Application Note:

High quality and sensitivity 3' scRNA-seq libraries in nanoliter volume

Abstract

Single-cell RNA sequencing (scRNA-seq) assays must combine both sensitivity and accuracy to capture and reverse-transcribe diverse transcripts in their relative proportions from a single cell. In this study, it was shown that combining *cellenONE*® for single cell isolation and nanoliter reagent dispensing with *cellenCHIP*™ as a nanowell substrate for library preparation provided an ideal platform for cost efficient and high sensitivity transcriptomic analyses from single cells.

Materials & Methods

cellenCHIP™ is comprised of four 96-wells arrays that are each barcoded with 96 unique oligo dT primers using *sciFLEXARRAYER S3* (SCIENION AG, Germany) and air-dried overnight. The chips were barcoded in large batches and stored. Each well contained a unique oligo dT primer with a preserved sequence for cDNA amplification, an individual well barcode (WB) and unique molecular identifier (UMI) to respectively trace back sequencing reads to each corresponding well and quantify the number of reads for each transcript. The oligo barcodes were rehydrated through dispense of lysis and reverse transcription (RT) buffer containing template-switch oligos (100 nl/well) using cellenONE® (Cellenion, France). Single human and mouse cells (HEK293T and NIH3T3) were then isolated using cellenONE® into prefilled wells. A checkerboard pattern of human and mouse cells containing different positive and negative controls was used. For each set of 96 wells, 3 positive controls for each cell type were prepared by dispensing exactly 5 cells per well. Four different negative controls (3 wells per control) were prepared as follow: (i) dispensing a single human cell without RT enzyme, (ii) no cell dispensing, (iii) dispensing culture medium without any cell, and (iv) dispensing a single human cell with RT buffer that contained RNase. After cell isolation, wells were sealed (Microseal 'F' and 'B', Bio-Rad, USA) and incubation was performed on an *in situ* block inside a GS1 thermocycler (G-storm, UK) at 42°C for 90 min. After unsealing, *cellenCHIP*™ was inverted and barcoded cDNAs from 96 single cells were pooled by centrifugation into a recovery funnel prior to transfer to microcentrifuge tubes. The cDNA was amplified for a maximum of 18 PCR cycles, and amplified cDNA was used to generate Illumina sequencing libraries with one-sided tagmentation and PCR amplification. The libraries were then QC'd for size distributions (Bioanalyzer, Agilent) and sequenced using pair-end 18-8-50 (NextSeq 500, Illumina).

Results and Discussion

To investigate sensitivity and reproducibility of the protocol, two independent biological replicates (Rep_1 and Rep_2) were prepared using single human and mouse cells dispensed in a checkerboard pattern across each array. Sequencing reads were demultiplexed and traced back to each isolated single cell thanks to known well barcode (WB). The **total mapped reads per cell in each replicate were 92,000 and 147,000 on average**, with mapped reads from individual cells ranging from about 50,000 to 200,000 for the first replicate and about 50,000 to 350,000 for the second replicate (Figure 2).

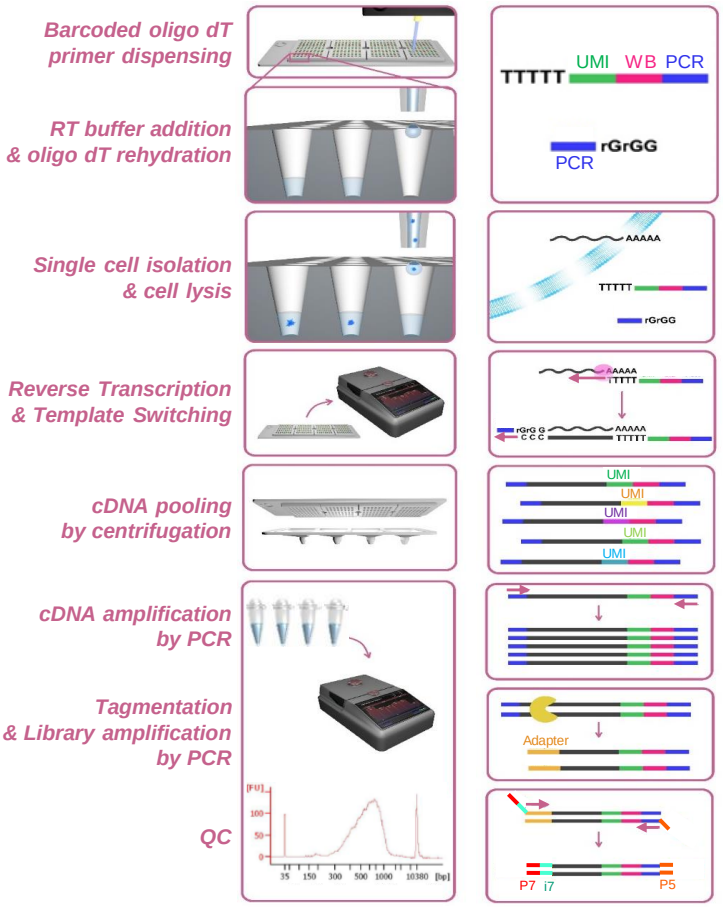

Figure 1. *3' RNA-seq workflow in cellenCHIP*™*.*

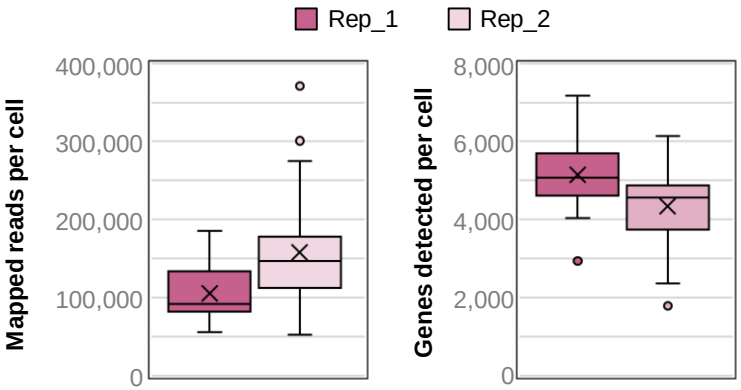

Figure 2. *Sequence data metrics were calculated for human single cells from the HEK293T cell line, where the box boundaries indicate 25th and 75th percentiles; center line represents the median, cross indicates the mean, whiskers shows ± 1.5x interquartile range, while points are actual values of outliers, with n = 36 for Rep_1 and n = 34 for Rep_2.*

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These mapped reads were then used to identify expressed genes in each cell (genes with at least 1 UMI). On average, the protocol **achieved a coverage of about 4,500 to 5,000 genes per single cell**, and a maximum of **up to 7,000 genes per single cell** (Figure 2), which underlies the **high sensitivity of this 3' RNA-seq protocol** for gene detection. **High reproducibility** was also observed between the two biological replicates.

Usage of UMIs in the oligo dT primers is crucial to eliminate effects of PCR amplification bias, which is particularly important in single cell studies where many PCR cycles are often required for whole transcriptome amplification. After PCR, molecules sharing the same UMI were assumed to be derived from the same input molecule.

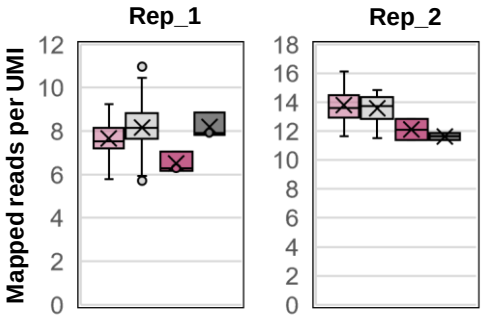

Figure 3. *The number of mapped reads per UMI for human single cells (left boxplot), mouse single cells (2nd boxplot), 5 human cells (3rd boxplot), and for 5 mouse cells (right boxplot) for each technical replicate (Rep_1, Rep_2), where the box boundaries indicate 25th and 75th percentiles; center line represents the median, cross indicates the mean, whiskers shows ± 1.5x interquartile range, and points are actual values of outliers, with n = 36 for Rep_1 and n = 35 for Rep_2*

To examine the quality of this 3' RNA-seq protocol, the number of mapped reads per UMI were calculated for both single cell containing wells and positive control wells. **High reproducibility within the biological replicates** were observed, with on average, 7 and 13 mapped reads per UMI for the first and second replicates, respectively (Figure 3).

To further investigate the quality and robustness of this 3' RNA-seq protocol using both ***cellenCHIP™*** as a nanowell substrate and ***cellenONE®*** as a nanolitre and single cell dispenser, a checkerboard pattern of human and mouse cells containing different positive and negative controls was included.

For each well, the percentage of reads per cell that mapped human and mouse genomes was calculated to determine if any contamination between wells occurred. For all wells, **the vast majority of reads per cell only mapped to the corresponding genome** with human transcript found in wells containing human cells and vice versa for wells containing mouse cells (Figure 4a). Such observations confirmed that **no detectable cross contamination** between ***cellenCHIP™*** wells or within ***cellenONE®*** dispensing nozzle occurred while performing this protocol.

Lastly, number of genes detected per cell were compared for single cell containing wells, positive controls (5 cells per well), and negative controls. As expected, **more genes were detected for the positive control wells** (Figure 4b) which contained multiple cells and thus lead to production of more cDNA per well. Additionally, **very few genes were detected across all four negative controls** (Figure 4b) and their number were consistent within the four different controls. Such observations confirmed the absence of contamination between wells and within buffers, absence of gDNA amplification, and absence of free floating mRNA in the cell suspension.

Altogether, these observations highlight the **quality, sensitivity and reproducibility of this 3' RNA-seq protocol**.

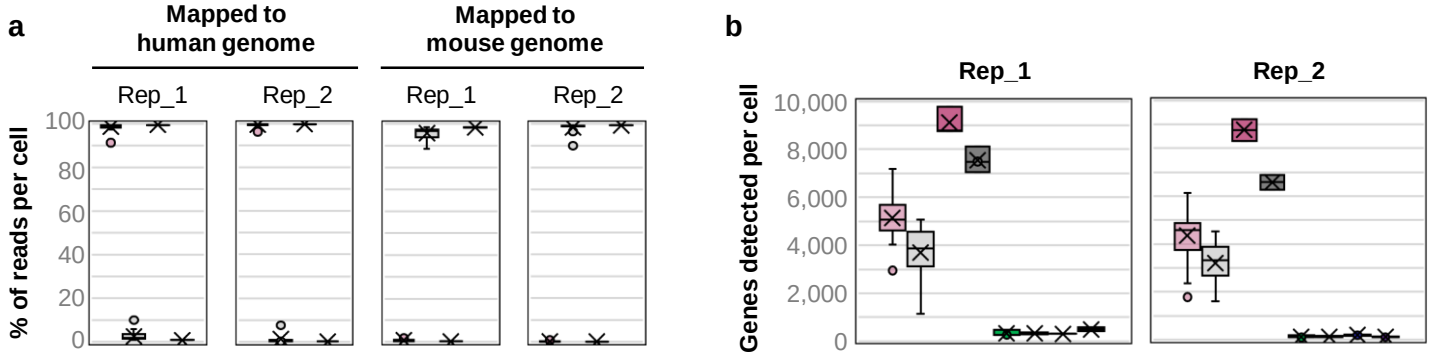

Figure 4. a. *Percentage of reads per cell that uniquely mapped to human genome (left panel) or mouse genome (right panel) for human single cells (left boxplot), mouse single cells (2nd boxplot), 5 human cells (3rd boxplot), and for 5 mouse cells (right boxplot).* ***b.*** *Number of detected genes per cell for human single cells (left boxplot), mouse single cells (2nd boxplot), 5 human cells (3rd boxplot), 5 mouse cells (4th boxplot), human single cells without RT enzyme (5th boxplot), no cells and no culture medium (6th boxplot), no cells with culture medium (7th boxplot), and for human single cells with RNase (right boxplot) for each technical replicate (Rep_1, Rep_2). The box boundaries indicate 25th and 75th percentiles; center line represents the median, cross indicates the mean, whiskers shows ± 1.5x interquartile range, and points are actual values of outliers.*

Conclusions

This 3' RNA-seq protocol combining ***cellenONE®*** technology for nanoliter dispensing and single cell isolation and ***cellenCHIP™*** as nanowell substrate enabled miniaturization of **whole transcription amplification in nanoliter volume** to generate **data with high sensitivity and low background**.

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