Automated viable circulating tumor cell (CTC) isolation enables efficient single cell multi-omics analysis in the clinical settings

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Abstract

Circulating tumor cells (CTCs) are rare cells found in the peripheral blood or other body fluids of cancer patients. Single cell multi-omics analysis of CTCs can provide critical information and insights for tumor heterogeneity, early detection, residual disease, recurrence, response and resistance to therapies etc. However, the adoption of the single cell analysis in the clinical settings have been lagging for many reasons including complicated and lengthy workflows to operate, low in automation and throughput, cell loss and inefficient cell picking, RNA and DNA degradation. To overcome these challenges, we have developed a full automated solution for CTC enrichment and single cell isolation.

Whole blood samples were collected from breast, prostate and lung cancer patients using BioFluidica blood collection tubes. CTCs were enriched with LiquidScan platform, microfluidic affinity selection of rare cells with EPCAM surface marker. Cell eluates released from microfluidic chips were further sorted and individual cells with EPCAM surface markers were placed into individual wells of PCR plates. The isolated single cells were processed with either whole genome amplification for DNA analysis or pre-amplification of RNA for transcriptome profiling. Cell viability and RNA integrity was assessed using bulk MCF-7 breast cancer cell line sample.

Enriched cells are 70% viable in average post LiquidScan sample processing, compared to >90% for freshly collected samples. The RNA RIN score for the enriched cells are ~6 in average. The whole blood samples are directly loaded to microfluidic chips and processed (capture, wash, release and elute) using Hamilton robot for automated processing with minimal hands on time. Red blood cell lysis was not required, and the sample processing time was ~3 hours per sample with up to 8 samples processed simultaneously per instrument. Success rate of DNA amplifications and RNA pre-Amplifications were both over 90% across all samples processed.

The LiquidScan platform provided a solution for fast, automated single viable cell isolation and make the downstream molecular analysis feasible in the clinical settings. Protocols for RNA pre-amplification and DNA sequencing including copy number analysis and targeted sequencing were developed. Development of single cell DNA methylation and proteomics assays are in progress.

Materials & Methods

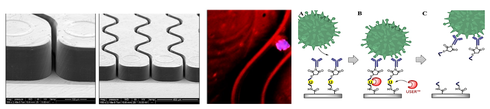
The Liquid Biopsy System is designed to minimize biomarker loss

Many biomarkers are lost through sample handling from collection through processing. Three major sources for sample loss or alterations are: 1) Degradation, 2) Complex processing including sample prep 3) Interaction with instrument components. BioFluidica has developed a platform that minimizes these sources of biomarker loss. **Whole Blood** (or plasma for exosome isolation) is directly introduced into a microfluidic chip surfaced with antibodies designed for specific biomarker capture.

Figure. LiquidScan instrumentation has "Hamilton Inside" (First image). The blood sample tube is placed on the robot, and the operator can walk away. Chips (Third image) are housed on the LiquidScan module (second image), up to eight per run. Whole blood is introduced directly from the sample tube into the biomarker isolation chip. Blood is slowly injected (pushed) into one port on the chip and simultaneously withdrawn on the other port (a push-and-pull no loss system). The robotic system is scalable for fully automated processing. In this study, the microfluidic chip is surfaced with antibodies to specifically capture CTCs from whole blood.

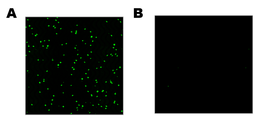
High Specificity Isolation of circulating cells

Any cell with specific cell surface markers can be isolated using the Liquid Scan Technology.

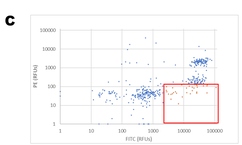


Sinusoidal microfluidic channels are programmed with specific antibodies or mixtures of antibodies for selective cell capture. After elimination of the background, the specific cells are released from the chip by cleaving the linker enzymatically or through photoactivation.

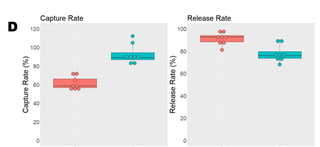
Fully automated sample processing for the isolation of rare biomarkers



A. Chip image with the CTCs captured on the chip
B. Chip image after the release of CTCs
MCF7 human breast cancer cells were labeled with a green fluorescent dye then mixed into blood and processed through LiquidScan microfluidic chips and imaged.



C. NamoCell Sorting Method.
Cells are eluted from LiquidScan into a NamoCell Pala Single Cell Sorting Chip. Cells are gated based on the absence of Negative Selection Markers (PE) and the presence of Target Cell Markers (FITC). Cells are sorted from the Sorting Chip into collections tubes in either single cell or pooled cell format for downstream molecular testing.

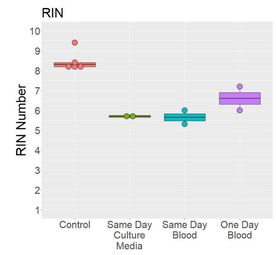


D. Capture and Release of different cell lines on antibody surfaced LiquidScan chips.
BeWo (placental choriocarcinoma) and MCF7 (breast cancer adenocarcinoma) were labeled and processed on the Hamilton LiquidScan System. Percent capture ranges from 60% to 90% and percent release ranges from 80% to 90%.

Cell Viability and RNA Quality Were Retained After Processing

Methods\Samples	Control	Same Day Culture Media	Same Day Blood	One Day Blood
Cell Type	MCF-7	MCF-7	MCF-7	MCF-7
Number of Samples	5	2	2	2
Number of Cells per Sample	10000	2000	2000	2000
Sample Buffer	NA	Culture Media	Healthy Donor Blood	Healthy Donor Blood
Storage Time	0 Hr	0 Hr	0 Hr	24 Hr
LiquidScan Processing *	No	Yes	Yes	Yes
RNA Extraction and Quality Assessment	RNA isolated using Zymo Direct-zol RNA Microprep Kit, RIN number was measured with Agilent Bioanalyzer. Cell viability was assessed using trypan blue staining.			

* Including capture and release of cells using LiquidScan and Sinusoidal EPCAM chips.

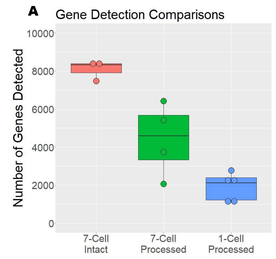


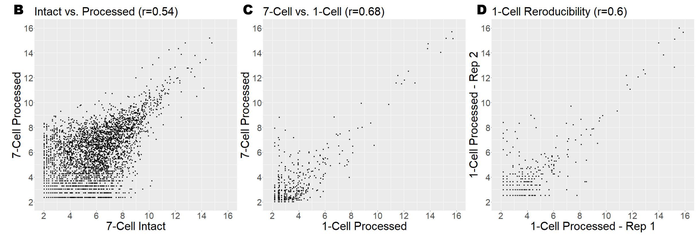
- RNA Quality: see RIN numbers above
- Same day blood cell viability: 75%

Single Cell RNASeq

Methods\Samples	7-Cell Intact	7-Cell Processed	1-Cell Processed
Cell Type	MCF-7	MCF-7	MCF-7
Number of Samples	3	4	5
Number of Cells per Sample	7	7	1
Sample Buffer	PBS	Culture Media	Culture Media
LiquidScan Processing *	No	Yes	Yes
Cell Sorting	Either 7 or 1 cell are sorted into collection tube using NamoCell cell sorter		
RNA pre-Amplification	Takara SMARTer Ultra Low Input RNA Kit for Sequencing - v3		
RNA Sequencing	Sequencing was done on Illumina NexSeq. RNASeq data analysis was performed using in-house RNASeq pipeline.		

* Including capture and release of cells using LiquidScan and Sinusoidal EPCAM chips.



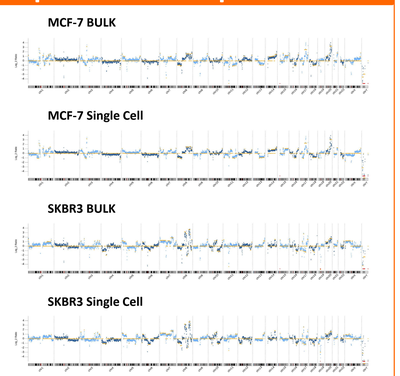


A. Average of 2000 genes were detected from single-cell samples. More genes are detected with intact samples and larger number of cells.
B. Intact and processed samples showed good correlation of RNA profile, indicating that sample processing has minimal impact on RNA profiling.
C. Example of single-cell and seven cell RNA profiling correlation, showing more genes were detected with 7-cell samples.
D. Example of single-cell RNA profiling correlation, with correlation coefficient (r) ranging from 0.48 to 0.6 for paired single cells comparisons.

Single Cell CNV Analysis is Compatible with LiquidScan

Methods\Samples	Bulk	Single-Cell
Cell Type	MCF-7, SKBR3	MCF-7, SKBR3
Number of Samples	2	6
Number of Cells per Sample	10000	1
LiquidScan Processing *	No	Yes
Cell Sorting	No	Yes, using NamoCell
DNA Preparation	Extracted using Zymo Quick DNA Miniprep Plus Kit	Whole genome amplified using Takara PicoPLEX single cell WGA kit
DNA Sequencing	Standard shotgun low pass whole genome sequencing was done on Illumina NextSeq. CNV analysis was performed using CNV-Seq method (BMC Bioinformatics. 2009 Mar 6;10:80. doi: 10.1186/1471-2105-10-80).	

* Including capture and release of cells using LiquidScan and Sinusoidal EPCAM chips.



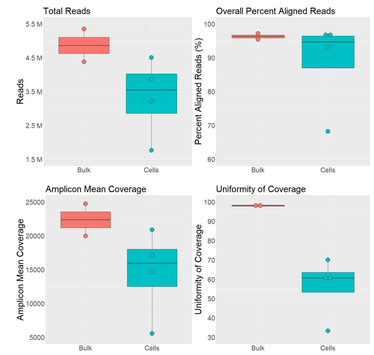
Single Cell CNV Analysis can be achieved with < 1 M Reads

Down Sampling was performed using MCF-7 single cell data

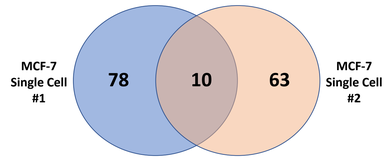
Single Cell Variant Detection

Methods\Samples	Bulk	Single-Cell
Cell Type	MCF-7	MCF-7
Number of Samples	1	2
Number of Cells per Sample	10000	1
DNA Preparation	Extracted using Zymo Quick DNA Miniprep Plus Kit	Whole genome amplified using Takara PicoPLEX single cell WGA kit
Amplicon Panel	AmpliSeq™ for Illumina Cancer Hotspot Panel v2	
Sequencer	Illumina NextSeq	
Analysis	Illumina BaseSpace DNA Amplicon App	

A. Sequencing Data Quality



B. WGA Error Correction



- 10 common variants were determined by both single cell WGA samples
- 10 out of 10 (100%) common variants were detected in MCF-7 bulk (no WGA) sample
- DNA WGA using PicoPlex has introduced amplification errors that was detected by sequencing, which require additional sample to verify and remove false positives

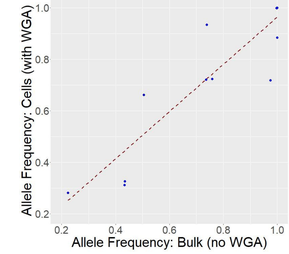
C. Confusion Matrix

Single Cell \ Bulk	Positive	Negative
Positive	10	0
Negative	4	193

Statistic	Value
Sensitivity	71.43%
Specificity	100.00%
Positive Predictive Value (PPV)	100.00%
Negative Predictive Value (NPV)	97.97%
Accuracy	98.07%

- Single cell variants were determined by using common variants detected by two independently WGA single cell samples.

D. Allele Frequency Consistency



A. The samples were sequenced with a minimum of 1.5 million reads, and the majority of the samples had over 90% overall percent aligned reads. The minimum amplicon mean coverage was over 5000. However, the uniformity of coverage of single-cell samples was significantly lower than that of bulk samples.
B. WGA introduced a significant amount of amplification errors, which can be reduced by independently sequencing multiple cells.
C. When using a bulk sample as a reference, single-cell sequencing had a 71.43% sensitivity and 100% specificity in detecting variants.
D. The allele frequencies detected by bulk sample and single-cell sequencing are highly correlated.

Summary

- LiquidScan is a fully automated system for CTC isolation from blood without preprocessing.
- Cell viability remains approximately 75% after LiquidScan sample processing.
- RNA extracted from cells processed with LiquidScan has a RIN number of about 6.
- Single-cell RNA profiling is feasible with an average of 2000 genes detectable from single cells, while significantly more genes can be detected with larger input cell numbers. Overall success rate with single-cell RNA sequencing is over 70%.
- Single-cell copy number analysis is feasible using cells processed with LiquidScan platform. The overall success rate with single-cell DNA sequencing is over 90%.
- Single-cell mutation analysis is also feasible. Using two independently whole genome amplified and sequenced cells, single-cell sequencing has 71.43% detection sensitivity and 100% specificity. The sensitivity can potentially be improved with additional cells.
- Molecular analysis (RNASeq, CNV and mutation analysis) of CTCs and exosome isolation from patient samples are ongoing.