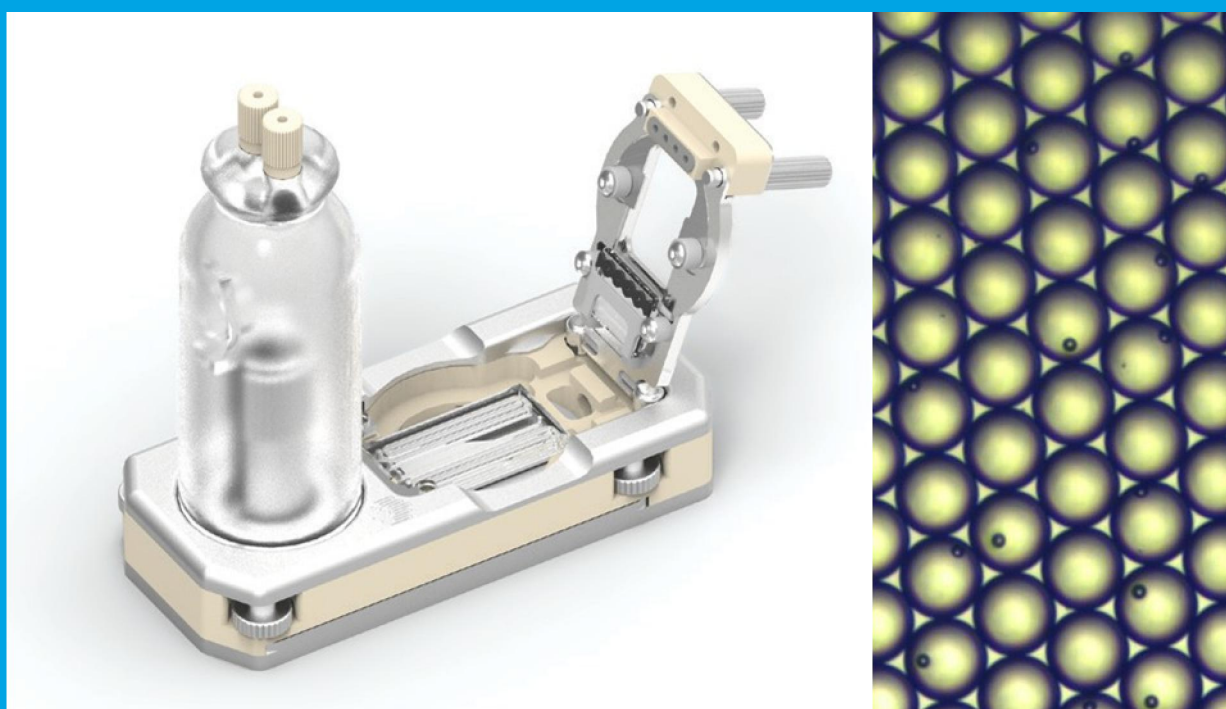


High Throughput Single Cell Pairing of Antibody or TCR Sequences

Using the μ Encapsulator to capture single cells in droplets with beads



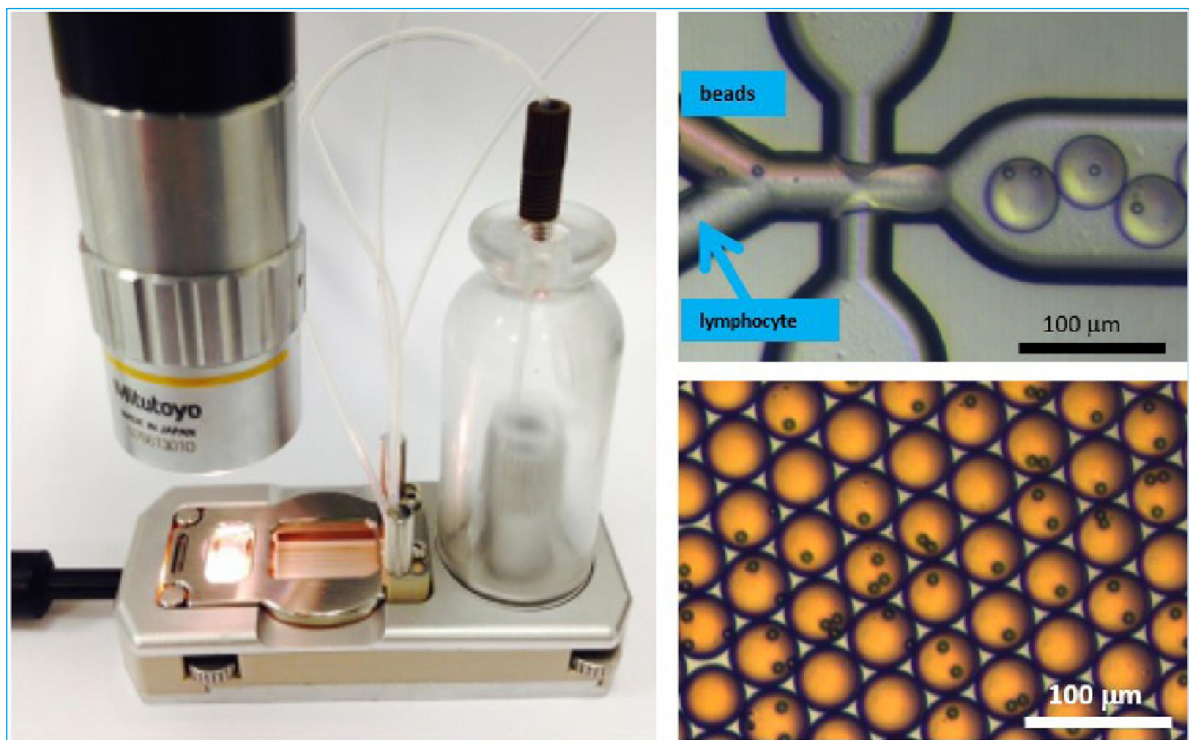
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Summary

Conventional tools in biology homogenise populations of cells, which means that in many processes, it is still not possible to observe and analyse crucial biological events (e.g. in immune responses, in complex tissues such as neural tissues, or events in tumourigenesis, metastasis, etc.). By capturing single cells in microfluidic droplets, it is possible to do high throughput single cell analysis, which opens up these processes to study.

As an example of the power of high throughput droplet microfluidics, a method for 'paired sequencing' of thousands of antibodies or T-Cell Receptors (TCRs) from blood samples was published recently (DeKosky et al., *Nat. Biotech.*, **31**:166, DeKosky et al., *Nat. Medicine*, **21**:86, and MacDaniel et al. *Nat. Protocols*, **11**:429). Implementing such methods in a lab may however not be straightforward as it often requires substantial multidisciplinary prowess (engineering, programming, biochemistry, microfluidics) to build a system.

To make high throughput single cell methods more widely available, Dolomite Bio has developed the μ Encapsulator 1, which greatly simplifies capturing single cells in microfluidic droplets. In this application note, we illustrate the use of the μ Encapsulator 1 for the simple, high throughput, capture of lymphocytes in 50 μ m droplets.



Introduction

In many biological processes, crucial events take place at the level of cells, and these cannot be understood without analytical tools that have single cell resolution. For instance, many crucial events in immune or vaccine responses take place at the level of single T and B cells, but are inaccessible to conventional bulk solution methods such as PCR, or to the cell surface markers used in FACS.

A series of papers published recently describes isolation of thousands of paired antibody coding sequences from thousands of lymphocytes using either micro-well slides (340,000 wells) or in microfluidic droplets (DeKosky et al., *Nat. Biotech.*, **31**:166, DeKosky et al., *Nat. Medicine*, **21**:86, and MacDaniel et al. *Nat. Protocols*, **11**:429). Isolated cells were lysed with poly(dT) beads in microfluidic droplet compartments to capture mRNA from single cells on beads. The mRNA beads were recovered, washed, and subjected to Reverse Transcription (RT). The resulting single cell cDNA beads were then singly encapsulated with PCR mix (Figure 1). Overlap PCR was performed to link the antibody coding sequences from single cells (Figure 2) and the amplified linked products were subjected to high-throughput sequencing.

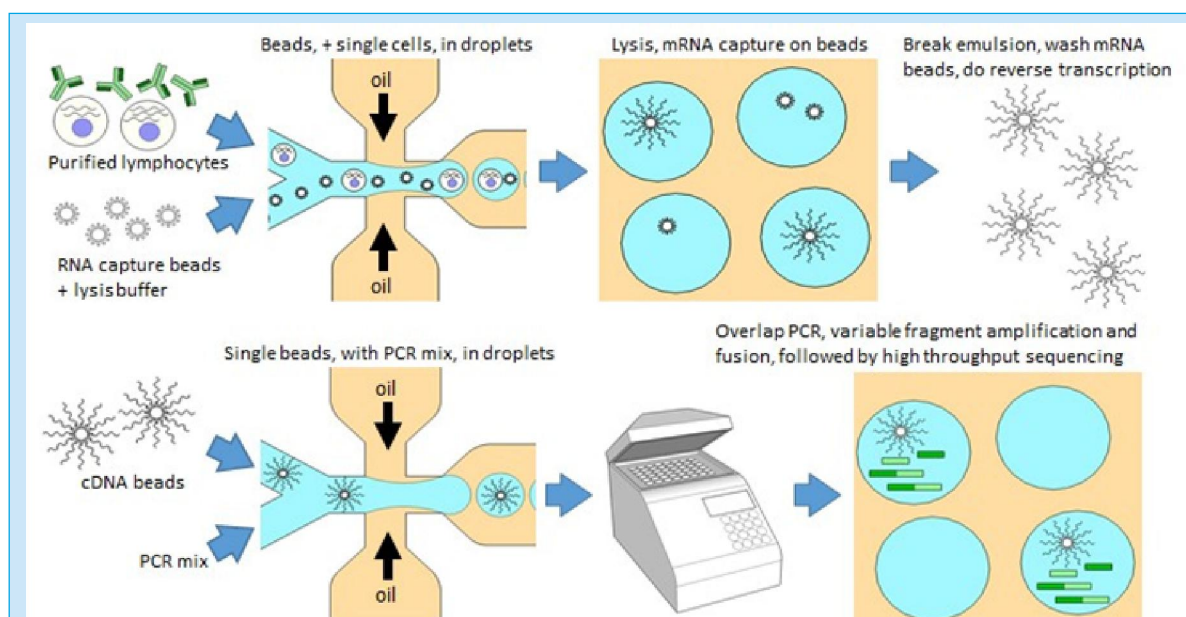


Figure 1. High-throughput sequencing of paired antibody or TCR coding sequences.

Single cells are isolated in droplets with mRNA capture beads (e.g. poly(dT) beads). Inside the droplets cells are lysed and the RNA is captured on the co-encapsulated beads. Following mRNA capture, the emulsion is broken, the beads are washed and Reverse Transcription is performed. The beads are then singly re-encapsulated with PCR mix containing specific primers to achieve overlap PCR and fusion of TCR or antibody coding sequences.

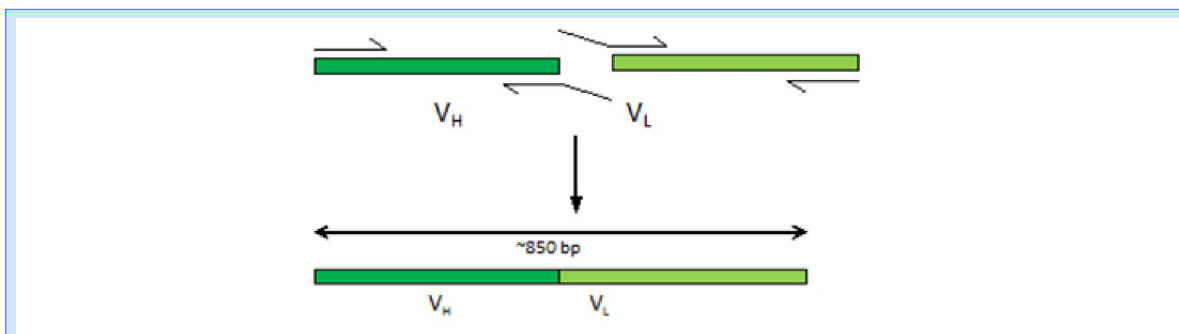


Figure 2. Overlap PCR of antibody coding sequences. Primers with overlapping regions are used to fuse both heavy and light variable regions during PCR amplification, resulting in an 850-bp long product. V_H = heavy variable region, V_L = light variable region.

When encapsulating cells in microfluidic droplets, single cells are typically captured in 50 - 125 μ m diameter (65 pl – 1,000 pl) droplet reactors, together with mRNA capture beads. The beads are coated with poly(dT) oligo, which both capture mRNA and prime reverse transcription. In order to obtain high quality data, it is generally necessary to minimise the number of droplets that contain more than one cell (cell ‘doublets’ and greater). Currently, the simplest and most robust process is limiting dilution, such that approximately 1 in 10 droplets contains a cell, and less than 10% of those contain cell doublets. To minimise cell doublets it is also important to encapsulate cells at a constant and controlled rate. Less intuitively, it is useful to keep the volume of droplets constant. A 50 μ m droplet has a volume of 65 pl, and a 60 μ m droplet has a volume of 113 pl – almost double the volume, and almost double the probability of a cell doublet. The number of beads is less critical, because having two beads in a droplet only results in sampling the cell twice, which is often not important. Bead doublets will generally not skew statistics, because the probability of a bead doublet is not correlated with the properties of the cell (the beads and cells come down separate channels). DeKosky et al (2015) aimed for ~55 poly (dT) beads per droplet.

In practice, high-throughput capture of single cells in microfluidic droplets is somewhat cutting edge and technically demanding. Injecting samples of beads and cells into a microfluidic system, getting them to flow through the microfluidics, and be encapsulated at controlled rates in droplets, can be difficult to get working reliably. To simplify high-throughput single cell methods, Dolomite Bio, which specialises in ‘Productising Science’ and delivering microfluidic solutions for research biologists, has developed the μ Encapsulator 1. It is a compact and easy-to-use module on which the encapsulation of single cells with mRNA capture beads in droplets can be implemented. The μ Encapsulator 1 securely holds a droplet-generating chip, convenient sample reservoirs where samples can be easily loaded, and an output reservoir. For applications where temperature control is important (e.g., for chilling the samples during processing) the μ Encapsulator 1 module can be mounted on the Dolomite Bio Temperature Control Unit (TCU100), to temperature control the reservoir chip and droplet generating chip as well as the output reservoir. The use of pressure pumps to drive fluids onto the microfluidic chip results in the production of highly monodisperse droplets, which helps minimising cell doublets.

This application note reports some results of high-throughput encapsulation of lymphocytes and mRNA capture beads in 50 μ m droplets.

Materials and Methods

Cell encapsulation system. The cell encapsulation system includes pulseless Dolomite Bio P-Pumps, microfluidic connectors and a μ Encapsulator 1 module that holds a droplet-generating chip, a sample reservoir chip and an output reservoir (Figure 3). The system is driven from a PC via the Flow Control Centre software.

Flow Control Centre Advanced software. FCC Advanced controls Dolomite Bio hardware, either manually, or via “FCC protocols”, which are programs that automatically control a system. Droplet formation on the μ Encapsulator 1 was performed with the FCC ‘ μ Encapsulator protocol’.

High speed microscope. The Dolomite Bio high speed microscope is a simple compact microscope, with a convenient long working distance lens. There are no eye-pieces, to protect users from the very bright light source.

Chips. The droplet chip connects directly to the reservoir chip via ports and a gasket. The droplet chip has filters with rounded pillars that are designed to let cells through without any interference.

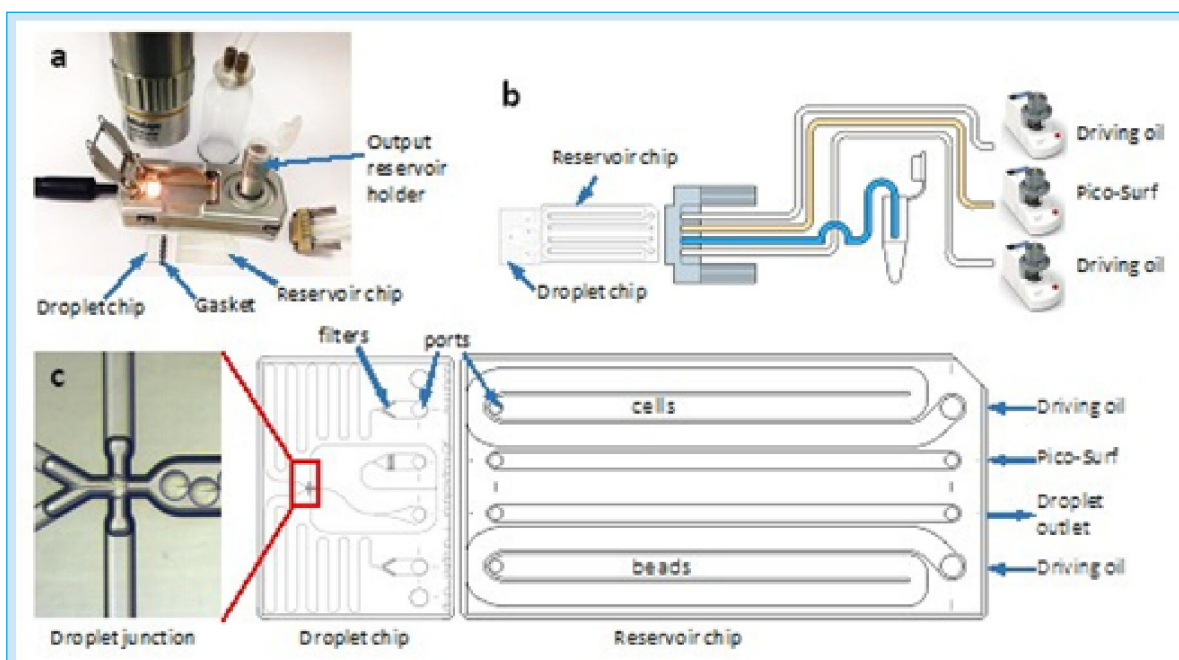


Figure 3. Connections for the μ Encapsulator 1. (a) The μ Encapsulator 1 module contains the droplet chip and a reservoir chip, plus a holder for the output reservoir. The reservoir chip has a pair of 100 μ L reservoirs, e.g. one for the cell suspension and one for beads + lysis buffer. The two chips are joined via a gasket. (b) Schematic, showing how the chips are connected to the pumps. The emulsion oil, Pico-SurfTM, is loaded into one of the P-pumps, and flows through a bypass in the reservoir chip. In the experiments described in this application note, we used HFE 7500 as a driving liquid for the samples. (c) A close-up of the droplet-generating junction, and a larger drawing of the two chips. The cells and beads samples are loaded into the reservoirs, and are driven onto the chip by the driving oil, here HFE 7500, which is in the P-pump reservoirs.



Preparation of lymphocytes. All solutions (including oil and solvents) were filtered with 0.2 μ m syringe filters. 300 μ l of whole blood was mixed with 300 μ L of PBS, gently layered onto 500 μ L of Histopaque 1077 in a microcentrifuge tube, and centrifuged at 700 g (3,000 rpm) for 15 min in a microcentrifuge. The top was gently pipetted off and discarded, and the interface, which contained the lymphocytes, was pipetted to a fresh microcentrifuge tube. The lymphocytes were washed by diluting with 1 mL isotonic buffer and pelleting at 700 g for 3 minutes. The supernatant was gently pipetted off and discarded, and the lymphocytes re-suspended in 100 μ L of cell suspension buffer (1X PBS + 0.1% BSA), adjusted to 1.07 g/mL with OptiPrep™ (Sigma) (Table 1). Cells were counted and diluted approximately 1 cell per 10 droplets to minimise the number of droplets containing two or more lymphocytes (which are around 5 – 10% of the density-prepared leukocytes). We generally made 50 μ m droplets, which have a volume of 65 pL. To obtain 1 cell/10 droplets, taking into account that the cells stream was mixed 1:1 with the beads stream, cells were suspended at around 3×10^6 cells/mL (2X (1 mL/(65pL X 10 droplets))).

Table 1: Concentration of density media, to achieve target density

Target Density g/mL	1.04	1.05	1.06	1.07	1.08	1.09	1.10
% Optiprep (1.32 g/mL)	12.5%	15.6%	18.8%	21.9%	25.0%	28.1%	31.3%

Preparation of beads. We used either 1 μ m poly(dT) magnetic beads or 10 μ m streptavidin-coated polystyrene beads. Beads functionalised with streptavidin or with carboxyl groups are useful for conjugating on custom primers (e.g., sequence-specific or longer capture probes).

The 1 μ m magnetic beads (NEB S1419S) are similar to those used by DeKosky et al (2015) and are convenient because they do not need to be re-suspended in density media - they are small and do not sediment appreciably during the experiment (> 1h). The concentration of the bead stock solution was determined by counting in a hemacytometer. An aliquot of beads was then transferred to a microcentrifuge where the beads were pelleted using a magnet and resuspended in lysis buffer (100mM Tris pH 7.5, 500mM LiCl, 10mM EDTA, 0.1% Triton, 5mM DTT, at around 9×10^8 beads/mL (~30 beads/droplet after mixing with cells).

The polystyrene beads (Bangs Laboratories, CP01N) have a density of ~1.06 g/mL. This low density allows the beads to be made neutrally buoyant in solutions of Optiprep™, to ensure that beads do not sediment too quickly at the bottom of the reservoir chip. Beads were pelleted at 3,000 rpm for 1 min, and resuspended in lysis buffer, adjusted to 1.06 g/mL with Optiprep™ (100mM Tris pH 7.5, 500mM LiCl, 10mM EDTA, 0.1% Triton, 5mM DTT, 18.8% Optiprep). Beads were counted, then diluted to the desired concentration of around 3×10^7 beads/mL (1 bead/droplet after mixing with cells), which resulted in ~80% of droplets being occupied by a bead during encapsulation.

Encapsulation. 1.6 mL of the emulsion oil (2% Pico-Surf™ 1 in Novec oil) was pipetted into a 2 mL microcentrifuge tube, and placed in the reservoir of a P-pump using a Dolomite Bio vessel holder. Two other P-pumps were loaded with vials of fluorocarbon oil (HFE 7500) as an inert driving liquid. The junction and reservoir chips were loaded into the μ Encapsulator 1, 100 μ L of cell suspension was pipetted into one serpentine reservoir and 100 μ L of bead suspension into the other reservoir. The system was controlled by



a μ Encapsulator 1 program in the Dolomite Bio FCC Advanced software. Encapsulation was run at 3-4 kHz (3 – 4,000 droplets/second), with flow rates of 7 μ L/minute each for the bead and cell suspensions, and 50 μ L/minute for Pico-Surf™.

To view droplets, 10 μ L of emulsion was either loaded into a disposable C chip hemacytometer and imaged, or imaged in a bridge slide.



Results

The aim of this note was to test the reliability of the μ Encapsulator 1 for encapsulation of cells and beads. Specifically, it was important that the μ Encapsulator 1 could reliably feed beads and cells onto the droplet generating chip without clogging, and that the beads and cells fed from the sample reservoir at a constant rate, so that the encapsulation rate could be readily controlled.

The cells and beads are both denser than water, so they will tend to sediment out at a high rate, which will change the encapsulation rate of cells or beads per droplet. To counteract this, we used Optiprep™ to make the cells and beads neutrally buoyant in the respective buffers. It is also generally useful to process the samples quickly, to minimise residual sedimentation, and because the microfluidics generally performs better at high flow rates, clogs less, and the sample is processed faster.

We first encapsulated lymphocytes. One reservoir was filled with a lymphocyte suspension, and the other reservoir with dye, to more easily visualise flow and mixing. The droplets were monodisperse, and their size could be readily controlled, by decreasing or increasing the flow of Pico-Surf™, from 50 μ m to around 70 μ m. To obtain a clear movie for estimating the variation in droplet size, we slowed the flow rate down to 20 Hz. At these flow rate ratios, the droplets were 63 μ m with a standard deviation of 0.71 μ m, which equates to 131 pl with a standard deviation of 4.4 pl, or 3%. We reached flow rates of 7 μ L/minute for each of the aqueous streams (i.e. a combined aqueous flow rate of 14 μ L/minute), and around 50 μ L/minute for the Pico-Surf™. This equates to a droplet rate of around 3.5 kHz (3,500 droplets/second with 65 pL droplets). Lymphocytes were encapsulated at a rate of around 1 cell/10 droplets, for an encapsulation rate of 350 lymphocytes/second. At these flow rates, the 100 μ L sample, containing ~300,000 lymphocytes, was processed in around 15 minutes, in 3 X 10⁶ droplets of 65 pL volume.

It is important that the cells feed at a constant rate into droplets, otherwise the frequency of cell doublets will either increase, confounding the data, or decrease, reducing the throughput. To determine this, we next counted cells per droplet at the beginning (0 minute), middle (7 minutes) and end (14 minutes) of the encapsulation of the sample (Table 2 and Figure 4c). As can be seen, the encapsulation rate is constant during encapsulation, and the rate of cell doublets stays low. Importantly, among droplets containing cells (278), 97% had one cell (269/278), and only 3% (9/278) had two or more.

We next encapsulated beads in the μ Encapsulator 1. We tested a number of concentrations of beads. At 1.5 beads per 65 pL droplet (4.6×10^7 beads/mL), the beads fed well from the reservoir chip into the junction chip, and through the junction, so long as the flow rate was kept above about 2 – 5 μ L/minute. At higher concentrations, or lower flow rates, the beads tended to clog in the upstream channel, between the junction and the port. Interestingly, we never observed beads clogging between the reservoir and junction chips, or in the port. At a concentration of 1.5 beads/droplet, around 80% of the droplets had a bead. We encapsulated beads at 3 kHz, at either 1.5 beads/droplet (Table 3), or 1 bead per 10 droplets (Table 4), and counted beads/droplet at the beginning (0 minutes), middle (7 minutes) and end (14 minutes) of the encapsulation of the sample (Figure 4d). As can be seen, the encapsulation rate was constant during encapsulation.

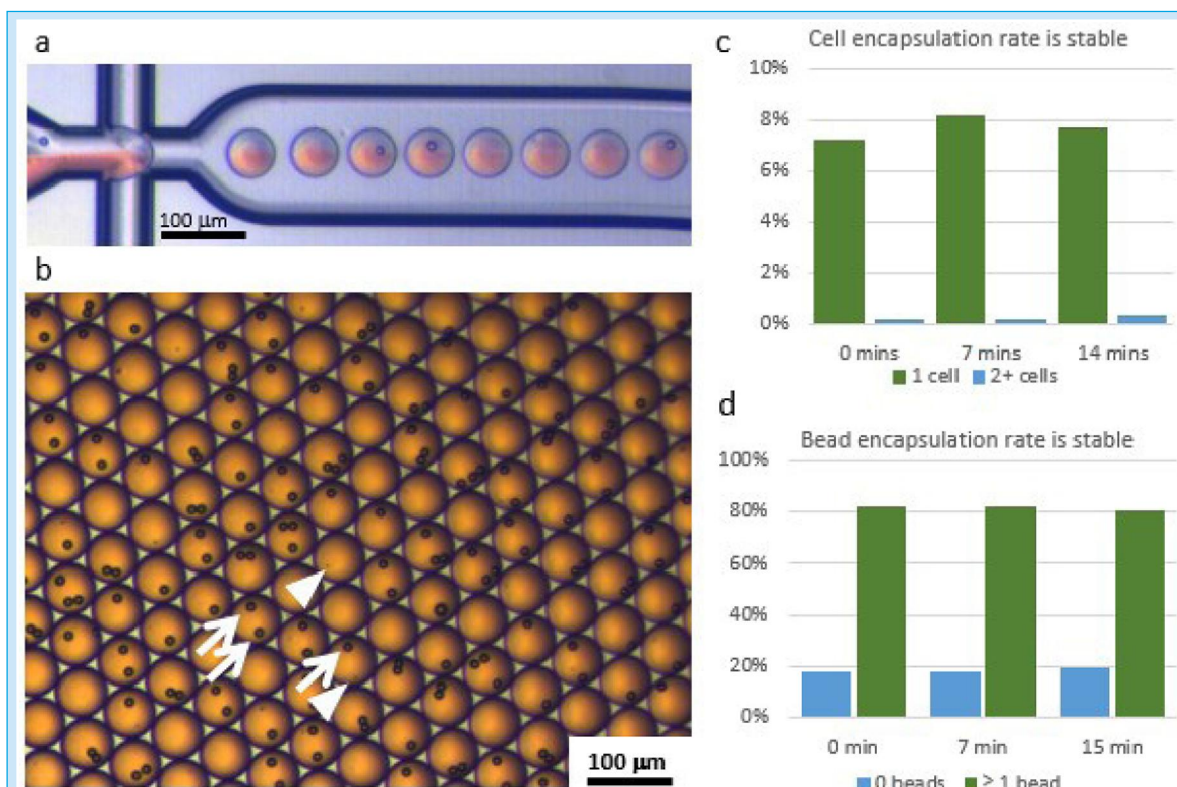


Figure 4. Encapsulation of polystyrene beads with single cells. (a) 10 μm beads being encapsulated with a dye stream in 50 μm droplets in a 50 μm junction chip. Note the mixing between the dye and bead suspension streams. (b) Single lymphocytes (arrowheads) encapsulated with beads (arrows) in 50 μm droplets. (c) Diagram showing stable cells/droplet encapsulation rate. Note that, of droplets containing cells (7 to 8% of all droplets), 97% had one cell, and only 3% had two or more. Droplets were made at 3 kHz, and collected at the beginning (0 minutes), middle (7 minutes) and end (14 minutes) of an encapsulation run. (d) Diagram showing stable beads/droplet encapsulation rate of 1.5 beads/droplet, where 80% of the droplets are occupied by a bead. Droplets were made at 3 kHz, and collected at the beginning (0 minutes), middle (7 minutes) and end (14 minutes) of an encapsulation run.

We also co-encapsulated cells and beads (Figure 4b) together. The cells were encapsulated at 1 cell/10 droplets, and the beads at 1.5 beads/droplet, resulting 80% of the droplets being occupied by a bead. This was straightforward, and around 300,000 cells were processed into 3 million monodisperse 65 pL droplets in 15 minutes. Of the 300,000 cells, 80%, or 240,000 cells, were in a droplet with at least one bead. Therefore, the resulting beads, each decorated with the mRNA from a single cell, can be processed to make single cell cDNA libraries representing 240,000 cells. The libraries can be amplified, then subjected to high throughput sequencing.

Table 2: Droplets with given numbers of cells

	0 cell	1 cell	2 cells	3+ cells
0 min	852	67	1	1
20 min	976	87	2	0
60 min	1367	115	4	1

Table 3: Droplets with numbers of beads from bead encapsulation at 1.5 beads/droplet

	0 bead	1 bead	2 beads	3 beads	4 beads	5 beads
0 min	87	145	192	61	5	1
7 min	97	159	196	88	5	4
15 min	106	165	183	76	7	2

Table 4: Droplets with number of beads from bead encapsulation at 1 bead/10 droplets

	0 bead	1 bead	2 beads
0 min	668	68	10
7 min	655	61	6
14 min	755	81	7

We ran another test where 1 μ m magnetic beads were encapsulated at around 30 beads/droplets to mimic the DeKosky et al (2015) protocol. The beads were easy to flow (Figure 5a) and encapsulate in 65 pL droplets (Figure 5b). They also did not sediment at the bottom of the sample reservoir during the experiment despite the absence of density media.

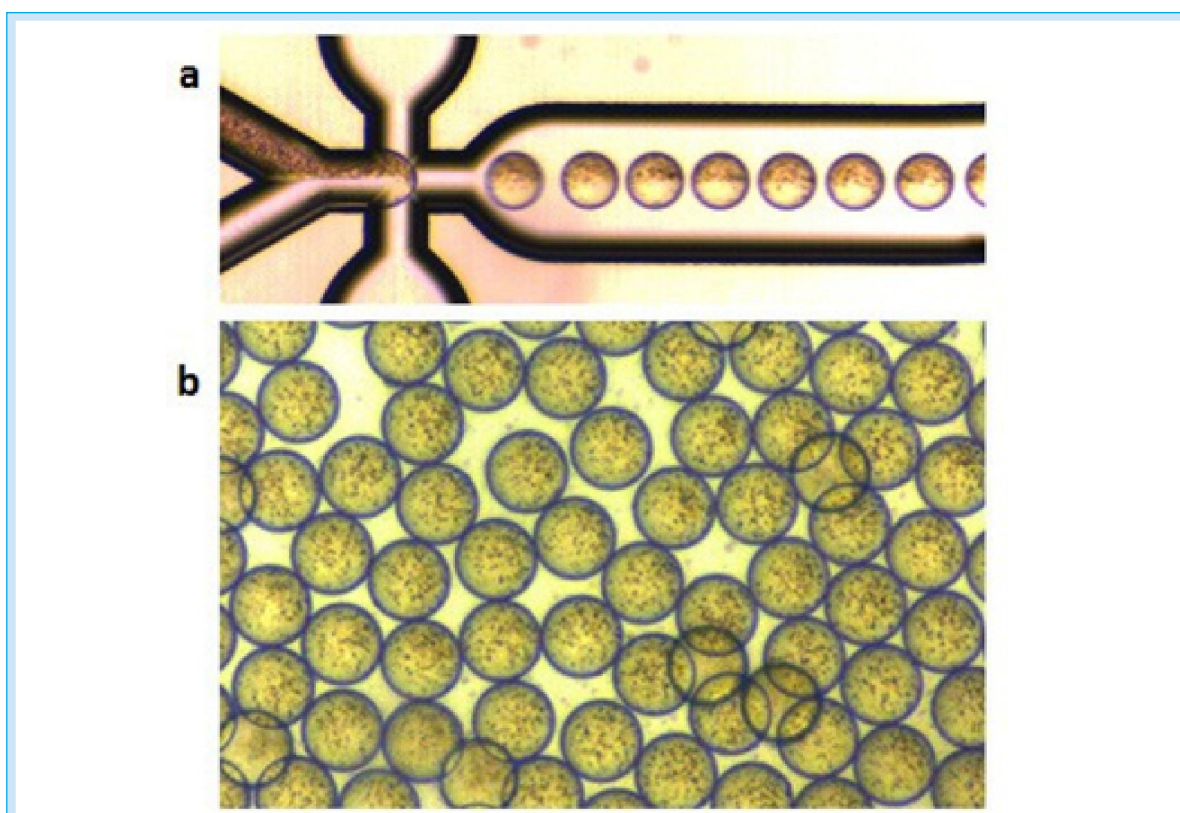


Figure 5. Encapsulation of beads with single cells. (a) 1 μ m magnetic mRNA capture beads being encapsulated with an aqueous stream in 50 μ m droplets in a 50 μ m junction chip. (b) 50 μ m droplets containing around 30 magnetic beads.



Conclusion

The reliable and fast encapsulation of thousands of single cells in microfluidic droplets allows the high-throughput analysis of thousands of single cells in parallel. This development opens the way for many exciting new applications including paired-sequencing of antibody or TCR coding sequences. However, microfluidics is still a somewhat cutting-edge technology and it may be difficult for researchers to assemble a 'home-built' system that runs consistently for a useful length of time, with good performance.

The μ Encapsulator 1 offers a simple solution for the encapsulation of single cells with reagents including mRNA capture beads. This way it allows the straightforward processing of large numbers of cells for making single cell cDNA libraries. In this application note we showed that it is straightforward to encapsulate up to 240,000 single cells in less than 15 minutes, with mRNA capture beads in monodisperse droplets with highly consistent volumes. We also showed that two different types of beads can be flowed into the droplet-generating chip, demonstrating the flexibility of the μ Encapsulator 1 system. The rapid production of single-cell libraries minimises incubation time of cells reducing possible interference with the cells expression profiles. Added to this, the possibility to mount the μ Encapsulator 1 module onto the TCU100 allows the precise temperature control of cell samples, helping prevent early cell death and loss of data.

The μ Encapsulator 1 is available now with a range of droplet-generating chips suitable for producing droplets of different sizes: 50 μ m, 30 μ m and 15 μ m.



Appendix A: System Component List

Orders from	Part No.	Part Description
US and Canada	3200554	μ Encapsulator System with Enhanced Control (110v, 60Hz, US) - Excludes Applications Pack
	3200531	High Speed Digital Microscope and Camera
UK	3200556	μ Encapsulator System with Enhanced Control (230V, 50Hz, UK) - Excludes Applications Pack
	3200531	High Speed Digital Microscope and Camera
Europe	3200558	μ Encapsulator System with Enhanced Control (230V, 50Hz, EU) - Excludes Applications Pack
	3200531	High Speed Digital Microscope and Camera
Japan	3200560	μ Encapsulator System with Enhanced Control (100V, 50-60Hz, JP) - Excludes Applications Pack
	3200531	High Speed Digital Microscope and Camera
Rest of the World	3200556	μ Encapsulator System with Enhanced Control (230V, 50Hz, UK) - Excludes Applications Pack
	3200531	High Speed Digital Microscope and Camera
	3200571	Installation and Basic Training (supplement for 2 days, on site RoW)

μ Encapsulator Consumables and Applications Packs

Part No.	Part Description
3200570	μ Encapsulator 1 Consumables Pack
3200573	Applications Pack for μ Encapsulator 1 - General 50 μ m Droplets
3200574	Applications Pack for μ Encapsulator 1 - Double Emulsions
3200575	Applications Pack for μ Encapsulator 1 - Smaller (15 μ m) Droplets
3200576	Applications Pack for μ Encapsulator 1 - Smaller (30 μ m) Droplets
3200577	Applications Pack for μ Encapsulator 1 - Comprehensive (Includes a Full Range of Chips)



μ Encapsulator Chips

Part No.	Part Description
3200562	μ Encapsulator 1 Sample Reservoir Chip (2x 100 μ l) Pack of 3
3200563	μ Encapsulator 1 - 2 Reagent Droplet Chip (50 μ m), fluorophilic, Pack of 3
3200564	μ Encapsulator 1 - 2 Reagent Droplet Chip (50 μ m), hydrophilic, Pack of 3
3200565	μ Encapsulator 1 - 2 Reagent Droplet Chip (50 μ m), hydrophilic, Pack of 3
3200566	μ Encapsulator 1 - 2 Reagent Droplet Chip (15 μ m etch depth), hydrophilic, Pack of 3
3200567	μ Encapsulator 1 - 2 Reagent Droplet Chip (30 μ m etch depth), fluorophilic, Pack of 3
3200568	μ Encapsulator 1 - 2 Reagent Droplet Chip (30 μ m etch depth), hydrophilic, Pack of 3

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