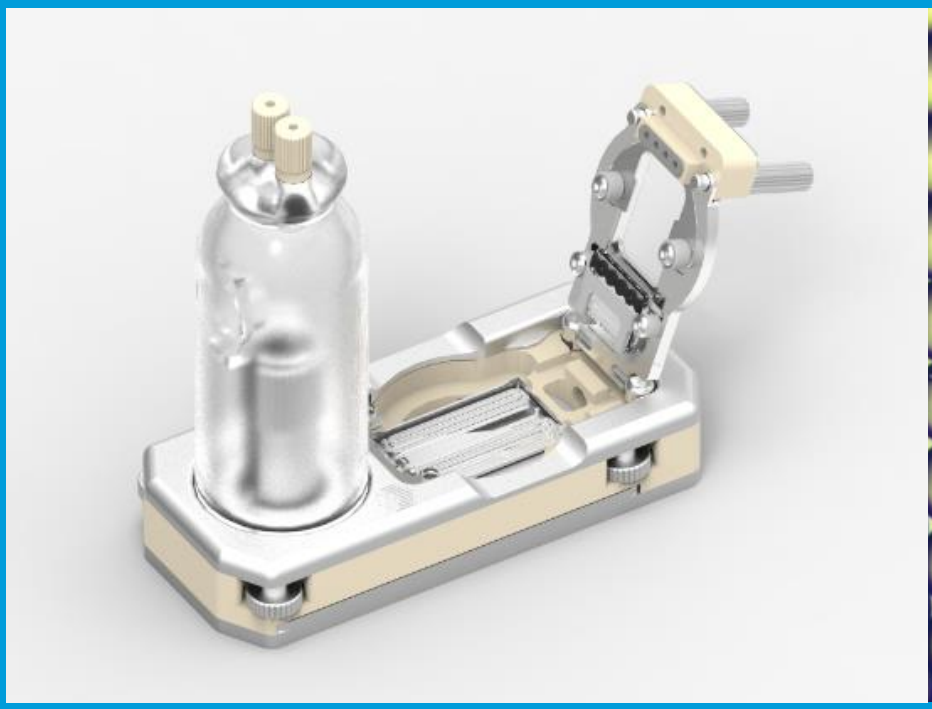
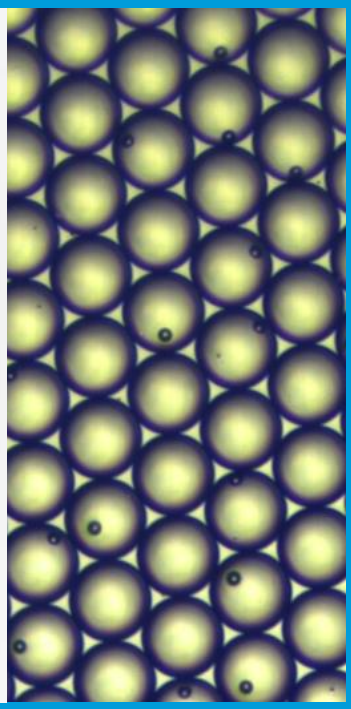
µEncapsulator 1 application note

Using the µEncapsulator 1 to capture single cells in droplets with beads





Summary

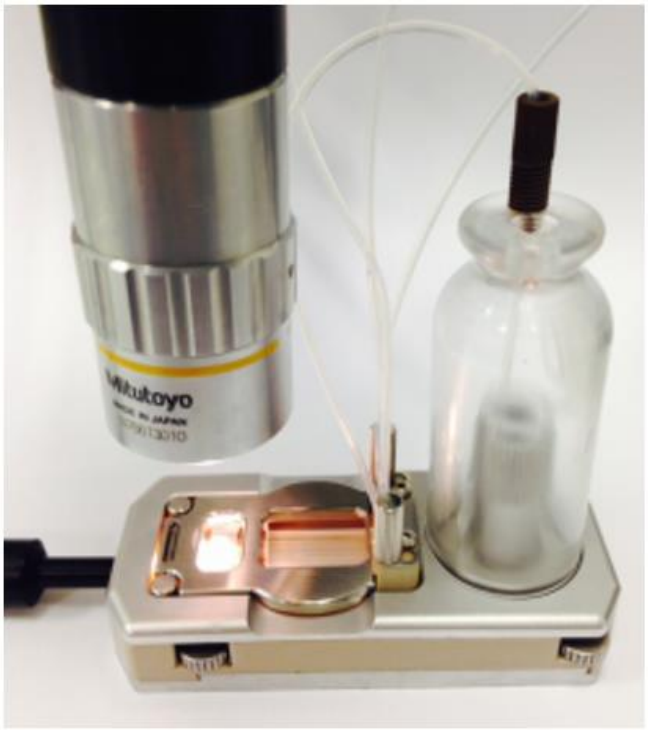
Conventional tools in biology homogenise populations of cells, which means that in many processes, it is still not possible to observe and analyse crucial biological events (e.g., in immune responses, in complex tissues such as neural tissues, or events in tumourigenesis, metastasis, etc.).

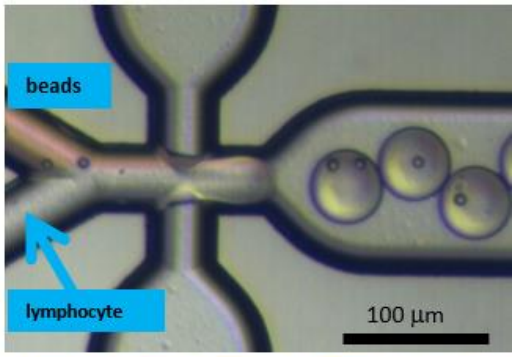
By capturing single cells in microfluidic droplets, it is possible to do high throughput single cell analysis, which opens up these processes to study.

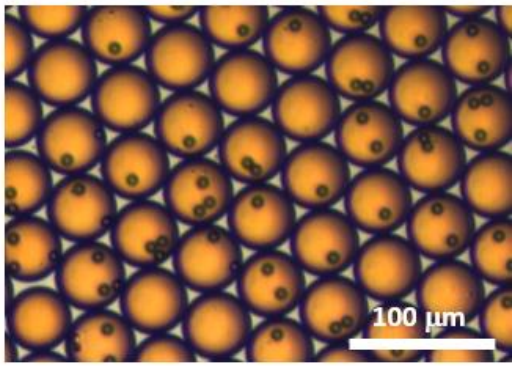
However, while proof-of-principle demonstrations of high throughput analysis have been published, microfluidic technology is far from mature. It often requires substantial multidisciplinary prowess (engineering, programming, biochemistry, microfluidics) to build a system, and to even get a proof-of-principle result. Getting a system running in a biology lab, and getting results from it day-in-day-out, may be impractical.

In order to make high throughput single cell analysis available to biologists, Dolomite has developed the μEncapsulator 1, which greatly simplifies capturing single cells in microfluidic droplets. If temperature controlled is desired, e.g., to chill samples, or form agarose droplets, the μEncapsulator can be mounted on Dolomite's Temperature Control Unit, the TCU100.

We illustrate the usefulness of the μEncapsulator 1 by demonstrating the simple, high throughput capture of lymphocytes in 50 μm droplets.







Introduction

In many biological process, 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 & B cells, but are inaccessible to conventional bulk solution methods such as PCR, or to the cell surface markers used in FACS. Similarly, the study of neural, tumour and embryonic tissues could benefit greatly from single cell analysis, such as single cell high throughput RNA expression profiling.

Isolating single cells in microfluidic droplets can enable these studies. Typically, single cells will be captured in 50 - 125 μm 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. Limiting dilution is used to ensure that the majority of cells are isolated in droplets that contain at most one cell, and at least one bead. To maximise the quality of data, it is important to minimise cell doublets, and it is therefore important to encapsulate cells at a constant and controlled rate. Less intuitively, it is useful to keep the volume of droplets constant. A 50 μm droplet has a volume of 65 pl, and a 60 μm droplet has a volume of 113 pl – almost double the volume, and almost double the probability of a cell doublet. Pressure pumps, such as the Dolomite P-pumps produce highly mono-disperse droplets, which helps with minimising doublets.

In one method, natively paired T cell receptors or antibodies were obtained from blood samples by singly encapsulating lymphocytes with oligo dT beads (DeKosky et al. "High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire." *Nature biotechnology* **31**:166). The cells were lysed to capture mRNA from single cells on beads, the emulsion is broken, and the mRNA capture beads were washed. The beads were then singly encapsulated with RT-PCR mix, and overlap PCR performed to link the T cell receptor or antibody chains from single cells. The amplified linked products can then be subject to high throughput sequencing. In another method (Macosko et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." *Cell* **161**:1202), cells were singly encapsulated with a barcoded bead library that has been prepared by split pool synthesis, such that each bead contains many copies of a single barcode. The oligo contains a 3’ poly (dT) sequence, such that after cDNA synthesis, the cDNAs from each cell will be labelled with a unique barcode. In this method, after washing, the beads can be subject to bulk RT-PCR, because the cDNA contents of each cell will be uniquely labelled with the barcode.

In practice, high throughput capture of single cells in microfluidic droplets is somewhat cutting edge and technically demanding, and can be difficult to get working reliably. For instance, a typical step is using mRNA capture beads to capture mRNA from individual cells. This is achieved by isolating individual cells in droplets, with one or more beads per droplet. Injecting samples of beads and cells into a microfluidic system, and reliably getting them to flow through the microfluidics, and encapsulated at controlled rates in droplets, can be frustrating.

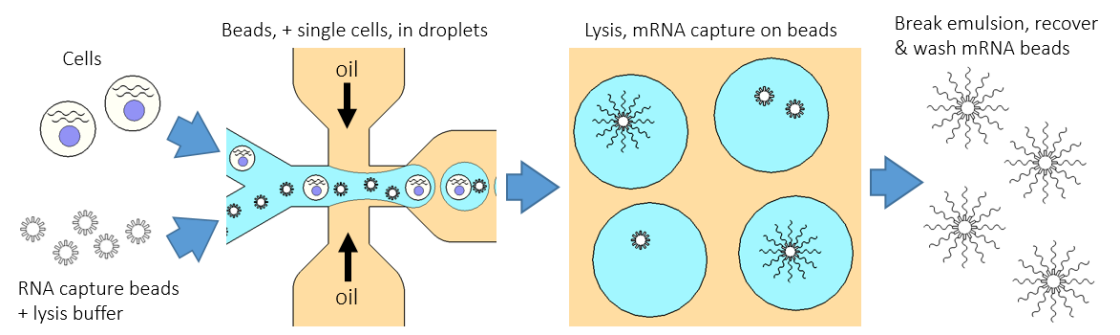


Figure 2. Capture of mRNA from single cells. Single cells are isolated in droplets with mRNA-capture beads. The beads are functionalised with a capture/primer oligo, such as poly (dT), or a gene-specific primer. The cells are lysed, and the RNA captured on the beads, such that each bead captures mRNA from only one cell. Cells are typically diluted to around 1 cell per 10 – 20 droplets to minimise the number of droplets containing two or more cells, because these are generally confounding or false positives.

In some applications, it is preferable to prioritise the cleanest data by also diluting the beads to one bead/10 droplets. This results in ~1% of droplets having both a cell and a bead. In other applications, high throughput is preferred, and 'oversampling' (by having more than one bead per cell) is tolerated because it is essentially randomly distributed, and doesn't skew the statistics. In this case, typically ~10% of droplets will have both a cell and a bead.

In order to simplify high throughput single cell methods, Dolomite has developed the μEncapsulator1, a compact module that simply and securely holds a droplet generating chip, convenient sample reservoirs, and an output reservoir. The droplet junction is readily observed with a microscope and high-speed camera. For applications where temperature control is important (e.g., for chilling the samples during processing) the μEncapsulator1 can be mounted on Dolomite's Temperature Control Unit, the TCU100, to temperature control the reservoir chip, droplet generating chip and output reservoir.

We illustrate the use of the compact chip holder by demonstrating the simple, high throughput capture of lymphocytes and beads in 50 μm droplets.

Materials and methods

Cell encapsulation system. The cell encapsulation system includes pulseless Dolomite P-Pumps, microfluidic connectors, and can be driven from a PC via the Flow Control Centre software.

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

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

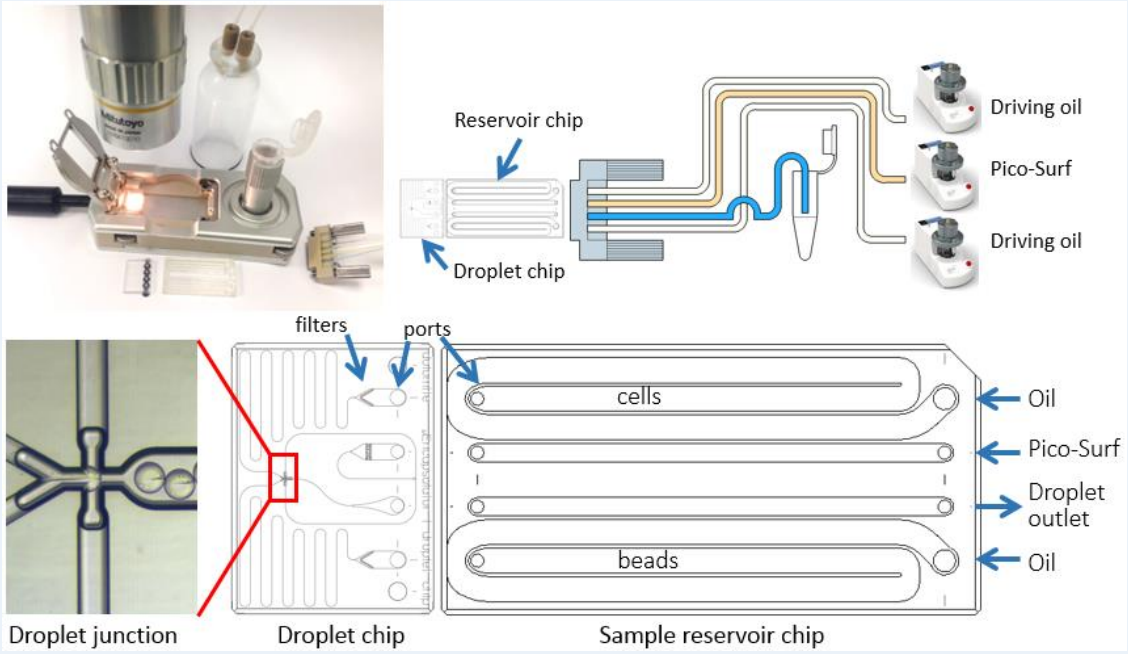


Figure 2. Connections for the μEncapsulator. a) The μEncapsulator contains the droplet chip and a reservoir chip, plus a holder for the output reservoir (a microcentrifuge tube). The reservoir chip has a pair of 100 ul reservoirs, e.g., for the cell suspension, plus one for beads + lysis buffer. The two chips are joined via a gasket. **b)** Schematic, showing how the chips are connected to the pumps. The Pico-Surf is in one of the P-pumps, and flows through a bypass in the reservoir chip. In these experiments, we used HFE 7500. **c)** A close-up of the droplet-generating junction, and a larger drawing of the two chips. The cells and beads samples are in the reservoirs, and are driven onto the chip by an inert driving oil, which is in the P-pump reservoirs.

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.

Preparation of lymphocytes. All solutions (including oil and solvents) were filtered with 0.2 μm syringe filters. 300 μl of whole blood was mixed with 300 μl of PBS, gently layered 500 μl of Histopaque 1077 in a microcentrifuge tube, and centrifuged at 700 g (3,000 rpm) for 15' in a microcentrifuge. The top was gently pipetted off and discarded, and the interface, which contains 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 μl of Dolomite cell suspension buffer, adjusted to 1.07 g/ml with Iodixanol. Cells were counted, then diluted to the desired concentration.

We generally made 50 μm droplets, which have a volume of 65 pl, so to obtain 1 cell/10 droplets, taking into account that the cells stream was mixed 1:1 with the beads stream, cells were generally suspended at ~ 3 X 10^6 cells/ml (2X (1 ml/(65pl X 10 droplets)).

Preparation of beads. We used 10 μm polystyrene beads, which have a density of ~1.06 g/ml (Bangs Laboratories, CP01N). These beads can be functionalised with 5' labelled primers, using systems such as biotion/avidin, or a carboxy/amine reaction. The low density of 1.06 g/ml allows the beads to be made neutrally buoyant in solutions of Optiprep™. Beads were pelleted at 3,000 rpm for 1 min, and resuspended in Dolomite lysis/hybridisation buffer, adjusted to 1.06 g/ml with Iodixanol. Beads were counted, then diluted or resuspended to the desired concentration of beads/ml. We typically suspended beads at around 3 X 10^7 beads/ml (~2 beads/droplet after mixing with cells), which resulted in ~80% of droplets being occupied by a bead.

Encapsulation. 1.6 ml of the emulsion reagent (2% Pico-Surf™ 2 in Novec oil) was pipetted into a 2 ml microcentrifuge tube, overlaid with 200 μl of mineral oil (to prevent gas dissolving in under pressure, and also evaporation of the oil), and placed in a 20 ml glass vial as a holder, in the reservoir of a P-pump. Two other P-pumps were loaded with vials of fluorocarbon oil (HFE 7500) as an inert driving liquid. The HFE 7500 was also overlaid with mineral oil.

The junction and reservoir chips were loaded into the μEncapsulator 1, and 100 μl of cell suspension was placed in one serpentine reservoir, and 100 μl of bead suspension in the other reservoir. The system was controlled by a μEncapsulator 1 program in the Dolomite FCC Advanced software. Encapsulation was generally run at 3-4 kHz (3 – 4,000 droplets/second), with flow rates of ~7 μl/minute each for the bead and cell suspensions, and ~50 μl/minute for Pico-Surf™.

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

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%
% Percoll (1.13 g/ml)	30.8%	38.5%	46.2%	53.8%	61.5%	69.2%	76.9%

Results

The aims of this note were to test the reliability of the μEncapsulator 1 for encapsulation of cells and beads. Specifically, it is important that the μEncapsulator 1 can reliably feed beads and cells onto the droplet generating chip without clogging, and that the beads and cells feed from the sample reservoir at a constant rate, so that the encapsulation rate can be readily controlled.

In order to capture data from single cells, single cells are commonly isolated with RNA capture beads in droplets, and lysed, so that each bead captures mRNA from a single cell. The emulsion is then broken, and the beads washed, ready for the next step.

Beads coated with poly (dT) oligos are often used to capture mRNA from single cells. The oligo (dT) both captures the mRNA (by hybridising to the polyA tail), and primes cDNA synthesis. In one approach, oligos can be 5' labelled (e.g., biotin, 5' Amino modification), and attached to appropriate beads (streptavidin or COOH-functionalised beads). In another approach, split-pool synthesis can generate barcoded oligo (dT) beads (e.g., DeKosky et al *Nature biotechnology* **31**:166), where each bead has multiple copies of a unique 'oligo barcode, sequence, followed by a 3' poly (dT).

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. 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 the properties of the cell (the beads and cells come down separate channels). DeKosky et al (*Nature biotechnology* **31**:166) aimed for ~55 poly (dT) beads per droplet, whereas Macosko et al (*Cell* **161**:1202) aimed for 1 bead/10 droplets, i.e., no more than 1 barcoded bead per droplet.

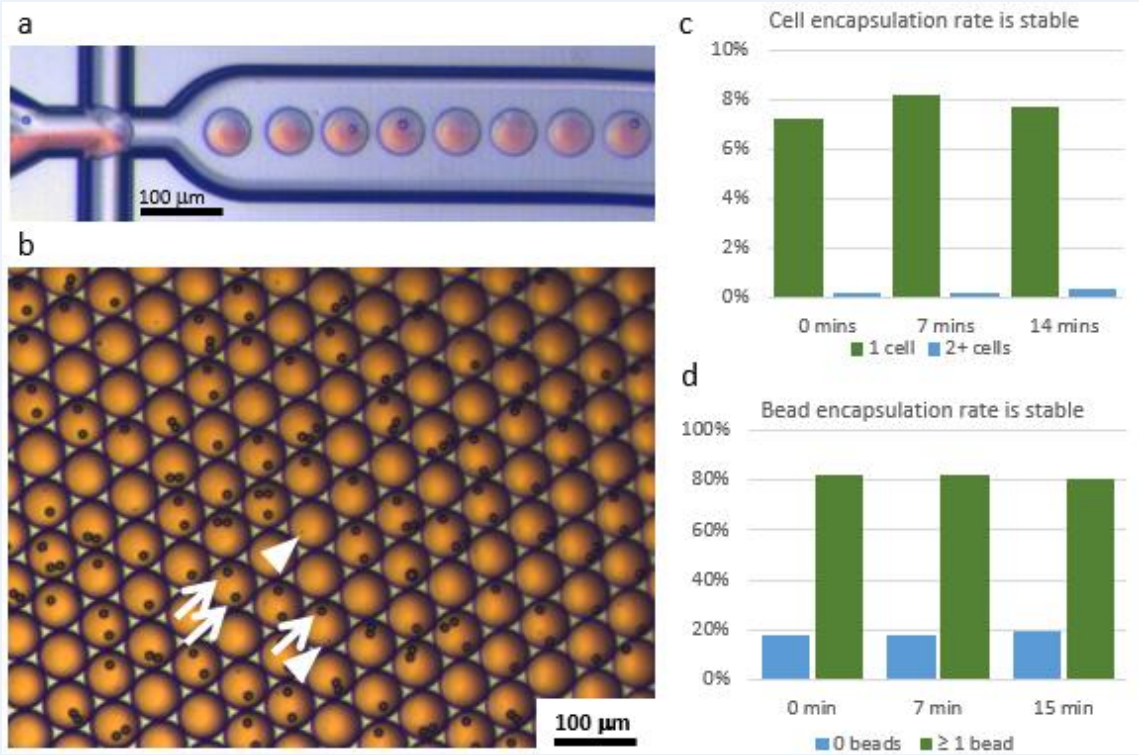


Figure 4. Encapsulation of beads with single cells. a) 10 µm beads being encapsulated with a dye stream in 50 µm droplets in a 50 µm junction chip. Note the mixing between the dye and bead suspension streams. **b)** Single lymphocytes (arrowheads) encapsulated with beads (arrows) in 50 µm droplets at 3 kHz. **c)** Graph, showing stable cells/droplet encapsulation rate. Note that, of droplets containing cells, 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)** Graph, 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.

The cells and beads are both more dense than water, so will tend to sediment out at anappreciable rate, which changes 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 also because the chips generally perform better at high flow rates, and clog 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 the size could be readily controlled, by decreasing or increasing the flow of Pico-Surf™, from 50 µm to around 70 µm. 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 µm with a standard deviation of 0.71 µm, which equates to 131 pl with a standard deviation of 4.4 pl, or 3%. We reached flow rates of 7 µl/minute (at 2.5 bar) for each of the aqueous streams (i.e., a combined aqueous flow rate of 14 µl/minute), and around 50 µl/minute (3.5 bar) for the Pico-Surf™. This equates to a droplet rate of around 3.5 kHz (3,500 droplets/second; (14 µl/60 seconds)/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 μl sample, containing ~300,000 lymphocytes, was processed in around 15 minutes, in 3 X 10^6 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 increase, confounding the data, or decrease, decreasing the throughput. To determine this, we next counted cells per droplet at the beginning (0 minutes), middle (7 minutes) and end (14 minutes) of the encapsulation of the sample (Table 1, Figure 4c). As can be see, the encapsulation rate is constant during encapsulation, and the rate of cell doublets stays low. Importantly, of 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 μEncapsulator 1. We tried a number of concentrations of beads. At 1.5 beads per 65 pl droplet (4.6 x 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 μl/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 2), or 1 bead per 10 droplets (Table 3), 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 see, the encapsulation rate is constant during encapsulation.

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 ~300,000 cells were processed into ~3 million mono-disperse 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 1: Droplets with given numbers of beads

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

Table 2: Droplets with numbers of beads from bead encapsulation at 1.5 beads/droplet

	0	**1**	**2**	**3**	**4**	**5**
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 3: 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

Conclusion

The μEncapsulator 1 allows the straightforward processing of large numbers of cells for making single cell cDNA libraries. In the example above, in 15 minutes, it is straightforward to produce single cell mRNA beads representing 240,000 cells.

Microfluidics is still a new technology, and many of the available systems are really proof-of-principle, and may be difficult to use day-to-day for biological experiments. It may be difficult to assemble the systems, or to get them running, or to run consistently for a useful length of time. It may also be difficult to introduce samples, or collect product. Workers may experience difficulty in getting cells and beads to flow reliably thorough the system without clogging, or at a reliable, controllable flow rate.

The μEncapsulator 1 is a compact, integrated module that has a droplet chip, a reservoir chip for sample and reagent, and mounts a microcentrifuge tube for sample collection. The junction can readily be imaged with a long working distance microscope, such as Dolomite's high speed microscope. The whole module can be mounted on a temperature control unit, Dolomite's TCU100, to control the temperature of the reservoirs, droplet chip, and the reservoir for the output droplets. The μEncapsulator 1 was designed to make easy leak-free connections, to readily inject and collect samples, and to have a simple flow path where cells and beads don't clog, and where the droplets can readily be recovered. Thus, it greatly facilitates experiments that require microfluidic droplets, such as high throughput single cell RNA sequencing, T cell receptor profiling, or isolation of monoclonal antibody sequences from blood samples.

The uEncapsulator 1 is available now with a chip suitable for producing 50um droplets. More chips will be added to cover a wider droplet size range and other encapsulation protocols.

Appendix A: System Component List

Part No.	Part Description	#
3200519	µEncapsulator 1 (coaxial illumination from microscope)	1
3200442	µEncapsulator 1 (illumination via gooseneck)	
3200444	µEncapsulator 1 - Sample Reservoir Chip	2
3200445	µEncapsulator 1 - 2R Droplet Chip - 50um (fluorophilic)	2
3200447	µEncapsulator 1 - Fittings Starter Kit	1
	µEncapsulator replacement parts	
3200446	µEncapsulator 1 – Gasket (FKM)	
3200454	FKM Seal (for linear connector)	
3200454	µEncapsulator 1 - Linear Connector Seal 4-way (FKM)	
	Droplet system	
3200016	Mitos P-Pump	3
3200095	Mitos Sensor Display	3
3200097	Mitos Flow Rate Sensor (30-1000µl/min)	1
3200098	Mitos Flow Rate Sensor (1-50µl/min)	2
2200480	USB Hub	1
3200118 (US) 3200117 3200128 (EU)	Mitos compressor 110V/60Hz Mitos compressor 230V/50Hz Mitos compressor EU 230V/50Hz	1
3200087	2-way In-line Valve	4
3200034	Pneumatic Connector Kit	1
	Microscope	
3200531	High Speed Digital Microscope	1
	Light Source (if not using microscope above)	
3200450	Gooseneck Light Guide	
3200451	Halogen Cold Light Source – 150W	

	Temperature controller	
3200428	Meros TCU-100	1
	Reagents	
3200215	Pico-Surf™1, 50ml, 2% in Novec 7500	1
3200204	Pico-Surf™1, 50ml, 2% in FC-40	
	Chip cleaning module	
3000024	Linear Connector 4-way	1
3000109	Top Interface 4 - way (4mm)	1
3000664	1/4 - 28 Modified Luer Fitting	4
3200063	FEP Tubing, 1/16" x 0.25mm, 10 metres	1

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