# Dolomite Bio Nadia Instrument Application Note for scRNA-seq

Encapsulating single cells with barcoded beads on the Nadia Instrument

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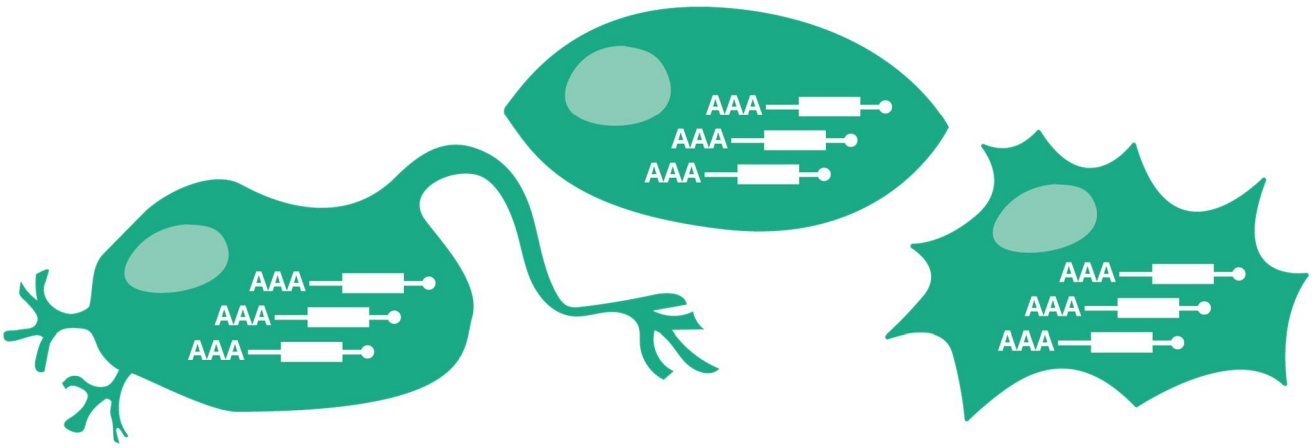
Author: JF2



# Contents

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## Summary

Dolomite Bio has developed the Nadia Instrument, a high-throughput platform for automated droplet generation from multiple biological samples in parallel. This application note describes the encapsulation of cells and beads using the Dolomite Bio Nadia Instrument and the generation of scRNA-seq libraries using the Drop-seq protocol (Macosko E., *et al*. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanolitre Droplets." *Cell* **161**:1202). In this protocol, tens of thousands of cells are individually encapsulated with uniquely barcoded mRNA capture beads (**Figure 1**), culminating in the robust and straightforward preparation of tens of thousands of single cell cDNA libraries.

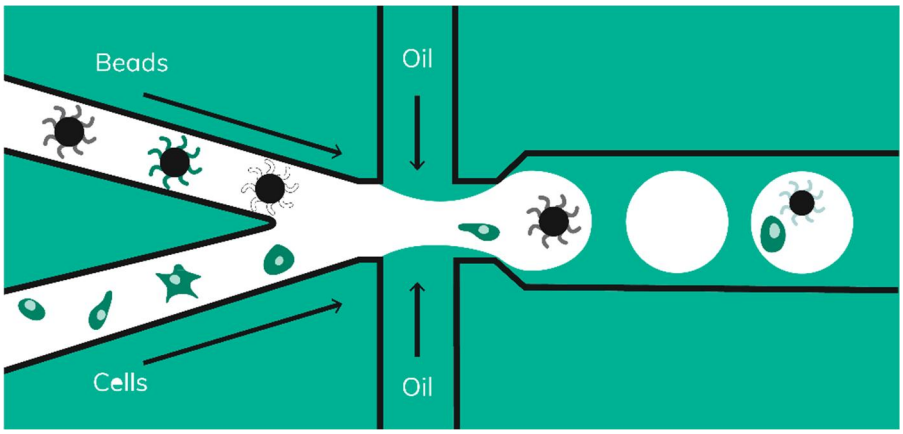


**Figure 1** Schematic of the encapsulation of barcoded mRNA capture beads with single cells. Tens of thousands of single cells are co-encapsulated with uniquely barcoded beads. The cells are lysed inside the droplets and their mRNA content is captured by the beads. These beads are subsequently recovered and processed for downstream analysis using NGS-technologies

# Introduction

Cell encapsulation within microfluidic droplets is an exciting, cutting edge technique that, for the first time, enables the analysis of thousands or even millions of single cells. This unprecedented analytical power enables the discovery of previously unidentified or cryptic cell types in a tissue of interest and also the characterisation of individual cells within biologically important processes.

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). Both techniques are highly tractable. The Drop-Seq protocol developed by Macosko *et al*., encapsulates a single cell alongside a single bead within a droplet. These beads have surfaces coated with DNA-oligos containing a (dT)30 stretch allowing for the capture of polyadenylated mRNA which is subsequently reverse transcribed into cDNA. Each bead also carries a unique DNA barcode that identifies not only individual cells but also individual mRNA transcripts derived from any given cell.

It is desirable that a single cell be encapsulated alongside a single bead within a droplet. This can be achieved through the dilution of cell and bead suspensions, but there is a fine balance to be struck between quality (fewer cells but data of higher quality) and quantity (higher throughput but data of lower quality). In this protocol, cell and bead suspensions are adjusted to achieve encapsulation in which 10% of droplets will contain a bead (i.e. 1 in 10 droplets) and 5% of droplets will contain a cell (i.e. 1 in 20 droplets). This translates to 10% of cells being encapsulated with a bead and very low cell doublet rates. A 15-minute run with 250 µl of bead suspension and 250 µl of cell suspension will result in the production of 6,000 individual cellular transcriptomes.

Cell encapsulation is fast becoming a mature technology having transitioned from early prototypes into robust instruments suitable for use in biological research that is at the cutting-edge. The bespoke, microfluidic systems first built by engineers as prototypes to carry out cell encapsulation were neither robust nor tractable for use in day-to-day research. Dolomite Bio is a world-leader in the design and manufacture of microfluidic systems and components for application in biology. Dolomite Bio specialises in 'Productising Science®', which aims to provide microfluidic solutions for research biologists and medical scientists. The Dolomite Bio Nadia Instrument is designed for the high throughput analysis of up to 8 single cell samples in parallel using a fully automated process.

This application note reports results obtained from the encapsulation of cells with barcoded beads for single-cell RNA sequencing using the Dolomite Bio Nadia Instrument.

# Materials and Methods

The objective of the tests conducted was to generate single cell libraries using the Drop-seq protocol on the Dolomite Bio Nadia Instrument. Measured parameters included the size of droplets and the rate of droplet formation. The captured mRNA was then further processed into cDNA and amplified. The quality of cDNA obtained was ascertained using a Bioanalyzer and further analysed using NGS.

**Droplet system.** The Dolomite Bio Nadia Instrument (Figure 2) is designed to allow high throughput analysis of single cells using droplet microfluidics. It produces highly monodispersed droplets through the use of three independent, ultra-smooth pressure pumps. The Nadia Instrument has been designed to guide the user through a chosen application using step-by-step instructions that are clear and effortless to follow.


***Figure 2* The Nadia Instrument** Before starting a run, beads are loaded into the bead-chamber of a Nadia Chip located on a Nadia Cartridge. The user then loads cells into the cell-chamber of the same Nadia Chip. Both chambers feature gentle agitation which maintains beads and cells in suspension. The aqueous suspensions of cells and beads mix at the microfluidic chip junction and are pinched off by an oil phase. This results in the formation of droplets that encapsulate both a cell and a bead. The droplets are collected in an output reservoir at approximately 1,300 droplets per second. Complete processing of a sample occurs in about 15 minutes and the user can run 1, 2, 4 or 8 Nadia chips in parallel on a single Nadia Cartridge.

**Preparation of beads.** 1 ml of lysis buffer was prepared (0.2 M Tris pH 7.5, 0.2 % Sarkosyl, 20 mM EDTA, 6 % Ficoll PM-400) with 50 mM DTT added prior to usage. ChemGenes beads were twice filtered through a 70 µm cell strainer and the bead concentration was determined using a haemocytometer. 150,000 beads were then spun down at 3,000 g for 1 min and the supernatant discarded. The beads were resuspended in 250 µl of lysis buffer (600 beads/µl) allowing for the encapsulation of 1 bead in every 10 droplets.

**Preparation of cells.** Human HEK and mouse 3T3 cells were trypsinised for 5 min with TrypLE as described in the Drop-seq protocol. The cells were collected and spun down for 5 min at 300 g. The pellet was resuspended in 1 ml of PBS-BSA (1x PBS, 0.01% BSA, made fresh before each experiment) and spun again for 3 min at 300 g. The cells were resuspended in 1 ml of PBS, passed through a 40 µm cell strainer and counted. A 1:1 mixture of both cell types was prepared at a concentration 300 cells/µl in 250 µl of PBS-BSA allowing for the encapsulation of 1 cell in every 20 droplets.

**Cell & Bead Encapsulation.** The run was initiated by placing a new Nadia chip cartridge on the Nadia Instrument. The instrument automatically recognised the cartridge and initiated an scRNA-seq run with on-screen instructions guiding the user through every step. The gasket was removed from the cartridge and 3 ml emulsion oil loaded into the white flashing well (Figure 3 A). Re-sealing the cartridge with its gasket and closing the Nadia Instrument initiated the primary cooling step. Preparation of beads and cell samples was finalized during cooling of the instrument. The 250 µl beads suspension was loaded first into the blue illuminated wells (Figure 3 B), followed by 250 µl cell suspension into the orange flashing wells (Figure 3 C). The gasket was put back in place and the instrument lid pushed down to trigger clamping. The instrument indicated after 16 min that the run was successfully completed. The emulsion (Figure 3 D) was left for another 10 min on the instrument at room temperature to ensure complete lysis of the cells inside the droplets. After the incubation time the gasket was removed from the cartridge and the emulsion transferred to a clean 50 ml Falcon tube. A 10 µl sample of the emulsion was taken to observe the quality of the emulsion under the microscope using a Neubauer-Improved haemocytometer.

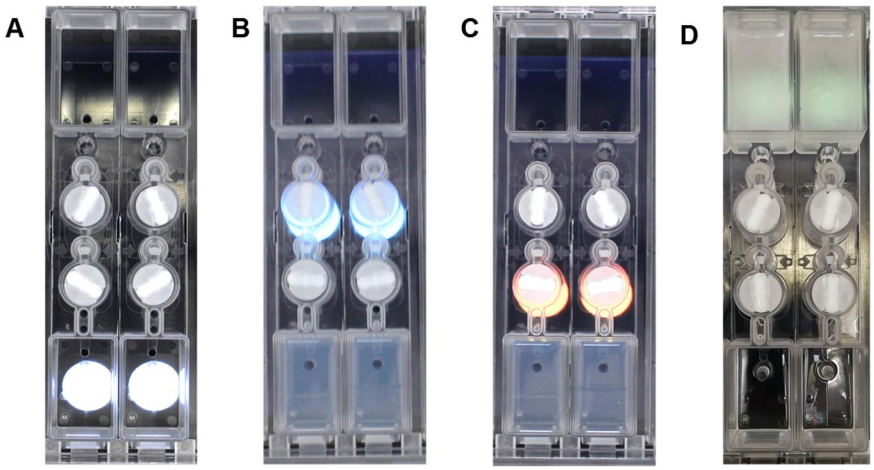


***Figure 3*** Guide lights underneath the Nadia cartridge chips indicate loading position of the emulsion oil (A), beads (B), cells (C) and indicate where to retrieve the emulsion (D) from after the run.

**Preparation and NGS-analysis of single cell DNA libraries.** The following steps were conducted as described in the Drop-seq protocol by Macosko and co-workers. An aliquot of 2,000 beads was used during PCR and this corresponded to approximately 100 STAMPs (Single-cell Transcriptome Attached to MicroParticles). The quality of DNA following PCR amplification and tagmentation was determined using a BioAnalyzer. The tagmented DNA libraries were sequenced on Illumina's NextSeq 500 instrument.

**Flowing beads and cells.** As noted by Macosko and co-workers, the flowing of beads in an encapsulator is somewhat challenging as beads are prone to settling on the bottom of the bead-chamber and are also susceptible to shearing. Dolomite Bio has therefore developed built-in stirrers that gently agitate the cell and bead suspensions. The stirrers avoid cell and bead damage by rotating gently and away from the walls or the bottom of the chambers. The quality of the droplet-emulsion obtained afterwards was ascertained through the loading of 10 µl of emulsion into a haemocytometer (C-Chip Neubauer improved, DHC-N01, Labtech) for observation under a microscope. Monodispersity was estimated by measuring the average droplet size and determining the number of beads per droplet (Figure 4).

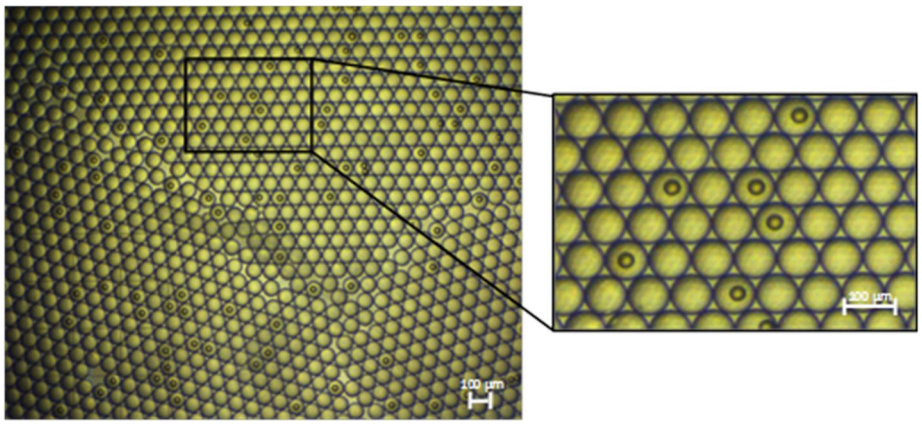


**Figure 4** Droplets produced through an scRNA-seq run on the Nadia Instrument

The average droplets size was 85 µm with a CV of 7 %. The number of beads per droplets was determined by a count of the numbers of beads encapsulated within a single droplet and this was performed across 450 droplets. The results are summarized in Table 1. Beads had been suspended at 620 beads/µl, corresponding to a theoretical encapsulation rate of 1 bead per 10 droplets (or 10 % of droplets). 50 droplets out of 450, or 11.0% of droplets contained a single bead. This was in agreement with theoretical expectations.

| Beads per droplets | % of droplets |
|---|---|
| 1 | 11 |
| 2 | 1 |
| 3 | 0.2 |
| 4 | 0.0 |
| 5 | 0.0 |

**Table 1**

**Cell viability.** To demonstrate that gentle stirring employed to maintain cells in suspension exerted no adverse effects, cellular viability was tested using 0.4% Trypan Blue Solution (#15250061, ThermoFisher Scientific). The tested sample contained MEF cells at a density of 6400 cells/ µl in a 1:1 solution of PBS-BSA + 0.4% Trypan Blue. Cells that had been stirred in the Nadia Instrument for 30 min at 4 ˚C were compared to cells that had been stored on ice for 30 min. 91 % of the cells were viable after stirring and 90 % of cells were viable when stored on ice. This demonstrated that the gentle stirring of cells does not adversely affect cellular viability.

**Preparation of the single cell DNA libraries for NGS-analysis.** After recovery of the emulsion from the instrument beads were treated as described in the Drop-seq protocol. After PCR-amplification and tagmentation a portion of the DNA-library was analysed on a BioAnalyzer to estimate size distribution and overall quality (Figure 5). The size distribution of the DNA-library correlated with an expected range of between 500 and 2500 bp **(**Figure 5 A).

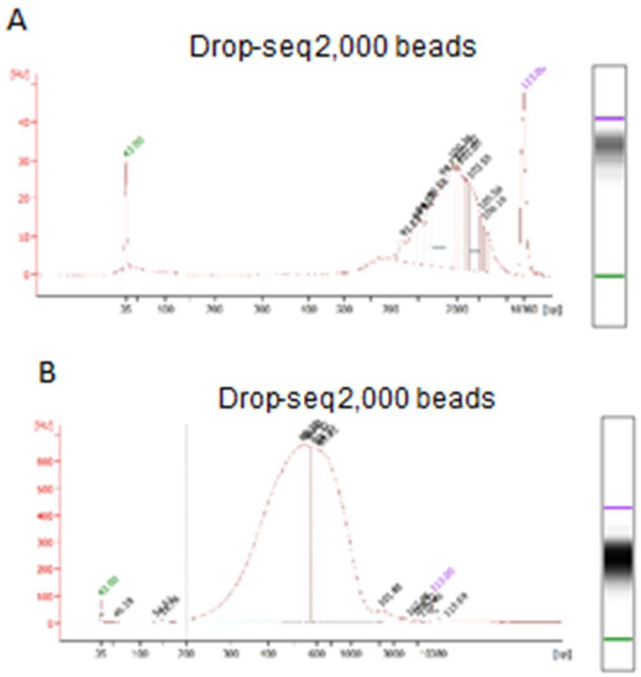


**Figure 5** BioAnalyzer results before and after tagmentation. (A) Electropherogram from analysis of a single cell DNA library prepared from 2,000 beads following PCR-amplification. (B) The quality of this single cell DNA library was examined again following

**Illumina sequencing and bioinformatic pipeline.** Sequencing was carried out on a NextSeq 500 instrument as described in the Drop-seq protocol. The "dropSeqPipe" (https://github.com/Hoohm/dropSeqPipe), a computational pipeline based on the original Drop-seq bioinformatic tool was used. The analysis was performed as described in the Drop-seq protocol but with additional improvements incorporated (software-bugs had been identified and rectified). The quality of the sequencing data was assessed based on knee- and Barnyard plots. The knee-plot allows for the estimation of the number of productive beads per sample (i.e. beads that have captured mRNA derived from a cell). Each bead can be associated with a unique cell barcode and a corresponding number of NGS-reads. The cell barcodes were arranged in descending order of reads and visualised alongside the cumulative fraction of reads in a knee-plot. An inflection-point can be observed at 114 and this corresponds well to the 100 cells predicted by Poisson statistics for 2000 beads. Estimates from use of a knee-plot suggested 114 STAMPS had been identified (Figure 6 A). The Barnyard-plot depicts STAMPs as individual data points each associated with a number of human or mouse transcripts (Figure 6 B). Most STAMPs had between 10,000 and 40,000 transcripts. An estimation of the doublet rate, which is an indicator of the quality of the scRNA-seq run can be made. For this dataset 72 STAMPs were associated with only mouse transcripts, 41 with only human transcripts and one single STAMP carried transcripts from both species. This equated to a doublet rate of 1.8 % (having after accounted for doublets from encapsulation of multiple cells from the same species). A gene expression-profile was obtained for each STAMP and the numbers of human and murine genes expressed per cell were highly comparable to published data from Macosko and co-workers (Figure 6 C).

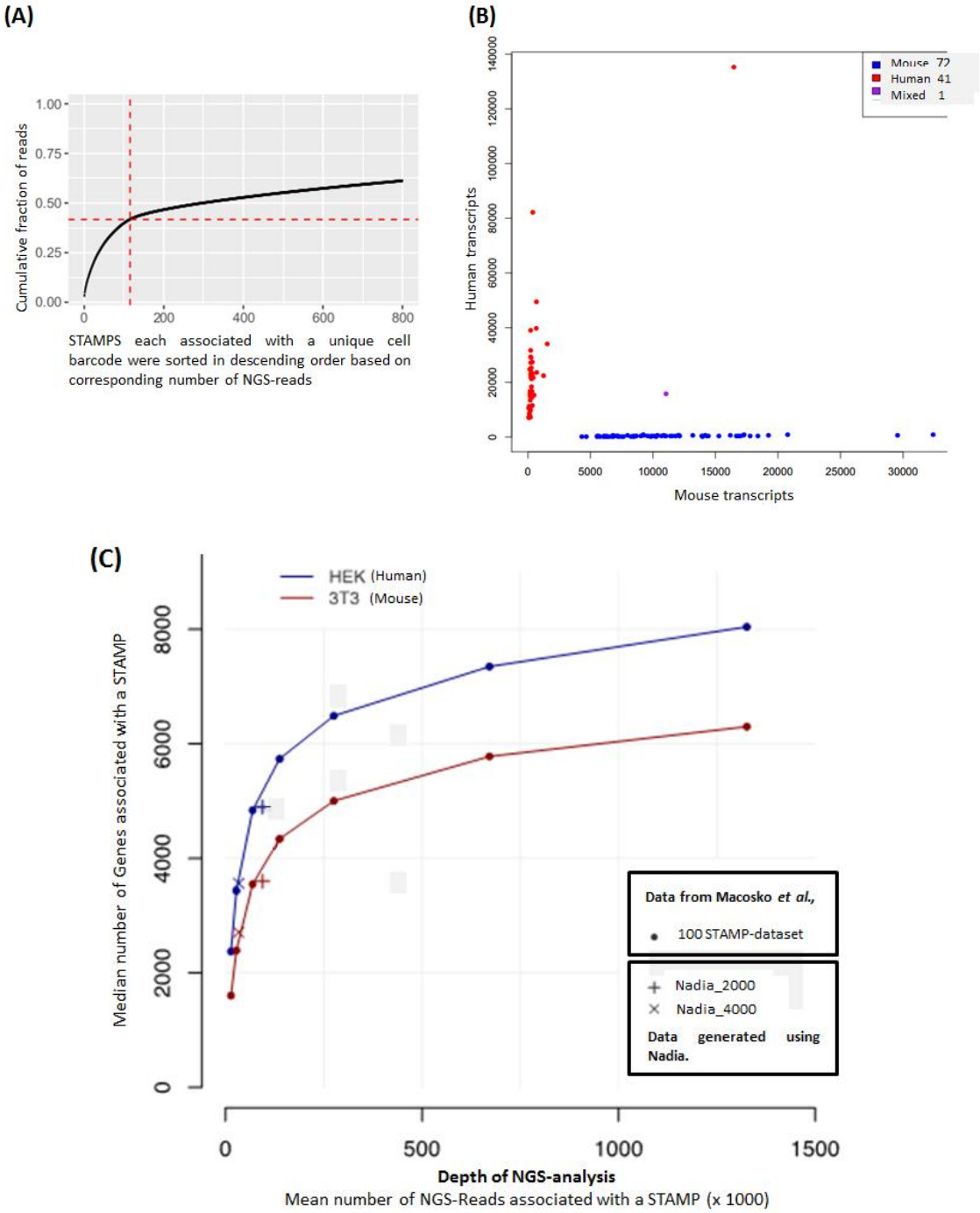


**Figure 6 Species mixing run on the Nadia Instrument**

(A) STAMPs were identified from a pool of 2000 amplified beads obtained after encapsulation of both human and mouse cells using the Nadia. Only a fraction of the beads had been encapsulated within droplets that contained only a single bead and cell, whilst the majority may have had contact with RNA from the extracellular milieu. In the above Figure, the cell barcodes were arranged in descending order of reads and visualised alongside the cumulative fraction of reads in a knee-plot. An inflection-point can be observed at 114 and this corresponds well to the 100 cells predicted by Poisson statistics for 2000 beads. (B) The significance of the inflection point estimated from use of a knee-plot was further validated with use of a Barnyard plot in the Figure above. This enables a visualisation of the species specificity of individual STAMPs with a dramatic drop in this specificity near the inflection point, at which there is a shift away from beads that had been encapsulated with cells towards the beads that had only been exposed to RNA in the extracellular milieu. (C) When compared with published data from Macosko and co-workers, the numbers of genes expressed by cells studied using Nadia (from use of both 2000 or 4000 amplified beads) were highly consistent at a given depth of NGS-analysis.

# Conclusion

Single-cell RNA-Seq protocols like Drop-seq offers an extremely powerful and exciting method for single-cell transcriptomics. This development allows for the first time, easy and straightforward access to large numbers of gene expression profiles derived from single cells. It is thus unsurprising that many scientists are eager to adopt this for their research.

It has been shown here that the Nadia Instrument can successfully encapsulate cells alongside barcoded beads within droplets containing lysis buffer. The device produced monodispersed droplets with approximately 5 % of droplets containing both a single cell and bead. It was also shown that gentle stirring of beads and cells on the Nadia Chips maintained beads and cells in suspension, facilitated encapsulation and did not damage cells. A high quality single cell DNA library was obtained from use of the Nadia Instrument and this gave rise to a dataset which after NGS-analysis was deemed to be of a high quality.

In conclusion, the RNA-Seq application using the Drop-seq protocol has been demonstrated with good results using Nadia Instrument.

# Product Information

| Instrument/Consumables | Order Number |
|---|---|
| Nadia Instrument | 3200590 |
| Nadia Training Cartridge | 3200605 |
| Nadia Cartridge for scRNA-Seq - 8 Samples (8x1) | 3200648 |
| Nadia Cartridge for scRNA-Seq - 8 Samples (2x2 & 1x4) | 3200649 |
| Nadia Cartridge for scRNA-Seq - 8 Samples (1x8) | 3200650 |
| Nadia Cartridge for scRNA-Seq - 40 Samples (40x1) | 3200651 |
| Nadia Cartridge for scRNA-Seq - 40 Samples (10x2 & 5x4) | 3200652 |
| Nadia Cartridge for scRNA-Seq - 40 Samples (5x8) | 3200653 |