Subpopulation heterogeneity demonstrated in circulating tumor cells isolated from breast cancer patients using ApoStream®, an antibody-independent cancer cell recovery device

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Abstract

Background: Current established methods of circulating tumor cell (CTC) isolation and identification rely on antibodies against epithelial specific markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK). The classical phenotypic definition of a CTC is a CK positive, CD45 negative, nucleated cell, yet several reports have shown that EpCAM and CK detect only a fraction of CTCs and are not sufficient to detect the heterogeneous subpopulations of CTCs. Moreover, subsets of primary tumor cells acquire features of invasiveness and transform into an aggressive phenotype. During this process, EpCAM and CK are down regulated or lost leaving a lethal population of CTCs undetectable and unstudied using antibody dependent CTC technologies. It is imperative to isolate CTCs in an unbiased, EpCAM independent manner and expand the phenotypic characterization of CTCs to elucidate the subpopulation heterogeneity. Here we used ApoStream®, a novel, antibody-independent device which exploits differences in the dielectric properties between cancer cells and normal blood cells to enrich CTCs from the blood of cancer patients. We demonstrate device performance and integration with additional methods to perform subsequent phenotyping and molecular marker analysis. Methods: The performance of ApoStream® was assessed using SKOV3 (ovarian cancer) and MDA-MB-231 (breast cancer) cell lines that have a high and low expression level of EpCAM, respectively, to demonstrate linearity and precision of recovery independent of EpCAM receptor levels. A side-by-side comparison of CellSearch® and ApoStream® was performed on 10 metastatic breast cancer patients. A multiplexed immunofluorescent assay and laser scanning cytometry (LSC) analyses were applied to identify multiple combinations of positive and/or negative staining for CK/CD45/DAPI cells, expression of EpCAM and vimentin. Results: In system precision performance studies, the average recovery of SKOV3 and MDA-MB-231 cancer cells spiked into approximately 12 million peripheral blood mononuclear cells obtained from 7.5 mL normal donor blood was 75.4 ± 3.1% (n=12) and 71.2 ± 1.6% (n=6), respectively[1]. The intra-day and inter-day precision coefficients of variation (CVs) of the device were both less than 3%. Linear regression analysis yielded a correlation coefficient (R2) of more than 0.99 for a spiking range of 4-2600 cells. ApoStream® consistently recovered significantly higher numbers of CTCs compared to CellSearch® (p<0.024). ApoStream® recovered varying numbers of CK+/CD45−/DAPI+, CK+/CD45+/DAPI+, CK−/CD45−/DAPI+ cells from each cancer patient sample tested. ApoStream® recovered both EpCAM+ and EpCAM− CTCs in 40% and 100% of patients, respectively. Vimentin+ CTCs were isolated from 90% of patients. Conclusions: The ApoStream® technology circumvents dependence on expression of EpCAM and recovers CTCs in high percentage of patients. ApoStream™ coupled with LSC analysis is a sensitive method for phenotyping and detecting biomarker expression in CTCs. These results demonstrate the broad applicability of ApoStream® for enrichment and molecular characterization of CTCs as a foundation for improved clinical applications of CTCs.

ApoStream® Technology

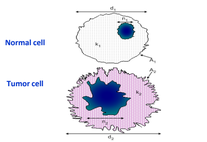


Figure 1. Dielectric properties (polarizability) of cells are dependent upon cell diameter, membrane morphology and conductivity.
Inherent differences in morphology of CTCs and normal cells result in different dielectric polarization charges when exposed to an AC electric current.

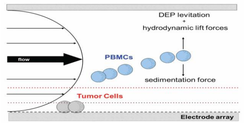


Figure 2. Dielectrophoretic, hydrodynamic and sedimentation forces are utilized to attract CTCs and repel normal cells from the chamber floor. CTCs are collected through a port located in the chamber floor while normal cells flow into a waste port.

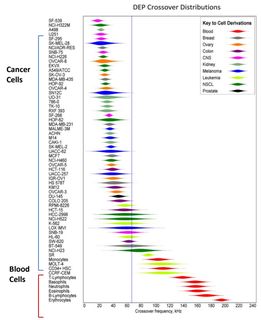


Figure 3. Cross-over frequencies from different tumor cell types including breast, colon, ovarian, lung and melanoma cell lines and from peripheral blood mononuclear cells (PBMCs) were determined.[2] The differences in cross-over frequencies between cancer and normal cells enable ApoStream ® to separate CTCs from normal cells.

ApoStream® Prototype Device

ApoStream® Instrument Performance

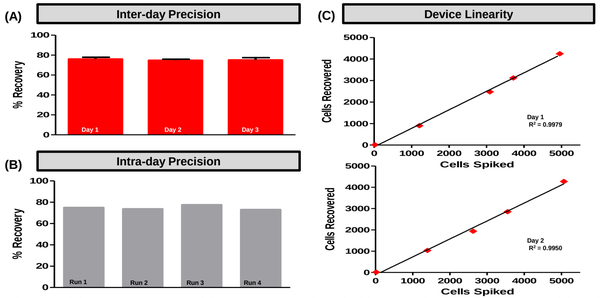


Figure 4. (A) Average recovery of SKOV3 cancer cells spiked into PBMCs shows inter-day precision of 75.4 ± 3.1%, CV = 3.3% (n = 12). (B) Recovery of SKOV3 cancer cells spiked into PBMCs shows intra-day precision of 71.2 ± 1.6 %, CV = 2.7% (n = 6). (C) Device linearity was demonstrated by spiking 4 to ~5000 SKOV3 cells into ~12 million PBMCs from 7.5 mL normal human donor blood.

Cancer Cell Line Recovery with ApoStream®

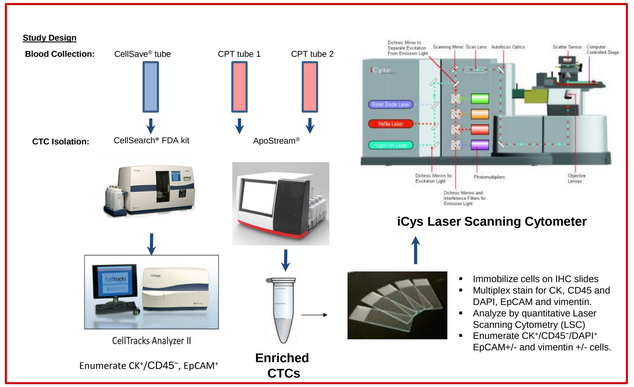
	Day of Run	Replicate	% Cancer Cell Recovery	Average % Cancer Cell Recovery	Standard Deviation	Coefficient of Variation (%CV)
Intra-day Precision (SKOV3 cells)	Day 1	Sample 1	75.0	74.9	2.0	2.7
		Sample 2	73.8			
		Sample 3	77.6			
		Sample 4	73.1			
Inter-day Precision (SKOV3 cells)	Day 1	Sample 1	77.4	76.2	0.7	0.9
		Sample 2	71.0			
		Sample 3	78.7			
		Sample 4	77.5			
	Day 2	Sample 5	75.0	74.9		
		Sample 6	73.8			
		Sample 7	77.6			
		Sample 8	73.1			
	Day 3	Sample 9	81.4	75.2		
		Sample 10	75.2			
		Sample 11	72.7			
		Sample 12	71.6			
Intra-day Precision (MDA-MB-231 cells)	Day 1	Sample 1	69.1	70.0	0.9	1.2
		Sample 2	70.8			
		Sample 3	70.0			
Inter-day Precision (MDA-MB-231 cells)	Day 1	Sample 1	73.4	72.4	1.1	1.5
	Day 2	Sample 2	72.6			
	Day 3	Sample 3	71.2			

Table 1. Intra-day and Inter-day precision of the ApoStream® device for the recovery of approximately 5000 SKOV3 and 500 MDA-MB-231 cells spiked into PBMCs from 7.5 mL of normal human donor blood.

	Number of Spiked Cancer Cells	Cancer Cells Collected After ApoStream™	% Cancer Cell Recovery	Average % Cancer Cell Recovery
SKOV3 cells	23	14	60.9	68.3
	19	14	73.7	
	5	4	80.0	
	4	2	50.0	
MDA-MB-231 cells	22	16	72.7	
	21	16	76.2	
	14	9	64.3	

Table 2. Cancer cell recovery from ApoStream® device for low number of cancer cells spiked into PBMCs from 7.5 mL of normal human donor blood.

Comparison of CTC Enrichment Methods



Gallery of Representative Images of CTCs

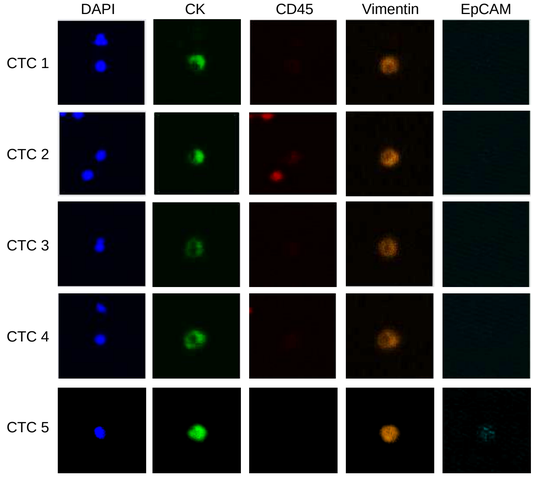


Figure 5. A multiplexed immunofluorescent assay and laser scanning cytometry were used to identify CK+/CD45−/DAPI+ CTCs and quantify EpCAM and vimentin expression in metastatic breast cancer patients

Heterogeneous CTC Phenotypes in BrCa Patients

Patient ID	Number of CK+/CD45-/DAPI cells per 7.5 mL of blood		ApoStream® (CK+/CD45-/DAPI+ cells)			
	CellSearch®	Apostream®	% EpCAM+ vimentin-	% EpCAM+ vimentin+	% EpCAM- vimentin+	% EpCAM- vimentin-
1	0	81	0	3	26	71
2	0	241	0	0	92	8
3	0	40	0	0	100	0
4	0	71	0	11	89	0
5	0	41	0	3	94	3
6	2	149	1	0	83	16
7	0	10	0	0	0	100
8	NA	176	0	0	74	26
9	NA	705	0	0	90	10
10	NA	772	0	0	31	69

Table 3. Distribution of EpCAM and vimentin phenotypes in CK+/CD45-/DAPI+ cells isolated from metastatic breast cancer patient blood by ApoStream®.
NA = no analysis, CellSearch® aborted sample.

Summary & Clinical Significance

- ApoStream® CTC isolation can be applied to all cancer types, including non-epithelial derived tumors because the basis for isolation is independent of antibodies to cell surface antigens like EpCAM.
- ApoStream® instrument performance and recovery data are robust and reproducible.
- Antibody-independent rare cell isolation by ApoStream® combined with phenotypic characterization allows identification of previously undetectable CTCs and enables insight into CTC population heterogeneity.
- A comparison between CellSearch® and ApoStream® showed greater CTC counts with ApoStream®.
- EpCAM+ and EpCAM-, CD45-, DAPI+ cells were detected.
- Vimentin expression was detected in 30% of EpCAM+ and 90% in EpCAM- cells.
- Understanding heterogeneity of CTCs will be key to achieving clinical utility as biomarkers.

References:
[1]Vishal Gupta, et al. ApoStream™, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. Biomicrofluidics 6, 024133, 2012.
[2]Sangjo Shim et al. Dielectrophoresis has broad applicability to marker-free isolation of tumor cells from blood by microfluidic systems. Biomicrofluidics, 7, 011808, 2013.

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