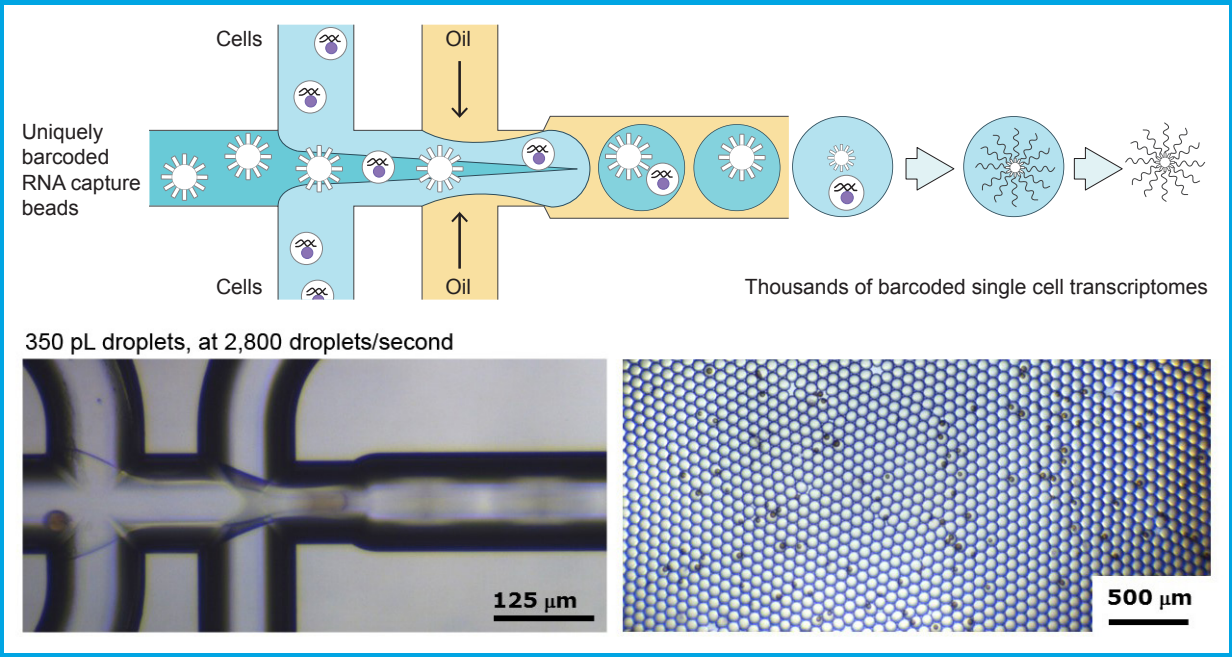
High Throughput Single Cell RNA-Seq Application Note

Encapsulating single cells with barcoded beads on the RNA-Seq chip



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Summary

Many biologically important processes take place at the level of single cells, including processes in neurobiology, development, immune responses and cancer. However, many traditional techniques, such as Western blots and PCR involve homogenising tissue samples, so can only deal with population averages, and much crucial information is lost. Droplet microfluidics offers the unique ability to isolate thousands to millions of cells in individual droplets. An exciting method for high throughput single cell RNA-Seq has been published recently (Macosko E., et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." Cell **161**:1202). In this protocol, called 'Drop-seq', tens of thousands of cells are individually encapsulated with uniquely barcoded RNA capture beads (Figure 1), leading to the robust and straightforward preparation of tens of thousands of single cell cDNA libraries. In this application note, we demonstrate the single cell RNA-Seq method on a Dolomite Bio droplet system.

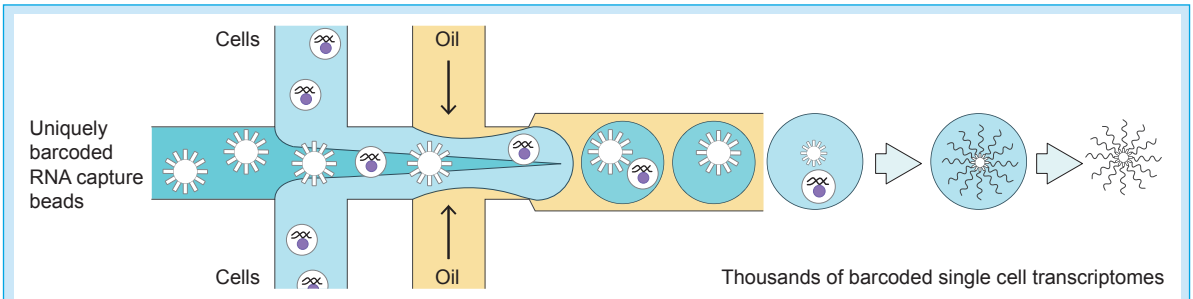

Figure 1. Schematic of the Drop-Seq single cell RNA-Seq method. Tens of thousands of single cells are encapsulated with uniquely barcoded mRNA-capture beads. The cells are lysed in the droplets, and beads capture mRNA from single cells. The single cell RNA-beads are recovered and processed for downstream high throughput sequencing.

Introduction

Cell encapsulation in microfluidic droplets is an exciting, cutting edge technique that, for the first time, enables expression analysis of thousands or even millions of single cells. This unprecedented analytical power allows the exhaustive discovery of previously unidentified or cryptic cell types in the tissue of interest, and also allows their behaviour to be followed through biologically important processes. Because the volume of the droplets is small – in the range of nanolitres to picolitres – mRNA capture or reverse transcription from single cells is efficient and reliable, and the technique is within the reach of most well-staffed research labs.

A pair of very exciting methods for high throughput single cell RNA-Seq was published recently (Macosko E., et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets." Cell **161**:1202; Klein, AM., et al. "Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells." Cell **161**:1187). The Drop-Seq protocol developed by Macosko et al. involves encapsulating single cells with single barcoded beads. The barcoded oligo bead library is constructed such that each bead has a unique DNA barcode sequence, but within a bead, the thousands of copies of oligo all contain an identical barcode sequence. The 3' end of the oligo has a poly(dT) stretch that captures mRNA and primes reverse transcription.

It is desired to encapsulate single cells with single barcoded beads, and this is achieved by limiting dilution. In general, there is a trade-off between cleaner data (more diluted cells and beads) and higher throughput, or more efficient capture of cells (more concentrated cells and/or beads). The dilution of cells or beads can be chosen based on the desired outcome of the experiment. If there are two cells in a droplet with a bead, then the bead will capture mRNA from two cells. In a discovery experiment, this could be spuriously interpreted as a new cell type. If the cell types are already known, it can be identified and discarded computationally. If there are two beads in a droplet with one cell, this is typically less confounding, because it only means the cell is identified or counted twice. As long as the cell identity is independent of the probability of being encapsulated with two beads (which it almost always will be), then it will not affect the statistics.

For cleaner data, a researcher may choose a dilution where 1 in 20 droplets contains a cell or bead. In this case, it means that 1 in 400 droplets contains a cell and a bead (1/20 X 1/20). This also means that only 1 in 20 cells will be encapsulated with a bead, and the rest will effectively be discarded. If a researcher desires to recover data from most of the cells, it may make sense to encapsulate cells at 1 cell per 20 droplets, but increase the bead concentration, so that more droplets contain beads, and more of the cells have their mRNA captured on beads. In this case, the droplets that contain one cell and two beads do not cause a problem.

Cells are typically encapsulated in aqueous droplets in biocompatible and inert fluorocarbon oils such as HFE 7500 containing a surfactant to stabilise droplets after formation.

Cell encapsulation is fast becoming a mature technology and now is rapidly transitioning from an engineering demonstration to a research tool being used by skilled researchers in many advanced biological laboratories. The original cell encapsulation microfluidic systems used in the initial research were rather cutting edge, bespoke experimental systems built by engineers within research laboratories. These prototype systems are not necessarily easy for biologists to create or to use in a day-to-day research work-flow.

Dolomite Bio is a world-leader in the design and manufacture of microfluidic systems and components for biology applications. Dolomite Bio specialises in ‘Productising Science®’, which aims to provide microfluidic solutions for research biologists. Dolomite Bio has complete cell encapsulation systems which are modular, and include components such as high pressure pumps and sensors, highly reliable glass microfluidic chips, easy-to-use microfluidic connectors, and automation software.

This application note reports some results of encapsulation of cells with barcoded beads for single-cell RNA sequencing using the Dolomite Bio cell encapsulation system.

Materials and Methods

The method was performed essentially as described in Macosko et al., except for modifications to run the Dolomite Bio droplet system, and modifications to improve bead 'flowability' and minimise bead shearing.

Droplet system. The cell encapsulation system uses pulseless Dolomite Bio P-Pumps, and can be driven from a PC via Dolomite Bio's Flow Control Centre (FCC) software. The beads were loaded into a sample loop, which allow them to be flowed without mechanical stirring to avoid fragmenting the beads. The upstream end of the sample loop had a four-way sample injection valve. Two-way valves ('shut-off' valve) were included on the oil and cell lines to prevent backflow on shutting the system down.

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. The microscope allows short exposure times (50 µs), which is useful in monitoring high speed droplet production.

Glass Single Cell RNA-Seq chip. A prototype PDMS Drop-seq chip was described in the literature (Macosko et al.). For standard products, glass is preferred, as the glass chips are robust, highly reproducible, and chemically inert. We therefore produced a glass Single Cell RNA-Seq chip (Figure 2a). The channels allow the use of robust, standard, leak-free connectors, while allowing the junction to be readily imaged. The chips had two identical microfluidic circuits per chip, and were fluorophilically coated. The schematic in Figure 2 shows a second-generation RNA-Seq chip that has improved filters, channel geometry (to prevent blockages) and an improved, more robust, coating. While the results described in this application note were obtained with the first-generation RNA-Seq chip, these results are directly transferable to the new chip because the microfluidic junction is the same.

To use the chip, the only adjustments required to the published Drop-seq protocol were to suspend the cells and beads at a higher concentration and to use slightly lower flow rates described in this application note.

After use, the chips were cleaned by sequentially flushing with 0.2 µm-filtered 10% detergent ('hand' washing up liquid), followed by filtered isopropanol then air, using a cleaning station.

Beads. The beads described in the Drop-seq protocol are around 30 µm in diameter but their size may vary slightly. For this reason and to avoid blockages on the chip, they were sieved through a 70µm cell strainer. They were then suspended in 6% Ficoll PM-400, 0.2% Sarkosyl, 0.02M EDTA, 0.2M Tris pH 7.5 and 0.05M DTT (as described in Macosko et al.). The bead concentration was counted in a hemacytometer. Following a test encapsulation, the droplets and beads were counted, and the necessary adjustment to the concentration to obtain 1 bead per 20 droplets was calculated.

Flowing beads using a sample loop. As noted in Macosko et al., the oligo beads sediment out of suspension rapidly. In the published protocol, a magnetic mixing disc was included in the syringes to stir the beads but this tended to fragment the beads. To avoid this fragmentation, Dolomite Bio developed a method of flowing beads using a sample loop (Figure 2d).

A sample loop was used to allow pumping of the beads without mechanical agitation and the consequent risk of mechanical shearing leading to broken beads which can then end up in more than one droplet. The sample loop was made of 10 m of 0.25 mm internal diameter (ID) tubing, with a volume of 0.5 ml, and was connected to the upstream sample injection valve with a nut and ferrule. Samples were injected into the loop with a 1 ml luer lock syringe. The sample was first aspirated into the syringe via a 16G blunt needle, and the needle was removed. The syringe was flicked to dislodge and discard bubbles, an important step, as bubbles sweep beads along in the sample loop, resulting in a higher local concentration of beads in front of the bubble. The syringe was then connected to the injection valve via a luer fitting. The beads settle rapidly, therefore, we found keeping the beads well suspended in the syringe during injection to be a critical step in efficiently running the chips without blockages. This can be easily achieved by gently inverting the syringe every 20 seconds or so during the injection process, to keep the beads in suspension. The beads were then efficiently injected into the sample loop at the desired concentration.

Cells. Sheep lymphocytes were suspended in 1X PBS + 0.01% BSA and counted in a hemacytometer. Following a test encapsulation, the cells and droplets were counted and the cell concentration adjusted as necessary to obtain 1 cell per 20 droplets.

Flowing cells using a remote P-pump chamber. The cell suspension was pipetted into a microcentrifuge tube in a remote P-Pump chamber. The microcentrifuge tube was rotated by placing a 20 mm stir bar in the chamber and putting the chamber on a stirrer (Figure 2c).

Cell & Bead Encapsulation. One P-Pump was loaded with the droplet oil, the ‘cells’ pump was connected to the remote chamber in which the cell suspension was loaded, and the ‘beads’ pump was loaded with lysis buffer. The buffer loaded into the ‘beads’ pump functioned as a ‘driving liquid’, to push the bead suspension out of the sample loop. The system was primed by pumping the buffer, cell suspension and oil until drops appeared at the connector. The pumps were then turned off, the injection valve turned to the sample injection position and the bead suspension was loaded into the sample loop. The flows were started essentially as described in Macosko et al., i.e. in the order cells>beads>oil, in order to avoid backflow of lysis solution into the cells tubing line and premature lysis.

Specifically, the pumps were controlled from a program in the Dolomite Bio Flow Control Centre (FCC) software, that rapidly turned on the ‘cells’ pump, then the ‘beads’ pump, then the ‘oil’ pump. This step could also be performed manually. As described in Macosko et al., the chip occasionally does not initiate droplet formation, but partially or completely ‘streams’ instead (Figure 3b). The high-speed microscope and camera enable the user to visualise droplets, and therefore confirm that droplet production has correctly initiated. If it has not, gently tapping the upstream tubing (leading from the P-Pumps to the chip) several times generally initiates droplet production. If droplet production has not begun, the aqueous flows should be reduced to 10 µL/min, then increased to the desired flow rate 30 µL/min again. Once stable droplet production is initiated (i.e. the droplets are stably forming just after the junction for longer than 5 – 10 seconds), then the outlet tubing should be moved to the collection vessel.

Table 1. Droplet sizes, and single cell library formation rates

droplet diameter, μm	88
droplet volume, pL	357
Flow rate for aqueous flows (μL/min)	2X 30
Droplet rate in droplets/second	2,803
cells/μL, for 1 cell/20 droplets	280
Single cell libraries/minute, at 1 cell/20 droplets, 1 bead/20 droplets	420

Droplet production successfully occurred at a flow rate of 30 μL/min for each of the aqueous flows, and 200 μL/min for oil. At these flow rates, the droplet formation frequency was approximately 2,800 droplets per second (Table 1).

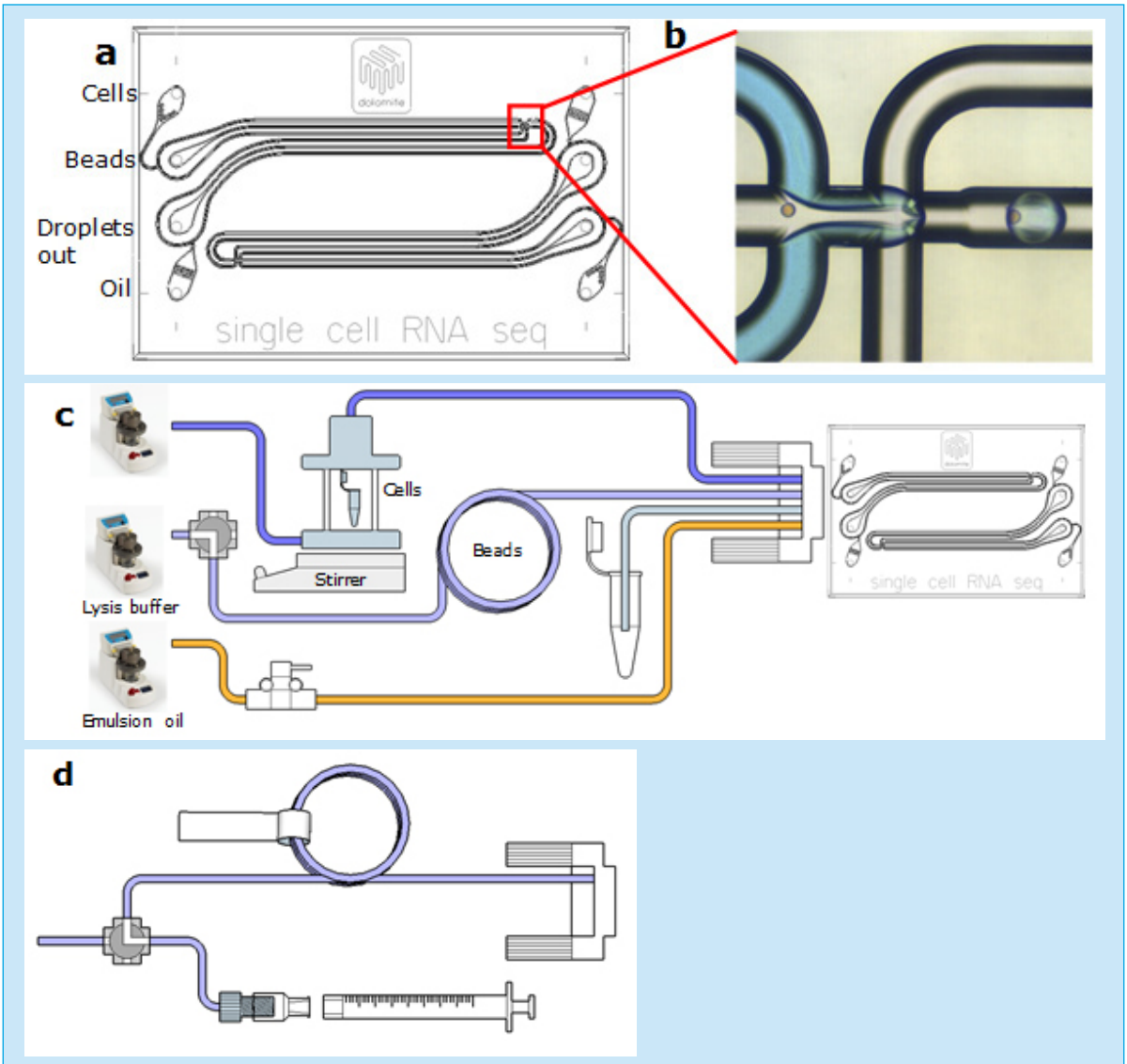

Figure 2. (a) Drawing of the chip, with two identical circuits. The ports are labelled ("cells", "beads", etc.) on the side of the chip for one circuit. (b) Photomicrograph of one of the junctions on the chip, making droplets with dye and beads. (c) Diagram illustrating how a magnetic stirrer was used to 'rotate' a microcentrifuge tube in a remote chamber. (d) Diagram illustrating the cleaning station. (e) Diagram illustrating the sample loop. The sample loop was loaded with 1-mL Luer lok syringe via a port on the injection valve.

Results

The objectives of the tests were 1) to develop a method of flowing beads resulting in no bead fragmentation, 2) to confirm that the glass chip produces droplets as described for the PDMS prototype, 3) to determine the optimal flow rates at which monodisperse droplets could be reliably formed, 4) to test that beads and cells were reliably encapsulated, and 5) to determine the droplet sizes that the chip reliably produces, the appropriate cell and bead suspension concentrations, and the consequent rate of cell encapsulation and single-cell library production.

Flowing beads. As noted in Macosko et al., flowing beads was somewhat challenging, as the beads sediment out quite rapidly.

A sample loop was tested (Figure 2d), with the intention of simplifying bead loading and minimising bead damage. Beads flowed from the sample loop had no noticeable damage. For this experiment beads were suspended at 3.1 X 105 beads/ml, corresponding to a theoretical encapsulation rate of 1 bead per 20 droplets. We found 662 droplets out of 13,300, or 5.0% of droplets, contained a bead, in good agreement with the expected result.

Testing flow rates for the production of droplets and encapsulation of beads/cells. To test for successful droplet production and cell/bead encapsulation, droplets were made at a series of flow rates (Table 2), with a cell suspension, beads suspended in lysis buffer, and droplet oil, essentially as described in Macosko et al.

Table 2. Flow and droplets rates for the Dolomite Bio RNA-Seq chip

Aq., X 2, µL/min	30	30	40	40
Oil, µL/min	100	150	200	200
Droplet dia., µm	110	99	88	85
Droplet vol., pL	697	508	357	322
Droplets/second	1,400	1,970	2,803	4,000
Cells/µL, for 1/20 droplets	144	200	280	311

Droplets were successfully produced and cells/beads were successfully encapsulated at all four combinations of flow rates (Figure 3a). The highest flow rates that produced mono-disperse droplets were 40 µL/min for each of the two aqueous phases, and 200 µL/min for the oil phase. At these flow rates, the optimal concentration of beads and cells to use to obtain 1 bead or 1 cell per 20 droplets was 311 beads/µL and 311 cells/ µL. The produced droplets were monodisperse, and did contain beads at approximately one bead per 20 droplets (Figure 3c and d; the cells cannot be seen, as they have been lysed by the detergent). However, it was noted that at 40 µL/min for the aqueous flows, the junction could occasionally stream, or jet (Figure 3b), and produce slightly polydisperse droplets. This happened more systematically at high flow rates (50 µl/min for each aqueous phase). For more robust applications and in cases where a lower throughput is acceptable, it is recommended to use slower flow rates, i.e. 30 µL/min for each of the two aqueous phases and 200 µL/min for the oil phase, for reliable droplet formation.

At the flow rates of 40 and 200 µl/min, the droplets were 85 µm in diameter, which equates to a volume of 320 pl. The droplet production rate was 4 kHz (4,000 droplets per second) At the flow rates of 30 and 200 µl/min, the droplets were 88 µm in diameter, which equates to a volume of 357 pl. The droplet production rate was 2.8 kHz (2,800 droplets per second).

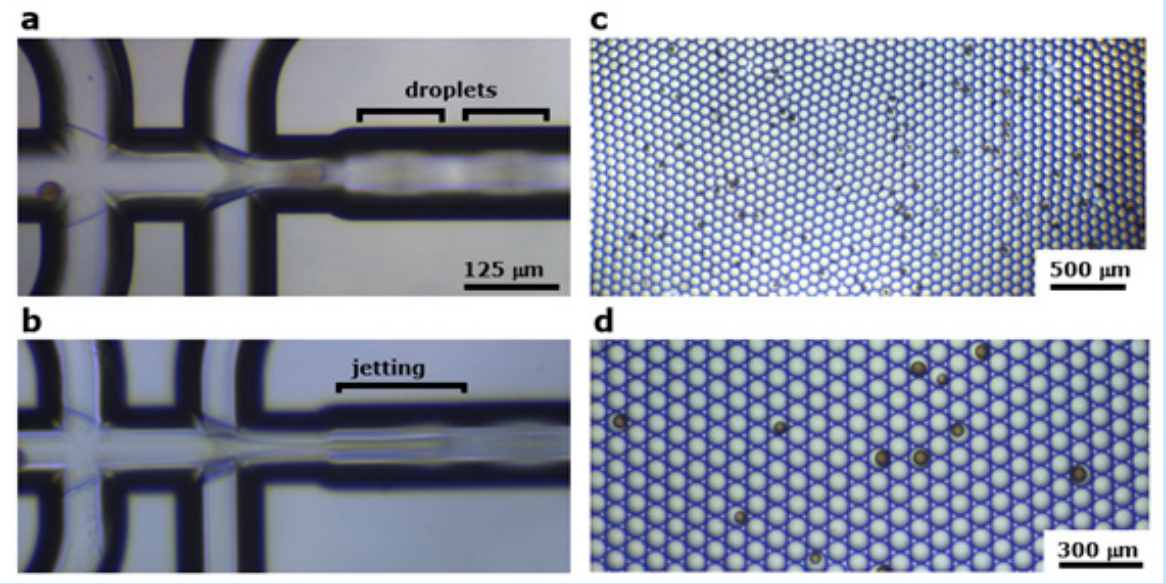

Figure 3. (a) Correct droplet formation just after the junction. (b) At high flow rates (50 µl/min each for the aqueous phases), the junction tended to 'stream' or 'jet', rather than form droplets, leading to polydisperse emulsions. (c) and (d) Emulsions imaged in a plastic hemacytometer slide. The cells are not visible, because they have been lysed.

In summary, droplets were successfully produced on the glass Single Cell RNA-seq chip. The highest flow rates that reliably produced mono-disperse droplets and the optimal beads/cells concentrations were determined (Table 3). Added to this, the chosen methods for the introduction of cells and beads were with a stirred microcentrifuge tube placed in a remote chamber and via a sample loop respectively (Figure 3 and Figure 4).

Table 3. Optimal flow rates and cells/beads concentrations

Aqueous phases, µL/min	30
Oil, µL/min	200
Beads/µL, for 1/20 droplets	280
Cells/ µL, for 1/20 droplets	280

Conclusions

The single-cell RNA-Seq protocol offers an extremely powerful and exciting method for single-cell transcriptomics. This development allows easy, straightforward access to large numbers of single cell expression profiles, for the first time. Consequently, many research groups are eager to adopt the workflow described in the Drop-seq protocol.

It was shown here that the glass Single Cell RNA-Seq chip can successfully encapsulate cells with barcoded beads in lysis buffer, producing monodisperse droplets using flow rates of 30 µL/min for each of the two aqueous phases and 200 µL/min for the oil phase.

In conclusion, the RNA-Seq application has been demonstrated with good results using a glass chip within a droplet system. The method has proven to be robust in the hands of reasonably skilled researchers. The results presented here show stable droplets, similar to those reported in the original publication, with the advantage of obtaining slightly smaller droplets, produced at a higher rate.

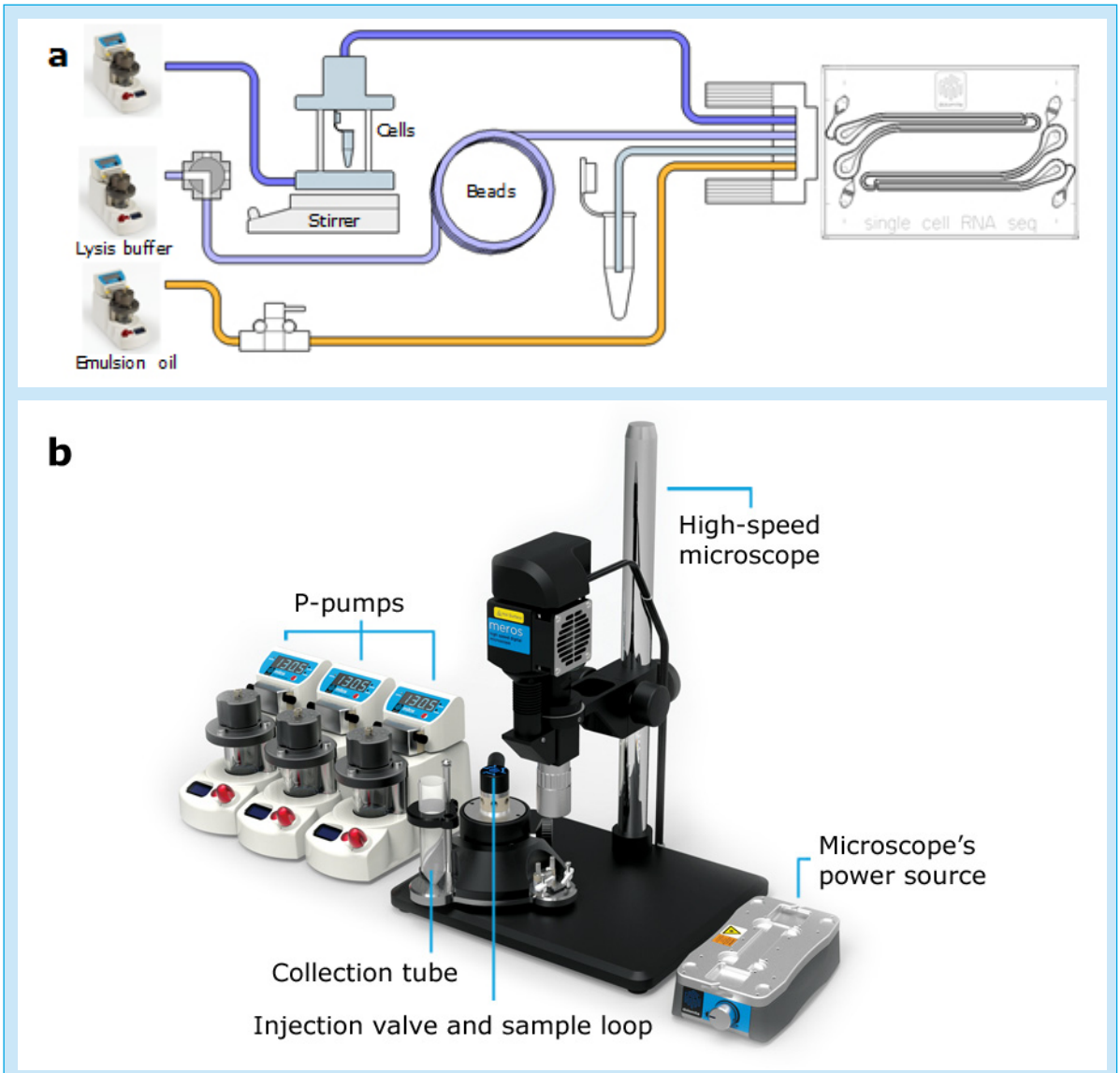

Figure 4. Chosen setup showed as a diagram (a) and a picture (b). Cells flow from a stirred microcentrifuge tube, and beads flow from a sample loop.

Appendix A: System Component List

Orders from	Part No.	Part Description	#
US and Canada	3200534	Single Cell RNA Seq System with Enhanced Control (110v, 60Hz, US)	1
	3200531	High Speed Digital Microscope and Camera	1
UK	3200536	Single Cell RNA Seq System with Enhanced Control (230V, 50Hz, UK)	1
	3200531	High Speed Digital Microscope and Camera	1
Europe	3200538	Single Cell RNA Seq System with Enhanced Control (230V, 50Hz, EU)	1
	3200531	High Speed Digital Microscope and Camera	1
Japan	3200540	Single Cell RNA Seq System with Enhanced Control (100V, 50-60Hz, JP)	1
	3200531	High Speed Digital Microscope and Camera	1
Rest of the World	3200536	Single Cell RNA Seq System (Enhanced Control, 230V, 50Hz, UK)	1
	3200531	High Speed Digital Microscope and Camera	1
	3200580	Installation & Basic Training (supplement for 2 days on site RoW)	1

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