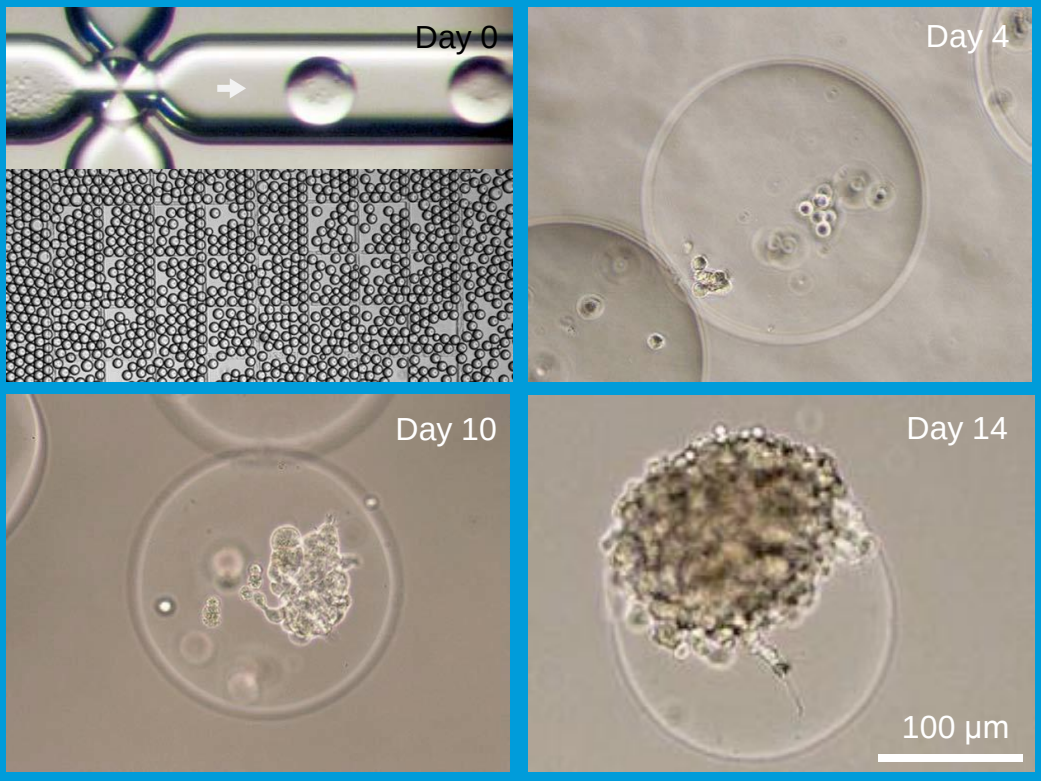
CHO Cell Encapsulation & Incubation in Gelatin Particles

Cell Encapsulation using Dolomite's Droplet System



dolomite

Summary

Cell studies by encapsulation, incubation, and manipulation in droplets is gaining popularity due to the ease afforded by droplet technology. Microfluidic systems further lend the advantage of producing extremely consistent droplets with size range limited to 1% size variation, making cell encapsulation still more attractive.

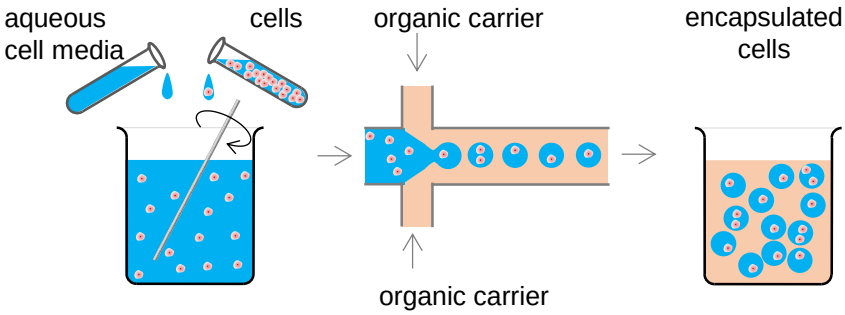
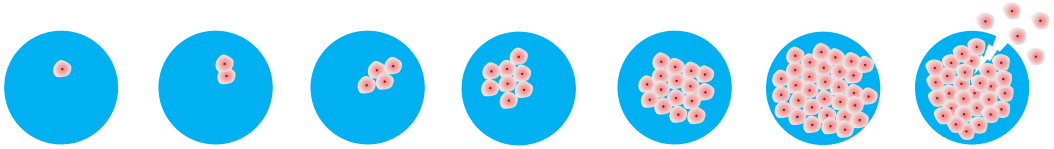


Illustration of cell encapsulation in gelatin hydrogel particles.

Cell encapsulation in picoliter volume droplets is demonstrated in this application note using Dolomite's Droplet system. Thereafter, the viability of the encapsulated cells is assessed by incubating and monitoring cell growth. A premixed cell suspension is made consisting of 10% Gelatin (highly viscous) in aqueous cell media. A cross linker is added shortly before start of test. This solution is used as the droplet phase in a droplet system to create approximately 200 µm diameter (~ 5 nanoliter) droplets at approximately 10 Hz. Pico-Surf2™ an immiscible fluorinated organic hydrocarbon carrier containing a bio-compatible surfactant stabilizes the emulsion. The gelatin upon curing becomes a soft solid hydrogel after which the particles are re-suspended in fresh cell medium. The cells continue to grow within this gel matrix.



Schematic of cell growth in encapsulated particles.

Cell viability is an important concern in encapsulation systems. To assess this, the cell encapsulated particles are incubated for 3 weeks. A few particles are taken intermittently and imaged using high magnification microscopy. Only a few sparse cells per particle are visible initially. At week 1, the single cells are seen to grown into clumps. The rate of increase in clump size rises with time – binary fission theory suggests growth rates to be exponential. At 3 weeks, the colony is seen to break out of the particles due to increased population density, as well as due to collagen degradation from the cell secretion.

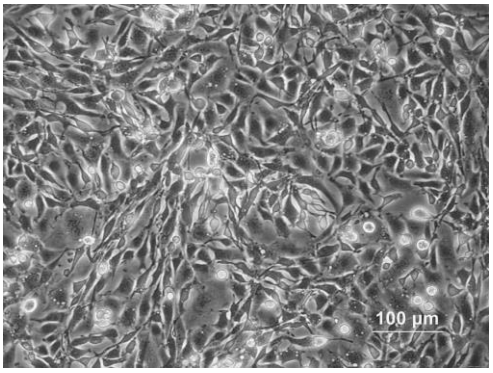
The results show that the Droplet system in the configuration presented in this application note is well suited for use in CHO cell encapsulation applications. The cells are shown to survive well the encapsulation process, with no visible adverse effect suggesting no material incompatibility.

Droplet Based Cell Study

A fundamental understanding of how cells work helps develop new vaccines, more effective medicines, and plants with improved qualities. Engineering solutions assisted cell biology has greatly assisted the human fertility programme, various forms of DNA testing, and forensic medicine. Cell studies rely heavily on averages of cell ensembles on the assumption of an underlying normal phenotype distribution.

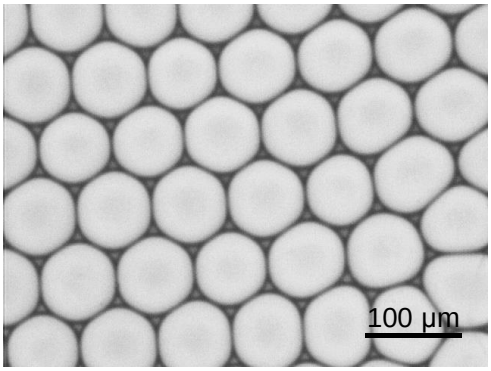
Small populations of cell observations can reveal many examples of heterogeneities even within isogenic cultures in terms of size, gene expression, and growth characteristics. The substantial diversity which actually exists within the same population is lost in the ensemble averaged observation. There is as a result growing interest in isolating, immobilizing and studying single cells with the prospect of addressing applications, such as personalized medicine and antibiotic resistance.

Chinese hamster ovary (CHO) cells are a cell line derived from the ovary of the Chinese hamster, often used in biological and medical research and commercially in the production of therapeutic proteins. They are also useful for studies of genetics, toxicity, and screening. As CHO cells are fast becoming the most popular mammalian cells used in recombinant protein expression, they are used in the study here as model surrogates to demonstrate encapsulation.



Chinese Hamster Ovary (CHO) cells.

Microfabrication technology has resulted in systems capable of producing emulsions of sizes tens to hundreds of micrometers. Reliable encapsulation of cells without affecting viability has promising applications in tissue engineering, high-throughput screening, clinical diagnostics, therapeutics and stem cell differentiation.



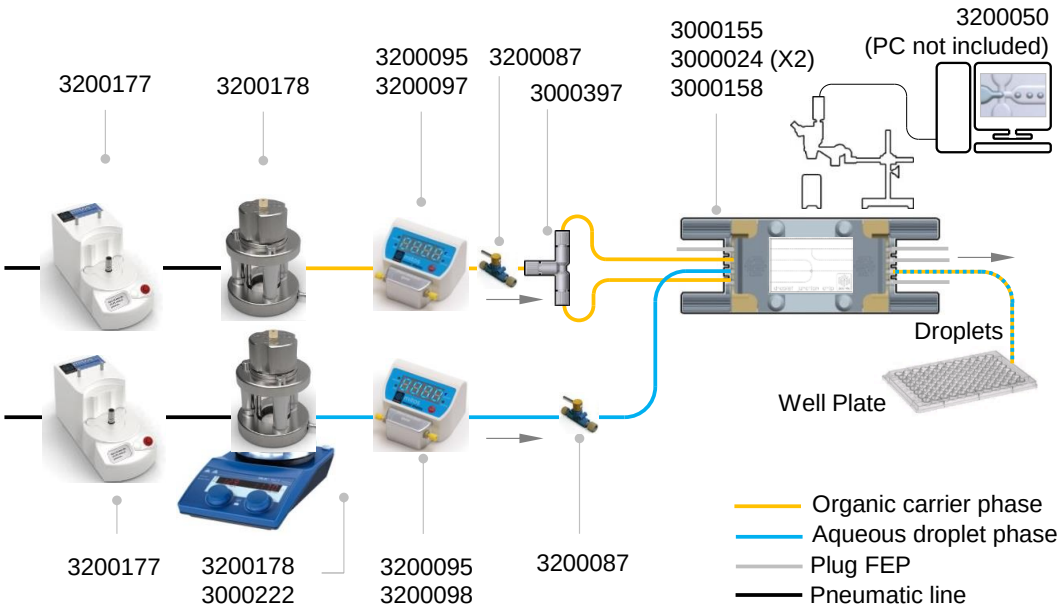
Water droplets in Pico-Surf2™.

Merging the fields of cell biology with droplet technology gives biologists a micromanipulation method to handle very small numbers, and possible single cells. When the droplet fluid is composed of cell media, continuous nourishment is provided to cells encapsulated within. The monodispersity enables quantitative control of solute concentrations, while encapsulation in droplets provides an isolated compartment for the single cell and its immediate environment. The high throughput allows the processing and analysis of the tens of thousands to millions of cells that are required to be analyzed to find rare cell types or access sufficient biological space for instance. The extremely low volumes of the droplets make very large screens economically viable.

Test Setup

Cell encapsulation setup with computer control and visualization.

A Dolomite Droplet System is used in a configuration selected specifically to allow for the requirements of cell encapsulation. The Remote Basic P-Pump for instance utilizes pressure based pumping while simultaneously allowing for continuous mixing. The pressure based pumping allows generation of highly monodisperse droplet sizes, while the continuous mixing feature reduces the likelihood of cells sedimenting in the fluid reservoir. This feature is achieved by the de-coupling of the pressurized fluid reservoir from the Pump itself, where the reservoir can be placed on a hot-plate/magnetic stirrer as illustrated in the accompanying schematic.



Schematic showing parts used for test setup. The P-Pump and sensors on each line are coupled to enable flow-control mode of pumping.

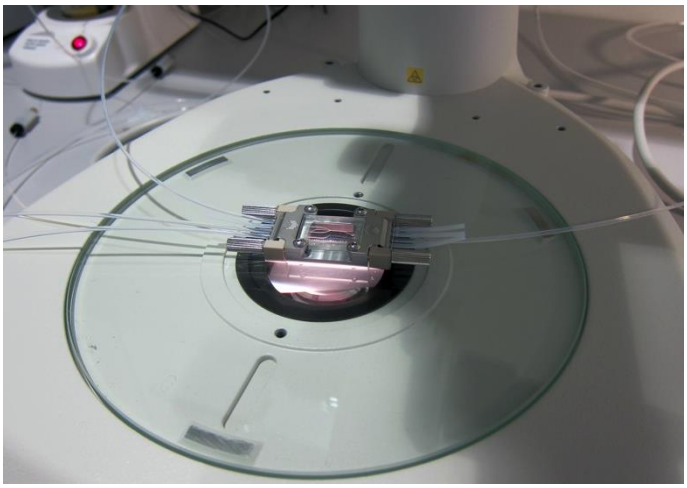
	Organic ID(mm); L(mm)	Aqueous ID(mm); L(mm)
Pump to Remote Chamber	Pneumatic tubing	Pneumatic tubing
Remote Chamber to Sensor	0.25; 300	0.10; 500
Senor to T-connector	0.25; 300	0.10; 1000
T to chip	0.25; 500 mm (×2)	
Chip to Outlet	0.25; 500	

The pumps were controlled using Flow Control Center – the software interface – and run in pressure control mode. The microfluidic device utilizes a flow focussing junction geometry where the droplet phase is non-wetting while the carrier phase wets the channel surfaces. A high magnification microscope continuously monitors the device junction. With consideration to balancing the fluid pressures at the droplet junction, the tubing sizes are

selected to provide the optimal fluidic resistance. These are specified in the accompanying table. All tubing is made of FEP, and outer diameter of all tubing is 1.6mm.

The use of a fluorocarbon carrier oil promotes the viability of cells while at the same time reducing the loss of cell components into the carrier phase. Pressurized air or nitrogen often dissolves into Pico-Surf2™ (a fluorinated hydrocarbon) in the P-Pump fluid reservoir. This dissolved gas is spontaneously released downstream as the pressure decreases, resulting in troublesome gas bubbles emerging in the channel. An alternative is to use Helium gas as this has much lower solubility in fluorinated oils. Alternatively, a method was used with some success involving the use of an overlay fluid as shown in the figure. The overlay fluid is HUVEC medium (red color) with 10% Dextran T1.5 (500 µl). This overlay fluid acts as an isolating layer between the gas and the oil. This method although successful, is risky as the fluid levels need to be closely monitored to ensure that there is no accidental infusion on-chip of wrong fluids.

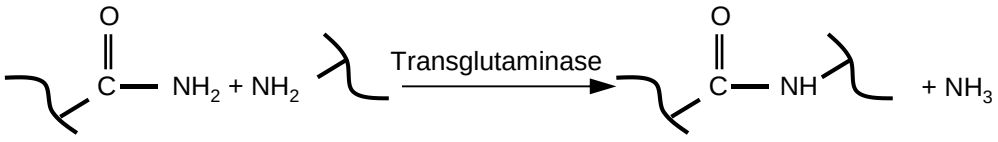
Eppendorf tube with 200 µL of overlay fluid on top of Pico-Surf2™ *oil*



Photograph showing the droplet generator microfluidic device assembled with the interface, connectors and fluidic tubing placed on a stereo microscope light stage.

Reagent Preparation

Transglutaminase, an enzyme with the ability to cross-link proteins has been shown to crosslink gelatin by involving the formation of covalent network junctions.



This acyl-transfer enzyme catalyzes transamidation reactions that lead to the formation of N-ε-(ϒ-glutamyl) lysine crosslinks in proteins.

These ‘‘chemical’’ gels can be contrasted with the ‘‘physical’’ gels that are formed by cooling gelatin solutions. A concentrated gelatin solution 10% (w/v) was prepared by dissolving 10 g gelatin into 100ml deionized water at temperature greater than 40°C.

The gelatin solution was prepared by melting/incubation at 65 °C. No pH adjustment was used, the added medium was assumed to buffer the solution. Gel formation was initiated by adding Transglutaminase 10 U/g-gelatin. All reactions were conducted at 35°C and a pH of 5.8–6. This was used as the starting medium for the droplet fluid to which cells were added.

Then CHO cells were included in the medium for the droplet source in order to generate cell-containing droplets. CHO medium was added to gelatin mix (0.25 ml mTr + 0.25 ml CHO medium + 0.5 ml 12.5 % Gelatin). CHO medium was used instead of water or casein to the gelatin mix and CHO medium from BMS/PRA carried added Pen/Strep. CHO cells from BMS, surplus cells after passage of CHO culture (1.88×10^6 cells/ml). The CHO cells were placed in a 6 well plate with shaking until use the same day. The organic medium was Pico-Surf2™ and used as is straight out of the bottle.

Chip Surface Conditioning

The microfluidic device used was a Dolomite hydrophilic chip (3000158), which underwent surface conditioning* as reported in a peer reviewed journal. The reagents were purchased from Sigma-Aldrich and from Aquapel® respectively as described in the paper. This surface conditioning step allows the use of Dolomite's hydrophilic chip with Pico-Surf2™ (fluorinated oils) following the two separate protocols in separate tests, both with successful outcomes.

* *Single-cell analysis and sorting using droplet-based microfluidics. Linas Mazutis, John Gilbert, W Lloyd Ung, David A Weitz, Andrew D Griffiths & John A Heyman.* ***Nature Protocols 8, 870–891 (2013) doi:10.1038/nprot.2013.046***

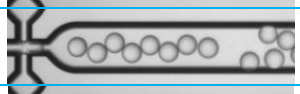
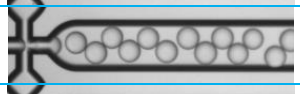
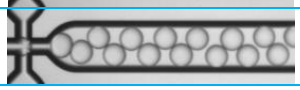
Results

Test Objective	Method
Check droplet generation	Water droplets in Pico-Surf™ 2 (2% in FC-40) carrier fluid.
Cell encapsulation in droplets	To add cell suspension to above test and image chip junction.
Harvesting of droplets and incubation in well plate	To remove samples intermittently and log visually signs of cell activity.

Droplet generation

The droplet system was setup as shown in an earlier section. To ensure that the flow resistances are setup optimally, droplets were made at reasonable conditions. This involved setting the carrier pressure to 1 bar, and varying the droplet pressure to a few different conditions. The resulting droplet generation was imaged and droplet size measured. These results are shown in the following table.

Aqueous phase	Pure Water
Organic phase	Pico-Surf™ 2 (2% in FC-40)

Organic Carrier		Aqueous droplet		Junction Image	Drop Dia
P	Q	P	Q		Φ
mbar	µl/min	mbar	µl/min		µm
1000	10	50	0.2		103
1000	10	100	0.6		106
1000	10	150	0.9		110
1000	10	300	1.9		118
1000	10	500	3.3		124
1000	10	750	5		138

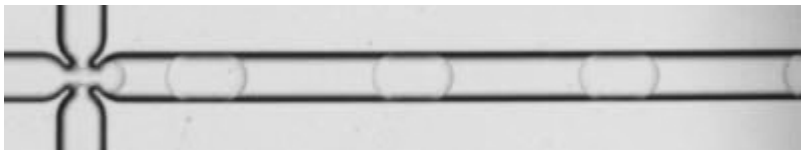
Cell Encapsulation in droplets

Once droplet generation is tested, the droplet phase is changed from pure water to cell medium. Due to the change in fluid (fluid properties such as viscosity and surface tension change) the droplet production changes slightly.

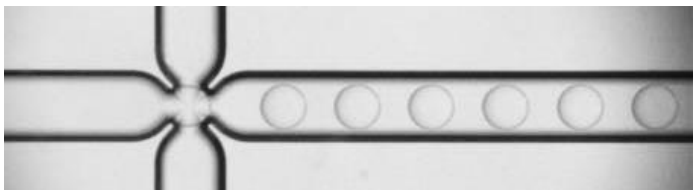
It is particularly worth noting that the droplet frequency is much lower when using gelatin compared with using plain water. This is mostly to do with the fact that the viscosity of a Gelatin solution is much higher, and hence at the same pressure, the Gelatin flow rate is lower. This however does not impact the droplet formation feasibility, however does impact droplet stability whereby contacting droplets fuse easier. For this reason, the droplet spacing was kept large so that there was a chance for droplets to cure/polymerize before contacting. A few conditions are chosen to use as test conditions.

A droplet source containing CHO cells was prepared by first mixing 0.25 ml transglutaminase solution with 0.25 ml CHO cell suspension and subsequently adding 0.5 ml gelatin solution. The melted gelatin solution was cooled appropriately by holding the volume in the pipette tip for a short time prior to mixing with the cells. Gel formation is initiated at mixing, and the use of the solution for droplet generation must be terminated before solidification becomes extensive (time window depending on the concentrations used).

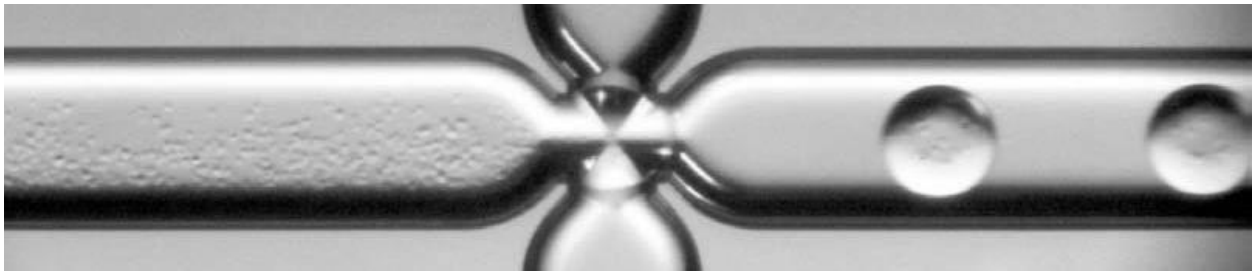
Aqueous phase	FreeStyle™ 293 expression medium + 6 % Gelatin + CHO Cells
Organic phase	2 ml Pico-Surf2™ (5% in FC-40) + 3 ml FC-70



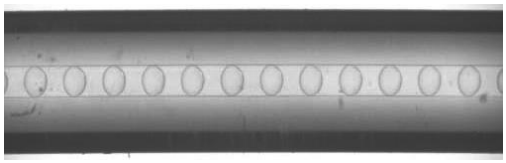
Droplet pressure = 200 mbar, Carrier pressure = 500 mbar.



Droplet pressure = 100 mbar, Carrier pressure = 400 mbar.



The presence of cells in the aqueous fluid to the left of the junction appears as tiny granules. To the right of the junction, these are seen encapsulated and contained completely within droplets.

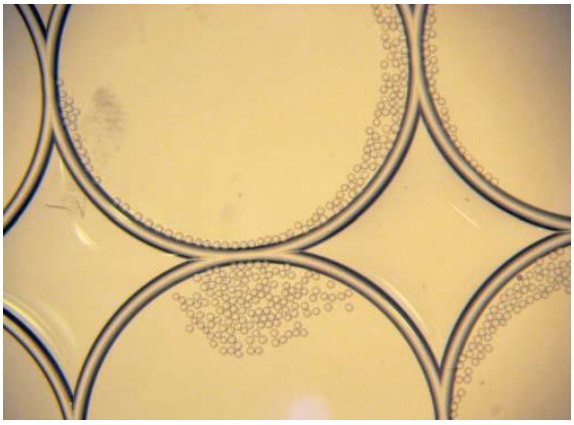


Droplets in the outlet tubing. They appear distorted as non-spherical because of looking through curved tubing.

There appeared to be slight fluctuation in the flux of cells coming onto the chip. This was most probably was due to a combination of factors – possibly cell sedimentation in reservoir, reduced cell entry into the tube. Some cells arrived in lumps, probably due to the adherent nature of the cells themselves.

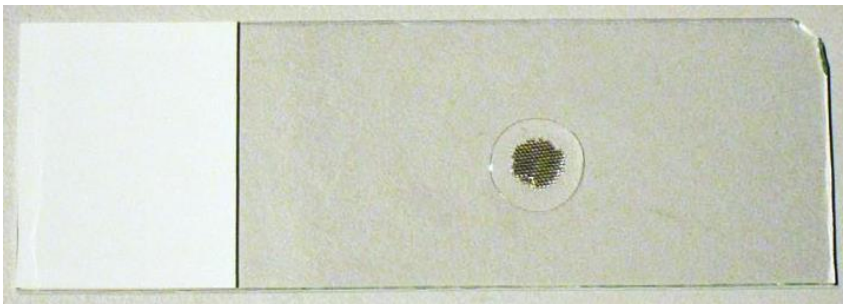
Harvesting of droplets and incubation in well plate

Droplets were collected onto a 96 plate lid (manual deposition of the chip outlet flow) On top of the lid is placed a “moisture chamber”, a tray plate with 3MM filter paper taped in sections into the tray, so the paper is kept in place while wet. Extra FC-40 was added to the lid surface to keep droplets floating on an oil surface (evaporation of fluorinated oil leads to mis-formed droplets, maybe the oil also forms a droplet surface layer that protects against evaporation). Droplets were harvested floating in FC-40 fluorinated oil. It was easy to transfer the droplets by pipetting. A 24 well plate was used for droplet collection, where 0.5 ml FC-40 was placed in a well before droplet collection started. Droplet floated on top of the FC-40 oil and formed a “ring layer”, where the oil adhered to the plastic well side, creating a ringed meniscus.

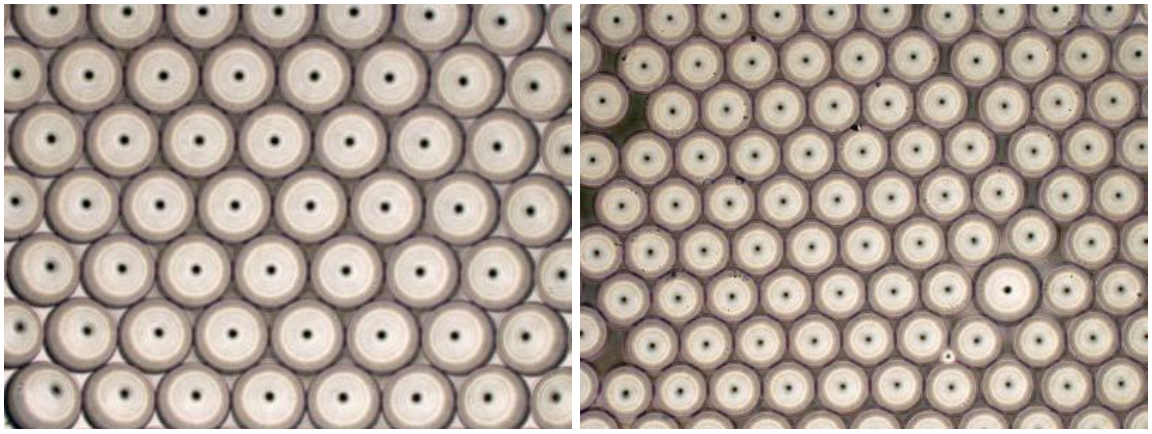


Droplets collected on 96 well plate lid and floating on a layer of fluorinated oil.

Some sample was pipetted off and placed on a glass slide and imaged under a bright field microscope.

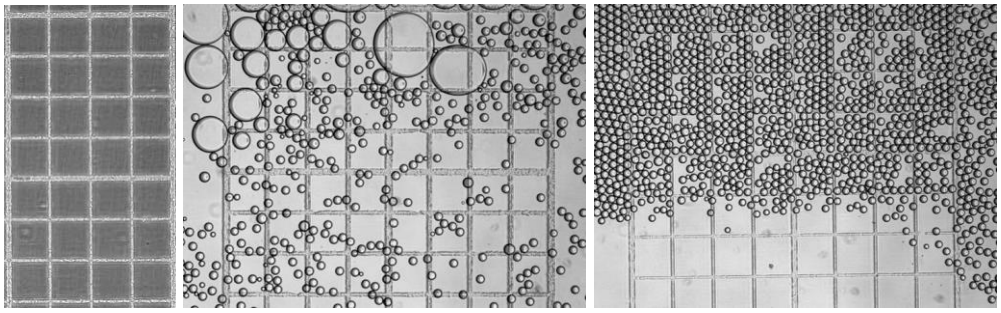


Chip outlet fluid with droplets deposited onto glass slide for microscopy by manually redirecting outlet tubing from collection to slide.



Left: Droplet diameter = 323 µm. Right: Droplet diameter = 226 µm. Less than 1 % droplet coalescence. Dark spots within droplets are droplet identifier for counting (not encapsulated content).

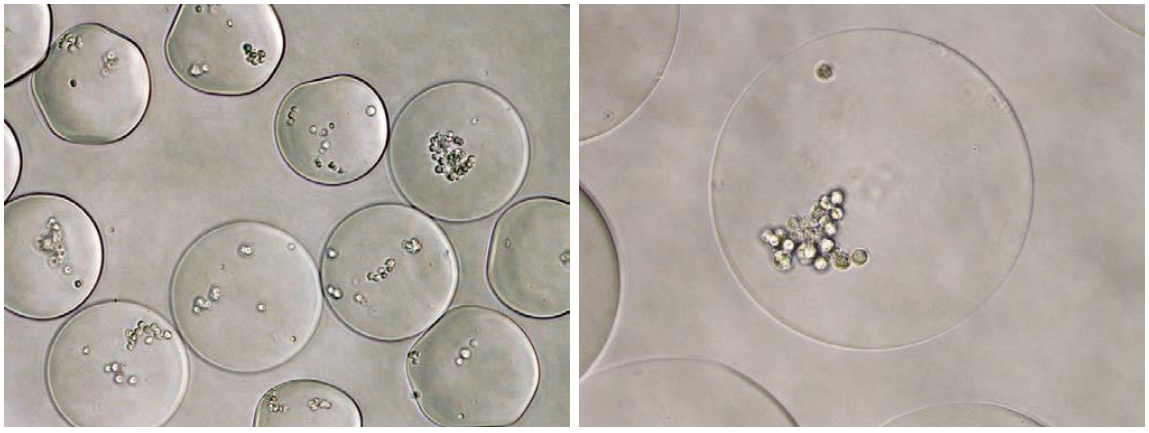
Chip output with droplets was applied onto a FastRead 102 counting slide and photographed using a microscope with a CCD camera. Each 4x4 grid contains a 10-4 ml volume with a chamber depth of 0.1 mm and with the dimensions 1x1 mm.



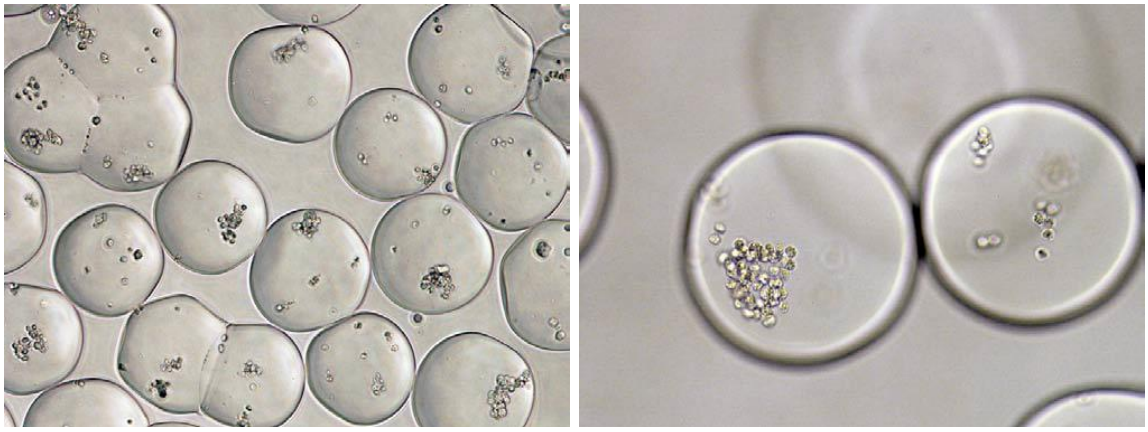
Left: FastRead 102 counting grid. 4 x 4 grid = 1 x 1 mm. Middle: Coalesced droplets seen on the outlet. Right: When the Gelatin is added, and droplets are cured into particles, then coalescence is avoided altogether.

Incubation and Cell Growth

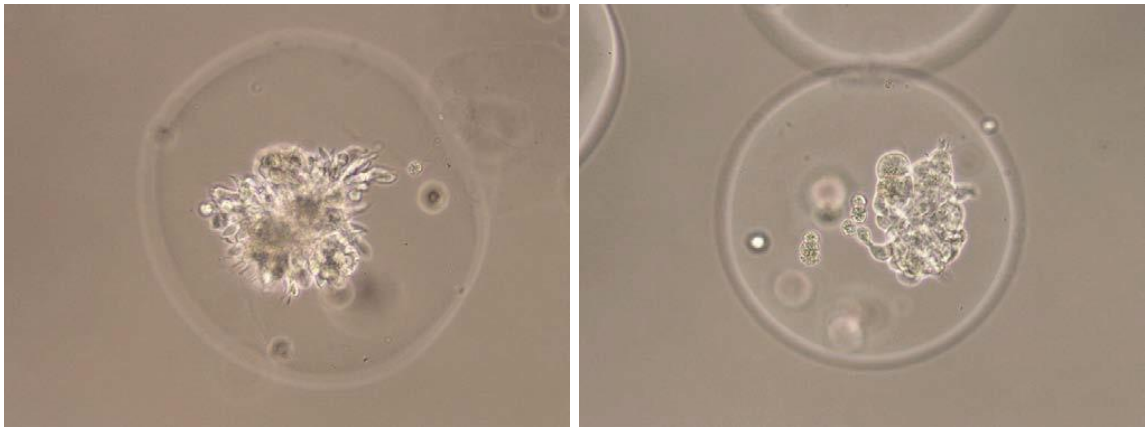
Droplets were incubated at room temperature for 2-3 hours for solidification (96 well plate lid with moisture chamber on top). After solidification the droplets were transferred to a 24 well plate (transfer using a surplus of FC-40 fluorinated oil and pooling into a single well). CHO medium was added to the well on top of the fluorinated oil (gentle addition to facilitate droplet “lift-off” without generation of liquid bubbles). Some droplets dissolved in the water phase, but the majority was found in the oil-water interface (no attempts to force the gelatin droplets into the water phase, as this would cause production of long lasting foam). Incubation at 37°C with shaking (CO_2 incubator). This was to allow continued growth of the embedded CHO cells (and with the hope, that they had survived the rather long period at room temperature). The next day droplets were removed from the oil phase and resuspended in CHO medium. The transfer was made using a P200 pipette and a microscope was used to visually control the correct picking of droplets. Several transfers were needed/used in order to remove the fluorinated oil and dissolved Pico-Surf2™ additive. A series of images below shows samples that were taken from the incubator at different amount of incubation time along with a brief description. Overall the cells appear to thrive well and showed good rate of growth.



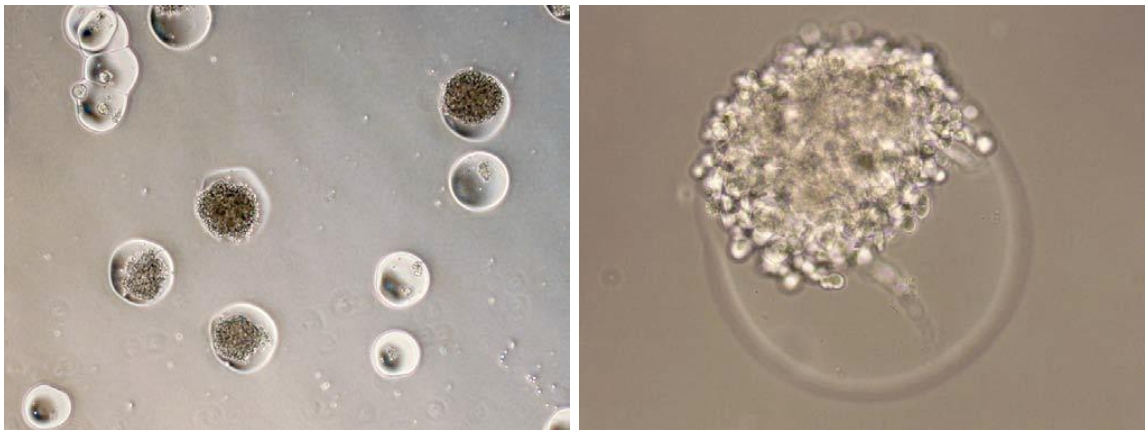
***Day 1**: A low number of cells are visible per particle. When droplets were collected into the 24 well plate (dissolved in FC-40 fluorinated oil), they were mostly in the form of single droplets. However, the overnight incubation seemed to generate additional confluent droplets, and maybe the oil phase promotes droplet agglutination as this was only seen for the droplets squeezed together in the oil-water interphase.*



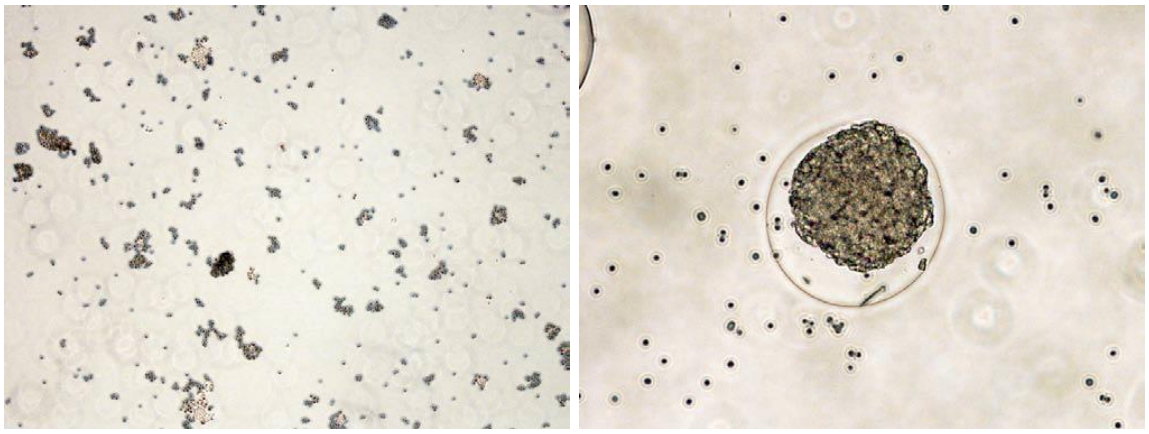
***Day 4**: The cell density per particle appears to increase. Difficult to say, if the number of CHO cells have increased (would indicate cell growth within the droplets).*



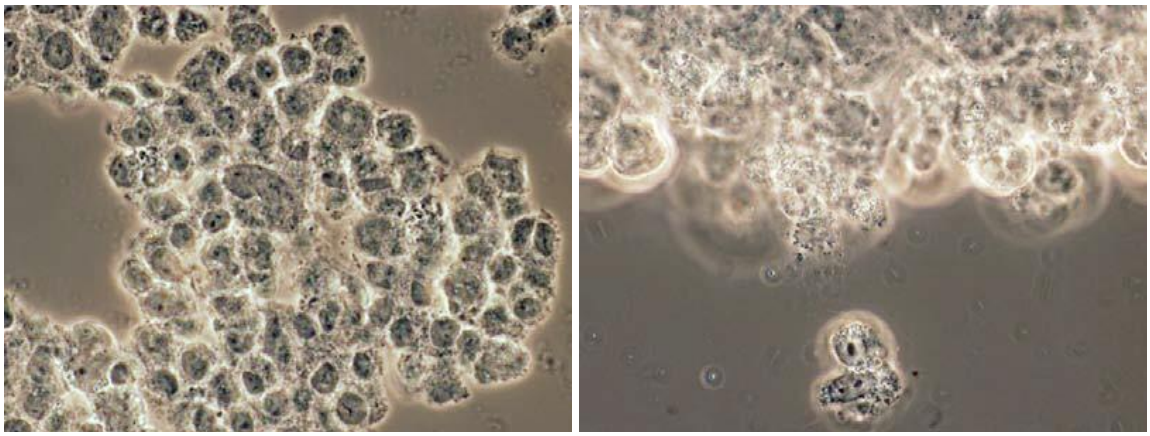
***Day 10**: New structures appear within the particle, somewhat similar to that of a colony of cells. Some cell aggregates show a CHO cell morphology that is quite different from those cultured in petri dishes.*



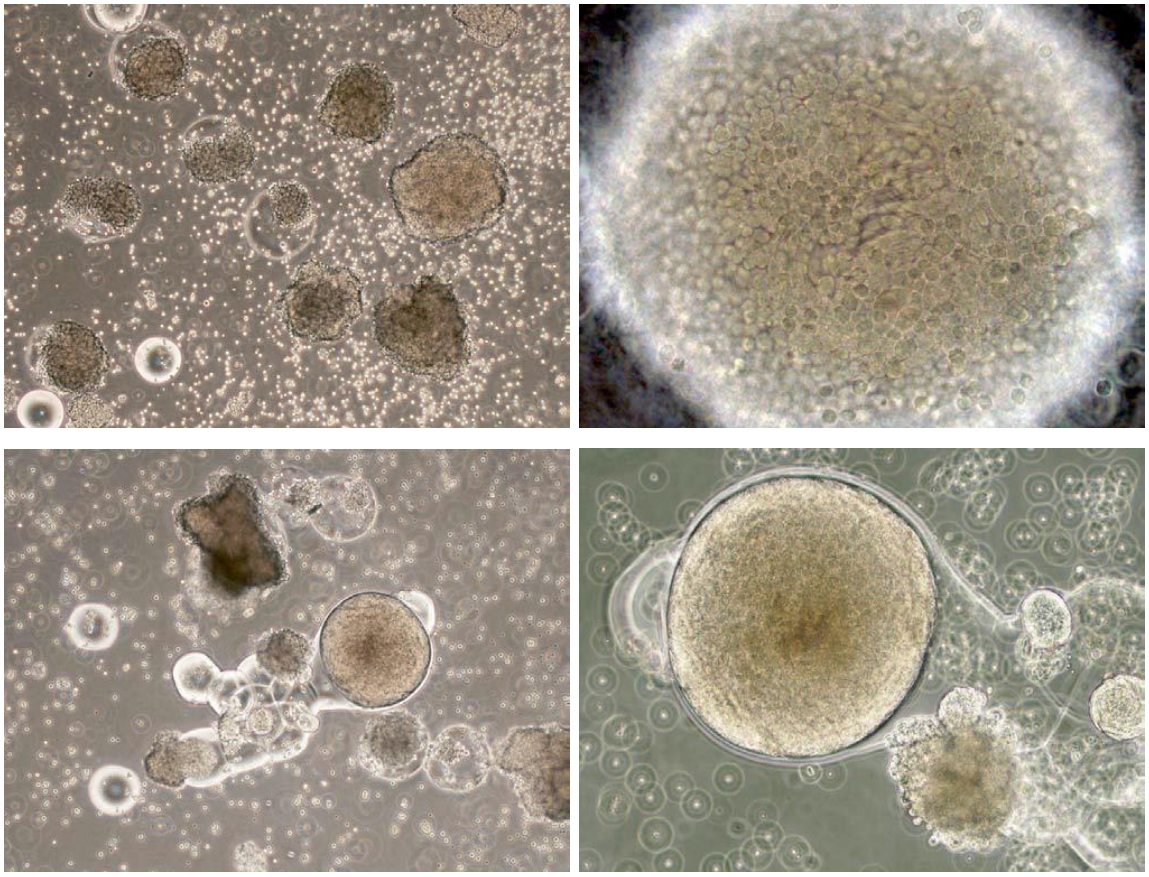
***Day 14**: Cell aggregates with increased size, clearly a result of CHO cell growth/cell division within the droplets. The cell density continues to increase with some particles starting to burst.*



***Day 17**: As more particles burst open, the number of cells found floating in the carrier oil is seen to increase. Increased size of cell aggregates that appear to "grow out" of the gelatin droplet. The medium also contains many CHO single cells, perhaps cells released from cell aggregates no longer enclosed in gelatin.*



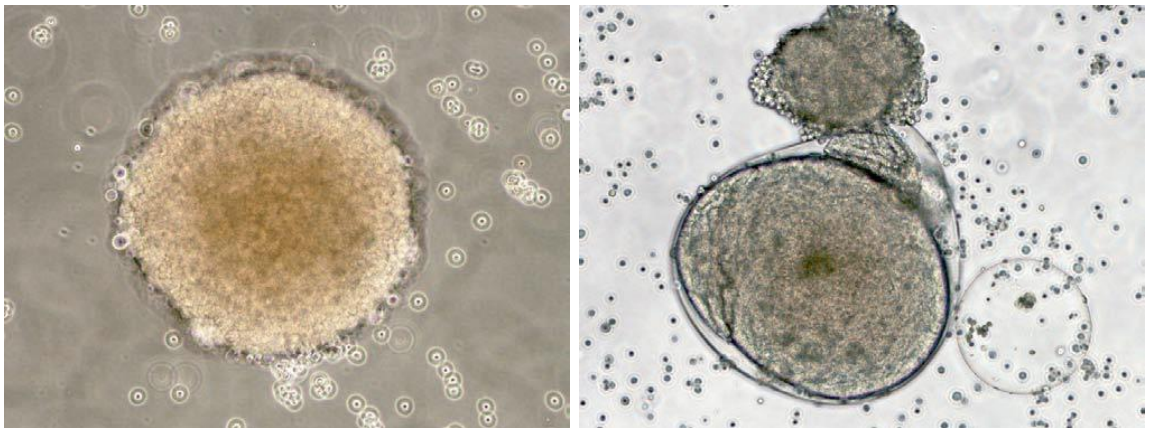
***Day 18**: Some floating cells were observed, possibly having broken free of the Gelatin particles. The gelatin in the particle simultaneously continues to degrade probably due to the excrement released by the cells. The cells continue to burst out of the particles and enter into the fluorocarbon oil phase. The floating cells look to be surrounded by some type of material (left side picture). Similar material is seen on the surface of the cell aggregates (right side picture). This material may be partially degraded gelatin or it's fragments produced by droplet decay.*



***Day 21**: Top – Most particles appear degraded and significantly distorted. The cells released into the surrounding fluid are seen to not clump together, so difficult to assess their viability. Large cell aggregates, many outside a gelatin droplet (perhaps due to droplet destruction via gelatin degradation). Large cell aggregates that are up to around 500 µm in diameter. Bottom: A large cell aggregates with a smooth cell surface within an intact/expanded gelatin capsule. At this point, the test is terminated.*

Manifestation of Difference in Cell Phenotypes

At later stages of growth (beyond the two week mark), most Gelatin particles were observed to start to deteriorate. There were some particles however that didn't burst open, and seemed to expand to be able to compensate for the increased volume of the growing cell colony contained within. Theoretical considerations predict that the collagen in the Gelatin particles degrades due to the waste produced by the cells. This was however not observed in all particles. The inference is that there is a difference in the waste produced between cells contained in different particles. Although this is a weak correlation, it is something that isn't directly visible in large colonies grown on agar plates, and could be potential for further investigation.



Microscopy using 10x objective. The different surface structure (smooth or rough) may be due to different protease expression, where a high level of gelatin degradation is associated with an "invasive" cell phenotype.

Conclusion

Encapsulation of CHO cells in aqueous droplets is achieved using Dolomite's Droplet System. The particles with encapsulated cells are then incubated. Intermittent observations of small samples taken from the incubator show the cells to be thriving and reproducing over time indicating good viability.

The pumping strategy consisted of a remote reservoir which was mounted on a hot plate along with continuous stirring action to keep cells suspended. The speed of the stirring was controlled and kept low thereby preventing shear induced cell rupture. The encapsulated Gelatin droplets were polymerized by cross linking with Transglutaminase to form solid hydrogel particles. The conversion from liquid to solid eliminated all possibility of coalescence and cross-talk chemical contamination between cells contained in the particles.

A system capable of delivering cell encapsulated hydrogel particles is demonstrated. Using a suspension of CHO cells, Dolomite's droplet system was used with a configuration best suited to preserve the cells in the gentlest manner. Subsequent to encapsulation in droplets, the viability of the cells was assessed. This was done to ensure that due consideration had been given to the sensitivity of the cells, and that no part of the droplet system posed hazards to their health. There were some issues with maintaining a steady of flux of cells, including some cases where cells were found to clump. There are expected to be resolved by tweaking the concentration along with some optimization involving reducing length of fluidic lines prior to chip. There is also the possibility of increasing the viscosity of the droplet phase with the expectation that the cells will sediment to a lesser degree.

Acknowledgement

Dolomite acknowledges the Eurostars Program funding for the project OPTISORT (E! 7790 OPTISORT). It is expected to provide 'Sorting and analysis platform for fragile mammalian cells using novel combination of optical and droplet technology'.

The areas of stem cell biology and mammalian cell based protein manufacturing are developing fast. The ability for gentle cell sorting technologies and methods is expected to enable customers exploit unprecedented applications of cell biology in technology.

Dolomite is collaborating with Danish project partners OptoRobotix (R&D performing SME) and Bioneer (technological service partner) as part of the Eurostars consortium.

The Eurostars Programme is powered by EUREKA and the European Community

APPENDIX A: System Component List

Part No.	Part Description	#
3200177	Mitos P-Pump Remote Basic	2
3200178	Mitos P-Pump Remote Chamber 30	2
3200097	Mitos Flow Rate Sensor (30-1000µl/min)	1
3200098	Mitos Flow Rate Sensor (1 - 50µl/min)	1
3200200	Mitos Sensor Interface	2
3000024	Linear Connector 4-way	2
3000155	Chip Interface H	1
3000158	Droplet Junction Chip (100µm etch depth)	1
3000397	T- Connector ETFE	1
3200272	Flow Resistor Kit	1
3200063	FEP Tubing, 1/16" x 0.25mm, 10 metres	1
3200300	FEP tubing, 1/16" x 0.1mm, 10 metres	1
3200197	USB to RS232 Adaptor Cable	2
3000222	Hotplate 110	1
3200087	2-way In-line Valve	2
3200050	High Speed Camera and Microscope System	1

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