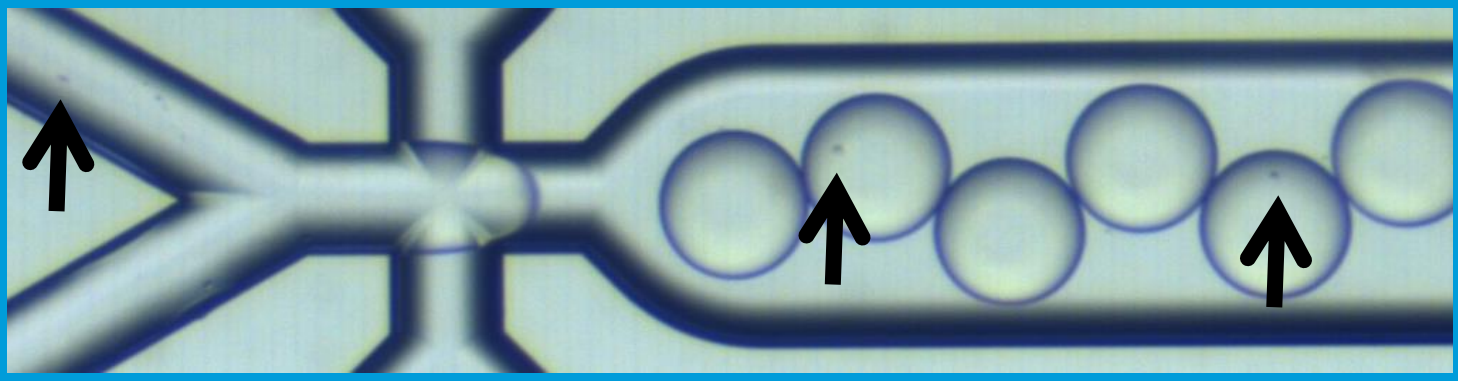
Capturing single cells in droplets using density media

Encapsulation of single cells is facilitated by the use of density matching media



Summary

Differences between sub-populations of cells are often biologically important (e.g., T cell clonotypes, tumour subpopulations), but traditionally, methods such as RT-PCR only allow analysis of a population average. Single cells can be co-encapsulated with a reaction mix such as RT-PCR, in picoliter aqueous droplets in oil, and the droplets can then function as micro-reactors, where each droplet contains amplicons from a single cell. For instance, leukocytes may be encapsulated to profile epitope binding sites in natively paired T-cell receptor or antibody coding sequences.

To minimise confounding droplets with two or more cells, cells are typically diluted so that only 1 in 10 droplets contain a cell. However, the number of cells per droplet tends to change over time, because the cells are more dense than the buffers, and sediment out. We show here that adjusting the density so that cells are nearly neutrally buoyant, using density media such as Percoll™ or Optiprep™, minimises the rate of cell sedimentation, and allows encapsulation of cells at a controlled rate of cells per droplet.

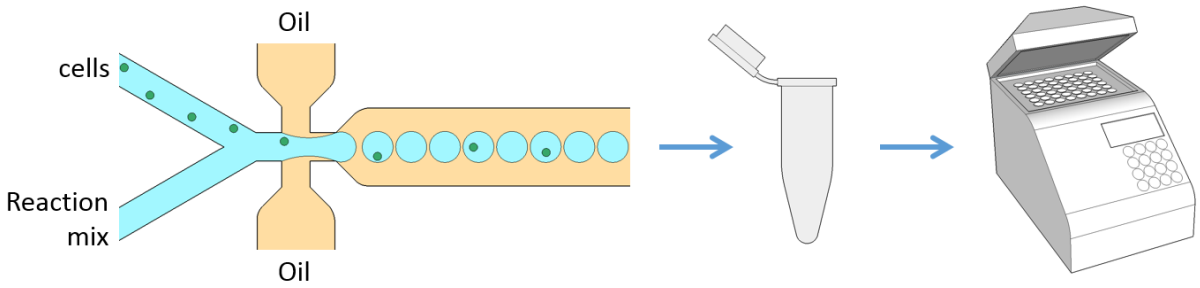


***Figure 1**. In a typical experiment, cells are combined with a reaction mix, such as an RT-PCR mix, and encapsulated in droplets in oil. The resulting micro-emulsion is collected, typically in a chilled PCR tube, and transferred to a PCR machine for RT-PCR.*

Introduction

It is often critical to be able to extract (linked) information from single cells. Applications include isolating natively paired epitope binding sites from T and B cells and high throughput single cell RNA sequencing.

For instance, to understand immune responses, it is important to know the T-Cell Receptor (TCR) clonotypes of T-cells. This requires isolating the natively paired TCR heterodimer chains (i.e., the TCR heterodimer from an individual cell). It would also be very useful to be able to isolate natively paired antibody light and heavy chains (e.g., to isolate candidate therapeutic antibodies). This can be done by encapsulating purified leukocytes in aqueous droplets in oil, so that there is one cell or less per droplet. The cells are co-encapsulated with an RT-PCR mix, and the resulting emulsion is put in a PCR machine for RT-PCR. Each droplet behaves as a micro-reactor, eventually containing the amplicons (PCR-amplified DNA fragments) from a single cell.

One of the primers may be immobilised on beads, and the beads co-encapsulated with individual cells, resulting in the amplicons from an individual cell being immobilised on an individual bead for, e.g., flow cytometry, or the beads may have a barcode library, such that the amplicons from an individual cell are identically tagged.

Alternatively, amplicons can be joined by ligation or overlap PCR, so that amplicons from a single cell remain physically linked after the emulsion is broken. The amplicons can then be submitted for one of the high throughput sequencing methods.

It is generally critical to minimise the number of droplets having multiple cells. In the example above, of obtaining populations of natively paired TCR heterodimer pairs from a blood sample, having two T cells in a droplet would make it impossible to determine the native TCR pairing. In order to minimise the proportion of droplets having multiple cells, it is common to dilute cells, to obtain approximately one cell per 10 droplets. However, because the cells are more dense than most buffers, they sediment fairly quickly. Because liquid is generally drawn from the bottom of the reservoir, the number of cells/droplet will increase when this fluid reaches the encapsulation chip. This can be mitigated by stirring or rocking the cell suspension reservoir, but a gentler, more reliable, mechanically simpler method is adjusting the density of the cell suspension medium, so that the cells have nearly neutral density.

Here we show that common density centrifugation media such as Percoll™ and Optiprep™, can be used to mitigate cell sedimentation in the reservoir, and that the density media do not affect droplet formation. This results in being able to maintain a steady rate of cell encapsulation, of around 1 cell per 10 droplets.

Materials and methods

Solutions were filtered through a 0.2 μm syringe filter to remove dust and fibres, which can block the chips. Cell suspensions were prepared with filtered solutions, but were not themselves filtered. The system was cleaned by flushing with air, filtered water, filtered acetone, then air.

Cells are typically diluted to around 1 cell for every 10 droplets. The cell suspension was prepared in a 1.5 ml Eppendorf tube, which was placed in a 20 ml glass vial (as a holder) inside the P-pump reservoir. We used a fluorophilically coated droplet incubation chip, which has a 50 μm junction, which produces approximately 50 – 55 μm droplets, and a 50 μm droplet has a volume of around 65 pl. For one cell per 10 droplets, we made test dilutions of 1 cell per 650 pl (i.e. 1.5 X 10^6 cells/ml), encapsulated them, counted cells/droplet, then adjusted the cell concentration. The resulting micro-emulsion was collected in a 1.5 ml Eppendorf tube.

As a reaction mix was not being flowed onto the chip, one of the reagent channels was sealed (by plugging the relevant hole in the connector). In the case where the cell suspension was being mixed 1:1 on the chip with another fluid (e.g., an RT PCR mix, see Fig. 1), the cell suspension would have to be more concentrated, to account for dilution by the reaction mix. Droplets were generated at around 100 Hz (100 droplets/second), at a

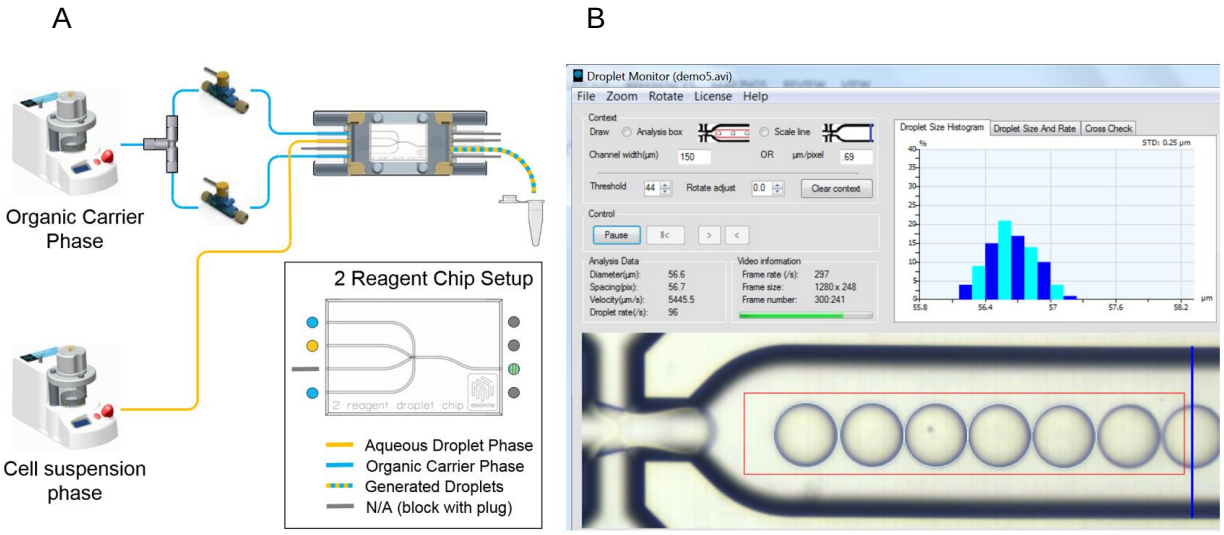


***Figure 2**. Cell encapsulation setup.*

A) The cell encapsulation setup consists of two P-pumps, flow meters, a chip, and associated connectors. The chip was imaged with a Dolomite high speed imaging system, comprising a light stage, microscope, and high speed camera.

B) Droplet size and frequency were determined using the Dolomite droplet monitor software. Note that the droplets are mono-disperse; the mean droplet diameter is 56.6 μm, with a standard deviation of 0.25 μm.

flow rate on the aqueous phase of around 0.5 ul/min (this was the total aqueous flow rate, because one of the two aqueous channels was plugged).

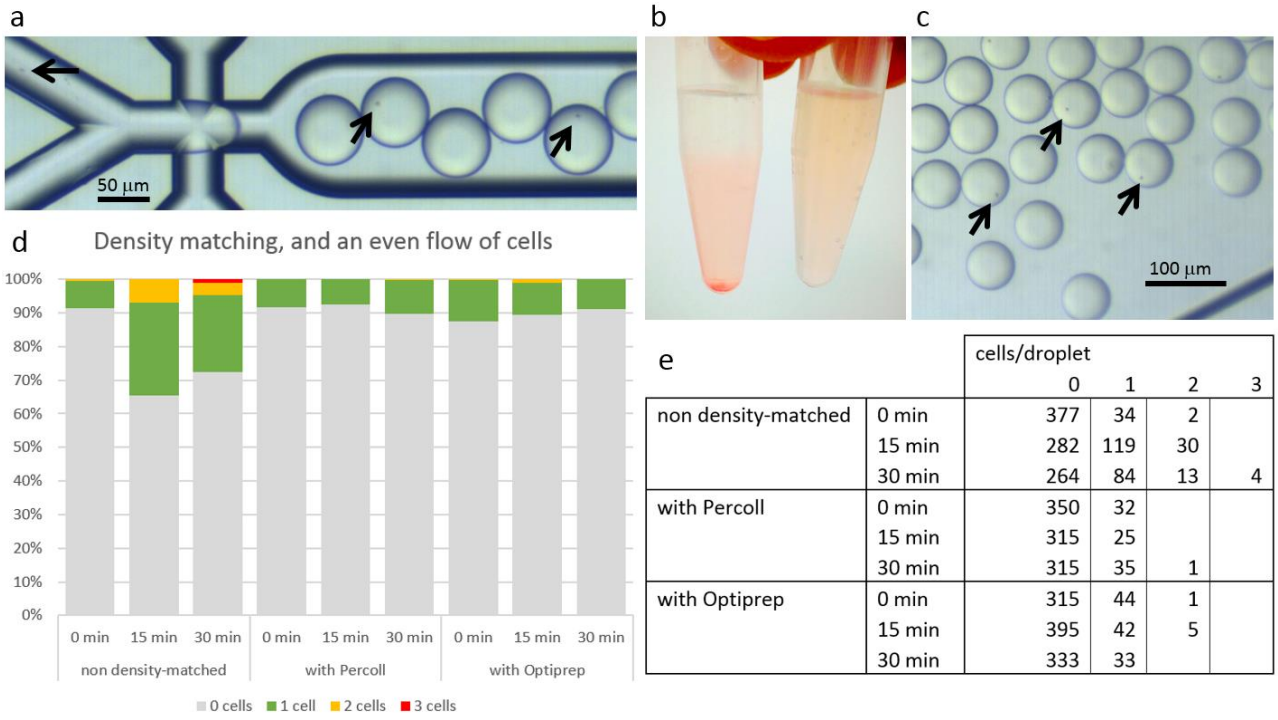
To simplify counting and analysis, sheep red blood cells (RBCs) were encapsulated in a droplet incubation chip. Photomicrographs (0.1ms exposure) were taken in the wide droplet incubation channels, where each photomicrograph included 50 – 100 droplets. Photomicrographs of droplets containing encapsulated cells were taken at 0 minutes, 15 minutes, and 30 minutes. Droplets containing 0, 1, 2 etc. cells were then counted.

RBCs have a density of 1.09 – 1.10 g/ml, so approximately 0.7 μl of sheep blood was suspended in DMEM alone, or DMEM adjusted to 1.09 g/ml with 69.2% Percoll™ (Sigma, P1644, density = 1.13 g/ml), or 28.1% Optiprep™ (Sigma, D1556, density = 1.32 g/ml). 20 X DMEM was added to a final concentration of 1X.

Cells may agglomerate or settle at ‘step-downs’, where the inside diameter of the flow path reduces suddenly, such as flowing from a fitting (like a valve or junction) down to 100 μm inner diameter (i.d.) tubing. Therefore, a simple flow path from the cell suspension reservoir to the chip was used, i.e., a single piece of 100 μm i.d. tubing from the reservoir to the chip.

Results

The objectives were to test that various density media kept the cells in suspension, and enabled cells to be encapsulated at a constant rate. We also wanted to determine



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		cells/droplet			
		0	1	2	3
non density-matched	0 min	377	34	2	
	15 min	282	119	30	
	30 min	264	84	13	4
with Percoll	0 min	350	32		
	15 min	315	25		
	30 min	315	35	1	
with Optiprep	0 min	315	44	1	
	15 min	395	42	5	
	30 min	333	33		

***Figure 3**. Cell encapsulation.*

A) Cells being encapsulated in the droplet incubation chip. The junction is 55 µm wide, and the downstream channel is 150 µm wide. The droplets are approximately 57 µm in diameter. Cells are indicated by arrows.

B). Density-matching minimises cell sedimentation. After an hour, the RBCs in saline (left tube) have sedimented markedly, whereas density-matched RBCs (right tube) are still well suspended.

C) A photomicrograph of droplets in the downstream wide incubation channel. These photos were used for counting droplets and cells. Some cells are indicated by arrows.

D) Graph, showing the influence of density matching on the even-ness of the cell encapsulation rate. In the non-density matched suspension, the rate of cells/droplet had already increased by 15 minutes, resulting in increased numbers of droplets with two or more cells. In contrast, using either Percoll™ or Optiprep™ to match the density resulted in an even flow of cells, by minimising cell sedimentation.

E) The table shows data from one of the runs.

whether the encapsulation media interfere with the formation, stability, or mono-dispersity of droplets. We used Percoll™ and Optiprep™, two media widely used for density gradient centrifugation, that are non-toxic to cells, and have low viscosities and osmolarities.

Cells were encapsulated in a chip with a 50 µm junction, which readily produces 40 – 60 µm droplets (30 – 110 pl). As the oil phase, we used 2% Picosurf™ 1 in Novec™ 7500, which is an oil/surfactant formulation developed for PCR. Fluorocarbon oils are similar to

hydrocarbon oils, except the hydrogen atoms are replaced with fluorine atoms. Fluorocarbon oils are biocompatible, will not partition organic compounds (e.g., hydrophobic peptides or hormones) like hydrocarbon oil would, make stable emulsions, and hold a high concentration of dissolved oxygen, which is useful if cells are to be kept alive. The chip was fluorophilically coated, to allow the fluorocarbon oil to coat the walls, and the aqueous phase to form droplets.

The droplet system produces highly mono-disperse droplets, which means highly reproducible reaction or analysis conditions, because a tightly controlled amount of reaction mix is included in the droplet. In Figure 2, it can be seen that the mean droplet diameter is 56.6 mm, with a standard deviation of 0.25 mm. This equates to a mean volume of 95 pl, with a standard deviation of 1 pl (i.e., approximately 1%). Therefore, cells can be encapsulated with density media, in highly mono-disperse droplets.

Cells were first encapsulated in DMEM culture medium alone, with no density medium. Cells were initially being encapsulated at a rate of approximately 1 cell per 10 droplets, i.e., 8% (34/413) of droplets had 1 cell, and 0.5% (2/413) droplets had 2 cells. However, as the experiment proceeded, the cells started to sediment to the bottom of the Eppendorf tube where the outlet tubing was, increasing the concentration of cells going into the outlet tubing. After 15 minutes, 28% of droplets (119/431) had one cell, and 7% of droplets (30/431) had two cells. At 30 minutes, 3.5% of droplets (13/365) had two cells, and 1% (4/365) had three cells. This illustrates the phenomenon of cell sedimentation causing a high rate of cells containing two or more cells/droplet, which is undesirable for many experiments.

We next adjusted the density to 1.09 g/ml with Percoll™ (69.2% Percoll™). Percoll™ did not noticeably affect droplet formation, and the droplets were stable on the chip for at least an hour. Initially, 8% of droplets (32/380) had one cell, and 0/382 had more than 1 cell. After 15 minutes, 7% of droplets (25/340) had one cell, and no droplets had two or more cells. At 30 minutes, 10% of cells (35/351) had 1 cell, and 0.3% of droplets (1/351) had two cells. This demonstrates that adjusting the density with Percoll™, so that the cells are nearly neutrally buoyant, can give an even encapsulation rate.

We also used Optiprep™ (28.1%) to adjust the density of the cell suspension. Optiprep™ also did not noticeably affect droplet formation, and droplets were again stable for at least an hour on the chip. Initially, 12% of the droplets (44/360) had 1 cell, and 0.3% (1/360) had two cells. At 15 minutes, 9.5% of droplets (42/442) had 1 cell, and 1.1% of droplets (5/442) had two droplets. At 30 minutes, 9% of droplets (33/366) had one cell, and no droplets had more than one cell, demonstrating that Optiprep™ is also useful for obtaining an even cell encapsulation rate, by matching the density of the medium to the cells.

Conclusion

Encapsulating single cells in ‘micro-reactor’ droplets in oil enables a number of analyses that are hard to achieve any other way, such as analysing and isolating natively paired epitope binding sites of T Cell Receptors or antibodies from blood samples and high throughput single cell RNAseq. We encapsulated cells using the fluoro-surfactant Pico-Surf™ 1 the fluorocarbon oil Novec™ 7500. This oil/surfactant combination is recommended for PCR, to maintain stability of droplets during thermal cycling. In order to maintain an even flow of cells, and an even encapsulation rate of 1 cell for every 10 droplets, we suspended cells in medium that was density matched to the cells, so that the cells were close to neutrally buoyant, to minimise cell sedimentation. For density matching, we used Percoll™ or Optiprep™, two density centrifugation media which are non-toxic to cells, and have low viscosities and osmolarities.

In principle, the distribution of cells in droplets should follow a Poisson distribution, so long as the probability of a cell arriving at the junction was independent of other cells arriving (e.g., the cells are not clumping). Therefore, the ratio of droplets with 1 cell to droplets with 2 cells should be 2/lambda, where lambda is average number of cells/drop. So if lambda = 0.1 (1 cell in every 10 droplets on average), the ratio of droplets with 1 cell to droplets with 2 cells should be 2/0.1 = 20, which is approximately what we see.

We show that neither Percoll™ nor Optiprep™ noticeably interfere with droplet formation or stability. Furthermore, whereas when cells are encapsulated without density matching, cells sediment rapidly, causing cell concentration to rise at the bottom of the reservoir, and the number of cells/droplet to increase above the target 1 cell per 10 droplets. However, adding Percoll™ or Optiprep™ to the cell suspension, to make the cells nearly neutrally buoyant, minimises cell sedimentation, and allows cells to be evenly encapsulated at the desired number of cells/droplet.

Appendix A: System Component List

Part No.	Part Description	#
3200016	Mitos P-Pump	3
3200118	Mitos Compressor 6bar (110V/60Hz)	1
3200097	Mitos Flow Rate Sensor (30 - 1000µl/min)	1
3200098	Mitos Flow Rate Sensor (1 - 50µl/min) (optional)	1
3200200	Mitos Sensor Interface	2
3200197	USB to RS232 Adaptor Cable	3
3200148	Linear Connector 7-way	1
3200294	Top Interface 7-way (4mm)	1
C000525A	2 Reagent Droplet Chip (50µm etch depth), fluorophilic	1
3000397	T-Connector ETFE	1
3200302	FEP Tubing, 0.8 mm x 0.25mm, 10 metres	1
3200304	FEP tubing, 0.8 mm x 0.1mm, 10 metres	1
3200063	FEP Tubing, 1/16" x 0.25mm, 10 metres	1
3200087	2-way In-line Valve	2
3200050	High Speed Camera and Microscope System	1

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