FLUXION

APPLICATION NOTE

Isolation and culturing of viable tumor cells using the IsoFlux™ System

OVERVIEW

- Many downstream analytical methods for circulating tumor cells (CTCs) depend on isolation of sufficient quantities of viable tumor cells.
- Culturing of CTCs can provide insight into the functional behavior of tumor cells, as well as serve as a platform for creating pure cell populations for further genomic analysis.
- Limited data has been shown for culturing of CTCs either directly from the patient, or following enrichment with a dedicated instrument.
- The IsoFlux System is an immunomagnetic enrichment device that uses no fixation, leaving the cells viable post-enrichment.
- Feasibility is demonstrated for culturing of tumor cells after enrichment with the IsoFlux System. Model experiments are shown using tumor cell lines spiked into healthy donor blood.
- A protocol is described for labeling proliferating cells and distinguishing them from contaminating leukocytes.

INTRODUCTION

The isolation of circulating tumor cells (CTCs) and other rare cells from patient blood is challenging due to the very low density, typically reported to be from a few cells to hundreds of cells in a typical blood draw volume (7.5mL), containing over 10^7 nucleated cells. While a number of different platforms have demonstrated CTC isolation, high efficiency recovery of viable cells has remained challenging. The CellSearch System (Veridex, J&J) is the most widely used clinical platform for CTC enumeration. CellSearch requires the cells to be fixed before the magnetic bead binding step (Cristofanilli, et al.). Fixation precludes assays that are dependent on cell health, such as RNA-based measurements, and subsequent tumor cell culture. Other more recent efforts have demonstrated recovery and culture of tumor cell lines on the isolation substrate itself, which can lead to difficulties in recovery (Desitter, et al.). In other research devices (iCTC-Chip), cell immobilization inside a channel and subsequent culturing of tumor model cells in the same channel has been demonstrated (Helzer, et al.).

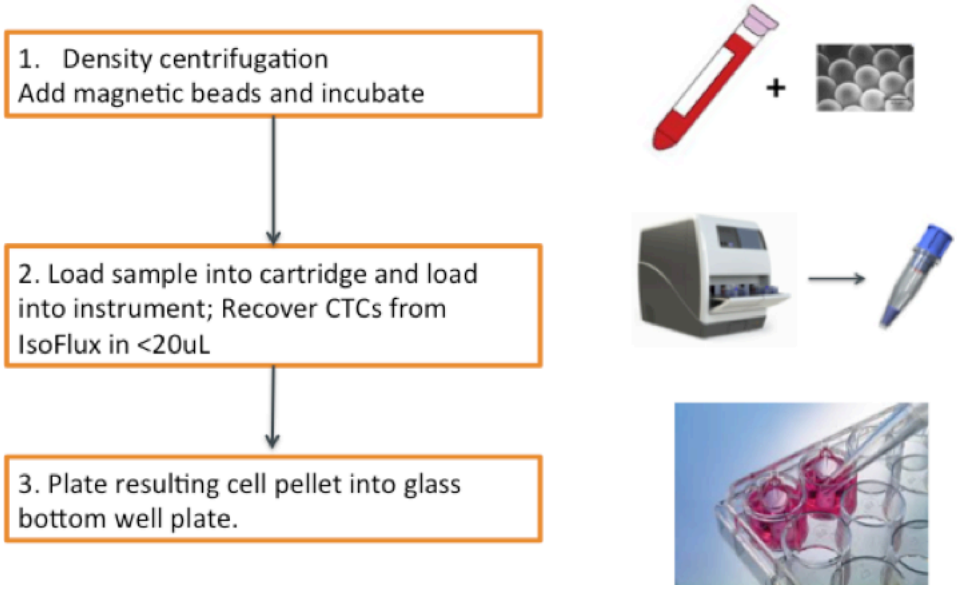
Here we report feasibility data of the cell culturing application using the IsoFlux System. The IsoFlux methodology is particulary well suited for this purpose since tumor cells are enriched and recovered with high efficiency and viability.

METHODS

A. Sample logistics and preparation - Whole peripheral blood from healthy donors (AllCells, Emeryville, CA) was collected in EDTA tubes. Samples were shipped at room temperature in an insulated shipping cooler. All samples were processed within 36 hours of the initial draw. Each 7mL of blood sample was processed to recover the peripheral blood mononuclear cell (PBMC) fraction. Briefly, Leucosep tubes (Greiner BioOne, Monroe, North Carolina) were prepared by adding 15mL of Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA). Blood samples were added and centrifuged for 15 minutes. The PBMC fraction was recovered and resuspended in 600µL of binding buffer (CTC Enrichment Kit, Fluxion Biosciences). Tumor cells (MDA-MB-231) labeled with CellTracker Red (Life Technologies, Foster City, CA) were spiked into the tube prior to addition of the magnetic beads. Immunomagnetic beads pre-conjugated with anti-EpCAM antibodies (CTC Enrichment Kit, Fluxion Biosciences) were added directly to the sample and incubated at 4°C with passive mixing on a rotator.

Figure 1: IsoFlux workflow for CTC culturing

Tumor cell recovery/viability experiments consist of three steps:
(1) Healthy blood collection in an EDTA blood tube, separation of the white blood cell fraction via FICOLL centrifugation, addition of the tumor cells, and magnetic bead coupling.
(2) CTC enrichment using the IsoFlux instrument; the positive fraction is recovered in an elution volume below 20µL.
(3) Cell recovery, placement in the tissue culture vessel, and viability assessment.



B. Separation - Following bead coupling, samples were loaded into the inlet well of the IsoFlux microfluidic cartridge. Samples were flowed through the magnetic separation region with each sample passing through the channel in about 15 minutes. After processing, the isolated target cells were recovered off of the isolation zone disk and dispensed into a microfuge tube and placed in culture.

C. Tumor cell culture – After sample extraction, cells were immediately seeded onto collagen coated glass bottom plates (24-well Sensoplate, Greiner BioOne, Monroe, North Carolina) filled with Leibovitz Medium (complete medium containing 10% FBS, Pen/Strep, HEPES, and Glutamax). The culture was maintained in a humidified 37°C incubator.

D. Proliferation assessment – Cells were imaged using an AxioObserver Z1 inverted microscope (Zeiss, Germany). After 2 days in culture, cells were fixed, permeabilized, and stained using Click-iT EdU cell proliferation kit (Alexa Fluor® 647 Kit, Life Technologies, Foster City, CA) and Hoechst dye (part of the IsoFlux CTC Enumeration Kit, P/N 910-0093) and imaged.

RESULTS

High sensitivity immunomagnetic recovery

A key feature of the IsoFlux System is improved sensitivity to the isolation antigen used. IsoFlux uses magnetic field focusing in a microfluidic environment to improve upon traditional immunomagnetic separation. This leads to significantly larger numbers of CTCs and more patients presenting CTCs, as compared to technologies like the CellSearch platform from Veridex (Stresemann, et al.). In addition to isolation efficiency, off-chip recovery is another important performance metric. Fluxion's CellSpot™ technology enables lossless recovery of cells from the microfluidic flow channels. Cells are deposited in a small area of the CellSpot substrate and extracted in very low dead volumes (2-5µL). The IsoFlux platform can isolate sufficient numbers of CTCs for downstream assays (CTCs>5 per 7mL) from over 75% of cancer patients with advanced disease (Fig. 2).

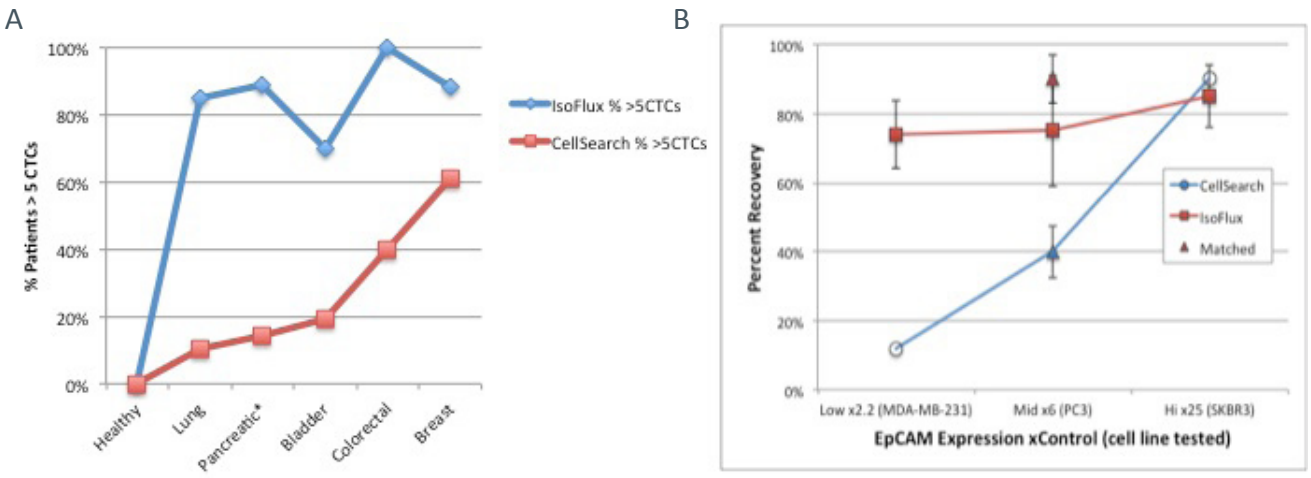


Figure 2. CTC recovery from patient samples and cell lines. A) High efficiency CTC recovery for patient samples. Percent of patients above a 5 CTC cutoff are shown for a number of different indications. IsoFlux recovery (blue) as compared to published data using the CellSearch system B) High sensitivity recovery and EpCAM expression. A comparison of recovery rates for three different tumor cell lines is shown. Mean results using the IsoFlux system are plotted with standard error bars (solid squares) for three cell lines: MDA-MB-231 (n=7), PC3 (n=38), and SKBR3 (n=8). Solid triangles represent matched sample results (n=4 samples each) that were run on either CellSearch (blue) or IsoFlux (red) using exactly the same cell counting protocols. Open circles represent literature data for CellSearch spike-in recovery from two of the cell lines studied: MDA-MB-231 (10) and SKBR3 (9) (reproduced from Harb et al.).

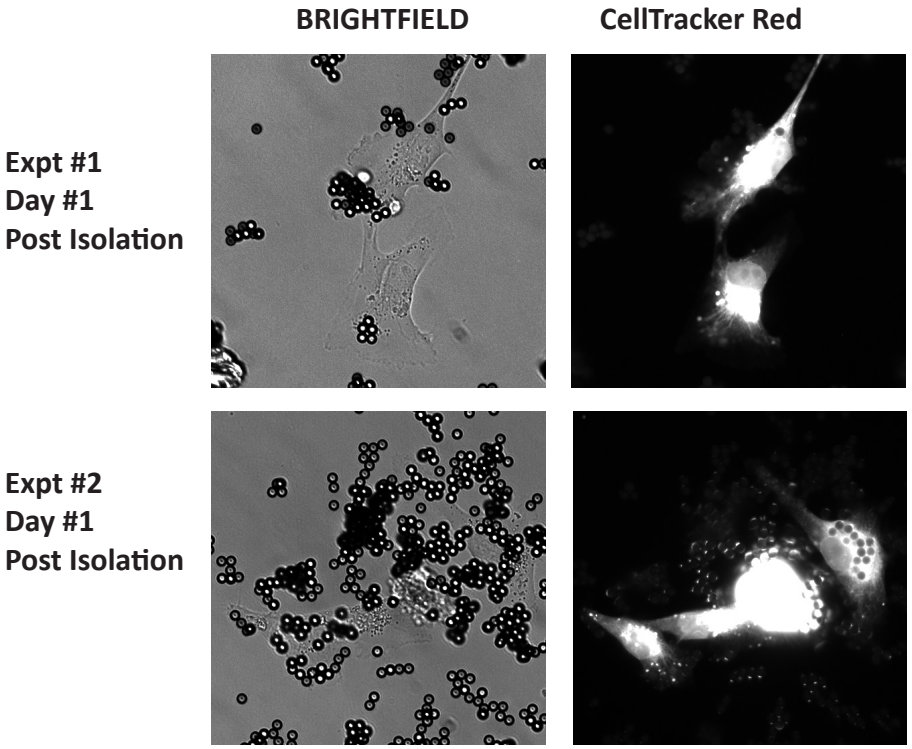


Figure 3. Cell attachment and spreading. Tumor cell attachment to the substrate and spreading was assessed after one day in culture. CellTracker Red was used to pre-stain MDA cells before spiking into healthy sample controls. Note that the cells are well attached, and flattened despite being bound by the magnetic beads used for separation. No microbial contamination was detected post isolation.

Cell proliferation in culture, post separation

After one day in the culture vessel, cell morphology was visualized via bright field as well as CellTracker dye microscopy (Fig. 3). On the second day, proliferation was assessed by EdU incorporation assay and Hoechst nuclear staining. Bright field microscopy showed post-isolation (Fig. 4, third row) cell spreading and morphological characteristics that are similar to MDA-MB-231 cells cultured pre-separation (Fig. 4, first and second rows). After separation, most cells that were positive for CellTracker (spiked cells) were also positive for EdU, and therefore proliferating.

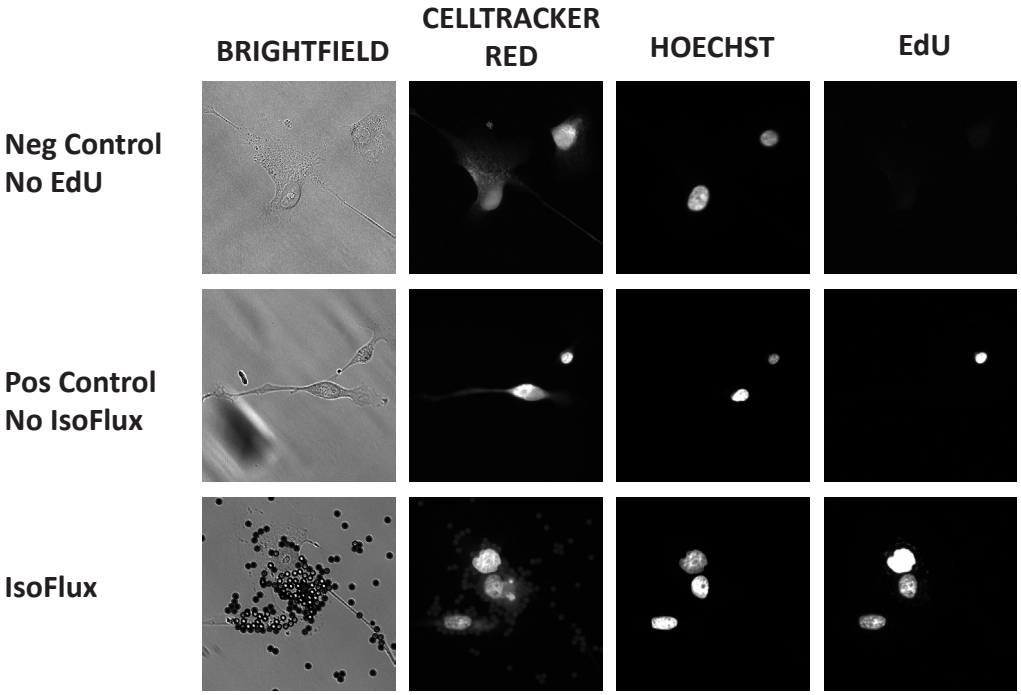


Figure 4. Proliferation assessment (Day 2). Cells were stained using the EdU proliferation kit. Note that a majority of the cells cultured pre-isolation (second row) and recovered post-isolation (third row) are labeled as proliferative (actively replicating DNA) using the EdU detection assay.

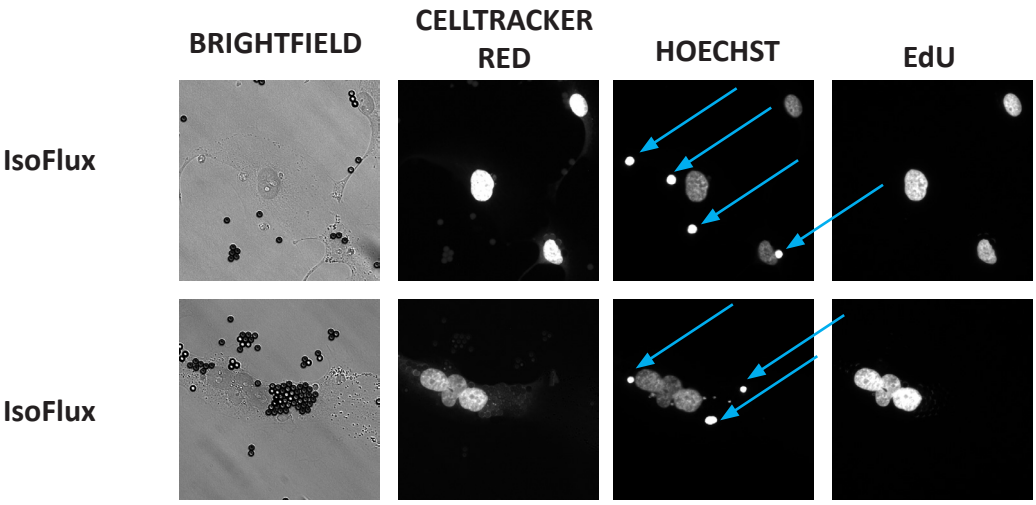


Figure 5. Proliferative properties of tumor cells as compared to leukocytes. Arrows point to leukocytes in the Hoeschst channel. Note that these cells are not proliferating (no signal in EdU channel).

Finally, the properties of recovered MDA-MB-231 cells were compared to background leukocytes. CellTracker Red (Fig. 5) identifies all of the pre-labeled tumor cells. The cells negative for CellTracker staining while displaying small nuclear morphology by Hoechst staining are putative leukocytes (Fig. 5). These cells are not stained by the EdU kit. This is consistent with the hypothesis that enriched tumor cells are proliferative but the contaminating leukocytes are not proliferative under short-term culture conditions.

CONCLUSIONS

We have developed a workflow that utilizes the IsoFlux CTC separation technology to isolate cells of tumor origin out of a blood draw without the use of fixation agents. The dimensions of the channels and flow rate have been optimized to protect CTCs from mechanical stresses that might alter their viability, physical structure or biochemistry, as well as to maximize the number of CTCs that can be recovered. Here we verify that tumor derived cells can preserve their viability and are not significantly damaged during separation process. Cells grow in culture when plated after the separation step. A dye identifying proliferating cells can be used to verify the presence of tumor cells and differentiate them from contaminating leukocytes in the isolated fraction. The data indicate that culturing of CTCs from clinical samples post-separation will also be feasible, but significantly more challenging due to the fact that a significant percentage of CTCs may be damaged while in circulation. Assays that depend on cell viability post isolation, such as RNA expression profiling, are also compatible with the IsoFlux system.

REFERENCES

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