Utilization of Dielectrophoresis for Antigen Independent Circulating Tumor Cell (CTC) Capture Allows for Detection of Heterogeneous Tumor Cell Populations

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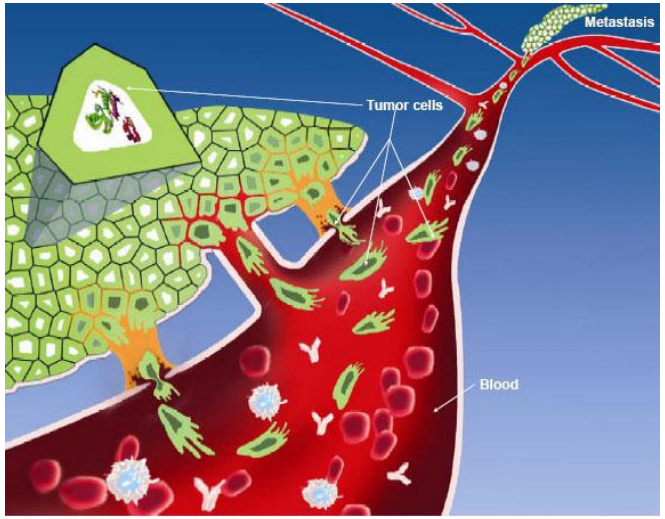
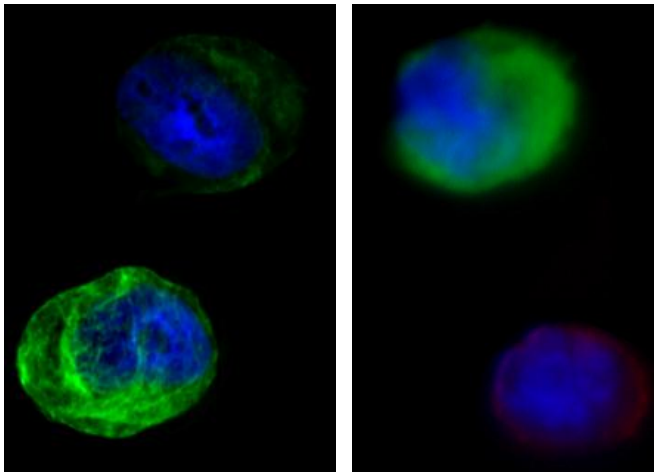
VCU MASSEY CANCER CENTER
Virginia Commonwealth University

CTCs - The “Liquid Biopsy”

Circulating Tumor Cells (CTCs)

- CTCs are cancer cells shed from either the primary tumor or its metastases that circulate in the peripheral blood and are more accessible and less invasive than tumor biopsies.
- The use of CTCs found in peripheral blood is currently cleared by the FDA as a prognostic test for breast, prostate and colorectal cancer (Veridex /J&J)
- CTCs are an attractive minimally invasive alternative to tumor biopsies for clinical applications enabling:
    a. Multiple time points - real-time monitoring vs. archival tissues
    b. Early stage detection
    c. Genetic analysis
    d. Dose/Schedule Selection
    e. Mechanism of action
    f. Patient Stratification
    g. Diagnostic Development
    h. Go/No Go Decisions





ApoStream™

- ApoStream™ cell isolation is based on dielectrophoresis (DEP) field-flow fractionation (DEP-FFF) technology developed at UT MDACC
  - Eight patents licensed by ApoCell in 2010

- Features of the ApoStream™ device:
  - Antibody independent method of isolating viable CTCs found in blood
  - Effective on large number of cancer cell types (to date, no known epithelial cancer cell types that were not amenable to DEP-FFF have been identified)
  - Isolated cells are intact, viable, and can be cultured
  - Recovered cells are suitable for multiple diagnostic applications

Apostream Alpha Prototype development was funded by the Division of Cancer Treatment and Diagnostics National Cancer Institute under the ARRA Program
(NCI Contract No. HHSN261200800001E)
Director: James H. Doroshow, MD
Associate Director: Joseph E. Tomaszewski, PhD

The Specific Goals of the DCTD-funded effort:

I. An instrument capable of separating CTC from blood for any cancer type
II. An instrument capable of separating CTC from blood of research animals
III. An instrument capable of operating with blood volumes as low as 100 uL
IV. An instrument capable of providing live CTC from patient blood

SAIC Frederick

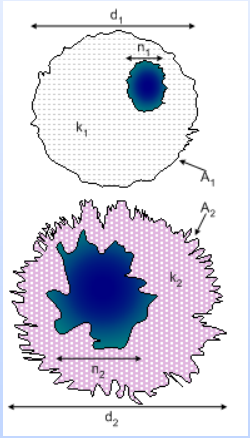
Frederick National Laboratory for Cancer Research

ApoStream™ Technology

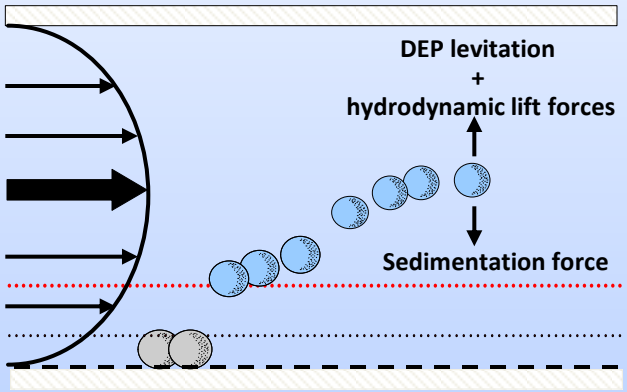
Theory of Operation

Normal cell

Tumor cell



Dielectric properties (polarizability) of cells are dependant upon cell diameter, membrane area, density, conductivity and volume. Inherent differences in morphology of CTCs and normal cells result in different polarisation charges when exposed to an AC electric current.



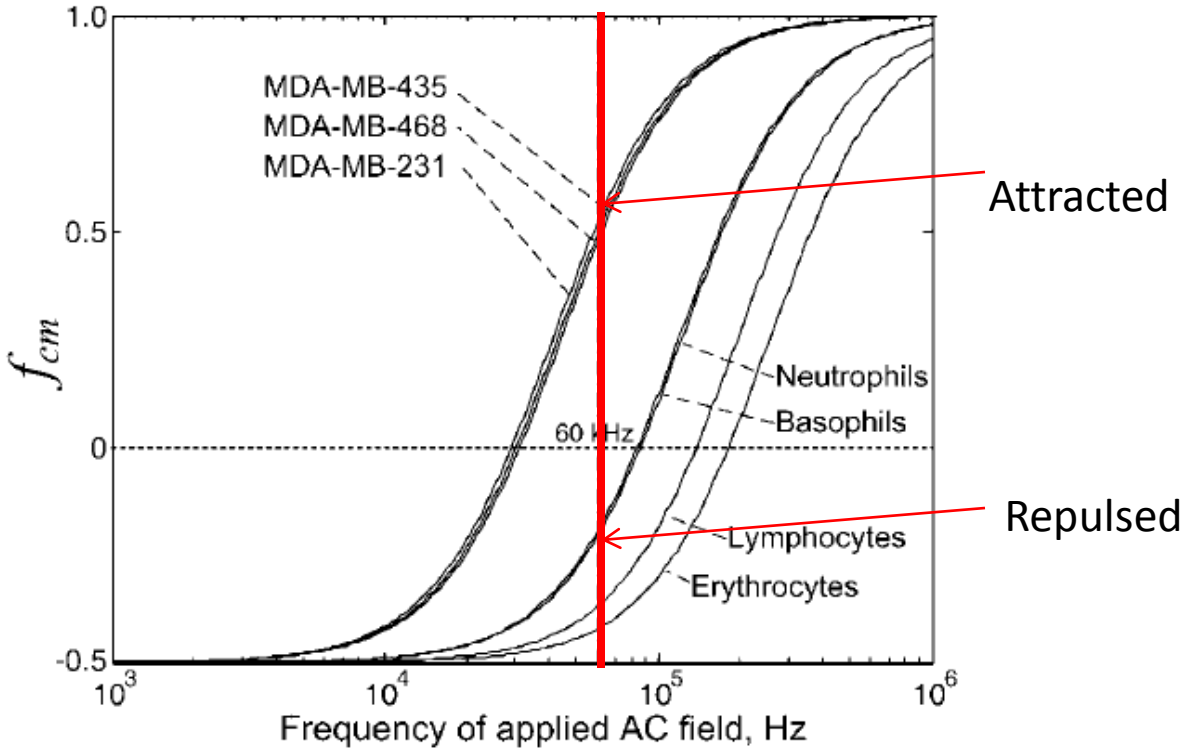
The force balance and levitation of various cells at different heights

Cell levitation is controlled by balancing DEP, hydrodynamic and sedimentation forces. CTCs are collected from the bottom of the flow chamber while the other cells flow into a waste collection port.

'For the separation of cancer cells from healthy blood cells, the ApoStream™ device operates in a modified form to conventional DEP-FFF, in that the cancer cells are attracted by positive DEP forces towards the electrode plane, and thus away from the bulk of the blood cells that are levitated by negative DEP into the fluid flow velocity profile.'

Vishal et al., ApoStream™, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. Biomicrofluidics 6, 024133 (2012).

Crossover Frequency



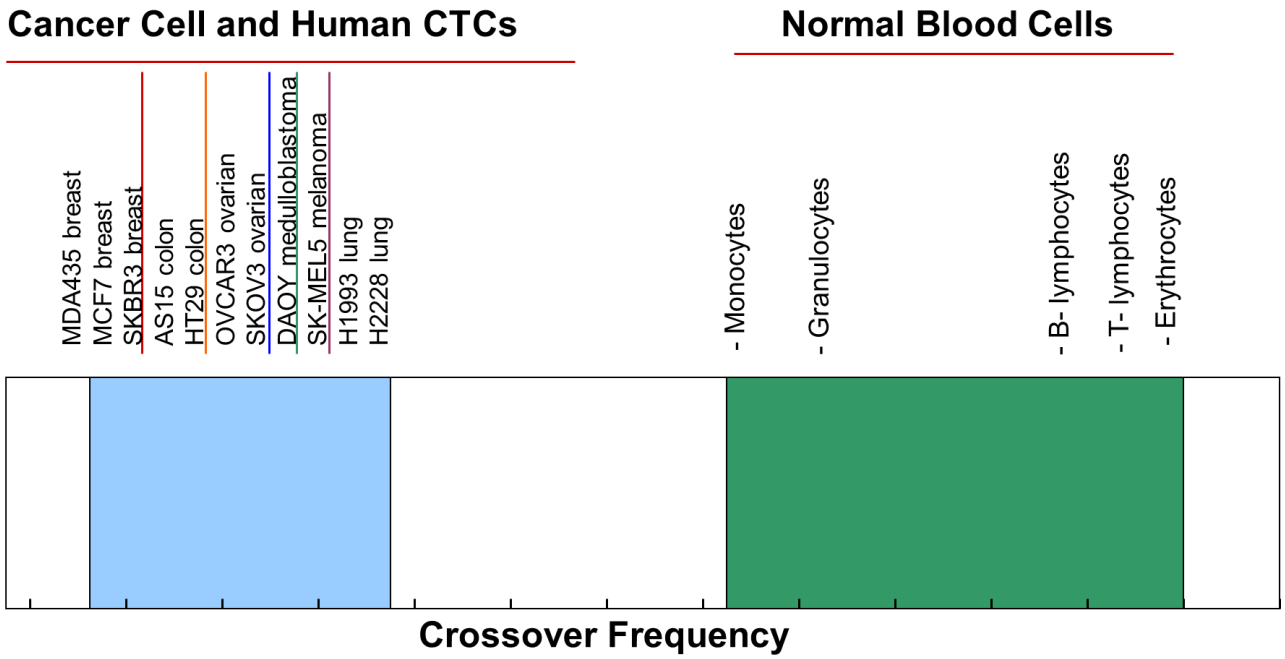
When the conductivity of the cell cytoplasm is much higher than, and the conductivity of the cell membrane is much less than, that of the suspending medium, the cell will exhibit negative DEP at low frequencies, positive DEP at higher frequencies, and no DEP at all at an intermediate crossover frequency

The total cell capacitance reflects plasma membrane area, which depends both on cell size and features such as chromatin density, rigidity, folds, and microvilli that all contribute to the differential responses to DEP forces.

P. R. C. Gascoyne et al. *Electrophoresis 2009, 30, 1388–1398*

DEP Crossover Frequency Forms the Basis for Separation of CTCs from Blood Cells

- The properties exploited by DEP are intimately associated with the cells dimensions and physicochemical properties
- DEP crossover frequency differs between cancer cells and normal blood cells
- This difference enables ApoStream™ to separate cancer cells from normal blood cells

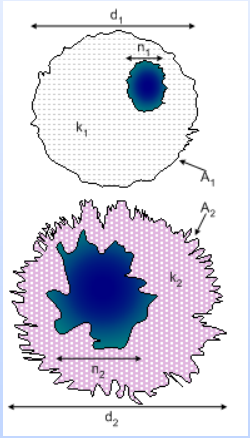


ApoStream™ Technology

Theory of Operation

Normal cell

Tumor cell



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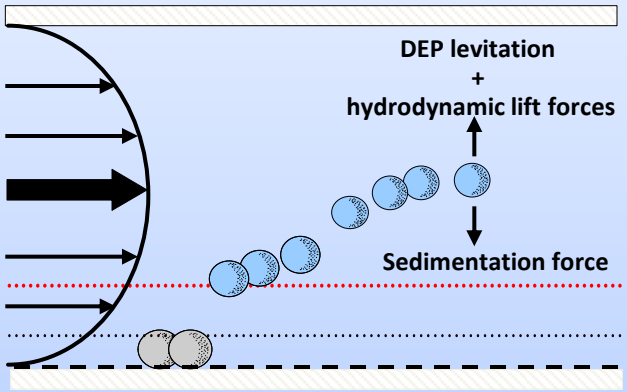

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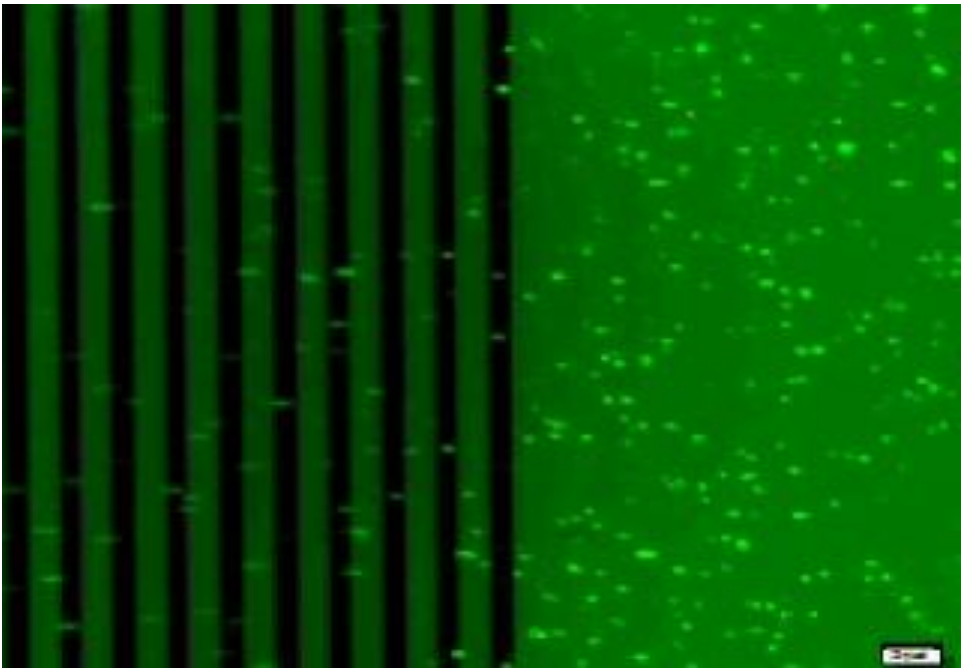
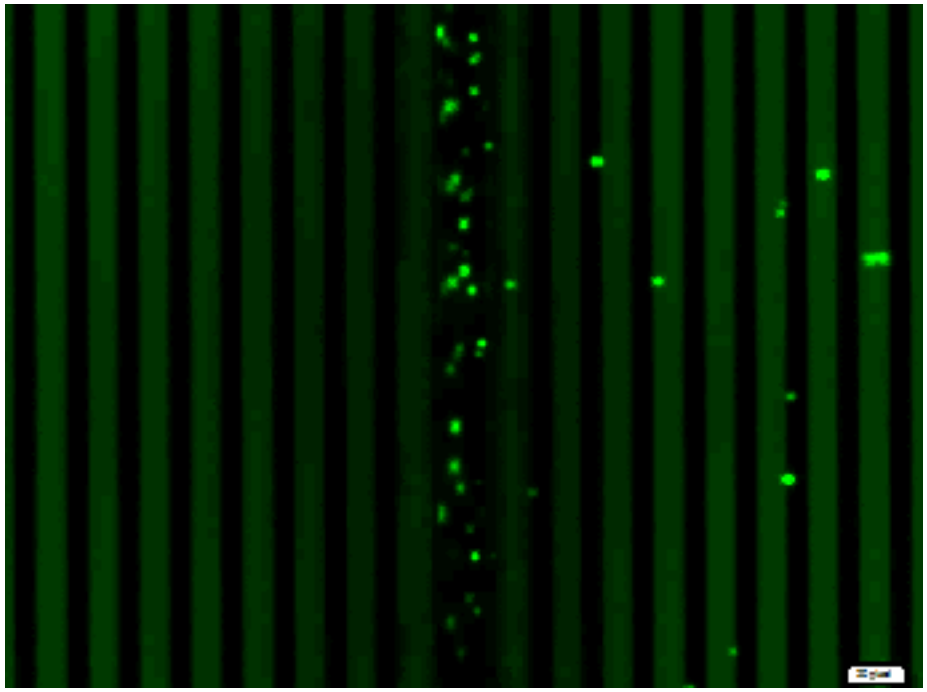
Video Footage of Spiked Cells





A single channel camera tagged PBMCs (left) or spiked CTCs (right)

Going Beyond CTC Enumeration

Cancer Patient Blood

ApoStream™ provides a non-invasive molecular snapshot, or "Real-time Biopsy" using CTCs, of the disease status and/or tumor response and reduces the need for invasive tumor biopsies.

Protein Quantification
IHC
Immunoflourescence

- Phenotyping
- PD Biomarkers
- Phosphorylation
- Signaling pathways

FISH Analysis

- HER2
- PTEN
- AR
- c-Met
- IGF1R
- EGFR
- EML-ALK
- TMPRSS-ERG

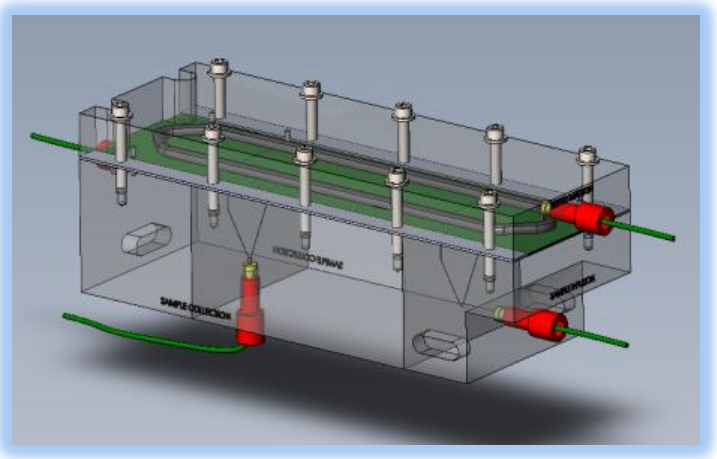
Genetic Mutations

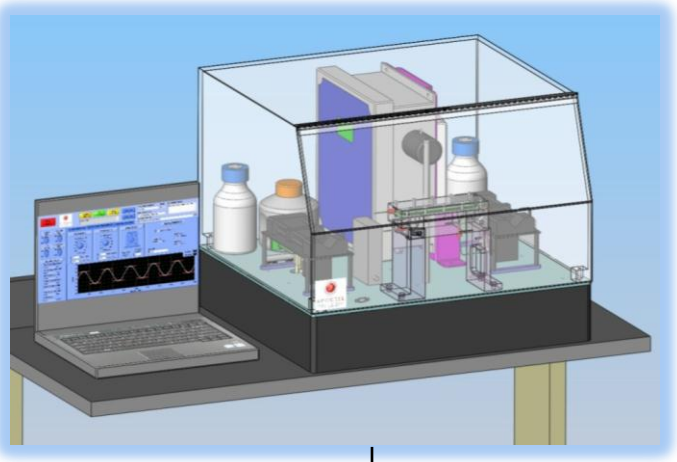
- K-RAS
- B-RAF
- EGFR
- BCR-ABL
- PI3K

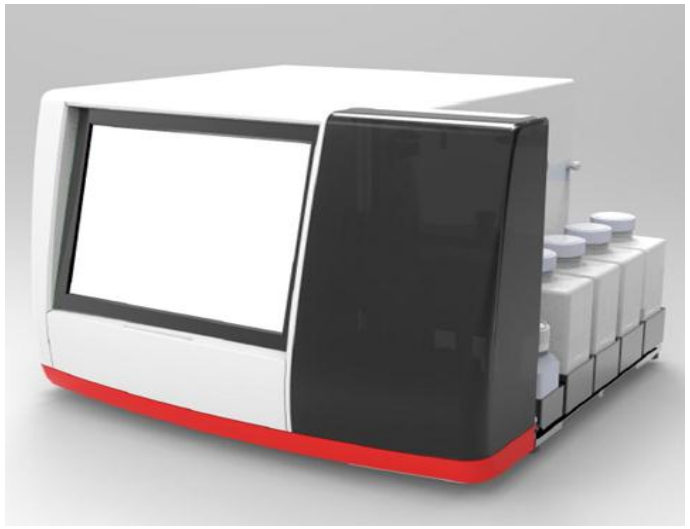
Gene Expression

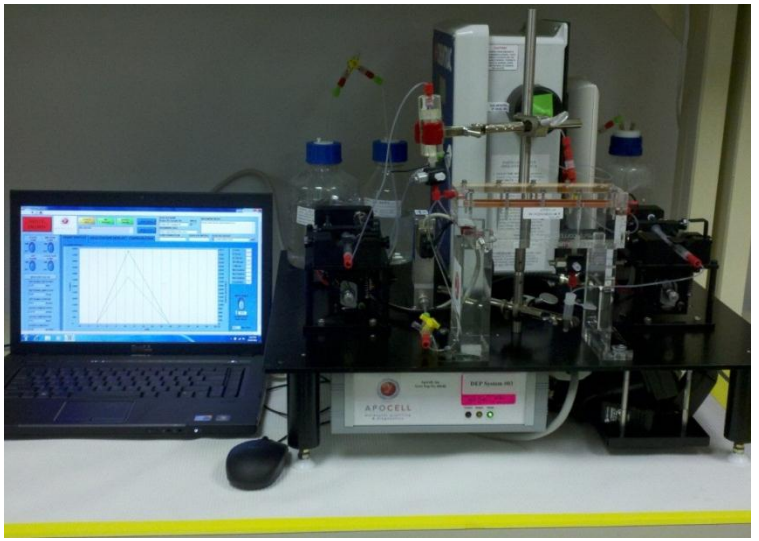
- TaqMan® quantitative RT-PCR
- Gene Expression Profiling

ApoStream™ Platform Development









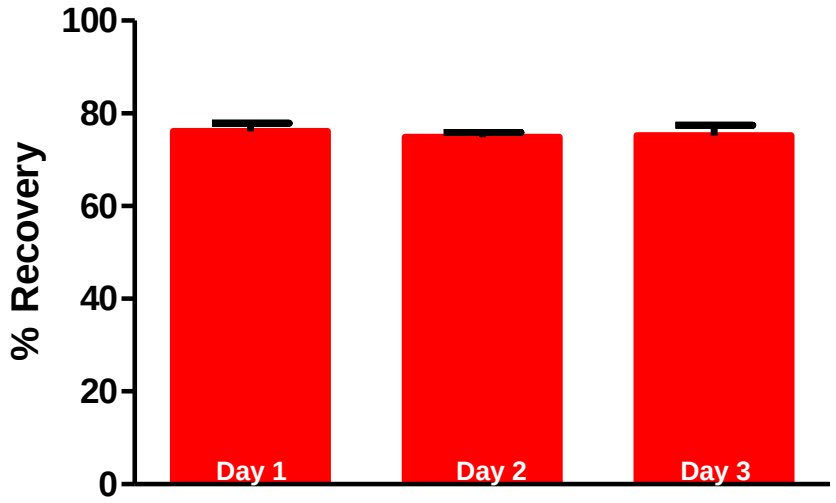
Currently integrated into
16 clinical trials (Phase I-III)

ApoStream™ Technology
Beta Prototype

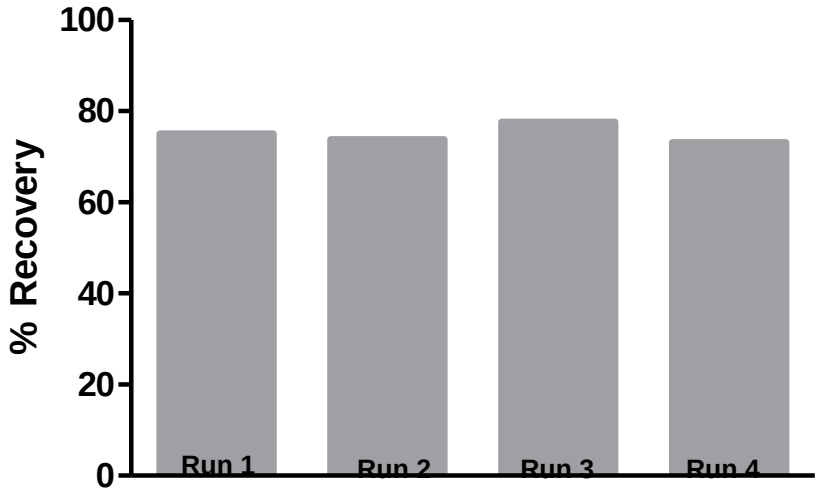
Current prototype design shipped to National Cancer Institute & Massey Cancer Center, VA, in Dec 2012

ApoStream™ Performance Evaluation

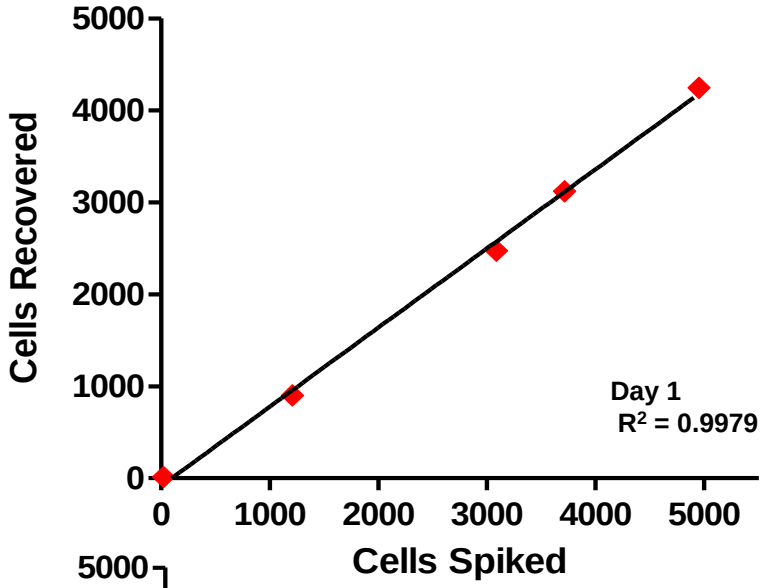
Inter-day Precision

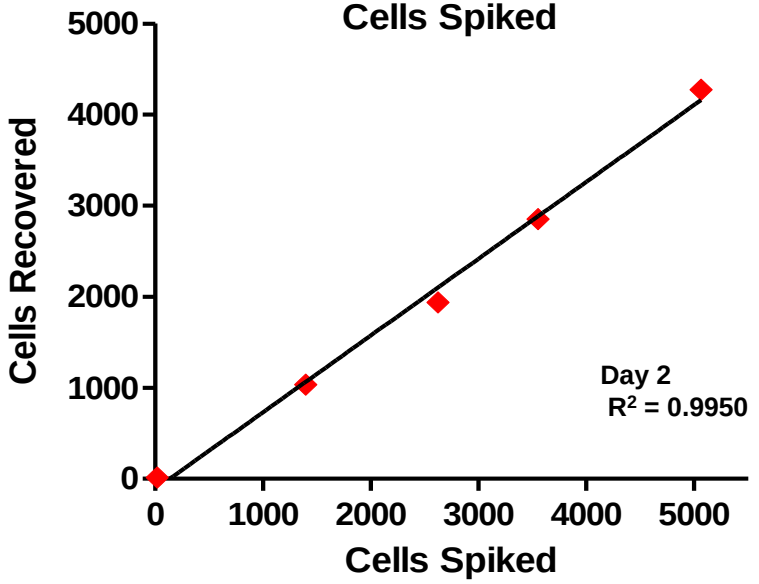


Intra-day Precision



Device Linearity

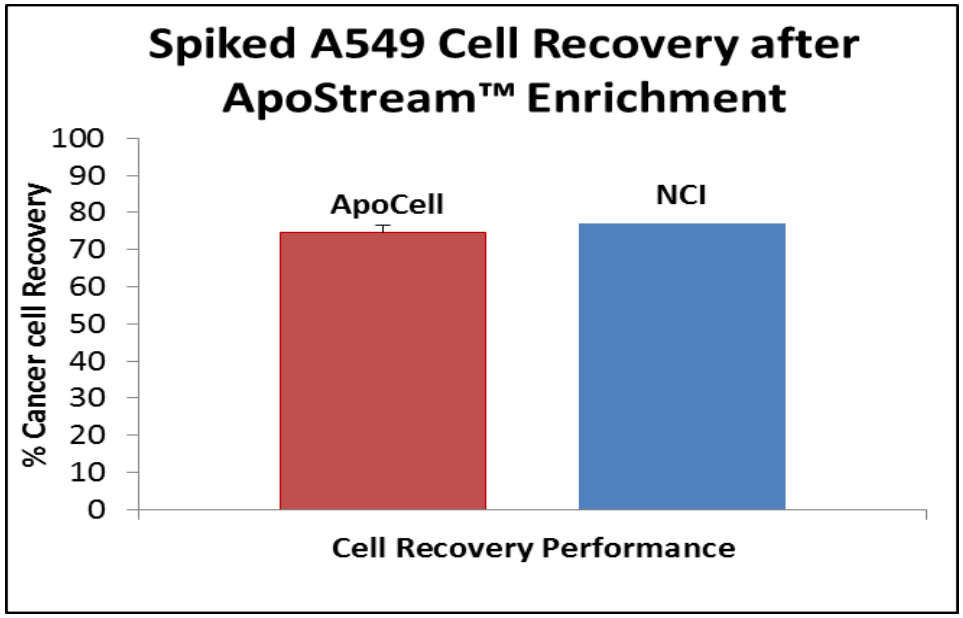




ApoStream™ Performance Across Multiple Sites

Spiking study with A549 cell line (human lung adenocarcinoma)

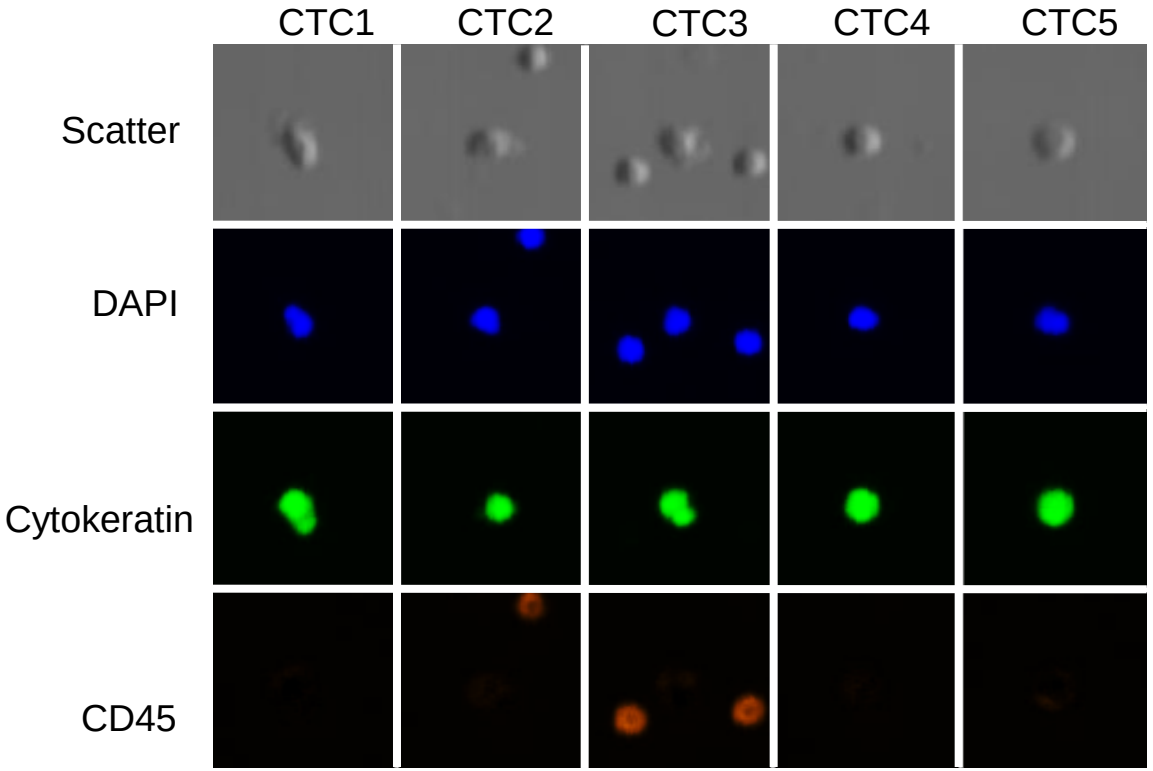
- A549 cells were spiked into PBMCs from 7.5 mL of normal human donor blood
- Process via ApoStream™
  - ApoCell average recovery = 74.5 ± 2% (mean ± SD)
  - NCI average recovery range 62-77 %



NCI results courtesy of Priya Balasubramanian, PhD

* Funded by NCI Contract No. HHSN261200800001E

Phenotypic Identification of CTCs Isolated Using ApoStream™



ApoStream™ isolates conventional $CK^{+}/CD45^{-}/DAPI^{+}$ CTCs from blood of NSCLC

After enrichment, cells are stained for markers of interest (CK, DAPI, CD45, additional markers) and then imaged by laser scanning cytometry.

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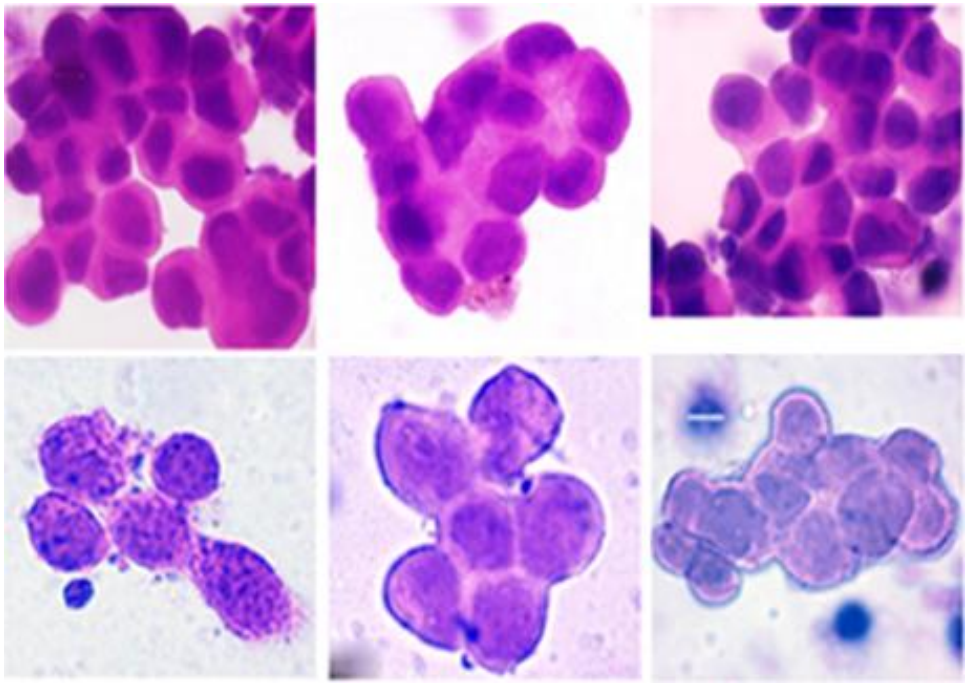
ApoStream™ Isolation of Both Single Cell and Tumor Cell Aggregates

H&E Staining of ApoStream™ Enriched Lung Cancer CTCs



- Tumor cell clusters can be isolated from blood of some patients
- Tumor cell clumps may be vital for cancer cell survival in circulation
- Tumor cell aggregates may be mediators of collective invasion

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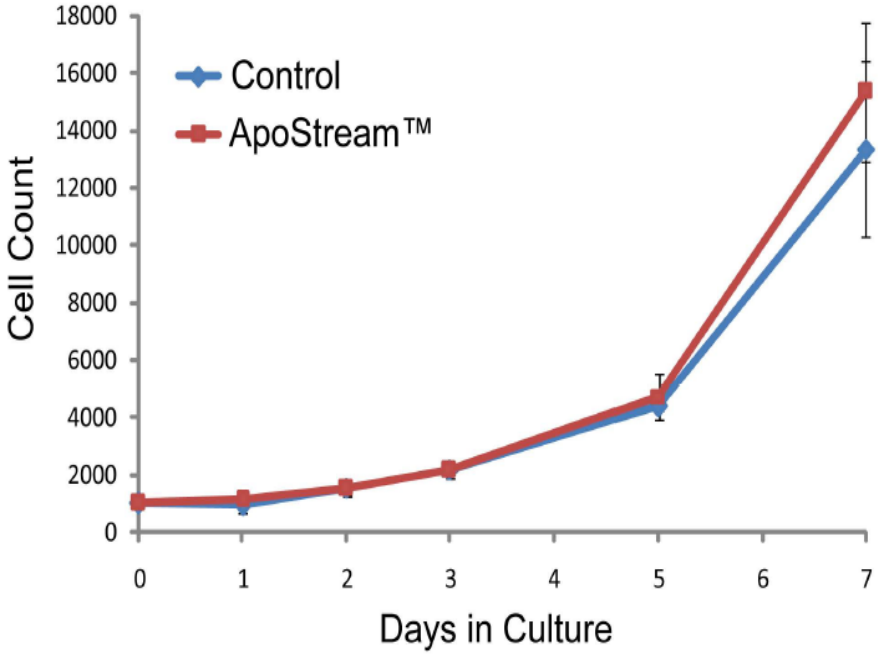
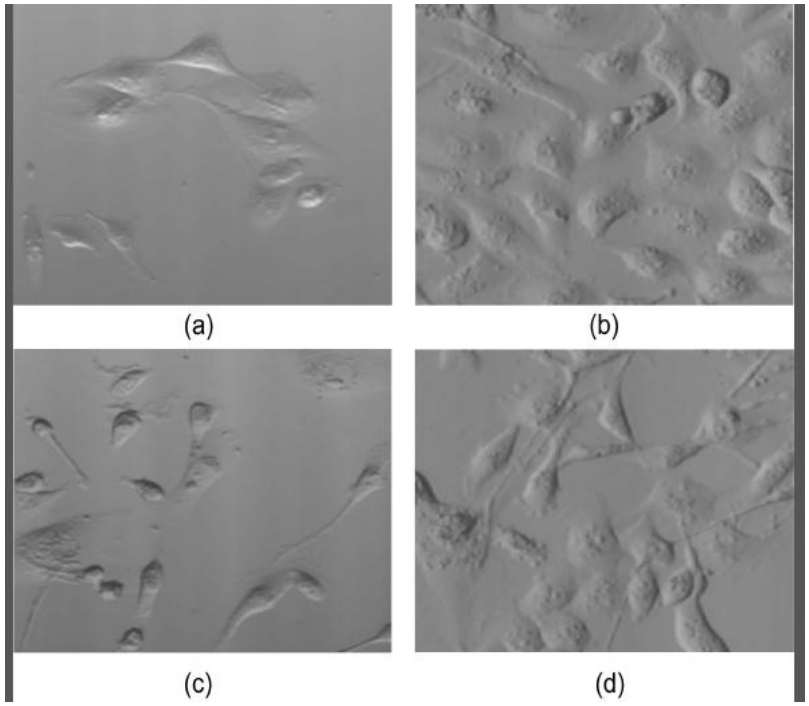
ApoStream™ Captured Cells Retain Viability MDA-MB-231 Breast Cancer Cells



ApoStream™ recovered MDA-MB-231 cancer cells show exponential growth and no difference compared to control cells



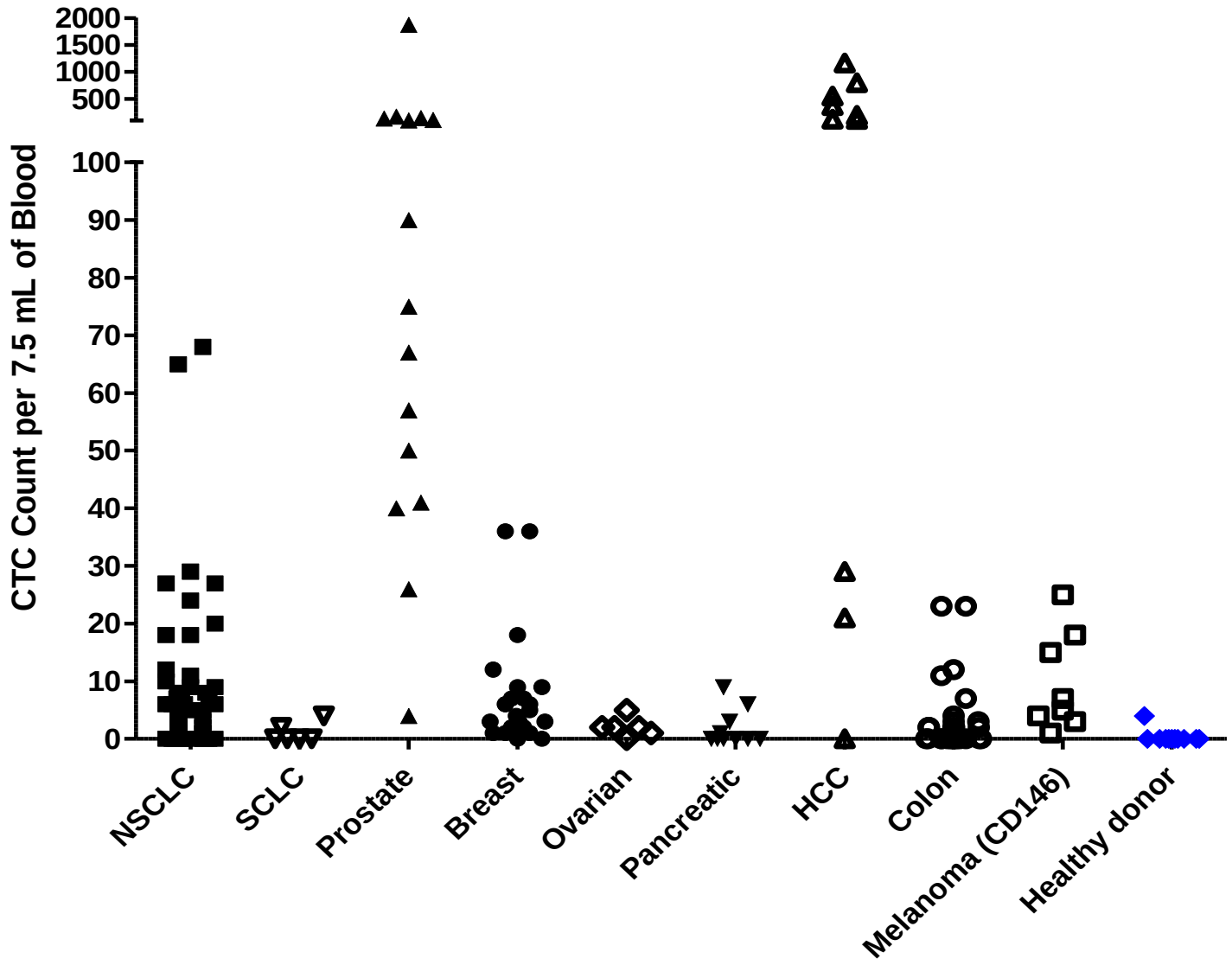
Images of cultured MDA-MB-231 cancer cells at day 2 and day 7: (a,b) control cells (no ApoStream™ separation); (c,d) cells captured with ApoStream™

Prostate Cancer Comparison

Patient	CellSearch® CK+/CD45-DAPI+	ApoStream™ CK+/CD45-DAPI+
1	19	116
2	0	41
3	0	90
4	1	40
5	1	174
6	1	138
7	41	67
8	11	75
9	8	152
10	21	50

- All cell counts obtained by the ApoStream™ technique were higher than CellSearch® ($p<0.01$).
- All 10 patients had detectable CTCs by ApoStream™, while only 80% of patients had detectable CTCs with CellSearch®
- Apostream™ captured
  - mean of 94.3 cells
  - median of 82.5 cells (range 40-174). By comparison,
- Cellsearch® captured
  - mean of 10.3 cells,
  - median of 4.5 cells (range 0-41).

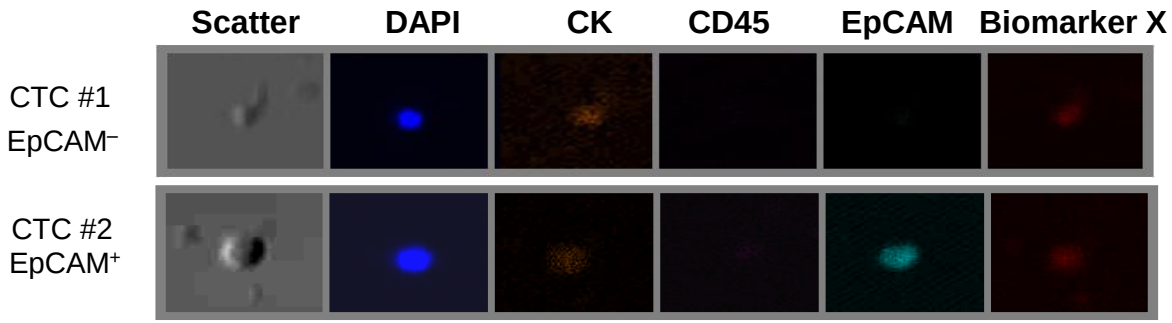
ApoStream™ isolates CTCs from Multiple Cancer Types



ApoStream™ Allows for Recovery of EpCAM negative Circulating Tumor Cells

Phenotypes	*CTC Count from NSCLC patients*		
	Patient A	Patient B	Patient C
CD45-CK+	10	31	52
CD45- CK+ EpCAM+	0	0	0
CD45- CK+ EpCAM-	10	31	52

- ApoStream™ isolates both EpCAM-positive and EpCAM-negative
- ApoStream™ recovered CTCs that would have been missed by EpCAM-based capture methods
- A larger population of potential CTCs exist in NSCLC patients that are CK^- and $CD45^-$ (other phenotypes under investigation)



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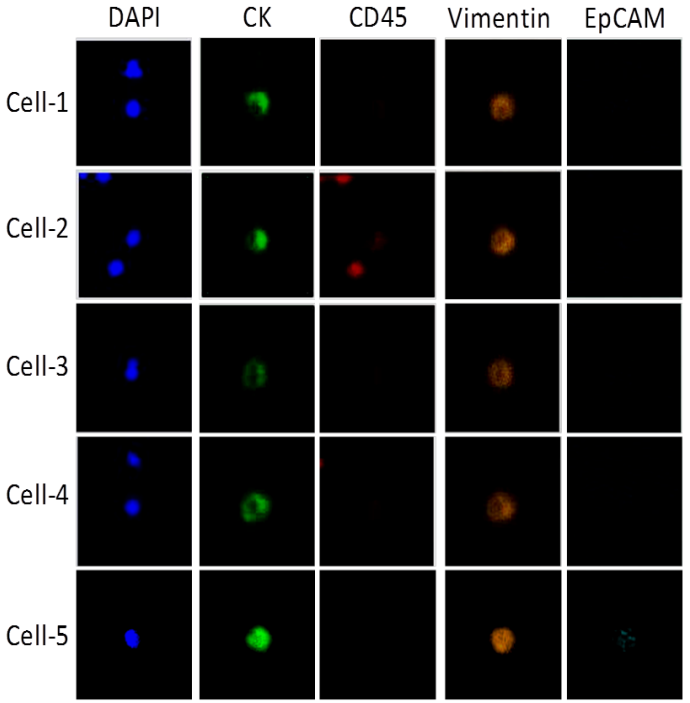
ApoStream™ Recovers High Yield of Breast Cancer CTCs

Patient ID	Number of $CK^+/CD45^-/DAPI^+$ cells per 7.5 mL of blood	ApoStream™ ($CK^+/CD45^-/DAPI^+$ cells)			
	ApoStream™	% $EpCAM^+$/ $Vimentin^-$ cells	% $EpCAM^+$/ $Vimentin^+$ cells	% $EpCAM^-$/ $Vimentin^+$ cells	% $EpCAM^-$/ $Vimentin^-$ cells
1	81	0	3	26	71
2	241	0	0	93	8
3	40	0	0	100	0
4	71	0	11	89	0
5	41	0	3	94	3
6	149	1	0	83	16
7	10	0	0	0	100
8	176	0	0	74	26
9	705	0	0	90	10
10	772	0	0	31	69



Gallery of images of cells isolated by ApoStreamTM and stained with Abs against CK, CD45, vimentin and EpCAM

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Science
AAAS
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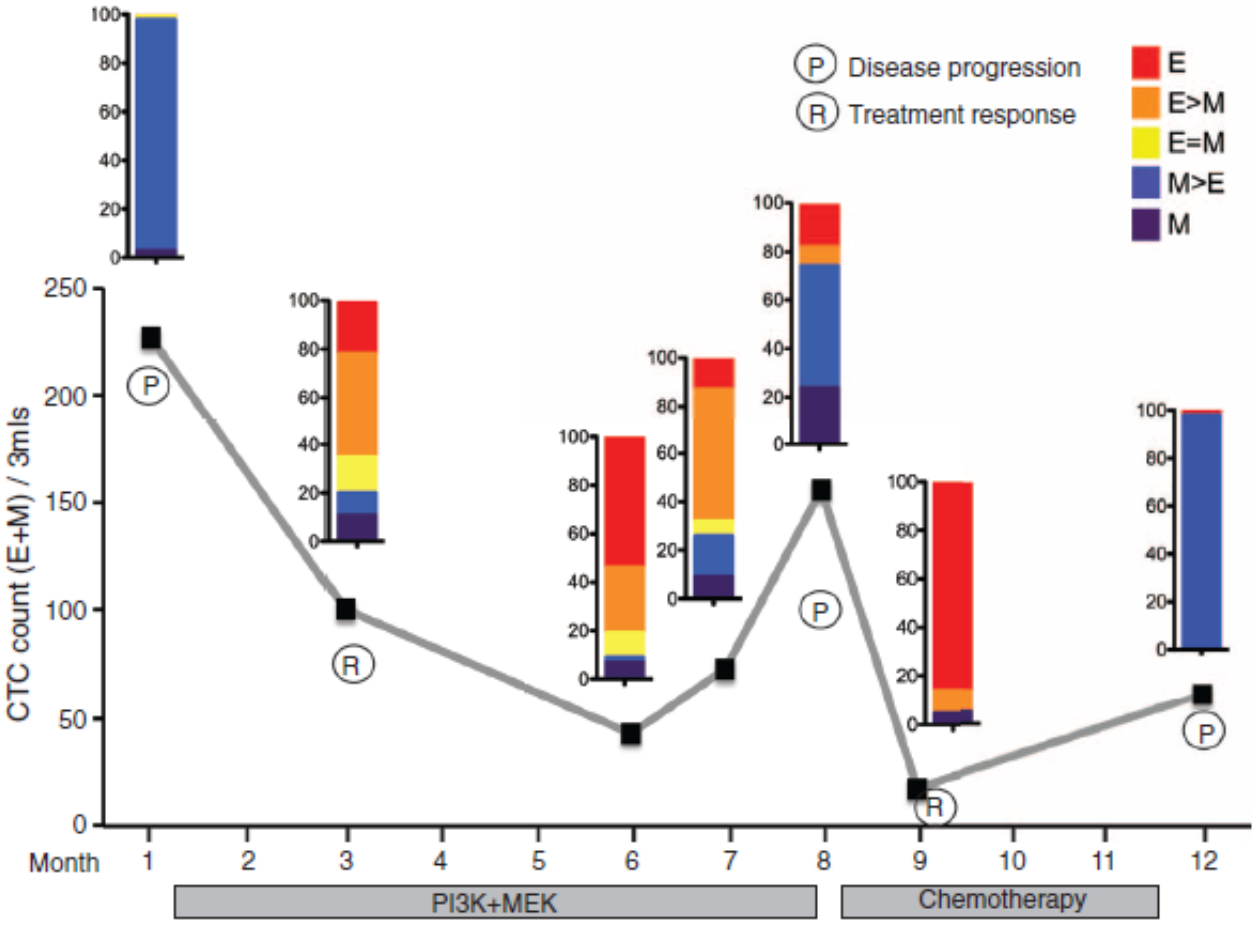
Science **339**, 580 (2013);
Min Yu, Daniel Haber, Shyamala Meheswaram *et al.*

Circulating Breast Tumor Cells Exhibit Dynamic Changes in Epithelial and Mesenchymal Composition



The microfluidic HB (herringbone)–chip to capture CTCs from blood with an antibody cocktail directed against EpCAM, EGFR, and HER2.

Color-coded quantitation of EMT features based on RNA-ISH staining is shown above each time point. RNA-ISH evaluated expression of seven pooled epithelial (E) transcripts [keratins (KRT) 5, 7, 8, 18, and 19; EpCAM; and CDH1 (cadherin 1)] and three mesenchymal (M) transcripts [FN1 (fibronectin 1), CDH2 (cadherin 2), and SERPINE1/PAI1 (serpin peptidase inhibitor, clade E)]

M+ clusters were detected at time points 1, 8, and 12.

Longitudinal monitoring of EMT features in CTCs from an index patient.

ApoStream™ readily captures large numbers of CTCs from tumors not suited for CellSearch® analysis.

Hepatocellular Carcinoma (HCC) is not a high- EpCAM expressing tumor, and as such current antigen based immunomagnetic capture methods are not very successful.

Patient	Serum AFP level (ng/ml)	Macrovascular Invasion	Extrahepatic disease	CTC count by CellSearch® DAPI+/CD45- /CK+	CTC count by ApoStream DAPI+/CD45- /CK+	AFP+ cells by ApoStream DAPI+/CD45- /CK+	AFP- cells by ApoStream DAPI+/CD45- /CK+
1	36,995	Yes	Abdominal Lymph-adenopathy	**0**	**0**	0	0
2	728	No	No	**1**	**21**	13 (62%)	8
3	78	Yes	No	**0**	**125**	90 (72%)	35
4	60	No	Prior tumor rupture	**0**	**554**	540 (97%)	14
5	2,278	Yes	No	**0**	**1,165**	1049 (90%)	157
6	3049	Yes	Abdominal wall implants	**0**	**29**	0	29
7	31,522	Yes	Abdominal Lymph-adenopathy	**0**	**380**	376 (99%)	4
8	29	No	No	**0**	**198**	0	198
9	4,083	No	No	**0**	**121**	52 (43%)	69
10	19,219	Yes	Abdominal Lymph-adenopathy	**0**	**803**	746 (93%)	57

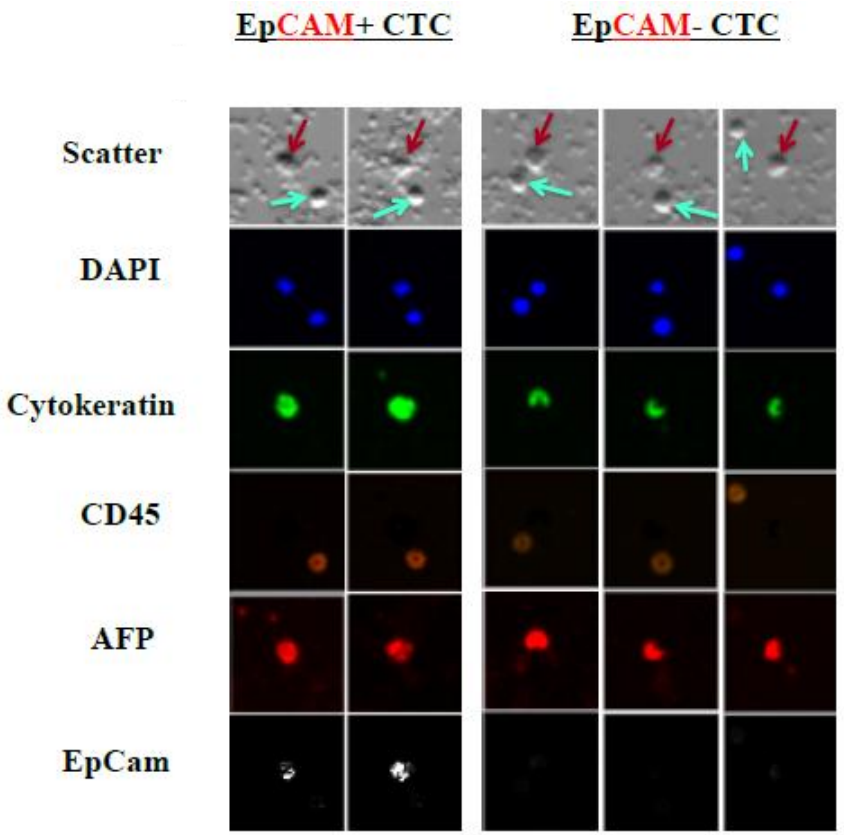
Examples of CTC Heterogeneity Identified with ApoStream™

Patient	Serum AFP	CTC count by ApoStream DAPI+/CD45- /CK+	AFP+ CTCs	AFP – CTC	EpCAM+ CTCs DAPI+/CD45- /CK+	EpCAM – CTCs DAPI+/CD45- /CK+
10	19,2019	803	746	57	87 (11%)	716 (89%)

Note that while the majority of these proposed CTCs are EpCAM negative, there are still a significant number of cells that are EpCAM posititve, yet no cells were identified in this patient with the CellSearch® technique.

The exact reasons why almost no CTCs were captured with CellSearch® is not fully understood.

These cells may have weaker expression of EpCAM, below the threshold of adequate antibody based ferrofluid catpure. It may be due to various processing steps in the CellSearch® methodology



Various other nucleated cell populations identified, not meeting any currently validated CTC definitions

Patient	Serum AFP level (ng/ml)	CTC count by CellSearch DAPI+/CD45- /CK+	CTC count by ApoStream DAPI+/CD45- /CK+	DAPI+ CD45- CK- AFP+ "Double Negative"	DAPI+ CD45+ CK+ TOTAL "Double Positive"	DAPI+ CD45+ CK+ AFP+	DAPI+ CD45+ CK+ AFP-
1	36,995	**0**	**0**	14	1	1	0
2	728	**1**	**21**	9	10	9	1
3	78	**0**	**125**	85	235	184	51
4	60	**0**	**554**	17	1047	978	69
5	2,278	**0**	**1,165**	1	1902	622	1280
6	3049	**0**	**29**	0	203	0	203
7	31,522	**0**	**380**	0	1418	367	1051
8	29	**0**	**198**	0	42	0	42
9	4,083	**0**	**121**	0	131	23	108
10	19,219	**0**	**803**	0	3755	3472	283

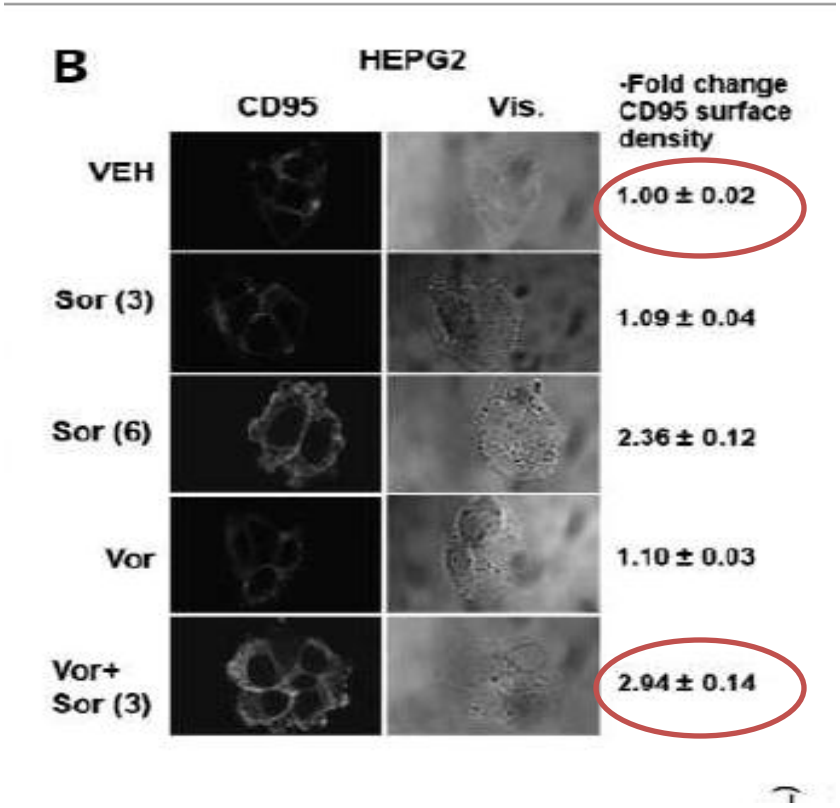
Conclusions from pilot project and next steps

- Within these 10 patients, at least 7 cell populations were identified:
  - AFP+ Classic CTC (DAPI+/CD45-/CK+/EpCAM+)
  - AFP- Classic CTC
  - AFP+ Putative CTC, (DAPI+/CD45-/CK+) but EpCAM-
  - AFP- Putative CTC, but EpCAM-
  - Unknown CD45-/CK-/AFP+ "double negative" cell type, ?EMT
  - Unknown CD45+/CK+/AFP+ "double positive" cell type
  - Unknown CD45+/CK+/AFP- "double positive" cell type

- Next steps will be to better characterize these cell populations, attempt to correlate with known mutations in the patient's primary tumor. (beta-catenin and p53 are 2 of the most common somatic mutations in HCC)

Ongoing Correlative Studies

- In preclinical models, the combination of sorafenib with the HDACi vorinostat increased tumor cell death though upregulation and activation of the extrinsic death receptor CD95 (FAS)
- Clinical trial testing the combination in patients with primary HCC. (NCT01075113)
- Correlative studies will enumerate CTCs before and after combination treatment, and will look at CD95 surface density on CTCs before and after combination treatment.



Cells were plated in eight-well chamber slides and were treated with vehicle (DMSO), sorafenib (3 and 6 µmol/L), or vorinostat (500 nmol/L), as indicated.
Cells were fixed 6 h after exposure and surface levels of CD95 determined by immunohistochemistry.

Park MA, Reinehr R, Häussinger D, Voelkel-Johnson C, Ogretmen B, Yacoub A, Grant S, Dent P. Sorafenib activates CD95 and promotes autophagy and cell death via Src family kinases in gastrointestinal tumor cells. Mol Cancer Ther. 2010 Aug;9(8):2220-31

Next Steps

- Continue to assess sub-populations of cells meeting criteria as potential CTCs, to determine if the current CTC definition needs to be expanded.
  - EMT molecular profile
  - Vascular mimicry profile
  - Collective invasion vs single cell invasion

- Develop CTC culture systems

- Gain additional experience with CTC analysis for pharmacodynamic endpoints and “proof of concept” in drug development in early phase clinical studies.
  - Planned analysis of LC3 autophagic vesicles in CTCs for patients treated with on MCC13874- pemetrexed+sorafenib
  - Planned analysis of EMT/Epithelial markers in metastatic epithelial tumors treated with XRT with or without lapatinib.

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