UKD Universitätsklinikum Düsseldorf

hhu Heinrich Heine Universität Düsseldorf

Circulating Tumor Cells (CTCs)

Nikolas Stoecklein

Experimental Surgical Oncology
Dept. for General, Visceral and Pediatric Surgery
Medical Faculty of the Heinrich-Heine-University
Düsseldorf, Germany

CTC History

1869: First Description of cancer cells in blood

146 *Hospital Reports.* [May,

HOSPITAL REPORTS.

MELBOURNE HOSPITAL.

A case of Cancer in which cells similar to those in the Tumours were seen in the blood after death. Reported by THOMAS RAMSDEN ASHWORTH, Resident Physician.

(WITH ENGRAVING.)

Richard J——, æt. 38, was admitted on Oct. 9th, 1868, suffering from what was understood to be "Rheumatism and Debility." He died of Marasmus on the 10th of the following March.

He had a number of subcutaneous tumours (about thirty) situated over the anterior wall of the thorax and abdomen, varying in size from that of a bean to that of a small orange. There was one between the scapulæ, and another on the inner side of the left thigh about four inches above the knee joint. In none of them had any softening as yet taken place.

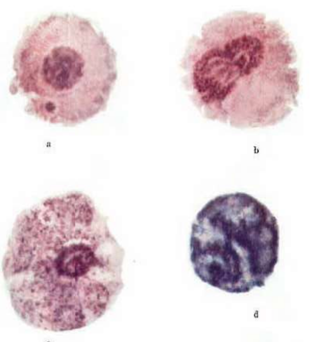
These tumours on section, thirty hours after death, were found to consist of a thick opaque jelly-like substance, of an amber colour, having a thin fibrous covering, and in places a delicate fibrous stroma running through the substance of the tumours themselves. Examined by the microscope, under a power of four hundred diameters, they presented an unusual structural character. The jelly-like substance appeared perfectly transparent dotted over at nearly equal distances with what presented the appearance of cells containing one ormore nuclei (Fig I.) Not being able toaccount for the appearance of these cells being equidistant from each other, some solution of magenta dye was added, and it was then seen that the cells were in reality only the nuclei containing nucleoli of large and beautifully pellucid cells, highly refractive, and not containing a trace of granular matter outside the nucleus They were mostly circular in shape, a few being slightly ovoid. (Fig II.)

These tumours being so numerous, and evidently malignant, it was determined to examine the blood also, in the belief that it might possibly throw some light upon their multiplication in different parts of the body. A portion was accordingly obtained from the internal saphena vein of the right leg which was quite free from any tumour.

This blood was dark and fluid, when viewed by the microscope a little of the magenta solution being added, it was seen to contain comparatively few red corpuscles, these being mostly shrivelled and stellate, large numbers of white corpuscles, often aggregated; together in masses, besides these some cells like white corpuscles only larger, together with patches of granular matter which took the dye; but the most singular circumstance was that occasionally cells were seen exactly in shape, size, and appearance like to those of the tumours. On one examination three were seen in the field of vision at the same time.—(Fig III.)

The Australian Medical Journal 14: 146-147, 1869.

1930-1960: First systematic analyses of CTCs



a, b, c. Typical cells observed in carcinoma cases. d. Atypical cell.

Pool & Dunlop *Am J Cancer* 1934;21:99-102.

582 MOORE, SANDBERG AND SCHUBARG Annals of Surgery October 1957

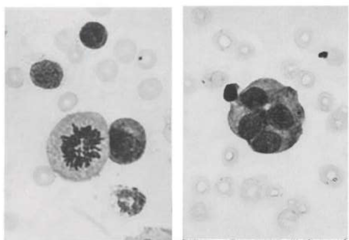


FIG. 3. #76578. Carcinoma of the pancreas. Tumor cells undergoing mitosis are not uncommon. Polyploid numbers of chromosomes and abnormal configuration of the spindle are frequent. Blood sample obtained after surgical manipulation; premanipulation specimen was negative.

FIG. 4. #77914. Carcinoma of sigmoid colon with metastases. A multi-nucleated tumor cell from a blood sample obtained before surgical manipulation from a mesenteric vein.

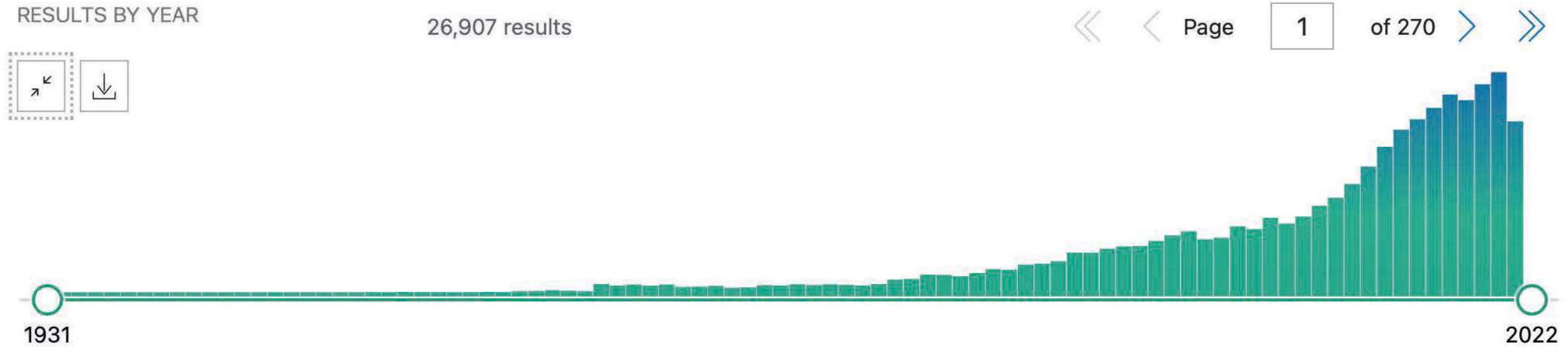
cytes and plasma cells, exfoliated endothelial cells, and deep-staining cells of the erythrocyte series cause one to stop and examine them under higher power, thus lengthening the time spent on each slide. Patients with advanced disease, benign or malignant, are more apt to have immature and "atypical" cells. In one of nine patients with benign disease, a large cell was identified in the preliminary screening as a definite tumor cell. A preliminary paper with further technical details is in press.*

OBSERVATIONS

In general, tumor cells have a "different" appearance than cells of the hemopoietic system which is difficult to analyze. They are usually much larger than normal cellular elements, and, in fact, size is such an important criterion (see Figs. 1 through 6) that probably some single small tumor cells have been called negative. Table I illustrates the comparitive difference in the average diameters of tumor cells and adult neutrophil leukocytes. Two diameters at right angles of three undistorted cells were averaged for each patient. Neutrophils were measured from each slide so as to control variations resultant to fixation, preparation, and staining. Next in importance is the morphology and the staining properties. For example, breast cancer cells are often very little larger than promyelocytes but are distinguishable by their immature nucleus, large nucleoli, blue-gray cytoplasm which is often foamy, and the presence of fine azurephillic granules. Tumor cells may undergo mitosis in the blood (Fig. 3), and

1961: "*Clinical Significance* of Cancer Cells in the Circulating Blood: Two- ot Five-year Survival". Ann Surg., 154:362

CTC History



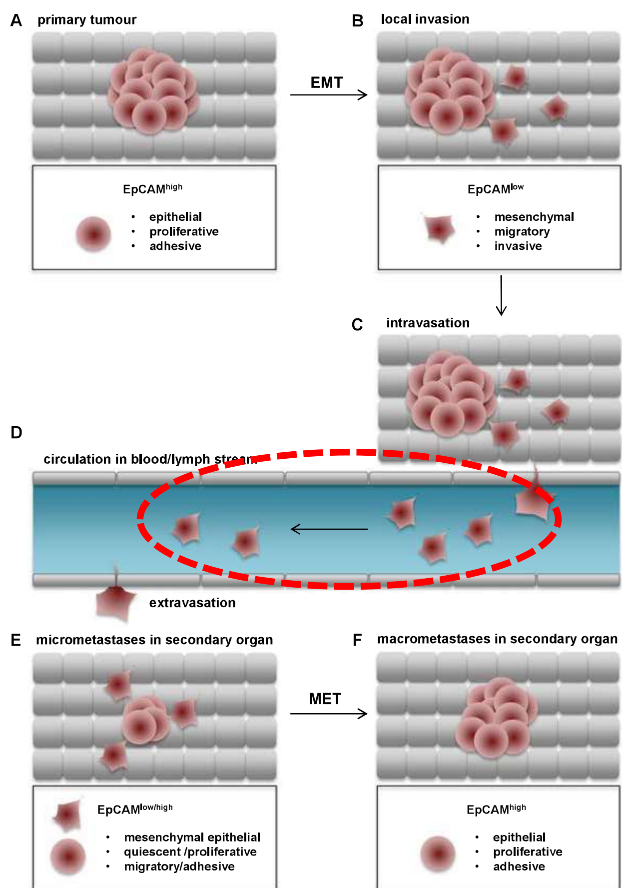
5-6 new papers per day!

Source: https://pubmed.ncbi.nlm.nih.gov/

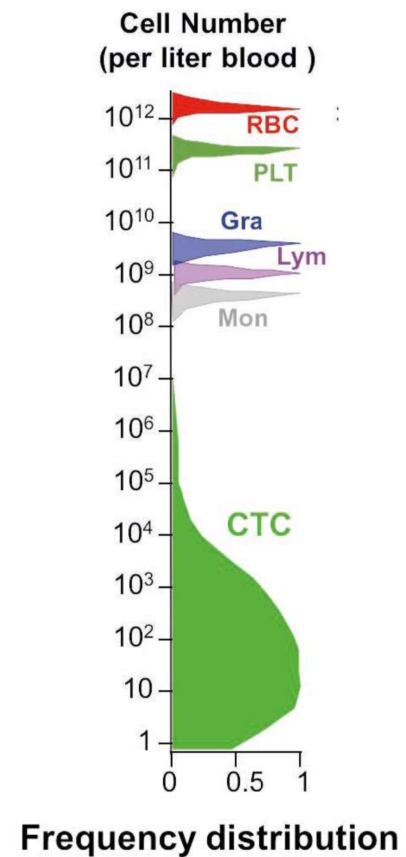
Blood based diagnostics as clinical routine in leukemias

Thanks to Dr. Johannes Fischer, ITZ, University Hospital Düsseldorf

CTCs: epithelial cancer cells that entered the blood stream



Gires & Stoecklein 2014, Cell Mol Life Sci



Stoecklein, ..., Terstappen, Expert Review Mol Diagnostics 2016

CTC based diagnostics in epithelial cancers

CHALLENGES

- **CTCs are extremely rare**
- **no cancer-specific markers available**
- **analysis of a small volume of blood (~10 mL)**

Blood based diagnostics in epithelial cancers

Source: https://www.triz.co.uk/

A typical sample of a M1 Breast CA patient (7.5 mL):

Erythrocytes: ~ 38×10^9

Platelets: ~ 2.2×10^9

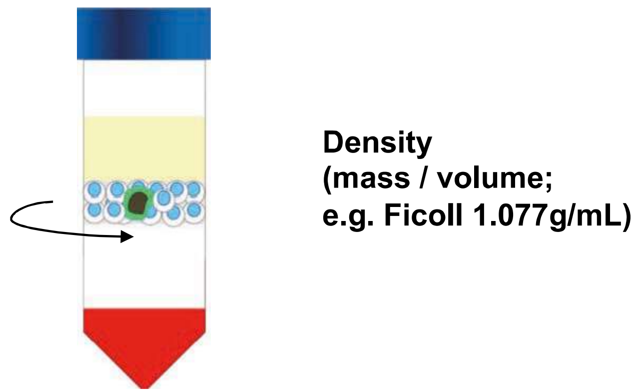
WBC: ~ 45×10^6

CTC: 5

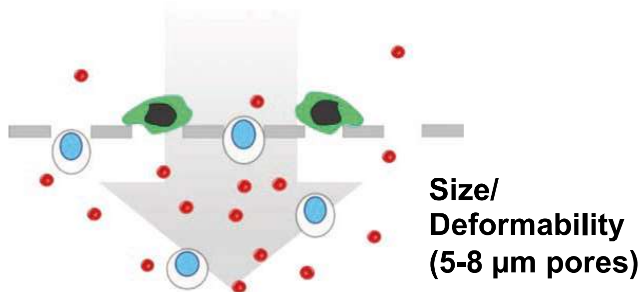
→ Enrichment required!!!

CTC Enrichment Strategies

Biophysical Properties

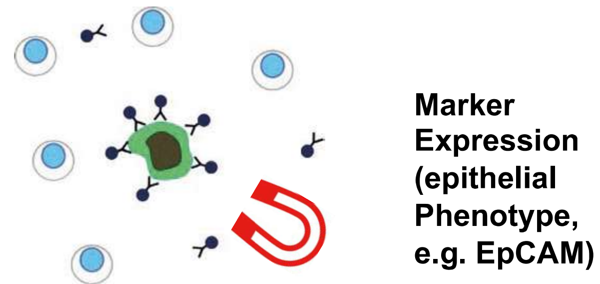


Density
(mass / volume;
e.g. Ficoll 1.077g/mL)

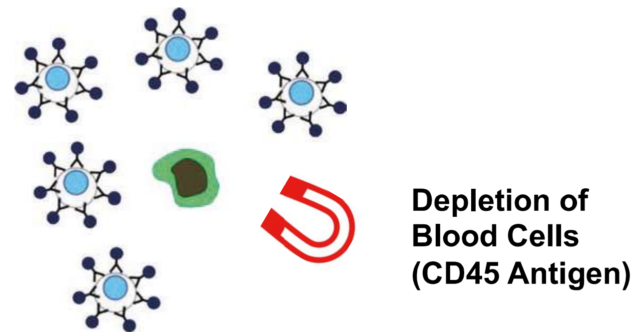


Size/
Deformability
(5-8 µm pores)

Molecular Properties



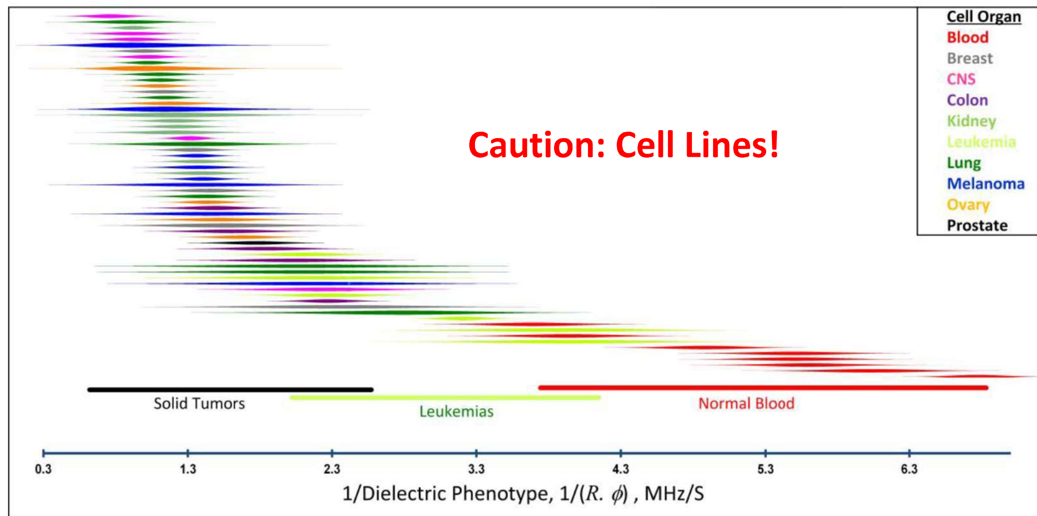
Marker
Expression
(epithelial
Phenotype,
e.g. EpCAM)



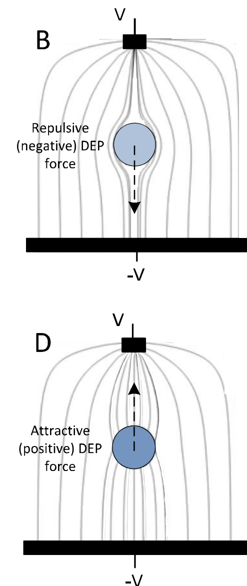
Depletion of
Blood Cells
(CD45 Antigen)

CTC Enrichment Strategies

Physical Properties



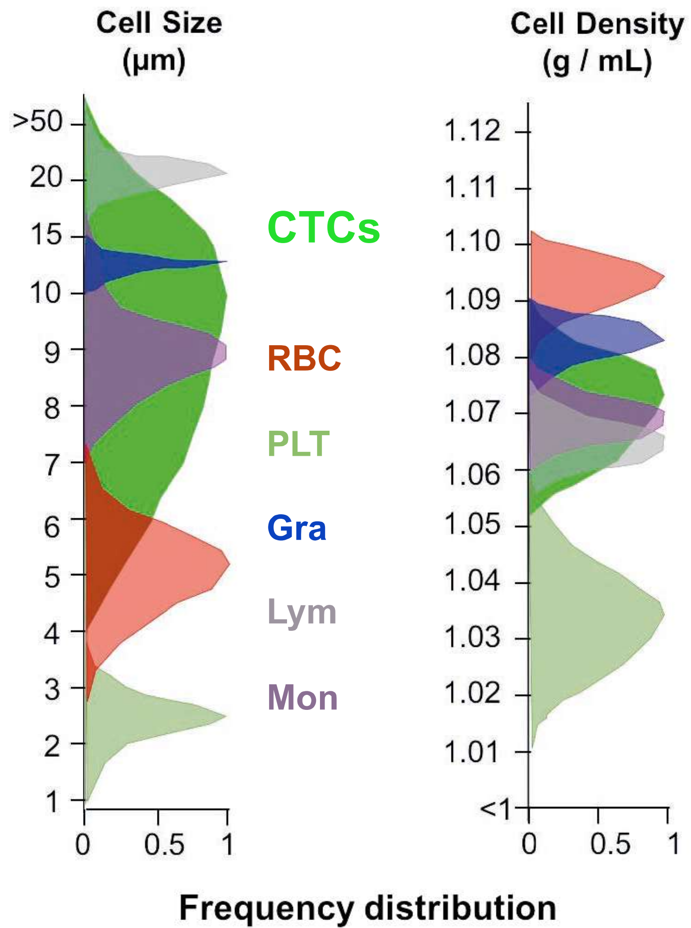
Taken from: Gascoine and Shuim, *Cancers* **2014**, *6*, 545-579; doi:10.3390/cancers6010545



Enrichment by dieelectrophoretic (DEP) force

The challenge detecting CTC in peripheral blood

Physical Characteristics of CTCs



Stoecklein, ..., Terstappen, Expert Review Mol Diagnostics 2016

The challenge detecting CTC in peripheral blood

Molecular Characteristics of CTCs

- **change their phenotype → e.g. loss of EpCAM**
  (e.g. Gorges, BMC Cancer 2012; Yu, Science 2013)

- **display heterogneous expression pattern → e.g. HER2 & ER expression**
  (Fehm, Breast Cancer Res Treat 2010, Babayan 2013)

- **mRNA expression profiling revealed intra-patient heterogeneity**
  (e.g. Powell, PlosOne 2012; Yu, Science 2013; Steinert, Cancer Res 2014)

- **genetic analysis showed intra-patient heterogeneity**
  (e.g. Steinert, Cancer Res 2014; Neves Clin Chemistry, 2014; Polzer EMBO Mol Med 2014)

The challenge detecting CTC in peripheral blood

CTCs are a heterogenous cell population!

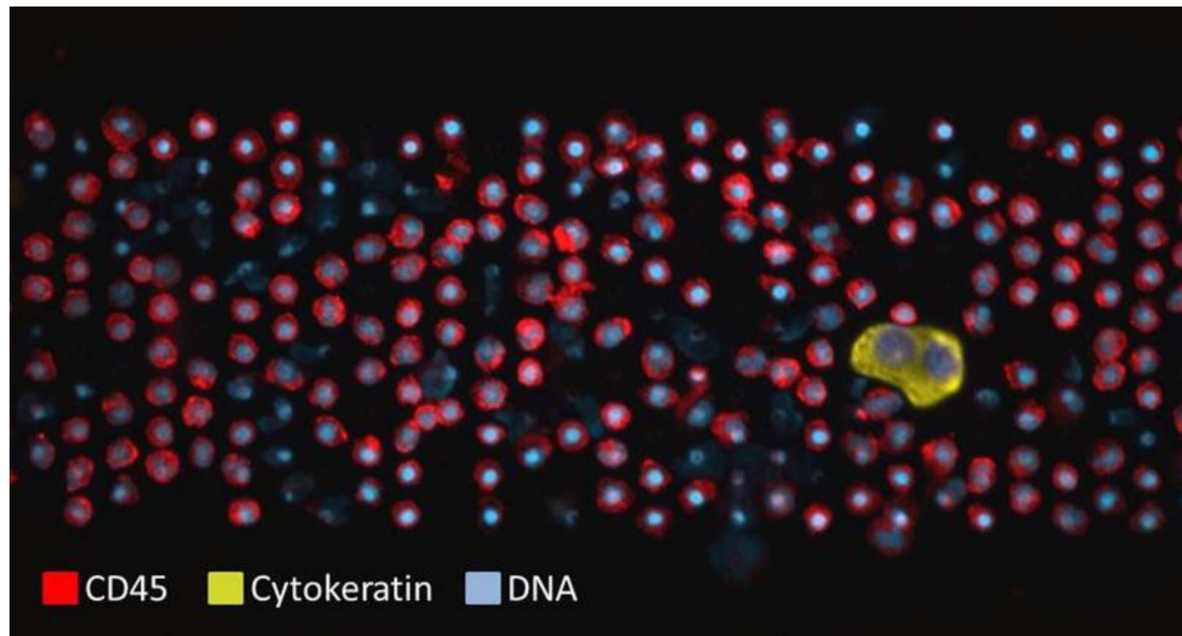
→ no CTC enrichment 100% effective...

e.g. because CTC loss due to

- smaller CTCs in Filtration (e.g. < 8 µm)
- EpCAM$^{low/neg}$ cells in EpCAM-dependent approaches

The challenge detecting CTC in peripheral blood

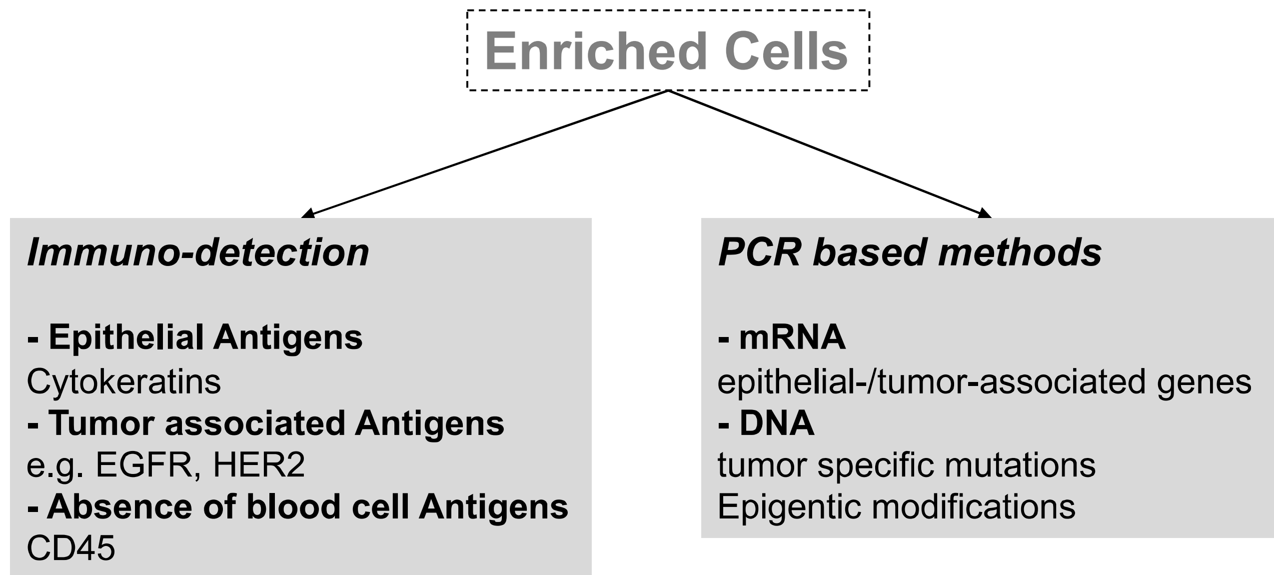
No enrichment methods delivers pure CTCs!



http://www.vycap.com/circulating%20tumor%20cells_files/image006.jpg

CTC detection strategies

Further qualification required



Current standard: Immuno-detection

EpCAM+/DAPI+/CK+/CD45- Phenotype

CTC detection via mRNA (after immunomagnetic enrichment - AdnaTest)

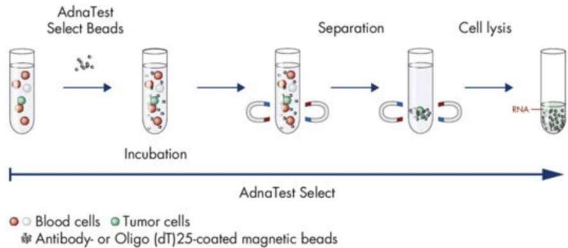


Figure 1. AdnaTest ProstateCancerSelect: Immunomagnetic cell selection with multiple tumor associated antibodies. In the first step, the CTCs in the blood are enriched (AdnaTest Select). This is achieved using antibody-coated magnetic particles (beads). Several antibodies are used, which bind with high specificity and affinity to the corresponding cancer cells. The enriched cells are lysed and subsequently purified several times to extract mRNA.

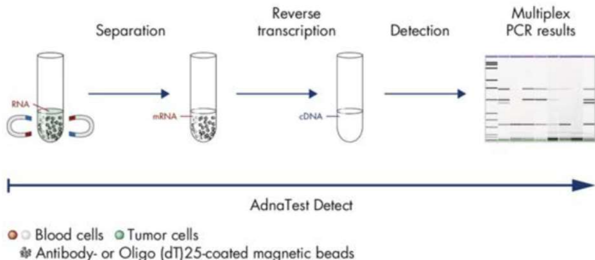


Figure 2. AdnaTest ProstateCancerDetect: Multiplex PCR of various cancer associated tumor markers. In a second step the enriched cells are examined by RT-PCR for tumor associated expression patterns. The mRNA strands are reverse transcribed into cDNA. Subsequently, several associated tumor markers can be amplified using multiplex PCR and visualized.

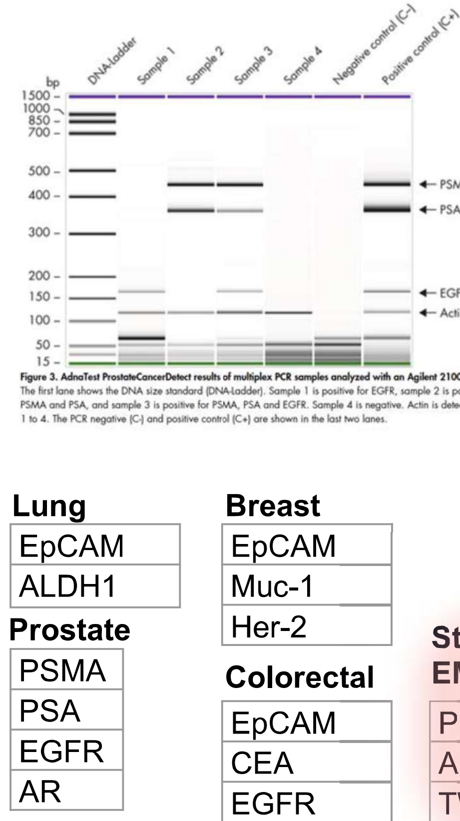


Figure 3. AdnaTest ProstateCancerDetect results of multiplex PCR samples analyzed with an Agilent 2100 Bioanalyzer. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is positive for EGFR, sample 2 is positive for PSMA and PSA, and sample 3 is positive for PSMA, PSA and EGFR. Sample 4 is negative. Actin is detected in samples 1 to 4. The PCR negative (C-) and positive control (C+) are shown in the last two lanes.

Lung
EpCAM
ALDH1

Prostate
PSMA
PSA
EGFR
AR
Ar-V7

Breast
EpCAM
Muc-1
Her-2

Colorectal
EpCAM
CEA
EGFR

Stem cell/ EMT
PI3Kα
Akt-2
TWIST1

?

EDTA → 4 hrs
BD ACDA tubes → 30 hrs

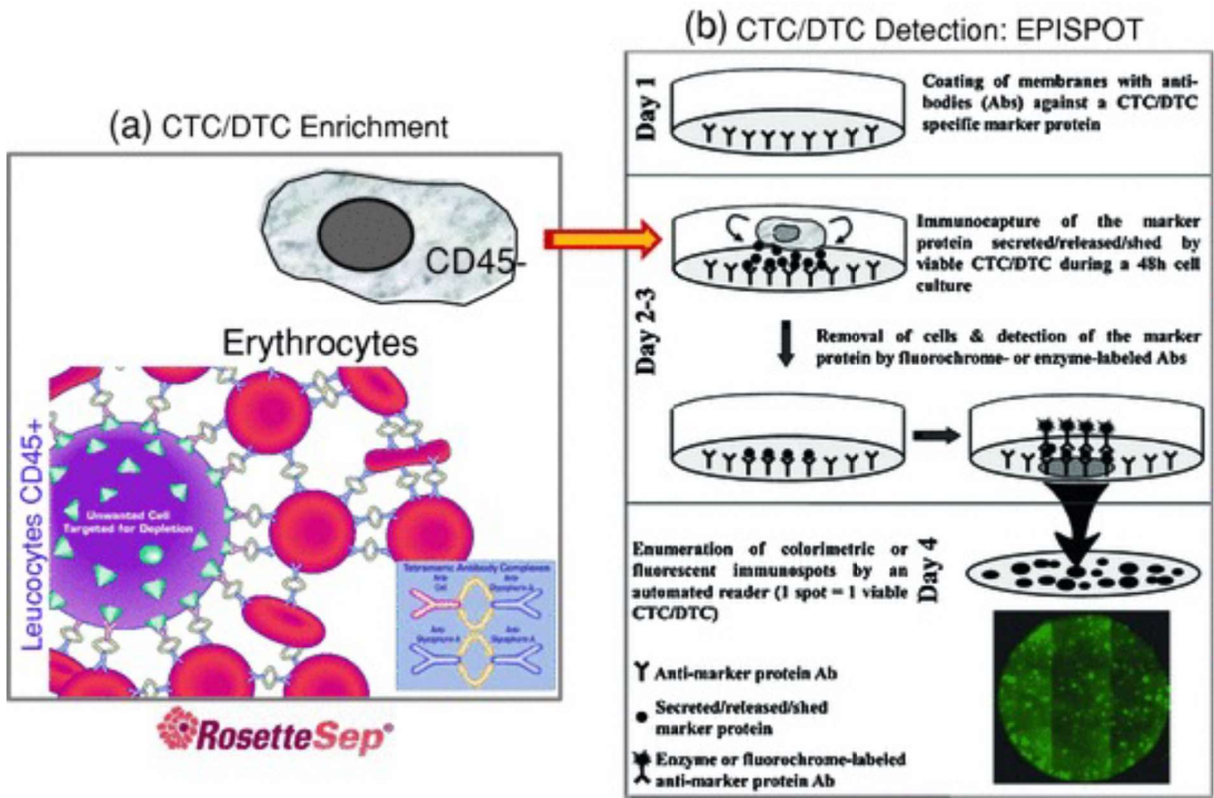
Blood must be stored at 4–8°C immediately

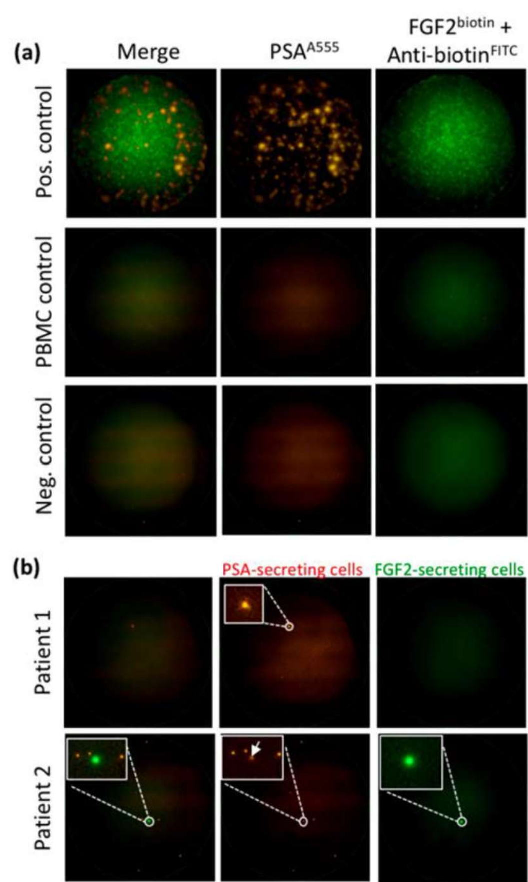
Sensitivity
2-5 CTCs / 5 mL
Specificity around 95%

ACDA: Anticoagulant Citrate Dextrose Solution A

Hauch et al Anticancer Res 2007; Information taken from https://www.qiagen.com/ (03/2022)

EPISPOT Assay: Detection of viable CTCs (secretion)



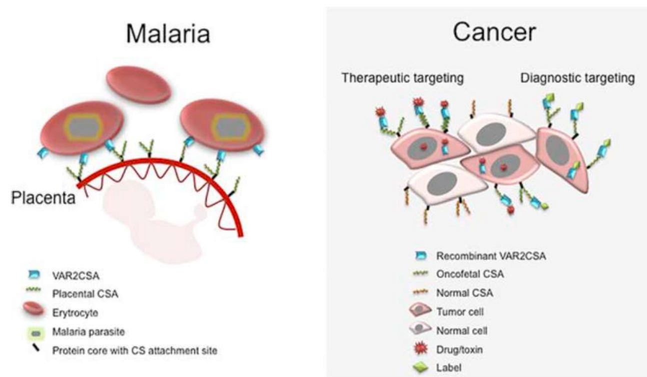


Alix-Panabières, Recent Results in Cancer Research 2012, Budna-Tukan et al Cancers 2019

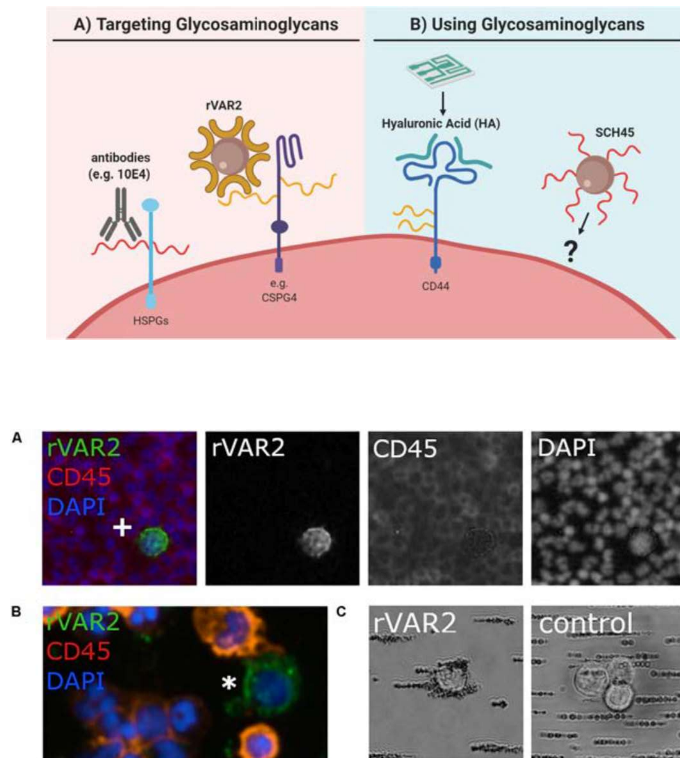
VAR2 and oncofetal chondroitin sulfate (ofCS)

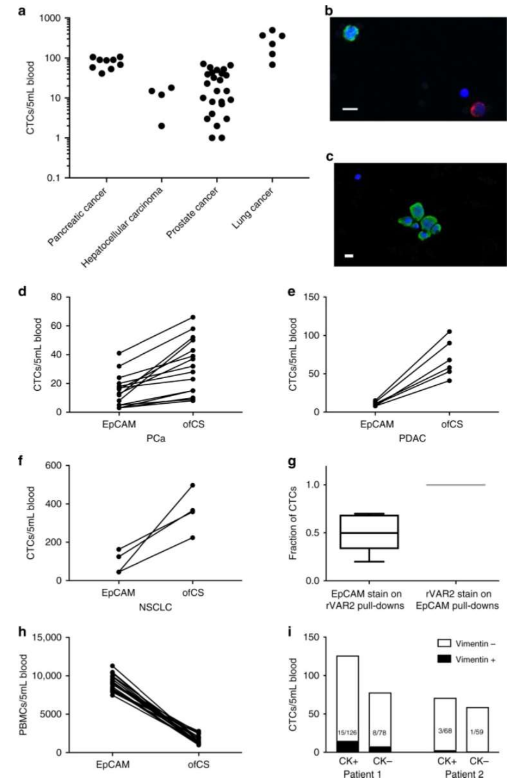
- chondroitin sulfate proteoglycans (CSPGs)
→ cell membrane / ECM

- CS → attached to >30 different proteoglycans



Malaria Research → Ali Salanti‘s group
Copenhagen, DK





Taken from Ahrens et al Front. Cell Dev. Biol., 2020, Agerbæk et al , Nature Comm 2018

>40 CTC Assays available...

Immunoaffinity Assays (molecular)

Table 1 — CTC technologies.

Subcategory	Technology	Selection criteria	Key features	References
Immunoaffinity — Positive Enrichment *Antibodies targeting tumor-associated antigens are tethered to magnetic particles (immunomagnetic) or the device surface to capture CTCs.*				
IM	CellSearch®	EpCAM	FDA-Approved	(Cristofanilli et al., 2004; Hayes et al., 2006; Riethdorf et al., 2007)
	AdnaTest	Antibody Cocktail	CTCs captured then multiple cancer markers measured by RT-PCR	(Andreopoulou et al., 2012; Müller et al., 2012; Musella et al., 2015)
	MACS	EpCAM	Pos/Neg enrichment, high surface area to volume	(Miltenyi et al., 1990; Pluim et al., 2012)
	MagSweeper	EpCAM	High purity, can process WB, 9 mL/h throughput	(Deng et al., 2014; Kim et al., 2014; Lohr et al., 2014; Powell et al., 2012; Talasaz et al., 2009)
Microfluidic (μF) Micropost Arrays	CTC-Chip	EpCAM	Micropost array optimized for cell-antibody contacts, 1–2 mL/h	(Nagrath et al., 2007)
	GEDI	PSMA/HER2, Size	Size-based separation minimizes contamination	(Galletti et al., 2014; Kirby et al., 2012)
	OncoCEE	Antibody Cocktail	Staining procedure labels capture antibodies	(Mikolajczyk et al., 2011)
Microfluidic Surface Capture	Herringbone Chip	EpCAM	Microvortices mix sample, clusters observed, 4.8 mL/h	(Stott et al., 2010)
	GEM	EpCAM	Microvortices mix sample, 3.6 mL/h	(Sheng et al., 2014)
	Graphene Oxide Chip	EpCAM	Planar geometry, 1–3 mL/h	(Yoon et al., 2013)
	Modular Sinusoidal Microsystem (commercialized by BioFluidica)	EpCAM	Three modules: selection, impedance, and imaging, clusters observed, >86% purity, >7.5 mL/h	(Kamande et al., 2013)
Microfluidic IM	Ephesia	EpCAM	Self-assembly of magnetic μm-beads into columns, >3 mL/h	(Autebert et al., 2015; Saliba et al., 2010)
	Magnetic Sifter	EpCAM	Vertical flow configuration, 10 mL/h	(Earhart et al., 2013)
	LiquidBiopsy	Antibody Cocktail	Automated, sheath flow minimizes non-specific binding, continuous flow, 5 mL/h	(Winer-Jones et al., 2014)
μF, IM	Isoflux™	EpCAM	Automated, continuous flow	(Harb et al., 2013).
μF, IM	CTC-iChip	EpCAM, Size	Pos/neg enrichment, size-based separation debulks WB, inertial focusing aids in magnetic deflection, 8 mL/h	(Karabacak et al., 2014; Ozkumur et al., 2013)
IM, *in vivo*	GILUPI CellCollector™	EpCAM	Can process large volumes of blood	(Saucedo-Zeni et al., 2012)
Immunoaffinity — Negative Enrichment *Antibodies targeting leukocyte-associated antigens are tethered to magnetic particles or the device surface deplete unwanted background cells.*				
IM	EasySep™ Human CD45 Depletion Kit	CD45	Simple, easy-to-use batch separation	(Liu et al., 2011)
	QMS		Continuous flow, high-throughput	(Lara et al., 2004; Wu et al., 2013)
	MACS			(Giordano et al., 2012; Lara et al., 2006)
μF, IM	CTC-iChip	CD45, CD66b, Size		(Karabacak et al., 2014; Ozkumur et al., 2013)
Biophysical — Density Gradient Centrifugation *Sample placed on top of a separation medium and centrifuged to separate different cell populations based on their relative densities.*				
	Ficoll-Paque®	Density	Inexpensive, easy-to-use	(Weitz et al., 1998)
	OncoQuick	Density, Size	Porous membrane above separation media for additional separation by filtration	(Balic et al., 2005; Clawson et al., 2012; Lagoudianakis et al., 2009; Müller et al., 2005; Obermayr et al., 2010; Rosenberg et al., 2002)

Biophysical Assays

Table 1 (continued)

Subcategory	Technology	Selection criteria	Key features	References
	RosetteSep™ CTC Enrichment Cocktail	Density, Antibody Cocktail	Antibody-labeling alters cell density	(He et al., 2008)
	Accucyte Enrichment and CyteSealer™	Density	Sequential density fractionation enriches target cells. Additional CyteFinder™ and CytePicker™ modules for high-throughput imaging and single-cell recovery	(Campton et al., 2015)
Biophysical — Microfiltration in Two and Three Dimensions *Size-based cell separation using pores or three-dimensional geometries.*				
2D, Track-etched	ISET®	Size, Deforma-bility	Filters fixed samples through 8-μm pores, 10–12 wells can process 1 mL each, clusters observed	(Chinen et al., 2013; Farace et al., 2011; Hofman et al., 2011; Ilie et al., 2014; Krebs et al., 2012; Pailler et al., 2013; Vona et al., 2000)
	ScreenCell®		Hydrophilic surface, fixed/live samples, 7.5/6.5-μm pores	(Desitter et al., 2011; Freidin et al., 2014)
2D, Lithography	CellSieve™		Constructed from a transparent, flexible, non-fluorescent photoresist, 7-μm pores	(Adams et al., 2014a, 2014b, 2015)
	Flexible Micro Spring Array (FMSA)		Constructed from parylene-C, can process WB, captures viable cells, clusters observed	(Harouaka et al., 2014; Kaifi et al., 2015)
3D	FaCTChecker		Constructed from parylene-C, captures viable cells between membrane layers	(Zhou et al., 2014)
	Parsortix		Viable cells released by reversing flow	
	Resettable Cell Trap		Pneumatically-controlled microvalves	(Qin et al., 2015)
	Cluster Chip		Triangular pillars designed for CTC clusters	(Sarioglu et al., 2015)
Biophysical — Inertial Focusing *Cells are passively separated by size through the application of inertial forces in microfluidic devices that affect positioning within the flow channel.*				
	Vortex	Size	No RBC lysis required, captures viable cells, easy to manufacture, clusters observed	(Sollier et al., 2014)
	ClearCell® FX		RBC lysis required, 1–1.5 mL/min, captures viable cells, easy to manufacture	(Khoo et al., 2015, 2014; Warkiani et al., 2014)
Biophysical — Electrophoresis *Separates cells based on their electrical signature using an applied electric field.*				
	ApoStream®	Electrical Signature	DEP-FFF, continuous flow, captures viable cells, >10 mL/h	(Gupta et al., 2012; Shim et al., 2013)
	DEPArray™		Requires pre-enrichment, allows recovery and manipulation of viable, single cells through DEP cages	(Carpenter et al., 2014; Fabbri et al., 2013; Fernandez et al., 2014; Manaresi et al., 2003; Peeters et al., 2013; Polzer et al., 2014)
Biophysical — Acoustophoresis *Separates cells based on acoustophoretic mobility, which is size dependent, by exposing them to acoustic waves.*				
	Acoustophoresis Chip	Size	Acoustic pre-alignment and separation	(Antfolk et al., 2015a, 2015b; Augustsson et al., 2012)

(continued on next page)

Table 1 (continued)

Subcategory	Technology	Selection criteria	Key features	References
Direct Imaging Modalities *Technologies developed to i) improve the efficiency of imaging or ii) replace enrichment through high-speed fluorescent imaging.*				
Pre-Enrichment Required	Microfluidic Cell Concentrator (MCC)	None	Passive pumping concentrates samples ~5x	(Casavant et al., 2013)
	ImageStream®	CK, CD45, DRAQ5	Hybrid of flow cytometry and fluorescence microscopy, 5000 cells/sec	(López-Riquelme et al., 2013; Zuba-Surma and Ratajczak, 2011)
Enrichment-Free	EPIC	CK, CD45, DAPI	Automated digital microscopy, clusters observed	(Marrinucci et al., 2012; Nieva et al., 2012)
	FASTCell™	CK, CD45, DAPI	Fiber-optic array allows larger field-of-view, 25M cells/min, low resolution	(Das et al., 2012; Krivacic et al., 2004; Somlo et al., 2011)
	CytoTrack	CK, CD45, DAPI	Special glass disc scanned as it spins, clusters observed, 100 M cells/min	(Hillig et al., 2015)
In vivo	Photoacoustic flow cytometry (PAFC)	Absorption spectra	Non-invasive label-free interrogation of large blood volumes	(Galanzha and Zharov, 2013)
Functional Assays *Viable CTC enrichment based on bioactivity of cells, such as protein secretion or cell adhesion.*				
	EPISPOT	Protein secretion	Discriminates between viable and apoptotic CTCs using protein secretion	(Alix-Panabières, 2012; Alix-Panabières and Pantel, 2015; Ramirez et al., 2013)
	Vita-Assay™	Cell adhesion matrix (CAM)	Enriches for viable CTCs based on preferential CAM adhesion. Clusters observed.	(Friedlander et al., 2014; Lu et al., 2010)

IM — immunomagnetic, μF — microfludic, WB — whole blood. Companies that have commercialized the technologies above are mentioned in the text.

Ferreira et al. Mol Oncol. 2016 Mar;10(3):374–94.

Exhaustive Reviews on CTC platforms / Chips

Ferreira et al Mol Onc
DOI: 10.1016/j.molonc.2016.01.007

Pei et al LabChip 2020 DOI: 10.1039/D0LC00577K

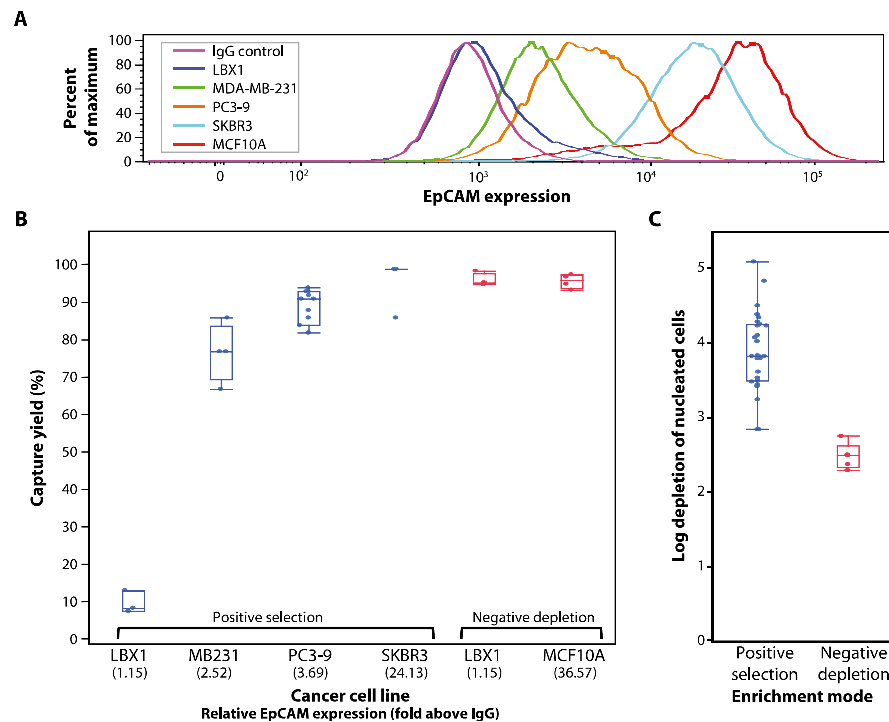
Rushton et al Cancers 2021 DOI:
10.3390/cancers13050970

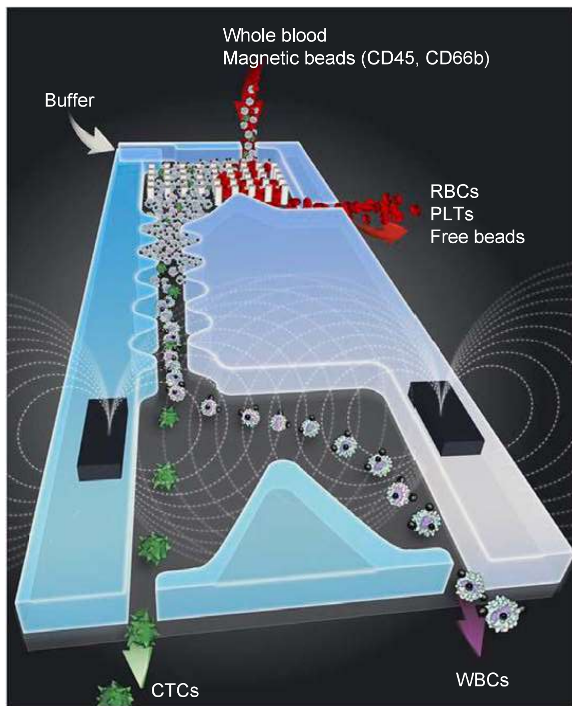
Microfluidic CTC detection devices

The CTC-iChip

Module 1:
CTC-iChip1
(deterministic lateral displacement)

Module 2:
CTC-iChip2
(inertial focusing and magnetophoresis)

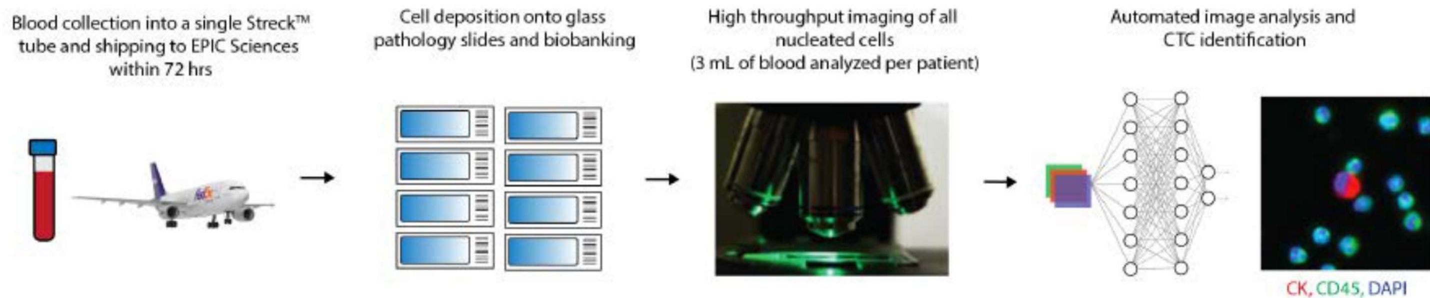




Processing rate of 8 ml of whole blood/h

Taken from Karabacak et al. Nature Biotechnology 2014 & Ozkumur et al. Science TM 2014

Epic Sciences integrated analysis platform (fee for service - centralized analysis)



Circulating Tumor Cell Definition

- Has epithelial lineage (CK+)
- No leukocyte lineage (CD45-)
- Has an intact nucleus (DAPI+)
- Clusters of CTCs are counted as 1 event
- The reported CTC number is from or per 3 mL of blood
- Median turn-around-time is 4 days from blood collection

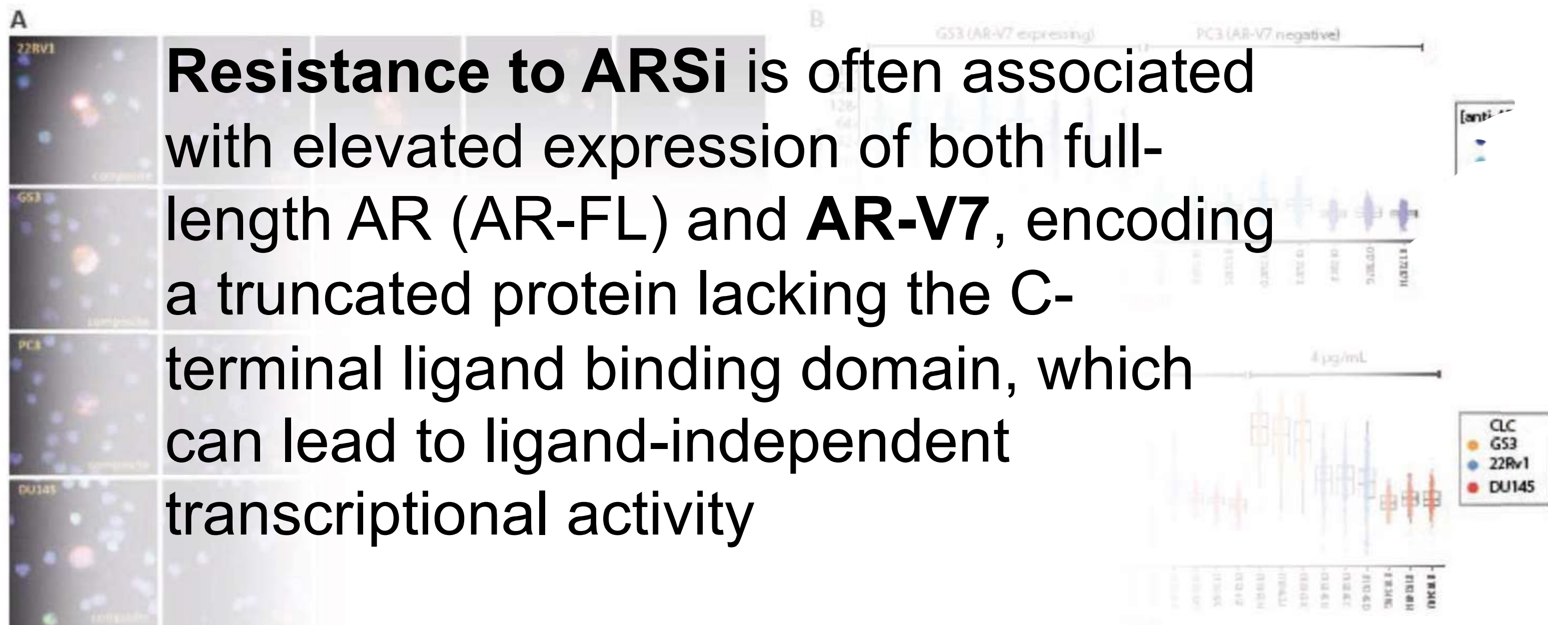
Workflow schematic of the Epic Sciences platform and definition of a CTC in this analysis. Within 72 hours of collection, red-cells are lysed and nucleated cells are plated onto glass pathology slides and can be stored long-term at -80 C. At analysis slides are stained with DAPI, CK, and CD45. Each cell image is automatically processed and CTCs are detected *in silico*. 3 mL of blood is analyzed per patient.

Reference: Werner *et al.* Analytical Validation and Capabilities of the Epic CTC Platform: Enrichment-free Circulating Tumour Cell Detection and Characterization J Circ Biomarkers 2015. PMID: 28936239

- Streck Tube → up to 7 days at 15 °C to 30 °C
- Red blood cell (RBC) lysis → ammonium chloride
- nucleated cells deposited on up to 12 glass slides per sample → concentration of 3 × 10^6 cells/slide
- frozen at −80 °C at Epic before analysis
- Enumaration
- Ar-V7 nucleus Test
- Profiling possible

ASCO 2020 Abstract #312459

Epic Sciences integrated analysis platform → AR-V7



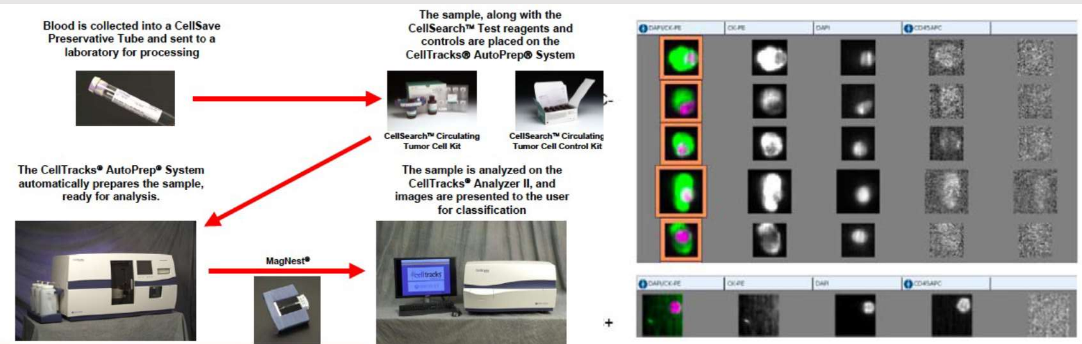
Resistance to ARSi is often associated with elevated expression of both full-length AR (AR-FL) and **AR-V7**, encoding a truncated protein lacking the C-terminal ligand binding domain, which can lead to ligand-independent transcriptional activity

→ Detecting AR-V7 in CTCs might therefore be helpful to **predict ARSi resistance** and improve therapy selection in mCRCP.

Lu et al J Circ Biomark. 2020

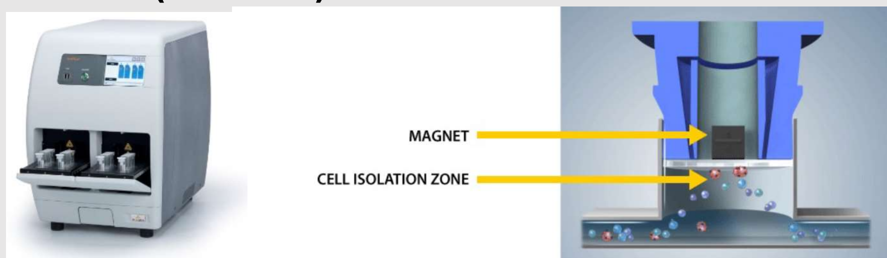
CTC technologies (commercially available and in our lab...)

CellSearch (Menarini) → FDA cleared



Racila et al. Proc Natl Acad Sci U S A. 1998, Tibbe et al. Nat Biotechnol. 1999 Dec;17(12):1210-3., Allard et al. Clin Cancer Res. 2004

Isoflux (Bioflux)



Herb et al Trans Ocol 2013, Ma et al Int J Mol Sci. 2016,
Sanchez-Lorenzi et al Transplant Proc. 2015

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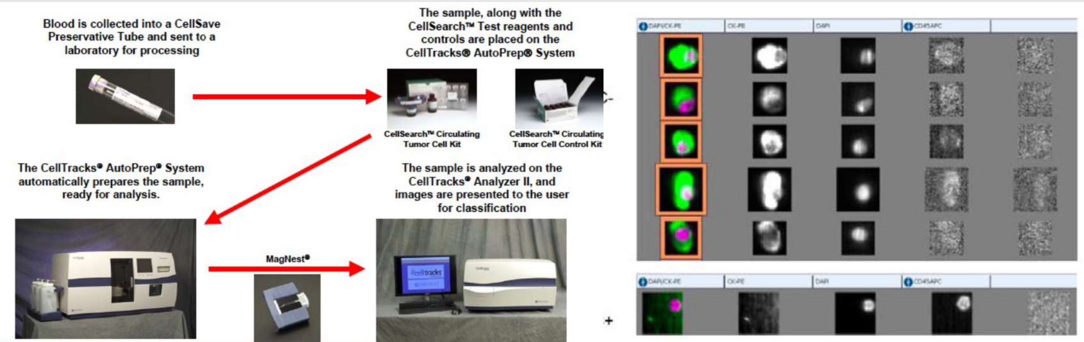
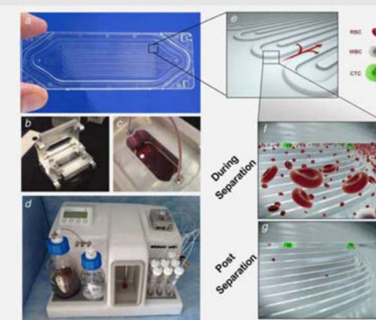


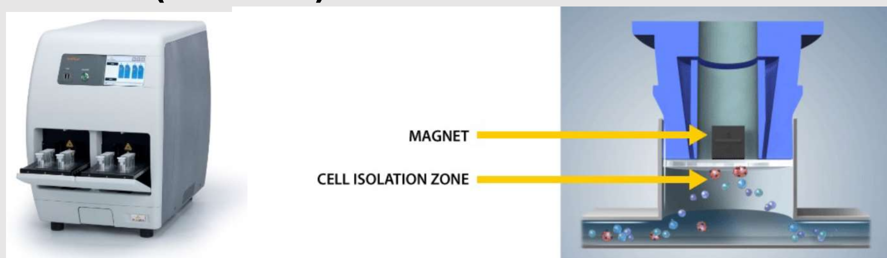
Racila et al. Proc Natl Acad Sci U S A. 1998, Tibbe et al. Nat Biotechnol. 1999 Dec;17(12):1210-3., Allard et al. Clin Cancer Res. 2004

Parsortix (Angle)



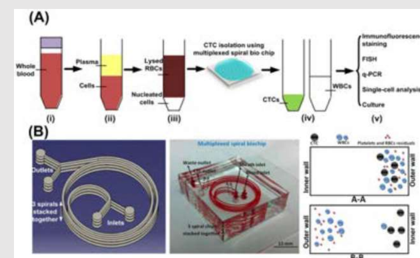
Hvicha et al Int J Cancer. 2016, Chudiak et al Analyst. 2016, Xu et al PLoS One. 2015

Isoflux (Bioflux)



Herb et al Trans Ocol 2013, Ma et al Int J Mol Sci. 2016, Sanchez-Lorenzi et al Transplant Proc. 2015

ClearBridge FX (Clearbridge)

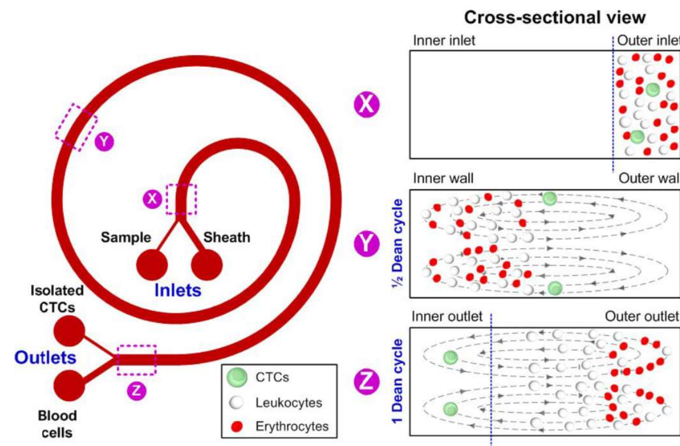


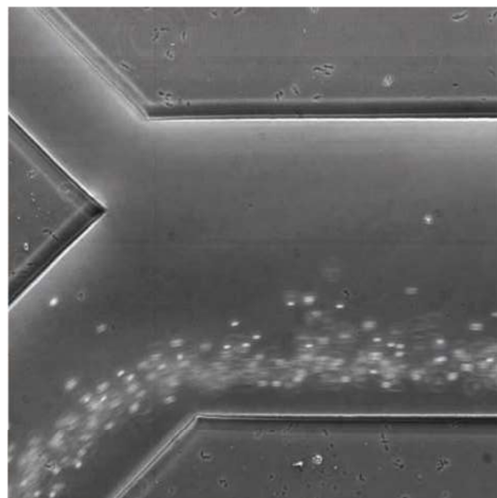
Tan et al Oncotarget. 2016, Warkiani et al Nat Protoc. 2016, Khoo et al PLoS One. 2014

Challenges in CTCs analysis – Enrichment

CTChip® FR + ClearCell System (ClearBridge BioMedics)

Clearbridge
BioMedics
Bringing clarity to cancer





- Dean vortices focus CTCs based on **physical attributes (size and stiffness)**
- **Label-Free**
- **Intact and viable** CTCs

Sources: Tan et al Oncotarget. 2016, Warkiani et al Nat Protoc. 2016, Khoo et al PLoS One. 2014