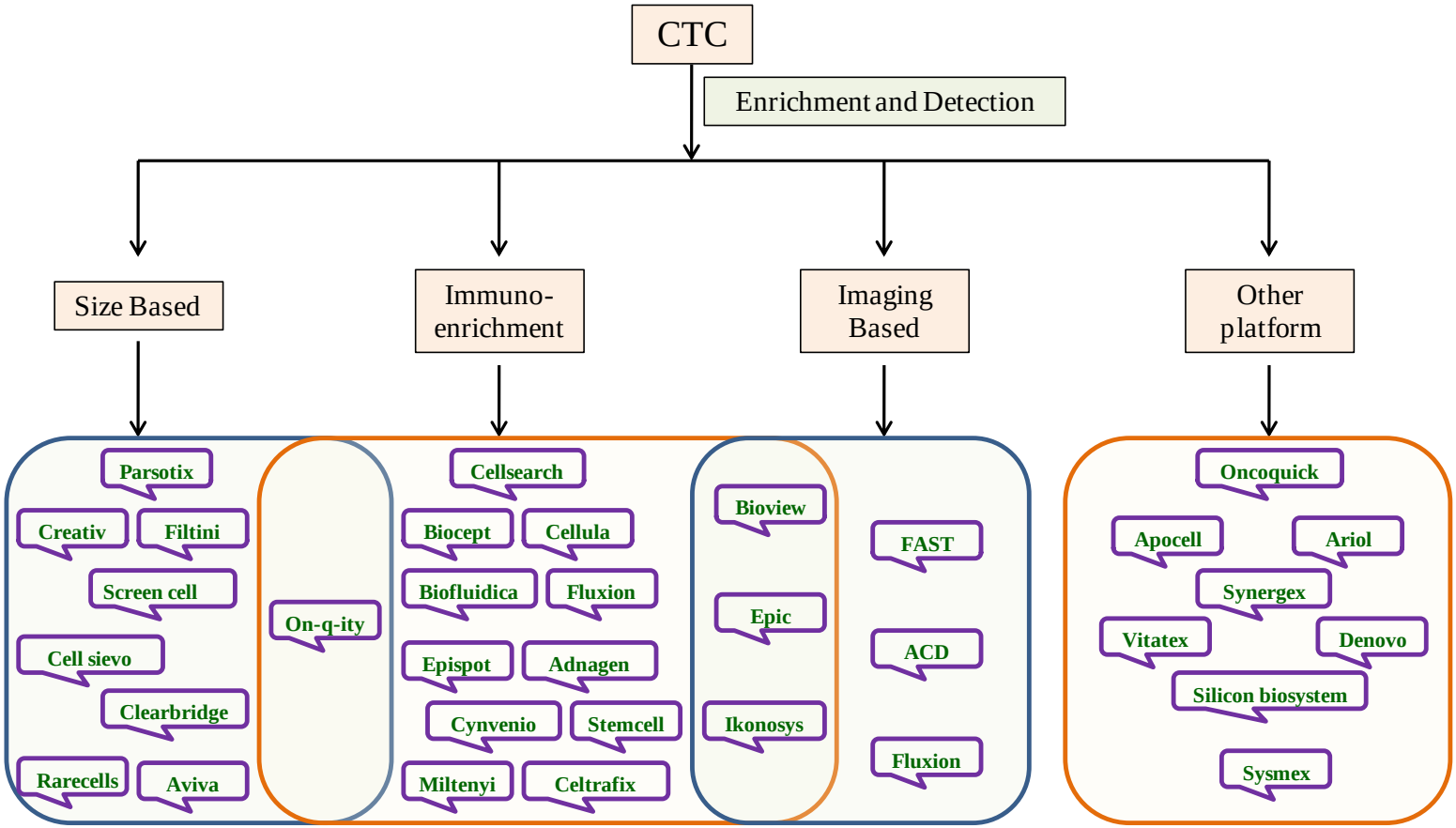
Supplementary Data

Supplementary Data 1

Cancer continues to be one of the leading causes of death globally. Most mortality from cancers is due to metastatic disease, in which cancer spread around the body. Cancer progression is conventionally analyzed by imaging techniques such as computed tomography scan, positron emission tomography, magnetic resonance imaging (MRI) and biopsies. However, these are cost intensive and involve complex procedures for close and frequent follow up of the patient. Further, these methods are also limited in sensitivity and ineptitude to detect small change in tumor size. Recently, various studies have demonstrated that the circulating tumor cells (CTCs) can be used as independent predictor of outcome in cancer patients and can provide good measure of prognosis and overall survival. CTC identification has gained much interest recently and number of commercial players has plunged into the research and development of CTC based platforms to improve cancer patients' life. Herein, various research approaches used by commercial players for CTC detection and analysis with advantages and disadvantages has been elaborated.

There are large numbers of well-established and startup companies who are investing very heavily and working very hard to bring CTC based technology into market for the cancer patients. Among these companies, few have kept their focus only on cell enrichment while others are working on both cell separation and their detection. Further, some companies are not working directly in CTC but providing common platform such as imaging platform for cell identification. Supplementary Fig. 1 categorizes the various types of such

companies based on their approach toward CTC technology. The following sections will describe the details of key stand on CTC isolation technologies and their current contributions.

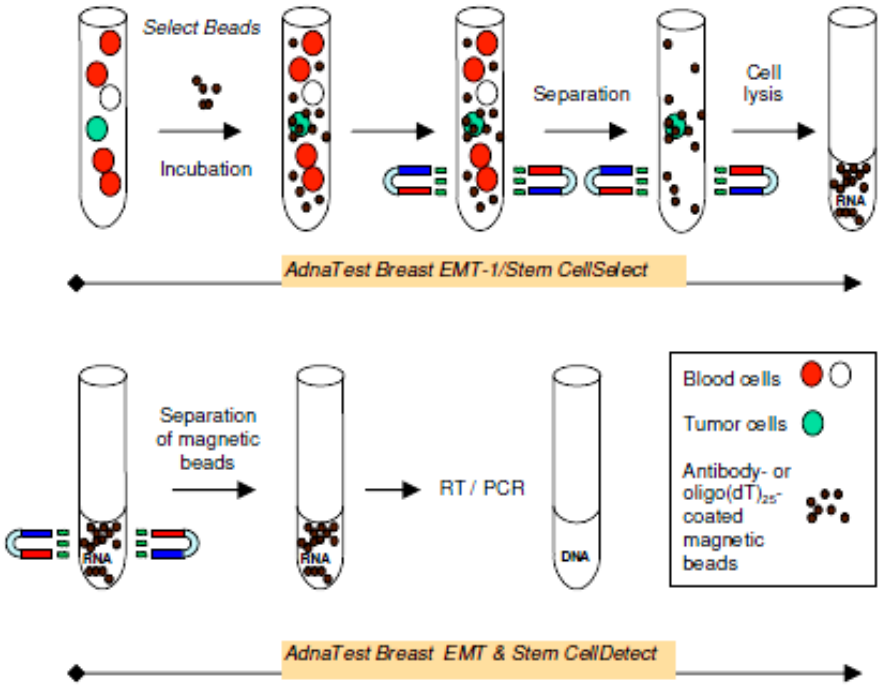


Supplementary Fig 1. Schematic representing companies working on the different domains of CTC Technologies

AdnaGen *(http://www.adnagen.com/)*

AdnaGen's proprietary 'combination-of-combinations-principle' allows the reliable and very sensitive detection of CTCs. In their approach cancer cells are first enriched *in vitro* from cancer patient's blood samples (EDTA or AdnaCollect) using magnetic bead conjugated antibodies. The labeled cells are extracted by a magnetic particle concentrator (AdnaMag and AdnaMag-S) and are subsequently lysed (Supplementary Fig. 2).[1] The cell lysate is used to get mRNA for further analysis.

The isolated mRNA is transcribed into cDNA that can be amplified in a following multiplex-PCR. The multiplex PCR detection step analyses the tumor associated gene expression of a variety of relevant tumor markers to make sure that the selected cells are cancer cells. Tests are offered as reagent sets for the detection of cancer cells in human blood. The combination of a variety of selection markers (antibodies) and a set of molecular tumor markers (mRNAs) takes care of the heterogeneity of the cancer cells gene expression preventing false negative and false positive results due to either illegitimate transcription in normal cells or individual changes in gene expression in general and during chemotherapy



Supplementary Fig. 2. Schematic overview of sample preparation based on AdnaGen's Technology.[2]

ApoCell *(http://www.apocell.com/)*

ApoCell was founded in 2004 to commercialize biomarker technologies for the effective monitoring of cancer drugs by measuring biomarker expression patterns in tumor biopsy specimens. ApoCell's ApoStream™ technology,[3] deploys the inherent difference of circulating tumor cells and other rare circulating cells from peripheral blood mononuclear cells (PBMCs) in morphology and dielectric

properties within a microchannel flow field to isolate CTCs using dielectrophoresis field flow fractionation (DEP-FFF) (Supplementary Fig. 3). The fractionation of cell types is made possible with the differential flow rates relative to distance from the electrode.

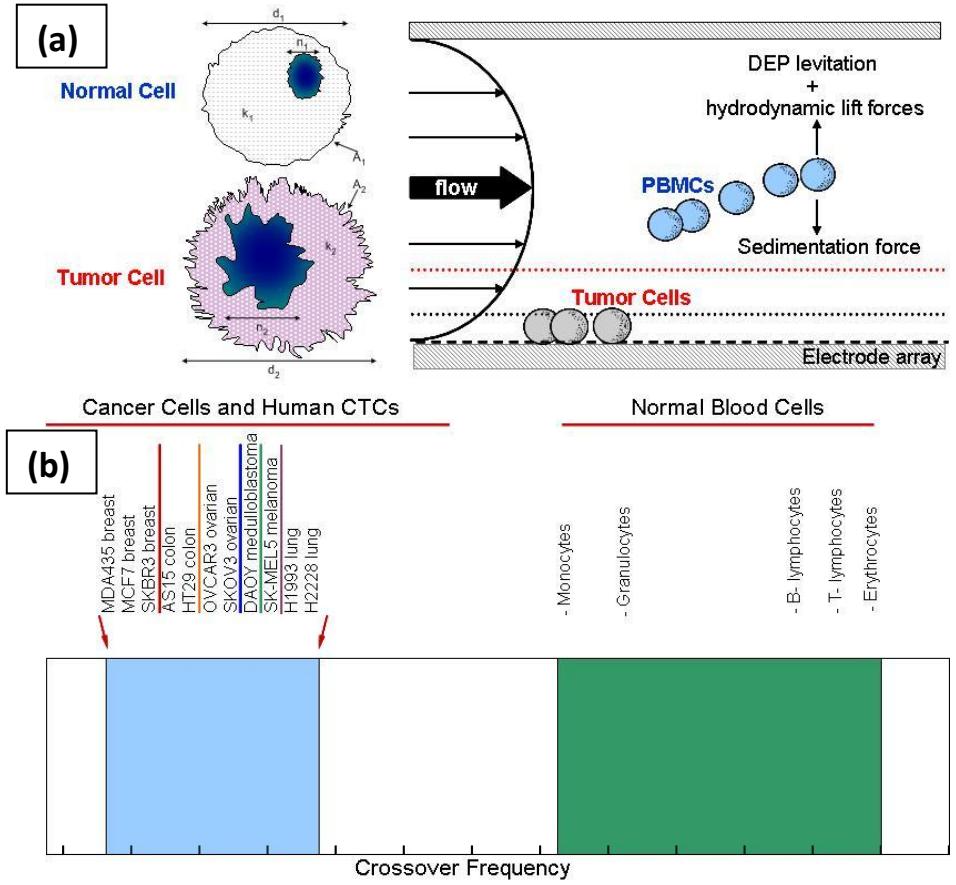
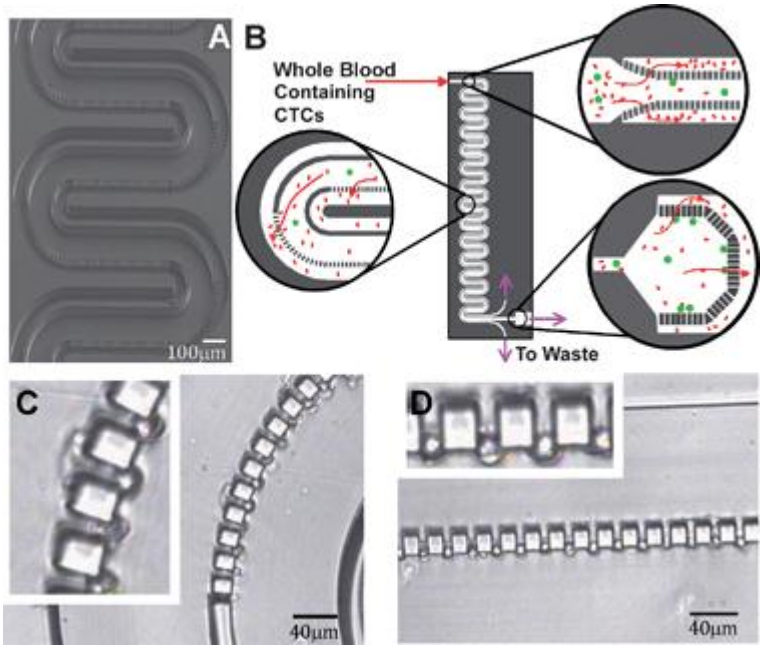


Fig 3 (a) Schematic pertaining to the ApoStream™ technology. **(b)** Crossover frequency determined for different cell types.[4]

Aviva biosciences *(http://avivabio.com/products/selectionfilter.php)*

Aviva bioscience founded in 1999 is currently developing innovative rare cell enrichment technologies, particularly focused on circulating tumor cells (CTC).[5] Their system includes a micro fabricated and nano-fabricated channel to separate cells. Device utilize features that reduces the hydrodynamic pressure experienced by the cells during separation, isolation and concentration process and therefore reduce the likelihood of cell lyses or other damage to the cells. For their system they have shown the recovery as high as 90% at 25 ml min^{-1} (Supplementary Fig. 4).[6, 7]



Supplementary Fig. 4. Schematic and images showing the operation of a CTC-isolation biochip.[6, 7]

BioCep ***(****http://www.biocep.com/)*

BioCep, an Israeli medical device research company, has developed a Cell Enrichment Process (CEP) system for cell separation from various clinical sources. BioCep utilize significance of the magnetic, closed circuit, generated "virtual mesh" with immunomagnetic marked cells for target cell separation without any surface contact with the targeted cells. Also it uses continuous, linear separation areas created by use of tubing as both transport and separation vessels. Tests have shown that the CEP renders 96-99% pure cells with recovery of 80-99% and viability of 99% in 5-15 min for 10^9 cells. BioCep is currently developing applications in non-invasive prenatal testing and stem cell separation, based on its advanced technology. The company will market its CEP system as research use only device and will provide consumable kits to its customers.[8]

Biocept *(http://www.biocept.com/)*

Biocept Laboratories specializes in oncology tests, specifically for circulating tumor cells (CTCs). They promote their unique technology (CEE^{TM}-cell enrichment and extraction) and family of laboratory tests (OncoCEE^{TM}) to high value oncology expectations. Their cell enrichment technology exploits the placement of posts and flow rates through mathematical modeling to enhance isolation and capture of extremely rare cells within a micromechanical system (MEMS) channel.[9] By using the CEE^{TM} attachment chemistry, the CTCs are captured which can be immobilized and heated to temperatures for denaturation and hybridization of direct labeled DNA FISH probes to targeted sequences. The cells can also be retrieved through high shear from within the microchannel and concentrated for

Taqman real-time PCR assessment of single gene mutations or deletions. Thus, over 70 % of cells are captured at antigen densities greater than 30,000 sites/cell.[10]

BioFluidica Microtechnologies *(http://www.biofluidica.com/)*

BioFluidica Microtechnologies LLC, formed in 2007 develops and commercializes medical diagnostics, pathogen detection, and personalized medicine. Their CTC detection technology was developed at Louisiana State University.[11] BioFluidica's CTC detection system consists of a portable instrument that works in conjunction with a disposable test-specific polymer cartridge the size of a standard microscope slide. The cartridge has a nano-engineered, high-aspect ratio capture bed. Monoclonal antibodies or aptamers, specific for antigenic integral membrane proteins (EpCAM and/or others) expressed by the target cells, are immobilized on the surfaces of this capture bed. Once the entire sample has been processed, the fluidic channels are rinsed to assure high purity of the subsequent elution of the target cells. Then the target cells are released from the capture surface and swept through a highly specific, single-cell conductivity sensor, where they are counted and then collected. This counting method eliminates the need for cell labeling and expensive optical detection. Because these cells are intact and viable, they are then available for molecular and/or other analyses. The instrument housing for BioFluidica's CTC detection system contains all the equipment, sample and bulk chemicals needed to process the sample in the polymer cartridge. Typically, the instrumentation include micropumps, a sample container, bulk reagent containers, electronics for the electrical conductivity sensor, and a data acquisition card that can be connected to a USB port. The instrument housing is approximately 1 cubic foot in size, and lightweight. Their applications are focused on CTC detection and molecular diagnostics.[12]

CellSearch *(https://www.cellsearchctc.com/)*

Veridex, LLC, a Johnson & Johnson Company was founded in 2004 to provide physicians with the only regulatory–cleared high-value *in-vitro* diagnostic oncology products to benefit patients through earlier disease detection.[13] The CellSearch® system consists of the CellTracks Autoprep to immnomagnetically enrich cells expressing EpCAM from 7.5 ml of blood and fluorescently label enriched cells with DAPI, CD45-APC and CK-PE. After which it resuspends the cells in the cartridge placed in the CellTracks Magnest which is placed on the CellTracks Analyzer II. Then the Analyzer system acquires images using a 10X NA 0.45 objective with filters for DAPI, PE and APC. The computer generated images are reviewed to confirm CTCs as nucleated $DAPI^+$ cells, lacking CD45 and expressing CK-PE. The accuracy, precision, linearity and reproducibility of the CellSearch system have also been reported by various researchers.[14, 15] More recently, the data of the first randomized, double-blind, placebo controlled Phase III trial (metastatic prostate cancer clinical trial) to evaluate CTCs as a biomarker for overall survival was presented at the 47^{th} American Society of Clinical Oncology (ASCO) annual meeting.

CellSievo ***(****http://www.biospectrumasia.com/biospectrum/influencers/1536/cancer-diagnostics-device-kick-starts-cellsievo)*

CellSievo is a Singapore-based start-up set up in 2011 to commercialize a non-invasive cancer diagnostics device for personalized cancer management. SureCELL developed by CellSievo is a micro-fabricated silicon microsieve device, which utilize CTCs in human blood as a biomarker and effectively isolate them from patients' whole blood and report its quantity.[16] Isolated cells can be easily eluted

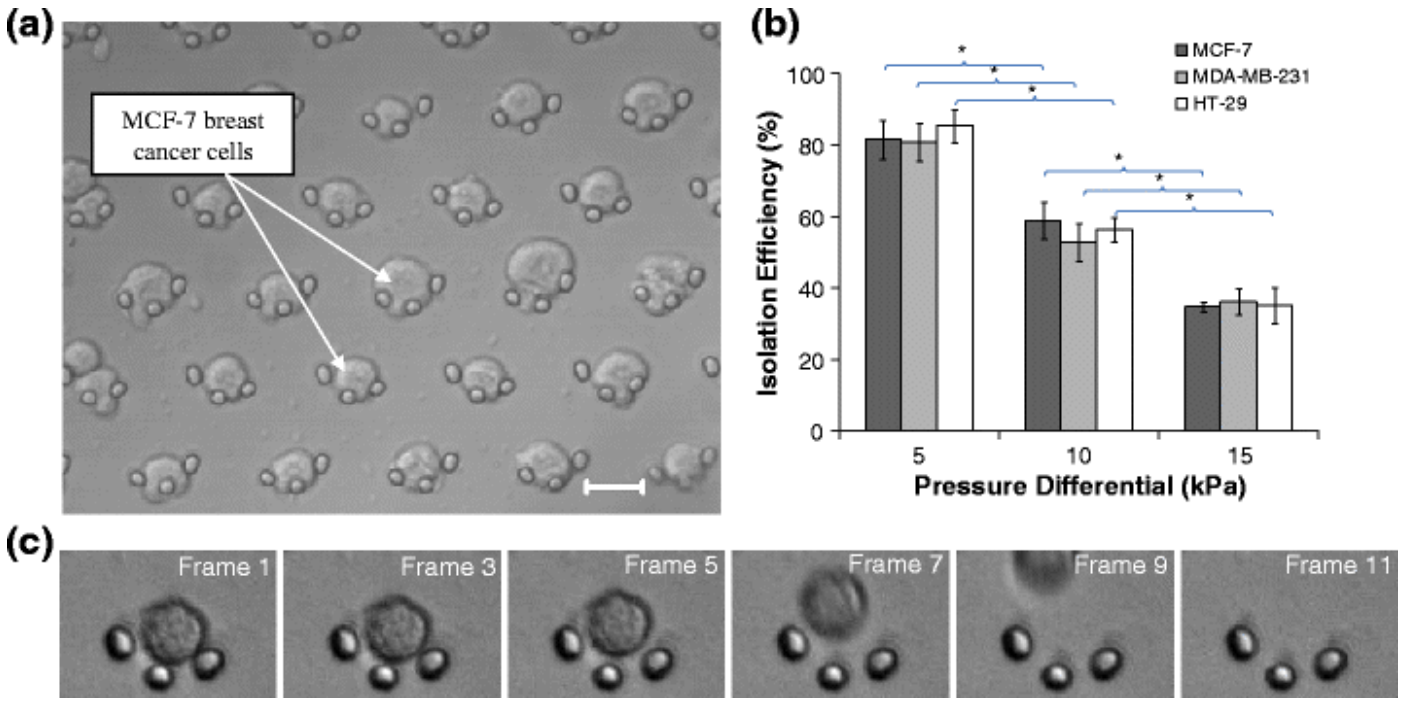
out for further molecular analysis. System is based on size based separation of CTCs where device allows blood cells to freely flow through it, while restraining and capturing the targeted cancer cells on the micro sieve surface. Captured cells are stained with fluorescence dyes, which automatically get identified under a microscope with a companion image processing program.

Celltrafix *(http://www.celltraffix.com/)*

CellTraffix has developed platform technologies for the selection and manipulation of a broad range of target cells, including CTC, found in the bloodstream for modification, collection, or elimination. Unlike existing products based on antibodies or other molecular tools, their system manipulates target cells by mimicking the mechanisms of cell trafficking employed by the body itself. The core platform technology involves a flow-mediated adhesion system, built on a biocompatible device substrate. Their system utilizes a class of molecules called selectins for adhering and rolling target cells either in vitro (in benchtop research kits or clinical diagnostics) or in vivo (in implantable devices), replicating the cellular trafficking mechanisms of the human body. Once target cells adhere and begin rolling, they can either be purified or removed, or therapeutically modified using a secondary set of signaling molecules on the device surface.[17] CellTraffix is engaging in commercial discussions with selected parties with an intend to pursue both co-development and/or joint-licensing relationships for therapeutic applications.

Clearbridge BioMedics *(http://www.clearbridgebiomedics.com/)*

Clearbridge BioMedics is a spin-off from National University of Singapore (NUS) that specializes in novel platforms with applications in oncology research and diagnostics. The ClearCell™ System (Supplementary Fig. 5) comprises patent-pending CTChips® which are microfluidic biochips able to effectively detect and isolate wholly-intact CTCs from small quantities of patient blood samples.[18] The CTChips® contains thousands of crescent shaped microstructures that can isolate CTCs without antibody or magnetic beads. By fluid dynamics, the cells are trapped on the microstructures and reversal of microfluidic flow allows cell retrieval for further molecular analysis. The ClearCell™ system aims to be the next generation non-invasive "liquid biopsy" approach for cancer screening, diagnosis, staging, personalized medication and treatment monitoring. The isolation efficiency of their system is at least 80 % with breast and colon cancer cells.[19]



Supplementary Fig. 5. Images exhibiting the isolation of CTCs using CTChips® [19]

Creatv Microtech *(http://www.creatvmicrotech.com/)*

Creatv Microtech is a privately-held company founded in 1996. Their business is based on high-aspect ratio microfabrication and ultra-sensitive bio detection. Highly efficient isolation of CTCs from peripheral blood is rapidly achieved through the novel precision microfilter called CellSieve™.[20] These microfilters have uniform 8 µm diameter pores in a polymer with approximately 90,000 pores imprinted on a 9 mm diameter area in a standard 13 mm filter format. The filtration efficiency using CellSieve™ were efficient and reproducible with 98 % ± 2 % for fixed and 85 % ± 2 % for unfixed MCF-7 cells wherein contamination of blood cells on the microfilter is on an average of 1000 cells per sample and zero for red blood cells.

Cynvenio biosystems *(http://www.cynvenio.com/)*

Cynvenio biosystems is developing an automated and integrated system that includes upstream CTCs isolation by using combination of microfluidics sheath flow technology and magnetic separation, and the downstream DNA analysis by using quantitative allele-specific PCR (Q-PCR) method.[21] The sample is collected by using special kit supplied by the company and then delivered to the Cynvenio lab for entire process, including CTC isolation and enumeration, and molecular analysis. In the future, the company is targeting to provide an automated instrument, which offers CTC recovery and three staining processes on-chip, including DAPI, CD45-, Cytokeratin, for imaging analysis along with downstream PCR based molecular analysis.[22]

Epic Biosciences *(http://www.epicsciences.com/)*

Epic Biosciences is a San Diego-based, start-up formed in mid-2008. They have taken a high-definition CTC technology from Dr Kuhn to commercialize. Dr Kuhn and diagnostic pathologist Kelly Bethel has recently unveiled what Dr Kuhn calls a "next-generation technology" for detecting and analyzing CTCs in patients' blood samples. Their approach involves spreading a layer of all nucleated cells found in a blood sample onto a glass surface, and adding fluorescent antibodies to cytokeratin, an essential component of CTCs. The technology then uses a digital microscope and an image-processing algorithm to scan the slide for clumps of aberrant fluorescence.[23] The process requires high-performance computing to help analyze and manage the data, and high-definition imaging to help cellular pathologists identify and analyze any of those fluorescent clumps that signify circulating tumor cells.

Fluxion *(http://www.fluxionbio.com/)*

Fluxion, founded in 2005 in South San Francisco area, developed IsoFlux Rare Cells Access System for CTCs detection.[24] This technology is called CellSpot™. In this technology, the CTCs are pre-treated with magnetic beads. When these labeled CTCs pass through the microchannels, they get pulled upwards and away from the flow stream by a localized magnetic field when other components are remaining within the flow. Then, the recovered CTCs (usually less than 20 µL were transferred to a centrifuge tube for downstream analysis, such as FISH and PCR.[24]

Miltenyi Biotech *(http://www.miltenyibiotec.com/)*

Miltenyi Biotech has developed a technology called MACS® Technology for cell isolation. MACS® Technology offers various separation strategies, which provide the perfect basis for the isolation of almost any cell type from any species and various sample materials. In their system desired cells in a single-cell suspension are magnetically labeled with MACS® MicroBeads. The sample is then applied to a MACS column placed in a MACS separator to separate the cells.[25] The unlabeled cells pass through while the magnetically labeled cells are retained within the column. The flow-through can be collected as the unlabeled cell fraction. After a short washing step, the column is removed from the separator, and the magnetically labeled cells are eluted from the column. In this method separation column plays an important part. MACS Columns contain a matrix composed of ferromagnetic spheres covered with a cell-friendly coating. When placed on a magnetic separator, the spheres amplify the magnetic field by 10,000-fold, thus inducing a high gradient within the column. This is crucial for isolation of cells which are only minimally labeled, leaving enough epitopes free for concurrent antibody staining. The space between the spheres is several times larger than primary and most cultured cells. This allows the cells to freely flow through the column. Magnetically labeled cells are held in suspension within the column and do not actually "bind" the column matrix. This suspension minimizes stress on the cells and allows for efficient sterile washing by avoiding cell aggregation. Their system can also be used to isolate a particular target cell type in an unlabeled, i.e., untouched form by magnetically labeling the non-target cells and depletion. During separation, the unlabeled target cell type is collected in the flow-through fraction.

Oncoquick *(http://www.greinerbioone.com)*

Oncoquick® is a product developed by Greiner Bio-one for CTCs enrichment purpose. It is a density gradient based separation approach. A porous barrier was placed within the 50 mL tube that contains 15 to 30 mL whole blood sample. After centrifugation of sample, the layer of CTC and platelets can be separated from the leukocytes and erythrocytes. The average recovery rate is 72% when the average repeatability is 83% with average detection limit of 1.46 CTCs per 20 mL whole blood sample.[26]

ON-Q-ITY *(http://www.on-q-ity.com/)*

On-Q-ity, which stands for "**On**cology + **Q**uality + Clar**ity"** is an innovative diagnostic company focused on novel technique for circulating tumor cell (CTC) capture and characterization. They are employing technology commercialized out of Mehmet Toner's lab at the Massachusetts General Hospital, which involves microfluidic approach. The OnQChip™ dual capture microfluidic chips capture CTCs by both size and affinity. Chips are manufactured with a hot embossing procedure which produces an open chamber containing ~100,000 posts from a plastic slide. The OnQChip™ modified with an antiEpCAM, capture cells and filter the blood. Captured cells on the chip were then fixed, permeabilized, and stained with a pan-cytokeratin PE MAb, an anti-CD45 Alexa647 antibody, and DAPI for fluorescent visualization under 5x magnifications using the OnQScan™ fluorescent microscope system.[27]

On-Q-ity's portfolio also includes a number of DNA repair biomarkers for monitoring the effectiveness of therapy. Captured cells can be lysed on the chip and RNA, miRNA, genomic DNA and proteins can be extracted and analyzed by QPCR.[28] Expressions of up to 50 different gene markers have been successfully measured in many clinical samples.

Recently, they presented their data in poster sessions at the American Association of Cancer Research (AACR) Annual Meeting in Chicago validating their new dual-capture circulating tumor cell (CTC) platform technology. Based on their prior investigation, the company is currently analyzing and characterizing CTCs in blood to identify cancer cells and gene mutations to provide drug developers and physicians with clear guidance for improved cancer diagnosis, treatment monitoring and earlier recurrence detection for their patients. Further, On-Q-ity is commercializing a microfluidic approach developed at MGH for capturing rare circulating tumor cells for quantification and analysis. Lab Corp has negotiated the rights to market cancer diagnostics firm On-Q-ity's circulating tumor cell (CTC) platform to the biopharma industry for use in cancer drug discovery and development applications.

RareCells *(http://www.rarecells.com/)*

RareCells Inc. was founded in July 2012 to commercialize the patented isolation technology by Size of Epithelial/Throphoblastic Tumor cells (ISET) to isolate Circulating Rare Cells in blood,[29] ISET provides the high sensitivity platform for the isolation of CTCs from whole blood treated within 4 h of collection. After which the isolated CTCs can be characterized by immunolabelling, FISH, Tunel and molecular RNA and DNA analyses etc. This technology enables the search for gene mutation in CTCs as identified by the cytopathological analysis (ex: K-ras, HER2 etc.) for theranostics.[30]

Screen cell *(http://www.screencell.com/)*

ScreenCell was founded in 2006 for creating technologies that allow CTCs to become potential end points in future oncology therapeutic arsenals by filtering out healthy live tumor cells.[31] Such live tumor cells are enabled for molecular biology, cell culture and enumeration & cytomorphology evaluation. ScreenCell® filtration device is a small compact and low-cost non-invasive technology for isolating CTCs from whole blood.[32] They provide a full range access to phenotypical, genotypical and functional characterization of CTCs and Circulating Tumor Microemboli (CTMs). Their isolation technology avoids any bias introduced by antibodies and generating false negatives paving way for direct and simple access to molecular biology

Silicon biosystem *(http://www.siliconbiosystems.com/silicon_website.page)*

Silicon Biosystems has developed a chip called DEP Array based on their proprietary lab-on-a-chip technology platform designed to individually identify, manipulate and sort specific cells within a heterogeneous population. The base of DEP Array™ is a microelectronic active silicon substrate embedding control circuitry for addressing each individual dielectrophoretic (DEP) cage. In their approach, the electric field is generated by a silicon chip directly interfaced to a micro-chamber containing living or non-living particles in liquid suspension.[33] The micro-chamber is confined between the chip surface and a conductive transparent lid spaced tens of microns apart. The chip surface implements a two dimensional array of micro-locations, each consisting of a surface electrode, embedded sensors and logic. The electrodes induce suitably closed μDEP cages in the spatial region above selected micro-sites, within which single particles may be trapped and levitated individually. Further, the step by step, DEP potential cages can be moved around the device plane concurrently and independently, thus grabbing and dragging single cells and/or micro-beads to or from any micro-chamber location.

The device embeds more than 300000, 20 μm x 20 μm electrodes, which can be used to create up to 76,800 DEP cages in a tiny volume of about 11 μl. Due to the small size of the electrodes in the chip, DEP cage sizes can be set to accommodate one single cell, thus enabling individual manipulation of a large number of cells. In their system samples including 1 to 100,000 cells in suspension can be managed on a single microelectronic chip, which ensures independent motion control for each cell by means of powerful dedicated software. Cells have been found to maintain viability with completely intact DNA and unmodified proliferation capability. The unprecedented flexibility and selectivity afforded by this device represent a breakthrough in biological research and analysis. This DEP Array technology has been fully developed and is now commercially available.

StemCell Technologies *(http://www.stemcell.com/)*

StemCell Technologies Inc is a privately-owned biotechnology company that develops specialty cell culture media and cell separation products primarily. StemCell grew out of the Media Preparation Service (originally created in 1981) of the Terry Fox Laboratory for Hematology/Oncology Research at the British Columbia Cancer Agency. In 1993, the Media Preparation Service was spun off as StemCell Technologies Inc. StemCell Technologies offers a wide range of optimized cell separation products for the isolation of cancer cells with their cell separation platforms (high purity and recovery), RosetteSep®, EasySep®, and RoboSep®. They are based on surface antigen immunochemistry based cell separation. They also have column based immunomagnetic negative selection of human epithelial tumor cells StemSep® which is a semi-automated mesofluidic system for epithelial tumor cell isolation.[34]

Synergex corporation *(http://www.synergxcorp.com/pacs.htm)*

Synergx Corporation, a wholly-owned subsidiary of Morphogenesis, Inc., is an emerging cell therapy company which began operations in 1996. They utilized synergy of two cutting-edge technologies, one of which detects and isolates circulating tumor cells and the other one takes the tumor cells and transforms them into a potent immunological therapy to create an unprecedented approach in management of malignant conditions. For cell separation they have developed a unique cell separation system, called Polymer-Antibody Cell Separation **(**PACS™**)**.[35] The system uses cell-specific antibodies with a special polymer that has no affinity for the stem cells themselves, thus eliminating the non-specific collection of unwanted cells which is a common problem and limitation of existing cell separation devices. The unique nature of the polymer also allows the PACS™ method to separate multiple cell types in one operation. With the hardware/software components of the PACS™ device completed, the prototype cell separation cartridge is expected to go to design freeze and beta testing at multiple sites this year.

Sysmex *(http://www.sysmex.co.jp)*

Sysmex Corporation has developed a sensitive technology for detecting living tumor cells freely suspended in blood using a virus that replicates and emits fluorescence in tumor cells. In 2010 they have started full-scale research jointly with the National Cancer Center Hospital (Location: Chuo-ku, Tokyo, Japan) to verify clinical usefulness of a technology. In their research work,[36] they detected CTC in breast cancer patients by using the telomerase-specific replication-selective adenovirus OBP-401 which once transferred replicate in

telomerase expressing cells and emit fluorescence. In their 50 metastatic patients study 21 patients (42%) were identified as positive with the OBP-401 assay and 27 patients (54%) with the Cell Search assay.[36]

Vitatex Inc *(http://www.vitatex.com/)*

Vitatex Inc is a Biotech Company focusing on research and commercialization of rare cell enrichment and diagnostics product. Their products fall among Vita-Cap™ and Vita-Assay™ categories. Vita-Cap™ are modified blood collection tubes (6ml tube format) that use proprietary cell adhesion matrices (CAM) to capture,[37] and preserve viable rare circulating cells in a single step from blood or tissue fluids. Vitatex claim that Vita-Cap™ enriches rare cells in 1-ml of blood to 1-10% purity of a specific rare cells compared to background blood cells. Vita-Assay™ (16-well slide format) is a cell culture plate that also uses CAM to capture and preserve viable rare circulating cells after removal of red blood cells from blood or bone marrow. Vita-Assay™ is useful when further culturing of the rare cells is required.[38] Both products enable molecular characterization of the enriched cells using analytic tools. They have 3 application platforms namely Cell Separation Technologies for cancer diagnostics, Cell Separation Technologies for anti-cancer drug discovery and Cell Separation Technologies for discovery research.

Apart from the above mentioned technologies, Celula has developed a mvs360 cell sorter chip for separating rare cells.[39] Chip enable >90% purity with recovery from upto 500000 cells in 50 µl solution in 5 to 60 min time. Parsortix CTC separation device from ANGEL's, targets for CTC recovery in relation to lung, pancreatic and colo-rectal cancers.[40] Instead of enriching CTCs in patient's blood by either CTCs size or surface epithelial markers verification, California-based ACD developed a multi-fluorescent RNA in situ

hybridization (ISH)-based CTC detection system named CTCscope™.[41] By employing this technology, they are not only able to perform CTCs counting, but also the molecular profiling and viability checking of the CTCs. ACD filed more than 8 patents along with technologies in-licensed from Affymetrix. Both companies are in partnership in order to deliver the CTCScope system by year 2013.[41] Filtini, located in Menlo Park, CA, provided a CTCs size-based isolation platform by using specialized microfilter membrane made by Parylene.[42] The technologies will be applicable to different cancer patients, including prostate cancer, breast cancer, colon cancer and pancreas cancer.

In order to overcome the drawback of distinguishing viable from apoptotic cells by both immunocytochemistry and RT-PCR, a new technique has emerged offering the most significant discrimination for CTC analyses.[43, 44] This technique was designated EPISPOT (for epithelial immunospot) and based on the secretion or active release of specific marker proteins using an adaptation of the enzyme-linked immunospot (ELISPOT) technology. The EPISPOT assay offers the advantages that only viable tumor cells will be detected and that protein secretion can be detected at an individual cell level.

Some companies such as Ariol, Bioview etc are not directly working on CTC enrichment and detection but providing imaging or other type of platform for cell detection. BioView provides an automated cell imaging and analysis system to fulfill the gap. Their system is now available for procurement.[45] Ariol CTC platform provides a fully automated method of scanning slides for CTCs and imaging cells in three different fluorescent channels plus bright field by classifying the CTCs being positive for cytokeratin, negative for CD45 and positive for nuclear staining.[46] The cell morphology is observed through bright field imaging for discrimination of cells from artifacts

like cell debris or cell fragments. Their system forms the complete platform with increased automation, standardization of scoring and sensitivity for analyzing samples for CTCs.[46] Supplementary Table 1 summarizes the target specification of various CTC technologies.

Supplementary Table 1. Benchmarking and the target specification of various CTC technologies

List of companies	**Target Approach Methodology**			**Process time**	**Sample volume (ml)**	**Patient sample studies**	**Remarks**	**Refs**
	Isolation	**Detection**	**Identification**					
Adnagen	Multiple Ab on beads	RT-PCR	RT-PCR	NA	15	Yes	Enrichment based on Ab coated beads (positive selection)	[1]
ApoStream	Dielectric properties	Optical	Staining			Yes	Independent of EpCAM expression	[3]
BIOCEPT	Ab coated posts	Optical	RT-PCR	7-9 h	8-10	Yes	Enrichment based on Ab coated posts and microfluidics	[9, 10]
Cell Search	Ab beads	Optical	Staining	3-7 days	7.5	Yes	Isolation based on positive selection may be misleading	[13-15]

Celltraffix	Cell trafficking mechanism						Isolation by flow mediated adhesion system	[17]
Clear Bridge Biomedics	Biorheological property	Optical	Staining	50 min	1.6	Yes	Enrichment based on biorheological property of blood cells	[18, 19]
Creatv Microtech	Size exclusion	Optical	Staining	<2 min	10 ml	Yes	Isolation based on Size exclusion without affecting the phenotype	[20]
Cynevenio	Magnetic Ab beads	Optical	staining	2.5 hr	5ml/hr	Yes	Isolation via ultrahigh magnetic gradient within the flow chip	[21]
Oncoquick	Density based	-	-	45 min	15-50 ml	Yes	70% recovery	[26]
On-qity	Both size based and affinity based	Optical	Staining			Spiked samples	Isolation based on positive selection (both size and affinity)	[27, 28]

Rarecells	Size based	Optical	Staining	3 min	1-10 ml	Yes	Isolation based on size based separation	[29, 30]
ScreenCell	Size based	Optical	Staining	50 s	1 ml	Yes	Enrichment based on size based separation	[31, 32]
Silicon biosystem	Dielectric properties	Electrical			11 µl			[33]
Sysmex	Telomerase specific replication-selective adenovirus	Optical	Staining		7.5 ml	Yes	Isolation of CTCs by infecting the blood sample ,lysing RBC and shrinking leukocytes	[36]

Supplementary Data 2: Author's view on CTC technologies

2.1 Technical specifications for CTC systems based on clinical requirements

Successful CTC systems aim to satisfy the criteria set forth by clinical requirements. Many aspects of the clinical requirements can be gathered from various publications and industry reports (Supplementary Data 1 and Supplementary Table 1).[47-60] Recently, a focus group comprising key thought leaders put forward a list of recommendations for technology developers.[47-62] Following is our view on list of key parameters with recommended specifications.

2.1.1 Blood volume

The current FDA-approved CTC system, CellsearchTM, uses 7.5 mL of blood. Cellsearch is currently the benchmark for CTC assays. Although, other assays have demonstrated capability to isolate CTCs from smaller blood volume, due to the stochastic nature of CTC's occurrence, larger volumes are recommended. A 100% sensitive system capable of processing 5-10 ml of blood could be ideal. Current literature describes various systems capable of processing anywhere from a few hundred microliters to few ml's of blood, with a throughput ranging from few minutes to a few hours have been described. The commercial systems however, typically have a range of 1-10 ml. Lower volume of blood draw is generally considered favorable to the patient. However, higher volume will likely prevail given that finding sufficient number of CTCs to enable molecular analysis will have a significant impact on patient outcome. Thus, a system which is scalable and has the ability to handle a range of volumes upto a few 10's of mls could be considered to be advantageous.

2.1.2 Standardization

Standard conditions for pre-analytical, analytical and post-analytical assay i.e for specimen collection, testing performance parameters, data collection and analysis have been recommended for CTC technology developers.[62] Such standard protocols for analytical and clinical validation of CTC assays and qualification of CTC-based biomarkers are mandatory for the next steps in evaluation of these technologies. Developing and applying these standards requires coordinated clinical trial resources, managing the collection and distribution of samples, and evaluation of test results, as well as input from engaged scientists and the FDA.

2.1.3 Turn around time

A generic need in diagnostics is for a shorter turnaround time. Currently, the FDA-approved Cellsearch™ has a turnaround time of 3-7 days. In light of the existing technology and the chronic nature of the disease, a day or less could be considered appropriate.

2.1.4 Point of test

Current trend in diagnostics is towards point of care tests. In that sense, a point of care test for CTC will be in line with the industry trend. However, the current market leader is not point of care and yet widely used. Thus, bench top systems in central labs or physician office labs are useful. POC CTC test will have an advantage over the bench top systems if they offer comparable performance.

2.1.5 Reliability

Several techniques for enrichment and detection have been described in literature; however there is lack of a gold standard in CTC technology. Thus, our current understanding of CTCs and their clinical relevance is biased by the technology used to isolate and identify them. Ideally, no CTC should be missed, including CTCs that do not express specific biomarkers and those that do not fall under specific physical characteristics. Additionally, the captured CTC population must be of high purity, uncontaminated with WBCs and RBCs. Moreover, the isolated cells should be morphologically preserved and viable for downstream molecular analysis.

2.1.6 Cost

The FDA-approved system is estimated to cost 450 USD/test, which is substantially less than the current standard of care (radiological imaging), which is over $1000. Many commercial technologies seem to be pricing CTC tests between 100 to 400 USD. From the payers perspective, lower cost of the assay without performance compromise will be desirable. Supplementary Table 2 summarized analytical parameter requirements for any commercial CTC technology.

Supplementary Table 2. Analytical parameter requirements for any commercial CTC technology.

Parameter	**Performance requirement**
Sample size	5-10 ml

Enrichment and detection time	<1 day
Enrichment Efficiency	>90%
Detection limit	Upto 1CTC/5ml blood
Detection range	1-10000 /ml of blood
Downstream analysis	Viable CTCs should be maintained for downstream molecular analysis

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