Cancer accounts for millions of deaths worldwide each year. Around 14.1 million new cancer cases were diagnosed in 2012 alone and 8.2 million cancer-related deaths were seen in the same year. These figures are expected to reach 22 million and 13 million respectively by 2030. According to the GLOBOCAN 2012 report (Lozano et al. 2012), lung cancer is the most prevalent cancer, followed by breast cancer, colorectal cancer, prostate cancer and stomach cancer.

Cancer cells are capable of rapid and uncontrolled growth, coupled with a myriad of genomic mutations. Cancer cells in a tumour are typically heterogeneous, evolving and expanding from different clonality of tumour populations that harbour different mutational characteristics and phenotypic aggressiveness. The growth of cancerous cells in situ is not necessarily life-threatening. However, metastasis where cancer cells disseminate through blood as circulating tumour cells (CTC), and invade distant sites to form metastatic lesions can be detrimental. As high as 90% of all cancer related deaths are attributed to metastasis (Chambers, Groom, and MacDonald 2002). Not surprisingly, the presence of CTCs is correlated with poor prognosis and dismal survival in multiple cancer types (de Bono et al. 2008; Cohen et al. 2008).

Although surgical removal and chemotherapy have been successful in many early stages of cancer, many patients still battle with tumour relapse or drug resistance. Tumour heterogeneity allows insidious populations of some cancer cells to escape chemotherapy treatments. Complex branching of various cancer subtypes with varying molecular signatures also does not allow for a single treatment that fits all cases. Cancer heterogeneity demands precise patient stratification with genomic and expression profiling so that therapeutic strategies can be tailored to each patient. A classic example is the identification of HER2 amplified subtype patients which paved the way for a monoclonal antibody-based treatment (Trastuzumab) of such patients. A recent example depicts the importance of PD-1 expression status in the efficacy of treatment response of PD-1 inhibitors.

In addition, genome-wide profiling of the patient tumour provides knowledge of the mutation status of cancer (Laird 2010). However, access to primary tumour tissue can be challenging for some cancer types such as pancreatic and hepatocellular carcinoma. Sequential biopsy of tumour is also difficult in lung and head/neck cancers. This limits the possibility of serially monitoring the mutation profile of these cells.
“Liquid Biopsy” – A glimpse of tumor in blood

Liquid biopsy offers a simple and less invasive means to complement the current standard of multiple tissue biopsies or when biopsy material is absent or inadequate (Alix-Panabières and Pantel 2013). Liquid biopsies can easily be performed at most clinics, without expensive medical procedures. CTCs provide information on the metastatic aggressiveness and molecular insights of the primary tumour or foreboding metastatic lesion (Bidard et al. 2014; Hou et al. 2012). Despite significant clinical relevance, one central challenge that lies in isolating CTCs from peripheral blood comes from its extremely rare occurrence; roughly 1-10 CTC can be found in 10⁹ blood cells. Their rarity, coupled with highly heterogeneous nature in expression profiles and morphologies, have thus far limited the use of CTCs for clinical monitoring and cancer management (Alix-Panabières and Pantel 2014).

In the past decade, many endeavours for more precise and robust isolation of CTCs were initiated. Most methods can be categorized into two broad classifications: affinity and non-affinity based approaches. Non-affinity based techniques rely primarily on physical biomarkers, for eg. size, shape and deformability. Affinity based approaches isolate CTCs via cell surface markers, allowing immune-mediated binding of CTCs on functionalized surfaces, including magnetic beads, microchannels and microstructures. CellSearch®, for instance, is the only commercialized product for the use of clinical diagnostics approved by U.S. Food and Drug Administration (FDA). It is based on the recognition of cell surface expression of epithelial cell adhesion molecule (EpCAM) by antibody-coated magnetic beads.

Although affinity-based approaches have higher purity of CTC-enriched fractions, targeting surface antigen is less desirable due to the highly heterogeneous nature of CTCs. Besides, retrieval of live and intact CTCs from immuno-captured surface is challenging. This outcome undermines the potential use of CTCs for different downstream assays. More importantly, using affinity-based techniques such as EpCAM may miss out the most aggressive population of CTCs that might have undergone the Epithelial-Mesenchymal Transition (EMT) process, which is known to promote invasiveness and drug resistance in cancer (Thiery 2009). As there are no universal markers available for capturing CTCs, a new method in addition to the dependence of immune-affinity is warranted.
Our Solution –
Label-free CTC enrichment

Figure 1. Schematic of isolation of CTCs in ClearCell® FX System. Smaller hematologic cells RBCs ~8 µm; leukocytes ~8-15 µm are affected by the Dean Drag and migrates to outer wall after one Dean cycle. Larger CTCs ~15-20 µm experience strong inertial lift forces as indicated by the blue arrows and is focused along the microchannel inner wall.

The ClearCell® FX system is an automated CTC enrichment system that leverages on an elegant microfluidic technology to offer a new perspective in clinical cancer diagnostics. The system is driven by the CTChip® FR1, a microfluidic biochip to isolate CTCs based on size, deformability and inertia. The isolation principle takes advantage of the inherent Dean vortex flows present in curvilinear channels for CTC enrichment, termed Dean Flow Fractionation (DFF). The ClearCell® FX system enables effective and rapid separation without compromising the quality of retrieved cells. With high-throughput capability, the ClearCell® FX system can process 7.5 mL blood in less than an hour, and isolates wholly intact and viable CTCs with higher purity than most other enrichment methods. Automated and entirely label-free, the ClearCell® FX system aims to empower doctors and researchers by bringing clarity to cancer so that patients can receive the timely, tailored treatment they need.

CTC enrichment principle

The principle of separating CTCs from background WBCs is based on inertial focusing in a curved microchannel (Hou et al. 2013). In curvilinear (spiral-shaped) channels, particles experience additional lateral Dean drag force due to presence of transverse Dean flows arising from the centrifugal acceleration of fluid flow in curved channels. The influence of centrifugal acceleration in radial direction results in the formation of two symmetrical counter-rotating vortices (top and bottom) across the channel cross-section, also known as Dean vortices (Figure 1). The magnitude of these secondary flows is characterized by a non-dimensional Dean number (De) and cells flowing in curvilinear channels experience a lateral drag force (F_D) which entrain and drive them along the direction of flow within the Dean vortex. Larger cells in curvilinear microchannels also experience appreciable inertial lift forces apart from the Dean drag force that equilibrates them near the inner wall.
ClearCell® FX offers a myriad of possibilities for downstream analyses

Take a few as examples, papanicolaou staining (PAP) and Hematoxylin and Eosin staining (H&E) are conventional histopathology tools used for detection and embedding of pathological tissue specimen. Fluorescence in situ hybridisation (FISH) is an established cytogenetic technique used in diagnostic laboratories for tumour profiling and screening of genetic aberrations such as chromosomal translocations, amplifications and deletions. While sometimes acquiring tissue sample for these assays may be difficult, ClearCell® FX opens up a new window using liquid biopsy for ease of repeated and serial monitoring and workflow integration.

Besides, understanding the molecular changes may guide the appropriate therapeutic interventions. The integration of ClearCell® FX with molecular diagnostic tools such as quantitative polymerase chain reaction (qPCR) and NGS is compatible with ultra-high purity protocol. Patient derived xenografts (PDX) of CTCs and culture of CTCs to establish in vitro primary culture or tumour spheroids allow for downstream assays for drug panel testing in drug discovery and guided therapy. This population of primary CTCs can be a valuable source to understand the malignant phenotype, or allow functional assays via xenotransplantation for personalized medicine.
Visualizing and Counting CTCs

Figure 3. Representative images of cancer cell identified with immunofluorescence staining (Top). Typical recovery of cancer cells in spiked samples (Bottom Right and Middle) and CTC count in clinical sample from different cancer types (Bottom Left).

To mimic clinical sample for consistent and reproducible recovery of CTCs using ClearCell® FX, a series of concentrations of H1975 human lung adenocarcinoma cell line, pre-labelled with cell tracker were spiked into 7.5 mL of healthy blood followed by the standard ClearCell® FX processing (Figure 3). Results showed stable recovery of H1975 cells in three different orders of magnitude from a few tens to thousands. In addition to lung cancer, different origin of cancer cell lines were investigated such as MCF-7 human mammary gland cancer and low-EpCAM expressing PC3 human prostate cancer, and results showed consistent recovery above 50%. Immunofluorescence labeling of spiked CTCs showed cytokeratin-positive (CK⁺), nucleus-positive (DAPI⁺) and leukocyte marker-negative (CD45⁻).

There are numerous clinical applications of CTCs. Enumeration and characterisation of CTCs could serve as to monitor treatment response and prognosis in patients. Supporting studies have shown that the significant presence of CTCs (≥5 CTCs for metastatic breast and prostate, and ≥3 CTCs for colorectal cancer) is inversely associated with increased progression free survival and overall survival.

The ClearCell® FX system obviates the need of a single biomarker and samples a heterogeneous CTC population without bias. The ClearCell® FX is also applicable for various cancer origins (Figure 3). A preliminary study to understand the dynamic range of CTCs over a cohort of 60 metastatic patients at various stages is performed across Lung, Breast and Prostate cancers. Results showed CTC counts range from 0 to 382.
Ultra-high Purity Enrichment

Figure 4. Average WBCs (7.5 mL blood) pre-enrichment are in the range of tens of millions. Enrichment of CTCs is 10,000x for standard protocol, and ~100,000x for ultra-high purity protocol (Left). Tumour cell purity achieved with H1975 cells spiked into 7.5 mL blood; high purity >5% can be achieved over different spike cell numbers (Right).

ClearCell® FX also provides unparalleled capability in removal of background WBCs from peripheral blood. Given the extremely rare occurrence of CTCs in the blood, the removal of background WBCs is critical in order for ease of downstream detection and analysis. Through a well-established ClearCell® FX workflow, rapid WBC enrichment in less than an hour can be easily achieved (Figure 4). Results showed enriched sample with a total of less than 30,000 WBCs (~10,000 fold change) through a standard ClearCell® FX enrichment. However, for sensitive molecular analysis such as Next Generation Sequencing (NGS), an ultra-high purity protocol can deliver enriched output of less than 1,000 WBCs. A series of concentration of H1975 cells were spiked into 7.5 mL blood to investigate the recovery of ultra-high purity protocol. Stable recovery of spiked cells generally maintained above 40% across different spiking concentration. Depending on the amount of CTCs, the purity (ratio of CTCs versus WBCs) can range from 2% to 20%, amenable for NGS.

To our knowledge, ClearCell® FX ultra-high purity protocol offers unparalleled purity than most size-based and affinity based techniques that typically range from 10⁴-10⁵ cells. ClearCell® FX is compatible with existing NGS technology, in detection of DNA mutations in CTCs, as low as 2% (Table 1).

<table>
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<tr>
<th>Sequencing Metrics</th>
<th>NCI-H1975 Variants</th>
<th>HCT-116 Variants</th>
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<td>Sample ID</td>
<td>Depth Coverage EGFR T790M</td>
<td>EGFR L858R</td>
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<td>250 cells (1)</td>
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Table 1. Mutations and variant frequency detected by NGS. Table shows the results of variants detected in NCI-H1975/HCT-116 spiked blood samples. Average sequencing depth and coverage of each sample are 6869 (s.d. 2317) and 97% (s.d. 0.99) respectively. NCI-H1975 carries known cancer hotspot mutations in EGFR T790M and EGFR L858R, which are detected in all spiked samples, except for one of the 10-cell spikes. Known mutations in HCT-116, namely SMO V404M, KRAS G13D and PIK3CA H1047R are also detected in the spiked samples, with variant frequency that correlates with the amount of cells spiked. None of these mutations are detected in the negative controls (whole blood or 0 cell spike samples).
Wholly-Intact and Viable Cells

There has been burgeoning interest to extrapolate the potential of CTC growth for drug testing and research purposes. There have been numerous efforts to optimize growth medium conditions, growth factor supplements, cell culture surfaces and hypoxia. Importantly, there is a need to minimize the damage and stress on the cells during CTC isolation, so that it does not perturb the innate nature of the CTC. The ClearCell® FX system isolates CTC in a fast but gentle method, with >90% cell viability as confirmed with MCF-7 and NCI-H1975 cells (Figure 5). As demonstrated with spiked MCF-7 cells, the proliferative potential of the cells is maintained after ClearCell® FX enrichment (Figure 5). Tumour spheroids are useful tools for drug assays, as they have been shown to recapitulate cancer growth more closely than monolayer cultures and reflects more accurate response to drug treatments (Fennema et al. 2013). The formation of tumour spheroids formation are also relevant to research in cancer stem cells and its associated features, such as its metastatic malignancy and EMT process (Pastrana, Silva-Vargas, and Doetsch 2011). We have confirmed tumour spheroids formation can be easily accomplished after ClearCell® FX processing. (Figure 6).

Figure 5. Average cell viability of cells before and after processing (Left). Representative images of GFP pre-labelled MCF-7 cells post ClearCell® FX processing on Day 1 and Day 7 (Right).

Figure 6. Tumour spheroid cultured in 2% Matrigel after ClearCell FX processing. Representative green fluorescent protein (GFP) (upper panel) and corresponding bright field (upper panel) images of MCF-7 were taken on Day 1 (left) and Day 7 (right), showing formation of tumour spheroids by Day 7.
This white paper presented a label-free CTC enrichment of heterogeneous populations of cancer cells using ClearCell® FX system. ClearCell® FX allows fast processing of 7.5 mL blood sample through a semi-automated workflow in less than an hour. Wholly intact and viable CTCs are conveniently collected from sample output in liquid suspension format that facilitates ease of downstream molecular diagnostics and pathology assays. Leveraging cutting-edge inertial microfluidic separation, excessive WBCs can be depleted up to 100,000 fold. Further purification of WBCs can be obtained via ultra-high purity protocol to achieve sequencing ready output purities. This rapid, convenient and reliable way to enrich CTCs is envisioned to provide new insights for clinicians and researchers to study CTCs in a brand new perspective.

Key Features

- Label-free CTC isolation
- Up to 100,000X enrichment of CTCs
- Wholly intact, viable cells
- Path-lab ready
- Large blood volume enrichment
- Fast processing time of less than an hour
- Semi-automated workflow
- Easy and ready downstream integration

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