Application Note
Evercode Whole Transcriptome

Single Cell Sequencing Built for High-Throughput Screening

Introduction

High-throughput compound screening enables the rapid identification of promising therapeutic candidates. Incorporating single cell RNA sequencing (scRNA-seq) enables the precise characterization of cellular responses at the single cell level.

Achieving such resolution with hundreds of conditions necessitates a flexible platform that can deliver high resolution on a dynamic range of cell inputs, from thousands to millions, while accommodating hundreds of samples and perturbations.

While existing methods offer some capability, they struggle with cumbersome workflows, scalability, and affordability.

The Evercode™ combinatorial barcoding approach is an instrument-free technology that allows researchers to profile single cells across hundreds of conditions at any scale in a single run—unlocking insights into drug responses, off-target effects, and cellular heterogeneity.

In this study, we demonstrate the effectiveness and flexibility of Evercode v3 chemistry in processing over 1,000 samples and 20,000 perturbations concurrently in 10 million peripheral blood mononuclear cells (PBMCs) within a single experiment (Figure 1).

EXPERIMENTAL DESIGN

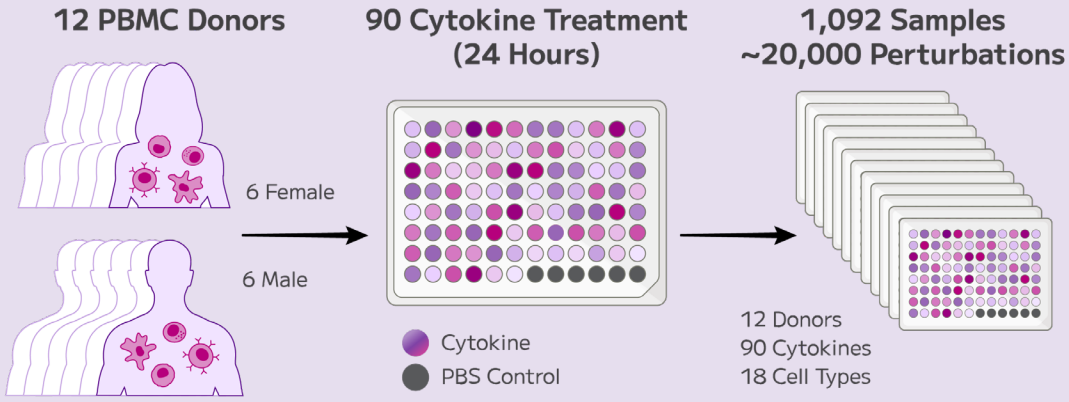

Figure 1. Study design: PBMCs from twelve donors were incubated 24 hours with 90 cytokines and a PBS as control, resulting in 1,092 total samples and about 20,000 perturbations across 18 cell types within the PBMCs.

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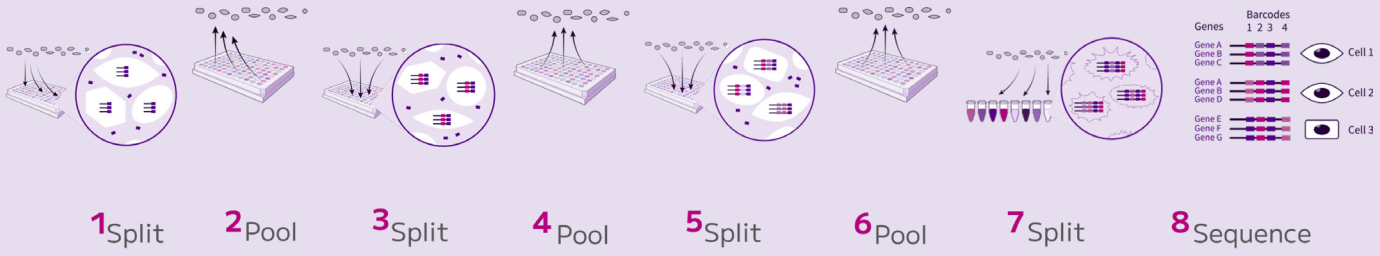

Figure 2. In the Evercode Whole Transcriptome workflow single cells undergo 3 rounds of splitting and pooling. During each split a plate-specific barcode is appended to each cell's transcriptome. When the cells are pooled, they are mixed and randomly split into the next plate where another barcode is appended.

Materials and Methods

Cytokines Perturbation

Peripheral blood mononuclear cells (PBMCs) from 6 male and 6 female donors were obtained after Ficoll separation and then cryopreserved.

Samples were thawed in a 37°C water bath, transferred into a 50 mL tube and diluted with warm FBS media, centrifuged, and washed with cold FBS. After thawing, >90% viability across all samples was verified. For each donor, 1 million cells were seeded in a 96-well plate, for a total of 12 plates. Cells were incubated 24 hours with 90 different cytokines and 6 PBS negative controls, resulting in a total of 1,092 experimental conditions. After 24 hours the PBMCs were collected and transferred into a deep-well plate, washed with PBS and centrifuged.

Once the supernatant was removed, cells were fixed with the Evercode Cell Fixation v3 High Throughput Plate-Based Workflow which was semi-automated using the INTEGRA Assist Plus platform. Fixed samples were aliquoted into PCR plates, and stored at -80°C. The day prior to running the Evercode Whole Transcriptome assay, aliquots of fixed samples were thawed in 37°C water bath and counted.

Evercode Split Pool Combinatorial Barcoding

A scaled-up workflow with enhanced automation was applied to fixed samples. Each sample received a unique barcode through a reverse transcription reaction. The samples were then pooled and split twice more to receive additional barcodes. Finally, the samples were redistributed into sublibraries, where a fourth barcode was added after cell lysis.

The combination of barcodes obtained after four rounds of split and pool assigns a unique identity to each cell, with a virtually non-existing chance of 2 different cells going through the same path and acquiring the same combination of barcodes. (Figure 2).

The sequencing libraries were made using a Hamilton liquid handler instrument and sequenced using the Ultima Genomics Sequencing platform targeting ~31,000 mean reads per cell.

Data Analysis

Clustering was performed using Scanpy, followed by cell type annotation based on canonical markers. Individual cell subsets were downsampled, and differential gene expression (DGE) analysis was conducted on each subset. Cytokine-perturbed gene expressions were compared to PBS-treated control cells to identify significant differences.

Differential Gene Expression

Differential gene expression analysis was conducted to determine the number of genes upregulated in response to cytokine treatment across all cell types. For each donor and cell type, the number of genes with a positive log fold change greater than 0.3 and an adjusted p-value less than 0.001 relative to the PBS control was calculated.

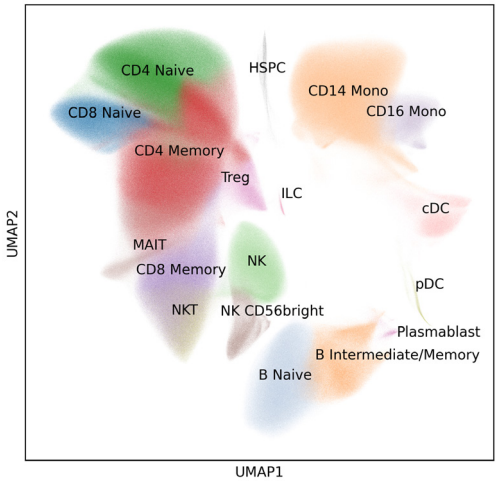

Figure 3. Dataset of 9,697,974 human PBMC responses to 90 different cytokines in 18 cell types.

The dataset generated 9,697,974 PBMCs across 18 cell types, including rare and small subsets - plasmablasts, hematopoietic stem and progenitor cells, innate lymphoid cells, and dendritic cell precursor, typically challenging to identify in smaller-scale experiments (Figure 3).

Differential expression analysis was performed to calculate the number of genes upregulated in response to cytokine treatment across all cell types. Cytokine treatment induced significant transcriptional changes. A heatmap was generated to display the average number of differentially expressed genes per cell type and cytokine treatment. The magnitude of the PBMC response to cytokine perturbation varied depending on the PBMC subtype and sampling abundance (Figure 4).

Figure 4. Magnitude of PBMC response to cytokines induction depends on cell subtype and/or sample size. The number of differentially expressed genes in each cell type was compared to PBS-treated controls. Values are the average number of differentially expressed genes across the 12 donors.

Increased Number of Cells Capture Biological Complexity

To illustrate the necessity of analyzing a larger number of cells to capture biological complexity and uncover novel functions, different numbers of CD16+ monocytes—a smaller monocyte subset 5-10%—were downsampled and analyzed for cytokine perturbation.

Within a subset of 78 CD16+ cells, the effects of 90 cytokines observed were very limited. However, the number of differentially expressed genes increased substantially with more cells included in the analysis, peaking at 2,500 cells.

This demonstrates that the differential gene expression induced by cytokine treatment is more effectively detected at a larger scale (Figure 5).

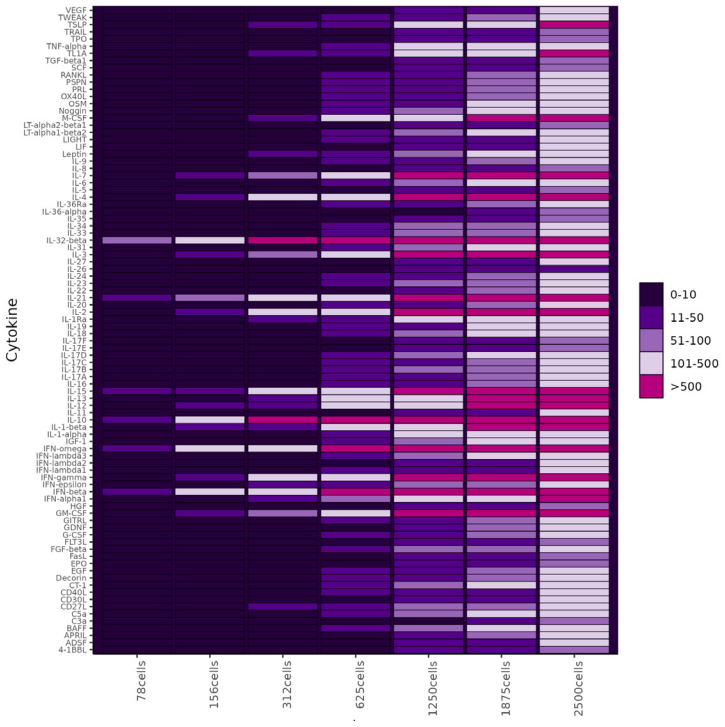

Figure 5. Cytokine-induced expression changes are better detected at increased scale, as the number of detected differentially expressed genes increases with the number of cells analyzed.

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Single Cell Resolution Unveils Hidden Cellular Responses in High-Throughput Screening

The response of CD16+ Monocytes and CD4 Memory T cells—two small subsets—to IFN-ω was extrapolated from the PBMC dataset. While the two cell types exhibited an overall similar response to IFN-ω for some genes, individual cells displayed distinct behaviors: subsets of genes were upregulated in both CD4 Memory cells and CD16 Monocytes, exclusively in CD4 Memory cells, or solely in CD16 Monocytes (Figure 6).

These results demonstrate a nuanced biological response that can be appreciated within a small subset from a very large dataset. Such layers of cellular behavior would be overlooked with bulk RNA screening.

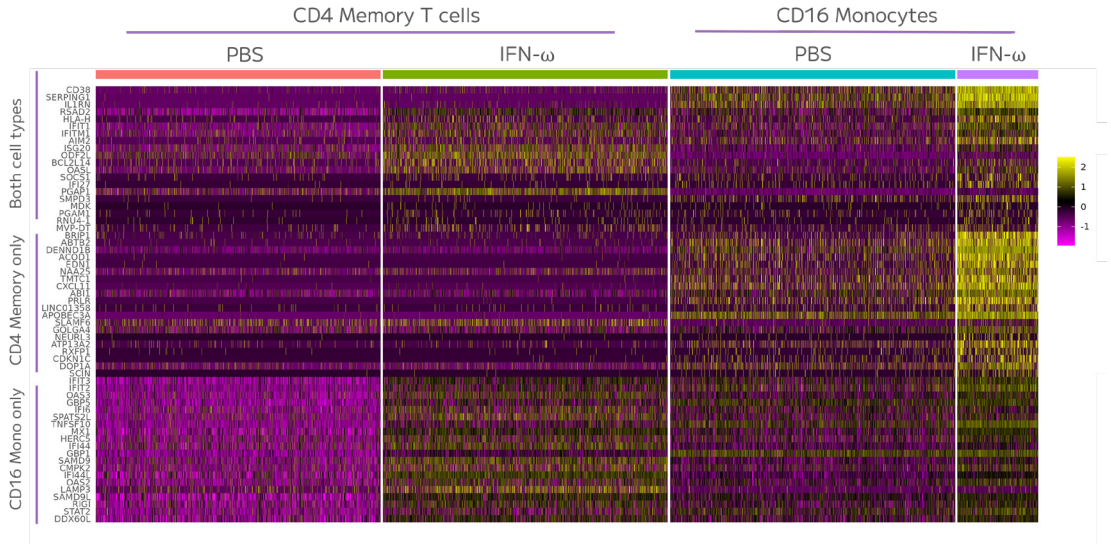

Figure 6. Heatmap showing expression of genes that are upregulated in response to IFN-ω. Subsets of genes here include genes that are upregulated in both CD4 Memory and CD16 Monocytes, CD4 Memory cells only, or CD16 Monocytes only.

Conclusion

This pioneering experiment, demonstrated the feasibility and advantage of incorporating single cell sequencing at scale into high-throughput screenings. This capability allows users to design large, complex, and comprehensive experiments, encompassing thousands of samples and millions of cells simultaneously.

The flexibility and scalability of the Evercode approach address longstanding challenges in single cell RNA sequencing by overcoming technical and cost barriers that have historically limited experimental design and sample size. By allowing researchers to fix their samples, this approach enables the preservation of biological states, streamlines sample processing, and facilitates studies across multiple time points—all without sacrificing flexibility or scalability.

This remarkable technology not only enhances the throughput of scRNA-seq but also provides a robust platform for comprehensive drug screening and toxicological assessments. Its ability to process such large datasets with high precision opens new avenues for understanding complex biological systems and accelerates the discovery of novel therapeutics.