Single-cell barcoding and sequencing using droplet microfluidics

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Single-cell RNA sequencing has recently emerged as a powerful tool for mapping cellular heterogeneity in diseased and healthy tissues, yet high-throughput methods are needed for capturing the unbiased diversity of cells. Droplet microfluidics is among the most promising candidates for capturing and processing thousands of individual cells for whole-transcriptome or genomic analysis in a massively parallel manner with minimal reagent use. We recently established a method called inDrops, which has the capability to index >15,000 cells in an hour. A suspension of cells is first encapsulated into nanoliter droplets with hydrogel beads (HBs) bearing barcoding DNA primers. Cells are then lysed and mRNA is barcoded (indexed) by a reverse transcription (RT) reaction. Here we provide details for (i) establishing an inDrops platform (1 d); (ii) performing hydrogel bead synthesis (4 d); (iii) encapsulating and barcoding cells (1 d); and (iv) RNA-seq library preparation (2 d). inDrops is a robust and scalable platform, and it is unique in its ability to capture and profile >75% of cells in even very small samples, on a scale of thousands or tens of thousands of cells.

INTRODUCTION

Cell-to-cell heterogeneity has been a major confounding factor in the phenotypic profiling of healthy and diseased tissues[1,2]. Single-cell technologies seek to turn this heterogeneity from a problem into a strong advantage for studying cell and tissue physiology. Unlike bulk assays, measurements of heterogeneity through single-cell assays provide far higher phenotypic resolution and do not require prior knowledge of the subtypes of cells within a sample, or of how to fractionate them[3]. These measurements allow *de novo* identification of cell types within tissues and detection of biomarkers in rare cell types[4]; they can be used to identify heterogeneous responses or to compare cell states between complex samples without requiring prior knowledge of their population structure[5]. Variations between cells in bulk populations can also be used to reconstruct differentiation trajectories or cell cycle dynamics[6–9]. We are probably only beginning to appreciate the range of diverse applications for single-cell transcriptional profiling in basic and clinical research[10–13]. It is therefore no surprise that single-cell profiling has gained considerable attention in the past few years[14,15], triggering substantial efforts to develop various analytical techniques to isolate, amplify, and sequence the genetic material of single cells[10]. Despite great progress in this field, however, one major limitation remained a challenge until the introduction of droplet microfluidic technology—the cell throughput. Using state-of-the-art technologies such as FACS into microtiter plates[9,16,17], a few hundred or, with great effort, a few thousand cells could be isolated and sequenced. These numbers are generally not sufficient for extensive analysis of heterogeneous cell populations. For example, a tumor is composed of many cell types, and thorough analysis requires sequencing of core biopsies that typically yield ~10^4 single cells[18]. Furthermore, performing single-cell isolation and subsequent nucleic acid amplification in microtiter plates requires relatively large amounts of reagents (typically ~20 µl per cell), which becomes prohibitively costly for high-throughput studies.

Microfluidics has been established as an enabling technology in single-cell studies[19–24]. Recent reports have shown that reduced reaction volumes improve the yield of cDNA[25] and reduce technical variability[26], making microfluidics very appealing for a variety of single-cell applications. The commercially available Fluidigm C1 platform, developed for automated capture and processing of single cells, facilitated the use of the technology and has been successfully adapted to various biological applications[1,5,27–31]. However, the number of cells that can be analyzed with one chip is typically in the range of tens of cells or, more recently, several hundred cells, setting a limit for analysis of large cell populations. Identifying rare cells in a heterogeneous population that do not have proper surface markers requires much higher throughput capabilities, which are not offered by commercial systems[3,6,31,32]. In addition to providing a low throughput, the efficiency of capturing cells within microfluidic chambers is typically low (~1–10% of the input) and is cell-size-biased, which is a serious drawback for primary and heterogeneous clinical samples. The use of microfluidic devices with patterned nano-wells has been shown to increase cell capture efficiencies[33,34], yet poor gene recovery and cross-contamination issues between individual compartments remains a challenge.

Droplet microfluidics technology circumvents the aforementioned limitations and opens new possibilities for the field of single-cell biology[35–37]. Microfluidic droplets function as independent microreactors that are functionally equivalent to wells (or tubes), yet the volume of a droplet is roughly a thousand to a million times smaller. Obviously, such a massive reduction in reaction volume provides huge savings in reagents cost when performing large numbers of reactions in parallel. In addition, unlike conventional microtiter plates or valve-based microfluidics, droplets are intrinsically scalable: the number of reaction 'wells' is not limited by the physical dimensions of the chip but scales linearly with the emulsion volume. Another notable advantage is that cell capture efficiencies with droplet microfluidics

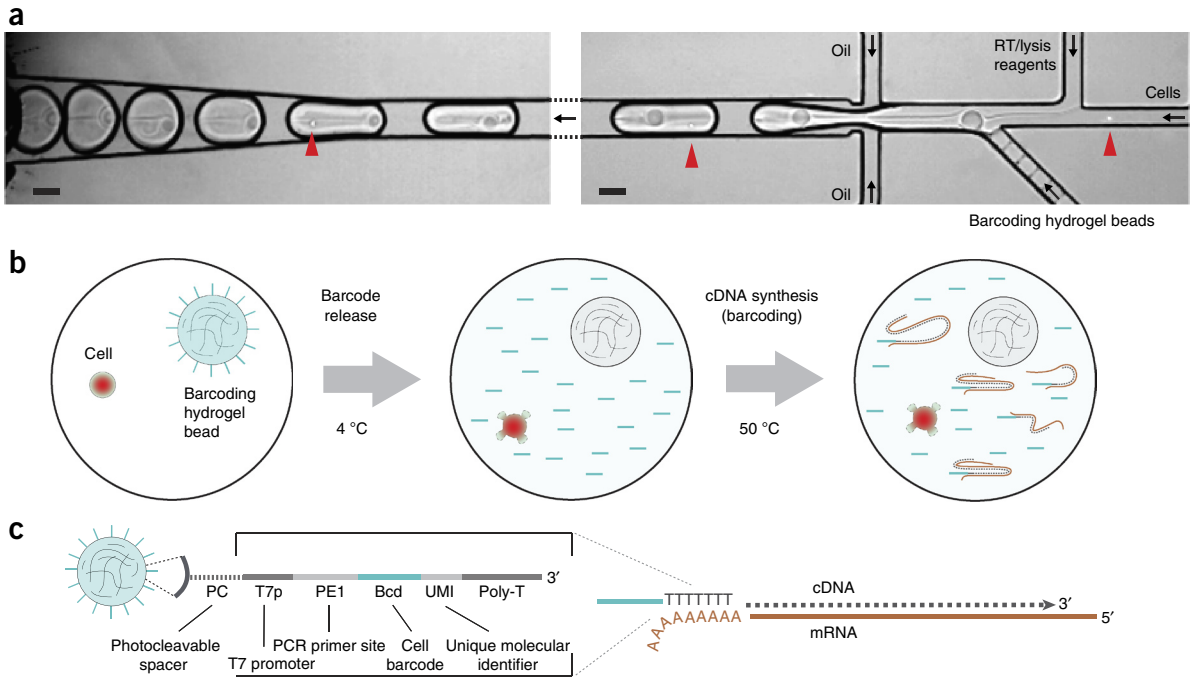

Figure 1 | Single-cell transcriptome barcoding in drops. (**a**) Coencapsulation of cells, barcodes (delivered by hydrogel bead), and RT–lysis reagents into microfluidic droplets. Red arrowheads show individual cells, and black arrows indicate flow direction. After the desired number of cells is collected, barcoding primers are released from hydrogel beads by photocleavage to initiate reverse transcription of mRNA. Scale bars, 100 µm. (**b**) Schematic of single-cell transcriptome barcoding in drops. After cell and hydrogel bead encapsulation, the barcoding cDNA primers are released from the beads using >350-nm UV light (which is not damaging to DNA/RNA), followed by mRNA capture and reverse transcription. (**c**) Schematic of barcoding cDNA primers attached to hydrogel beads. mRNA is brown and cDNA is the gray dashed line.

are extremely high; nearly all cells in a sample can be loaded in droplets, which is an important consideration when working with primary samples. Furthermore, different microfluidic modules can be used to manipulate droplets in a sophisticated, yet highly controllable, manner[38]. Large numbers of droplets (>10^7) can be generated at astonishingly high rates (>20,000 droplets per s)[39–41], their size can be tuned precisely[42,43], new reagents can be introduced into preformed droplets at defined time points[44–49], and droplets can be split[50,51] and sorted[52–59], therefore opening new opportunities for single-cell genomics. A number of single-cell applications have already benefited from droplet microfluidics to assay different biomolecules[56,57,60–71], amplify selected genes[72–75], and sort individual cells of interest at high-throughput rates[56,57,76]. For single-cell genomics studies, droplet microfluidics can bring a much needed throughput necessary to profile large (>10,000) numbers of cells.

Our method, known as inDrops (for indexing droplets), makes use of droplet microfluidic technology to index individual cells at a rate of >12,000 cells/h in nanoliter-scale droplets[6]. The basic principle of the technology is easy to understand: a mixture of cells is encapsulated into microfluidic droplets together with barcoding oligonucleotide primers (attached to HBs), and a mix of RT and lysis reagents (**Fig. 1**). The mRNA released from lysed cells remains trapped inside the same droplet and is tagged (barcoded) with oligonucleotide primers during the RT reaction. After barcoding, the material from all cells is pooled by breaking the droplets, and the cDNA library is processed for next-generation sequencing. In this process it is essential that each droplet carry primers encoding only one unique barcode, which should be different from the barcodes in other droplets. To achieve this, we developed a combinatorial approach to produce a library of barcoding hydrogel beads (BHBs) that are coencapsulated with the cells. Each bead carries covalently coupled, photoreleasable primers encoding one predefined barcode. The barcode in this context encodes two parts: a 'cellular barcode', which indicates the cell from which mRNA is captured, and a 'unique molecular identifier' (UMI), which provides a quantitative measure of absolute transcript levels in a given cell. It is noteworthy that the delivery of deformable HBs can be efficiently synchronized, allowing nearly 100% loading of a single bead per droplet[77]. This is an important distinction of inDrops, ensuring that individual cells randomly arriving into droplets will nearly always be exposed to one, unique DNA barcode. For capturing rare primary cells, this feature is of critical importance as compared with those of alternative methods based on random delivery of barcoding beads[32].

In this work, we describe the detailed steps for building and using an inDrops platform for whole-genome single-cell RNA sequencing. The protocol can be divided into five parts: the first two parts involve hydrogel bead and DNA barcode synthesis, which is carried out infrequently and in advance; the other three parts involve cell barcoding, library preparation, and data analysis. We expect that additional methods will eventually appear based on the inDrops technique, and their development will benefit from a detailed description of this protocol. The platform could eventually be extended by increasing flow rates and barcode number, library protocol optimization, or application to other single-cell profiling assays such as DNA sequencing, assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq), or chromatin immunoprecipitation sequencing (ChIP-seq).

Advantages and limitations in comparison with other methods

There are several advantages and limitations to consider when choosing inDrops for single-cell RNA sequencing[10]. As already discussed, droplet-based methods are currently unparalleled in the number of cells that can be processed, and thus any application requiring whole-genome sequencing of thousands or tens of thousands of cells would indicate the use of inDrops[6] or a similar droplet-based method[32]. For limiting samples, in which the total input consists of <200,000 cells (e.g., fine-needle aspirations, tumor biopsies, or rare stem cell populations already purified by FACS), inDrops may be the only suitable method of choice, as it can barcode a high fraction (~60–90%, typically 75%) of cells passing through the device. Another droplet-based approach, known as Drop-seq, offers most of the advantages of inDrops, but it barcodes only 2–4% of the cells in a sample[32].

The inDrops platform also has the ability to capture cells of practically any size and shows no size bias, an important advantage as compared with microfluidic approaches that make use of hydrodynamic traps to capture cells (e.g., the Fluidigm C1 chip). The method has short capture times, allowing thousands of cells to be captured in under an hour and 1,000 cells barcoded in a few minutes. The cost of library preparation is low, with costs estimated at ~$0.06 per cell (**Supplementary Table 1**), and will drop for larger-scale applications.

There are also important limitations of the inDrops method. One major trade-off is between high cell numbers and per-cell sequencing depth. Because the cost of sequencing is invariant, sequencing transcriptomes of many thousands of cells to saturation rapidly becomes limiting. Measurements of inDrops sensitivity suggest that it is three-fold less sensitive when ten-fold fewer reads are collected per cell as compared with lower-throughput methods (~100–200 K reads per cell for inDrops as compared with 1–2 million reads per cell using the Fluidigm C1 system)[6,26]. Thus, in experiments in which the important differences between cells are subtle and cell subpopulations of interest are not rare, lower-throughput methods might be preferred. As the cost of sequencing drops, this trade-off will diminish in importance. Further optimization of cell lysis conditions, mRNA capture, and cDNA amplification should improve the method sensitivity and the diversity of transcripts recovered from individual cells.

There are several other situations in which inDrops would not be the method of choice. inDrops assigns a random barcode to each cell, and thus it is not possible to associate transcriptomics data with individually tracked cells. Thus, inDrops is not suitable for experiments aiming to link specific cell transcriptomes with previous data collected on each cell (e.g., morphology, lineage, position, and fluorescence). Another constraint is that inDrops requires at least 2,000 cells of input material (and in practice an amount >10,000 cells is preferred), and thus it is not suitable when the initial sample of cells consists of just tens or hundreds of cells (e.g., if cells are selected by hand or laser dissection).

In addition, as an alternative to assembling their own inDrops platform, users may wish to purchase a commercially engineered system and reagents. One company, 1CellBio, holds licenses to inDrops and provides all required equipment and consumables; other companies (e.g., 10× Genomics) provide researchers with a variant of the inDrops technique. The commercial solutions suffer from the same advantages and disadvantages described above, although some researchers may prefer the support and simplicity of a commercial system. In contrast to a commercial solution, the current protocol is much lower in cost, offers higher flexibility, and allows assay innovation. There is still a huge window for innovation of droplet microfluidic technologies, and this protocol will thus particularly appeal to laboratories wishing to tinker with aspects of the method in an affordable and controllable manner. Single-cell methods are likely to improve in quality substantially in the coming years, and major breakthroughs will likely come from the deep knowledge and creativity of academic laboratories working with open systems such as those described here.

Experimental design

The inDrops protocol involves five parts: (i) microfluidic chip manufacturing, (ii) BHB synthesis, (iii) single-cell transcriptome barcoding in drops, (iv) library preparation for next-generation sequencing, and (v) sequencing data analysis. Chip manufacturing and hydrogel synthesis are carried out well in advance of the biological application at hand; a single batch of HBs can then serve to barcode millions of cells, lasting for several weeks or months of inDrops analysis, depending on the frequency of use.

Microfluidic device fabrication. A detailed protocol for microfluidic device fabrication has been described previously[57]. Briefly, this multistep process begins with a computer-assisted design (CAD) drawing of the microfluidic device design, which is then printed on a transparent film (mask) and used to fabricate a master with channels as embossed features. The master serves as a reusable mold to transfer the microfluidic pattern onto poly(dimethyl siloxane) (PDMS) slabs, which are then bonded to a glass slide. Treating microfluidic channels with a hydrophobic coating completes microfluidic device manufacturing. The protocol reported here requires two separate microfluidic devices, one for hydrogel bead generation, and the other for cell encapsulation and barcoding (CAD designs provided as **Supplementary Data 1**). Users may wish to skip these steps and instead purchase functional devices from commercial manufacturers (1CellBio (http://1cell-bio.com) in the United States or Droplet Genomics (http://dropletgenomics.com) in Europe).

BHB synthesis. The principle of hydrogel bead synthesis relies on microfluidic emulsification of an acrylamide:bis-acrylamide solution supplemented with an acrydite-modified DNA primer that is covalently incorporated into the hydrogel mesh. DNA primers on polymerized HBs are then barcoded using a combination of a split-and-pool method and a primer extension reaction, as depicted in **Figure 2**. The microfluidic device used to produce HBs has a flow-focusing junction at which the continuous stream of aqueous phase is emulsified into a stream of highly monodisperse droplets, which are collected off-chip and polymerized into HBs. This microfluidic approach allows for the generation of HBs of a precise size, which is important for subsequent steps to ensure a high capture efficiency of cells in downstream single-cell barcoding experiments. The most important advantage provided by HBs is their use for synchronized and evenly spaced delivery into droplets[77]. Because hydrogel-based beads are deformable, they can be close-packed, making their injection into a microfluidic device highly controllable. By matching the speed of bead injection with the speed of droplet generation, it is possible to set

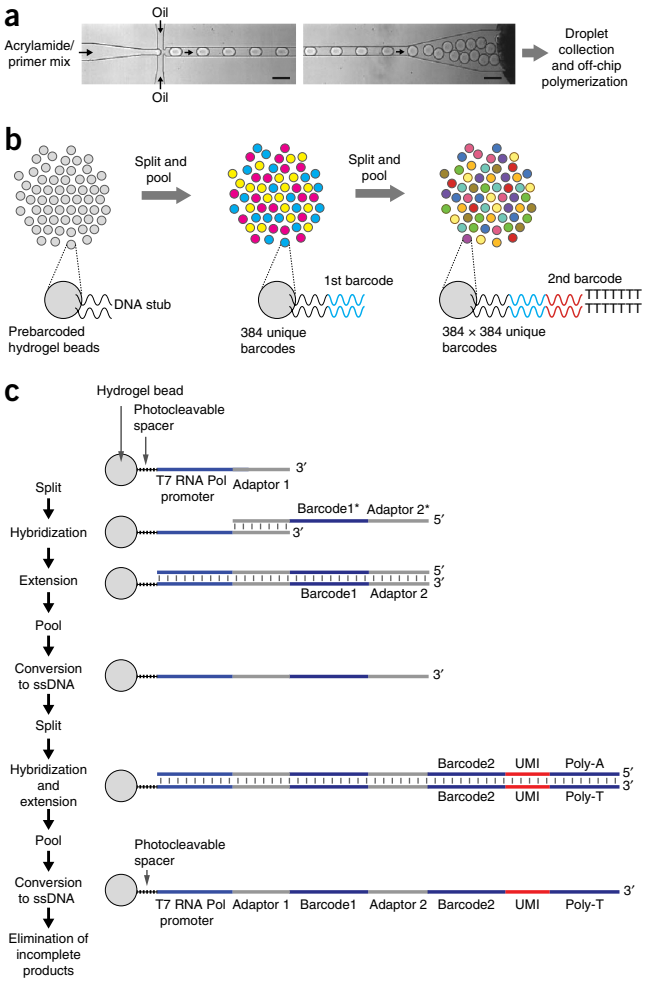

Figure 2 | Synthesis of barcoding hydrogel beads. (**a**) Hydrogel bead generation and collection. An aqueous acrylamide/bis-acrylamide solution carrying acrydite-modified DNA oligonucleotide is emulsified using a microfluidic device to yield highly monodispersed droplets, which are collected off-chip and polymerized into hydrogel beads. Scale bars, 100 µm. Image adapted with permission from ref. 6, Cell Press. (**b**) Illustration of the 'split-and-pool' approach, with a geometric increase in barcode diversity at each step. The diversity of unique barcodes can be increased by performing more than two rounds of split-and-pool synthesis, and/or using more than 384 barcodes at each round. (**c**) Primer extension reaction to incorporate barcode sequences into DNA oligonucleotides attached to hydrogel beads. Pol, polymerase; UMI, unique molecular identifier[79].

conditions in which nearly every droplet would be loaded with one barcode (hydrogel bead). For applications dealing with single cells, such straightforward control over bead injection is highly desirable because it reduces the number of droplets that have a cell but no barcoding bead, and it simplifies the overall operation of a device. Moreover, as compared with solid beads, HBs provide a higher number of attachment sites for DNA primers, thus increasing the concentration of single-stranded DNA (ssDNA) that is delivered into droplets. This protocol describes the synthesis of HBs containing ~10^9 photoreleasable DNA primers, yet this number can be adjusted by changing the initial concentration of acrydite-modified oligonucleotides. The barcoding bead library is generated by the use of oligonucleotide building blocks (barcodes) that are incorporated into the hydrogel mesh by primer extension in a combinatorial split-and-pool manner (**Fig. 2b**,**c**). For primer extension, we recommend a thermostable DNA polymerase (e.g., Bst and Bsm) that possesses 5′→3′ DNA polymerase activity and lacks 5′→3′ and 3′→5′ exonuclease activities. In addition, the enzyme is active at 60 °C, which helps minimize unspecific annealing of DNA primers. At the final step, the double-stranded DNA (dsDNA) produced during primer extension is converted into a single-stranded form by alkaline denaturation. However, it is easy to envision certain applications such as ChIP-seq, ATAC-seq, or Hi-C, in which HBs carrying double-stranded DNA oligonucleotides could be applied to barcode fragments of genomic DNA. In such cases, the poly-T 3′ end of the primers would be replaced by an adaptor sequence for tagmentation[78], and alkaline denaturation would be omitted. The modular nature of barcoding primers allows other adaptations as well: for example, poly-T could be replaced with a gene-specific sequence for targeted RNA-seq.

Our protocol describes two-step synthesis of a library of 147,456 unique barcodes (repeated across 40 million HBs per synthesis batch), but this library size can be increased in a straightforward manner by incorporating an additional primer extension step. Each DNA primer incorporates a cellular barcode, as well as a UMI sequence that allows correction of PCR amplification bias during data analysis[79]. Once synthesized, the BHBs can be stored for an extended period of time. Users may wish to skip these steps and instead purchase BHBs with similar characteristics from 1CellBio.

Single-cell transcriptome barcoding in drops. This is the key step of the protocol, which can be performed routinely with ~1 h of setup time per day. In this step, cells are captured in droplets with RT–lysis reagents and with BHBs (**Fig. 1** and **Supplementary Video 1**). After collection, droplets are exposed to >350-nm light to release RT primers from the barcoding beads; this step is critical for efficient mRNA capture in droplets. Only droplets containing both a hydrogel bead and a cell result in an RT product (**Fig. 1b**); droplets that do not contain cells do not contribute to the final barcoded library. However, ambient mRNA can contribute to background noise, and therefore great care must be taken to prepare clean cell suspensions before encapsulation. To prevent cell sedimentation in the syringe or in tubing, we typically adjust the density of the medium to that of cells with Optiprep solution.

The microfluidic device used to barcode individual cells (**Fig. 1a**) contains four inlets for (i) BHBs, (ii) cell suspension, (iii) RT and lysis reagent mix, and (iv) carrier oil, and one outlet port for encapsulated cell collection. To reduce potential flow fluctuations that arise because of the mechanics of syringe pumps, fluid resistors in the form of serpentine channels are incorporated into the device, whereas passive filters at each inlet prevent channels from clogging. The device consists of two junctions: the first for bringing the RT–lysis reagents, cells, and barcoded beads together, and the second for cell and bead coencapsulation, where droplet generation occurs (**Fig. 1a**). Due to laminar flow, the mixing of cells and reagents occurs only after encapsulation, preventing premature cell lysis. Depending on the flow rates used, droplet volume can be tuned precisely in 1–5 nl range at a rate of ~100 drops per s. During development, we found that the RT reaction can be strongly inhibited when droplets are

smaller than 1 nl, in agreement with previous reports[80]. To minimize cases in which two or more cells enter the same droplet, cells are diluted and injected into the device at a concentration corresponding to one cell in every ~10 droplets. Keeping cell occupancy low ensures that droplets containing two or more cells are extremely rare. The flow rates recommended here produce ~170,000 droplets per hour, leading to >15,000 barcoded cells per hour. This protocol describes ways to achieve >90% hydrogel bead loading efficiency, although any fraction above 60% can provide adequate data, and typical runs in our experience have a 75–90% loading efficiency. For certain cell types, or for future applications, parameters such as the flow rates and the droplet size can be easily controlled.

Library preparation for next-generation sequencing. After generating barcoded cDNA from single cells, the droplets are broken and cDNA is processed for next-gen sequencing. The general strategy used follows that developed in Jaitin *et al.*[16] and Hashimshony *et al.*[81], but several steps have been added or revised as compared with previous protocols. Detailed steps shown in **Figure 3** include initial purification of the cDNA library and second-strand synthesis, followed by *in vitro* transcription (IVT) using T7 RNA polymerase. The amplified antisense RNA is fragmented using zinc-ion-mediated cleavage, and converted into a DNA library ready for Illumina sequencing by a second RT reaction and a few cycles of PCR. This library preparation step takes ~2 d and includes two quality control (QC) steps. As detailed in **Figure 3**, a library index is added during the final PCR to allow pooling of multiple libraries for sequencing in a single run. Our protocol is designed to work with all Illumina sequencing platforms and can possibly be adapted to work with different sequencers.

Although the library protocol itself is flexible and can be adapted for other applications, we draw attention to two general considerations: first, it is critical to take precautions during library preparation to minimize material loss during all steps between droplet breakage and library amplification. Each molecule in the preamplified library represents a unique cell transcript, and thus losses lead to lower sensitivities for the method, seen in lower UMI counts per cell. Second, we recommend splitting the droplet emulsion into aliquots before breaking droplets, with each aliquot processed into an independent library. Splitting ensures that ample archived aliquots remain should any errors affect library preparation. To minimize cases in which two cells acquire the same barcode, the number of cells in each aliquot should ideally not exceed 2% of the total number of available barcodes—i.e. ~3,000 cells per aliquot for the synthesis scales described here. Increasing the combinatorial scale of split-and-pool hydrogel bead synthesis can alleviate this limit if necessary.

Sequencing data analyses. Raw sequencing reads are first processed through a low-level pipeline that generates a table of gene expression counts, followed by a high-level analysis to identify cell population structure and gene expression correlations. The high-level analysis is platform independent and is not discussed in this article. Scripts and documentation for the low-level analysis are provided as the **Supplementary Software**.

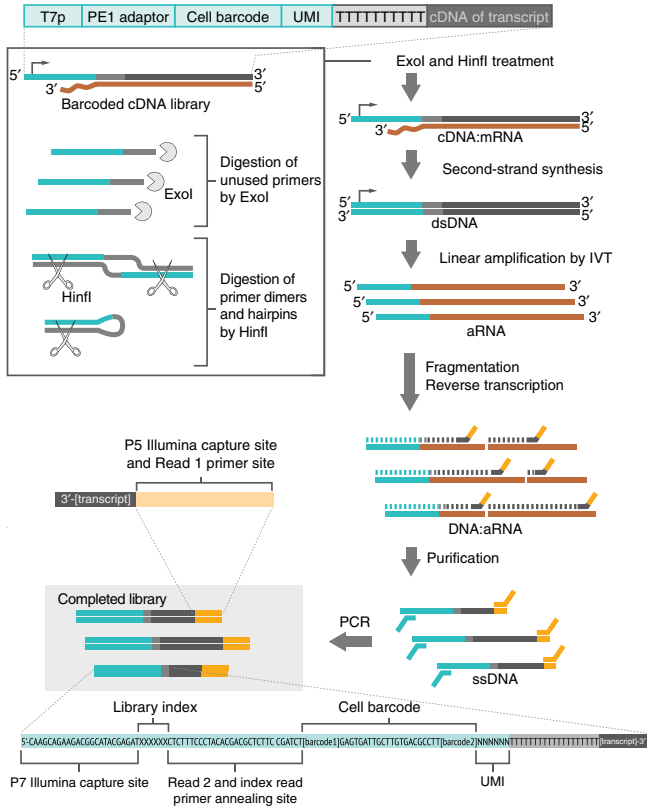

Figure 3 | Library preparation. Library preparation starts with enzymatic cleanup of the reverse transcription reaction product. The 3′–5′ exonuclease ExoI is used (to digest unused ssDNA primers) together with the dsDNA-specific restriction endonuclease HinfI (which cleaves the T7 promoter (T7p) sequence in primer dimers and hairpins). Note that the target library is immune to both HinfI and ExoI, as it is in the form of a cDNA–mRNA hybrid with the T7p sequence as ssDNA. Second-strand synthesis generates dsDNA that is used in an *in vitro* transcription (IVT) reaction to linearly amplify the cDNA library. The resulting amplified RNA (aRNA) is fragmented by zinc-ion-catalyzed hydrolysis, and a reverse transcription reaction followed by limited-cycle PCR is used to convert the aRNA into a dsDNA library compatible with Illumina sequencing platforms.

Level of expertise needed to implement the protocol

The core steps for barcoding single cells with the inDrops platform require a background in molecular biology covering standard procedures such as nucleic acid purification and enzymatic reactions, and basic skills in microscopy. Users do not require deep microfluidics expertise, as the protocol provides complete instructions on operating the microfluidic device. However, users with no microfluidics background might expect to spend 2 weeks becoming fully proficient with device operation. Other aspects of this protocol may require more specialized expertise: fabrication of PDMS-based microfluidic devices requires cleanroom training; automation of barcode synthesis requires use of a liquid-handling robot; and single-cell data analysis requires bioinformatics expertise. However, if the user chooses to purchase ready-made microfluidic devices (to replace Step 1 of PROCEDURE), experience in microfabrication becomes unnecessary. Ready-to-use BHBs are also available from commercial vendors (e.g., 1CellBio), further facilitating the implementation of the inDrops platform.

MATERIALS

REAGENTS

- Mineral oil (Sigma-Aldrich, cat. no. M5310-1L)
- Nuclease-free water (Life Technologies, cat. no. 10977-023)
- EDTA (0.5 M; Thermo Fisher Scientific, cat. no. 15575020)
- Tris–HCl, pH 8.0 (1 M; Thermo Fisher Scientific, cat. no. 15568025)
- Tris–HCl, pH 7.0 (1 M; Thermo Fisher Scientific, cat. no. AM9851)
- Tween-20 (Fisher Scientific, cat. no. BP337-100)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787-100ML)
- NaCl (Sigma-Aldrich, cat. no. S3014-1KG)
- KCl (Sigma-Aldrich, cat. no. P9541-1KG)
- Carrier oil (e.g., Droplet Genomics, droplet stabilization oil, cat. no. DS003; RAN Biotechnologies, cat. no. 008-FluoroSurfactant-2wtH-50G; or alternative)
- 1H,1H,2H,2H-Perfluorooctanol (Alfa Aesar, cat. no. B20156) **! CAUTION** Wear appropriate personal protective equipment and avoid contact with skin when handling this reagent. Use it in a well-ventilated area.
- Exonuclease I (ExoI, 20,000 U/ml; Thermo Fisher Scientific, cat. no. EN0581; or NEB, cat. no. M0293L)
- Exonuclease I reaction buffer (10×; NEB, cat. no. B0293S)
- FastDigest HinfI restriction endonuclease (HinfI; Thermo Fisher Scientific, cat. no. FD0804)
- FastDigest Buffer (10×; Thermo Fisher Scientific, cat. no. B64)
- Deoxynucleotide solution mix (dNTP; 10 mM each; NEB, cat. no. N0447L)
- 3M Novec 7500 Engineered Fluid (HFE-7500 oil, 3 M; Novec, cat. no. Novec 7500) **! CAUTION** Avoid direct contact; it may cause respiratory, skin, and eye irritation.
- DNA oligonucleotides (e.g., IDT and TriLink). Refer to **Supplementary Tables 2**, **3** and **4** for a complete list of oligonucleotides. See also Reagent Setup.

Barcode synthesis

- 5′-BAAAAAAAAAAAAAAAAAAA-3′ DNA oligonucleotide (standard desalting; it can be ordered from IDT or other vendors)
- 5′-/6-FAM/AGATCGGAAGAGCGTCGTGTAGG GAAAGAG-3′ DNA oligonucleotide (standard desalting; it can be ordered from IDT or other vendors)
- 5′-/6-FAM/AAGGCGTCACAAGCAATCACTC-3′ DNA oligonucleotide (standard desalting; it can be ordered from IDT or other vendors)
- 5′-/6-FAM/BAAAAAAAAAAAAAAAAAAA-3′ DNA oligonucleotide (standard desalting; it can be ordered from IDT or other vendors)
- 5′-/acrydite/photocleavable spacer/ CGATGACGTA ATACGACTCACTATAG-GGATACCACCATGGCTCTTTCCCTAC A CGACGCTCTTC-3′ DNA oligonucleotide (HPLC-purified; it can be ordered from TriLink, IDT, or other vendors).
- Acrylamide solution (AA; 40% (wt/wt); Sigma-Aldrich, cat. no. A4058-100ML) **! CAUTION** AA is a potent neurotoxin. Use the reagent in a well-ventilated area and wear appropriate personal protective equipment.
- AA/bis-acrylamide solution, 40% (wt/wt), molar ratio 19:1 (AA/BIS; Sigma-Aldrich, cat. no. A9926-100ML) **! CAUTION** AA and bis-acrylamide are potent neurotoxins. Use the reagent in a well-ventilated area and wear appropriate personal protective equipment.
- Span-80 (Sigma-Aldrich, cat. no. S6760-250ML)
- Hexane (≥99%, mixture of isomers, anhydrous; Sigma-Aldrich, cat. no. 227064-1L) **! CAUTION** Hexane is highly flammable and toxic.
- *N,N,N′,N′*-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T7024-50L) **! CAUTION** TEMED is harmful if inhaled or swallowed; it is corrosive and causes burns. Use the reagent in a well-ventilated area and wear appropriate personal protective equipment.
- APS (Sigma-Aldrich, cat. no. A9164) **! CAUTION** APS is an oxidizing agent; it may ignite combustible materials. It is harmful if swallowed. Wear appropriate personal protective equipment when handling this reagent.
- Isothermal Amplification Buffer (10×; NEB, cat. no. B0537S)
- Bst 2.0 DNA polymerase (Bst; 8,000 U/ml; NEB, cat. no. M0537L)
- Barcoded DNA oligonucleotides (refer to **Supplementary Tables 3** and **4** for complete list)
- Brij-35 (30% (wt/wt); Thermo Fisher Scientific, cat. no. 20150)
- NaOH solution (10 M; Sigma-Aldrich, cat. no. 72068-100ML)

Barcoding transcriptomes of single cells and library preparation

- 5′-TCGGCATTCCTGCTGAACCGCTCTTCCGATC TNNNNNN-3′ DNA oligonucleotide (standard desalting, hand-mixed; it can be ordered from IDT or other vendors)
- 5′-CAAGCAGAAGACGGCATACGAGATXXXXXXCTCTTT CCCT ACACGA DNA oligonucleotide, where XXXXXX is an index sequence for multiplexing libraries (see **Supplementary Table 2**; standard desalting; it can be ordered from IDT or other vendors).
- 5′-AATGATACGGCGACCACCGAGATCTACACGGTCTCGGCA TTCC TGCTGAAC -3′ DNA oligonucleotide (standard desalting; it can be ordered from IDT or other vendors)
- 5′-GGCATTCCTGCTGAACCGCTCTT CCGATCT-3′ DNA oligonucleotide (HPLC purification; it can be ordered from IDT or other vendors)
- 5′-AGATCGGAAGAGCGTCGTGTAGGGAAAGAG-3′ DNA oligonucleotide (HPLC purification; it can be ordered from IDT or other vendors)
- 5′-CTCTTTCCCTACACGACGCTCTT CCGATCT-3′ DNA oligonucleotide (HPLC purification; it can be ordered from IDT or other vendors)
- PBS (10×; Thermo Fisher Scientific, cat. no. AM9625)
- SuperScript III Reverse Transcriptase (SuperScript III, 200 U/μl; Thermo Fisher Scientific, cat. no. 18080-044) **▲ CRITICAL** In our experience, the primer dimer amount produced with this reverse transcriptase is the lowest as compared with other brands.
- RNaseOUT Recombinant Ribonuclease Inhibitor (RNaseOUT, 40 U/μl; Thermo Fisher Scientific, cat. no. 10777-019)
- Trypan blue (0.4% (wt/vol); Thermo Fisher Scientific, cat. no. T10282)
- Ethanol (Koptec, cat. no. V1016) **! CAUTION** Ethanol is highly flammable.
- OptiPrep Density Gradient Medium (OptiPrep; Sigma-Aldrich, cat. no. D1556-250ML)
- Igepal CA-630 (Sigma-Aldrich, cat. no. 56741)
- 1 M $MgCl_2$ (nuclease-free; Thermo Fisher Scientific, cat. no. AM9530G)
- BSA (2% (wt/vol); Thermo Fisher Scientific, cat. no. B14)
- Agencourt AMPure XP magnetic beads (AMPure beads; Beckman Coulter, cat. no. A63881)
- NEBNext mRNA Second Strand Synthesis Module (NEB, cat. no. E6111S)
- HiScribe T7 High Yield RNA Synthesis Kit (NEB, cat. no. E2040S)
- BioAnalyzer RNA pico chip and reagents (Agilent, cat. no. 5067-1513)
- BioAnalyzer HS DNA chip and reagents (Agilent, cat. no. 50674626)
- RNA Fragmentation Reagents (Ambion/Life Technologies, cat. no. AM8740)
- PrimeScript Reverse Transcriptase (Takara Clontec, cat. no. 2680A)
- Kapa 2× HiFi HotStart PCR mix (Kapa Biosystems, cat. no. KK2601)
- EvaGreen Dye (20× in water; Biotium, cat. no. 31000-T)
- 5× First Strand Buffer (Thermo Fisher Scientific, cat. no. 18080-044)
- Aquapel (Aquapel, cat. no. 47100) **! CAUTION** This material is highly toxic and moisture sensitive. Work in a fume hood and wear appropriate protective clothing and equipment when handling it.

EQUIPMENT

- Inverted fluorescence microscope (e.g., Nikon Ti-U Eclipse) equipped with FITC set of filters
- Red band-pass filter (wavelength cutoff at ≥ 600 nm; e.g., BP635 Light Red Bandpass Filter from Midopt)
- Digital camera (complementary metal oxide semiconductor high-speed camera such as the Fastec IL-3, Phantom V7.0 series, or similar)
- Syringe pumps (four; Harvard Apparatus, cat. no. 702226, or alternatives from, e.g., Cetoni and KD Scientific)
- Pump control software (custom script in LabVIEW v15.0; National Instruments, http://www.ni.com/labview/)
- Micro gear motor (Firgelli Automation, cat. no. FA-GM-6V-30, for keeping cells suspended)
- Gear motor drive hub (Firgelli Automation, cat. no. FA-HUB-3MM)
- Magnetic stirring bar (2 × 2 mm; Spinbar, cat. no. 371210008, or VWR, cat. no. 58948-377)
- Cold Rush Cold Therapy system (Ossur, cat. no. B-232000010)
- Refrigeration copper tubing (1.8-inch outer diameter; e.g., Cardel Industries, cat. no. REF-1/8)
- Tygon S3 Laboratory Tubing (3.2-inch inner diameter; VWR, cat. no. 89403-850)
- High-Intensity UV Inspection Lamp (UVP, cat. no. 95-0127-01)
- Liquid handler (e.g., Hamilton Microlab STAR-let)
- Heat-block for 1.5-ml tubes (e.g., VWR, cat. no. 12621-084)
- 4-block PCR apparatus compatible with 96-well PCR plates (e.g., MJ Research Tetrad PTC-225 Thermal Cycler)
- Rotary incubator (37 °C) for 15-ml and 50-ml tubes (e.g., VWR, cat. no. 47746-130)

- Tube rotator (e.g., VWR, cat. no. 10136-084)
- 2100 Electrophoresis Bioanalyzer Instrument (Agilent, cat. no. G2939AA)
- Refrigerated centrifuge for 1.5-ml and 2-ml tubes (e.g., Eppendorf, cat. no. 5424 R)
- Swing-bucket centrifuge for 15-ml and 50-ml tubes (e.g., Eppendorf, cat. no. 5810)
- Vortex (e.g., Scientific Industries, cat. no. SI-0236)
- 12-channel pipette (p300, e.g., Eppendorf, cat. no. 3122000060)
- Liquid-handler-compatible 50-µl pipette tips (nuclease-free; e.g., Hamilton Co-re, cat. no. 235964)
- Liquid-handler-compatible 300-µl pipette tips (nuclease-free; e.g., Hamilton Co-re, cat. no. 235950)
- Gel-loading pipette tips (Research Products International, cat. no. 148622)
- 15- and 50-ml serological pipettes
- 25-ml Reagent Reservoirs (VWT, cat. no. 89094-662)
- 300-ml Reagent Reservoirs (Thermo Scientific, cat. no. 1200-1300)
- 96-well round-bottom plates (Corning, cat. no. 3359)
- Flat 96-well PCR plates (VWT, cat. no. 82006-640)
- Aluminum microplate sealing tape (RNase, DNase free; Sigma-Aldrich, cat. no. CLS6570-100EA)
- 1.5-ml DNA LoBind tubes (Eppendorf, cat. no. 022431021)
- 0.5-ml DNA LoBind tubes (Eppendorf, cat. no. 0030108035)
- 0.2-ml PCR tubes (e.g., VWT, cat. no. 53509-304)
- Magnetic racks for 0.2-ml and 1.5-ml tubes (e.g., Permagen, cat. no. MSR1224)
- Sterile 1-ml syringes, Slip Tip (BD, cat. no. 309626)
- Sterile 1-ml low-dead-space syringes (for cell mix; B Braun, cat. no. 9166017V-02)
- Sterile 3-ml syringes, Luer-Lok Tip (BD, cat. no. 309657)
- Sterile 60-ml syringes, Luer-Lok Tip (BD, cat. no. 309653)
- 25 G × 5/8 needles (0.5 mm × 16 mm; BD, cat. no. 305122)
  ▲ **CRITICAL** The specific needle gauge size has to match the inner diameter of the microtubing used.
- Microtubing (i.d. 0.38 mm × o.d. 1.09 mm; Scientific Commodities, cat. no. BB31695-PE/2) ▲ **CRITICAL** Tubing size has been matched to microfluidic device port size.
- Black tubing (i.d. 1.6 mm; Saint-Gobain, cat. no. AFL00003)
- Hemocytometer (e.g., Bulldog Bio, cat. no. DHCN420)
- Aluminum foil (e.g., Ted Pella, cat. no. 43–100)
- Tweezers (blunt end; e.g., Fisher Scientific, cat. no. 16-100-111)
- Complete Filtration Units (filter + bottle; 500 ml; 0.2-µm PES membrane; VWR, cat. no. 10040-436)
- Syringe filters (25 mm diameter; 0.2-µm PTFE membrane; VWR, cat. no. 28145-495)
- Kimwipe delicate task wipers (Kimwipes; Kimberly-Clark, cat. no. 34155)
- 10-µm, 20-µm, and 85-µm strainers (e.g. pluriSelect, cat. nos. 43-50010-03, 43-50020-03, and 43-50085-03, respectively)
- 70-µm strainer (e.g., Falcon 70-µm Cell Strainer; Corning, cat. no. 352350)
- Corning Costar Spin-X column (pore size 0.45 µm, cellulose acetate membrane; e.g., Corning, cat. no. 8162)

REAGENT SETUP

10% (vol/vol) Tween-20 Combine 90 ml of nuclease-free water and 10 ml of Tween-20. This solution is stable for at least 6 months at room temperature (20 °C).

10% (vol/vol) Triton X-100 Combine 90 ml of nuclease-free water and 10 ml of Triton X-100. This solution is stable for at least 6 months at room temperature.

10% (vol/vol) Igepal CA-630 Combine 9 ml of nuclease-free water and 1 ml of Igepal CA-630. This solution is stable for at least 6 months at room temperature.

1 M NaCl Dissolve 58.44 g of molecular-biology-grade NaCl in ~900 of nuclease-free water, and adjust the final volume to 1 l. This solution is stable for at least 1 year at room temperature.

2 M KCl Dissolve 149.1 g of molecular-biology-grade KCl in ~900 of nuclease-free water, and adjust the final volume to 1 l. This solution is stable for at least 1 year at room temperature.

20% (vol/vol) 1H,1H,2H,2H-Perfluorooctanol in HFE-7500 oil (20% (vol/vol)) Combine 8 ml of HFE-7500 oil with 2 ml of 1H,1H,2H, 2H-perfluorooctanol (PFO). ! **CAUTION** Wear appropriate personal protective equipment and avoid contact with skin when handling this reagent. Use it in a well-ventilated area.

DNA elution buffer Combine 49.5 ml of nuclease-free water, 500 µl of 1 M Tris–HCl (pH 8.0), and 10 µl of 0.5 M EDTA (pH 8.0). Make 1-ml aliquots and store them at −20 °C for up to 1 year.

RNA elution buffer Combine 49.5 ml of nuclease-free water, 500 µl of 1 M Tris–HCl (pH 7.0), and 10 µl of 0.5 M EDTA (pH 8.0). Make 1-ml aliquots and store them at −20 °C for up to 1 year.

Tris–EDTA–Tween buffer Combine 480 ml of nuclease-free water, 5 ml of 1 M Tris–HCl (pH 8.0), 10 ml of 0.5 M EDTA, and 5 ml of 10% (vol/vol) Tween-20 in nuclease-free water. Filter the solution through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

Hydrogel bead wash buffer Combine 980 ml of nuclease-free water, 10 ml of 1 M Tris–HCl (pH 8.0), 200 µl of 0.5 M EDTA, and 10 ml of 10% (vol/vol) Tween-20 in nuclease-free water. Filter the solution through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

Barcode synthesis Prepare the following solutions.

4× Acrylamide/bis-acrylamide solution contains 3.6 ml of acrylamide/bis-acrylamide (AA/BIS), 2.58 ml of AA, and 3.82 ml of nuclease-free water. Filter the solution through a 0.2-µm membrane. The solution can be stored at 4 °C for at least 6 months.

10% (wt/vol) ammonium persulfate should be freshly prepared. Prepare a 10% (wt/vol) ammonium persulfate solution in nuclease-free water and filter it through a 0.2-µm membrane. Aliquots of 100 µl can be stored at −20 °C for up to 6 months.

Tris-buffered saline–EDTA–Triton buffer is prepared by combining 822 ml of nuclease-free water, 10 ml of 1 M Tris–HCl (pH 8.0), 137 ml of 1 M NaCl, 1.35 ml of 2 M KCl, 20 ml of 0.5 M EDTA, and 10 ml of 10% (vol/vol) Triton X-100 in nuclease-free water. Filter the solution through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

Acrydite-modified primer (5′-/acrydite/photocleavable spacer/ CGATGACGTA ATACGACTCACTATAG GGATACCACCATGGCTCTTT CCCTACA CGACGCTCTTC-3′) should be diluted to 250 µM in DNA elution buffer. This solution is stable for at least 1 year at −20 °C. ▲ **CRITICAL** The primer is light-sensitive. Avoid any exposure of the tube to UV or bright light.

1% (vol/vol) Span-80 in hexane is prepared by combining 99 ml of hexane and 1 ml of Span-80 in a fume hood. Store the solution in a glass bottle in a fume hood. This solution is stable for at least 6 months at room temperature.

STOP-25 buffer is prepared by combining 880 ml of nuclease-free water, 10 ml of 1 M Tris–HCl (pH 8.0), 50 ml of 0.5 M EDTA, 10 ml of 10% (vol/vol) Tween-20, and 50 ml of 2 M KCl. Filter the solution through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature. Prechill the solution to 4 °C before use.

STOP-10 buffer is prepared by combining 1,820 ml of nuclease-free water, 20 ml of 1 M Tris–HCl (pH 8.0), 40 ml of 0.5 M EDTA, 20 ml of 10% (vol/vol) Tween-20, and 100 ml of 2 M KCl. Filter the buffer through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

Denaturation solution is prepared by combining 242 ml of nuclease-free water, 3.75 ml of 10 M NaOH, and 4.2 ml of 30% (wt/wt) Brij-35. Filter the solution through a 0.2-µm membrane (e.g., using Complete Filtration Units). ▲ **CRITICAL** The solution must be freshly prepared just before use, starting from solid NaOH or a 10 M NaOH stock. ! **CAUTION** NaOH is highly corrosive; wear appropriate personal protective equipment when handling this reagent.

Neutralization buffer is prepared by combining 385 ml of nuclease-free water, 50 ml of 1 M Tris–HCl (pH 8.0), 10 ml of 0.5 M EDTA, 5 ml of 10% (vol/vol) Tween-20, and 50 ml of 1 M NaCl. Filter the buffer through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

Hybridization buffer is prepared by combining 407 ml of nuclease-free water, 5 ml of 1 M Tris–HCl (pH 8.0), 100 µl of 0.5 M EDTA, 5 ml of 10% (vol/vol) Tween-20, and 82.5 ml of 2 M KCl. Filter the buffer through a 0.2-µm membrane. This solution is stable for at least 6 months at room temperature.

QC buffer is prepared by combining 24 ml of nuclease-free water, 250 µl of 1 M Tris–HCl (pH 8.0), 500 µl of 0.5 M EDTA, 250 µl of 10% (vol/vol) Tween-20, and 25 ml of 2 M KCl. This solution is stable for at least 6 months at room temperature.

BA19 oligo is 5′-BAAAAAAAAAAAAAAAAAAA-3′ (standard desalting); prepare a 1 mM solution in nuclease-free water. This solution is stable for at least 1 year at −20 °C.

FAM-PE1 probe is 5′-/6-FAM/AGATCGGAAGAGCGTCGTGTAGG GAAAGAG-3′ (standard desalting); prepare a 100 µM solution in DNA elution buffer. This solution is stable for at least 1 year at −20 °C.

FAM-W1 probe is 5′-/6-FAM/AAGGCGTCACAAGCAATCACTC-3′ (standard desalting); prepare a 100 µM solution in DNA elution buffer. This solution is stable for at least 1 year at −20 °C.

FAM-BA19 probe is 5′-/6-FAM/BAAAAAAAAAAAAAAAAAAA-3′ (standard desalting); prepare a 100 µM solution in DNA elution buffer. This solution is stable for at least 1 year at −20 °C.

Barcode plates (eight 96-well plates) should be ordered with the DNA oligonucleotide sequences listed in **Supplementary Tables 3** and **4**. We typically use a 10-nmole-scale normalization and standard desalting, and order oligonucleotides dissolved to a final concentration of 50 µM in TE buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) from IDT. The two sets of 4 × 96-well plates are referred to as 'Barcode 1 plates' and 'Barcode 2 plates' in PROCEDURE.

Barcoding transcriptomes of single cells and library preparation

1×PBS with 30% (vol/vol) OptiPrep is prepared by combining 6 ml of nuclease-free water, 3 ml of OptiPrep, and 1 ml of 10× PBS. Store the solution at 4 °C for up to a month.

Cell mix is prepared by combining (on ice) 150 µl of 1× PBS and 300 µl of 1× PBS with 30% (vol/vol) OptiPrep, and then vortexing the mixture well. Add 150 µl of a 320,000 cell/ml single-cell suspension, and gently mix by pipetting up and down. Prepare immediately before use at Step 109, and keep the solution on ice. ▲ **CRITICAL** Cell preparation is cell-type-dependent and is not covered in detail in this protocol. It should be optimized by the reader to guarantee high cell viability (>90%). For example, certain cell types should not be cooled on ice before encapsulation into droplets.

1.3× RT premix is prepared by combining 2.2 ml of nuclease-free water, 1.13 ml of 5× First Strand Buffer, 680 µl of 1 M Tris–HCl (pH 8.0), 680 µl of 10 mM dNTP mix, and 356 µl of 10% (vol/vol) Igepal CA-630. Make 20 aliquots of 240 µl and store them at −20 °C for up to 6 months.

2× Bead concentration mix is prepared by combining 6.1 ml of nuclease-free water, 4.2 ml of 5× First Strand Buffer, and 210 µl of 10% (vol/vol) Igepal CA-630. Make 20 aliquots of 500 µl and store them at −20 °C for up to 1 year.

Blocking buffer is prepared by combining 9.3 ml of nuclease-free water, 105 µl of 1 M Tris–HCl (pH 8.0), 2.1 µl of 0.5 M EDTA, 525 µl of 10% (vol/vol) Tween 20, and 525 µl of 2% (wt/vol) BSA. Prepare 0.5-ml aliquots and store them at −20 °C for up to 1 year. Upon thawing, use the aliquot within a day, and then discard it.

PE2-N6 primer is 5′-TCGGCATTCCTGCTGAACCGCTCTTCCGATC TNNNNNN-3′ (standard desalting, hand-mixed); prepare a 100 µM solution in DNA elution buffer. This solution is stable for at least 1 year at −20 °C.

PE1/PE2 primer mix is prepared by combining equal volumes of 10 µM PE1 primer and 10 µM PE2 primer in DNA elution buffer.

PE1 primer: 5′-CAAGCAGAAGACGGCATACGAGATXXXXXXCTCTTT CCCTACACGA-3 (purification: standard desalting), where XXXXXX is an index sequence for multiplexing libraries. See **Supplementary Table 2** for a list of 24 PE2 primer variants. Typically, from one (i.e., no multiplexing) to six indexes are used.

PE2 primer: 5′-AATGATACGGCGACCACCGAGATCTACACGGTCTCGGCA TTCCTGCTGAAC-3 (purification: standard desalting).

Sequencing

Custom read 1 primer is 5′-GGCATTCCTGCTGAACCGCTCTT CCGATCT-3′ (HPLC); prepare a 100 µM solution in DNA elution buffer or nuclease-free water. This solution is stable for at least 1 year at −20 °C.

Custom index read primer is 5′-AGATCGGAAGAGCGTCGTGTAG-GGAAAGAG-3′ (HPLC); prepare a 100 µM solution in DNA elution buffer or nuclease-free water. This solution is stable for at least 1 year at −20 °C.

Custom read 2 primer is 5′-CTCTTTCCCTACACGACGCTCTT CCGATCT-3′ (HPLC); prepare a 100 µM solution in DNA elution buffer or nuclease-free water. This solution is stable for at least 1 year at −20 °C.

EQUIPMENT SETUP

Microfluidics platform The setup of the droplet microfluidics platform has been reported previously[57], except that in this work four syringe pumps are needed to deliver (i) cells, (ii) lysis and RT reagents, (iii) BHBs, and (iv) carrier oil. **Figure 4** depicts the inDrops platform, in which the major components are an inverted bright-field microscope (i), syringe pumps (ii–v), a digital camera (vi), and a computer (vii). Loaded syringes are connected to the microfluidic device (viii) via tubing attached to the needle and are placed close to the microscope to reduce the length of tubing. It is useful to place the cell infusion pump (iii) above the microscope stage (e.g., using monitor arm) to further shorten the distance that cells travel from the syringe to the chip. This is particularly important when working with large and fragile cells. Furthermore, cells in the syringe are gently agitated with a micro-flea to prevent sedimentation and clumping. Syringe pumps for BHBs (ii) and RT–lysis mix (vi) should be placed vertically because syringes containing these reagents are prefilled with HFE-7500 oil, which we use to overcome the dead volume of the syringe and tubing. Depending on the type of syringe pump, the infusion rate can be controlled manually or by using pump-control software. We typically encapsulate cells into 3–4 nl droplets at a throughput of 5.5 cells per s and record the encapsulation process with a digital camera (vi) using a 10× objective (NA 0.3, WP 16 mm) and 100-µs exposure time. We recommend that the minimal camera requirements be 100 fps at 1.3-MP resolution, with a sensor sensitive enough to give bright images with a <1,000 µs exposure time. For fast cell encapsulation (>100 cells per s), a high-speed camera (e.g., CMOS-based Phantom V7, Fastec IL-3) is a preferable option. It is noteworthy that most of the bright-field microscopes are equipped with powerful Xe or Hg lamps that emit a fraction of light at near UV and can damage the photocleavable linkage used to bridge barcoded DNA primers to HBs. Therefore, to prevent premature primer photocleavage, the incoming light from the microscope lamp must be filtered with a red band-pass filter (x). In addition, the tubing used to deliver HBs (ix) should be inserted into a black tube (e.g., Saint-Gobain, i.d. 1.6 mm, cat. no. AFL00003), or alternatively wrapped in aluminum foil to protect photosensitive primers from ambient light (**Supplementary Video 2**). The encapsulated cells are collected into a 1.5-ml tube prefilled with 300 µl of mineral oil (to prevent evaporation) and placed in an ice rack (xi). After encapsulation, the tube will contain three phases (xiii): mineral oil (top), encapsulated cells (middle), and carrier oil (bottom).

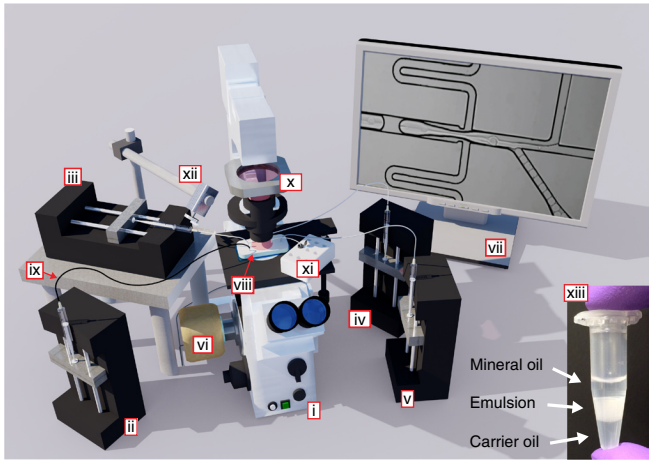

Figure 4 | The inDrops platform. (i) Inverted bright-field microscope; (ii–v) syringe pumps; (vi) fast speed camera; (vii) computer; (viii) microfluidic chip; (ix) barcoding hydrogel bead containing tubing, protected from ambient light by insertion into a second, opaque tubing, or alternatively, wrapped in aluminum foil; (x) red band-pass filter (≥600 nm); (xi) ice-cold rack containing the collection tube; (xii) cell mixer; (xiii) photograph of the collection tube showing three distinct phases.

Cell mixer To ensure that cells remain in suspension, place a micro-stirring bar (2 × 2 mm) inside the cell syringe, and agitate it using a neodymium magnet attached to a 6 V gear motor (xii, **Fig. 4**). The mixing can be controlled by connecting it to an adjustable power source or a 6 V battery with a potentiometer. Cells can be also encapsulated without using a cell mixer, but the cell occupancy may change over time, depending on the cell type.

Syringe cooling system During the experiment, syringes carrying cells and RT–lysis mix must be cooled. We use ice-cold water jackets, or ice-cold water circulating through a copper coil wrapped around the syringes (**Supplementary Fig. 1**). Using pliers, form a copper coil that tightly fits a 1-ml syringe. Ice-cold water is delivered via flexible tubing (e.g., Tygon S3 Laboratory Tubing) and fittings as required, and circulated using a circulator pump (e.g., Cold Rush Cold Therapy system; Ossur, cat. no. B-232000010).

PROCEDURE

Microfluidic device fabrication ● TIMING 2–3 d

1| Follow the protocol reported previously[57] to manufacture the PDMS-based microfluidic devices whose design is indicated in **Figure 5**. Channels of the hydrogel bead generation device (**Fig. 5a**) and the cell encapsulation device (**Fig. 5b**) should be 50 and 80 µm deep, respectively. CAD files for the microfluidic devices are provided as **Supplementary Data 1**. Ready-to-use microfluidic devices can be also obtained commercially (from 1CellBio, http://1cell-bio.com, or Droplet Genomics, http://dropletgenomics.com).

Barcode synthesis: hydrogel bead production ● TIMING 14 h

2| Prepare 2.5 ml of carrier oil–TEMED mix by combining 2.5 ml of carrier oil and 10 µl of TEMED. Vortex the mixture well.

3| Transfer the carrier oil–TEMED mix to a 3-ml syringe. To transfer, load the oil–TEMED mix into a p1000 pipette tip, insert the tip firmly into the Luer tip of the syringe, and suck the liquid into the syringe by withdrawing the plunger (see **Supplementary Video 3** for Steps 3–5).

4| Attach a 25-gauge needle and connect a piece of tubing (~40 cm (~16 inches)) using tweezers.

5| Hand-prime the syringe: hold the syringe upright, and then slowly push the plunger until the liquid fills half of the tubing. Then mount the syringe on a syringe pump.

6| Pump-prime the syringe: run the syringe pump at 2,000–5,000 µl/h until the liquid reaches the end of the tubing.

7| Prepare 1 ml of acrylamide–primer mix by combining the following reagents. Mix the solution by vortexing and centrifuge at 1,000*g* for 5 s at room temperature.

Component	Amount (µl)	Final
Nuclease-free water	420	
1× TBSET buffer	100	0.1×
4× AB solution	250	1×
Acrydite-modified DNA primer (250 µM)	200	50 µM
10% (wt/vol) APS	30	0.3% (wt/vol)
Total	1,000	

AB, acrylamide/bis-acrylamide.

▲ CRITICAL STEP Acrydite-modified DNA primers are light-sensitive, as they contain a photocleavable (1-(2-nitrophenyl)-ethyl-based) moiety. At all times protect the DNA primers from exposure to UV light (<400 nm). Noticeable primer deactivation may occur at prolonged (≥20 min) exposure to ambient white light illumination. Therefore, perform Steps 7–79 in dim illumination and away from UV light sources.

8| Transfer the acrylamide–primer mix to a 3-ml syringe prefilled with 300 µl of HFE-7500 oil (**Supplementary Video 3**).

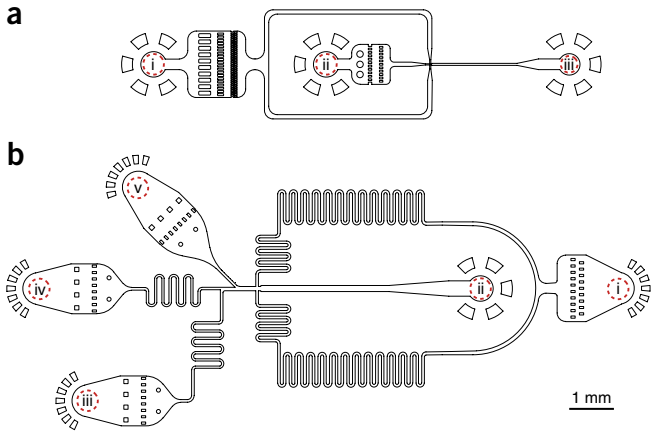

Figure 5 | Design of the microfluidic devices. (**a**) Design of the hydrogel bead generation device. The numbers indicate the carrier oil inlet (i), the acrylamide–primer mix inlet (ii), and the outlet for droplet collection (iii). The depth of microfluidic channels is 50 µm. (**b**) Design of the cell encapsulation device. The numbers indicate the carrier oil inlet (i), the collection outlet (ii), the inlet for RT–lysis mix (iii), the cell inlet (iv), and the inlet for barcoding hydrogel beads (v). The depth of the microfluidic channels is 80 µm.

Box 1 | Estimating droplet volume and diameter

To calculate droplet volume and diameter, record a short (1–2 s) video at the outlet, and then play it back at a slower frame rate and count 10–20 droplets. Droplet volume in picoliters (pl) is given by $V = \frac{Ft}{c_1 N}$, where F is the sum flow rate (microliters per hour) of all aqueous phases (e.g., hydrogel beads, cell mix, and RT–lysis mix), t is the time (s) to count N droplets, and c_1 is a factor to correct for unit differences $\left(c_1 = 3.6\frac{\text{sec}\cdot\mu l}{pl\cdot h}\right)$.

Before performing an actual experiment, we recommend a test run of the microfluidic device to determine optimal imaging conditions (such as frame rate and exposure) for the specific camera used. If no camera fast enough for counting droplets as they are being formed is available, droplet size can be determined from still images of droplets at the outlet of the microfluidic device using the equation $V = \frac{\pi}{12}[2D^3 - (D-h)^2(2D+h)]$, where h is the height of the channel and D is droplet diameter (μm). Alternatively, a hemocytometer can be used to trap and measure the droplet volume using the expression $V = \frac{4}{3}\pi R^3$, yet care must be taken to ensure that all dimensions of the imaging chamber are larger than $2R$, where R is the droplet radius.

9| Connect a piece of tubing (~40 cm (~16 inches)), and prime the syringe as described in Steps 4–6. Cover the syringe and tubing with foil.
▲ **CRITICAL STEP** Syringes prefilled with HFE-7500 oil should be placed into vertical pumps (**Fig. 4**) with the needle pointing upward. This will prevent the HFE-7500 oil from entering the tubing before the entire acrylamide–primer mix is pushed out of the syringe.

10| Connect a piece of tubing (~15 cm (~6 inches)) to the outlet (**Fig. 5a**, port no. ii), and insert the tubing into a 2-ml collection tube prefilled with 300 μl of mineral oil. Cover the collection tubing and 2-ml tube with aluminum foil.

11| Connect the syringes (via tubing) to corresponding inlets of the hydrogel bead generation device (**Fig. 5a**) and place it on the stage of a microscope equipped with a red band-pass filter (≤650 nm cutoff). Use flow rates of 900 μl/h and 1,800 μl/h for the acrylamide–primer mix and carrier oil, respectively. When using this 50-μm-deep microfluidic chip (**Fig. 2a**), hydrogel droplets of 125 pl (62 μm diameter) should be generated at ~2,000 droplets per s (**Supplementary Video 4**).
▲ **CRITICAL STEP** Microscope light should be filtered through a red band-pass filter to minimize damage to photocleavable primers.

12| Once flow rates in the system are stabilized (~3–5 min), record the droplet size (**Box 1**) and, if necessary, adjust it by changing the flow rate of the carrier oil. Droplet diameter should be in the 58- to 63-μm range, ideally 60 μm.
? TROUBLESHOOTING

13| After 30 min of operation, replace the collection tube to prevent it from overflowing.

14| Once hydrogel bead generation is complete, transfer both tubes to 65 °C and incubate overnight protected from light. During this step, the aqueous acrylamide droplets will polymerize into HBs.
■ **PAUSE POINT** If you are not proceeding to Step 16 immediately, HBs can be stored at 4 °C protected from light for at least 1 week.

15| Repeat Steps 2–14 to produce a desired amount of HBs. For example, the barcode synthesis protocol (Steps 16–79) is described for a 5-ml batch.

Barcode synthesis: cleanup ● TIMING 2 h
16| Remove the top (mineral oil) and bottom (carrier oil) phases from the 2-ml collection tubes, leaving ~500 μl of polymerized HBs per tube. Add 500 μl of Tris-buffered saline–EDTA–Triton (TBSET) buffer on top of the creamy-looking emulsion.

17| To release polymerized HBs from the emulsion, add 1 ml of 20% (vol/vol) PFO per tube, vortex well, and centrifuge at 5,000*g* at room temperature for 30 s. HBs should appear as a milky (or semitransparent) phase.

18| Remove the bottom 20% (vol/vol) of the PFO phase and repeat Step 17 until the milky phase is transformed into a solid, well-packed, translucent mass on top of the 20% (vol/vol) PFO phase.

19| Remove the bottom 20% (vol/vol) of the PFO phase and add 1 ml of 1% (vol/vol) Span-80 in hexane per tube. Mix vigorously by vortexing and centrifuge at 5,000*g* at room temperature for 30 s. This time, the HBs will appear as a solid mass at the bottom of the tube. Remove the top hexane layer.

20| Repeat the previous step.

21| Add 1 ml of TBSET buffer to each 2-ml tube and resuspend HBs by vortexing. Combine HBs from all tubes and split equally into two 15-ml tubes.

22| Fill the 15-ml tubes with TBSET buffer, vortex the tubes, and centrifuge them at 3,000*g* at room temperature for 3 min. The HBs will sediment, whereas traces of hexane from Step 20 will appear as a milky layer on the top. Carefully aspirate the supernatant, keeping the tip of the serological pipette at the liquid–air interface.
▲ **CRITICAL STEP** If the HBs do not sediment, extend the centrifugation time.
? TROUBLESHOOTING

23| Repeat Step 22 three times until the top milky layer disappears.
■ **PAUSE POINT** After the third wash, the HBs can be kept at 4 °C, protected from light, for several months.

24| Measure the hydrogel bead diameter using the hemocytometer-based approach described in **Box 1**. The bead diameter should be ~70 µm, as they swell in TBSET buffer.
? TROUBLESHOOTING

25| Pass the HBs through a 70-µm cell strainer attached to the top of a 50-ml tube. Use ice-cold TBSET buffer to facilitate the passage of the beads through the mesh.
▲ **CRITICAL STEP** The use of a strainer allows the removal of dust particles and occasional large HBs (>75 µm), both of which interfere with proper microfluidic operation at Steps 118–120.
? TROUBLESHOOTING

26| Centrifuge the pass-through HBs at 3,000*g* at room temperature for 10 min. Remove the supernatant, leaving a final volume of ~20 ml.
▲ **CRITICAL STEP** If the HBs do not sediment, extend centrifugation time and/or speed.

27| Distribute the HBs evenly into four 15-ml tubes (~5 ml per tube). Collect the HBs remaining in the 50-ml tube by washing the inner walls with ~1 ml of TBSET buffer.
▲ **CRITICAL STEP** HBs sediment faster in 15-ml tubes as compared with 50-ml tubes and allow shorter centrifugation times in the subsequent steps of barcode synthesis.
■ **PAUSE POINT** The HBs can be stored at 4 °C for up to 6 months protected from light.

Barcode synthesis: first split-and-pool round ● TIMING 5 h
28| Wash the HBs contained in 15-ml tubes with hydrogel bead wash (HBW) buffer three times. To wash, fill the tubes with HBW buffer, resuspend the HBs by vortexing, centrifuge at 1,000*g* at room temperature for 3 min, and carefully remove the supernatant. The HBs will swell to ~2 ml per tube.
▲ **CRITICAL STEP** If no swelling is observed, perform additional washes.

29| Combine all HBs into a single 15-ml tube (~8–10 ml). Rinse the empty tubes with ~1 ml of HBW buffer to collect the HBs adhered to the inner walls.

30| Centrifuge the tube for 1 min at 1,000*g* at room temperature and remove the supernatant, leaving a final volume of 12 ml.

31| Add 2.9 ml of 10× Isothermal Amplification Buffer and vortex well. Increased ionic strength will cause the HBs to shrink. Centrifuge the tube 1 min at 1,000*g* at room temperature and remove the supernatant, leaving a final volume of 9.4 ml.

32| Prepare the hydrogel bead mix by combining the following reagents:

Component	Amount (ml)	Final
Nuclease-free water	2.3	
Hydrogel bead suspension from Step 31	9.4	
dNTP (10 mM each)	0.812	650 µM each
Total	12.5	

33| Transfer the hydrogel bead mix from Step 32 to a 25-ml reagent reservoir.

34| Using a multichannel pipette, load a round-bottom 96-well plate with 125 µl of hydrogel bead mix per well. The remains of the hydrogel bead mix can be saved for another run by mixing with 5 volumes of Tris–EDTA–Tween (TET) buffer; the solution can be stored up to several months at 4 °C protected from light.

35| Place the round-bottom plate with the hydrogel bead mix into a liquid handler.

36| Thaw the four 96-well Barcode 1 plates at room temperature (see Reagent Setup and **Supplementary Tables 3** and **4**), and centrifuge for 1 min at 1,000*g* at room temperature to collect the condensate.

37| Put the plates into a PCR machine set at 70 °C (lid 105 °C) for 40 s (primer-dimer denaturation and heat-induced convection), and then at 4 °C for 20 s. Rapidly peel off the seal. Discard the seal immediately.
▲ **CRITICAL STEP** Take great care to avoid cross-contamination between wells.

38| Place the four Barcode 1 plates and four 96-well PCR plates (Reaction plates) into the liquid handler.

39| Using the liquid handler, mix the HBs with the barcoded DNA oligonucleotides. Resuspend the HBs contained in the round-bottom 96-well plate (Step 34) by pipetting up and down a few times, and then dispense 30 µl of hydrogel bead mix into every well of each reaction plate. Transfer 13.5 µl of DNA oligonucleotides from each well of the Barcode 1 plate into the four reaction plates. Mix by pipetting up and down.

Detailed method files for use with a Hamilton Microlab STAR-let liquid handler and Hamilton Method Editor v4.4.7740 software are provided as **Supplementary Data 2**.

40| Seal the reaction plates and transfer them to a four-block PCR machine for incubation at 85 °C for 2 min, 60 °C for 120 min, and 4 °C indefinitely. Keep the lid temperature at 100 °C.

41| While the reaction is incubating at 60 °C, proceed to Steps 42–44.

42| Carefully seal the four Barcode 1 plates with adhesive seal, and mark the remaining volume. Place the plates in a plastic bag to avoid frost accumulation and store them at –20 °C for up to 3 years.

43| While the reaction plates are incubating at 60 °C, prepare the following isothermal reaction mix. Gently mix by inverting the tube 15 times. Keep on ice.

Component	Amount (ml)	Final
Nuclease-free water	6.85	
10× isothermal amplification buffer	0.8	1×
Bst 2.0 DNA polymerase (8,000 U/ml)	0.35	350 U/ml
Total	8	

44| Transfer the isothermal reaction mix to a 25-ml reagent reservoir and split into a round-bottom 96-well plate, 80 µl per well. Keep the plate on ice.

45| After 20 min of incubation at 60 °C, pause the PCR machine program, take the reaction plates out of the PCR machine, and remove the seals (as described in Step 37).

46| Using the liquid handler, transfer 15 µl of the isothermal reaction mix to each well of the four reaction plates, and mix. Discard the tips after each transfer.

47| Seal the reaction plates with fresh tape and return them to the PCR machine. Continue the isothermal reaction at 60 °C for 60 min. Longer incubations have no harmful effect. During this step the barcoded DNA primers in each well will be incorporated into the hydrogel matrix via a primer extension reaction catalyzed by Bst 2.0 DNA polymerase.

48| Cool the reaction plates on ice for 1 min, remove the adhesive seals, and transfer the plates to the liquid handler.

49| Place two reservoirs in the liquid handler: a 300-ml reservoir filled with 150 ml of STOP-25 buffer (source reservoir) and a 300-ml reservoir filled with 50 ml of STOP-25 buffer (collection reservoir).

50| Using the liquid handler, stop the primer extension reactions in each of the four reaction plates. Using 200- to 300-µl tips, transfer 40 µl per well of STOP-25 buffer from the source reservoir to each of the four reaction plates, and combine the mix from all reaction plates in the collection reservoir. Rinse the empty wells with 100 µl of STOP-25 buffer, and transfer to the collection reservoir. There is no need to change tips during this step.

51| Using a serological 50-ml pipette, transfer the contents of the collection reservoir to three 50-ml tubes covered in foil. Rinse the collection reservoir with 10 ml of STOP-25 buffer and transfer to one of the 50-ml tubes.

52| Adjust the volume of all tubes to 50 ml with STOP-25 buffer, and leave the tubes rotating (5–50 r.p.m.) at room temperature for >30 min. The tubes can also be incubated at room temperature without rotation.
■ **PAUSE POINT** The HBs can be stored at 4 °C overnight.

Barcode synthesis: postbarcoding washes ● TIMING 4 h

53| Centrifuge the 50-ml tubes containing the HBs at 300*g* at room temperature for 15 min.

54| Using a serological pipette, remove the supernatant, leaving a total volume of ~5–10 ml per tube. When using 50-ml tubes, we always observe some HBs in the supernatant. Do not discard the supernatant at this step: transfer it to fresh 50-ml tubes, and collect the HBs by repeating centrifugation at 300*g* at room temperature for 15 min.
▲ **CRITICAL STEP** Do not use excessive centrifugation force, as the HBs may clump.

55| Combine the HBs from all 50-ml tubes into one, mix by pipetting up and down, and split equally into four 15-ml tubes. Rinse the 50-ml tubes with 5 ml of STOP-10 buffer to collect the beads that are adhered to inner walls.

56| Wash the HBs three times in STOP-10 buffer. To wash, fill 15-ml tubes with STOP-10 buffer, rotate them for 15 min at room temperature, centrifuge for 3 min at 300*g* at room temperature, and remove the supernatant. Make sure to leave <3 ml total volume per tube after the last wash.
■ **PAUSE POINT** The HBs can be stored at 4 °C for up to 6 months.

57| Prepare 250 ml of fresh Denaturation solution (Reagent Setup).

58| Fill the four hydrogel-bead-containing 15-ml tubes with denaturation solution, rotate them for 10 min at room temperature, centrifuge for 3 min at 300*g* at room temperature, and discard the supernatant.

59| Wash the HBs three times in denaturation solution. To wash, fill 15-ml tubes with denaturation solution, invert and flick the tubes to resuspend all beads, incubate for 1 min at room temperature, centrifuge for 3 min at 300*g* at room temperature, and remove the supernatant.

60| Repeat the previous step for two washes using neutralization buffer.
■ **PAUSE POINT** If stopping here, wash the HBs three times in TET buffer with 3-min centrifugation at 1,000*g* at room temperature, and store them protected from light at 4 °C for up to 6 months. If the beads are to be used on the same day in the second split-and-pool round of barcode synthesis, skip the washes in TET and proceed directly to the washes in HBW (Step 61).

Barcode synthesis: second split-and-pool round ● TIMING 1 d

61| Wash the HBs three times in HBW buffer with 3-min centrifugations at 1,000*g* at room temperature, as described in Step 28.

62| Combine all HBs in a single 15-ml tube. Rinse the empty tubes with HBW buffer to collect the HBs adhered to the walls. At this step (postdenaturation at Steps 58–59), the HBs will swell up to 12 ml.

63| Repeat Steps 30–60 using the Barcode 2 plates.

Barcode synthesis: enzymatic cleanup ● TIMING 1 d

64| Perform HBs washes in HBW buffer four times, as described in Step 28.

65| Transfer 30 µl of packed HBs to a 1.5-ml tube filled with 270 µl of TET buffer. Label the tube 'HBs before cleanup' and store them at 4 °C protected from light until QC (Step 80).

66| To each of the four 15-ml tubes (containing ~2-5 mL of HBs), add the following components in listed order, and then rotate them at 37 °C for 2 h.

Component	Amount (ml)	Final
10× FastDigest buffer	1.5	1×
Nuclease-free water	Up to 14.7	
FastDigest HinfI	0.3	
Total	15	

67| Spin the tubes down at 300*g* at room temperature for 3 min, and remove the supernatant.

68| Wash the tubes with hybridization buffer three times. To wash, fill the tubes with hybridization buffer, resuspend the HBs by vortexing, centrifuge at 1,000*g* at room temperature for 3 min, and carefully remove the supernatant.

69| Combine the content of all 15-ml tubes into one 50-ml tube.

70| Fill the 50-ml tube with hybridization buffer, invert a few times, and centrifuge at 300*g* at room temperature for 10 min. Remove the supernatant, leaving a final volume of 22.5 ml.

71| Add 500 µl of 1 mM BA19 oligo. Mix well to resuspend the HBs.

72| Rotate the 50-ml tube at room temperature, protected from light, for 30 min.

73| Transfer half (11.5 ml) of the hydrogel bead/BA19 oligo mix to a second 50-ml tube. Pipette slowly to minimize sticking of the hydrogel beads to the serological pipette.

74| To remove primers missing poly-T tails, add the following reagents to each of the two 50-ml tubes:

Component	Amount (ml)	Final
Hydrogel bead/BA19 oligo mix from Step 73	11.5	
10× ExoI buffer	3.3	1×
Nuclease-free water	18	
ExoI (20 U/µl)	0.44	0.27 U/µl
Total	33	

Rotate the tubes for 2 h at room temperature, protected from light.

75| Stop the ExoI treatment by filling each 50-ml tube with STOP-25 buffer and inverting a few times.

76| Perform washes in STOP-10 buffer, followed by BA19 oligo removal by alkaline denaturation, as described in Steps 53–60.

77| After neutralization of sodium hydroxide (Step 60), filter the HBs through a 85-µm strainer placed on top of a 50-ml tube, as described in Step 25.

78| Centrifuge the 50-ml tube at 1,000*g* at room temperature for 5 min, remove the supernatant, and distribute the HBs into two 15-ml tubes.

79| Wash the HBs three times with TET buffer, as described in Step 28. Prepare aliquots of the concentrated hydrogel bead suspension in 1.5-ml nuclease-free tubes.
■ **PAUSE POINT** At this step, combinatorial barcode synthesis is complete, and the HBs are referred to as BHBs. BHBs can be stored at 4 °C, protected from light, for up to 6 months. Before first use, perform the QC checks described below.

Barcode synthesis: QC by capillary electrophoresis ● TIMING 30 min

80| In two PCR tubes, prepare two samples for comparison—'Before ExoI': 8 µl of water + 2 µl of close-packed HBs before cleanup (from Step 65), and 'After ExoI': 8 µl of water + 2 µl of close-packed BHBs after cleanup (from Step 79). The HBs can be close-packed by letting them sediment by gravity or by centrifugation for 3 min at 1,000*g* at room temperature.

81| Release photocleavable barcoding primers from HBs by UV treatment for 7 min (**Supplementary Video 5**).

82| Remove HBs using 0.45-µm filter columns, and centrifugation for 5 min at 16,000*g* at room temperature.

83| Incubate the flow-through at 90 °C for 5 min, and then place on ice.

84| Run a BioAnalyzer HS DNA assay on 1 µl of each flow-through. See ANTICIPATED RESULTS for examples of electropherograms.

Barcode synthesis: QC using fluorescent probes ● TIMING 1.5 h

85| In two 1.5-ml tubes, prepare two samples for comparison—'Before ExoI': 1.4 ml of QC buffer + 10 µl of close-packed HBs before cleanup (from Step 65), and 'After ExoI': 1.4 ml of QC buffer + 10 µl of close-packed HBs after cleanup (from Step 79).

86| Vortex the mixture, centrifuge for 1 min at 1,000*g* at room temperature, and remove most of the supernatant. Perform Steps 87–92 with each sample in parallel.

87| Adjust the volume of QC buffer to a final of 120 µl.

88| Resuspend the HBs by pipetting up and down, and distribute the suspension into three 1.5-ml tubes, at a volume of 36 µl per tube, and label them 'PE1', 'W1', and 'BA19' (**Fig. 6**).

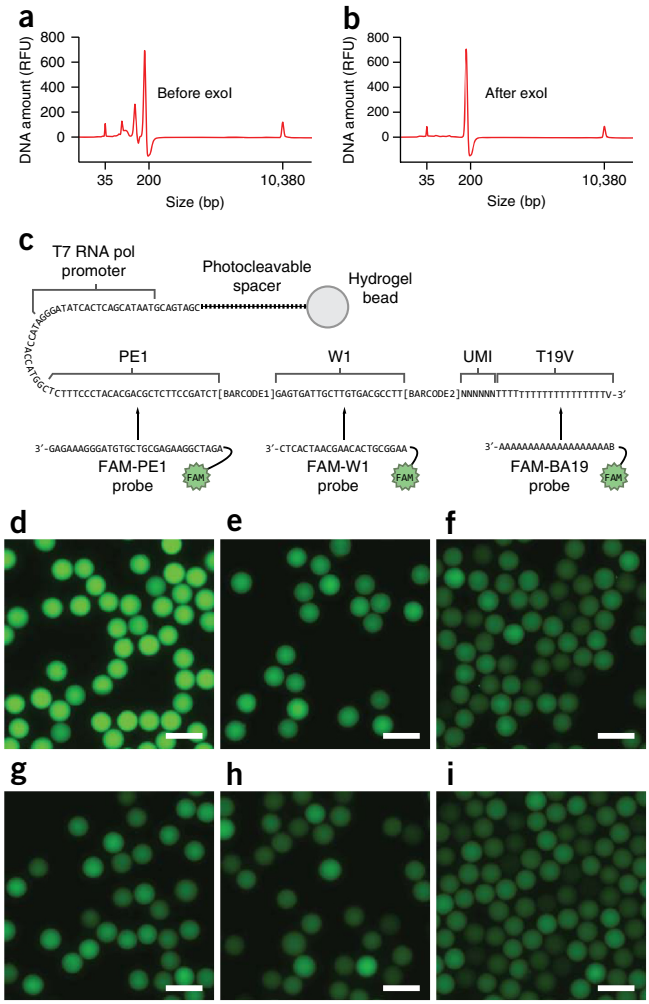

Figure 6 | Quality control of barcoded hydrogel bead synthesis. (**a**,**b**) BioAnalyzer electropherograms of barcoding cDNA primers photoreleased from hydrogel beads before (**a**) and after (**b**) ExoI treatment (Step 84). The highest-molecular-weight peak (that typically appears at 170–190 bp) represents the full-length barcoding primer (actual size 134–136 nt), and lower-molecular-weight peaks are synthesis intermediates that are hydrolyzed by ExoI. Peaks at 35 and 10,380 bp are gel migration markers. Note that the BioAnalyzer HS DNA assay is optimized for double-stranded DNA, and thus the ladder cannot be used for estimating primer length. (**c**) Sequence of fully assembled barcoding primer. (**d**–**i**) Fluorescent confocal imaging of barcoding hydrogel beads after hybridization of probes targeting PE1 (**d**,**g**), W1 (**e**,**h**), and T19V (**f**,**i**) sequences. Fluorescent *in situ* hybridization results before (**d**–**f**) and after (**g**–**i**) ExoI treatment are compared. Scale bars, 100 µm. RFU, relative fluorescence units.

89| Add 4 µl of the 100 µM FAM-PE1, FAM-W1, and FAM-BA19 probes to the 'PE1', 'W1', and 'BA19' tubes, respectively. Mix by pipetting up and down, and incubate the tubes at room temperature for 20 min.

90| Remove excess probe by washing the HBs three times with QC buffer. To wash, add 1.4 ml of QC buffer, vortex the mixture, centrifuge for 1 min at 1,000*g* at room temperature, and remove the supernatant. At the end of the last wash, remove the supernatant, leaving an ~40-µl total volume in each tube.

91| Record digital images of HBs from each tube under a fluorescence microscope equipped with a filter set for FITC (**Fig. 6d–i**).

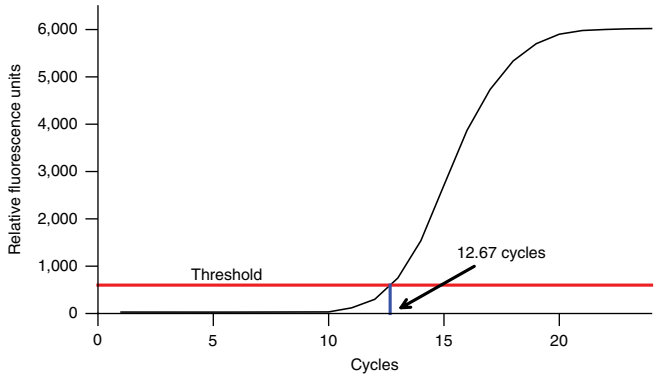

Figure 7 | Setting the qPCR threshold. The *x* axis shows 24 temperature cycles with the higher (65 °C) primer annealing temperature. In this example, Ct (cycle threshold) was determined to be 12.67.

92| Measure the normalized intensity for each bead ((total fluorescence)/(area of hydrogel bead)) using an image analysis software (such as Fiji[82]). See ANTICIPATED RESULTS for typical QC results.

Barcoding of transcriptomes of single cells: BHB preparation ● TIMING 20 min

93| Transfer 300 µl of close-packed BHBs obtained in Step 79 to a 1.5-ml tube prefilled with 1 ml of HBW buffer. 300 µl of close-packed BHBs is sufficient for barcoding 20,000–30,000 cells.
▲ **CRITICAL STEP** At all times until barcoding, HBs can become encapsulated into droplets; protect them from direct light, close window blinds, and use dim illumination.

94| Briefly vortex the tube, centrifuge at 1,000*g* at room temperature for 1 min, and transfer the supernatant to a clean 15-ml tube, wrapped in foil. Label the 15-ml tube 'Recovered BHBs'.

95| Repeat the previous step three times, filling the 1.5-ml tube with HBW buffer during each wash. After the last wash, remove the supernatant, leaving an ~500-µl total volume (use the mark on the tube as a reference).

96| Add 500 µl of 2× bead concentration mix (Reagent Setup), vortex well, and centrifuge the BHB suspension at 5,000*g* at room temperature for 1 min.

97| Using a p1000 pipette, transfer as much of the supernatant as possible to the 15-ml 'Recovered BHBs' tube. 300 µl of close-packed BHBs from Step 93 should now concentrate to ~150–200 µl.

98| Spin down the tube at 5,000*g* at room temperature for 1 min, and use a fine gel-loading pipette tip remove any remains of the supernatant and transfer it to the 'Recovered BHBs' tube.
▲ **CRITICAL STEP** Make sure to remove all of the supernatant.

99| Attach a 25-gauge needle to a 1-ml syringe, and connect ~90 cm (~3 feet) of tubing using tweezers. Push the plunger all the way through until it reaches the 0-ml position.

100| Insert the open end of the tubing into the bottom of the concentrated BHBs (Step 98), and fill the tubing with BHBs by gently pulling the plunger. Stop once ~3/4 of the tubing is filled with concentrated BHBs (see also **Supplementary Video 2**, for Steps 100–103). The leftover BHBs can be mixed with 1 ml of TET buffer and returned to the 'Recovered BHBs' tube.
▲ **CRITICAL STEP** Make sure that the end of the tubing remains immersed in BHBs during the procedure to avoid aspirating air. If it happens, gently push the plunger to remove the air gap and resume the loading.

101| Using a razor blade, cut the open end of the tubing right where the BHBs start. Place the tubing with the BHBs on your bench.

102| Fill a 1-ml syringe with 500 µl of HFE-7500, attach a 25-gauge needle, remove air bubbles by pushing the plunger, and connect it to the open end of tubing that contains BHBs. Remove the empty syringe from the opposite end.
▲ **CRITICAL STEP** When you are connecting the syringe with HFE-7500 to the tubing, avoid any air gaps.

103| Insert the BHB tubing into an opaque tubing of larger diameter (e.g., 1.6 mm), or alternatively wrap the BHB tubing in foil, and mount the syringe with HFE-7500 onto a vertical syringe pump with the needle facing upward ((ii) in **Fig. 4**).

Barcoding of transcriptomes of single cells: RT–lysis mix preparation ● TIMING 10 min

104| Estimate the volume of RT–lysis mix to prepare. Count 15 µl of RT–lysis mix per ~1,000 cells and ~10 µl for priming. For example, if you plan to encapsulate and barcode two samples of 10,000 cells, prepare 320 µl of RT–lysis mix.

105| On ice, prepare the RT–lysis mix by combining the following components:

Component	Amount (µl)	Final
1.3× RT premix	240	1×
$MgCl_2$ (1.0 M)	3.6	11 mM
DTT (0.1 M)	22	7 mM
RNaseOUT (40 U/µl)	22	2.7 U/µl
SuperScript III (200 U/µl)	32	20 U/µl
Total	320	

▲ **CRITICAL STEP** Add RNAseOUT and SuperScript III just before encapsulation to reduce the time on ice.

106| Transfer the RT–lysis mix to a 1-ml syringe prefilled with 300 µl of HFE-7500, connect a piece of tubing (~40 cm (~16 inches)), and hand-prime the syringe (as described in Steps 3–5 and **Supplementary Video 3**).

107| Mount the syringe onto a vertical pump ((iv) in **Fig. 4**) and ensure cooling using a copper coil with circulating ice-cold water or an ice jacket (**Supplementary Fig. 1**).
▲ **CRITICAL STEP** Cooling of the RT–lysis mix is critical to prevent the heat-induced inactivation of the RT enzyme.

Barcoding of transcriptomes of single cells: cell preparation ● TIMING 10 min

108| Confirm that your cell sample meets the following requirements, which we recommend verifying on a separate day before a single-cell transcriptome barcoding experiment. The cell suspension should have no, or very few (<5%), cell doublets or clumps. Cell strainers can be used to filter out cell clumps. Ensure that the sample is free from cell lysate, as it contains RNA, which cross-contaminates the sample. Cell viability in 1× PBS needs to be high (ideally 95–100%), and it should remain stable (not more than a 5% drop) for at least 30 min on ice.

109| On ice, prepare the cell mix, which consists of 80,000 cells per ml in 1× PBS with 15% (vol/vol) OptiPrep (Reagent Setup). For the first runs, we recommend using 600 µl of cell mix. When the number of available cells is very low (e.g., a total number of 1,000 cells), use a final volume of at least 100 µl.

110| Place a magnetic stir bar into a low-dead-space 1-ml syringe. If, because of limited cell availability, the total volume of cell mix is <500 µl, the use of a flea is not necessary.

111| Transfer the cell mix from Step 109 to the syringe containing the magnetic stir bar.

112| Hand-prime the cell syringe (as in Step 5) and mount it onto the pump equipped with a magnet attached to a rotor (see Equipment Setup and **Fig. 4**). Turn on the rotor. Use a copper coil with circulating ice-cold water or an ice jacket to cool the cell mix in the syringe (**Supplementary Fig. 1**).
▲ **CRITICAL STEP** Adjust the rotation speed so that the stir bar spins gently. Vigorous mixing can damage cells.

Barcoding of transcriptomes of single cells: system setup ● TIMING 5 min

113| Fill a 1-ml syringe with 1 ml of carrier oil. Attach tubing, hand-prime, and mount onto a horizontally held syringe pump, as described in Steps 3–5 ((v) in **Fig. 4**).

114| Set the correct syringe diameters on the syringe pumps. For example, when using the 1-ml syringes called for by this protocol, set the diameter at 4.78 mm.

115| Pump-prime the syringe with BHBs at 500 µl/h. Once the BHBs start moving, stop the pump immediately. Due to compression, the BHBs will keep moving slowly for a few minutes (for ~3–5 cm of tubing distance). Once the BHBs stop moving, cut the empty end of the tubing and connect to the appropriate port of the microfluidic chip (**Fig. 5b**, port no. v).
▲ **CRITICAL STEP** Make sure that the tubing containing the BHBs is fully covered in foil or opaque tubing (except for a few millimeters that go into the port).

116| Pump-prime the remaining syringes at 2,000 µl/h, and connect the tubing to the corresponding inlets of the microfluidic device (**Fig. 5b**).
▲ **CRITICAL STEP** Make sure that the red band-pass filter is placed in the path of microscope light source ((x) in **Fig. 4**).

117| Connect a 1.5-ml tube to the device outlet (**Fig. 5b**, port no. ii) via ~15 cm of tubing. The 1.5-ml tube is a waste tube that will be replaced by a collection tube at Step 120 once the system is primed.

Barcoding of transcriptomes of single cells: cell encapsulation ● TIMING 30–60 min
118| Once all syringes are connected to appropriate ports, fine-tune the flow rates of the inDrops system:

	Priming BHBs	Priming the rest of the system	Close-packing BHBs	Working flow rates
Cell mix	50 µl/h	250 µl/h	50 µl/h	250 µl/h
Barcoding hydrogel beads	150 µl/h	20 µl/h	50 µl/h	40 µl/h
RT/lysis mix	50 µl/h	250 µl/h	50 µl/h	250 µl/h
Carrier oil	50 µl/h	360 µl/h	360 µl/h	360 µl/h
Additional instruction	As BHBs reach the inlet, immediately switch to 'Priming the rest of the system'	Once air is displaced from the system, switch to 'Close-packing BHBs'	Once beads are close-packed, proceed to 'Working flow rates'	Adjust the flow rate of BHBs to reach 85–95% occupancy

119| Once flow rates are stabilized, allow the system to run for 2–3 min until the 'priming emulsion' is displaced from the tubing connected to the outlet of the microfluidic device.
? TROUBLESHOOTING

120| Start collecting the emulsion into a 1.5-ml collection tube prefilled with 300 µl of mineral oil and held in a cooling rack or ice bucket on the microscope stage ((xi) in **Fig. 4**).
▲ **CRITICAL STEP** Make sure that the collection tube remains on ice at all times until Step 128.

121| Adjust the flow rate of BHBs by increments of 5 µl/h to reach 85–95% occupancy (**Box 2**).
▲ **CRITICAL STEP** Avoid having two BHBs per droplet.
? TROUBLESHOOTING

122| Confirm the droplet size as described in **Box 1**. Using the flow rates provided at Step 118, the drop size should be 3.0–3.5 nl, and it can be adjusted by changing the flow rate of the carrier oil.

123| Confirm the cell count as described in **Box 2**.

124| Calculate the time of encapsulation required to collect the desired number of cells, as described in **Box 2**. Using the cell concentration and flow rates described in this protocol, and with 90% BHB occupancy, it takes less than 4 min to coencapsulate ~1,000 cells with barcoding BHBs. You can also estimate the fraction of productive droplets containing more than one cell. Using the cell concentration and flow rates described in this protocol, it should be ~5%. This number can be linearly decreased by reducing cell concentration (Step 109), but this comes at the expense of longer encapsulation time.

125| Follow the encapsulation on a computer screen in a snapshot mode, which can be achieved by setting the camera display rate to 2 frames per s.
? TROUBLESHOOTING

Box 2 | Calculating the inDrops platform parameters

Using the flow rates, 90% BHB occupancy, and cell dilution listed in this protocol, the throughput of the system is ~45 droplets per s or ~5 barcoded cells per s. We recommend performing a test run using mock reagents to become familiar with the flow parameters of the system and to determine the optimal imaging conditions, such as frame rate, exposure, and playback speed with the specific camera used.

Hydrogel bead occupancy refers to the percentage of droplets containing a bead, and can be easily determined from still images or a short (~1 s) video recorded at the outlet of the microfluidic device.

Cell count (cells/ml) is most conveniently determined at the cell inlet. Record an ~10-s-long video, count the number of cells flowing through, and calculate the cell count using the following equation:

$$C = c_3 \frac{N}{F_{cells} t},$$

where F is the flow rate of the cell syringe (250 µl/h), t is the time (s) to count N cells, and c_3 corrects for unit differences

$$\left(c_3 = 3600 \frac{\text{sec}}{\text{h}}\right).$$

Time of encapsulation (min) to collect a desired number of cells is given as follows:

$$T = c_4 \frac{N_{\text{target}} \eta t}{N},$$

where N_{target} is the desired number of cells to barcode, η is BHB occupancy, t is the time (s) to count N cells, and c_4 corrects for unit differences

$$\left(c_4 = \frac{\text{min}}{60\text{sec}}\right).$$

The fraction of droplets containing more than one cell should be kept as low as possible, as such droplets will give rise to artificial cell types. Those droplets that have both a cell and a barcoding hydrogel bead are considered productive. Cell arrival into droplets is a Poisson process, and the fraction of productive droplets containing more than one cell can be estimated from the Poisson distribution:

$$P(X \geq 2 \mid X \geq 1) = \frac{1 - e^{-\lambda}(1 + \lambda)}{1 - e^{-\lambda}},$$

where λ is the average number of cells per droplet. λ is given by

$$\lambda = CV \frac{F_{\text{cells}}}{F}$$

where C is the cell count (cells/nl), V is the droplet volume (nl), F_{cells} is the flow rate of the cell syringe (250 µl/h), and F is the sum flow rate of BHBs, cell mix, and RT–lysis mix (typically 540–560 µl/h).

126| Once the desired number of cells is encapsulated, unplug the tubing from the outlet (leave the other end in the collection tube), stop the pumps, and let the emulsion in the tubing drain into the collection tube by gravity.

Barcoding of transcriptomes of single cells: RT ● TIMING 3 h

127| To release photocleavable barcoding primers from the BHBs, place the collection tube on ice and expose it to 6.5 J/cm^2 of 365-nm light. When using the UV light source provided in the MATERIALS section, place the lamp ~3–5 cm above the tube and expose the tube for 7 min (**Supplementary Video 5**).

▲ **CRITICAL STEP** Keep the emulsion on ice at all steps during and after encapsulation.

128| To initiate the RT reaction, transfer the tube with the UV-exposed emulsion to 50 °C for 2 h followed by 15 min at 70 °C.

129| Once the RT reaction is complete, transfer the tube to room temperature and remove as much light mineral oil (top phase) and HFE-7500 (bottom phase) as possible.

? TROUBLESHOOTING

130| Divide the emulsion into fractions containing the desired number of cells; use 0.5-ml DNA LoBind tubes. For example, if 4,000 cells were barcoded, divide the entire volume of the emulsion in two equal parts to get 2,000-cell libraries. Using the conditions described in this protocol, 35 µl of emulsion typically contains ~1,000 barcoded cells.

131| Release the barcoded cDNA from the droplets by adding one drop (~3 µl) of 20% (vol/vol) PFO on top of the emulsion, and then add 40 µl of HFE-7500 oil.

? TROUBLESHOOTING

■ **PAUSE POINT** Store post-RT libraries at –80 °C. They are stable for at least 3 months.

Library preparation: digestion of unused primers and primer dimers ● TIMING 2 h

132| Up to eight libraries can be conveniently processed in parallel. Thaw post-RT libraries on ice. Centrifuge the tubes at 4 °C for 5 min at 19,000*g* to pellet cell debris.

133| Without aspirating the HFE-7500 oil (bottom phase), transfer the post-RT material (top phase) to a nucleic acid purification column, such as Corning Costar Spin-X (pore size 0.45 µm, cellulose acetate membrane) and collect the flow-through fraction into a 1.5-ml DNA LoBind tube by centrifuging for 1 min at 16,000*g* (at 4 °C). After centrifugation, leave the filter column inserted into the collection tube until Step 136.

▲ **CRITICAL STEP** At all steps before linear amplification by IVT (Step 141), block the pipette tips to minimize material loss due to adsorption. Blocking is performed by immersing the tip into blocking buffer, and pipetting up and down once. If large drops remain attached to the tip, flick the pipette to remove them.

134| Prepare the digestion mix. For each 70 µl of post-RT material, prepare 100 µl of digestion mix.

Component	Amount (µl)	Final
Nuclease-free water	79	
10× FastDigest buffer	9	0.9×
ExoI (20 U/µl)	5	1 U/µl
FastDigest HinfI	7	
Total	100	

135| Add 100 µl of digestion mix on top of the HFE-7500 oil in the original 0.5-ml tube and mix gently by pipetting up and down to collect the remains of the post-RT material attached to the inner walls of the tube.

136| Transfer the digestion mix to the nucleic acid purification column containing the bulk of the sample (from Step 133) and centrifuge the filter column at 4 °C for 5 min at 16,000*g*, thus combining the digestion mix and the sample in the 1.5-ml LoBind collection tube. After centrifugation, discard the filter membrane containing the BHBs.

137| Incubate the flow-through fraction for 30 min at 37 °C.

138| Purify the reaction product (in the form of a cDNA:RNA hybrid) using AMPure beads. Use a 1.2× volume of AMPure beads (e.g., to 100 µl of digestion reaction product, add 120 µl of AMPure beads) and elute into 17 µl of nuclease-free water. Follow the instructions from the AMPure Purification Kit. Detailed steps for AMPure purification are also provided as **Supplementary Method 1**.

Library preparation: second-strand synthesis and linear amplification ● TIMING 16–18 h

139| Set up second-strand synthesis (SSS) using reagents from the NEBNext mRNA Second Strand Synthesis Module. On ice, add 2 µl of 10× SSS buffer to the 17 µl of eluate from Step 138. Mix by vortexing, centrifuge at 1,000*g* for 5s at room temperature, add 1 µl of SSS Enzyme, and mix by gently flicking the tube.

140| Incubate at 16 °C for 2.5 h, followed by 20 min at 65 °C.

141| At room temperature, top the 20 µl of SSS product with the following reagents for IVT (HiScribe T7 High Yield RNA Synthesis Kit):

Component	Amount (µl)	Final
SSS product	20	
Nuclease-free water	12	
10× reaction buffer	8	1×
ATP, CTP, GTP, and UTP (100 mM)	8 each	10 mM each
T7 RNA Polymerase Mix	8	
Total	80	

142| Mix, centrifuge at 1,000*g* for 5s at room temperature, and incubate at 37 °C for 13–15 h on a PCR machine with a heated lid (50 °C).
■ **PAUSE POINT** After 15 h, the IVT reactions can be kept at 4 °C for up to 2 h.

143| Purify with a 1.3× volume (104 µl) of AMPure beads (Step 138) and elute into 19 µl of RNA elution buffer.
■ **PAUSE POINT** If you are pausing here, store the samples at –80 °C for up to 6 months.

144| On ice, transfer 10 µl of the eluate from the previous step to a fresh tube and label it 'Post-IVT back-up'.

145| Run a BioAnalyzer RNA Pico assay on 1 µl of Post-IVT back-up and transfer the remaining aliquot to –80 °C. See ANTICIPATED RESULTS for examples of post-IVT RNA traces of successful libraries.
? TROUBLESHOOTING
■ **PAUSE POINT** At this step samples can be stored at –80 °C for up to 6 months.

Library preparation: fragmentation of amplified RNA ● TIMING 20 min
146| Prepare STOP mix. For one sample, combine the following, vortex the mixture, and place it on ice.

Component	Amount (µl)
RNA elution buffer	9.8
AMPure beads	26.4
Stop solution from the RNA Fragmentation Kit	1.2
Total	37.4

147| Allow the PCR machine to reach 70 °C (lid: 100–110 °C)

148| On ice, add 1 µl of 10× RNA Fragmentation Reagents to 9 µl of purified IVT product from Step 143. Flick the tube, centrifuge at 1,000*g* for 5s at room temperature, and immediately incubate the mixture at 70 °C for exactly 3 min.
▲ **CRITICAL STEP** Fragmentation time might vary between 2 and 3 min depending on IVT material amount, RNA quality, and sample handling.

149| Transfer the fragmentation reaction on ice and immediately add 34 µl of STOP mix (from Step 146). Mix well, and spin the mixture very briefly (~1 s) to collect drops from walls of the tube. Avoid sedimentation of magnetic beads.

150| Incubate the samples for 5 min on ice, and then place them in a magnetic rack and continue the AMPure purification, as described above (Step 138). Elute in 8 µl of RNA elution buffer and transfer to a PCR tube.

Library preparation: RT using random hexamers ● TIMING 2.5 h

151| On ice, combine the following reagents, vortex the mixture, and then spin it down.

Component	Amount (μl)	Final
Purified fragmented amplified RNA (from Step 150)	8	
dNTP (10 mM each)	1	500 μM each
PE2-N6 primer (100 μM)	2	10 μM
Total	11	

152| Incubate the mixture at 70 °C (lid 105 °C) for exactly 3 min, and cool it immediately on ice.

153| On ice, add the following components (to a total volume of 20 μl):

Component	Amount (μl)	Final
Mixture from step 152	11	
Nuclease-free water	3.5	
5× PrimeScript buffer	4	1×
RNaseOUT (40 U/μl)	1	2 U/μl
PrimeScript reverse transcriptase (200 U/μl)	0.5	5 U/μl
Total	20	

154| Mix gently. Incubate the reaction mixture at 30 °C for 10 min, followed by 42 °C for 1 h. Heat-inactivate the mixture at 70 °C for 15 min.

155| Add 20 μl of nuclease-free water, mix, and save 20 μl as backup (label it as 'pre-PCR backup' and store it at –80 °C for up to 6 months).

156| Purify the RT product with 1.2× (24 μl) AMPure beads, and resuspend it in 10 μl of DNA elution buffer.

Library preparation: diagnostic quantitative PCR ● TIMING 1.5 h

157| Perform a quantitative PCR (qPCR) to determine the correct number of cycles for PCR amplification at Step 161. Combine the following components on ice:

Component	Amount (μl)	Final
Eluate from Step 156	0.5	
Nuclease-free water	6.5	
2× Kapa HiFi HotStart PCR mix	10	1×
20× EvaGreen Dye	1	1×
PE1/PE2 primer mix (5 μM)	2	0.5 μM
Total	20	

158| Perform the following qPCR thermal cycling program:

Cycle number	Denaturation	Annealing	Extension	Termination
1	98 °C, 2 min			
2–3	98 °C, 20 s	55 °C, 30 s	72 °C, 40 s	
4–27	98 °C, 20 s	65 °C, 30 s	72 °C, 40 s (with fluorescence acquisition)	
28				4 °C, hold

EvaGreen Dye requires the same excitation/emission settings as for SYBR Green.

159| Set the qPCR threshold within the exponential phase of amplification, as shown in **Figure 7**. Correct the resulting cycle threshold (Ct) value to account for input differences between the qPCR (Steps 157–158) and library PCR (Steps 160 and 161). The library input is 19 times higher, and assuming a 100% PCR efficiency, the difference corresponds to $\log_2 19 = 4.25$ cycles. For example, if the number of cycles determined by qPCR is 2 + 12.67, the required number of PCR cycles for the library is 2 + (12.67–4.25) = 2 + 8.

Library preparation: library amplification by PCR ● TIMING 2 h

160| Combine the following components on ice:

Component	Amount (μl)	Final
Eluate from Step 156	9.5	
Nuclease-free water	0.5	
2× Kapa HiFi HotStart PCR mix	12.5	1×
PE1/PE2 primer mix (5 μM)	2.5	0.5 μM
Total	25	

▲ **CRITICAL STEP** If multiple libraries are to be sequenced in one sequencing run (i.e., multiplexed), use different PE1/PE2 primer mix variants to introduce standard Illumina library indices (Reagent setup).

▲ **CRITICAL STEP** Whenever you are multiplexing libraries, two of the indices must be index 6 and index 12 (see Reagent Setup and **Supplementary Table 2**). This requirement ensures proper signal registration during the index read using Illumina sequencing platforms.

161| PCR thermal cycling program:

Cycle number	Denaturation	Annealing	Extension	Termination
1	98 °C, 2 min			
2–3	98 °C, 20 s	55 °C, 30 s	72 °C, 40 s	
4–11/15[a]	98 °C, 20 s	65 °C, 30 s	72 °C, 40 s	
16			72 °C, 5 min	
17				4 °C, hold

[a]The number of cycles at 65 °C annealing temperature is determined at Step 159 and is typically between 8 and 12.

If you are working with multiple libraries that require different numbers of cycles, set the PCR machine to perform the highest number of cycles required, and manually remove individual libraries from the PCR machine. For example, if library X requires 8 cycles, at the end of the 72 °C step of cycle 8, pause the cycler and place library X on ice. Next, resume the PCR program. Once all libraries have undergone the required number of PCR cycles, transfer all tubes back to the PCR machine for 5 min at 72 °C.

162| Add 25 μl of DNA elution buffer to the PCR product.

163| Purify using a 0.7× volume (35 μl) of AMPure beads, and elute in 20 μl of DNA elution buffer. The eluate is the final sequencing-ready library.

164| Test library quality by running a BioAnalyzer DNA HS assay on 1 μl of eluate. Libraries should look like a smooth hump starting at 200 bp and ending at 1.5 kb, and have an average size of 400–600 bp. See ANTICIPATED RESULTS for examples of successful libraries.

? TROUBLESHOOTING

■ **PAUSE POINT** Store the final libraries at –20 or –80 °C; they should be stable for at least 1 year.

TABLE 1 | Sequencing parameters for different Illumina sequencers.

Sequencer	Multiplexing?	Kit	Read1	Index read	Read2
MiSeq	Yes	MiSeq Reagent Kit v3 (150 cycles)	89 cycles	6 cycles	55 cycles
NextSeq	Yes	NextSeq 500/550 High-Output v2 Kit (75 cycles)[a]	37 cycles	6 cycles	51 cycles
NextSeq	No	NextSeq 500/550 High-Output v2 Kit (75 cycles)[a]	43 cycles	0 cycles	51 cycles
HiSeq 2500	Yes	HiSeq SBS Kit v4 (2 × 50 cycles)	61 cycles	6 cycles	51 cycles

[a]92–94 cycles can actually be performed using the '75 cycle' Kit.

Sequencing ● TIMING 1 d

165| Quantify, dilute, and mix (if multiplexing) the libraries according to standard procedures.

166| Sequence the libraries on an Illumina sequencer using custom sequencing primers (Reagent Setup). Read 2, which is the cell barcode and UMI read (**Fig. 3**), should be at least 50 bp. Distribute the rest of the cycles between the Index read (if multiplexing) and Read 1 (transcript read). See **Table 1** for examples.

▲ **CRITICAL STEP** The optional PhiX control library will not be sequenced when using custom sequencing primers. To use the PhiX Control, contact Illumina. Do not mix custom index read primers with standard index read primers.

Sequencing data processing ● TIMING 1 d

167| Process raw reads using the script and instructions provided in the **Supplementary Software**. The output is a genes-versus-cells UMI-filtered count matrix.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Steps	Problem	Possible reasons	Solution
12, 119, 125	Fluctuation in droplet size	Air bubbles in the system	Verify that no air bubble is trapped at the passive filters of the inlet ports. Increasing the flow rate of the selected phase by 1,000 µl/h for 1–2 s may facilitate air-bubble escape. Gently press the PDMS device to facilitate air-bubble release Stop encapsulation and remove bubbles from the tubing and/or syringes. Repeat priming as described in Steps 5 and 6
	Liquid jetting: the liquid steam extends into the droplet stabilization channel and produces droplets of variable size	Incorrect flow rate	Increase the flow rate of carrier oil until jetting disappears
	Wetting: the aqueous phase 'sticks' to the walls of the droplet stabilization channel, resulting in polydisperse drops	Poor coating of microfluidic channels	Treat microfluidic channels with fresh Aquapel. Inject the solution into the channels, leave for 10–30 s, rinse the channels with HFE-7500 oil, and then flush with pressurized air or nitrogen. A detailed protocol is provided in Mazutis *et al.*[57]
12, 119, 121, 125	Droplets coalesce at the outlet of the device	Insufficient surfactant concentration	Use a fresh batch of carrier oil
		PDMS or dust particles cause droplet coalescence	Use a new microfluidic device
		Igepal CA-630 concentration is too high	Droplets are typically stable in the presence of up to 1% (wt/vol) Igepal CA-630. A higher concentration of detergent might destabilize droplets

(continued)

TABLE 2 | Troubleshooting table (continued).

Steps	Problem	Possible reasons	Solution
22	White pellet at the bottom of the tube after centrifugation	Remains of HFE-7500 oil	At Step 18, make sure to perform enough washes for the HBs to form a translucent mass To rescue a contaminated batch of HBs, carefully and without disturbing the white pellet, transfer the bulk of the HBs to a fresh 15-ml tube
24	Satellite droplets (variable size, smaller, and different color than HBs)	Remains of hexane and/or HFE-7500 oil	HFE-7500 contamination: see above Hexane contamination: at Step 22, make sure to hold the tip of the serological pipette at the liquid–air interface when removing the supernatant. To rescue a contaminated batch of HBs, perform another 2–3 washes in TBSET as described in Step 22
25	The bulk of the HBs do not pass through the strainer	The HBs are slightly too large and clog the mesh	Try the following approaches that cause hydrogel beads to shrink: (i) refrigerate hydrogel beads in the strainer at 4 °C for 5 min (ii) Use cold TBSET supplemented with 1 M NaCl to wash the strainer
121, 125	Inconsistent flow of BHBs	Hydrogel bead inlet is blocked by dust	Remove tubing from the collection tube. Using an Allen wrench or blunt-end tweezers as a pointer, and gently press the PDMS at the location of the blockage. If the dust blockage is not released, switch to a new device
		Polydisperse BHBs	Hydrogel beads of >70 µm might cause temporary blockage of the channel and disturb smooth injection of the BHBs. Pass the BHBs through a 70-µm strainer (Step 25)
		Air in BHB tubing	Verify that there are no air gaps in the BHB tubing. Repeat hydrogel bead loading (Steps 93–103)
129	An aqueous layer between the mineral oil and the emulsion	Merged droplets	Use a p200 pipette to remove the aqueous layer between the mineral oil and the emulsion
131	The emulsion is not breaking	Insufficient amount of PFO	Add up to 20 µl of PFO, and flick the tube. If needed, increase PFO concentration further
145	Large peak at ~200 nt	Incomplete digestion of primer dimers due to HinfI failure (Steps 133–137)	Make sure that the sample for HinfI digestion is free of PFO (Step 134) Restart library preparation with a fresh batch of HinfI
145	The yield of amplified RNA (400–4,000 nt) is low	Low amount of starting material (<500 cells and/or cells containing little mRNA)	Proceed further and use the BioAnalyzer electropherogram of the final library (Step 164) as a validation of successful library preparation (ANTICIPATED RESULTS)
		RNA degradation following premature cell death	Verify cell viability before the experiment (Step 108)
164	Average library size is <400 bp	Fragmentation (Step 148) went on for too long	Repeat library preparation from Step 146 using the post-IVT backup, and make sure to perform fragmentation at 70 °C for exactly 3 min. Some cell types require a shorter shearing time (~2 min). Run a BioAnalyzer RNA Pico Chip to confirm the size of sheared RNA

(continued)

TABLE 2 | Troubleshooting table (continued).

Steps	Problem	Possible reasons	Solution
164	High-molecular-weight products (at 2,000 bp and above)	Too many PCR cycles at Step 161	Repeat library preparation from Step 156 using the pre-PCR backup, and perform 1–2 PCR fewer cycles at Step 161
164	No DNA library detected	Library was lost during purification because of an incorrect concentration of AMPure reagent	Repeat library preparation from the pre-PCR backup (Step 156), making sure to completely resuspend the AMPure beads before use by vortexing vigorously. If the problem persists, optimize the AMPure amount by increments of 0.1× at Step 163, but avoid products of <200 nt (as determined by BioAnalyzer HS DNA assay at Step 164)

● **TIMING**

Microfluidic device fabrication

Step 1, 2–3 d, or the device can be purchased

Barcode synthesis

Steps 2–15, hydrogel bead production: 14 h
Steps 16–27, cleanup: 2 h
Steps 28–52, first split-and-pool round: 5 h
Steps 53–60, postbarcoding washes: 4 h
Steps 61–63, second split-and-pool round: 1 d
Steps 64–79, enzymatic cleanup: 6 hrs
Steps 80–84, QC by capillary electrophoresis: 30 min
Steps 85–92, QC using fluorescent probes: 1.5 h

Barcoding of transcriptomes of single cells

Steps 93–103, BHB preparation: 20 min
Steps 104–107, RT–lysis mix preparation: 10 min
Steps 108–112, cell preparation: 10 min
Steps 113–117, system setup: 5 min
Steps 118–126, cell encapsulation: 30–60 min
Steps 127–131, RT: 3 h

Library preparation

Steps 132–138, digestion of unused primers and primer dimers: 2 h
Steps 139–145, second-strand synthesis and linear amplification: 16–18 h
Steps 146–150, fragmentation of amplified RNA: 20 min
Steps 151–156, RT using random hexamers: 2.5 h
Steps 157–159, diagnostic qPCR: 1.5 h
Steps 160–164, library amplification by PCR: 2 h

Sequencing and raw data processing

Steps 165 and 166, sequencing: 1 d
Step 167, sequencing data processing: 1 d
Box 1, estimating droplet volume and diameter
Box 2, calculating the inDrops platform parameters

ANTICIPATED RESULTS

This protocol describes the establishment of a droplet microfluidics platform for high-throughput single-cell RNA-seq experiments using the inDrops technique. Upon completion of the procedure described above, one will have produced an amount of BHBs sufficient to barcode 500,000–1,000,000 single cells, and will be able to perform inDrops experiments starting from a suspension of cells and ending with a genes-versus-cells UMI-filtered count matrix.

Below are described QC results from the synthesis of a 5-ml batch of BHBs (**Fig. 6** and **Table 3**), and typical single-cell RNA-seq results using inDrops (**Figs. 8** and **9**; **Table 4**).

Barcode synthesis—QC by capillary electrophoresis (Step 84). After combinatorial barcode synthesis (Steps 28–79), the 3′5′ exonuclease ExoI is used to remove reaction intermediates that did not undergo the first or second primer extension

reaction (Steps 64–79 and **Fig. 2c**). As shown in **Figure 6a**,**b**, successful barcode synthesis and ExoI treatment results in a single peak in the BioAnalyzer electropherogram of primers photoreleased from the HBs. This QC check can also be performed by denaturing PAGE (**Supplementary Method 2**).

Barcode synthesis—QC using fluorescent probes (Step 92). **Table 3** summarizes results of barcode synthesis QC by fluorescent *in situ* hybridization (FISH). PE1, W1, and T19V sequences are shared by all full-length barcoding primers (**Fig. 6c**), and their presence is confirmed using fluorescent probes in three separated FISH reactions (**Fig. 6d–i** and Steps 85–92). This QC check is performed on HBs before and after ExoI treatment (Steps 68–79). The sample before ExoI treatment provides information about the efficiency of each primer extension step, which is measured as the relative fluorescence of FAM-W1 and FAM-BA19 probes as compared with the FAM-PE1 probe. The overall efficiency of both primer extension steps is typically >30%. In the example provided in **Table 3**, the efficiencies of the first and second steps were 58% and 62%, respectively, yielding an overall efficiency of 36%, or ~10^9 copies of full-length barcoded primers per hydrogel bead. The sample after ExoI treatment is used to quantify the variability in primer amount between ready-to-use barcoded HBs. Upon successful barcode synthesis, the coefficient of variation (CV) of FAM-BA19 probe fluorescence across all HBs should be less than 25%, and the CV of all three probes should not differ by more than 20%. A higher CV in fluorescence typically indicates suboptimal settings of the liquid handler (Steps 39–50) or pipetting errors.

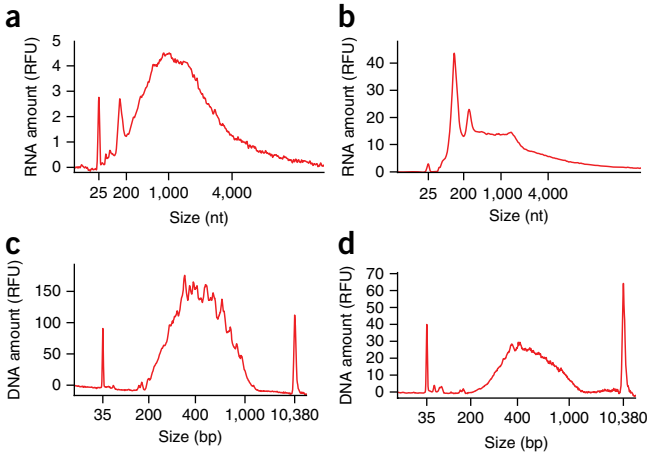

Figure 8 | BioAnalyzer electropherograms of amplified RNA (Step 145) and sequencing-ready DNA libraries (Step 164). Traces were obtained from neural organoid culture cells (**a**,**c**) and blood cells (**b**,**d**). Peaks at 25 nt (**a**,**b**), 35 bp, and 10,380 bp (**c**,**d**) are gel migration markers. Library concentration and average fragment size are 475 bp and 20 nM, respectively, in **c**, and 530 bp and 5 nM, respectively, in **d**.

BioAnalyzer electropherograms (Steps 145 and 164). The profile of amplified RNA (aRNA) electropherograms (Step 145 and **Fig. 8a**,**b**) is highly variable depending on the cell source (e.g., primary versus culture cells), library size, and initial biological sample quality. A successful library typically shows a distinguishable signal between 200 and 4,000 nt, although it may be less pronounced with some samples. A single peak or two peaks at ~200 nt are indicative of primer dimers/hairpins that survive digestion by ExoI and HinfI (Steps 133–137 and **Fig. 3**), although they are eventually eliminated by size-selective purification using AMPure beads in subsequent steps of library preparation. DNA electropherograms of final libraries (Step 164) are more reproducible among samples, and serve as a better estimate of library quality as compared with aRNA traces. A successful library should be distributed between 200 and 2,000 bp, with an average fragment size between 400 and 600 bp. Library concentration depends on the number of PCR cycles applied (Step 161), and it is typically between 3 and 10 nM (as determined by BioAnalyzer, **Fig. 8d**), although concentrations up to 20 nM are also acceptable (**Fig. 8c**). However, it is noteworthy that overcycling libraries can cause preferential amplification of a fraction of amplicons, and require deeper sequencing to detect rare molecules.

Typical single-cell RNA-seq results using inDrops. We typically barcode transcriptomes of 3,000–30,000 cells in one run (Steps 118–131), and further process in parallel multiple libraries of 1,000–3,000 cells (Steps 132–166). **Figure 9** summarizes intermediate (i.e., BioAnalyzer electropherograms) and final results of sequencing a 2,000-cell library of immune cells.

TABLE 3 | Quality control of barcode synthesis by fluorescence *in situ* hybridization.

	Before ExoI treatment			After ExoI treatment		
Probe	**FAM-PE1**	**FAM-W1**	**FAM-BA19**	**FAM-PE1**	**FAM-W1**	**FAM-BA19**
Number of hydrogel beads analyzed	354	428	435	207	1,085	375
Mean fluorescence across beads, RFU/px	23,273	13,465	8,383	9,683	8,787	7,834
Standard deviation of fluorescence across beads, RFU/px	2,673	1,853	1,880	1,930	1,746	1,800
Coefficient of variation	11%	14%	22%	20%	20%	23%
Fraction of PE1 probe fluorescence	100%	58%[a]	36%[b]	100%	91%	81%

px, pixel; RFU, relative fluorescence units.
[a]Barcode yield after the first round of primer extension. [b]Overall barcode yield.

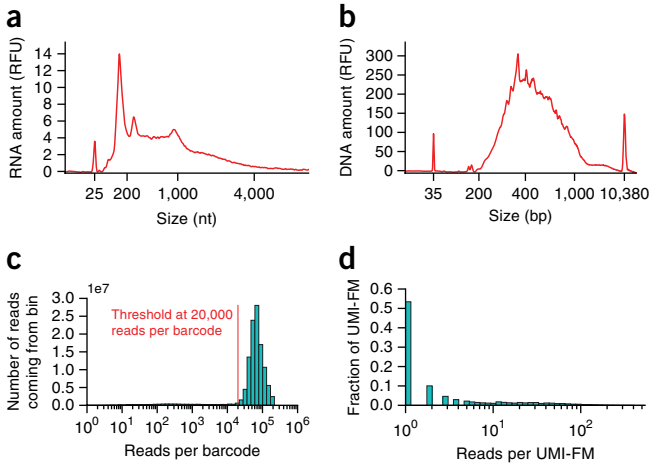

Figure 9 | Results of sequencing a library of 2,000 immune cells. (**a**) BioAnalyzer electropherograms of amplified RNA (Step 145). The peak at 25 nt is the gel migration marker. (**b**) BioAnalyzer electropherograms of sequencing-ready DNA libraries (Step 164). Peaks at 35 and 10,380 bp are gel migration markers. (**c**) Histogram of reads per cell barcode. Note that units of the *y* axis are the number of reads coming from the histogram (rather than the number of barcodes having a given value of reads per barcode). A good-quality library should have a distinct peak on the right side of the histogram (the exact position of the peak being a function of sequencing depth, library size, and cell type). This peak represents high-abundance barcodes associated with actual cells. A threshold is set manually (at 20,000 reads per barcode in the case of this specific library), and only barcodes passing the threshold are kept for further analysis. The number of detected cells should agree with the estimated library size (at Steps 124 and 130). (**d**) Oversequencing histogram, showing the distribution of reads per UMI-filtered molecule (UMI-FM—i.e., a unique [cell barcode] × [gene] × [UMI] combination). The shape of the histogram suggests that deeper sequencing should allow the detection of more unique molecules. However, the coverage represented by this histogram was sufficient to reveal rich biological information, and distinguish clear cell clusters (data not shown).

The average library size was 586 bp, and it was multiplexed with another five libraries and sequenced in two NextSeq runs (NextSeq 500 High Throughput 75 cycle v2 kit). A total of 154,518,684 reads were obtained for the library (Step 167). Barcodes associated with actual cells were selected by manually placing a threshold in the barcode abundance histogram shown in **Figure 9c**. This filters out barcodes represented by small numbers of reads that are due to RNA contamination in the cell sample and/or premature barcoding primer cleavage during transcriptome barcoding in drops (Steps 93–126). The number of retained barcodes—which corresponds to the number of cells detected—should agree with the estimated library size. For example, we detected 1,725 cells in the library described here. After filtering for barcode abundance and read quality, 85,196,231 reads were retained for alignment to the reference transcriptome and gene expression quantification. The total number of UMI-filtered molecules (UMI-FMs) was 5,098,601, resulting in 2,956 UMI-FMs per cell on average. With this particular sample, this was sufficient to address the biological questions of interest. On average, each UMI-FM was represented by 10.1 aligned reads. However, the shape of the oversequencing histogram (**Fig. 9d**) suggests that deeper sequencing would allow the detection of additional unique molecules because a large fraction of UMI-FMs are represented by just one read.

The results of sequencing one specific library are described above; **Table 4** summarizes sequencing results of two projects focusing on cells of different origin and showing results at a scale of tens of thousands of cells.

TABLE 4 | Examples of inDrops RNA-seq results using cells of different origin and different per-cell sequencing depth.

Type of sample	Mouse primary immune cells (data not shown)	Mouse embryonic stem cells (data from Klein *et al.*[6])
Flow-estimated number of barcoded cells (Steps 124 and 130)	35,400 (18 libraries of 1,000–2,400 cells)	1,000
Sequencing platform	NextSeq	HiSeq
Number of cycles (transcript read/index read/cell barcode read)	35/6/51	119/0/54
Total number of raw reads across all libraries	1,554,529,911	602,681,732
Number of cells detected	25,408	919
Number of raw reads per cell	61,183	655,802
Percentage of reads used for alignment after filtering	53%	39%
Percentage of reads aligned to reference and used to generate UMI-FM counts.	58%	54%
Number of reads used to generate UMI-FM counts per cell	18,802	136,930
Number of UMI-FMs per cell	2,350	14,508
Average number of reads per UMI-FM	8.0	9.4

UMI-FM, unique molecular-identifier-filtered molecule.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS A.M.K. and L.M. developed the original inDrops method. A.M.K., A.V., and V.S. developed the bioinformatic pipeline for raw sequencing data analysis. R.Z., A.M.K., and L.M. analyzed data provided in the Anticipated Results. D.Z. designed library PCR primers and custom sequencing primers. R.Z., J.N., A.M.K., and L.M. wrote the manuscript.

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