



Enrichment and Isolation of Uncontaminated Breast Cancer Cells from Human Blood Samples

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Cancer cells in peripheral blood, known as circulating tumor cells (CTCs), play an important role in tumor dissemination. CTCs are shed from either the primary tumor or its metastases and circulate in the peripheral blood of patients; thus they can be regarded as “liquid biopsies” of metastasizing cells. Although the exact origin and physiology of CTCs is unknown, a fraction of these cells are thought to be viable metastatic precursors capable of initiating a clonal metastatic lesion. The molecular characterization of CTCs is important because it may provide insights into the molecular biology of metastasis, the association of their molecular profiles with treatment outcomes, and reveal the presence of potential therapeutic targets. The process of CTC enrichment represents a significant step toward the isolation of CTCs from whole blood. Given that there are approximately 5×10^9 erythrocytes and 10^7 leukocytes per ml of whole blood, purification must represent a reduction of many orders of magnitude. In this study, we show a method to isolate CTCs which combines a microfluidic cell separation device (Parsortix™ system) as an enrichment method followed by the DEPArray selection system. The Parsortix™ system is versatile in its ability to separate, capture and harvest CTCs in an enriched form compatible with the needs of downstream processes such as DEPArray. Here we demonstrate proof of principle for enrichment of CTCs via the Parsortix system using breast cancer cells spiked healthy blood donor sample. For these experiments, eight milliliters of blood was processed. The cells were harvested and stained to identify tumor cells in the enriched harvest product. Cells were stained with fluorescently labeled monoclonal antibodies specific for pan cytokeratin (CK-8/18/19-PE), leukocyte common antigen (CD45-APC), and nuclear stained with [4',6-diamidino-2-phenylindole (DAPI)]. With spiked samples, tumor cells were isolated by DEPArray selection, which yielded a pure population of tumor cells for molecular characterization. Tumor cells were defined by presence of a clear DAPI-stained nucleus, CK-PE-positive cytoplasm and CD-45-APC negative. We demonstrated that by using a combination of enrichment and isolation/selection methods, we are able to isolate single, uncontaminated tumor cells to achieve single cell molecular analysis. The use of single cells is emerging as a powerful approach to molecular analysis in oncology, and this study demonstrates its potential application with circulating tumor cells.

Objective

Evaluate the Parsortix™ system for enrichment of cancer cells from human specimens. Isolate single tumor cells from human blood to perform molecular analysis.

Methodology & Results

Pre-enrichment using the Parsortix™ system

A total of 100 breast tumor cells, FC-IBC02, were spiked in 8 ml of blood from a healthy donor. The Parsortix™ system (research use only), was used to separate breast tumor cells from the blood – i.e. red blood cells, white blood cells (Figure 1 and Figure 2). Cells were recovered in a volume of 1.2 ml of PBS buffer, centrifuged, and the cell pellet was suspended in 50µl of PBS and fluorescently labeled with CK-PE, CD45-APC and nuclear stained with DAPI (Figure 3).

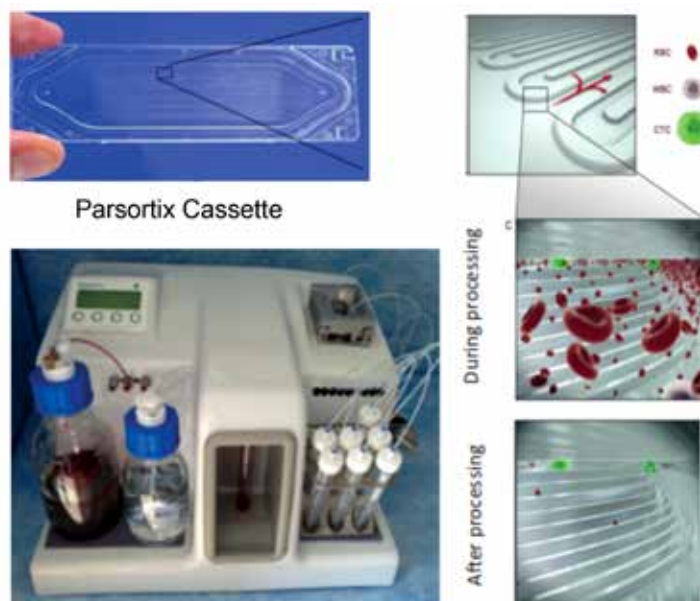


Figure 1. Parsortix system. Illustration of separation and cell capture principle.

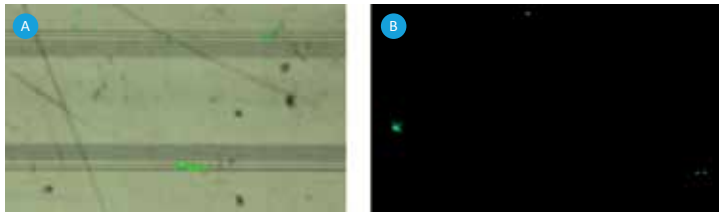
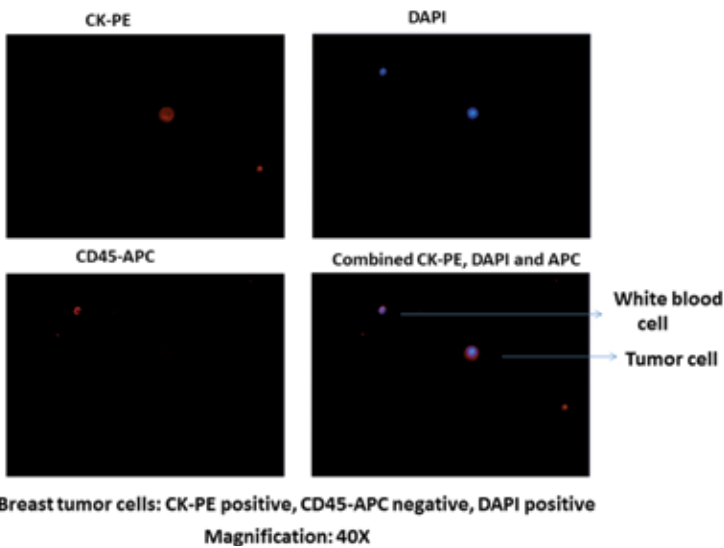


Figure 2. Pre-labeled human breast tumor cells inside the Parsortix™ cassette. **A)** Fluorescence bright field merged micrograph of captured cells. Cells were pre-labeled with CFSE (green fluorescent dye); **B)** Fluorescence micrograph of harvested cells.



Single cell isolation using the DEPArray™ system

After labeling, the cell pellet was washed twice with SB115 buffer (Silicon Biosystem, CA) and suspended in a volume of 14 μ l of SB115 buffer. The cells were loaded in the DEPArray cartridge to facilitate single cell isolation as we previously described (Figure 4)⁽¹⁾. The DEPArray chip consists of various microelectrodes that create electric cages into which individual cells are trapped and, by alternatively activating and deactivating the microelectrodes, the cells are moved to a position in the chip that allows their recovery. Cells are automatically detected by the system and assigned a unique cell ID. Cells that were CK-PE positive, CD45-APC negative and positive for DAPI were identified as breast tumor cells (Figure 5); WBC were identified as CD45-APC positive, CK-PE negative and positive for DAPI (Figure 6). A third population of cells CK-PE positive, CD45-APC positive and DAPI+ was also identified (Figure 7). Single cells from the three identified populations were collected for molecular studies.

Figure 3. Cells purified using the Parsortix™ system before being loaded in the DEPArray cartridge. Before loading the cells, an aliquot (1 μ l) was visualized under the fluorescence microscope.

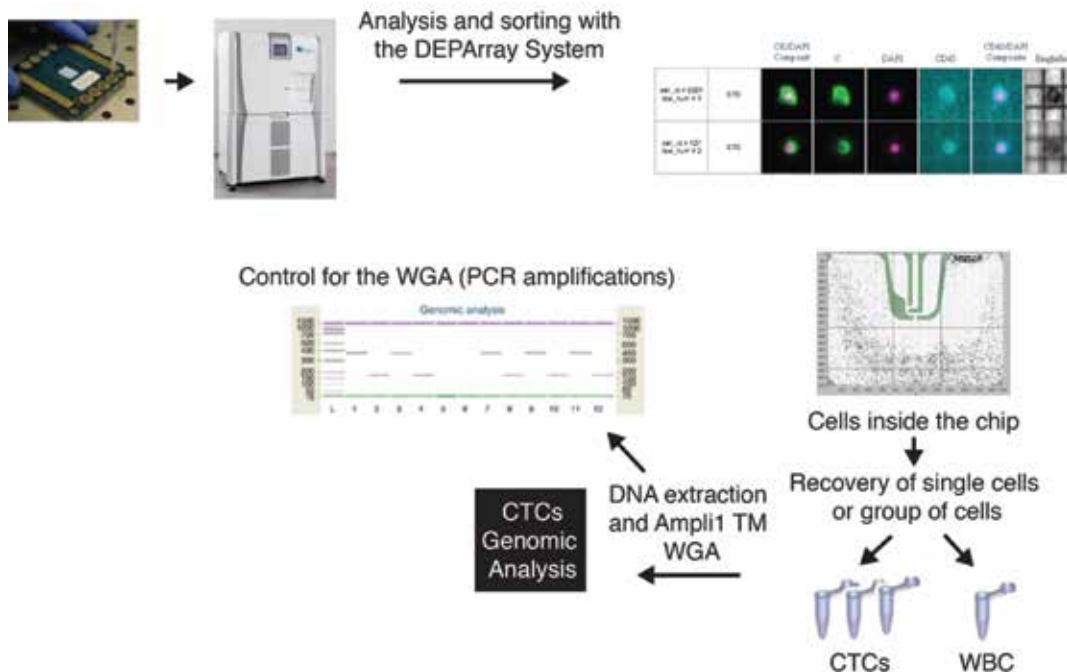


Figure 4. Single cells isolation using the DEPArray™ system.

Results

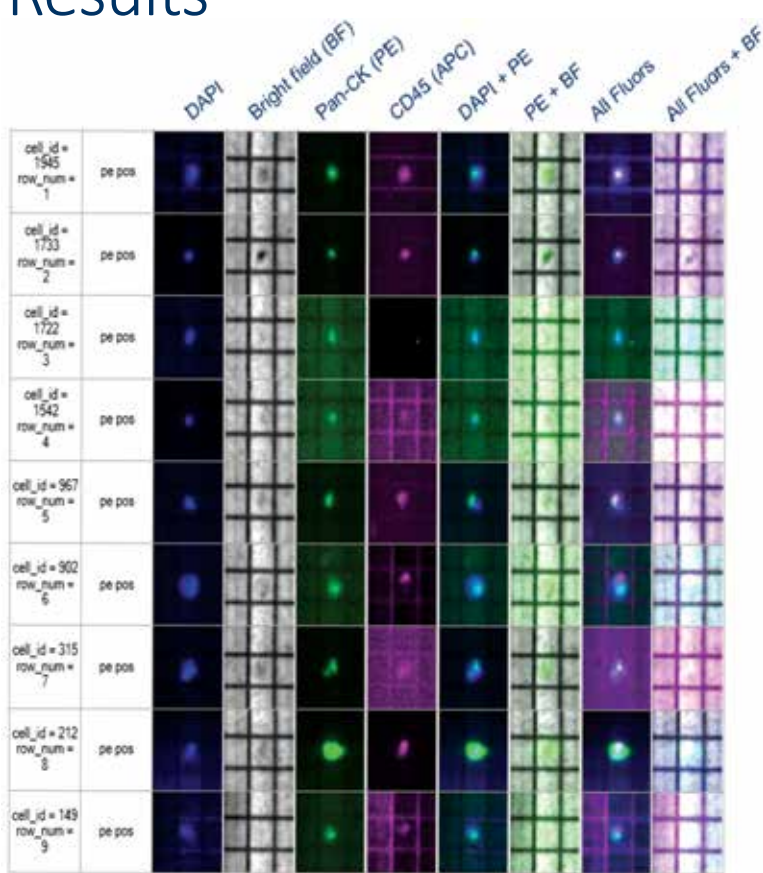


Figure 5. Tumor breast cancer cells FC-IBC02 CK-PE positive, CD45-APC negative and DAPI+.

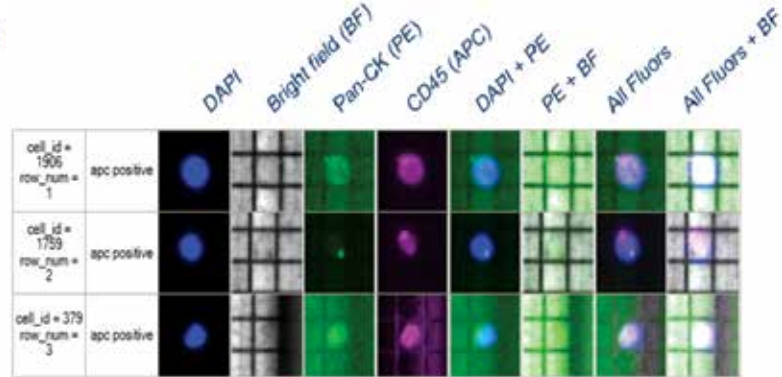


Figure 6. White blood cells CD45-APC positive CK-PE negative and DAPI+ .

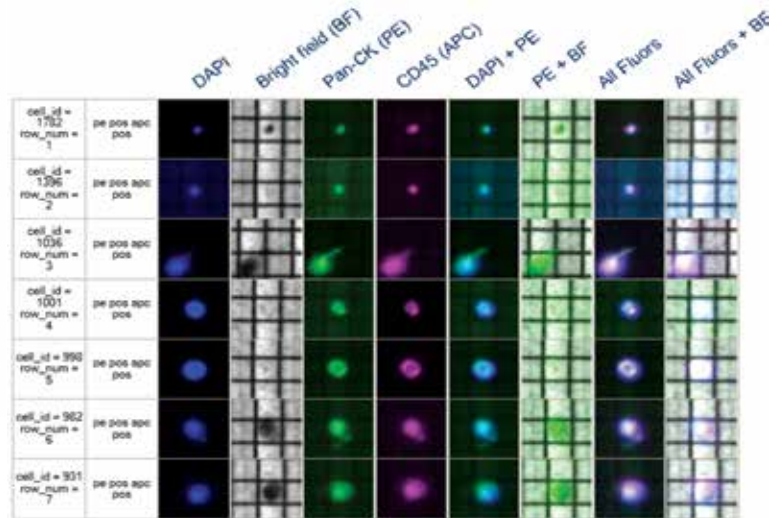


Figure 7. Cells from the blood CK-PE positive, CD45-APC positive and DAPI positive (dual positive).

Whole genome amplification (WGA) of DNA from single cells

To allow genotyping analysis, WGA was performed using the Ampli1™ WGA kit (Silicon Biosystems, CA) as we described⁽¹⁾. As control for the WGA, products were subjected to an end-point PCR for control genomic sequences (Ampli1 QC kit, SB); products were analyzed on the Agilent 2100 Bioanalyzer using the DNA 1000 kit.

ESR1 and p53

Estrogen receptor alpha (ESR1) and p53 were amplified by PCR (Figure 8) and the products were sequenced in the ABI 3130XL as described before⁽¹⁾.

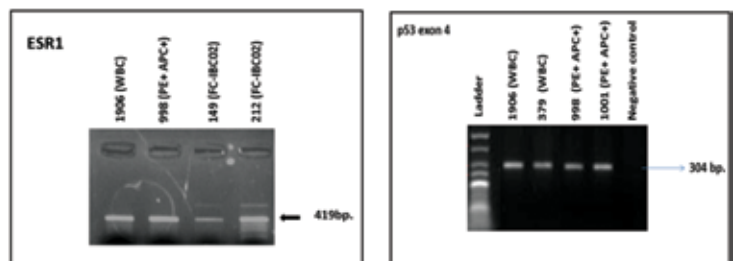


Figure 8. ESR1 and p53 amplification from single cells. The whole genome amplification (WGA) product from each single isolated cell was used to amplify ESR1 and p53. Cell IDs are indicated.

Enrichment of CTCs from metastatic breast cancer patients using Parsortix™ system

Eight milliliters of blood from metastatic breast cancer patients were processed by the Parsortix™ system. The purified cells were harvested from the cassette in 1.2ml of PBS buffer (200µl then 1ml). The cells were centrifuged fixed and stained with CK-

PE, CD45-APC and DAPI and visualized under the fluorescence microscope. Circulating tumor cells (CK-PE positive, CD45-APC negative and DAPI+) from two patients are shown (Figure 9).

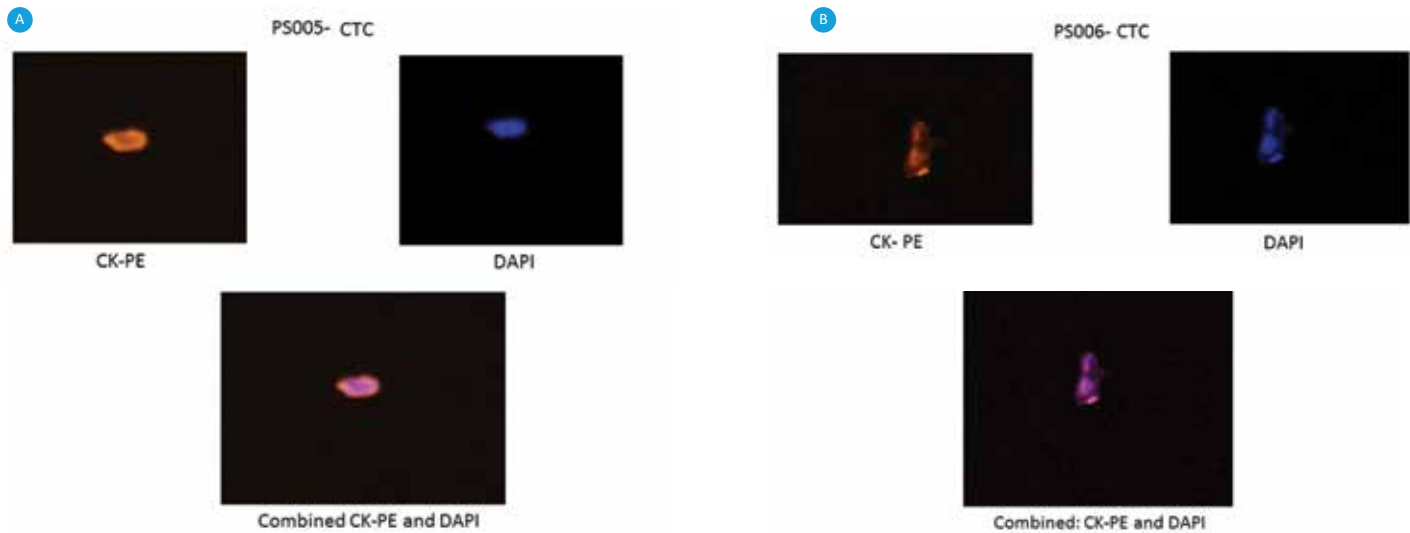


Figure 9. Circulating tumor cells from two metastatic breast cancer patients (A and B).

Conclusions and Final Remarks

- Circulating tumor cells from metastatic breast cancer patients were successfully enriched from blood using Parsortix™.
- The system detected a sub-population of double positive cells exhibiting Cytokeratin and CD45 proteins. Such cells have been reported previously but their biological significance has not yet been confirmed.
- The Parsortix™ cell capture and enrichment approach is not directly biased by the presence or absence of EpCAM, and thus can capture cells independent of EpCAM expression.
- Using the Parsortix™ system for pre-enrichment and DEPArray™ for single cell isolation allows for the downstream molecular analyses of a highly enriched population of single cells. Future work will deploy this technique to enable next generation sequencing of single CTCs from patient blood.

References

1. Fernandez et al. "TP 53 mutations detected in circulating tumor cells present in the blood of metastatic triple negative breast cancer patients", *Breast Cancer Res.* 2014, 16:445



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