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Towards an optimal and unbiased approach for tumor cell isolation

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Abstract Our current understanding of clinical significance or the lack thereof of circulating tumor cells (CTCs) is biased by the technology used to isolate these rare cells. Despite the presence of a vast number of academic and commercial technologies, the lack of a standardized and optimized platform has been widely noted. We present a negative enrichment approach, integrating WBC depletion and chemical-free RBC depletion in the same setup without the need for centrifugation, washing or multiple sample handling steps. This approach achieves an average of > 90% recovery of spiked tumor cells and > 99% total WBC depletion in whole blood across multiple cell lines, in a simple and easy-to-use assay. The results presented herein and ongoing improvements aim to fulfill the need for a highly reliable, unbiased, standardized, and optimized CTC isolation platform, using component technologies that are validated for cell isolation.

Keywords *Circulating tumor cells (CTCs) · Cancer cell isolation · Metastasis ·
Negative enrichment · White blood cell (WBC) depletion*

Abbreviation CTC: Circulating tumor cells; WBC: White blood cell; RBC: Red blood cell; EMT: Epithelial to mesenchymal transition; TAC: Tetrameric Antibody Complexes; GFP: Green florescent protein; PBS: Phosphate Buffered Saline; EpCAM: Epithelial cell adhesion molecule

Introduction

The significance of CTCs for clinical cancer management has been widely recognized (Rolle 2005; Zieglschmid et al. 2005; Kahn 2004; Racila 1998). In the last decade CTC research has accelerated dramatically, as evidenced by the number of publications rising exponentially from less than 100 in the late 1980s to over 2800 in 2010 (Cote 2010). This is also accompanied by heightened commercial activity, with the existence of more than 100 companies worldwide catering to the market for CTC isolation products and services (RI technologies 2012). Despite such rapid development, it has been widely noted by clinical-thought leaders that there is a lack of standardization and optimization of assays, and that our current understanding of clinical significance of CTCs is biased by the technology used to isolate and detect these cells (Riethdorf and Pantel 2008; Budd 2009). For example, in 292 metastatic cancer patients, CTCs were not detected in 36% of the patients using the CellSearch® technique (Kasimir-Bauer et al 2012). Several publications based on novel technologies have demonstrated substantially better performance than CellSearch®, which is currently used in many clinical trials to establish the validity of CTCs as a therapeutic efficacy marker (Marrinucci et al 2012; Desitter et al 2011; Lim et al 2012; Denis et al 2012; Kim et al 2011; Gupta et al 2012; Vona et al 2000; Tan et al 2010). This leads to the scenario that if no CTCs are detected in a clinical sample, is it because of true absence of CTCs or due to the limitation of the technique used? This has led to calls for standardization and evaluation of technology platforms that are optimally suited for CTC isolation (Parkinson et al 2012).

The gold standard, which refers to the most optimal technology for CTC isolation, must first and foremost aim to isolate the maximum number of viable cells without relying on subjective (subjective to tumor heterogeneity and evolution) markers such as specific antigens or a specific size. Thus the most important performance parameter becomes the recovery efficiency. Secondly, various degrees of purity are acceptable depending upon the contamination

tolerance of downstream molecular analysis methods. Nevertheless, a method that targets the highest purity is considered to be superior (Lara et al 2004; Tong et al 2007). Thus, the second important performance parameter is the purity of isolated cells. This is followed by implementation parameters such as reproducibility, robustness, ease of use (automation), cost and test turnaround time (TAT). Due to the heterogeneous and mutagenic nature of tumor cells (Paterlini-Brechot and Benali 2007), which affect their biological and physical phenotypes (Bednarz-Knoll et al 2011), an ideal CTC platform should not depend on subjective markers such as antigen expression or physical parameters. An ideal CTC platform could be one that is based on a negative enrichment approach (Yang et al 2009), which aims to remove all “normal” blood cells while retaining the “abnormal cells” for cytometric and molecular analyses.

The current technologies available for CTC isolation have been widely reviewed (Chen et al 2012; Zhe et al 2011; Yu et al 2011; Sun et al 2011; Gerges et al 2010; Hou et al 2010; Paterlini-Brechot and Benali 2007). In general, the contemporary CTC platforms are mainly categorized into a) antibody/antigen-based, which rely on a single or a combination of antibodies and b) cell morphology-based, which rely on size and deformability. It has been widely noted that antibody/antigen-based approaches are susceptible to undercounting of CTCs due to cells undergoing Epithelial to Mesenchymal Transition (EMT) and hence not considered to be optimal (Paterlini-Brechot and Benali 2007; Allard et al 2004; Goeminne et al 2000; Gorges et al 2012). The size-based approaches are susceptible to the overlapping of size and density between the CTCs and WBCs, (Zhe et al 2011; Park et al; 2012; Meng et al 2004) hence are unable to overcome the recovery/purity trade-off. Although physical filtration methods are easy to implement, fast and relatively cost effective, they are unable to fulfill the need for the most optimal technology for CTC isolation due to the inherent trade-off. A negative depletion approach, which avoids labeling the cells or defining their size, has been recommended as an optimal approach (Yang L et al 2009).

The negative depletion approach involves eliminating the RBCs, typically by chemical lysis followed by depletion of white blood cells by immunomagnetic method, leaving behind the “abnormal cells,” which can then be identified by immunohistochemical staining or molecular analysis. Many of the currently developed techniques involve multiple steps such as density gradient

centrifugation and RBC chemical lysis that contribute to the risk of losing CTCs and adversely affect the cells of interest (Park et al 2012; Yang et al 2009; Marrinucci et al 2012; Marrinucci et al 2010; Cho et al 2012; Tong et al 2004; Lara et al 2004). Chalmers et al have demonstrated a negative enrichment method with an isolation efficiency of 84% and 2.7 log₁₀ enrichment (Yang et al 2009; Lustberg et al 2012). This approach requires multiple sample processing steps such as chemically RBC lysis, centrifugation, washing and isolation of peripheral blood mononuclear cells (PBMCs) using Ficoll density gradient separation. It has been reported that RBC lysis and density gradient centrifugation steps can lead to 10% and 30% cell loss of spiked tumour cells, respectively (Tong et al 2007; Lara et al 2004). Therefore, multiple sample processing steps are especially prone to compounding of cell loss with each additional step, resulting in reducing overall efficiencies. Thus, a better implementation of a negative enrichment approach that reduces the number sample handling steps, avoids centrifugation or use of chemicals and use of specialized instruments, is needed.

We present a two-step process combining WBC depletion and chemical-free RBC depletion, directly from 2 mL of whole blood. In this approach, 2 mL of whole blood is conjugated with anti-CD45 antibodies and magnetic particles in a syringe barrel, which is then placed inside a permanent magnet. The end of the syringe barrel is connected to a filter membrane holder containing a precision micro-fabricated slit membrane via the luer connector. Upon activation of the valve at the end of the syringe barrel, WBC-depleted blood flows through the precision membrane, which selectively allows passage of RBCs and retention of nucleated cells. Thus in a single step with minimal sample handling, WBCs, RBCs and platelets can be depleted effectively. This method yields an average tumor cells isolation efficiency of 94% with 2.25 log enrichment across multiple cell lines. The process was done in 1 hour, in 2 steps, without any manual sample handling. Fig 1 illustrates the advantages of the newly developed approach over other published works on negative enrichment. Our method dramatically simplifies assay implementation with elimination of several steps, while offering better performance.

(Figure. 1)

Materials and Methods

Immunomagnetic Separation

WBCs are CD45-positive and can be depleted by means of immunomagnetic approach (Yang et al 2009; Lustberg et al 2012). EasySep Human Whole Blood CD45 Depletion Kit (StemCell Technologies, Canada) was incorporated in our experiments. The kit consists of two-part components: Anti-CD45 Tetrameric Antibody Complexes (TAC) and EasySep® Magnetic Nanoparticles. This kit was used for immunomagnetic WBCs depletion during the sample preparation in this work.

Sample Preparation

Blood samples taken from healthy donors were used to spike the cancer cell lines for conducting the experiments. BD Vacutainer® K2 EDTA (Catalog# 367863, BD) blood tubes were used for venipuncture collection. EDTA acts as an anticoagulant that prevents the blood clotting. The EDTA tubes did not contain any preservative or fixative agents. 2 ml of blood was diluted with 2 ml of dilution buffer in a 5-mL syringe barrel (Catalog#302135, BD) for each experiment. 100 μ l of Anti-CD45 TAC (Catalog#18289, STEMCELL Technologies) was conjugated with 100 μ l of magnetic nanoparticles (Catalog#18289, STEMCELL Technologies) and incubated for 10 min. Then, the conjugated mixture was added to diluted blood and incubated for 30 min. “The Big Easy” EasySep® Magnet (Catalog#18001, STEMCELL Technologies) was placed around the barrel to separate the labeled cells, mainly WBCs for 15 min. After incubation, the sample was passed through micro slit membrane for RBC depletion. WBC depletion was determined by counting the cells before and after the immunomagnetic depletion procedure using an automatic haematology analyzer. (Horiba Micro ES60, Horiba).

Micro Slit Membrane Fabrication

Micro slit membranes were fabricated for this work by using Parylene-C. The 25 mm diameter circular membrane consists of periodically arranged, precision etched slits with slit dimension of 5.5 μ m in width and 40 μ m in length. The choice of 25 mm membrane was to allow for high throughput blood flow with

least fluidic resistance. It is important to minimize the fluidic resistance (pressure drop across the membrane), as it affects the viability and morphology of the cells and can potentially force target cells to pass through (Tong et al 2010; Bo et al 2010). The 25mm membrane was also designed to be compatible with a doubly-supported, commercial filter holder (Catlog#F0102-BA, SPI Supplies), thus reducing overhead manufacturing cost of fixture as well as creating a stable and repeatable experimental setup. Previous work involving slit filters (Tong et al 2010) used higher pressures to force WBCs and RBCs through the membrane, whereas the objective of this work is to capture as many nucleated cells as possible on the membrane, including WBCs. Thus minimal pressure was applied to drive blood, which leads to well-preserved viability and morphology of isolated cells.

The fabrication of RBC elimination slit-membrane was performed at the Institute of Microelectronics (IME). This design was preferred over other micro filter designs for cell separation due to its unique advantages in retaining PBMCs at a high flow rate, while eliminating RBCs (Ji et al 2008; Zhu et al 2007; Sajay et al 2012). Silicon wafer deposited with 1 μ m thick silicon dioxide (SiO₂) by using plasma-enhanced chemical vapour deposition (PECVD) was used as a substrate. A monolayer of adhesion promoter, gamma-Methacryloxypropyltrimethoxysilane (A-174 silane) was functionalized on the substrate to promote subsequent poly-p-xylylene (parylene-C) deposition. 10 μ m-thick Parylene-C was deposited by Parylene Deposition System (PDS 2010 Labcoter 2, Specialty Coating System, Inc). A layer of 100 nm thick Chromium (Cr), serving as a hard mask, was deposited on top of Parylene-C film via electron beam evaporation. With assistance of photolithography, thin Cr layer was defined by CEP-200 Chrome etchant with an optimum etching time of 50 seconds. Subsequently, micro slits were developed on Parylene-C membrane via reactive ion etching (RIE). Finally, filter membrane patterned with rectangular micro slits was released from silicon dioxide substrate by using buffered oxide etchant (BOE) or hydrogen fluoride (HF) vapour.

The main purpose of this membrane design is to maximize the depletion of platelets, RBCs, and smaller-sized leukocytes such as lymphocytes, monocytes, and granulocytes that escaped from upstream immunomagnetic depletion, while maximizing retention of other nucleated cells. The rectangular slit, as opposed to

the commonly used circular pore design, also helps to alleviate the pressure build up on the cells, as the cells do not fully occupy the porous structure. RBCs being 1000-times more deformable can easily re-orient themselves to pass through the opening while nucleated cells will not pass through easily. Hence, the rectangular slit design maximizes the flow rate when it minimizes the required pressure to drive the flow. This preserves the viability and morphology of target cells.

The membrane has an active diameter of 21 mm and a thickness of 10 μm . Fig. 2 shows the different views of the Parylene micro slit membrane. Parylene was employed as membrane material because it possesses high bio-compatibility, excellent mechanical properties with Young's modulus (4 GPa) and an inert chemical property, which is resistant to moisture and most chemicals. In addition, Parylene deposition is conformal and yields membranes of uniform thickness.

(Figure. 2)

Spiking of cancer cells

Two types of cancer cell lines were used in this work, namely, green florescent protein (GFP)-tagged MCF-7 human breast cancer cells (Cell Biolabs, USA) and GFP-tagged A549 lung cancer cells (Cell Biolabs, USA). These are stable cell lines with reporter protein that exhibited bright green fluorescence when exposed to blue excitation light. The MCF-7 is useful for breast cancer studies because it has retained several ideal characteristics that are particular to the mammary epithelium. The A549 cell line was derived from the human alveolar basal epithelial cells and was used for studying lung cancer. The MCF-7 cells in this work were bigger than the A549 cells with average cell sizes of 17.6 μm and 13.6 μm , respectively. These cell lines were chosen to test the robustness and applicability of our system to a wider range of cancer types.

Cancer cell lines were spiked into whole blood from healthy donor before any blood processing procedures in order to simulate the actual screening of cancer patient blood. Cultured cancer cell lines were harvested and resuspended in 1X Phosphate-Buffered Saline (PBS) solution (Catlog#10010, Invitrogen) with a concentration of 1 million cells per mL. The cells were stained with trypan blue to check for the cell count, average size and viability using an automated cell counter (Luna, Logos Biosystems). Lower concentration of cells was obtained by serial dilutions using 1X PBS. In addition, manual haemocytometer was

employed in order to obtain the actual number of cells within a sample volume of 10 μL . In the end, 20 μL of cells was added to 2 mL of whole blood to prepare a sample spiked with 200 ± 10 cells.

Experimental Setup

Fig 3 shows the illustration of our CTC isolation system. Prior to beginning the blood processing, all of the tubing and connections were checked to ensure a leak-free setup. The experimental setup was primed with blocking buffer solution (1X PBS containing 5% BSA) to eliminate air bubbles and to prevent any non-specific binding of cells within fluidic pathway.

(Figure. 3)

Blood sample was flown with a constant air pressure source (MV20 pump, Ibidi) controlled by manufacturer's software. The pressure source was measured by a calibrated external pressure gauge (Catalog#717-100G, Fluke). In addition, the applied pressure was monitored in real time by the manufacturer's software. The sample was contained in a 5-ml syringe barrel (Catalog#302135, BD) that is connected to the membrane holder via the luer connection. The micro slit membrane was supported by a mesh-like structure within the holder to prevent buckling of the membrane under fluid pressure.

WBC depleted blood sample was introduced to the membrane holder by applying the pneumatic pressure after opening the pinch valve (shown in the Fig. 3). The filtration process of 4-mL assay took less than 4 minutes resulting in a high flow rate of $\sim 1\text{mL}/\text{min}$. 2 ml of washing buffer, 1X PBS, was introduced to clear the membrane surface. After the microfiltration process, the cells captured onto the micro-slit membrane were stained with nuclear stain by flowing through 1 μL of Hoechst 33342 dye (Catalog#H1399, Invitrogen) in 499 μL of 1X PBS and incubated for 2 minutes. Subsequently, the micro-slit membrane was washed with 1 mL of washing buffer to eliminate unbound counter staining and minimize the background noise. All of the reagents were introduced sequentially at a constant pressure of 3.5 mbar in order to ensure a continuous flow.

Finally, the micro-slit membrane was carefully removed from its holder and then transferred to a microscopic slide to be inspected under a fluorescent microscope at 20X magnification.

Cell Identification

The micro slit membrane was carefully removed and placed flat on an ordinary microscope slide for cell inspection. An upright microscope (BX61, Olympus) with a motorized xy stage was used for imaging and inspection of cells. Image capturing was performed by 12-bit monochrome Rolera XR fast 1394 CCD camera (QImaging, Canada). Cells expressing positive GFP and Hoechst were counted as cancer cells. U-MWU2 and U-MWIB2 filter sets were used to visualize Hoechst and GFP probes respectively. Motorized stage was controlled by a joystick to scan through the entire membrane. In addition, the software (Image Pro-Plus MDA, Media Cybernetics) was employed to stitch individual frames to create a single image file for analysis of cells that were captured on the membrane. Cell counting and image capturing was performed using a 20X objective lens.

Evaluation of Depletion, Recovery and Enrichment

The depletion of WBCs, cancer cell recovery and enrichment of cancer cells on the membrane were determined as following:

$$\text{WBC depletion (\%)} = \frac{W_i - W_f}{W_i} \times 100$$

$$\text{Capture efficiency (\%)} = \frac{C_R}{C_S} \times 100$$

$$\log_{10} \text{enrichment} = \log \left(\frac{\frac{C_R}{N_f}}{\frac{C_S}{N_i}} \right)$$

Where W_i is the total number of WBCs in the original sample, W_f is the number of WBCs in the sample after immunomagnetic depletion. Both W_i and W_f were obtained by Horiba Micros ES60 automated haematology analyzer. C_S is the number of cancer cells spiked into the blood sample prior to experiment, and C_R is the number of cancer cells counted on the micro-slit retention membrane. N_i is the total number of nucleated cells in the sample prior to the experiment, which is equal to W_i . And, N_f is the number of nucleated cells retained and counted on the micro slit membrane. The final counting of cells on the membrane was performed using a Matlab-based automated image processing algorithm, CellC (Selinummi et al 2005).

Results and Discussion

Upstream Immunomagnetic WBC Depletion

WBC depletion using TAC has been previously reported (Yang et al 2009), which involved RBC lysis and centrifugation that require additional chemical reagents, laboratory equipment, skilled personnel and causing potential risks of cell loss. In comparison, we developed an optimized protocol to deplete WBCs directly in whole blood, thus eliminating several steps that could potentially cause cell losses. Fig. 4A shows the WBC depletion efficiency as a function of concentration of TAC in whole blood. The effect of blood dilution on WBC depletion efficiency was also studied. Fig. 4B shows the WBC depletion efficiency as a function of dilution factor (0 mL, 1 mL, 2 mL and 4 mL of dilution buffer solution) and two different antibody concentrations (50 and 100 $\mu\text{L/mL}$ of TAC) added to 2 mLs of blood. Data from Fig. 4 leads to the conclusion that the diluting ratio 1:1 of the blood sample to the dilution buffer gives an average of 90% of WBC depletion at an optimal cost and performance trade-off. Based on the analysis of 24 healthy volunteer samples, a spread of 85% and 95% WBC depletion was observed.

(Figure. 4)

Optimization of CTC Isolation Process

In typical size-based isolation of CTCs, the goal is to allow passage of all normal blood cells while capturing the tumor cells, purely relying on size as the criteria. This kind of approach has many pitfalls. First, there is a significant overlap between the size and density of WBCs and CTCs. Thus the size-based systems have an inherent recovery/purity trade-off. Second, cells that are undergoing EMT have been reported to assume blood-cell like characteristics, in terms of size and deformability. Such characteristics would make size-based technique highly susceptible to loss of CTCs, similar to that of EpCAM based techniques (Riethdorf and Pantel 2008; Pantel et al 2009; Cristofanilli and Braun 2010). Third, the circular pore design can damage the cell morphology severely as the cells experience the blocking force of flow across the membrane. Finally, patients have varying levels of WBCs as compared to normal individuals due to their response to therapy (Kim et al 2012; Fujiwara et al 2011). Due to the variation,

samples from patients with high levels of WBCs demonstrate physical blockage of pores on the membrane. Hence, such instances require high pressures to clear up the clog in order for the flow to continue. In our case, without WBC depletion, it took 10 minutes and 25 mbar of pressure to run the 2-ml blood sample. Without increasing the pressure, the flow stalled. This was probably the reason why typical size-based methods used large sample dilutions, typically 10X or more (Zheng et al 2011). The higher pressure also can force the target cells to pass through the pores. Thus, the CTC yield will potentially be biased by the WBC content and volume of the sample.

CTC Isolation

The micro slit membrane used for retaining CTCs was made by established silicon micromachining techniques, which produced precise geometries with good reproducibility. The slits in the membrane were arranged to maximize the fill factor (39%) without compromising the mechanical strength of the membrane. Higher fill factor coupled with large membrane area, reduces the flow resistance, thus enabling flow at minimal pressure (3.5 mbar). RBCs have no nucleus and are highly deformable – 1000 times more deformable than leukocytes. They are able to easily realign and squeeze through the slits even at small driving pressures. The design criterion for the membrane was to maximize the nucleated cell retention, including WBCs. Because the membrane was directly coupled with upstream WBC depletion, WBC contamination was minimal in our assay.

(Figure. 5)

Fig. 5 shows the size ranges of variety of cells within a blood sample compared to the sizes of tumor cells with the indication of pore size of current techniques. A slit size of 5.5 μm was determined to be optimal for CTC isolation. Dimensions higher than 5.5 μm led to loss of tumor cells due to cells wedging in the slits and undergoing deformation. Constant pressure flow was selected over constant flow rate to ensure minimal pressure on cells and to preserve their morphology and viability.

To demonstrate tumor cell detection, human whole blood spiked with GFP-tagged MCF-7 and A549 cells, upon WBC depletion was flowed through the micro slit membrane and stained with Hoechst. Fig. 6A and 6B shows the summarized result of capture efficiency of tumor cells spiked into whole blood.

An average capture efficiency of 97.8 % for GFP-tagged MCF-7 and 90.3 % for GFP-tagged A549 (n=5) was found, respectively. The choice of these two cell lines was to demonstrate the capability of the platform to isolate a wide range cell types with good recovery efficiency. Fig. 6E shows the fluorescent images of stained nucleated cells by Hoechst, captured GFP-tagged MCF-7 cells and merged image for tumor cells identification. It is evident from the images that this system is capable of capturing tumor cells with well-preserved morphology.

Cell recovery and purity

We have previously reported that the applied pressure has critical effect on the recovery efficiency of cancer cells and depletion of WBCs (Sajay et al 2012). Morphology of the recovered cells also depends heavily on the applied driving pressure (Tong et al 2010). In order to achieve high CTC recovery with good cell morphology, a lower pressure is desirable. But at lower pressure, depletion of WBCs by forcing them through porous membranes will cause more unwanted cells retained on the surface of membrane (Sajay et al 2012). This can subsequently lead to a lower filtration flow rate of sample, clogging, loss of target cells and/or decreased purity of the captured CTCs. This limitation was overcome by depleting the WBCs by means of immunomagnetic separation, prior to elimination of RBCs via a slit membrane.

Since an average of 90 % of WBCs were depleted upstream before the sample was flown through the membrane, a smoother and uninterrupted flow was achieved at a high flow rate of about 1 mL/min. This enabled processing a higher volume of whole blood (2 ml), than other reported CTC isolation assays (Tong et al 2010; Zheng et al 2011; Desitter et al 2011; Lim et al 2012). Fig. 6C and Fig. 6D show the fluorescent image (Hoechst) of nucleated cells captured by micro slit membrane with (6C) and without upstream WBC depletion (6D). The effect of upstream WBC depletion is appreciable from comparison of Fig 6C and 6D. The less number of WBCs explains the smoother sample flow and higher purity of CTCs enrichment obtained using this system. Without pre-depletion of WBCs, it was not possible to complete processing 2 mL of blood through the system due to clogging. Based on the number of WBCs on the membrane after sample processing we obtained 2.25 log₁₀ enrichment over nucleated cells.

(Figure. 6)

(Table. 1)

Comparison

A variety of techniques have been developed for the isolation of CTCs with different performance parameters. A complete performance comparison between published methodologies is difficult due to incomplete information (Mocellin et al 2006). The reported analytical data is generated under highly controlled conditions. For example, 2 and 5 cells spiking experiments were reported, where the cells were handpicked and micropipette just before filtration (Desitter et al 2011; Vona et al 2000). In another instance, spiking was performed right before the final step of processing (Yang et al 2009). This type of analytical data is not fully representative of the clinical sample processing. Thus, the true comparison between technologies can only be arrived at by independent control studies.

Many sample preparation steps are typically employed for CTC isolation. Such steps include RBCs lysis, centrifugation, cells washing and resuspension, etc. These steps are necessary to improve the purity of the recovered cells to facilitate downstream molecular analysis. However, every additional step and sample handling compounds the probability of rare cell loss. Additionally, manual steps are difficult to replicate in a standardized fashion in multi-center clinical trials, rising concerns about assay standardization.

Table 1 compares this work with other relevant CTC isolation assays. Negative enrichment based on cell size suffers from recovery/purity tradeoff, due to the size overlap between CTCs and other blood cells. In size-based filtration methods, many require a high degree of dilution in order to reduce the flux of millions of blood cells flowing through the filtration device, which will block and eventually choke the flow, thus requiring higher fluid drive pressures. Higher sample dilution ratio increases the assay footprint, prolongs assay time and potentially contributes to cell loss. Chalmers et al demonstrated an isolation efficiency of 83% at 2.7 log enrichment using a 7-step process involving RBC chemical lysis and centrifugation. When generating the analytical data to calculate the cell recovery, the tumor cells were spiked after RBC depletion with lysis buffer. This overestimated the recovery of cells, as the potential cell loss due to RBC dilution was not factored-in. In this work we successfully demonstrated

high isolation efficiency (94%) and enrichment (2.25 log₁₀) of the tumor cells in a two-step protocol directly from whole blood in an hour.

Conclusion

The motivation of this work was to address the need for a highly reliable, simple and easy-to-use assay for tumor cell isolation. We successfully implemented a novel negative enrichment approach employing upstream immunomagnetic depletion which is directly coupled to downstream chemical-free RBC depletion, in a simple two-step protocol. In order to prevent any loss of the target cells due to extensive sample processing and handling, such as RBCs lysis, density gradient centrifugation, cells washing and resuspension, among others, we developed a simple yet effective protocol which includes immunomagnetic depletion of ~90% WBCs directly from whole blood, followed by platelet and RBC depletion using a large area and high fill factor rectangular slit membrane. Using this system, we have demonstrated a high tumor cell capturing efficiency >90% across multiple cell lines. Our approach neither relies on antibodies nor size as the criteria for CTC isolation, thus, addressing the two major concerns of contemporary CTC techniques. This technique yields several benefits, such as high throughput of ~ 1 mL/min, lower fluid drive pressure of 3.5 mbar for maximal cell viability, minimal instrumentation and the ability to achieve an average of 2.25 log₁₀ enrichment in a simple 2-step protocol in 1 hour. Further work is ongoing to achieve 4-log depletion of WBCs while maintaining >90% recovery efficiency. This will be followed by pilot clinical trials to isolate CTCs from clinical patient samples.

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Figures for manuscript: Towards an optimal and unbiased approach for tumor cell isolation

Fig. 1 Comparison between contemporary approaches of negative selection of CTC and our new approach. Our newly developed method successfully eliminated the multiple handling processes from either 7-step or 9-step to two steps to reduce the risks of losing target cells.

Fig. 2 Photograph of micro slit membrane with slit dimension $5.5 \times 40 \mu\text{m}$ with thickness of $10 \mu\text{m}$ and a fill factor of 39 %. (A) Membrane image taken by digital camera (B) membrane image taken at 20x optical microscope (C) membrane image taken at 50x optical microscope.

Fig. 3 Illustration of CTC isolation system. A constant air pressure of 3.5 mBar was applied to gently push the sample from syringe barrel to the micro slit membrane and then to the waste.

Fig. 4 Results of newly developed upstream WBC depletion. A. WBC depletion (in percentage) as a function of different amount of antibody loading into whole blood. B. Results of WBC depletion (in percentage) obtained at different dilution factors under antibody loading of $50 \mu\text{L/mL}$ and $100 \mu\text{L/mL}$, respectively.

Fig. 5 Distribution of size of hematopoietic cells and comparison of pore size of current techniques. The width of our slit is $5.5 \mu\text{m}$, which is smaller than other current technologies.

Fig. 6 Results of tumor cell isolation. A. Capture efficiency of GFP-tagged MCF-7 lung cancer cell line. B. Capture efficiency of GFP-tagged A549 breast cancer cell line. C. Image showing nucleus of PBMCs on membrane for experiment done with immunomagnetic WBCs depletion (255 cells). D. Image showing nucleus of PBMCs on membrane for experiment done without immunomagnetic WBCs depletion (1122 cells). E. MCF-7 cancer cells captured on micro slit membrane and imaged under fluorescence. From the left to right, they are nucleus stained cells, GFP-tagged cells and merged image of those two. Software (Image Pro-Plus MDA, Media Cybernetics) was used to apply the pseudo color on these cell images.

Fig 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Table Title

Table 1 Comparison of different tumor cell isolation systems

Cell Isolation Design	Sample Preparation	CTC Recovery	Capture Purity	Remarks
This work	2mL blood diluted with 2mL dilution buffer. Upstream immunomagnetic WBCs depletion integrated with CTC isolation process.	97.8% for MCF7; 90.3% for A549	2.25 log enrichment	- 2 step process without manual handling - high isolation efficiency and high purity - Chemical-free RBC depletion without centrifugation
CTC enrichment by using 2D Parylene-C membrane micro-filter with 6 μm $\times$ 40 μm slots (Tong et al 2010).	1mL whole blood without dilution	90%	200 fold enrichment; 70-fold for 5 μm wide slot	- Small sample volume - rely on slit size to discriminate between cells thus subject to recovery/purity trade-off.
CTC enrichment by using polycarbonate filter with 6.5 μm circular holes (Desitter et al 2011).	1mL whole blood diluted with 7 or 8 mL buffer containing RBC lysing reagent and with or without fixative. After filtration, centrifugation may apply before CTCs recovered	91.2% for 5 cells spiking; 74% for 2 cells spiking	Not reported	- RBC lysis and centrifugation were applied that could potentially cause cell loss - Recovery/purity trade-off.
CTC enrichment by using SOI based filter with 10 μm circular holes (Lim et al 2012).	1mL whole blood diluted with 2mL PBS	>80%	200-6000 WBCs observed through cases	- Rely on pore size to discriminate between WBCs and CTCs thus subject to recovery/purity trade-off.
CTC enrichment by using Silicon-on-glass filtration device with 10 μm slots (Park et al 2012).	Started with 1mL whole blood with 2mL Ficoll-Hypaque gradient medium. CTCs were pre-conjugated with microbeads by anti-EpCAM.	~99% for MCF7; ~89% for DMS-79	Deplete 99.81% erythrocytes and 99.996% leukocytes	- Centrifugation was employed which can cause cell loss - Only applicable for EpCAM positive cells.
CTC enrichment by depleting normal cell (Yang et al 2009; Lustberg et al 2012).	Started with 1mL whole blood with 25mL RBC lysing reagent. Then, centrifugation was applied to obtain the buffy coat. Finally, immunomagnetic WBC separation was applied.	~84%	2.7 log enrichment	- RBC lysis and centrifugation were applied that could lead to cell loss. - involves very high dilution (26X).
CTC isolation/detection by fiber-optic array scanning technology (Marrinucci et al 2012; Robert et al 2004).	2mL whole blood sample was collected and processed with RBC lysis and centrifugation steps.	~100%	Not reported	- RBC lysis and centrifugation could lead to cell loss. - No steps described to improve the purity, which minimizes the possibility to use this platform for further molecular analysis.