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Marker-Free Microfluidic Enrichment Enables Full-Length mRNA Transcriptome Analysis of Single Live Circulating Tumor Cells from Six Breast Cancer subjects

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Introduction

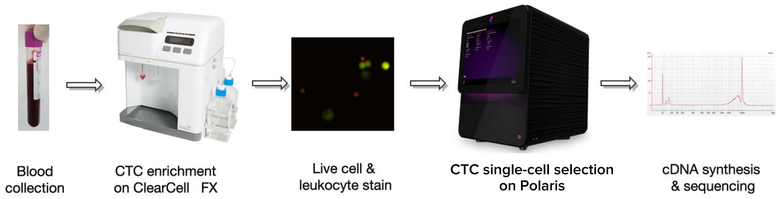
Existing technologies enrich circulating tumor cells (CTCs) with some level of contaminating white blood cells (WBCs) that pose a challenge to comprehensively characterizing pure CTCs. We addressed this challenge by integrating two commercially available microfluidic systems. Integration of the Clearbridge BioMedics ClearCell® FX System and the Fluidigm Polaris™ system enabled us not only to develop a marker-free workflow to isolate CTCs but also to seamlessly prepare amplified cDNA from CTCs for full-length mRNA-seq analysis. Marker-free methods for isolating CTCs are attractive because they provide an opportunity to analyze a larger set of CTCs that may otherwise be missed due to variable or no expression of protein (label) markers.

Understanding genetic and functional heterogeneity in CTCs allows us to gain insight into the mechanisms underscoring metastasis, drug resistance, and tumor aggressiveness. The ClearCell FX system processes blood samples from cancer subjects and enriches for CTCs in a marker-free antibody-independent manner. The low level of nonspecifically isolated white blood cells from ClearCell FX is further depleted on the Polaris system by negative enrichment of viable CTCs. This unique integration of systems will enable researchers to perturb single CTCs in a controlled environment, monitor and measure the response due to perturbation, and link these response measurements to downstream genomic and transcriptomic analysis.

Methods and Materials

Integrated ClearCell FX and Polaris workflow

Enrichment of circulating breast tumor cells: CTCs from 7.5 mL of peripheral blood sample from breast cancer subjects were enriched using ClearCell FX.



Isolation and mRNA-seq preparation of target single cells: To differentiate larger blood cells from putative CTCs, we stained the enriched cells with Alexa Fluor® 647-conjugated CD45 and CD31 to identify leukocytes and endothelial cells, respectively. Calcein AM (live cell marker) and CellTracker™ Orange (universal cell marker) were added to identify live cells. Single CTCs were selected on Polaris system, lysed, and reverse-transcribed, and cDNA were preamplified on the Polaris integrated fluidic circuit (IFC).

Sequencing and analysis: Sequencing libraries were generated using the Nextera® kit and sequenced on Illumina® MiSeq™. We successfully processed blood samples from six subjects. Sequenced data showed high-quality metrics, with read depth of up to 2.5 million reads (MiSeq), with a low percentage of mapped reads to ribosomal RNA and mitochondrial RNA.

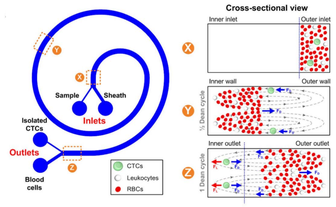
Conclusions

1. Demonstrated feasibility of integrating two microfluidic platforms to capture single CTCs for transcriptome and functional study in a label-free manner from blood sample until mRNA-seq data analysis.
2. Unsupervised hierarchical clustering of gene expression data shows clustering by subject, but considerable heterogeneity is also observed among the CTCs from the same subject.
3. First mRNA-seq dataset of EpCAM (CD326) negative triple-negative breast cancer (TNBC) CTCs.
4. Unique set of gene expression noted for TNBC CTCs when compared to CTCs from subjects with other hormone status.

ClearCell FX: CTC enrichment from subject blood

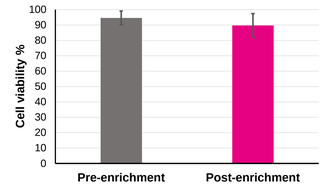
System features

- Label-free isolation method
- Enrichment of CTCs based on size, inertia
- Retrieval of wholly intact and viable cells
- High purity, $5\log_{10}$ depletion of white blood cells
- Process high blood volume of 7.5 mL
- Fully automated CTC enrichment platform



Isolation by size

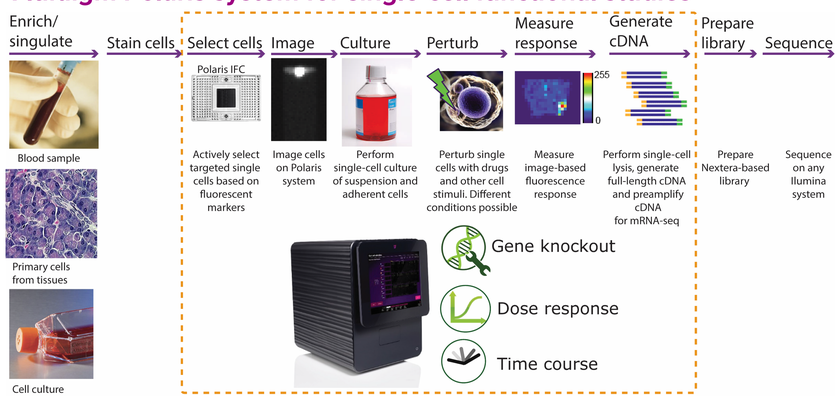
- Smaller blood cells [red blood cells (RBCs) ~8 µm; leukocytes ~8–15 µm] are affected by the Dean drag and migrate to outer wall.
- Larger CTCs (~15–20 µm) experience strong inertial lift forces as indicated by the red arrows and are focused along the microchannel inner wall.



Cell viability

- High cell viability (89.7%) after sample enrichment of MCF-7 and H1975 cells by ClearCell FX, as shown by trypan blue assay (n=5) (figure on left).

Fluidigm Polaris system for single-cell functional studies



Cell selection

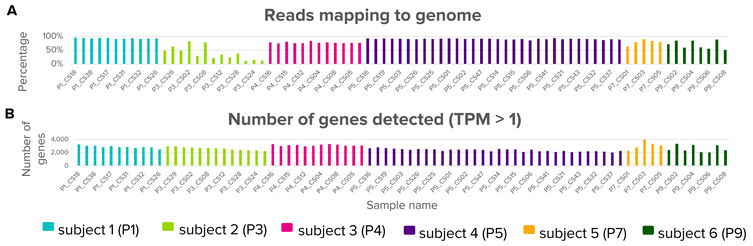
- Cells can be selected based on universal tracker, viability stain, labeled antibody to cell surface receptor, or fluorescent reporter gene.
- Select 48 target single cells (3% to 100% of target to total cell population) across 3 fluorescent channel's.
- Accepts input of 300–8,000 cells per inlet (5 inlets/25 µL per inlet)

Single-cell transcriptomics

- mRNA-seq analysis: preamplified full-length mRNA transcriptome/cDNA

Results

Sequencing performance metrics of 81 CTCs from six subjects

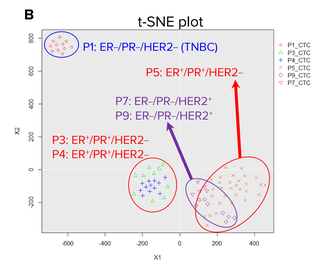


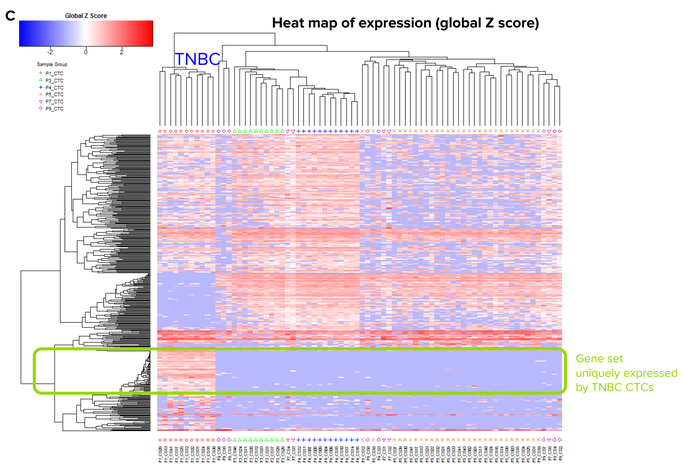
High-quality sequencing data were obtained for CTCs from six subjects (MiSeq). (A) Reads mapping to genome. (B) The number of genes detected for 81 CTCs, ranging from 2,000 to 3,966.

Dimensionality reduction and hierarchical clustering of CTCs from ER⁻/PR⁻/HER2⁻ (TNBC), ER⁺/PR⁺/HER2⁻, and ER⁻/PR⁻/HER2⁺ subjects.

A

Subject	Hormone receptor status	Number of CTC
P1	ER–/PR–/HER2–	11
P3	ER+/PR+/HER–	13
P4	ER+/PR+/HER–	12
P5	ER+/PR+/HER–	32
P7	ER–/PR–/HER2+	5
P9	ER–/PR–/HER2+	8
	Total	**81**





(A) Hormone receptor status of breast cancer subjects. (B) A t-distributed stochastic neighbor embedding (t-SNE) plot shows clear separation of TNBC CTCs from other subject CTCs. (C) Unsupervised hierarchical clustering shows heterogeneity among CTCs.

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