Comparison of Evercode™ WT v4 and Chromium™ Single Cell Gene Expression Flex v2 (Apex) Kit in Fixed Human PBMCs

Introduction

A head-to-head evaluation of single cell RNA profiling technologies with fixation was performed between droplet-based microfluidics (10x Genomics™ Chromium Single Cell Gene Expression Flex Apex Kit) and combinatorial barcoding (Parse Biosciences Evercode WT v4). The evaluation was conducted using fixed cells from human PBMCs, a widely used sample type in scRNA-seq benchmarking studies without tissue dissociation.

Comparison Highlights

- Head-to-head comparison shows >4x higher overall cell retention.

- >60% higher median transcripts detected per cell including diverse RNA biotypes.

- Probe-based methods produce inconsistent measurements in comparison to RT-based methods.
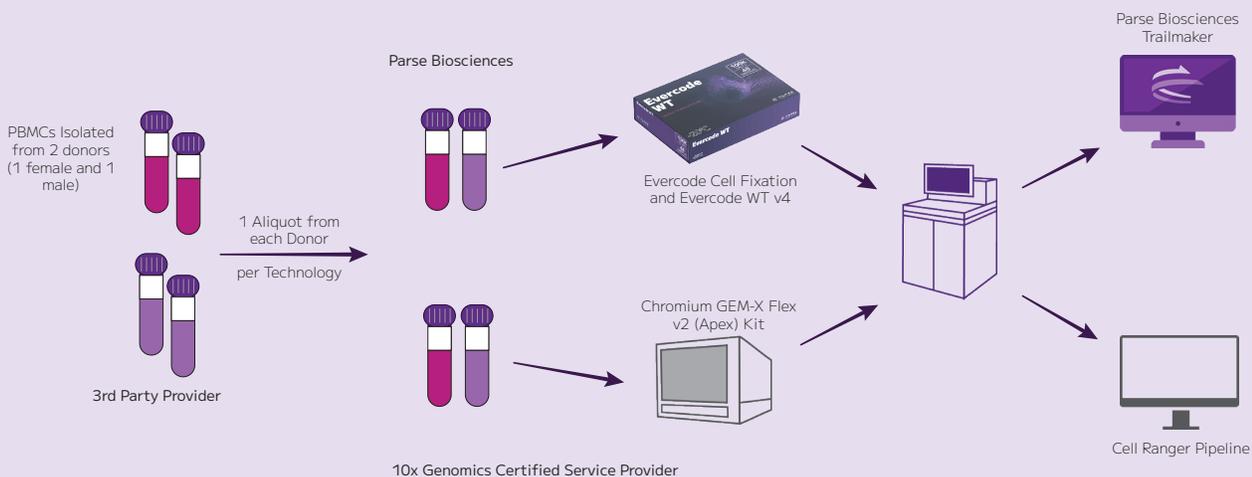
EXPERIMENTAL DESIGN

Figure 1. Experimental Design. Frozen PBMCs from two donors were obtained from a third-party provider. Aliquots derived from each donor were distributed to separate laboratories for processing. One aliquot per donor was shipped to a 10x Genomics Certified Service Provider for cell preparation and library construction using the Chromium GEM-X Flex v2 (Apex) kit. Parallel aliquots were processed by Parse Biosciences using the Evercode WT v4 workflow. Sequencing libraries from both technologies were sequenced on the same sequencer model, and data were analyzed using each manufacturer's respective analysis pipeline.

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A. Reverse Transcription-Based Capture Design: Evercode WT v4
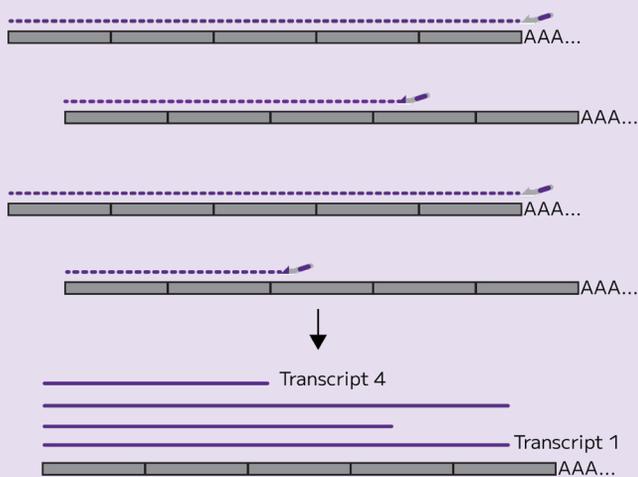
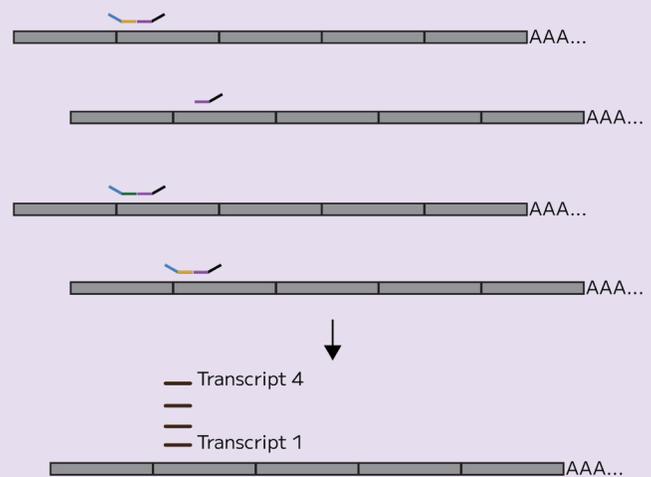B. Probe-Based Capture Design: Chromium Flex v2 (Apex)

Figure 2. Overview of Two Different Gene Capture Methods for Single Cell Gene Profiling Assays. (A) Evercode WT v4 employs poly-dT and random hexamer primers to capture genes which cover known and novel or uncharacterized transcripts with broad gene body coverage. (B) The probe-based method targets predetermined regions in the transcriptome designated for protein-coding genes. Mapped reads by Chromium Flex v2 is limited to probe-target regions (<100 bp).

Methods

Sample Collection

PBMCs were obtained as frozen cryovials from a third-party provider. Aliquots from two donors (one female, one male) were distributed to participating labs for processing and library preparation. To ensure comparable starting material across technologies, all workflows used aliquots from the same donor cryovial lots.

10x Genomics Chromium Flex v2

A Chromium GEM-X Single Cell Flex v2 & Gel Bead Kit was used to fix, partition and prepare sequencing libraries. All activities were performed according to the vendor procedures.

Parse Biosciences Evercode WT v4

The cells were fixed with Evercode Low Input Cell Fixation v4. Whole transcriptome sequencing libraries were prepared using Parse Biosciences Evercode WT v4 (Evercode WT v4) without any alterations to the protocols.

Sequencing and Data Analysis

10x Genomics libraries were sequenced on an Illumina© Novaseq™ X by the certified service provider, and the Parse Biosciences libraries were sequenced on the same instrument model. The 10x Genomics data were analyzed with Cell Ranger™ v10.0.0, and the Parse Biosciences data were analyzed with the Parse Biosciences analysis pipeline v1.7.0.

RNA Biotypes and Correlation Analysis

The genes identified in both Chromium Single Cell Gene Expression Flex v2 (Chromium Flex v2) and Evercode WT v4 were analyzed, along with the RNA biotypes associated with them, sourced from Gencode Release M23. Pseudobulk gene expression correlation analysis was conducted on matching samples processed with Chromium GEM-X Single Cell 3' v4, Chromium Single Cell Gene Expression Flex v2, and Evercode WT v4.

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Higher Cell & Gene Recovery with Evercode

Results

Overall Cell Retention and Assay Time

With a new magnetic bead–based fixation and barcoding workflow, Evercode v4 delivers higher cell recovery, enabling researchers to maximize data from precious samples. Replacing centrifugation with a streamlined bead-based process reduces hands-on time and pipetting steps and is highly automation-friendly, making it accessible to labs with varying levels of single-cell experience. Compared to the centrifugation-based Chromium Flex v2 cumulative workflow, Evercode achieved 4.7× higher overall cell retention while requiring less than half the total assay time (Figure 3).

Sensitivity

Improved sensitivity enhances the detection of lowly expressed genes, leading to a more thorough cell type annotation. At a read depth of 40,000 reads per cell, Evercode WT v4 detected 62% more median transcripts per cell than Chromium Flex v2. Of this increase, 43% came from protein-coding genes and 19% from other RNA biotypes. (Figure 4).
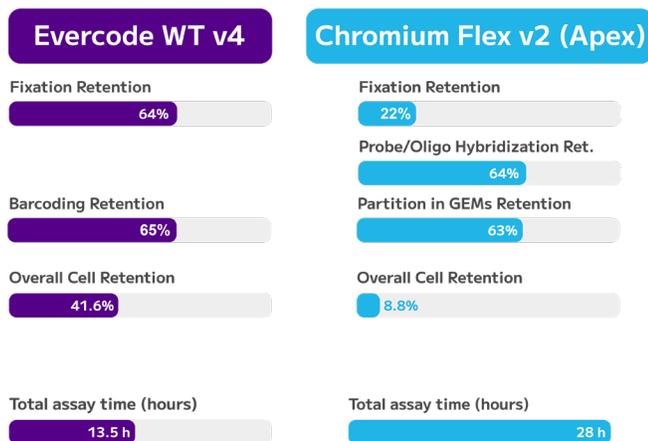

Figure 4. Detected Transcripts and RNA Biotypes Comparison. Total transcripts detected in human PBMCs across sequencing depths, including protein-coding genes and other RNA biotypes. Other biotypes comprise lncRNA, miRNA, snRNA, snoRNA, miscRNA, pseudogenes, and Ig/TCR genes.

RNA Biotypes

Identifying diverse RNA biotypes improves our understanding of gene regulation, cellular function, disease mechanisms, and therapeutic targets. While Chromium Flex v2 is designed to capture only protein-coding genes, analysis of median transcripts detected in human PBMCs showed that Evercode WT v4 consistently detected more genes across all RNA biotypes, including protein-coding genes (Figure 4).

Correlation of Gene Expression Profiles

When choosing to switch assays for sample fixation, it is crucial to thoroughly assess the potential implications and challenges to maintain the integrity and validity of the data. The comparison between RT-based Chromium 3′ v4 and Evercode WT v4, utilizing both fresh and fixed versions of human PBMCs, revealed a strong correlation with r^2 value of 0.87 (Figure 5A; Evercode™ WT v4 and Chromium™ GEM-X

Figure 3. Cell Retention & Assay Time. Overall retention rates were calculated by multiplying stepwise retention across all samples, and total assay times were based on vendor recommendations for four PBMC aliquots.
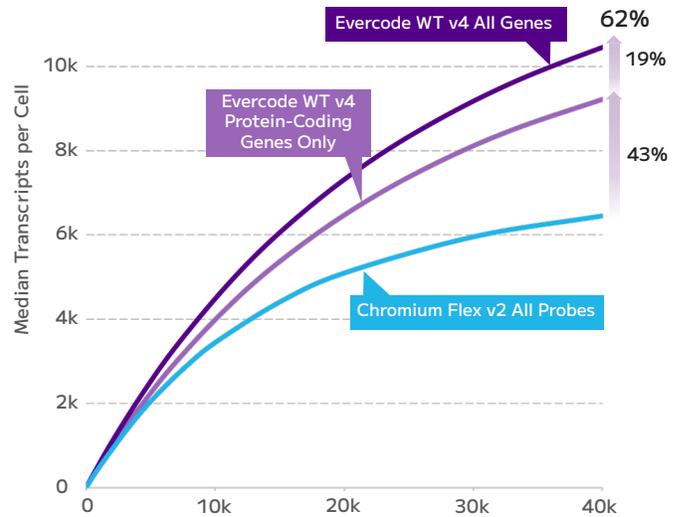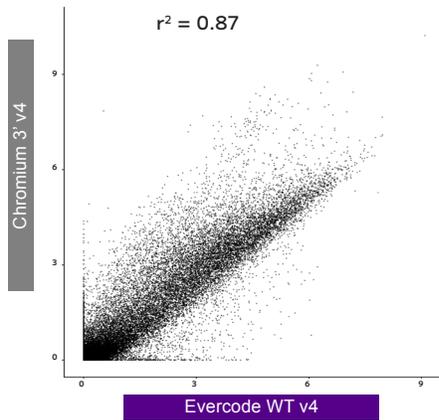
Probe-Based Assays Produce Inconsistent Measurements

A. Reverse Transcription-Based Capture Designs Comparison

$r^2 = 0.87$

Chromium 3' v4 (y-axis) vs Evercode WT v4 (x-axis)

B. Probe-Based vs RT-Based Capture Designs Comparison

$r^2 = 0.73$

Chromium 3' v4 (y-axis) vs Chromium Flex v2 (x-axis)
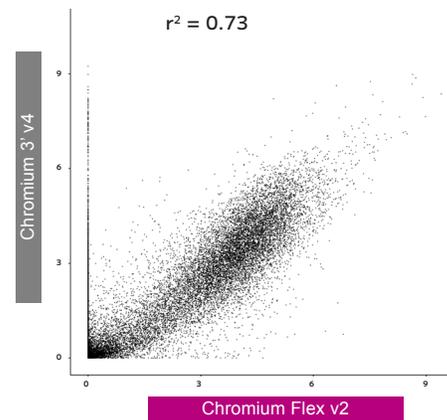
C. Heatmap depiction of genes with significant variations in expression levels between Chromium 3' v4 and Chromium Flex v2

Rows: Evercode WT v4, Chromium 3' v4, Chromium Flex v2

RNA ln(tpm+1)

Genes: TOMM6, IGLC1, EN284976, SRXN1, CD8B2, GP1BB, MUSTN1, H2BC9, WDR83OS, IGFLR1, OOEP, ANGPTL1, DLEU7, PTPRN2, ZNF226, SPAG6, HECW2, PZP, DMGDH, H2BC15, POLD4, CCDC200, A2M, ZNF429, SH3D19, TRIM69, FAM104A, AMZ1, ANKUB1, CFAP210, CC21, ATP8B1, TARS1, MEI1, SLAC4A10, KRBOX5, LRRC37A2, GABARAP, UBE2V2, CNOT7, RBM8A, H2AC6, CACNA2D3, RNF34, CS, ZNF431, NASP, TEX14, POM121C, PPP2R1A, MAPRE1, SRSF10, XRCC6, SINHCAF
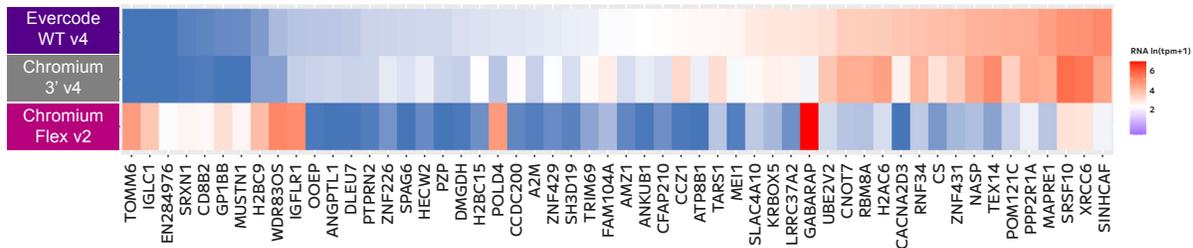
Figure 5. Probe-based Technologies Show Poor Correlation with Other Technologies and Contain Many Outlier Genes.
(A) Pseudo-Bulk correlation of common genes comparing Chromium 3' v4 vs Evercode WT v4 using the same sample.
(B) Pseudo-Bulk correlation of common genes comparing Chromium 3' v4 vs Chromium Flex v2 using the same sample.
(C) Many genes showed substantial differences between Chromium Flex v2 and Chromium 3' v4, despite concordance of the same genes between Evercode WT v4 and Chromium 3' v4.

Single Cell 3' Kit v4 in Human PBMCs). Comparison of Chromium 3' v4 against Chromium Flex v2 with probe-based capture showed weaker correlation with r² value of 0.73 (Figure 5B). Protein-coding genes with significant differences between Chromium assays predominantly aligned with the profiles identified by Evercode WT v4, indicating a stronger correlation between Evercode WT v4 and Chromium 3' v4 compared to Chromium Flex v2 Apex (Figure 5C).

Conclusion

In this evaluation, Evercode WT v4 outperformed Chromium Single Cell Flex v2 in gene body coverage, RNA biotype diversity, median transcripts detected, and overall cell retention, while also offering a shorter workflow. It showed stronger correlation to Chromium GEM-X 3' v4 than Chromium Flex v2 Apex and delivered more novel information per cell in fixed human PBMCs.

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