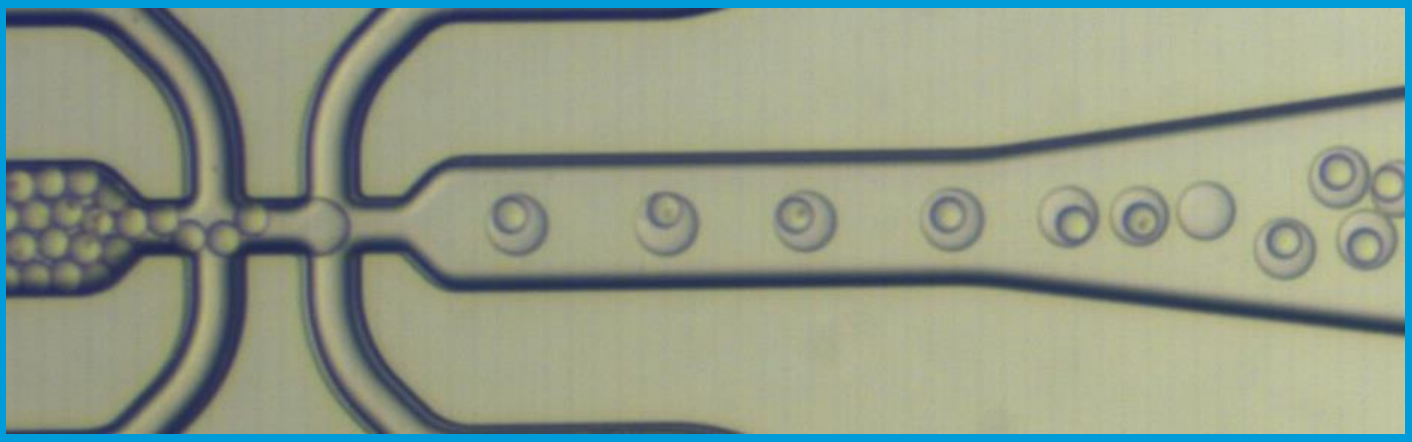
Cells in double emulsions for FACS sorting

Capturing individual cells in 30 μm double emulsions, suitable for FACS sorting



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Summary

Very high throughput can be achieved in molecular biology reactions by emulsifying or ‘dropletising’ the reaction in an oil carrier, so that millions of droplets each behave as discrete micro-reactors. Using microfluidic droplet generating chips, it is possible to capture individual cells in micro-droplets containing highly reproducible volumes of a reaction mix. If a fluorogenic substrate is available, and the droplets can be sorted in a FACS machine, then very large libraries can be screened, and multiple generations of screening can readily be carried out. Small double emulsion droplets, in the size range of 25 – 35 μm, can be useful for sorting, because the continuous ‘outer’ phase is aqueous and compatible with a FACS, and the 30 μm droplets are small enough to pass undamaged through the sorting cell. Here, we investigate the use of a two-chip system for making 30 μm double emulsion droplets.

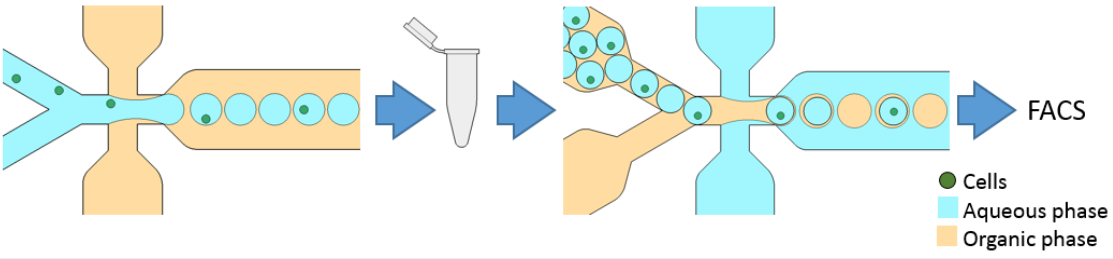

Figure 1. Double emulsion schematic. The double emulsion is produced in two stages. On the first chip, cells are captured in a single (water-in-oil) emulsion. In the second chip, the first emulsion is further emulsified in an aqueous solution, resulting in aqueous droplets contained in an oil ‘shell’, suspended in an aqueous continuous phase.

Introduction

An example of an application that can use FACS sorting of libraries is directed evolution for protein engineering. This is a powerful method of protein engineering for a variety of applications, ranging from improving the activities, selectivity and stability of enzymes used as industrial biocatalysts to improving antibodies and other therapeutic proteins, to developing proteins with novel activities. Directed evolution generally relies on expression of a library in a host, such as *E. coli*.

The throughput of library screening can be very greatly increased by capturing individual cells from the library in tiny droplets, which can behave as discrete reactors. For example, a 20 μm droplet has a volume of 4 pl, so converting a 100 μl sample into 20 μm droplets

results in 2.5 X 10^7 'micro-reactors'. If there is a fluorogenic substrate available, and the droplets can be sorted by FACS, then a very large number of clones can be screened.

When reactions are emulsified with oils, the resulting aqueous reaction droplets can behave as millions of discrete 'micro-reactors'. Using microfluidic flow focussing junctions, it is possible to make droplets that are highly 'mono-disperse', i.e., with very little variation in volume. This is important because the volume determines how much reaction mix is in each droplet, and a highly reproducible droplet volume can mean a highly reproducible amount of reaction product or signal.

Using microfluidics, it is possible to make 'double emulsion' droplets or w/o/w (water-in-oil-in-water) droplets. Thus the droplets can contain an aqueous core, which is a droplet of reaction mix containing a single cell, surrounded by a 'shell' of oil, with the droplets being suspended in an aqueous phase. If the droplets are of a suitable size (i.e., around 25 – 35 μm), they can be fed into a FACS for sorting.

In this application note, we investigate a two-chip system for making 30 μm double emulsion droplets, containing individual cells, suitable for FACS sorting.

Materials and methods

We made double emulsion (water in oil in water) droplets in the range of 30 μm diameter, in a two-stage process, where water-in-oil (w/o) droplets were made on a first chip and collected in an Eppendorf tube. The w/o emulsion was further emulsified in a second chip, with an aqueous solution as the continuous phase, to turn the droplets into water-in-oil-in-water (w/o/w) droplets, i.e., an aqueous core with an oil shell in an aqueous continuous phase. All emulsions were made with Pico-Surf™ blends, which produce stable, biologically compatible emulsions.

All liquids (except for cell suspensions) were filtered through a 0.2 μm syringe filter, to remove dust and fibres, which clog chips. The chips and tubing were cleaned immediately after use with filtered, 1% hand dishwashing liquid ('Fairy dishwashing liquid', P&G), which is primarily a blend of ionic and anionic detergents.

Microfluidic setup. P-pumps etc. Cells and emulsions can clog at fittings, flow meters, etc. Therefore, we used a sample loop composed of 500 mm of tubing connected to the chip. 500 mm sample loops of 0.25 mm i.d. tubing have a volume of ~50 μl, and those of 0.5 mm i.d. have a volume of ~200 μl. As small an inner diameter as possible is preferred. At a given flow rate, a smaller inner diameter tubing will have a higher linear flow velocity,

which will tend to sweep cells and emulsions along, rather than allowing them to settle on the walls of the tubing.

To load the sample loop, it is disconnected from the chip connector and upstream fitting, and connected to a 1 ml syringe filled with Novec™ 7500. First, a negative pressure is applied with the syringe to suck in a small 1 – 5 mm bubble, so that the interface between the driving fluid and sample can be seen. Then the end of the tubing is dipped into the sample, and a negative pressure is applied with the syringe to suck the sample into the sample loop, stopping before the bubble reaches the syringe. The sample loop is then reconnected to the chip connector, which is connected to the chip. Once that end is relatively sealed, the syringe is disconnected, and that end of the sample loop is reconnected to the upstream fitting.

The use of the sample loop adjacent to the chip allowed the flow meters to be used in the system, without flowing cells or emulsions through them. This allowed the system to regulate and maintain a constant flow rate, which is important for keeping the droplet size constant over time. The fluorocarbon oil Novec™ 7500 was used as an inert driving fluid. When driving a water in oil emulsion, the emulsion sample was separated from the Novec™ 7500 driving fluid by a bubble.

First stage (water in oil) emulsion. The first chip was fluorophilically coated, with a 15 μm etch depth, with junctions that were 15 μm deep and 18 μm wide, producing droplets approximately 15 μm in diameter, or 2 pl in volume. In order to produce a cell suspension with approximately 1 cell per 10 droplets, sheep blood was diluted to approximately 1 red blood cell per 20 pl, or $2x10^8$ cells/ml. Two different cell suspension media were tested. The first cell suspension medium was 1X DMEM, and 28.1% Optiprep™ (so that the red blood cells were approximately neutrally buoyant). The second, low salt isotonic cell suspension medium was 0.25X DMEM, 210 mM sucrose and 21.6% Optiprep™. The continuous phase was either Pico-Surf™ 1 or Pico-Surf™ 2. The micro-emulsion was collected in a 1.5 ml Eppendorf tube. The emulsion floats on the Pico-Surf/fluorocarbon oil blend, because the fluorocarbon oils are more dense than water. Excess Pico-Surf/oil was pipetted out from under the emulsion, and the emulsion stored at 4°C.

Second stage (water in oil in water) emulsion. Second stage, hydrophilic (i.e., uncoated glass), chips were either also etched to either 15 μm or 20 μm depth. The second stage chips were '2 reagent' or double junction chips, to allow for injecting spacer oil (Pico-Surf). 20 μm etch depth chips had a junction that was 20 μm deep and 23 μm wide. The emulsion from the first stage was loaded into the sample loop. The aqueous 'sheath' fluid was 0.1% TWEEN 80, 150 mM NaCl. The double emulsion was collected in a 1.5 ml Eppendorf tube. The ratio of first stage emulsion to spacer oil was between 1:1

and 1:4. Using more spacer oil resulting in thicker ‘shells’, and more oil droplets with no aqueous core. Using less spacer resulted in oil droplets containing two aqueous droplets. The ratio of aqueous continuous phase (TWEEN + NaCl) to first stage emulsion + spacer was around 20:1 to 10:1. Less aqueous phase resulted in oil droplets containing two or more aqueous droplets.

Sample loop, and loading. Both the cell suspension and the first stage emulsion were loaded into a sample loop that was downstream of the flow meter, and immediately upstream of the chip. The sample loop allowed the use of the flow meter without flowing the samples through it. The first stage water-in-oil emulsion also fed better from the sample loop than from a micro-centrifuge tube in the P-pump chamber. To load the sample loop, it was disconnected from the chip and upstream valve. The head fitting on the sample loop was connected via a ¼” 28 luer adapter to a 1 ml syringe that contained dye. The sample loop was flushed with dye, then the free end of the loop was dipped into the sample, and suction applied by the syringe to back-fill the sample loop. The free end of the sample loop was re-connected into the chip connector, the chip connector was connected to the chip, then the syringe was disconnected and the upstream end of the sample loop was re-connected to the upstream valve.

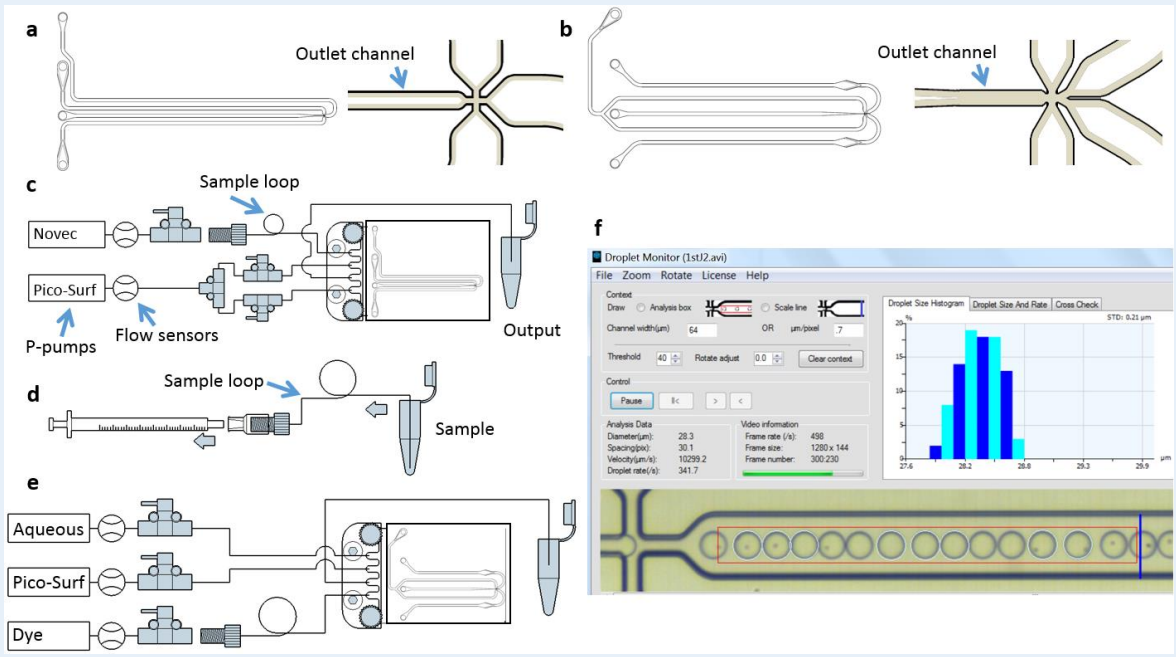

Figure 2. Experimental setup. A) The 'one reagent' chip used for generating the water-in-oil emulsions, with a magnified view of the junction. B) The '2 Reagent Chip', one fo the chips that was used in the second stage for converting the emulsions to double emulsions. C) The setup for making first stage emulsions. Only two P-pumps were connected, one with the surfactant/emulsion blend (Pico-Surf), and one with inert driving fluid (Novec™ HFE 7500 in this case). D) Sample loop. The sample was loaded as shown, into a 25 μl sample loop, composed of 500 mm of 0.25 mm inner diameter FEP tubing. E) Setup for second stage, to make water-in-oil-in-water double emulsions. An aqueous dye solution was used as the driving fluid, and Pico-Surf™ was injected to space the water-in-oil droplets out. F) Analysis of 'first stage' single emulsion droplets in the Droplet Monitor software. At this flow rate ratio, 28.3 um droplets were formed. Note that droplets were monodisperse.

Results

The objective of this note was to investigate i) capturing cells in small double emulsion droplets that are suitable for feeding into a FACS for sorting ii) the reproducibility of droplet size, which determines the amount of reaction product/droplet and iii) droplet stability.

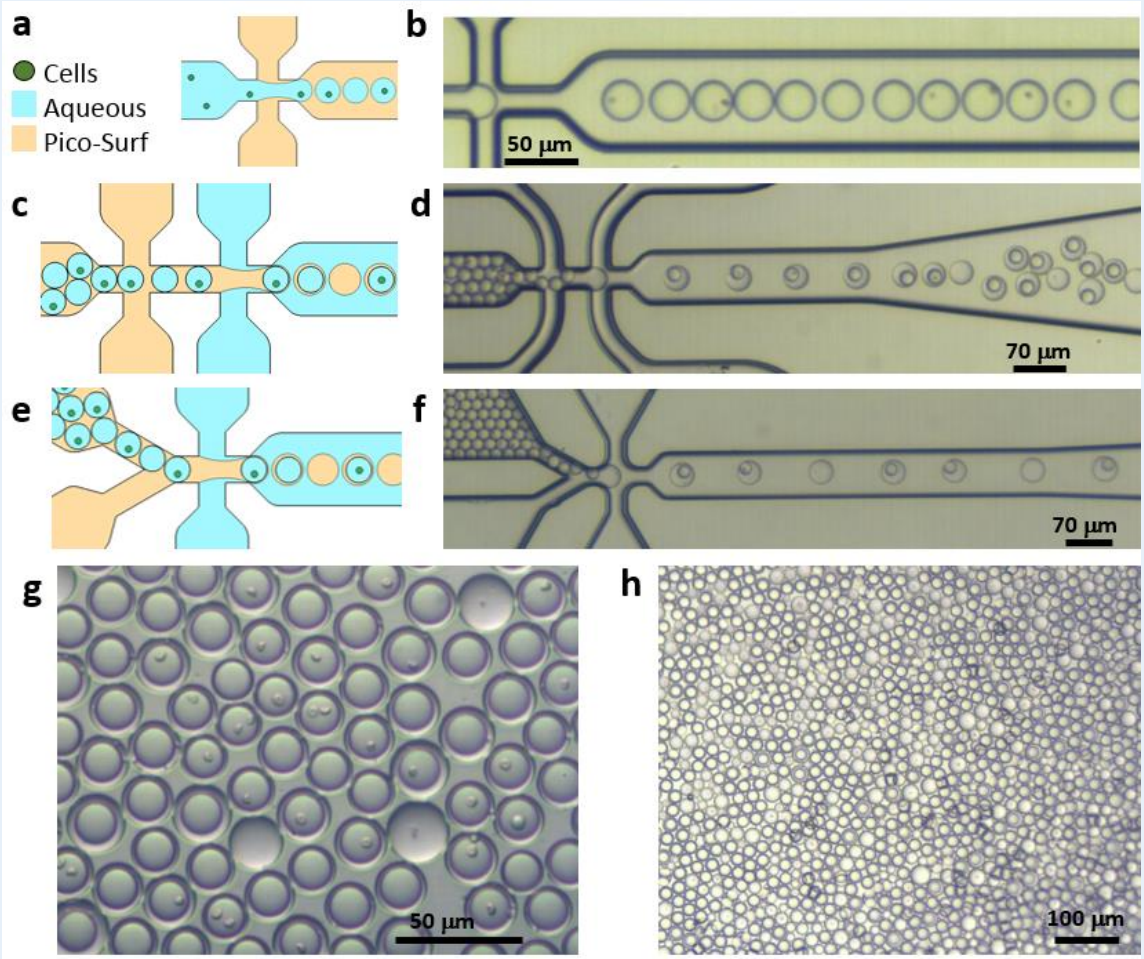

Figure 3. Cells in double emulsion droplets. A) Schematic of first stage single emulsion formation. An aqueous suspension medium (blue) containing cells (green filled circles) is turned into droplets in a Pico-Surf™ continuous phase at a flow focussing junction. B) Photomicrograph of aqueous droplets with cells formed at a junction. C) Schematic of double emulsion formation double junction 'Carrier Addition Chip', where spacer oil is injected symmetrically from both sides. D) Photomicrograph of double emulsion formation. E) Schematic of double emulsion formation on a '2 Reagent Chip', where spacer oil is injected from only one side. F) Photomicrograph of double emulsion formation. G) Double emulsion, with red blood cells. In this case, cells were included at a higher concentration of around 1 cell every three droplets. Note that the size of the aqueous core droplets (which determines the amount of reaction product per droplet) is constant, although the thickness of the oil shell is variable. Apparent 'specks' in the oil shells are reflections. H) Same double emulsion, after 7 days at 4°C. Note that the emulsion is stable, and the aqueous droplets are still mono-disperse.

In the first stage, a cell suspension was 'dropletised' in a fluorocarbon oil, in a 15 μm X 18 μm junction. Junctions usually readily make droplets with a diameter equal to 75% to 125% of the diameter of the junction, depending of the relative flow rates of the droplet phase (in this case, the cell suspension) and the carrier phase (in this case, Pico-Surf). In general, droplets are readily formed at droplet:carrier flow rate ratios of 1:10 to 1:20. The higher the relative flow rate of the droplet phase, the larger the droplets will be.

It is important that the droplets are mono-disperse, i.e., have a reproducible size, because this controls the amount of reaction mix in the droplet, and therefore the amount of reaction product or output signal. A movie was captured of a cell suspension being turned into a micro-emulsion at the junction at a low flow rate, and analysed with the Dolomite Droplet Monitor. The flow rates were low, so as to capture high resolution movies. At the low total flow, and the flow rate ratios used, the droplets formed were an average of 28.1 μm in diameter (12 pl volume), with a standard deviation of 0.21 μm (i.e., a standard deviation of 0.26 pl, or 2% in volume). Thus, the droplet volumes were highly reproducible.

Next, smaller droplets were made at a high flow rate, as the first stage in making the small double emulsions. The cell suspension was flowed at up to 0.5 μl/minute (at ~6 bar pressure (i.e., 6 atmospheres)), forming ~15 μm (2 pl) droplets at up to 4.7 kHz (i.e., 4,700 droplets per second), or 2.8 million droplets every 10 minutes. At this flow rate, the droplets were moving fast enough they were blurred even at exposure times of 50 μs. The cells in droplets in oil suspension was collected in an Eppendorf tube, and stored overnight at 4°C.

In the second stage, the water in oil emulsion was injected into second stage chips, to turn them into water-in-oil-in-water droplets. The aqueous droplets from the first stage pack together, so extra Pico-Surf™ is injected on the chip to space the droplets out before the junction. At the cross junction, the 'aqueous phase' (0.1% TWEEN 80, 150 mM NaCl) is injected at the sides, to form the double emulsion droplets. The spacer oil was fed in at around 1/3rd to 1/5th of the flow rate of the water in oil emulsion, and the flow rate of the outer aqueous phase was about 10 – 20 times higher than the first stage emulsion. The flow rates were adjusted to get one aqueous core droplet per oil droplet. Once this is established, double emulsion production is very stable, especially at higher flow rates.

When the chip is run slowly (10 – 100 Hz), the oil 'shells' are relatively thick (see Figure 2d and Figure 3, d & f). Also, at low flow rates, the resistance from the individual droplets in the first stage emulsion squeezing into the thin channels of the junction makes the emulsion feed slightly discontinuously, rather than smoothly, through the junction. In

general, the chips perform better at high flow rates (above approximately 100 Hz or a droplet phase flow rate of 50 nl/minute).

At higher flow rates, it is possible to reduce the relative flow of spacer oil, and thereby produce a higher ratio of 'filled shells' (i.e., oil droplets with aqueous droplets inside), and thinner shells, because the aqueous droplets feed more smoothly through the junction. The first stage emulsion was fed in at up to 620nl/minute, or 10,300 pl/second. The resulting droplets have an outer diameter of around 20 μm, which equates to a volume of 4 pl, so the droplet formation rate was around 2.6 kHz, or around 1.6 X 10^6 droplets in 10 minutes. The resulting double emulsion droplets were around 20 μm, and preserved the mono-disperse aqueous core droplets (Figure 3g). Interestingly, at these high flow rates, while the oil 'shell' was variable in thickness. The double emulsion was stored at 4°C for a week, and examined again. The droplets were stable and hadn't noticeably coalesced (Figure 3h).

Conclusion

The two-chip system reliably produced 30 μm mono-dispersed double emulsion droplets, depending on the flow rate ratios, and the absolute flow rates. Specifically, at low total flow rates, and high ratios of droplet:carrier phase flow rates, the droplets were up to around 33 μm in diameter. At higher flow rates, and lower relative droplet phase flow rates, the aqueous droplets were around 15 – 17 μm (i.e., 2 pl). Aqueous droplets could readily be formed up to around 5 kHz, and double emulsion droplets to 2.6 kHz. The droplets were stable at 4°C for at least a week.

Zinchenko *et al*. (*Anal. Chem*., **86**:2526) previously found that formation of water in oil emulsions was favourable with the fluorocarbon oil HFE 7500. However, if reactions were heated, it was preferred to exchange the oil to another fluorocarbon oil, FC40 (by pipetting out excess HFE 7500 from underneath the emulsion and replacing with it FC40, or by diluting the emulsion), because diffusion of small molecules between the droplets during heating was minimised with FC40. They also found that double emulsion stability during storage was favoured by an isotonic outer aqueous phase that had TWEEN 80 as a surfactant.

During formation of an emulsion, it is important to keep the flow rates of the droplet (e.g., cell suspension) and continuous (e.g., Pico-Surf) phases constant, because the flow rate ratio between the two phases is what determines droplet size. This is achieved by running the system in 'flow control mode' from the Dolomite Flow Control Centre software. If the system is instead run on constant pressure, then tiny variations in flow resistance result in

changes in the flow rate ratios, and consequently, variation in the droplet sizes being produced. In the current work, to avoid running samples through the in-line flow meter, samples were loaded into a sample loop downstream of the flow meter, and immediately upstream of the chip. Also, the first stage emulsion fed into the chip much better from the sample loop than from a micro-centrifuge tube.

In general, the system performed better at high flow rates that is around 0.5 μl/minute droplet phase flow, or 1 – 5 kHz. The flow of emulsions was smoother through the junctions, and cells and emulsions fed better through the tubing.

In summary, the two chip system for making small double emulsions reliably and readily makes highly mono-disperse aqueous core droplets that are important for reproducibility and low variance. When the first stage water-in-oil droplets are fed into the second chip, it is straightforward to adjust the flows of first stage emulsion, spacer oil, and out aqueous phase, to make oil droplets that carry a single aqueous core. Once these flow rates are established, the systems stably makes good double emulsion droplets, with mono-disperse aqueous core droplets.

Appendix A: System Component List

Part No.	Part Description	#
3200016	Mitos P-Pump	3
3200118	Mitos Compressor 6bar (110V/60Hz)	1
3200099	Mitos Flow Rate Sensor (0.4-7µl/min)	2
3200098	Mitos Flow Rate Sensor (1 – 50µl/min)	1
3200200	Mitos Sensor Interface	3
3200197	USB to RS232 Adaptor Cable	3
3200148	Linear Connector 7-way	1
3200294	Top Interface 7-way (4mm)	1
	2 Reagent Droplet Chip (15µm etch depth), fluorophilic	1
	2 Reagent Droplet Chip (20µm etch depth)	1
	Carrier Addition Chip (20µm etch depth)	1
3000397	T-Connector ETFE	1
3200307	End Fittings and Ferrules for 0.8mm Tubing (pack of 10)	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	3
3200050	High Speed Camera and Microscope System	1

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