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Short communication

Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients

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ABSTRACT

The isolation of circulating tumor cells (CTCs) using microfluidics is attractive as the flow conditions can be accurately manipulated to achieve an efficient separation. CTCs are rare events within the peripheral blood of metastatic cancer patients which makes them hard to detect. The presence of CTCs is likely to indicate the severity of the disease and increasing evidences show its use for prognostic and treatment monitoring purposes. We demonstrated an effective separation using a microfluidic device to utilize the unique differences in size and deformability of cancer cells to blood cells. Using physical structures placed in the path of blood specimens in a microchannel, CTCs which are generally larger and stiffer are retained while most blood constituents are removed. The placements of the structures are optimized by computational analysis to enhance the isolation efficiency. With blood specimens from metastatic lung cancer patients, we confirmed the successful detection of CTCs. The operations for processing blood are straightforward and permit multiplexing of the microdevices to concurrently work with different samples. The microfluidic device is optically transparent which makes it simple to be integrated to existing laboratory microscopes and immunofluorescence staining can be done in situ to distinguish cancer cells from hematopoietic cells. This also minimizes the use of expensive staining reagents, given the small size of the microdevice. Identification of CTCs will aid in the detection of malignancy and disease stage as well as understanding the phenotypic and genotypic expressions of cancer cells.

1. Introduction

Cancer is a leading cause of death, and in most cases, cancer deaths are the result of metastasis (Steeg, 2006) with malignant cancer cells spreading to distant sites. The lack of early warning at initial stages of the disease limits the effectiveness for cancer treatment (Chambers et al., 2002). Circulating tumor cells (CTCs) are disseminated from solid tumors that enter the blood circulation during hematogenous metastasis (Poste and Fidler, 1980) and recent clinical studies show that the quantity of CTCs in circulation is a good measure for prognosis and overall survival (de Bono et al., 2008; Pantel and Riethdorf, 2009; Slade and Coombes, 2007). It is also reported to have association to disease progression (Cohen et al., 2009; Cristofanilli et al., 2004) and treatment efficacy (Reuben et al., 2008; Urtishak et al., 2008). Hence, the blood specimens of cancer patients are a potential source of tumor cells. Furthermore, blood extraction is routinely performed for various health tests and also less invasive compared to surgical biopsies. The availability of blood samples is attractive for CTC enumeration to complement current techniques in the detection and monitoring of cancer.

The technical challenge to detect CTCs in peripheral blood lies in the rarity of these cells (Zieglschmid et al., 2005). The cell count can be as low as 1 cancer cell to 1 ml of blood which contains approximately 4.8–5.4 billion erythrocytes; 7.4 million leukocytes and 280 million thrombocytes (Fournier, 1998). Leading technologies in CTC enrichment from blood specimen uses affinity based techniques which employ antibodies that are expressed only on cancer cells (Cohen et al., 2008; Riethdorf et al., 2007). These methods face various drawbacks such as the need for pre-sample preparation and more importantly the specificity of the antibody selected for enrichment. Additional preparatory steps are likely to incur CTC losses while the lack of a universal biomarker for CTC enrichment limits

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the detection of CTCs for different cancer types (Allard et al., 2004; Sieuwerts et al., 2009). The technique is also laborious, complicated and potential important information about the subpopulations of these cells may also be lost. Furthermore, the isolated cells may no longer be viable after processing of the blood samples which limits the downstream applications that can be done on CTC subpopulations. These tumor cells hold important information to the metastatic process and will aid in understanding the disease better (Pantel et al., 2008; Slade and Coombes, 2007).

Using a microfluidic device, we aim to achieve an effective CTCs separation from peripheral blood using distinctive physical differences between cancer cells (Weiss, 1990; Weiss and Dimitrov, 1986) and blood constituents (Mohamed et al., 2004; Shelby et al., 2003). We had previously reported on the use of the microdevice in the isolation of breast and colorectal cancer cell lines and optimized the design with computational fluid dynamic simulations (Tan et al., 2009). In the current study, we extended the investigation to cover a wider range of cancer types to determine the versatility of the technique to handle different cancers as the disease is genetically heterogeneous (Braun et al., 1999; Reya et al., 2001; Shah et al., 2004). In addition, we demonstrated the sensitivity of the system with the successful recovery of low counts of cancer cells in numerous tested samples. From the peripheral blood of metastatic lung cancer patients, the platform is tested to detect CTCs and characterized for the isolation purity. Sample processing is user friendly, requires no pre-preparation of the sample and enrichment of cells can be achieved with a single processing step. The microfluidic device is also label free and will allow for the retrieval of viable CTCs after blood processing. All together, the system is attractive for applications in oncology research.

2. Methods and materials

2.1. Sample blood collection

Informed consent from healthy volunteers and cancer patients were taken before blood extraction. Blood samples from healthy volunteers served as controls and were also used in spiking experiments. Samples were stored in EDTA tubes (BD, Franklin Lakes, NJ, USA) prior to use and discarded after the experiment. Institutional Review Board approval and informed consent was obtained from patients with lung cancer accrued onto this study. 10 ml of blood was extracted each time into sodium EDTA tubes after discarding the first 0.5–1 ml of blood drawn.

2.2. Microdevice fabrication

The fabrication of the microdevice was done via soft lithography (McDonald et al., 2000) and the procedures are briefly described below. The design of the microdevice was first printed on a photo mask (Infinite Graphics Inc., Minneapolis, MN, USA), drawn using the software Cadence (Cadence Design Systems, Inc., San Jose, CA, USA). SU8-2025 (Microchem Corporation, Newton, MA, USA) was spin coated at 3200 rpm for 45 s on an 8 in. silicon substrate to achieve the thickness of 18–20 μm. Then it underwent an ultraviolet exposure of 120 mJ/cm^2 through the photo mask and followed by the photoresist development. A final hard bake was performed to ensure better adhesion of the photoresist to the substrate to form the final master mold. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) mixed according to manufacturer's recommendation was degassed and poured over the master mold. The mixture was then subjected to the curing conditions of 80 °C for 2 h in an oven. Fluidic ports were created using punches on the patterned PDMS after the removal from the master mold. The PDMS block together with a glass slide were then subjected to oxygen plasma treatment and bonded irreversibly. Tubings were finally inserted directly into the fluidic ports of the finished device to allow samples to be introduced.

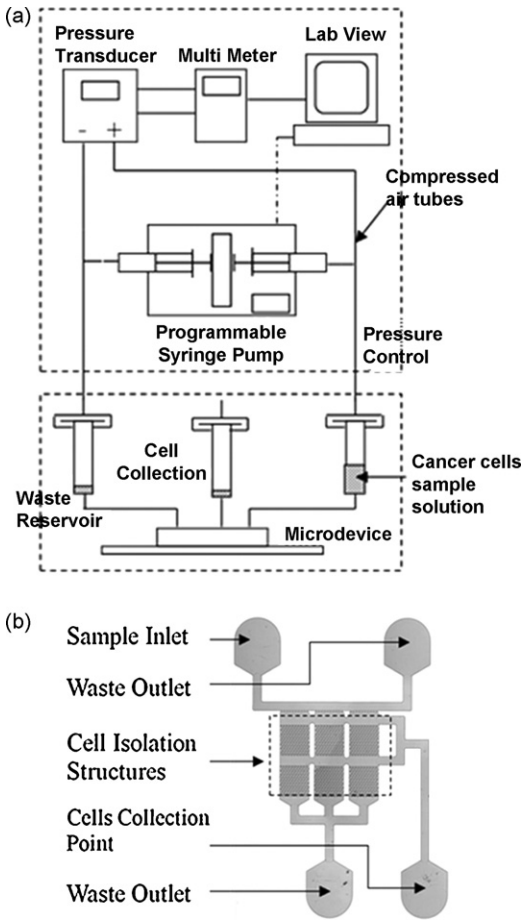

Fig. 1. Microdevice setup and design layout. (a) Schematic of semi-automated setup which is controlled by the computer to adjust the pressure differential to drive blood samples through the microdevice. (b) Overall microfluidic chip layout.

2.3. Experimental setup and apparatus preparation

The experimental setup and the design of the microdevice are shown in Fig. 1. The platform was upgraded from the previously reported (Tan et al., 2009) using manual pressure regulators. The current system used compressed air from two large syringes to produces pressured lines as shown in Fig. 1a. The differential pressure was measured by a pressure transducer which was feedback to the computer via a voltmeter to precisely control the pressure settings into the microdevice. A program written in NI Labview (NI, Austin, TX, USA) controlled the syringe pump (Harvard Apparatus, Holliston, MA, USA) and made minute adjustments every 100 ms in response to pressure drop. This allowed semi-automation in the blood processing and also made the entire system easily portable without requiring external pressure sources.

For apparatus preparation prior to samples processing, the microfluidic system was flushed with 5 mM EDTA (Sigma, St. Louis, MO, USA) buffer through the sample inlet as shown in Fig. 1b for 10 min at 120 μl/min. No other preparatory steps were required for the sample. The device was mounted on an inverted microscope (Leica Microsystems, Singapore) and cell enumeration was done manually after cell isolation. Image capturing of the cell isolation process was taken using a high speed camera (Photron, San Diego, CA, USA).

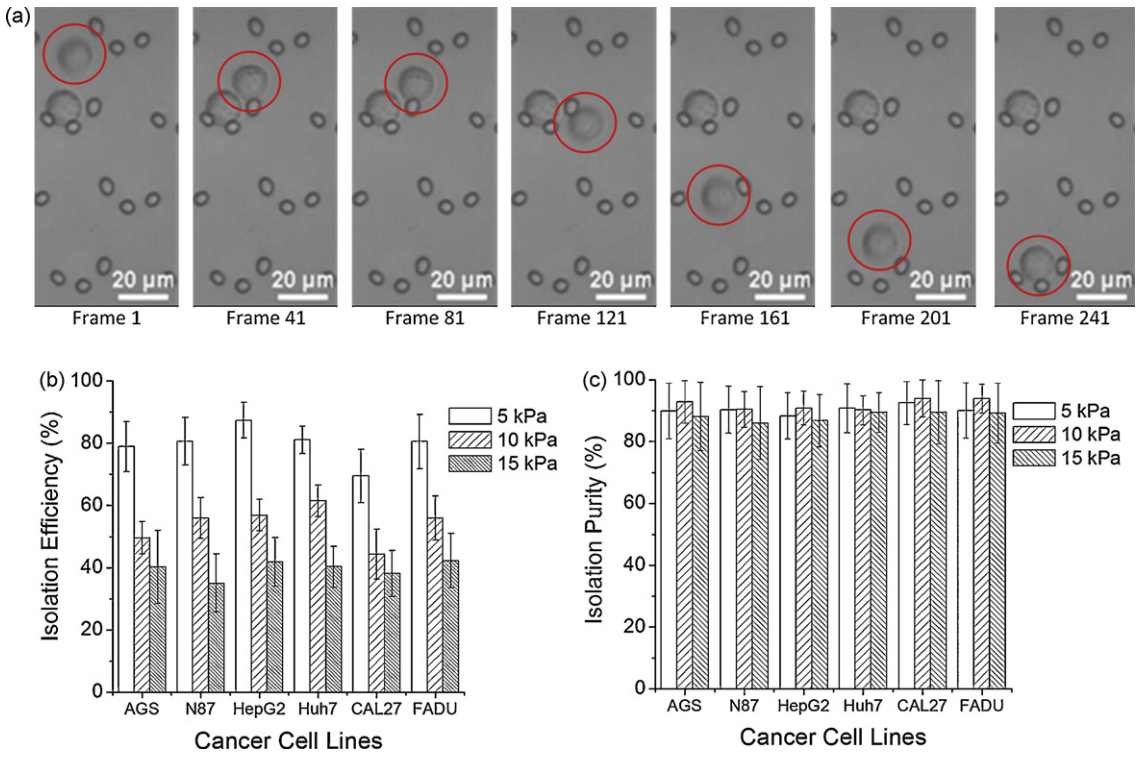

Fig. 2. Microdevice characteristics and real time imaging of the isolation process. (a) Time sequence images showing the capture of a cancer cell. The arrangement of the cell traps enables the capture of cells that circumvent occupied traps and prevents clogging in the microdevice. Images taken with a high speed camera at 1000 fps. (b) Tumor cell isolation efficiency of various cancer cell lines over a range of operating pressures. (c) Tumor cell isolation purity that shows low amount of noise after enrichment.

2.4. Cell culture and immunofluorescence staining of CTCs

The cell lines of human breast adenocarcinoma (MCF-7 and MDA-MB-231), gastric carcinomas (AGS and N87), heptocellular (HepG2 and HuH7) adenocarcinomas, tongue squamous carcinoma (CAL27) and pharynx squamous carcinoma (FADU) were used to characterize the efficacy of the microfluidic chip. AGS and N87 were cultured using RPMI 1640 (Sigma, St. Louis, MO, USA) while the rest used Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA). Both culture media were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin G/streptomycin/amphotericin (Gibco, Carlsbad, CA, USA).

For testing the cancer cell isolation purity in the microdevice, the isolated cells were stained for EpCAM (1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to identify cancer cells, CD45 (1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for leukocytes and 4′,6-diamidino-2-phenylindole (DAPI) to permit nuclei visualization. The protocol for performing immunofluorescence staining in the microdevice was as previously reported (Tan et al., 2009). For identification of CTCs in metastatic lung cancer patients, anti-cytokeratin antibodies (CAM5.2, BD Bioscience, San Jose, CA, USA) was used. A positive CTC count was defined to be cytokeratin positive, CD45 negative and DAPI positive.

2.5. Experimental tests with low cancer cell count

We attempted to ascertain the detection limit for the setup with low cell count in sample solutions. 1–3 cell(s) were suspended in 1 ml of 1× PBS and passed through the device. Isolation of individual cells from suspended state after trypsinization was done by either manual pipetting or using the fluorescence activated cell sorter (FACS, BD FACSAria II Cell Sorter, Franklin Lakes, NJ, USA) to automatically sort the cells.

3. Results and discussions

The microdevice utilizes the biorheological differences in the cell size and deformability of cancer cells to achieve an effective separation. For operations, the sample was pressure driven through the system from the sample inlet past the cell traps and the effluence was collected in the waste outlet (Fig. 1b). The pressure control allowed instantaneous manipulation of the fluid conditions in the microdevice. The layout of the cell traps is shown in Fig. 2a where cancer cells being generally larger and stiffer than blood cells will be retained. The selectivity of the system to isolate CTCs is high as blood cells are able to deform through the structures which greatly increases the isolation purity. Furthermore, the microdevice will retain all cells that are physically different from blood cells which meant a larger CTC yield than affinity based separation which enriches the samples for a particular subgroup of cells based on the biomarker used. The design of the traps also prevents build up of cells when the structures are occupied, unlike most direct filtration methods and allows processing of large sample volumes. Due to the rarity of CTCs in peripheral blood, processing of larger volumes of samples is required and the laminar flow around the cell traps channel cells away to prevent clogging due to lower fluid resistance on the adjacent sides. In addition, each trap is likely to hold only a single cell and this simplifies enumeration either optically or through an image processing software.

3.1. Microfluidic chip efficiency with cancer of different origins

The efficiency of the microfluidic chip is determined by the ratio of isolated cancer cells to the total number of cells flowing into the device. In the current study, 6 dissimilar cell lines from 4 different cancer origins were used to ascertain the microdevice characteristics. A diverse sample group of cell lines were used to ascertain the versatility of the technique towards the detection of CTCs. Cancer

cells were spiked into 1× PBS at the concentration of 100 cells per millilitre to mimic the rarity of CTCs in peripheral blood of cancer patients. Fig. 2b summarizes the efficiency of the microfluidic device in processing the samples. A total of 180 experimental runs were performed for the entire investigation, showing an average effective isolation rate of 80% for the 6 cell lines, at an operating condition of 5 kPa. The optimum driving pressure differential of 5 kPa was also coherent with earlier analyses for breast and colorectal cancer cells (Tan et al., 2009). The Student's *t*-test with a significance level of 0.01 confirmed that isolation efficiencies at 5 kPa were significantly higher than other operating pressures for all the samples.

With cancer cells spiked into blood samples from healthy volunteers, the quantitative analyses for the isolation purity can be determined. Spiking concentrations of 100 cancer cells per millilitre of blood which was further diluted in 5 mM EDTA buffer (1:2) were used in all the tests. Samples were diluted with EDTA buffer to prevent blood clotting. Standard staining protocols were followed in the identification of cancer cells from the isolated cell population in the microdevice. Fig. 2c shows the capture purity of the different cell lines over a range of operating conditions. A mean isolation purity of 89% was obtained at the operating pressure of 5 kPa and showed insignificant variations at larger operating conditions. A Student's *t*-test performed on each individual cell line showed no significant differences in the mean isolation purity over the range of operating pressures at $p < 0.01$.

Hence, CTC enrichment via size and deformability is versatile and effective for various cancer types, in contrast to affinity based separation which can be selective due to the specificity of the biomarkers used (Sieuwerts et al., 2009). The disease though complex in their molecular aspects presented lesser dissimilarities in the physical characteristics. The diverse range of cancer cell lines in our tests which yielded similar isolation efficiencies demonstrates that physical separation is a versatile technique to enrich CTCs from different cancer types with high isolation efficiency. Viable cells are also obtained with this separation technique and can be easily recovered by applying a backflow which had previously been demonstrated (Tan et al., 2009).

3.2. Microdevice detection limit

The rarity of CTCs in the presence of a multitude of blood cells is the main technical challenge to identify them. Therefore, the detection limit of the system is of interest as it represents the sensitivity of the microfluidic chip in CTC detection. Very low counts of cancer cells were added to 1× PBS, either with manual pipetting or FACS, and made to pass through the microdevice. We define a positive ratio for the number of trials for which at least one of the total number of cells spiked into PBS is retrieved, over the total cases. A total of 90 experimental runs were performed for all the cell lines.

From the results obtained with low cell count in spiked samples, the average positive rate is approximately 0.81 from all the eight different cancer cell lines that were tested (Supplementary Table 1). This is coherent with the microdevice efficiency characterization in Section 3.1. However it was observed that within cancer cells of the same origin, there exist distinct isolation rate variations. For instance, MCF-7 was found to have a higher positive detection rate at low cell numbers in the sample solution than MDA-MB-231 which was of the same cancer type. This can be attributed to a number of factors such as the heterogeneity of different cancer cell lines, random errors in FACS separation when preparing the spiked sample or a result of the setup with cancer cells adhering to tubes before entering the microdevice. Nonetheless, an overall effective rate of 81% is promising and significant enough to accurately detect CTCs from peripheral blood of cancer patients.

3.3. Detection of CTCs from patients with metastatic lung cancer

Having optimized the system with controlled parameters, the platform was tested on actual clinical blood specimens from patients with metastatic lung cancer. A total of 5 blood samples were taken from 5 patients. A successful negative control using blood samples from healthy volunteers (sample size 10) were performed as well (data not shown), showing no cells were retained in the cell traps.

In order to determine the sensitivity of the system and the variability for the tests with clinical samples from metastatic lung cancer patients, the amount of blood processed were varied to determine the linearity and reproducibility of the device. The aim is also to minimize the volume of blood required in each test to reduce the patients' burden during blood extraction. Fig. 3a illustrates the enumeration of CTCs from all 5 patients, with experimental splits of 1, 2 and 3 ml of whole blood simultaneously processed. Fig. 3b and c shows the isolated cells from one of the clinical blood samples. Fig. 3b shows an isolated cell directly after blood processing and Fig. 3c illustrates the isolated cell after immunofluorescence staining on chip to rule out false positives. During processing of the blood specimen, real time isolation of cells could be observed as the sys-

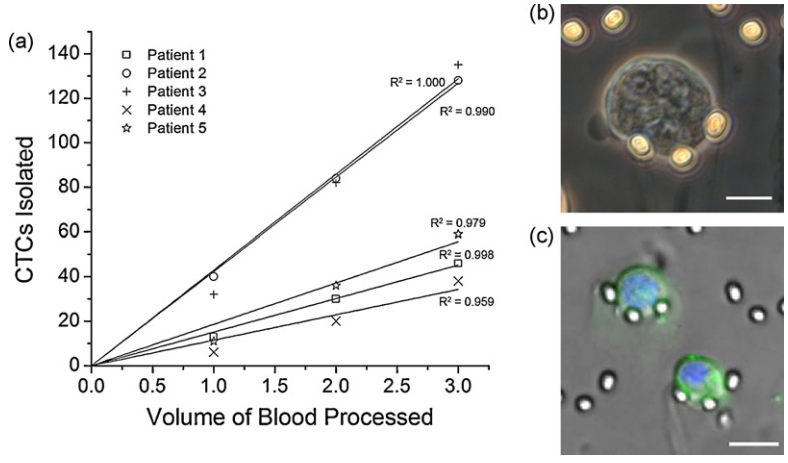

Fig. 3. CTC isolation from peripheral blood samples from metastatic lung cancer patients. (a) Comparison of CTC enumeration with different volume of blood from the same specimen. (b) Isolated CTC directly after blood processing. (c) Immunofluorescence staining that demonstrates the isolated cells being Pan-CK positive (green), CD45 negative (red) and DAPI positive (blue). Scale bar represents 10 μm. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

tem was optically transparent and compatible to be mounted on the microscope. Whole blood was also used directly without the need for any pre-preparation or dilution of the blood specimen. This simplifies sample processing procedures and is likely to increase the cell yield as losses are minimized due to the direct processing of blood. With 2 ml of blood from each samples, we achieved a 100% detection rate of CTCs from all the samples with an average of 50 cells captured (Supplementary Table 2). From Fig. 3a, we derived the goodness of fit with a linear model for each of the patient samples. The adjusted R^2 values in this group covered the range of 0.959–1.000 which is indicative of the microdevice reproducibility to detect CTCs. The results also suggest that a low amount of blood is required for a sensitive detection as compared to other leading technologies which need about 7.5–50 ml of blood. Table 2 in the supplementary data further summarized the isolation purity of the cell capture for each sample and achieving an average rate of 83% which showed minimal noise from leukocytes in the enriched specimen. This high signal to noise ratio is beneficial for downstream processing especially for molecular detection to avoid false positives. With an initial tumor cell concentration of approximately 1 cancer cell to 1 billion blood cells in whole blood, an enrichment factor of 4.9×10^9 was attained. It is also evident that physical dissimilarities between CTCs and the blood constituents are significant in peripheral blood of cancer patients for an efficient tumor cell enrichment and separation.

4. Conclusions

The microfluidic platform has shown successfully the isolation of various cancer cell types using differences in size and deformability of cancer cells and blood cells. The device which is also label free will address numerous concerns linked to affinity based isolation methods. No functional modifications are required for the system and operational procedures are straightforward. The microdevice is versatile to handle the heterogeneities of different cancer cells from diverse origins and is sensitive to detect small cancer cell count in the specimens. With clinical samples, there is positive CTC detection in preliminary studies which is also promising for the isolation of viable cells. The microdevice which separates CTCs in a single step, without requiring biochemical modifications is attractive to maximize the yield for downstream analyses to enhance the understanding of the disease, and complement current cancer detection and prognosis techniques.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.054.

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