Circulating Tumor Cell Enumeration on the MiSelect R System

Background

The metastasis pathway of cancer often involves hematogenous circulation. Capturing tumor derived cells in blood circulation can serve as more direct biological evidence of the tumor's capability to metastasize and/or be a signal of the existence of micro-metastasis. The presence of CTCs in breast cancer patients, as well as in many other types of cancer, is correlated with poor prognosis in both early and advanced breast cancer. Recent findings also show that the number of CTCs in metastatic breast cancer patients correlates better with progression-free survival and overall survival than the response determined only by radiological imaging.

The MiSelect R System (MiCareo Taiwan Co., Ltd.) for rare cell isolation and identification can be applied to CTCs for biomarker assessment and enumeration. By using the eDAR (ensemble-decision aliquot ranking) process, the MiSelect R System with the microfluidic SelectChip Dual cartridge, is able to sort, enrich, stain, and image CTCs.

Whole blood is labelled with the staining reagents in our SelectKit, which contains a phycoerythrin (PE) conjugated EpCAM antibody. Once the initial labeling is completed, the blood is loaded into the instrument, and the rest of the process runs automatically. As an aliquot of blood passes through the detection region in the microfluidic channel, a laser illuminates and excites any fluorescence labels present in the aliquot. If a signal is detected from the target cell, it along with the rest of the cells in the aliquot, are sorted into a separate area of the chip. Here blood cells, especially RBCs, are removed from the CTCs by an on-chip filtration system. After enrichment of the CTCs, fixation and staining reagents from the SelectKit are automatically added for imaging, identification, and enumeration of CTCs. Anti-panCK APC targets intracellular protein cytokeratin; DAPI stains for the cell nucleus; anti-CD45 FITC is specific for leukocytes.

Methods

1. Sample Preparation

Blood samples are collected into EDTA-coated vacutainer tubes and processed within 48 hours. The first tube is discarded to reduce contamination of samples with skin epithelial cells. For spiked assay, a predetermined number of cultured tumor cells is added into blood drawn from healthy subjects. The sample preparation is shown in figure 1 below.

Briefly, two 4 mL aliquots of blood are transferred from K2EDTA tubes into 50 mL conical centrifuge tubes. Whole blood samples are incubated with EpCAM for 20 minutes on a rocker. After staining, each blood sample is diluted with Isoton and then gently centrifuged to remove the free antibody. The blood is then processed using the MiSelect R System within 30 minutes of sample preparation.

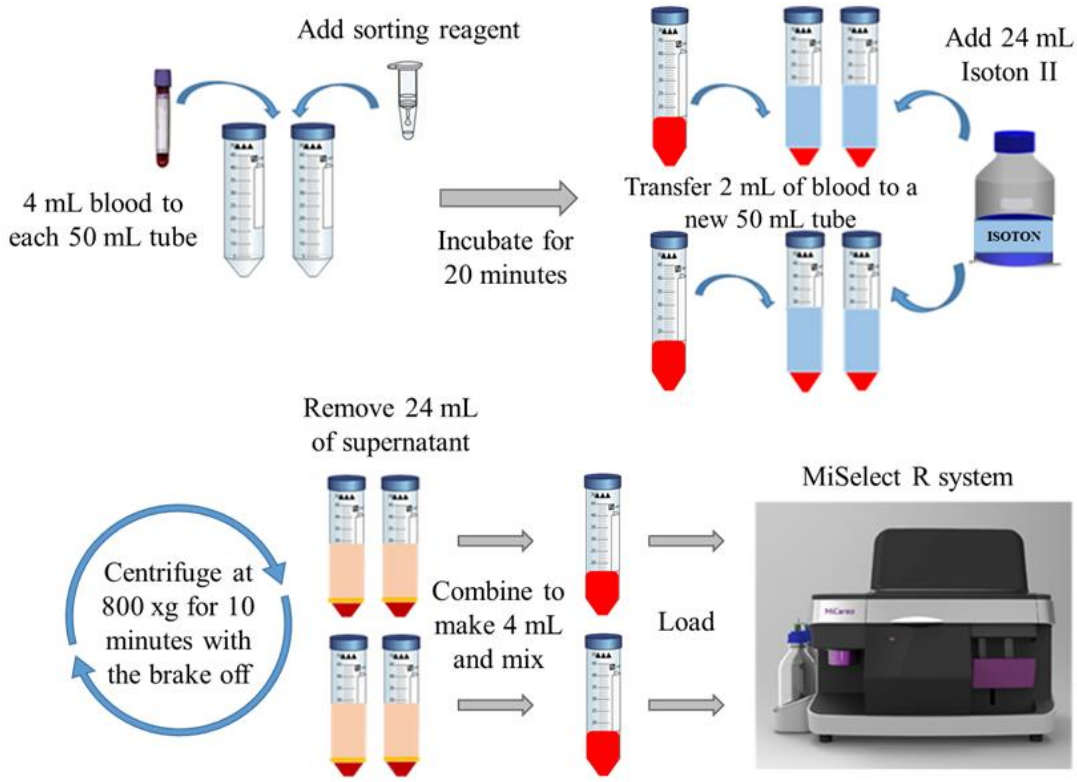


Figure1. Whole blood sample preparation

2. CTC identification with the MiSelect R

The MiSelect R System automatically labels and images the isolated cells with a panel of fluorescent antibodies for phenotypic profiling. A cell is classified as a CTC when its morphological features are consistent with that of a tumor cell and

it exhibits the phenotype EpCAM(+), CK(+), DAPI(+), and CD45(-). The CTC number is counted, analyzed, and recorded.

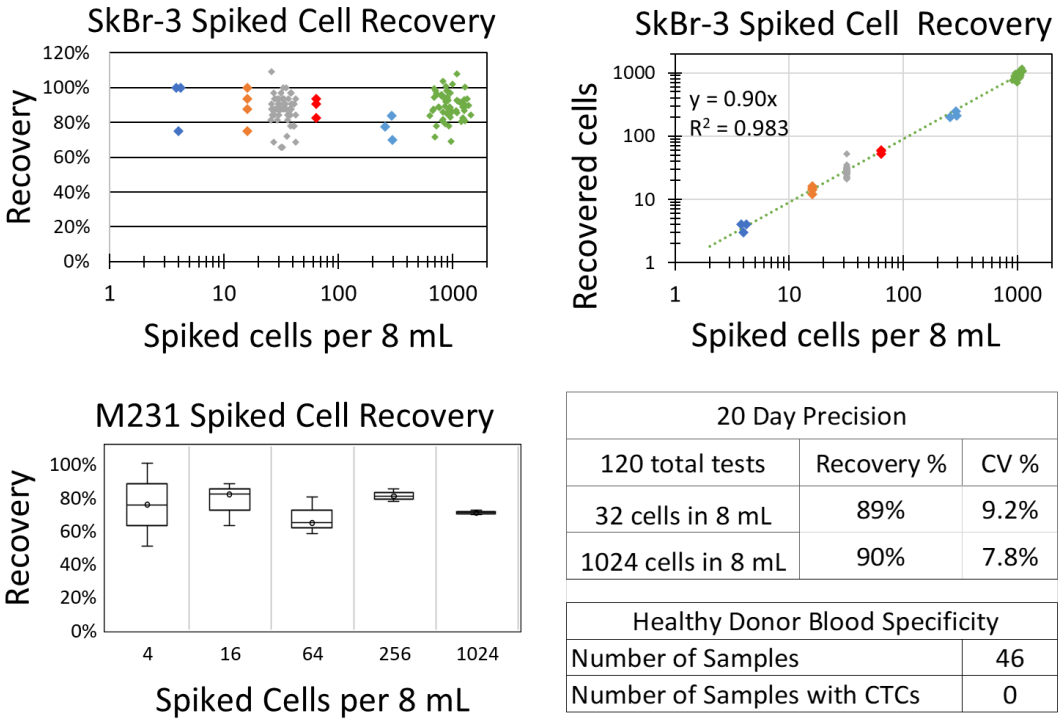
Results

1. Recovery Rate and Linear Measuring Range

To assess the analytical performance of the MiSelect R system, breast cancer cell lines, SkBr-3 (high EpCAM expressing cells) and MDA-MB-231 (low EpCAM expressing cells), are spiked into 8 mL of whole blood from healthy donors (figure 2).

A total of 139 spiking experiments was done with SkBr-3 cell concentrations of 4, 16, 32, 64, 256, and 1024 per 8 mL. Using linear regression analysis the average recovery rate is 90% with a R2 = 0.983, demonstrating a linear measuring range of 0 to 1024 cells per 8 mL.

For MDA-MB-231, 8 mL blood samples were prepared for each of the following cell spiking concentrations in triplicate: 2, 8, 32, 128, 512. Using linear regression analysis the average recovery rate is 71% with a R2 = 0.9957 demonstrating the high sensitivity of the optical detection for low EpCAM expressing cells.



20 Day Precision		
120 total tests	Recovery %	CV %
32 cells in 8 mL	89%	9.2%
1024 cells in 8 mL	90%	7.8%

Healthy Donor Blood Specificity	
Number of Samples	46
Number of Samples with CTCs	0

Figure 2. Precision, Recovery rate, Specificity, and Linearity of whole blood spiked with SkBr-3 and MDA-MB-231.

2. Precision and Repeatability

120 tests were performed over 20 days by three different operators to determine the instrument precision and reproducibility. The within-device coefficient of variation (CV) is 9.2% for the 32 cells per 8 mL tests and 7.8% for the 1024 cells per 8 mL tests.

3. Analytical specificity

The assay specificity is examined using blood from 46 healthy subjects. No CTCs are detected in any of the blood samples.

4. Combination of multiple antibodies for Circulating Tumor Cell recovery

Epithelial-to-mesenchymal transition (EMT) is a major process in which tumor cells gain migratory and invasive abilities. This is accompanied by a down-regulation of epithelial marker expression, e.g. EpCAM, and an up-regulation of mesenchymal-associated markers. Some CTCs lose part, or all, of their EpCAM expression during EMT. EGFR is a transmembrane receptor tyrosine kinase protein that is expressed in some normal epithelial, mesenchymal, and neurogenic tissues. Overexpression of EGFR is reported and implicated in the pathogenesis of many human malignancies, including non-small cell lung cancer. On the MiSelect R System a combination of multiple cell surface antibodies can be used together to target a wider range of circulating cells.

For example, the combination of EGFR and EpCAM enhanced the recovery rate of A549 cultured cells. Here, 200 A549 cells were added into 8 mL of blood drawn from healthy subjects. The recovery rate of the A549 cells increased from 34% to 68% with the addition of EGFR (Table 1).

200 cultured A549 cells in 8 mL of blood	Recovery using anti-EpCAM	Recovery using anti-EpCAM plus anti-EGFR
recovery rate (%) (n repeat)	34.2 ± 11.7% (n = 3)	67.8 ± 2.6% (n = 3)

Table 1. Recovery rate of cultured A549 cells with EpCAM alone and with the combination of EpCAM and EGFR.

5. CTC isolation from metastatic breast cancer patients

Samples from 11 metastatic Breast Cancer (mBC) patient are tested. A sample of at least 8 mL from each patient is processed according to our protocol for CTC enrichment and then loaded into the MiSelect R. After capturing, staining and imaging the CTCs, candidate cells are selected by our cell analysis software and confirmed by user review. DAPI(+), EpCAM(+), CK(+) and CD45(-) cells are considered as CTCs if their morphology is consistent with that of a tumor cell. Preliminary data analysis shows that CTCs are detected in 90.9% of the mBC samples analyzed with a range of 0 to 787 CTCs per 8 mL. Final data analysis is still under review and subject to change.

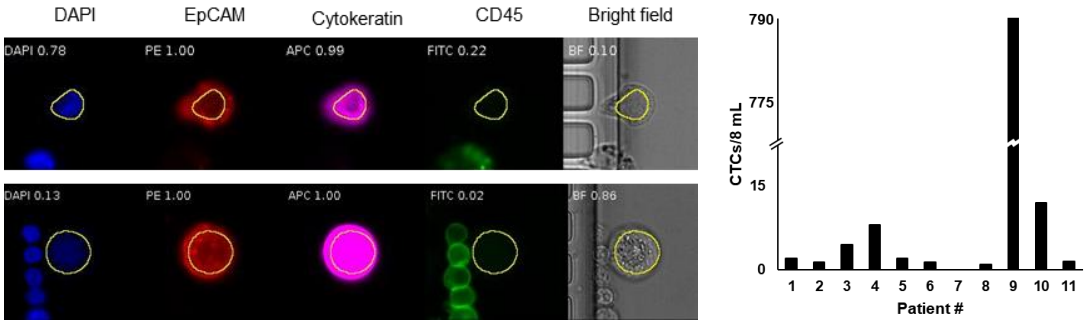


Figure 3. Images and count number of CTCs from mBC patients

Conclusion

The MiSelect R System shows excellent capability for single cell analysis of rare cells from whole blood. It demonstrates outstanding analytic performance and clinical sensitivity for CTC analysis as well as versatile capability for multiple-biomarker characterization.