ABSTRACT
The capture and subsequent treatment of Circulating Tumor Cells (CTCs) can provide cellular-level information for the study and treatment of metastatic cancer in a minimally invasive fashion. In the following manuscript, a microfluidic concentrator device is described and used for collecting CTCs isolated from whole-blood in order to enable downstream treatment and analysis of live CTCs. The device performance and operation of the device is evaluated and used in the development of protocols for CTC isolation, culture, and other functional assays.

KEYWORDS: Circulating Tumor Cells, CTCs, Isolation, Concentration, Microfluidics

INTRODUCTION
This paper reports a new device for the collection and subsequent treatment of circulating tumor cells (CTCs) obtained from the whole blood of breast and prostate cancer patients (Figure 1a). CTCs are of great interest in epithelial based cancers as they may provide a relatively non-invasive means of monitoring disease progression and response to treatment. Further, the ability to culture these cells would open the door to a host of assays that could give much needed insight into the molecular mechanisms of cancer metastasis. Despite the potential impact to the study of cancer and metastasis, the authors are only aware of one reported example of successful CTC culture [1]. The example from the literature used a collagen matrix to selectively isolate CTCs with an invasive phenotype. This selection process works to bias subsequent studies of CTC function an behavior and may prove to be detrimental to a broader understanding of CTCs and their role in metastasis. The method proposed here attempts to avoid removing of any CTC subpopulations and collects the cells into a microfluidic channel that provides the functionality necessary to subsequently treat and culture the cells. Beyond the specific application of CTCs, the proposed device facilitates study of other cell types that also exist in small quantities – approximately less than 50,000 per sample (e.g., subpopulations of cells isolated through procedures such as a FACS).

In this study, device operation and performance are evaluated. The device is then used in preliminary work aimed at establishing a protocol for the isolation and culture of CTCs. The device and protocol are aimed at minimizing perturbation of the cells to best promote downstream study of cell behavior. For this reason, the device and isolation protocol avoid the use of previously reported methods for concentrating or isolating specific cell types such as positive selection using functionalized surfaces, size exclusion/filtration, and electric charge as these methods can significantly bias downstream readouts of cell behavior.

THEORY
The microfluidic device is designed to take the purified CTC sample and increase the number of cells per unit volume of culture media while simultaneously collecting the cells into a microchannel device that can be used for subsequent culture and treatment. This concentration process is needed due to the limitations of centrifugation/resuspension alone for sample preparation. When dealing with rare cells such as these, small volumes are needed to achieve appropriate cell densities for assays such as those that involve culture. Without concentration, the number of cells, the cell surface density, or cell volume ratio of the culture can be inadequate to support cell signaling and growth. Further, resuspension volumes after centrifugation can often be much less than 50 μL, making it difficult to avoid issues of cell loss, pipetting error, and bubbles. The device can be used to concentrate a cell suspension approximately 50 times beyond that which can be achieved via centrifugation alone. This effectively makes the microenvironment of cells 50 times more sensitive to cell induced changes in soluble factor concentrations, thereby facilitating cell-cell signaling and dramatically increasing assay sensitivity. After seeding, the device also allows for subsequent culture and treatment of the cells using slow flow via passive-pumping, a method that requires only a single pipette to implement.

The device collects cells by leveraging the process of cell settling and non-specific interactions with the substrate of the collection region (Fig 1). Cells flow radially outward through the device via transport channels to enter the collection region. Within the collection region, flow is slow enough to allow the cells to settle to the substrate where non-specific interactions are able to keep the cells from leaving. The fluid in which the cells were suspended continues to flow to the output port leaving an increased concentration of cells in the collection region.

During concentration, cells can be lost to the output port and is a particularly important consideration when working with rare cells and is the primary readout of device performance used here. The two main parameters of device operation that influence cell loss are the volume of cell suspension used per addition and the ratio of the settling time to the time it takes for a cell to pass through the collection region (i.e., the residence time). The ratio is referred to here as \( t_{set}/t_{res} \).

EXPERIMENTAL
Device characterization was performed using LNCaP (lymph node carcinoma of the prostate) cell-line transfected with GFP. Cell suspensions were prepared to a density of 50,000 cells/mL. Cell loss was measured for the process of seeding and staining using a variety of pumping parameters and two different device designs. Fluid flow modeling was also performed to illustrate the effect of changing values of \( t_{set}/t_{res} \) as well as how the volume of each fluid addition can affect cell-loss.
OncoQuick literature suggests that the separation protocol captures > 70% of spiked tumor cells; it is likely that the addition of tumor cells from the whole blood of cancer patients. Given that we have observed spiked cell loss of < 50% and that traditional methods also linearly with respect to how much fluid is added and allows users to tune the density near a value of 1 for how many extraneous cells still exist after isolation.

DISCUSSION

Similar EpCAM staining intensities were observed in the case of the spiked LNCaP cells (data not shown). Fig 3b shows a cell obtained from the whole blood of a metastatic breast cancer patient using the isolation protocol outlined here that stain positively for EpCAM (epithelial cell adhesion molecule). When whole blood samples are spiked with tumor cells, they are spiked at a density of 100 cells/mL, which is similar to the density of CTCs observed in metastatic patients.

RESULTS

Fig 1 shows the device design (a) and a conceptual representation of the cell settling and concentration process (b). Cell surface density was measured after each of 10 fluid additions of 10 µL each using Design I and II. Cell surface density increased linearly (R² = 1.00) in each case. Cell loss in these designs using various passive pumping strategies is summarized in Fig 2c and 2d over simulation data (solid lines). The value of t_{res} is estimated from the flow rate measurements of passive pumping within each device. A model of passive pumping is matched to the data in order to obtain a flow rate vs. droplet-volume curve. The curve is then integrated to determine the volume-averaged flow rate, v, for each condition. The residence time is then calculated as w/v where w is the width of the collection region. The calculated values of t_{set}/t_{res} are then used to plot cell-loss data from many different experimental conditions.

Simulations are performed using COMSOL Multiphysics 3.4 (Burlington, Massachusetts, USA). Cell settling velocities are superposed with flow velocities through the device to estimate cell trajectories. Cells are considered lost if the cell does not reach the substrate before leaving the collection region or meets the collection region substrate in an area where the shear stress is > 0.1 dynes/cm². CTCs are isolated from whole blood using a sequence of centrifugation (OncoQuick, Greiner Bio-One GmbH, Frickhausen, Germany), red blood cell lysis (Boston BioProducts, Ashland, Massachusetts, USA), and negative selection using magnetic beads labeled with antibodies to CD45 (Invitrogen, Carlsbad, California, USA). Prior to processing, the sample is split into two aliquots, one of which is spiked with LNCaP cells transfected with GFP to evaluate the efficiency of the process (i.e., what percentage of the original number of LNCaP cells is recovered in the device after isolation). When whole blood samples are spiked with tumor cells, they are spiked at a density of 100 cells/mL, which is similar to the density of CTCs observed in metastatic patients.

Fig 3a and 3b show the results of CTC isolation and concentration. Fig 3a is an image of spiked LNCaP cells (green) that were recovered from whole blood. The GFP fluorescence is overlayed with the phase-contrast image to illustrate how many extraneous cells still exist after isolation. Preliminary calculations suggest that > 50% of the spiked LNCaPs are recovered in the concentration device. Fig 3b shows a cell obtained from the whole blood of a metastatic breast cancer patient using the isolation protocol outlined here that stain positively for EpCAM (epithelial cell adhesion molecule). Similar EpCAM staining intensities were observed in the case of the spiked LNCaP cells (data not shown).

DISCUSSION

Simulations suggest and data demonstrate that when using this method of cell concentration, it is desirable to operate near a value of 1 for t_{set}/t_{res}. Operating above 1 increases loss while operating below 1 needlessly reduces throughput. The device avoids the use of a functionalized surface, physical traps, and electric charge. Further, the flow velocities used here are on the order of cell settling velocities providing a gentle alternative to other macro- or micro-scale methods. The process of collection is also linear with respect to how much fluid is added and allows users to tune the density of the cells manually if needed.

Positive EpCAM staining in Fig 3b demonstrates that the isolation protocol and device can be used to find epithelial tumor cells from the whole blood of cancer patients. Given that we have observed spiked cell loss of < 50% and that OncoQuick literature suggests that the separation protocol captures > 70% of spiked tumor cells; it is likely that the addi-
tional steps of lysis, bead separation, and concentration are reasonably efficient. The bright field image in Fig 3c also suggests that the samples have relatively few extraneous cells compared to the spiked LNCaPs. The cost of the isolation protocol is roughly $60-$70 for 30 mL of whole blood.

Figure 2: The percent of cells lost to the outer ring using either 6 (a) or 15 (b) µL drops and Design I (blue) or II (red). All results are normalized to $t_{set}/t_{res}$. Blue and red lines indicate 3D flow simulation results of the device. Points indicate experimental measurements of cell loss (± SE). Cell settling velocity is calculated to be 2.7 µm/s (Stokes drag analysis). A single asterisk (*) indicates that there was no wait time after fluid flow stopped and the next fluid addition instead of the standard 2 min. A double asterisk (**) indicates that the wetted radius was determined using a 6 µL drop to produce significantly faster flow rates than for the standard wetted area created by a 15 µL drop.

CONCLUSION

The operation of a microfluidic device for concentrating dilute suspensions of cells into a microchannel suited for subsequent culture and treatment has been evaluated. The device can produce up to a 50 times increase in cell:volume ratio with less than 5% loss. The CTC isolation protocol presented here produces a relatively clean sample with a reasonable loss of spiked tumor cells for around $60-$70 per 30 mL of whole blood. The gentle processing and concentration without the use of specific adhesion ligands provides a basis for future functional studies of minimally biased circulating tumor cells.

ACKNOWLEDGEMENTS

The authors would like to thank Erwin Berthier and Dr. Scott Berry for their help and suggestions with modeling and dimensional analysis of the device. This work was supported in part by the Wallace H. Coulter Translational Research Partnership, the Department of Defense Prostate Cancer Research Program (DOD PCRP Idea Award, W81XWH-09-1-0192), and the University of Wisconsin Carbone Cancer Center.

REFERENCES


CONTACT

*D.J. Beebe, tel: +1-262-2260; dbbeebe@wisc.edu