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Miniaturization and Automation of a Full-length Single-cell RNA-seq Workflow

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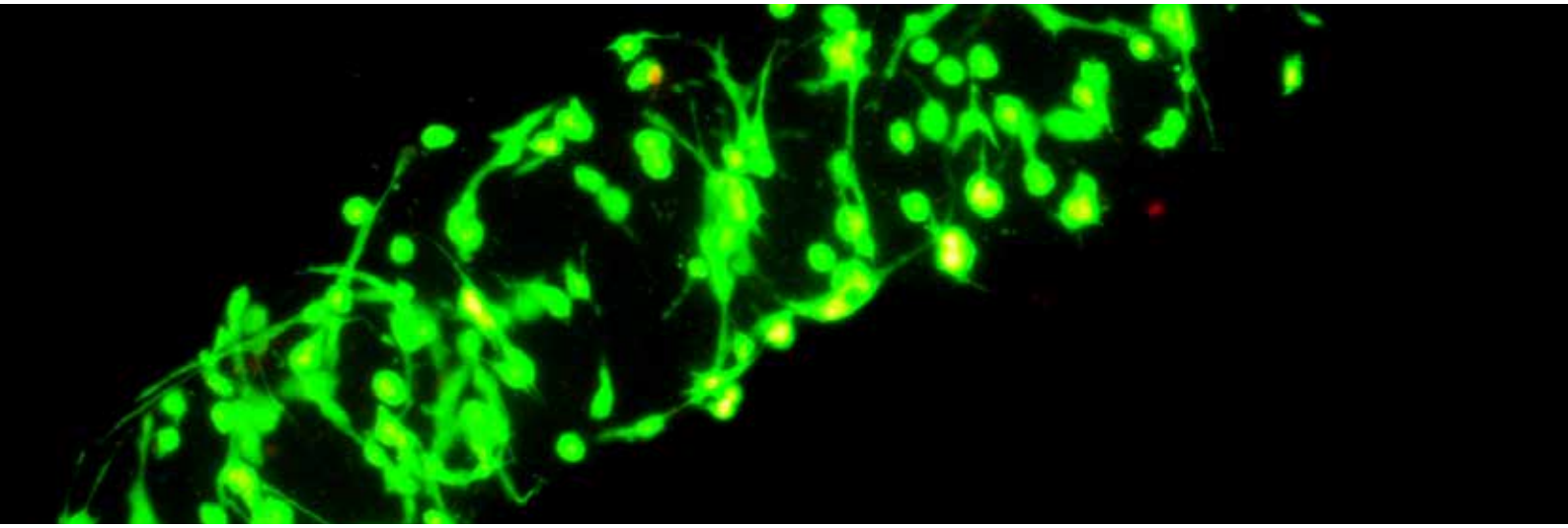

Abstract

Despite significant advances in microfluidic technologies, sensitive single-cell RNA sequencing workflows that generate full-length cDNA still remain plate-based. Miniaturization of these plate-based workflows has been increasingly implemented to reduce costs and increase throughput. We introduce a miniaturized, highthroughput workflow in which single HEK293FT cells were sorted by the f.sight™ into 384-well PCR plates, then libraries were prepared with the I.DOT. Using this workflow, the SMART-seq and NGS library prep protocols, including cDNA amplification, tagmentation and library indexing, were performed at 10-fold reduced reaction volumes. Cells were lysed in volumes as low as 1 µL thanks to the f.sight's precise cell dispensing.

Furthermore, the I.DOT's low-volume dispensing enabled the miniaturization of the library prep. We found that the cDNA concentrations correlated with the cell size, which can aid when selecting the optimal number of PCR cycles in cDNA amplification for a given sample. This miniaturized workflow generated sufficient cDNA for tagmentation, and the cDNA and tagmented cDNA had the expected size distribution. There were 125 cells sequenced and more than 94% of the reads mapped to the human reference genome. Additionally, nearly 10,000 genes per single cell were detected at 1 million reads per cell. Using the f.sight and I.DOT combination to create a workflow that is readily compatible with standard SMART-seq protocols also gives one the flexibility to establish other plate-based library prep protocols.

Introduction

Single-cell RNA sequencing (scRNA-seq) enables the characterization of gene expression at the single cell level. The standard scRNA-seq library preparation is traditionally performed manually, which is low-throughput and time consuming, especially as the number of cells assayed increases. Thus, there is a demand for the automation and miniaturization of plate-based scRNA-seq workflows to process larger numbers of cells and reduce costs while maintaining high-quality sequencing data. The scRNA-seq library workflow includes 1) sample prep; 2) single-cell isolation; 3) library prep; and 4) sequencing and data analysis (Figure 1). Single-cell isolation and library prep are two critical steps in the workflow as errors can have a negative effect on the resulting data. For single-cell isolation, it is important that cells are gently isolated to maintain viability as this significantly impacts data quality. Reaction miniaturization requires the precise dispensing of cells into reduced lysis volumes in the PCR well plates to ensure the RNA is released and available for subsequent steps. It is also important to have confirmation that only single cells are used for analysis. For library prep miniaturization, low volumes of reagents must be precisely and accurately dispensed into the PCR well plates to minimize technical variation between samples. As the number of cells increases, the automation of complicated liquid handling tasks such as normalization of DNA concentration or indexing of samples becomes more essential. Thus, the selected reagent dispensing method influences several metrics, including the number of samples processed, preparation time and sequencing data quality.

Here, we present a complete, miniaturized and automated scRNA-seq workflow using the f.sight for singlecell isolation and the I.DOT for library prep of HEK293FT cells in 384-well PCR plates. We showed that the f.sight can precisely dispense single cells into 1 µL of lysis buffer. Additionally, the f.sight provided visual confirmation that a single cell was dispensed. Using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio), we demonstrated that the I.DOT can perform miniaturized cDNA library prep at 1/10th the volume of a standard reaction. We discovered that the cell diameter, provided by the f.sight, correlated with cDNA concentration, and that the cDNA libraries had correct size distribution. The Nextera XT DNA Library Preparation Kit (Illumina) was used for next generation sequencing (NGS) library prep at 1/10th the standard reaction volume. Intricate tasks such as normalization and indexing were performed in minutes. The I.DOT generated sufficient and co rectly sized tagmented cDNA for sequencing. We pooled and sequenced 125 cells on the Illumina NextSeq 500 Sequencing System. We achieved high quality sequencing data with more than 94% of the reads mapped to the human reference genome, and almost 10,000 genes were detected in each individual cell at 1 million reads per cell. The combination of the f.sight and I.DOT creates a fast, flexible and efficient workflow to perform library prep of thousands of single-cell libraries in a day, making this time-saver accessible to the general lab user.

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Figure 1. *Miniaturized and high-throughput workflow for generating single-cell RNA sequencing libraries. After sample preparation, the f.sight isolates single cells into PCR well plates, which undergo library prep with the I.DOT. The resulting libraries are sequenced and further analyzed.*

reduce costs while maintaining high-quality sequencing data. The scRNA-seq library workflow includes 1) sample prep; 2) single-cell isolation; 3) library prep; and 4) sequencing and data analysis (Figure 1). Single-cell isolation and library prep are two critical steps in the workflow as errors can have a negative effect on the resulting data. For single-cell isolation, it is important that cells are gently isolated to maintain viability as this significantly impacts data quality. Reaction miniaturization requires the precise dispensing of cells into reduced lysis volumes in the PCR well plates to ensure the RNA is released and available for subsequent steps. It is also important to have confirmation that only single cells are used for analysis. For library prep miniaturization, low volumes of reagents must be precisely and accurately dispensed into the PCR well plates to minimize technical variation between samples. As the number of cells increases, the automation of complicated liquid handling tasks such as normalization of DNA concentration or indexing of samples becomes more essential. Thus, the selected reagent dispensing method influences several metrics, including the number of samples processed, preparation time and sequencing data quality.

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Materials and methods

Cell culture conditions and single-cell isolation

scRNA-seq was performed with HEK293FT cells (Thermo Fisher). Cells were cultured in DMEM high-glucose (Sigma-Aldrich) that was supplemented with 10% fetal bovine serum (Gibco) and 1% of the following: 10 mM NEAA (Gibco), 200 mM GlutaMAX (Gibco) and 10,000 U/mL Penicillin-Streptomycin (Gibco). Cells were cultured at 37oC at 5% CO2. Prior to single-cell isolation, cells were harvested with TrypLE (Gibco), washed with PBS and resuspended in PBS at a concentration of 1 x 106 cells/mL. Cell viability was determined and only samples that had a cell viability >90% were processed. Using a pipette fifty µL of the cell suspension was added to a dispensing cartridge and placed on the f.sight for cell dispensing. Plates with single cells were snap-frozen on dry ice and kept at -80oC until processing.

SMART-seq v4 and Nextera library preparation with the I.DOT

The I.DOT was used to perform scRNA-seq library prep with 1/10th of the original reaction volume of Takara Bio's SMART-seq v4 Ultra Low Input RNA Kit for Sequencing, which provides full-length cDNA with high sensitivity. Samples were annealed at 72oC for 3 minutes and placed on ice. The PCR program for first-strand synthesis and cDNA were performed as specified by the user manual for the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, 2019). A total of twenty PCR cycles were used for amplification followed by bead cleanup (AMPure XP, Beckman). cDNA was resuspended in 10 µL of water. The cDNA was normalized to 0.2 ng/µL with the I-DOT and was tagmented using the Nextera XT DNA Library Preparation Kit (Illumina) to prepare the samples for sequencing. Tagmentation was also performed at 1/10th the original volume. The tagmented cDNA underwent a final quality control (QC) and was pooled and processed for sequencing.

cDNA quality control

After the amplification of both the cDNA and tagmented DNA, the concentrations of the samples were measured with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) using 384-well microplates. Per well, 1 µL of a single cell's cDNA library was added to 20 µL of working solution. Lambda DNA was used as a control and serially diluted via the I-DOT to create the standard curve. The PicoGreen fluorescent intensity was measured by the Spark Microplate Reader (Tecan). To characterize the size distribution of cDNA and tagmented cDNA fragments, samples were processed through the Agilent Bioanalyzer.

Sequencing and data analysis

The pooled library was denatured and diluted as specified in the NextSeq 500 System Guide for high output kits. A total volume of 1.3 mL at 1.8 pM was loaded onto the sequencer. Here, we sequenced 75 bp single end (SE) reads. FASTQ files were demultiplexed and analyzed at 1 million reads per cell with zUMIs (Parekh, 2018). Sequences were aligned against the GRCh38.99 human reference genome.

Results and discussion

Precise dispensing of single cells in PCR well plates

The f.sight reliably isolated and dispensed single cells into low volumes of lysis buffer—a critical step for assay miniaturization. Here, 384-well PCR plates were prefilled by the I-DOT with 1 µL of lysis buffer. The harvested HEK293FT cells were resuspended in PBS at a concentration of 1 million cells per 1 mL. As stated, fifty µL of cell suspension was pipetted into a single-use cartridge for dispensing—sufficient to fill many plates. The smaller sample volume required by the f.sight makes it ideal when working with costly samples. Many protocols recommend resuspending cells in PBS as culture media can interfere with first-strand synthesis. However, PBS in larger quantities can also interfere with cDNA synthesis and PCR amplification (Takara Bio, 2019). Thus, the sorting buffer should be minimized in the reaction, which becomes more difficult as the total reaction volume is reduced. The f.sight generates small, free-flying droplets, which are only ~200 pL, ensuring that the PBS will have minimal impact on the assay performance and enabling extensive miniaturization of established protocols.

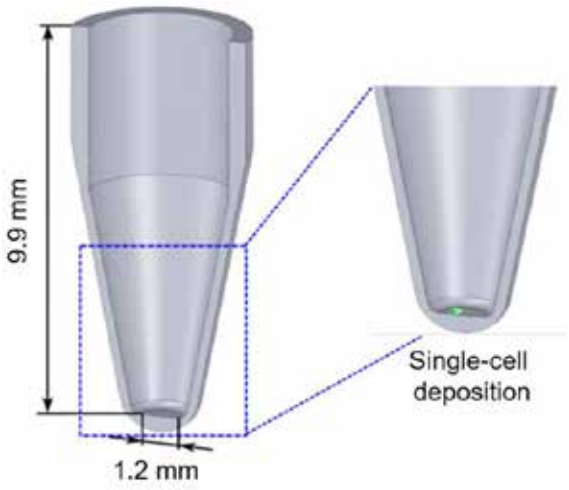

Figure 2. *The precise dispensing of single cells into the PCR well plates is critical for miniaturizing library prep. The f.sight uses an ionizer to remove electrostatic charges, which can deflect the droplet. The automated offset correction (AOC) accounts for any deviation from the target position. This enables precise cell dispensing without the need for manual alignment.*

SMART-seq v4 (Takara) per reaction	Full volume (µL)	1/10th volume (µL)
Lysis		
10x reaction buffer	1	0.1
Cell	1	~0.0002 (droplet)
dH_2O	8.5	0.950
Total	**10.5**	**1.05**
1st Strand cDNA Synthesis		
CDS Primer	2	0.2
5x first strand buffer	4	0.4
SMART-Seq v4 oligonucleotide	1	0.1
Rnase inhibitor	0.5	0.05
SMARTscribe RT	2	0.2
Total	**9.5**	**0.95**
cDNA Amplification		
2X SeqAmp PCR buffer	25	2.5
PCR Primer IIA	1	0.1
SeqAmp DNA polymerase	1	0.1
dH_2O	3	0.3
Total	**30**	**3**

Table 1. *Volumes of reagents in the SMART-seq v4 Kit in the standard reaction (30 µL) and 10-fold reduction (3 µL).*

While small droplet size is advantageous for miniaturization, these small droplets are more prone to deflection. The f.sight houses an ionizer to minimize electrostatic charges that could affect the droplet position. The f.sight is also equipped with a module for automated offset correction (AOC), which measures droplet position on a sensor. A target position on the sensor represents the center of a PCR well plate. Ideally, the droplet will land on the target, however, there are slight differences that can cause a droplet to offset. If the sensor detects an offset, this offset will be corrected so that the droplet will land at the center of the PCR well plate (Figure 2) (Riba, 2016). This ensures precise and accurate dispensing of single cells to plates with low volume of lysis buffer to ensure the cells are lysed, without the need to manually align the droplet to the PCR well plate.

To dispense the HEK293FT cells, a diameter range of 10 to 25 µm and a roundness of 0.5 to 1 were selected. During dispensing, cells were continuously imaged and an algorithm was used to select only for single cells that fall within the specified parameters (Stumpf, 2015; Gross, 2013). The few wells where multiple cells were unintentionally dispensed were easily determined by reviewing the images and, thus, excluded from further library preparation. The f.sight can also readily dispense multiple cells for a positive template control (PTC) or an empty droplet for a negative template control (NTC).

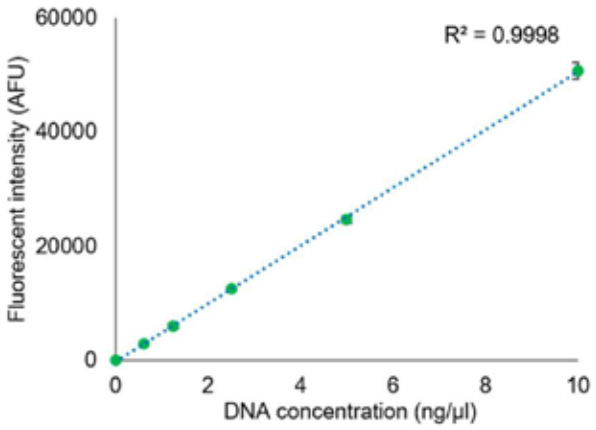

Figure 3. *The PicoGreen fluorescent assay used to generate the standard curve was miniaturized 100-fold. The I.DOT generated precise dilutions of lambda DNA with water, which ensured accurate calculation of the single-cell cDNA library concentrations.*

cDNA library generation with SMART-seq v4 Ultra Low Input RNA Kit and quality control

The I.DOT's low-volume dispensing successfully generated cDNA single libraries at 1/10th the volume of a standard reaction. cDNA library prep requires cell lysis, annealing, first-strand cDNA synthesis and PCR amplification of the cDNA (Table 1). Here, a standard reaction volume for SMART-seq v4 is a total of 30 µL, which was reduced to 3 µL by using the I.DOT to dispense the reagents. The reagents required for cDNA prep were preloaded into a source well plate. After dispensing the first-strand reagents, samples were placed on a cycler as specified in the SMART-seq v4 user manual for reverse transcription (Takara Bio, 2019). Next, the I.DOT dispensed the amplification reagents and the cDNA was amplified. Bead cleanup was performed, and the cDNA was resuspended in 10 µL of water and transferred to a fresh plate for QC.

QC was performed on the single-cell libraries to determine the cDNA concentration and the size distribution of the cDNA fragments. To measure the cDNA concentration, the PicoGreen fluorescence assay was used at 1/100th the volume of the standard reaction. Each single-cell library was placed into a source well plate and 1 µL was dispensed by the I.DOT into 20 µL of the PicoGreen working solution per well in a 384-well microplate. To calculate the concentrations, the I.DOT diluted the lambda DNA with water to generate the standard curve. Figure 3 shows a triplicate dilution series of the lambda DNA. The standard deviation between the triplicates was very low, signifying high reproducibility between dispensing runs. The I.DOT accurately dispensed reagents, which resulted in a correlation coefficient of 99.98% between the DNA concentration and fluorescent intensity. All libraries were measured with a microplate reader, and the unknown cDNA concentrations were determined via the standard curve. It is important to generate a reliable standard curve to ensure the calculated cDNA volumes are accurate.

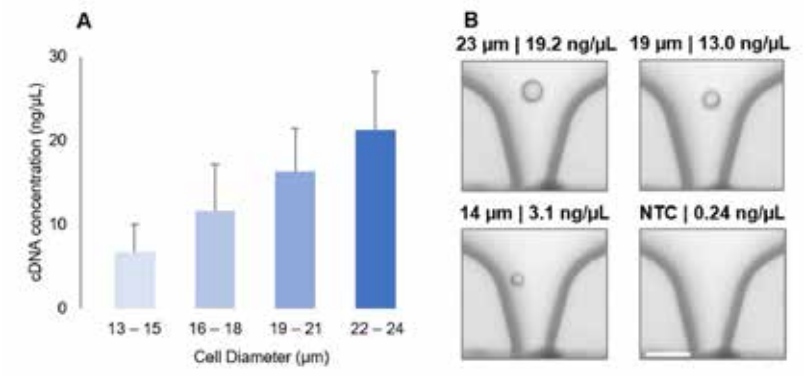

Figure 4. *(**A**) Cell size correlated with the cDNA concentration. (**B**) Representative images of single cells captured by the f.sight before dispensing and the cell size and corresponding cDNA concentrations. The negative template control (NTC) had minimal cDNA. Scale bar = 40 µm.*

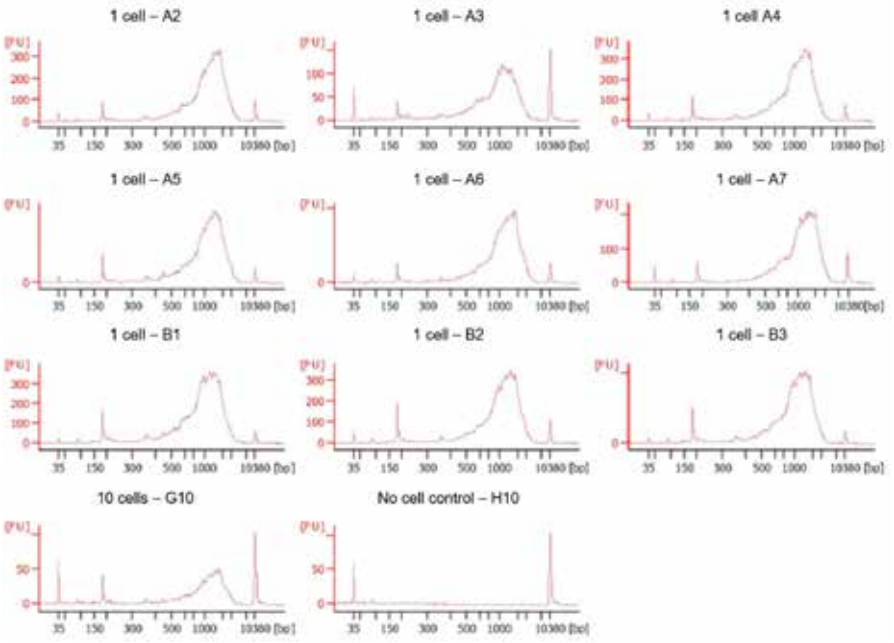

Figure 5. *Representative Bioanalyzer traces of single cells, a positive control (10 cells) and negative control (no cell). The miniaturized workflow produced single-cell libraries with the expected size distribution between 400 bp to 10, 000 bp and a peak at ~2,500 bp. No product was observed with the negative control.*

We found that the cell size correlated with the amount of cDNA generated (Figure 4A). Since the f.sight measures the diameter for each dispensed cell, the average cell size for the population can be determined and used to inform the number of PCR cycles required for cDNA amplification. The selected number of PCR cycles must be sufficient to proceed to tagmentation but not excessive to minimize bias. For our samples, the average cell diameter was 18 µm. However, the cell diameters ranged from 13 to 24 µm. Figure 4B shows representative example images of the single cells in the nozzle as detected by the f.sight during isolation and their corresponding cDNA concentration.

It is also important that the miniaturized samples result in a similar cDNA size distribution as a standard reaction with minimal sample degradation. For SMART-seq v4 library prep, the expected peak should be ~2,500 bp, and this was achieved in the miniaturized reactions. Figure 5 shows representative Bioanalyzer traces of single cells, the PTC and the NTC. Thus, the samples passed QC for both cDNA concentration and size distribution.

NGS library prep for sequencing with Nextera XT Kit

The I.DOT can easily normalize cDNA concentrations and index samples for NGS. The tagmentation reaction volume was also reduced 1/10th from a standard reaction volume of 50 µL to 5 µL (Table 2). Here, the source well plate that contained the cDNA libraries for concentration measurements was also used for normalization. By using the calculated cDNA concentrations, the I.DOT normalized the samples with water.

Figure 6. *(**A**) I.DOT software highlighting the normalization of cDNA (blue) with water (green) for each well in the 384-well plate. (**B**) I.DOT software can quickly index individual samples. Unique barcodes from the Nextera Kit (N7XX and S5XX) are defined in the software and the corresponding target well plate schematic that shows a unique set of indexes for each well.*

Nextera XT (Illumina) per reaction1	Full volume (µL)	1/10th volume (µL)
Tagmentation		
cDNA sample	5	0.5
TD buffer	10	1
ATM	5	0.5
Total	**20**	**2**
NT		
Tagmentation reaction	20	2
NT buffer	5	0.5
Total	**25**	**2.5**
Indexing Amplification		
Tagmentation reaction	25	2.5
Primer 1 (N7XX)	5	0.5
Primer 2 (S5XX)	5	0.5
NPM	15	1.5
Total	**50**	**5**

Table 2. *Volume of reagents in the Nextera XT Kit in the standard reaction (50 µL) and 10-fold reduction (5 µL).*

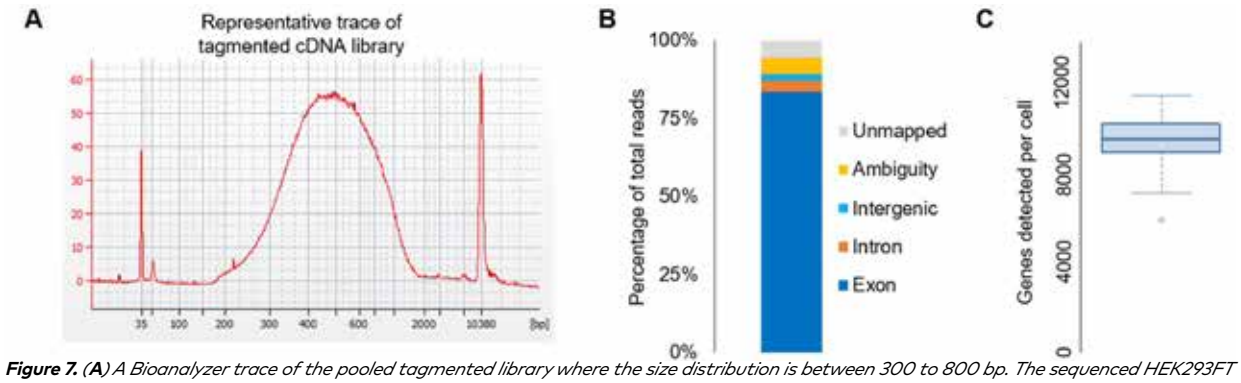

Figure 7. *(**A**) A Bioanalyzer trace of the pooled tagmented library where the size distribution is between 300 to 800 bp. The sequenced HEK293FT libraries had more than 94% of the reads mapped to the human reference genome, (**B**) and nearly 10,000 genes were detected in each individual cell at 1 million reads per cell (**C**).*

The libraries were adjusted to a concentration of 0.2 ng/µL, and 0.5 µL of each library was dispensed into a fresh plate for tagmentation. Figure 6A shows an image from the I-DOT software with 96 samples that were normalized. At the bottom right, the blue bars represent the sample volumes and the green bars are the water volumes dispensed to achieve the 0.2 ng/µL concentration in 0.5 µL. This is also represented in Figure 6A at the top right of the target well plate schematic.

After the tagmentation reaction, the samples were indexed to differentiate the single-cell libraries. Figure 6B shows an image from the I-DOT software of 96 samples that were indexed in a 384-well plate. Indexes required for barcoding (12 of the N7XX indexes and 8 of the S5XX indexes) were placed into the source wells. The dispensing program ensures that each well contains a unique set of indexes. The barcoding is automated and fast where 96 samples were barcoded in ~1 minute. Since the I-DOT can hold 96 different samples, the complex barcoding can be performed efficiently. A single I-DOT source well plate is sufficient to process dozens of 384-well plates with cDNA libraries. After indexing, the sample underwent a final PCR. Samples were pooled and the tagmented cDNA concentration was measured and analyzed by the Bioanalyzer to determine library size.

With 1/10th reaction volume miniaturization, **Figure 7A** shows the Bioanalyzer trace of the library with a size distribution of 300 to 800 bp, which indicates a successfully tagmented library for sequencing.

Sequencing and data analysis

The pooled library was denatured and diluted as specified in the NextSeq protocol. The library was sequenced 75 bp single end (SE) on a high output flow cell using the NextSeq 500 System (Illumina). After conversion to the FASTQ file format, the zUMIs pipeline was setup for filtering, demultiplexing, mapping and gene counting. Figure 7B shows that more than 94% of the reads of the 125 single HEK293FT cells mapped to the human reference genome and almost 10,000 genes per cell were detected on average (**Figure 7C**).

Conclusions

- When combined, the f.sight and I.DOT can create an automated, high-throughput workflow that can readily miniaturize standard plate-based protocols and generate high-quality sequencing data.
- Reaction miniaturization is enabled by the precise cell dispensing into low lysis volumes by the f.sight and the low volume dispensing of the I.DOT.
- It was found that cell size correlated with cDNA concentration and that the cell size data generated by the f.sight can be used to determine the optimal number of cycles for cDNA amplification.
- The I.DOT easily performed automated normalization and indexing of samples for further highthroughput processing.
- Due to the f.sight's gentle cell isolation and low input volume requirements, the workflow is ideally adaptable to work with high-value samples such as primary cells derived from patients or mice.

References

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