten	Fisher Scientific	10630694
Biopsy Punch 1.2 mm	Harris Uni-Core	
Cell Proliferation Dye eFluro 670	eBioscience	65-0840-85
CellTrace CFSE	Invitrogen	C34554
CellTrace Far Red Cell	Invitrogen	C34564
Eppendorf Tubes	Eppendrof Tubes	
Glass Slide	Sigma Aldrich	CLS294775X38-72EA
Harvard Pumps	Harvard Apparatus	C-400750; C-400727
HFE-7500 3M Novec Engineered fluid	Fluorochem/UK	51243
Kai Biopsy Punch 5 mm	Amstel Medical	1980130
Luer stub	Instechlabs/USA	LS20S
Mineral oil (Light)	Sigma Aldrich	M8410-1L
Phosphate buffered saline	Sigma-Aldrich	P4417-50TAB
Pico-Surf 1 (5%in Novec 7500)	Sphere Fluidics	020317-09
Plasma Asher	Emitech	K1050X
RPMI 1640 Medium	Gibco	11875093
Silicone Elastomer Base 184	Sylgard	9355218
Silicone Elastomer Curing Agent	Sylgard	9355218
Stainless steel catheter coupler	Instechlab/USA	SC20/15
TFE Teflon Tubing	Sigma-Aldrich	58696-U
Thinky mixer ARE-250		EX-4025F

## Comments/Description

Silane (toxic)

Syringe

Safe-Lok tubes 1 mL and 2 mL

Corning microscope slides, plain L × W 75 mm × 38 mm

Syringe pumps

Fluorinated oil

Luer stub, 20ga (pink) × 0.5in (12mm), non-sterile

Tablets

Surfactant

Plasma asher

PDMS base

Curing Agent

20ga × 15mm, non-sterile

PTFE Tubing L × O.D. × I.D. 50 ft × 1/16 in. × 0.031 in.

Conditioning mixture





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Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*We thank the editor for giving us the opportunity to go through our manuscript again. We have carefully checked the manuscript for any grammatical errors and spelling mistakes and revised it accordingly.*

2. Summary: Please write the Summary in complete sentences.

*We have revised our short summary and re-written it in a full sentence.*

3. Please spell out each abbreviation the first time it is used.

*We have checked all the abbreviations and spelled it out when it has been used for the first time.*

4. Please use SI abbreviations for all units: L, mL,  $\mu$ L, h, min, s, etc.

*We have checked all the SI units and made changes at places where they were not according to the convention.*

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*We understand this concern and have removed all the commercial names in the manuscript and updated it in the Table of Materials and Reagents.*

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

*We thank the editor for highlighting the use of aforementioned phrases. We have checked and edited the protocol accordingly and also, reduced the number of notes.*

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

1.5: Does the pre-cured PDMS mixture refer to the content in the cup?

*Yes pre-cured PDMS refers to the content in the cup and we have mentioned it in the protocol. (Protocol step 1.5)*

1.6: How large is the petri dish?

*Theoretically, a petri dish of any size can be used as long as it fits the silicon wafer, fabricated for the replica molding process. We use a large circular petri dish and have mentioned its dimension in the protocol. (Protocol step 1.4)*

3.1.2, 3.1.6: What volume of PBS is used to wash? Please specify throughout.

*The volume of PBS used is 1 mL, but the amount of PBS used varies depending on the sample solution. We have mentioned this in the protocol step 3.1.2 and protocol step 3.1.6..*

3.1.5: What volume of ice-cold FCS is added?

*The volume of ice-cold FCS used is 1 mL, but the amount of PBS used varies depending on the sample solution. We have mentioned this in the protocol step 3.1.5.*

3.1.7: Please specify the media used.

*The media used is RPMI and we have updated this information in step 3.1.7*

3.2.2: How many concentrations are prepared?

*We have mentioned in step 3.1.1 that two samples of Jurkat T cells were harvested. Using this we have prepared in step 3.2.2, an agarose solution to yield an agarose concentration of 2%. For both the harvested cell samples, the yielded agarose concentration was 2%.*

3.3.4: Please describe how to analyze using flow cytometry.

*We feel that the large variety of flow cytometers and analysis software that are available make it hard to make a standardized protocol. In the main text and in the figure legend we have added a more balanced description how the analysis was performed.*

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*We have made sure that all the steps are clear and each additional step is mentioned separately. We have also combined some of the smaller steps which are a continuation of the previous step.*

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

12. Figures 3-5: Please include a space between numbers and their corresponding units (i.e., 1 M/mL, 2 M/mL, 1.07 mm, etc.). Please define the error bars in the figure legend.

*We thank the editor for highlighting the unit conventions in the figures. We have modified our figures according to the new convention where we have mentioned cell concentration as follows: 1.0E6 cells/mL, etc. We added in the figure legend that the error bars represent the standard error of the mean (SEM).*

13. Figure 6: What does 10M cells mean? Please define the scale bar of panel C in the figure legend.

*In our figure, 10M means actually 10 million cells. As mentioned above, we have updated the writing convention in which we write the cell number and cell concentration (10.0E6 cells). Furthermore, we thank the editor for pointing out the missing scale bar information. We added that the scale bar represents 150  $\mu$ M.*

14. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. As suggested, we have removed all the trademark and registered symbol from the table.

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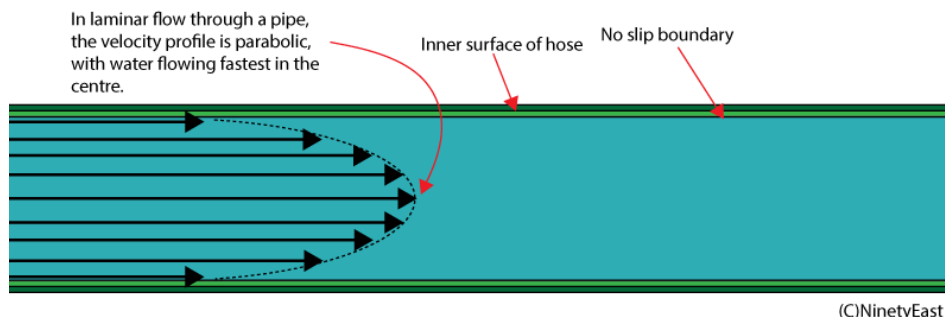
##### Manuscript Summary:

This manuscript has introduced a protocol for droplet microfluidic-based cell seeding which is the most fundamental step for droplet single cell technologies; this is highly valuable in the field. It utilized pipette tips sealed with mineral oil to prevent quick cell sedimentation, which has led to a more uniform cell distribution through the sample injection process. Compared with traditional methods, the reported one is efficient as it can achieve cell encapsulation ratio near that predicted by Poisson distribution. Thus, it is recommended for publication on JoVE. But before acceptance, we have several suggestions:

##### Major Concerns:

1. The cells are different in density with the cell culture media; thus with the setting shown in figure 3A, it is highly possible that cells distribution within the tube would change gradually with time, leading to nonuniform encapsulation efficiencies over time. We suggest the authors provide more information in this respect.

*We thank the reviewer for the thorough reading of our manuscript and we agree that this point deserves more explanation. The tubing used for cell seeding is of very small dimensions and can be compared to microchannels. The flow in the tubing is pressure driven with a parabolic velocity profile.*



*This implies that the maximum flow velocity is at the center of the tubing and minimum velocity is at the edges of the tubing. When only one cell flows through the channel, the cell should be located somewhat at the center where the velocity is highest. However, when a population of cells flows through the through the tubing, the velocity gradient causes the cells to be pushed towards the edges where they settle or slow*

*down, because the velocity at the boundary is close to zero. Thereby, the sedimentation or settlement of cells in tubing reduces the encapsulation efficiency. This theory of reduced cell encapsulation is supported by the observation that large aggregates of cells enter the device upon reaching the final microliters of the loaded cell suspension. We have updated this information in the manuscript as well.*

**Minor Concerns:**

1. There are several grammatical mistakes, please double check.

*We thank the reviewer for highlighting this concern. We have cross-checked our manuscript and made sure that the manuscript is free from grammatical mistakes.*

2. Some of the descriptions for the experiment steps are vague to the readers. Please improve them:  
a. In 2.3.8 the total volume of the sample is not clear.

*We thank the reviewer for the concern. We have updated this information in the protocol. Note: This protocol step is now 2.3.7. We have also updated this information in step 2.3.9. Furthermore, we have checked all protocol steps again to decrease potential ambiguity.*

b. In 3.2.4 note, the speed can be expressed in  $\mu\text{L}/\text{h}$ . Also, it is quite fast. Thus the total volume of the oil phase might be mentioned.

*As suggested, we have expressed the flow rates in  $\mu\text{L}/\text{h}$ . For our experiments we use 1-mL syringes. We typically fill the entire syringe with oil. The volume of the oil phase used, however, varies during the experiment and is dependent on the flow rates and the time duration for stable droplet production. With higher flow rates for the oil phase and longer duration for droplet production, the consumption of oil will be higher and vice-versa.*

c. In 3.2.20, the viability of cells incubated under such condition should be testified.

*We agree that this information is important, however the viability and biocompatibility was thoroughly tested in the paper by Chokkalingam, V. et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip 13, 4740 (2013). We have made a reference to this paper in the section that concerns the use of low-melting agarose for cell encapsulation.*

**Reviewer #2:**

Manuscript Number: JoVE57848

A pipette-tip based method for seeding cells to droplet microfluidic platforms

Remarks on protocol:

**Protocol**

The protocol steps are clearly written and easy to understand. I have only two small remarks on them that are listed below.

1.14: It is useful to mention the settings of the plasma asher, but you should also reveal the company and model name of the plasma asher used. It is not in the materials and equipment table.

*We have given the most important settings of the plasma asher used here. The other settings are as default and never get modified. (We have also updated the company and model name of the plasma asher in the material list).*

2.2.8. What is your recommended PDMS plug diameter for a 1000  $\mu\text{L}$  pipette tip?

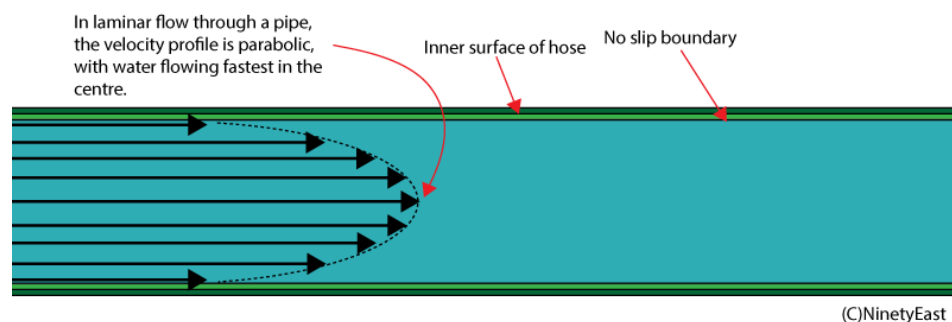


*For 1000  $\mu\text{L}$  pipette tip, plugs of diameter ranging between 5 mm and 7 mm can be used. With a plug of diameter 5 mm, a sample volume of around 400  $\mu\text{L}$  can be aspirated in the pipette tip. If a plug of larger diameter is used (7 mm), more sample volume can be aspirated (around 900  $\mu\text{L}$ ). We have added this information to our manuscript.*

### Representative Results

The results of the setup in Figure 4 are not completely comprehensible to me. In my understanding the cells should sediment to some extent towards the chip inlet, when they are injected in the tubing on top of the tripod. Thus, the approach is not too different from your presented pipette-tip based technique. However, you seem to have low encapsulation efficiency. Do you have an explanation for this? Have you tried different tube lengths? If you have sticky cells, which tend to the surface of the tubing you could also try to coat the walls with e.g. BSA as an alternative to Teflon tubing. And do you have any explanation for the increased efficiency in your pipette-tip based loading method?

*We agree with the reviewer that this point deserves more explanation. The tubing used for cell seeding is of very small dimensions and can be compared to microchannels. The flow in the tubing is pressure driven with a parabolic velocity profile. This implies that the maximum flow velocity is at the center of the tubing and minimum velocity is at the edges of the tubing. When we have only one cell flowing through the channel, the cell should be located somewhat at the center where the velocity is highest. However, when*



*a population of cells flows through the through the tubing, the velocity gradient causes the cells to be pushed towards the edges where they settle down because the velocity at the boundary is close to zero. The sedimentation or settlement of cells in tubing reduces the encapsulation efficiency. This theory of reduced cell encapsulation is supported by the observation that large aggregates of cells enter the device upon reaching the final microliters of the loaded cell suspension. Previously, we have tried loading cells via tubing either positioned horizontally or vertically and also tried various tube lengths. In these experiments there was no clear benefit in both the tube position and length. Furthermore, the length of the tubing that we used depended on our experimental setup and loaded cell suspension.*

*A pipette-tip, unlike a tubing, has variable dimension with larger diameter at the top and it converges at the bottom, resembling a nozzle. This means that the average velocity of the sample will be smaller at the top that gradually increases towards the narrower side. When the velocity is low at the top, it makes sure that all the cells get pushed towards the end (small changes in velocity from the center towards the end, smaller velocity gradient). As the flow reaches the end of the tip, the increased velocity pushes the cells out of the tip and the cells immediately enter the microfluidic chip. This variable flow velocity across the length of the pipette tip makes sure that the cells do not sediment to the edges of the tip and we obtain a much higher encapsulation efficiency as a smaller number of cells are lost.*

*In previous studies we have opted to use different types of tubing but also there have not observed significant or noteworthy differences.*

### Figures

I have no negative remarks on Figure 1 and Figure 2. But Figure 3 and 4, showing two conventional setups for droplet generation, need to be improved. Figure 3A has a poor quality and the caption is not very explanatory. The legend in Figure 3B has an untypical unit writing. Before I read the text, it was not clear to me that "1M/mL" means " $1 \times 10^6$  cells/mL". Maybe you should change it in the figure itself or write a better caption. Figure 4A is not sufficiently explained in the caption. It does not show how and where exactly the cells are injected in the tubing. Furthermore you do not explain what tube length you chose and why. Is there a reason why you have such an inconsistent number of replicates per concentration, e.g. in Figure 4C "2 M/mL (n=26)" and "12 M/mL (n=1)"?

Figure 5A is nicely illustrated. Therefore Figure 5B is redundant and can be left out because no new information is shown. What is the cell concentration for the results shown in Figure 5F? Figure 6D contains much information, which is not mentioned or explained in the caption or text at all.

*We thank the reviewer for highlighting the concerns with the figures. We have improved the resolution of the figure that illustrate the different setups. Also, we have changed the convention of writing the units in all the figures and used the following format:  $1.0 \times 10^6$  cells/ML etc. In Figure 4A, we have provided better description in the figure itself specifying where the cells are injected from in the tubing. For this method, we use the tube length depending on the setup. What is important is that the cell concentration should only be filled in one half of the tube length and this length can vary depending on the sample volume. We would like to apologize for the typo in the replicate number. We have updated this information in the figure. Finally, as suggested by the reviewer we omitted Figure 5B from the figure, mentioned the cell concentration in 5F and have added a more balanced description of figure 6 in the figure legend.*

#### Discussion

Did you use Teflon tubing for all your presented experiments? If so, you should mention it in the protocol and representative results part and not only in the discussion.

*We have used Teflon tubing for all the experiments, second method onwards. For all our subsequent experiments, including the ones in protocol, we adapted our methods to work only with Teflon tubing. We have updated this information in the manuscript.*

#### Reviewer #3:

##### Manuscript Summary:

The authors present a new methodology that uses pipette-tips to load cells to droplet-based microfluidic devices without the significant loss of cells.

##### Major Concerns:

1. If different type of cells are loaded together, will it still work?

*The microfluidic chip design used in our laboratory is a three-inlet device where the inner two inlets are used for seeding cell samples and media and the outer inlet is used for oil phase. For single-cell encapsulation, we use only one inlet and flush media or stimuli from another inlet, or split the cell concentration and flush same cell population from both the inlet to achieve desired encapsulation rate. Also, the two inlets can be used for loading two different cell types to probe cellular communication between two different cell types at single-cell level. We have addressed this concern in our discussion section and also highlighted that we can use this method to pair multiple cells in single droplet for understanding the influence of cellular microenvironment on different cell types. Overall, yes this method will work when different cell types are loaded together given both the loading techniques use pipette-tip based approach.*



2. Can this method apply to microparticles loading rather than cell?

*We believe that it is possible to use this method for seeding microparticles, that face similar loading statistics as cells, to microfluidic devices and we have mentioned it as a potential application in the introduction section.*

3. Cells always precipitate down to bottom of tip, how to solve this problem?

*We understand the concern of the reviewer and agree that cells do precipitate down to the bottom of the tip. This is the reason why we have to optimize the cell concentration to make sure that the amount of cells that precipitate to the bottom of the tip is minimum and interfere very less with the seeding procedure. This is the reason why work with a maximum cell concentration of 13 million cells per mL to have a high encapsulation efficiency. With increase in cell concentration, more cells will start to precipitate and settle at the bottom of the tip interfering with our experimental procedure.*