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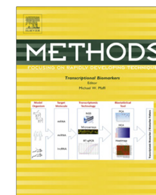

A negative selection methodology using a microfluidic platform for the isolation and enumeration of circulating tumor cells

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ABSTRACT

Circulating tumor cells (CTCs) exist in the peripheral blood stream of metastatic cancer patients at rates of approximately 1 CTC per billion background cells. In order to capture and analyze this rare cell population, various techniques exist that range from antibody-based surface marker positive selection to methods that use physical properties of CTCs to negatively exclude background cells from a CTC population. However, methods to capture cells for functional downstream analyses are limited due to inaccessibility of the captured sample or labeling techniques that may be prohibitive to cell function. Here, we present a negative selection method that leverages a Microfluidic Cell Concentrator (MCC) to allow collection and analysis of this rare cell population without needing cell adhesion or other labeling techniques to keep the cells within the chamber. Because the MCC is designed to allow collection and analysis of non-adherent cell populations, multiple staining steps can be applied in parallel to a given CTC population without losing any of the population. The ability of the MCC for patient sample processing of CTCs for enumeration was demonstrated with five patient samples, revealing an average of 0.31 CTCs / mL. The technique was compared to a previously published method – the ELISPOT – that showed similar CTC levels among the five patient samples tested. Because the MCC method does not use positive selection, the method can be applied across a variety of tumor types with no changes to the process.

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1. Introduction

The development of metastatic disease is a hallmark of nearly all solid tumor malignancies and is predictive of both morbidity and mortality in patients with advanced cancer [1]. Numerous theories have been proposed as to the underlying pathophysiology that leads to the development of metastatic lesions [2,3]. One such theory suggests that tumor cells develop the capacity to extravasate into circulation and implant at distant sites [4]. This theory, known as hematogenous spread, has been supported by the identification of DNA, RNA and whole cells in blood samples from patients with advanced cancer. These circulating tumor cells (CTCs) are rare events in whole blood, estimated to be approximately one CTC per one billion peripheral blood cells [5]. Over the last fifteen years, various technologies have been developed to permit the isolation and analysis of these rare cells [6–8]. These technologies can generally be divided between those that rely on isolation of cells based on expression of cell surface markers, called positive selection techniques, and technologies that rely on the removal of other cells in peripheral blood from a CTC population by distinct physicochemical properties that differentiate the two, thus known as negative selection techniques [9,10].

Within the realm of positive selection methodologies, a number of engineering solutions have been developed that use antibody based capture methods to isolate CTCs [7]. Many of these technologies rely on cell surface expression of the epithelial cell adhesion molecule (EpCAM). Successful isolation of CTCs has been achieved with EpCAM based identification using multi-color flow cytometry and fluorescent activated cell sorting techniques [11]. The Herringbone-CTC chip uses antibody-coated walls within a microfluidic device to both maximize interaction of CTCs with antibodies while simultaneously minimizing the manipulation of these cells with centrifugation and pipetting steps [12]. The only FDA cleared technology for the isolation of CTCs is the Veridex CellSearch™ technology which relies upon immunomagnetic particles bound to EpCAM antibodies [13,14]. Recently, changes in CTC number have been shown to predict for both survival and response to anti-cancer therapies in patients with metastatic prostate cancer [15–17]. Ongoing research efforts are attempting to qualify CTC enumeration, and

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changes in CTC numbers, as a surrogate biomarker for survival in clinical trials for patients with advanced prostate cancer [18].

Positive selection techniques have been criticized for their reliance upon cell surface expression of EpCAM to isolate CTCs as some tumors may downregulate expression of this marker during epithelial-mesenchymal transitions and other tumor types, such as renal cell carcinoma, do not commonly express EpCAM. To address this issue, recent technologies have been developed to isolate CTCs based on physicochemical properties distinct from peripheral blood mononuclear cells (PBMC) such as size, density or electrophoretic properties [13,19–23]. One example is a novel filter based technology developed to isolate epithelial cells based on the purported size differential between CTCs, white blood cells (WBC) and red blood cells (RBC) [19,20]. Another negative selection method developed by Dr. Kuhn and colleagues subjects a blood sample to RBC lysis followed by plating of all nucleated cells onto a proprietary microscopy slide, and revealed greater complexity and heterogeneity among these circulating events than previously anticipated [24,25].

While isolation and enumeration of CTCs has been the primary focus of these technologies, there is greater interest in the underlying biology of these cells. Recent work by Armstrong et al. performed fluorescent imaging of CTCs for markers other than EpCAM and identified a subset of CTCs that express markers of epithelial-mesenchymal transition (EMT) and may represent a cell population with greater potential to develop into a metastatic lesion [26,27]. EMT has also been implicated in the process of intravasation [28]. Further work by Danila et al. have found that CTCs from patients with prostate cancer can be analyzed for expression of cancer-specific genes including the androgen receptor and the fusion gene, TMPRSS2-ERG [9]. However, the major challenge confronting this field is the isolation and purification of these cells in a fashion that maximizes the capacity to interrogate rare cell populations by minimizing the risk of cell damage.

Here, we present a method that uses solely negative selection processes for the isolation of circulating tumor cells from the whole blood of metastatic cancer patients and a microfluidic device, termed the Microfluidic Cell Concentrator (MCC), for subsequent concentration and functional immunostaining analysis of all isolated cells. This method of negative selection for CTC isolation and analysis represents specific advantages to other CTC isolation methods. First, because the method does not rely on positive selection based on surface markers, this method allows for the evaluation of the heterogeneity of CTC surface markers, enabling the isolation of EpCAM-negative cells that may have undergone EMT. Second, isolated cells are free of antibodies or other tethering molecules, creating 'clean' cellular samples. Finally, the MCC results in cells isolated in a microfluidic compartment, where multiple analysis reagents can be applied in sequence without cell loss. We further evaluate the efficacy of our method using patient samples collected through an IRB-approved protocol (H-2009-0019). Prostate cancer patients with high circulating serum levels of prostate-specific antigen (PSA, typically indicating high tumor burden [29]) were collected and analyzed using the MCC methodology and shown to correlate with a previously published method of prostate CTC enumeration, the ELISPOT. The MCC method presented here represents an entirely negative selection methodology that can be used to collect patient CTCs from a variety of tumor types without the need to label, or otherwise isolate cells, using any specific pre-assumed characteristic of the cells.

2. Methods

2.1. Negative selection pre-processes

The macroscale methods for negative selection applied were in three steps representing the removal of approximately one billion non-target cells (Fig. 1A). Briefly, the first two steps involved the removal of erythrocytes from the sample and some peripheral blood mononuclear cell (PBMC) hematopoietic cell depletion with the OncoQuick buffycoat isolation method; the final step involved the removal of peripheral hematopoietic blood cells, identified as CD45+ cells. To evaluate the sample retention efficiency of this process, each blood sample was split. One-half was spiked with a known number of cells from an EpCAM+ prostate cancer cell line that constitutively expressed GFP. Each sample was then processed in parallel.

2.2. Bulk RBC removal

Blood was pooled and divided into spiked and non-spiked portions, with each portion brought to a final volume of 30 mL with 0.1% BSA and 1 mM EDTA in PBS (called 'Buffer 1'). (Fig. 1A). 1500 Lymph Node Carcinoma of the Prostate cells transfected to produce intracellular green fluorescent protein (LNCaP + GFP) cells were added at this stage to the spiked sample to quantify processing efficiency. Blood samples were then placed into an OncoQuick® tube (Greiner Bio One, Monroe, NC) and centrifuged at 400*g* at 4 °C for 30 min with no brake to isolate the buffycoat layer of mononuclear cells. All volume above the filter in the OncoQuick® tube was then isolated and placed into a new tube filled to a final volume of 50 mL using Buffer 1, ensuring that Buffer 1 comprised at least half of the final volume. At this stage, 10 μL of the collected fluid was removed and placed into a hemocytometer to quantify the number of PBMCs. Tubes were then centrifuged at 300*g* at RT for 10 min as a wash step to remove platelets. The cell pellet in this step and all subsequent cell pelleting steps was aspirated to approximately 100 μL to avoid aspiration of the pellet. The pellet was then resuspended in a Human RBC lysis Buffer (Boston BioProducts, Ashland, MA) in a 1:10 ratio of PBS to RBC lysis buffer by volume and allowed to sit for 30 min at room temperature. Following RBC lysis, the tubes were centrifuged at 300*g* and RT for 10 min and transferred to a 1.5 mL centrifuge tube, and Buffer 1 was added to bring the final volume of each sample to 1 mL.

Protocol for OncoQuick:

1. Mix whole blood 1:1 with Buffer 1.
2. Place blood into OncoQuick tubes carefully.
3. Centrifuge at 400*g* and 4 °C for 30 min with no brake.
4. Remove entire volume above filter of OncoQuick tube.
5. Place into new 50 mL conical tube, fill to 50 mL with Buffer 1.
6. Remove 10 μL of fluid, place into hemocytometer to obtain a PBMC cell count.
7. Centrifuge at 300*g* and room temperature for 10 min.
8. Remove supernatant to approximately 100 μL.

Protocol for RBC Lysis:

1. Add 1 mL Human RBC lysis buffer to 10 mL PBS.
2. Resuspend 100 μL CTC-containing volume with mixture of Human RBC lysis buffer.
3. Let rest for 30 min at room temperature
4. Centrifuge at 300*g* and room temperature for 10 min.
5. Aspirate fluid to approximately 100 μL, transfer to a 1.5 mL centrifuge tube, add Buffer 1 until the final volume of each sample is 1 mL.

Note:

- Any tube that will contact the CTCs should be pre-filled and blocked with Buffer 1 at least for one hour prior to use.

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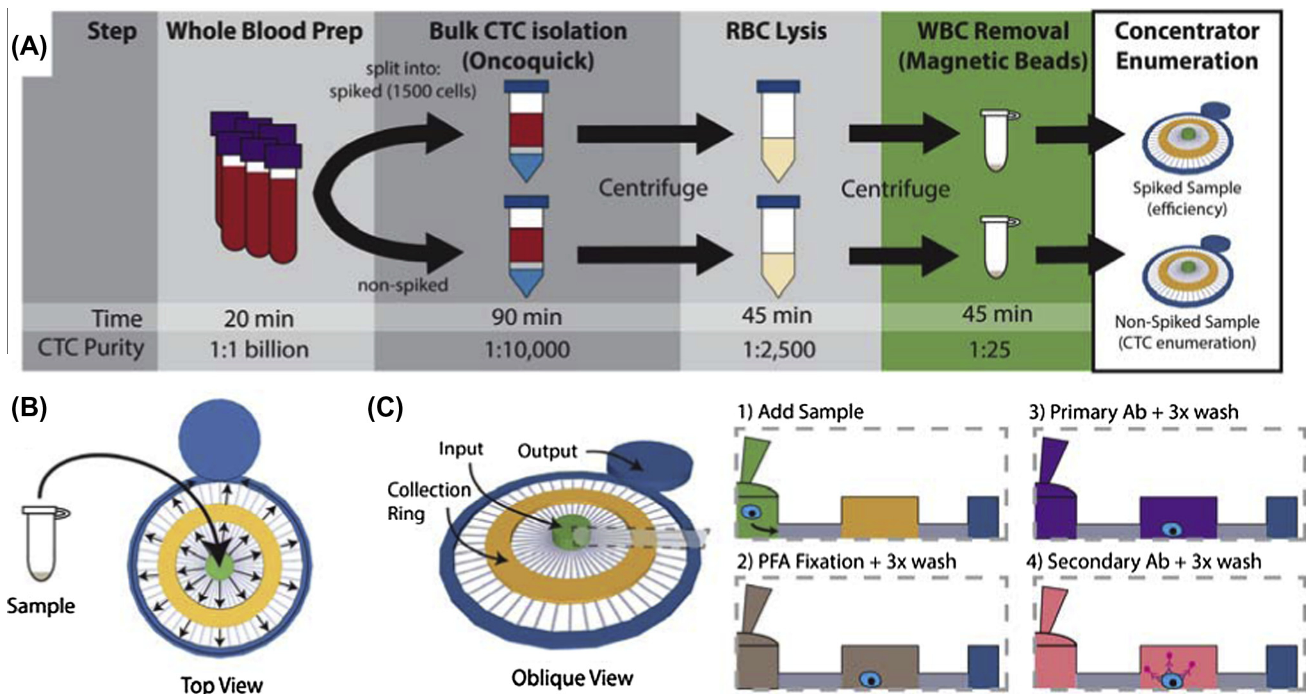

Fig. 1. (A) Summary of blood processing method. Whole blood was split into two parallel processed samples, one containing spiked LNCaPs for efficiency studies and the other remained non-spiked for CTC enumeration. (B) Top view schematic of the MCC showing the isometric flow pattern created when pipetting sample to the center chamber extending radically to the output ring. The passive pumping pressure created between the input and output droplets is evenly distributed through the transport channels, allowing for an increased throughput for sample processing. (C) Device loading and post-processing methodology is shown for the EpCAM primary and secondary staining within the MCC.

2.3. CD45 negative selection for WBC removal

Magnetic beads with bound anti-human CD45 antibody (Invitrogen, San Diego, CA) were added to each tube according to PBMC counts at a density of $1 \times 10^{\wedge 8}$ beads (250 μL of $4 \times 10^{\wedge 8}$ beads/mL stock solution) per $2 \times 10^{\wedge 7}$ cells to remove WBCs from the sample. Tubes were placed on a rocker for 30 min at 4 °C to allow beads to bind PBMCs. Magnetic isolation removed WBCs bound to magnetic beads, and samples were collected and placed into new 1.5 mL centrifuge tubes and centrifuged at 300*g* for 10 min. Supernatant was removed, leaving approximately 100 μL to avoid aspiration of cells at the bottom of the tube. Each suspension was then brought to 200 μL using Buffer 1.

Protocol for CD45 negative selection:

1. Add 250 μL (of a $4 \times 10^{\wedge 8}$ beads/mL solution) per $2 \times 10^{\wedge 7}$ - cells to remove WBCs from the sample.
2. Place on a rocker for 30 min at 4 °C.
3. Place in a magnetic tube rack, remove fluid into a new 1.5 mL centrifuge tube.
4. Centrifuge at 300*g* and room temperature for 10 min.
5. Remove supernatant to approximately 100 μL, resuspend to 200 μL using Buffer 1.

3. CTC isolation and quantification

The Microfluidic Cell Concentrator (MCC) method has been demonstrated previously to have the ability to enrich a cell sample in a manageable viewing window with low cell loss. As such, the method is well suited for use with CTC collection, where the device is an enabling combination of (1) high sample retention (only 1% of cells are lost using the MCC method [30]) and (2) downstream methods for cellular interrogation.

To best leverage these benefits of the MCC for CTC research, we decided to apply a negative selection technique to isolate CTCs, and use the MCC to concentrate the final sample and perform staining techniques. Negative selection methods have the advantage of isolating cells without requiring the use of a specific marker, opening CTC research to the possibility of cellular plasticity (EMT, stemness). However, most macroscale negative selection methods are limited by centrifugation and resuspension techniques; the final step of which creates a ~50 μL solution that contains at best tens of cells. Using the MCC, this final solution can be concentrated by approximately 5× into a compartment that facilitates washing, staining, and imaging steps with minimal risk of loss.

3.1. Microfluidic Cell Concentrator for cellular analysis

Cell suspensions following pre-processing steps were placed into the MCC for cell enrichment and downstream processing. Devices were fabricated by passively bonding a polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) to a tissue culture treated polystyrene omni-tray (Nunc, Rochester, NY) as described previously [30]. Channels created by the PDMS negative form the device in Fig. 1B, which is designed to use passive pumping to create fluid flow within the device by having a larger output droplet than input droplet, creating a pressure drop due to differences in surface tension between the two. In this specific device, an input carried fluid through 50 small transport channels (29 μm tall ×100 μm wide) into a collection region where cells can settle out due to relatively high gravitational settling to flow speeds in this chamber (shown as yellow ring). Non cell-containing fluid then flowed into the outer ring and was aspirated from the output port (shown as blue ring). The collection region is a circular region with a 7.5 mm diameter, a channel width of 1.25 mm, and a channel height of 750 μm. Devices were sterilized by washing with ethanol for 4 h and were filled with ethanol followed by 3 full device volumes of cell culture

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media to prepare devices for CTC collection. Addition of samples to the MCC involved a 15 μL drop being placed on the input every 2 min until the entire sample was processed.

MCC Filling Protocol:

1. Sterilize channels by filling with a solution of 70% ethanol for 4 h.
2. Wash device with 3 full device volumes (120 μL) of cell culture media.
3. Add samples to the MCC by 15 μL drops every 2 min until the entire sample is processed.

Note:

- It is important to allow the device to pump fluid fully between additions.
- When the output drop of the MCC becomes too full, the device operation slows. This should be alleviated by removing 30 μL of fluid from the output drop upon accumulation of 2 × 15 μL fluid additions, utilizing the 2 min wait-time between additions to do so.

3.2. Staining of CTCs within MCC

Cells were processed in the MCC using the ability of the device to wash and stain non-adherent cell types without washing the cells away as previously described [30]. Cells were fixed by flushing the device with 3 × 15 μL additions of 4% PFA in PBS for 12 min. Cells were then washed with 3 × 15 μL additions of PBS. Blocking before staining was achieved by washing the cells with 3 × 15 μL additions of 1% BSA in PBS for 20 min followed by 3 × 15 μL additions of primary antibody for EpCAM (AbCAM ab32392, 1:100 dilution) and a 24 h incubation at 4 °C. Cells were then washed with 3 × 15 μL of 1% BSA in PBS before adding the secondary goat anti-mouse Cy5 antibody (AbCAM ab6563, 1:200 dilution) for 24 h at 4 °C. Devices were washed 6 × 15 μL and imaged using an automated protocol taking 36 images in 3 channels (Phase, GFP, and Cy5) using MetaMorph® (Molecular Devices, Sunnyvale, CA) to visualize the full collection chamber. For analysis, images were loaded into data organizational software being developed by our lab called Je'Xperiment (abbreviated JEX, http://sourceforge.net/projects/jextools/). In JEX, images were stitched to form the full circular device (Fig. 2A), the background was then subtracted, and spiked cells were enumerated by visual inspection. Because anti-CD45 beads autofluoresced in the Cy5 spectrum, manual enumeration was required for EpCAM quantification. The ability to differentiate EpCAM+ cells from beads was validated in a separate experiment where the GFP positive LNCaP cells were spiked into a normal blood sample, processed and counted, revealing a 95.9 ± 6% correlation in the number of GFP+ and EpCAM+ cells ($n = 5$, Fig. 2B).

MCC Staining Protocol

1. Add 3 × 15 μL additions of 4% PFA in PBS.
2. Let sit for 12 min at room temperature.
3. Wash cells with 3 × 15 μL additions of PBS.
4. Block cells using 3 × 15 μL additions of 1% BSA in PBS for 20 min.
5. Add 3 × 15 μL of primary antibody for EpCAM, incubate at 4 °C for 24 h.
6. Wash cells with 3 × 15 μL of 1% BSA in PBS.
7. Add 3 × 15 μL of secondary goat anti-mouse Cy5 antibody for 24 h at 4 °C.
8. Wash cells with 6 × 15 μL of 1% BSA in PBS.
9. Image cells with an automated MetaMorph protocol for 36 images in 3 optical channels (Phase, GFP, and Cy5).

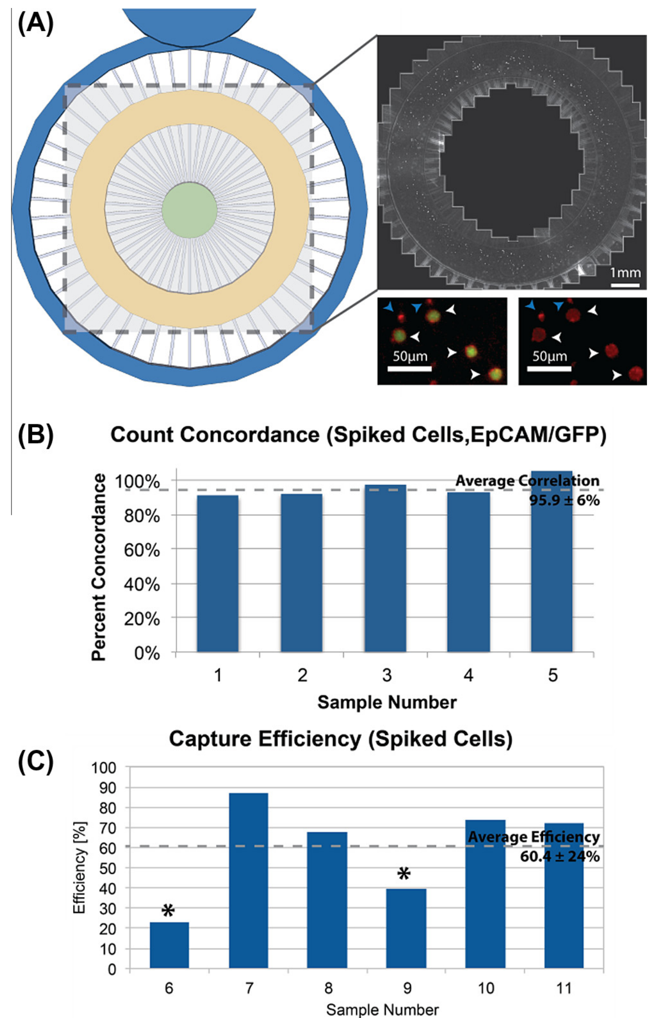

Fig. 2. (A) Top-view schematic of the MCC with the stitched fluorescent image shown expanded (right). Below, an image of spiked GFP-fluorescent LNCaPs (white arrows, green) that were stained for EpCAM (red) shown with and without the internal green marker to demonstrate the clarity of the surface EpCAM stain. Blue arrows show beads auto-fluorescing. (B) Count concordance shown between EpCAM cells over counted GFP cells to demonstrate robust staining and enumeration using spiked LNCaPs into a blood sample. Cells were spiked and counted using the red channel for EpCAM and compared to counts using the green channel for the LNCaP internal fluorescence. (C) LNCaPs were spiked into whole blood samples to evaluate the efficiency of the capture. Average efficiency of all processed samples was 60.4 ± 24.1%, however two of the samples (*) were confounded due to a device inconsistency (see Section 3.4).

10. Stitch channels and images for a full image, manually count EpCAM stained cells.

3.3. ELISPOT methods

As a validation of the negative selection concentrator methodology, samples were benchmarked against the published method of the ELISPOT [31]. The ELISPOT method used here observes individual cell production of prostate-specific antigen (PSA) and enumerates spots created by bound PSA proximal to the producing cells. To accomplish this validation, Millipore NC 96-well ELISPOT plates (#MAIPS4510, Thermo Fisher Science) were prepared by incubating overnight with 50 μL/well of 2.4 μg/mL PSA monoclonal antibody (#10R-P142E, Fitzgerald Industries International, Acton, MA). Plates were subsequently washed three times with PBS and blocked for 15 min with culture media. Fresh buffycoats from whole blood were then obtained via density centrifugation with

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Ficoll-Paque. The buffycoats were then resuspended at two million cells per mL in culture media RPMI 1600 (#MT10040CV, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% Human AB sera (HP1022, Valley Biomedical, Winchester, VA), 2% penicillin/streptomycin (#ICN1670249, Thermo Fisher Scientific), 1% sodium pyruvate (#MT25000CI, Thermo Fisher Scientific), and 0.1% β-mercaptoethanol (Thermo Fisher Scientific). Collected buffy coats were added to wells at 200,000 cells/well in a volume of 100 μL. Plates were incubated for 72 h at 37 °C, 5% CO_2. Following incubation, cells were discarded and plates were washed three times with PBS/0.1% Tween-20 and one time with PBS. Wells were developed with 50 μL/well of 0.5 μg/mL human PSA-HRP conjugated antibody (#61R-P142J, Fitzgerald Industries International) for 90 min. Following incubation, antibody solution was discarded and plates were washed as before. Horseradish-peroxidase TMB substrate solutions (#50-76-00, KPL Incorporated, Gaithersburg, MD) were mixed per manufacturer's instructions and 75 μL/well added to wells. Plates developed for up to 90 min or until visible blue spots appeared. Plates were washed three times with PBS, then three times with cool, running neutral-pH water and allowed to dry overnight protected from direct light.

ELISPOT Preparation Protocol:

1. Incubate 96-well Millipore NC ELISPOT plate with 50 μL per well of PSA monoclonal antibody.
2. Wash plates three times with 200 μL per well PBS.
3. Block plate wells with 200 μL per well culture media. Let stand at room temperature for 15 min.
4. Mix whole blood 1:1 with Buffer 1.
5. Underlay a Ficoll-Paque layer below the blood.
6. Centrifuge blood and Ficoll-Paque mixture at 400*g* for 30 min and no brake at room temperature.
7. Carefully pipette buffycoat layer into a new 15 mL conical tube. Remove 10 μL of fluid for a cell count via hemocytometer.
8. Resuspend buffycoat at a density of 2 million cells/mL in culture media RPMI 1600 supplemented with 10% human AB sera, 2% penicillin/streptomycin, 1% sodium pyruvate, and 0.1% β-mercaptoethanol.
9. Add 100 μL of the resulting cell suspension to each well.
10. Incubate plates for 72 h at 37 °C, 5% CO_2.

ELISPOT Readout Protocol:

1. Following incubation, discard cell solutions.
2. Wash plates three times with 200 μL of PBS and 0.1% Tween-20 and once with 200 μL of PBS.
3. Develop wells with 50 μL/well of 0.5 μg/mL human PSA-HRP conjugated antibody for 90 min.
4. Following incubation, discard antibody solution.
5. Wash plates three times with 200 μL of PBS and 0.1% Tween-20 and once with 200 μL of PBS.
6. Mix horseradish peroxidase TMB substrate solution per manufacturer's instructions. Add 75 μL/well.
7. Develop plates for up to 90 min, or until visible blue spots appear.
8. Wash plates three times with 200 μL PBS.
9. Wash plates with cool, running neutral-pH water.
10. Dry overnight protected from direct light.
11. Enumerate spots.

3.4. Efficiency analysis

To demonstrate retention of target cells in the MCC throughout the isolation and analysis, spiked samples were run in parallel to CTC enumerations. Enumeration of GFP-positive spiked cells

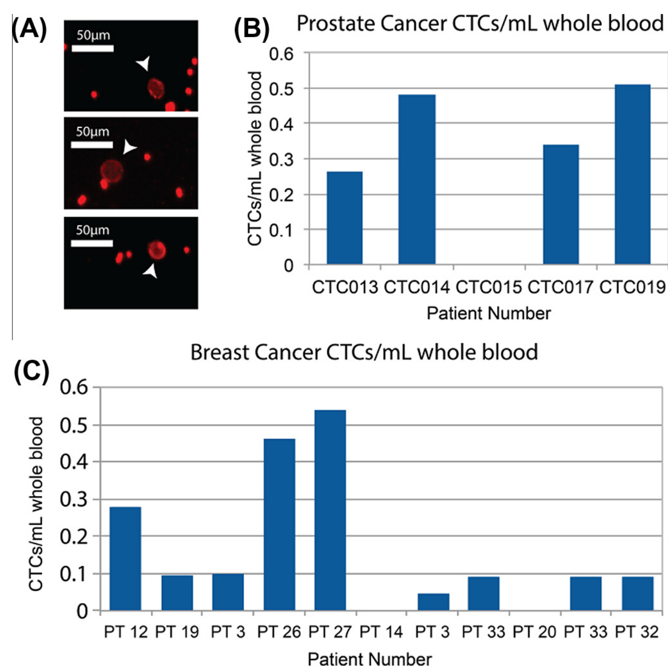

Fig. 3. (A) Individual spiked CTCs imaged within the MCC shown (arrows). (B) Prostate Cancer CTCs per mL of whole blood calculated based on enumerated cells within the concentrator. CTCs were detected and enumerated in each patient except for CTC015. (C) Breast Cancer CTCs per mL of whole blood calculated based on enumerated cells within concentrator. No CTCs were detected in either patients 14 or 20.

(LNCaPs) demonstrated a capture efficiency of 60.4 ± 24.1% (Fig. 2B) which also represented an 8 order of magnitude depletion of background cells. There were two samples that showed efficiencies significantly below this average, each representing missteps in sample processing due to operational inconsistencies. Specifically, the MCC was fabricated from PDMS and passively bonded to a plastic tray. The source of inconsistency for patient number 17 was caused by poor bonding of the device to the surface, allowing fluid and cells to flow freely under the device resulting in an increased spiked cell loss. The inconsistency found with patient number 13 was due to omission of a resuspension step. Further, measured numbers of EpCAM + LNCaP cells were compared to the number of GFP positive cells, and were found to result in counts that were 95.9 ± 6% concurrent (Fig. 2B). Importantly, the high concurrence of the counts demonstrates that enumeration resulted in counts that were accurate to epithelial cells within the sample and were not confounded by blood cells or beads that were not removed from the sample processing (red auto-fluorescence, Fig. 2B). These two pieces of data show that the process was gentle enough and rigorous enough to remove background cells without lysing cells of interest while sufficiently enriching the population for detection and enumeration.

3.5. Patient samples

Peripheral blood from patients with advanced prostate or breast cancer was collected after informed consent was obtained under a University of Wisconsin IRB approved protocol (XP08813). A maximum of 40 mL of blood was obtained at any given blood draw using EDTA vacutainers.

3.6. Cell culture

The lymph node carcinoma of the prostate (LNCaP) cell line was used to study the efficiency of this device by spiking this cell line

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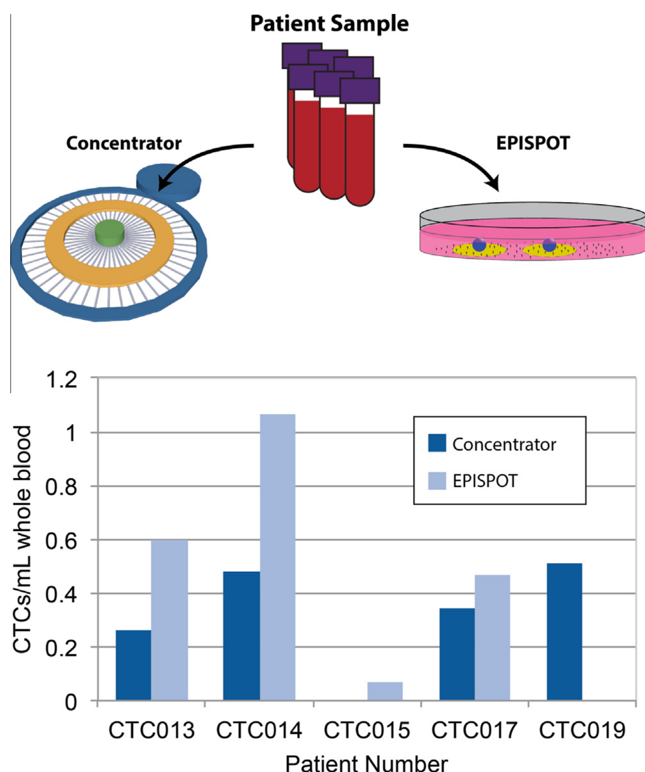

Fig. 4. Validation of Concentrator CTC enumeration via comparison with ELISPOT methodology. Blood was split upon collection and processed in parallel. Data representing CTC enumeration shown (below).

into whole blood prior to sample processing. The specific cell line used was stably transfected with green fluorescent protein (GFP) [32]. The cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 10 mM HEPES buffer, 1 mM sodium pyruvate, and 25 mM glucose. 1500 cells were used to evaluate device efficiency as it pertained to cell loss in each patient sample shown.

4. Conclusions

Benefits of the MCC are illustrated with this methodology when used as a final step to concentrate and isolate the CTC sample. The MCC provides a gentle and efficient method of concentrating the precious sample into a device suitable for downstream treatments and imaging, providing a beneficial alternative to basic centrifugation. Because cell numbers are so low, it is difficult to use centrifugation to concentrate and preserve the sample in a robust manner. The number of cells within the samples is insufficient to form proper pellets, causing visual detection of the pellets to be impossible during aspiration. As such, to prevent loss during aspiration steps, a higher volume is necessarily left above the pellet. The MCC in this way can concentrate the sample beyond that which can be achieved with subsequent centrifugation steps and perform staining procedures all while maintaining the cells within a manageable area for imaging and analysis.

To demonstrate the ability to use the MCC for patient sample processing and CTC enumeration, the method was applied to five patient samples. Surface staining showed high cell surface integrity (Fig. 3A). An average of 0.31 CTCs/mL (or on average 2 CTCs per 6 μL of blood collected) were detected among all five patient samples (Fig. 3B). The technique was further validated by parallel assessment of blood samples using the ELISPOT secretion-based detection technique ([31]), and showed similar levels of detection of CTCs using both methods (Fig. 4). The MCC method of CTC collection and enumeration was performed with a breast cancer clinical trial demonstrating the applicability of this method to other epithelial-based carcinomas (Fig. 3C). The MCC was successfully used to enumerate CTCs from patient samples and validated using ELISPOT enumeration, with resulting levels of CTC detection similar between the two. CTC enumeration was low relative to other collection techniques, possibly due to patient variability or low efficiency of the method for patient samples compared to contrived samples from cell lines. Importantly, the MCC method leaves these cells directly within an environment that could be used to culture or otherwise perform functional analyses on these non-fixed, unlabeled cells, expanding the information from these cells beyond enumeration. Because the MCC is designed to allow collection and analysis of non-adherent cell populations, multiple staining steps can be applied in series to a given CTC population without losing any of the population. As such, CTC analysis with this technique can extend into probing cellular surface heterogeneity, intracellular protein analysis, or nuclear staining for various markers, including those for proliferating cells (Ki-67), stemness markers (CD133), or for comparisons with the primary or metastatic cellular populations of a given patient. The negative selection process applies a new concentration and treatment method to minimize perturbation of the CTCs during isolation and analysis and works towards the goal of enabling capture of live CTCs for studies of CTC function and behavior.

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