Efficient Isolation and Accurate In Situ Analysis of Circulating Tumor Cells Using Detachable Beads and a High-Pore-Density Filter

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Cancer and Circulating Tumor Cells (CTC)

- Cancer is a prominent cause of death worldwide
- Most cancer deaths are due to metastases and not the primary tumor
- A cell is progressively genetically damaged and turns into a cell-bearing malignant phenotype

- **CTCs** are cells that have shed into the vasculature from a primary tumor and circulate in the bloodstream
- Indicate likelihood and severity of metastatic progression

Figure 1. Formation of metastasis
Detecting Primary Tumor and Metastasis

• Physical examination

• Traditional imaging methods
  - MRI, PET, CT, X-ray or ultrasound
  - detection limit is not sufficiently low to detect smaller metastasis

• Bone Marrow Aspirations to detect **Disseminated Tumor Cells (DTC)**
  - failure to demonstrate an independent prognostic value
  - biological functions of DTCs evolve overtime
  - painful sample collection
CTC the Liquid Biopsy: Potential applications and Clinical Impact

- Prognosis: CTC Enumeration
- Disease Monitoring: Characterization of Markers in CTC
- Identification, enumeration, and characterization of CTCs to assess cancer status and personalized anticancer therapy
- Understand the biology of the metastatic process

Yu M et al. JCB 2011;192:373-382

Figure 2: Potential Application of CTCs
Detecting CTC: Finding a needle in a haystack

- Very rare – one CTC per $10^9$ non-cancerous hematopoietic cells

Fig 3. The frequency of erythrocytes, platelets, leukocytes and CTC in blood of metastatic carcinoma patients and their cumulative probability
CTC ISOLATION, ENUMERATION, and CHARACTERIZATION: THE GRAND CHALLENGE

IDEAL CTC ISOLATION
High Efficiency
High Purity
High Cell Viability

IDEAL CTC ENUMERATION and CHARACTERIZATION
High sensitivity
High specificity
High reproducibility

1) Use a big needle and a small haystack
2) Look at the haystack
3) Look at the needle
## CTC Capture Technology

<table>
<thead>
<tr>
<th>METHOD</th>
<th>Principle</th>
<th>Advantage</th>
<th>Disadvantages</th>
</tr>
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</table>
| Antibody based-Enrichment Method            | Using specific antibodies for epithelial markers (Ex. either single-EpCAM or combination) | - semiautomated system  
- high reproducibility  
- high purity  
- can be used for molecular profiling | - multiple steps  
- non-viable cells  
- modest cell recovery  
- only EpCAM-positive CTCs detected  
- Expression levels vary  
- expensive |
| Cell Size                                   | CTCs are larger than most hematopoietic cells (Screen Cell, Clearbridge, CellSeivo, Rare cells, Screen Cell, Creative Microtech, Filtini, Parsortix) | low cost, live cells can be retrieved for culture purposes, different kits available for cell enumeration, immunolabeling, and molecular biology studies  
Easy procedure, availability of cells for additional studies, EpCAM-positive and – negative tumor cells are retained | low specificity due to potential leukocyte contamination  
Limited data for clinical validation  
large leukocytes are retained, CTCs are fixed, requires an automated filtration system |
| Density-Based                                | Separate mononuclear cell fraction fromm blood by centrifugation  
Ex. Ficoll-Hypaque or similar              | Easy, low cost, availability of cells for additional studies, EpCAM-positive and – negative tumor cells are retained | - low specificity, due to high contamination with leukocytes,  
- Low CTC recovery  
- limited data for clinical validation |
| Automated Enumeration and Identification     | Automated Microscopy (Bioview, Ikoisys, eDAR, Epic Sciences)          | Very sensitive  
Single step separation | Only EpCAM-positive CTCs detected  
Limited data for clinical validation |
## CTC Capture Technology

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| Antibody based-Enrichment Method       | Using specific antibodies for epithelial markers (either single-EpCAM or combination) (Ex. CellSearch, Veridex, Biocept, OnQity, Biofluidica) | - semi-automated system  
- high reproducibility  
- high purity  
- can be used for molecular profiling  
- FDA approved for predicting the prognosis | - multiple steps  
- non-viable cells  
- modest cell recovery  
- only EpCAM-positive CTCs detected  
- expression levels vary  
- expensive |
| Size-Based Capture                      | CTCs are larger than most hematopoietic cells (Clearbridge, CellSeivo, Rare cells, Screen Cell, Creative Microtech, Filtini, Parsortix) | - low cost  
- live cells can be retrieved for culture purposes  
- immunolabeling and molecular biology studies  
- easy procedure  
- EpCAM+ and – tumor cells are retained | - low specificity due to potential leukocyte contamination  
- large leukocytes are retained  
- limited data for clinical validation |

**Figure 4: Antibody-Based Systems**

![Antibody Based Systems](Image)
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| DENSITY-BASED          | Separate mononuclear cell fraction from blood by centrifugation Ex. Ficoll-Hypaque | - easy, low cost  
- availability of cells for additional studies  
- EpCAM-positive and – negative tumor cells are retained | - low specificity due to high contamination with leukocytes,  
- Low CTC recovery  
- limited data for clinical validation |

**Figure 5: Size-Based CTC Isolation**

| AUTOMATED ENUMERATION AND IDENTIFICATION | Automated Microscopy  
(Bioview, Ikoisys, eDAR, Epic Sciences) | Very sensitive  
Single step separation | Only EpCAM-positive CTCs detected  
Limited data for clinical validation |

**Figure 6: Density-Based CTC Isolation**
The problem with existing technologies

• As pointed out by the numerous review papers, all technology platforms existing today suffer from one trade-off or another.
  
  For example, antibody-based technologies are limited by the expression of specific antibodies.

• A universal CTC marker is yet unknown and given the heterogeneity of cancer, it is doubtful that a single universal cancer marker will be found.
A Novel Approach for Isolation and Subsequent In Situ Protein Expression Analysis - RIA (reversible bead attachment for cell isolation and analysis)

EpCAM antibody

Bead based capture for increased isolation efficiency

High pore density filter for enhanced enrichment and increased purity

hv 365 nm

Detachable bead for accurate CTC assessment
Synthesis of the Photo-cleavable antibody conjugated bead

Fabrication of high pore density membrane filter chip and fluidic block

Evaluation of RIA Platform
- Isolation Efficiency and Purity of CTC
- HER2 In situ Expression Analysis

Expression Markers Ex. HER2/neu
The Heterobifunctional Photocleavable Linker

Figure 7. a) Micrographs of control (top) and bead-attached SK-BR-3 cells (bottom).

Figure 8. Correlation of fluorescence intensities with bead coverage.

Figure 9. Fluorescence intensity of control and bead-attached cells.
**Initial Evaluation of the RIA System**

LED light source = 365 nm
Irradiation energy = 20 J cm\(^{-2}\)
98.4% cleavage efficiency
97% cells viable

**Figure 10.** Photocleavage efficiency and cell viability after different levels of light exposure

**Figure 11:** SEM image of a CTC isolated by the RIA platform.

Fluorescein isothiocyanate-labeled protein G
CTC Isolation using the RIA platform

Figure 12.
(a) Membrane filter chip used in the RIA platform is manufactured from SOI wafer to form evenly spaced uniform 8 μm diameter pores.

(b) The membrane filter chip is placed in a fluidic block attached to a syringe pump.
Increased efficiency of CTC Isolation from Cancer Cells with high EpCAM expression using the RIA platform

Figure 13. Distribution of cell diameters for four different cell types

- Average isolation efficiency increased from 59.3% using conventional size-based exclusion to 89.1% using the RIA platform

Figure 14. Comparison of isolation efficiencies between conventional size-based exclusion and RIA
Cells that do not express EpCAM were also isolated with 98% efficiency.

Figure 15. Frequency distribution of the diameter of MDA-MB-231 and MCF-7 cells and comparison of isolation efficiencies for these cells using the RIA platform and conventional size-based exclusion method.
### Table 1. Comparison of isolation efficiencies between conventional size-based exclusion and RIA per cell line.

<table>
<thead>
<tr>
<th>cell lines</th>
<th>method</th>
<th>size filtration only</th>
<th>RIA platform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AVE</td>
<td>stDEV</td>
</tr>
<tr>
<td>HCT-116</td>
<td>size filtration only</td>
<td>44.8</td>
<td>15.6</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>RIA platform</td>
<td>83.7</td>
<td>11.9</td>
</tr>
<tr>
<td>BT474</td>
<td>RIA platform</td>
<td>96.3</td>
<td>4.0</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>RIA platform</td>
<td>99.0</td>
<td>2.4</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>RIA platform</td>
<td>96.1</td>
<td>8.0</td>
</tr>
<tr>
<td>MCF-7</td>
<td>RIA platform</td>
<td>96.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Overall, the isolation efficiency and purity of the RIA platform were on par or better than other well-known CTC isolation methods.
### Isolation Efficiency and Purity of RIA Platform versus Commercial Isolation Method

<table>
<thead>
<tr>
<th></th>
<th>Isolation efficiencies (%)</th>
<th>Purity (leukocytes/mL)</th>
</tr>
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<tr>
<td></td>
<td>AVE</td>
<td>stDEV</td>
</tr>
<tr>
<td>RIA platform</td>
<td>96</td>
<td>2.8</td>
</tr>
<tr>
<td>Track etched membrane (ScreenCell)</td>
<td>76.5</td>
<td>27.6</td>
</tr>
</tbody>
</table>

Table 2. Comparison of the isolation efficiency and purity of the RIA platform with commercially available track-etched membrane filter using MCF-7 breast cancer cells.

- The isolation efficiency is better for the track-etched membrane but purity of the RIA platform was better.
- The difference can be attributed to the high pore density filter chip, which has a uniform distribution of pores compared to a track-etched membrane.
CTC analysis for Cancer Cell Markers

- CTC can be used to detect treatment targets
- Monitor the efficacy of targeted therapeutics
  Ex. Her2/neu expression in Herceptin (Trastuzumab) therapy

HER2 (Human Epidermal Growth Factor Receptor 2)
- Important biomarker and target of therapy for approximately 30% of breast cancer patients
- Common target in breast cancer cell therapy
- HER2 encoded by the ERBB2 gene.
- Amplification or over-expression of this gene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer
**HER2 in situ expression analysis**

\[ RIA \text{ HER2 SCORE (cell)} = \frac{\text{Average Foreground Intensity (analysis region)}}{\text{Average Background Intensity (non – ROI)}} \]

Bead does not affect in situ expression analysis

Fig 16: Comparison of fluorescence signal intensities in control and RIA-processed cells for cancer cells with different levels of HER2 expression
Fig 17. HER2 scoring standard generated from various breast cancer cells to categorize HER2 expression of isolated CTCs into three different levels: low, moderate, and high.
Verification of HER2 expression by Western Blotting

Western blot results were concordant with the expected levels of HER2 expression from literature, as well as RIA HER2 scores determined from those cell lines.

Fig 19. Western blot assessing HER2 expression in various breast cancer cell lines
CTC Visualized on Chip

Fig 20. a) Visualization of CTC clusters on the filter chip surface. (DAPI: blue; CK: green; HER2: red; CD45: pink)

b) Different levels of HER2 expression in CTCs isolated from patients.

CTC clusters were observed
CTCs were identified in all samples, with numbers ranging from 1 to 31 CTCs per mL. HER2 expression in isolated CTCs was quite heterogeneous. 10 of 12 samples containing CTCs with different HER2 expression levels. 67% overall concordance was observed between HER2 scores of CTCs and primary tissues.
SUMMARY

• The RIA platform is capable of isolating CTC from metastatic cancer patients
• CTCs were also characterized based on their in-situ protein expression levels.
• The novel platform that combining affinity-based and size-based exclusion assay improves isolation of small sized CTCs.
• Protein expression levels were accurately assessed without optical distortion because of the detachable beads.
• Utilization can be extended to a wide range of biomarker
• Short analysis time allows for testing of large sample volume
• Still requires staining for CTC identification and enumeration
1. The need for a Gold Standard Platform:

“The true potential of CTCs has yet to be realized because of limits in technology used to capture these cells and the lack of a complete understanding of metastasis. “

2. The use of CTC would require a change from the usual practice of oncologist in delivering treatment through to completion

3. SWOG 0500 clinical trial is investigating the benefit of early changes in therapy based on CTC testing by CellSearch:
   How do you interpret the result if the study fails?

CTCs and their characterization with novel techniques is one of the most promising research areas today, holding enormous potential for changing the way treatment is delivered and evaluate therapy to patients with cancer.
Thank you for your attention!