TUMOR CELL ISOLATION IN MICROFLUIDIC DEVICES FOR CANCER TREATMENT MONITORING

Z. Hugh Fan, Jose I. Varillas, Jinling Zhang, Kangfu Chen, and Thomas J. George, Jr.
University of Florida, Gainesville, FL 32611-6250, USA

ABSTRACT

This paper reports two microfluidic devices for isolating circulating tumor cells (CTCs) in blood samples of pancreatic cancer patients. These devices consist of micromixers or micropillars for enhancing the interactions between tumor cells and capture agents, which are immobilized onto the surfaces of microchannels and microfeatures. Both antibodies and aptamers, as well as their ensemble, have been explored for tumor cell isolation. The devices have been used for enumerating CTCs and we have correlated the CTC number with the anti-cancer treatment responses in a clinical trial.

INTRODUCTION

Cancer accounts for millions of deaths every year worldwide. More than 90% of cancer deaths result from metastasis, in which some cancer cells escape from the primary tumor, circulating in the bloodstream before spreading to distant organs. As a result, circulating tumor cells (CTCs) in the peripheral blood have a potential to become a biomarker for early-stage cancer diagnosis, prognosis, and metastasis prevention/treatment [1, 2]. However, CTCs are extremely rare in the bloodstream (about 10 CTCs in 1 mL of blood that consists of billions of healthy blood cells), making their detection technically challenging [1-3].

The state of the art for CTC detection is CellSearch® (Janssen Diagnostics), the only Food and Drug Administration (FDA)-approved method for CTC enumeration [4]. In CellSearch®, a blood sample is mixed with ferrofluid particles conjugated with antibodies against epithelial cell adhesion molecule (EpCAM). After magnetic selection, those isolated cells are verified as CTCs by a composite identification process using a nuclear DNA stain (DAPI or 4',6-diamidino-2-phenylindole), anti-cytokeratin (CK) to confirm epithelial origin, and anti-CD45 to exclude white blood cells.

Microfluidics could become a better platform [1-3] because of a significant decrease in diffusion distance, resulting in more interactions between CTCs and capture agents and accordingly higher capture efficiency. This enhanced capture efficiency, as well as a higher surface-to-volume ratio in microchannels for denser loading of capture agents, would address the challenge of CTC rarity.

In this work, we report two microfluidic devices we have developed for capturing tumor cells. We have studied using an ensemble of antibodies and aptamers for achieving higher capture efficiency than using antibody alone or aptamer along due to the multivalent binding of the ensemble with tumor cells. We have also employed the microfluidic devices for isolating CTCs in blood samples of pancreatic cancer patients and we found that the CTC number correlated with the anti-cancer treatment responses in a clinical trial.

EXPERIMENTAL SECTIONS

Microfluidic Device Fabrication

The microfluidic devices were made from either glass or polydimethylsiloxane (PDMS). All devices were designed to be in the size of a microscope slide. Fig. 1a shows a glass device consisting of eight parallel channels with an array of >59,000 isotropically-etched, elliptical micropillars. The geometric design of the micropillar array was inspired by the deterministic-lateral-displacement-based particle separation [5], in which the flow streamlines were distorted to enhance cell-micropillar interactions. The interpillar distance is 80 µm (center to center), with an 80-µm shift after every 3 rows in the direction of the minor axis.

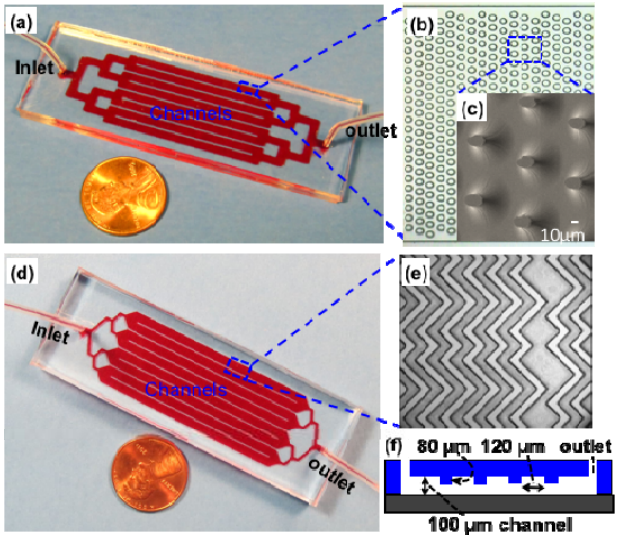

Figure 1: (a) Picture of a CTC device, consisting of 1 inlet, 1 outlet, and 8 parallel channels connected through bifurcation. The size of the device is 1" x 3". (b) Exploded view of a portion of one channel containing a large array of micropillars. (c) Scanning electron microscope (SEM) image of elliptical micropillars in the glass substrate. The micropillars are in elliptical shape, with a dimension of 30 µm (major axis) × 15 µm (minor axis) × 32 µm (height). (d) Picture of another CTC device, with an exploded view of a portion of one channel containing micromixers (e), and cross-sectional view of the mixer design (f).

Glass devices were fabricated using photolithography. A chrome photomask with a desired pattern was created and a chromium coating was used as an etching mask. The patterned glass substrate was chemically etched using a mixture of HF, HNO_3 and H_2O. The glass substrate was then sealed with a PDMS sheet, fabricated from Sylgard 184 reagents (Dow Corning, Midland, MI) according to the instructions of the

manufacturer.

Fig. 1d shows a PDMS device fabricated using soft lithography and a high resolution transparency photomask. A two-layer SU-8 structure was fabricated via two spin-coating/exposure steps and a single developing step. One layer of the design is for making microchannels while the other layer is for herringbone mixers [6]. The SU-8 master on a silicon wafer was used to create a PDMS structure by casting. Inlet and outlet wells were created at the channel ends by punching holes in the PDMS sheet, which was then bonded to a glass microscope slide.

Surface Modification

Each device was functionalized with aptamers or antibodies or aptamer-antibody ensemble through two-step surface modification: (1) physical adsorption of avidin (Invitrogen, Carlsbad, CA) onto the glass surface and (2) immobilization of biotinylated aptamers and/or biotinylated antibodies via biotin-avidin interaction.

Cell Culture

CCRF-CEM cells (human acute lymphoblastic leukemia) and Ramos cells (human Burkitt's lymphoma) were purchased from American Type Culture Collection (ATCC). They were cultured in RPMI medium supplemented with 10% FBS (heat-inactivated; GIBCO) and 100 units/mL penicillin-streptomycin (Cellgro, Manassas, VA). All cultures were incubated at 37˚C under 5% CO_2 atmosphere.

Patient Samples

Blood samples of patients with metastatic pancreatic cancer were obtained from the University of Florida (UF) Health after informed consent through a UF Institutional Review Board (IRB)-approved protocol. Specimens were collected into BD Vacutainer tubes containing anti-coagulant sodium heparin and were processed within 6 hours after being drawn. After CTC capture in the microfluidic device, three-color immunocytochemistry (DAPI, FITC anti-cytokeratin, PE anti-CD45) was conducted to identify CTCs from nonspecifically captured blood cells. Only cells that were DAPI+, CD45-, cytokeratin+, with an appropriate size and morphology, were counted as CTCs.

Experimental Setup

A sample was introduced into a microfluidic device by using a syringe pump. To avoid cell settling, a tiny magnetic stirring bar was placed inside a 1-mL syringe, with a stir plate beneath the syringe. The magnetic stirring bar kept cells in suspension while the cell mixture or blood was being pumped through the device. An Olympus IX71 fluorescence microscope (Olympus America, Melville, NY) with an automated ProScan stage (Prior Scientific, Rockland, MA) was used to image and count the captured cells on the device.

RESULTS AND DISCUSSION

Microfluidic Device Optimization

As mentioned in the Experimental Section, the device in Fig. 1a was designed by following the principle of the deterministic lateral displacement (DLD). DLD makes use of the asymmetric bifurcation of laminar flow around obstacles to achieve particle separation [5], but we primarily took advantage of the distortion of flow streamlines to enhance cell-micropillar interactions.

The device Fig. 1b was designed by following the principle of the herringbone-based microfluidic mixer [6]. However, we found that a micromixer with the traditional design dimension [6] had low cell purity, presumably due to geometric trapping of cells in the narrow grooves as illustrated in Fig. 2a. As a result, we studied the effects of the groove width on cell purity. Fig. 2b shows a cross-sectional view of a micromixer with a larger groove width, which was expected to have better cell purity due to less cell trapping. We found that cell purity was increased from 61% to 84% when the groove width was increased from 50 μm to 120 μm, as shown in Fig. 2c, while capture efficiency was maintained. The capture efficiency was calculated by dividing the number of the target cells captured in the device by the number of the target cells initially introduced into the device. Cell purity was calculated by dividing the number of the target cells captured in the device by the total number of cells captured (i.e., both specifically-bound target cells and non-specifically-bound control cells).

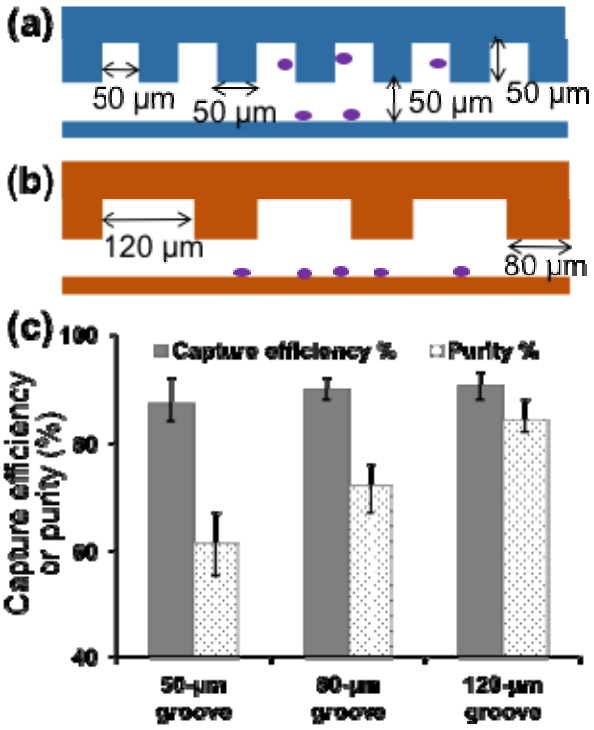

Figure 2: (a). A herringbone-based micromixer design (cross-sectional view) with a design of 50-μm groove width (100 μm groove pitch). The channel depth is 50-μm and the groove depth is also 50-μm. The purple dots represent cells captured inside a microchannel. (b) A micromixer design with wider grooves (120 μm groove width and 200 μm groove pitch). (c) Increases in cell purity when the groove width was increased from 50 μm (conventional narrow groove micromixers) to 80 μm and 120 μm. Error bars represent one standard deviation obtained from 3 repeat experiments.

Multivalent Binding for Tumor Cell Isolation

We used an aptamer-antibody ensemble to achieve multivalent binding and employed the ensemble for tumor cell isolation. Aptamers are typically 2-3 nm in diameter, with molecular weight of 8-15 kDa. Antibodies are typically 12-15 nm in diameter, with molecular weight of 150 kDa. The difference in their sizes could enable simultaneous binding of both aptamers and antibodies with receptor molecules on the irregular cell surfaces as shown in Fig. 3. This type of multivalent binding between capture agents and cell surface markers could lead to effective cell capture due to their cooperative manner, resulting in higher capture efficiency than using antibody or aptamers alone.

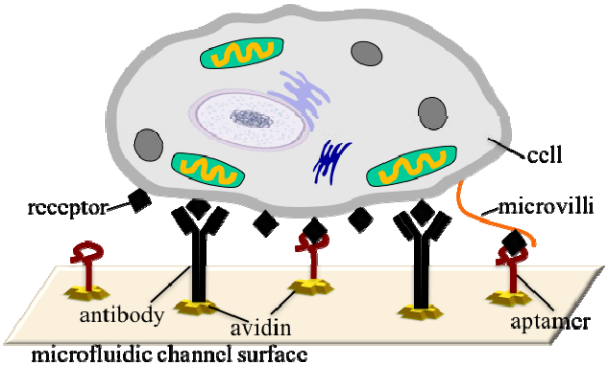

Figure 3: Schematic showing a tumor cell is captured by an ensemble of antibodies and aptamers on a microchannel surface. Multiple receptors on the cell membrane can bind strongly via multivalent interactions to the channel surfaces. Aptamers are in 2-3 nm, with molecular weight of 8-15 kDa while antibodies are in 12-15 nm, with molecular weight of 150 kDa. The drawing is not to scale.

The morphology of a cell and its surface structure with nanoscale microvilli and filopodia enable the aptamer-antibody ensemble to achieve more accessibility of receptors on cell surfaces and higher frequency of interactions between receptors and capture agents. In addition, an enhanced binding avidity through the multivalent binding between the tumor cell and capture agent would result in stronger interactions between the substrate and tumor cells, allowing a higher flow rate that has more ability to dislodge non-specifically-bound cells (accordingly increasing the purity of cells captured).

To verify the effects of multivalent binding, we immobilized a mixture of antibody and aptamer onto the microchannels. The aptamer used is sgc8, with a sequence of 5'-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GAT TTT TTT TTT-3'-biotin while the antibody used is the one against protein tyrosine kinase-7 (PTK-7). Both sgc8 aptamers and PTK-7 antibodies (anti-PTK7) have specific binding with CCRF-CEM cells. Fig. 4 shows the effects of the antibody-to-aptamer ratio on the capture efficiency of CCRF-CEM cells when they were introduced into the device. The ratio of 1:300 shows the highest capture efficiency in all of three flow rates we studied, indicating a certain number of aptamers are needed to accommodate each antibody molecule due to their difference in size.

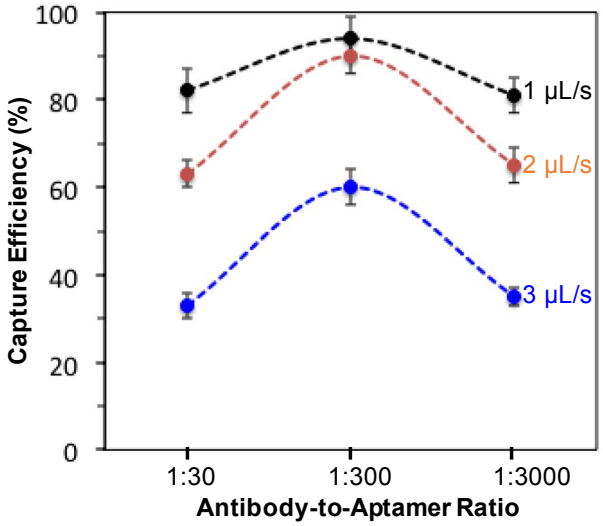

Figure 4: The effect of antibody-to-aptamer ratio on the cell capture efficiency. Under three flow rates studied (1, 2 and 3 µL/s), the ratio of 1:300 showed the highest capture efficiency among all conditions due to their most efficient cooperation in capturing tumor cells.

We also found that an ensemble of antibody and aptamers had higher capture efficiency than either antibody or aptamer alone. Fig. 5 compared the antibody-aptamer ensemble with antibody or aptamer alone for cell capture at a flow rate of 2 µL/s. The capture efficiency of CCRF-CEM cells is significantly higher for the ensemble than either antibody or aptamer alone. More importantly, the ensemble also produced the significantly higher cell purity when we isolated 10^3/mL CCRF-CEM cells among 10^6/mL control cells (Ramos cells).

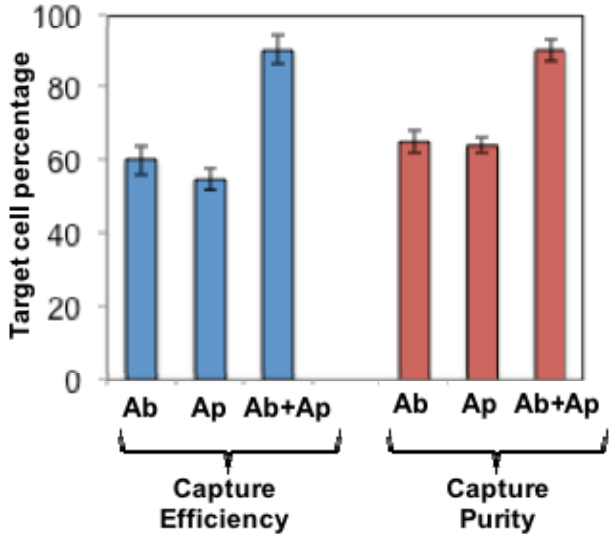

Figure 5: Comparisons in the capture efficiency and cell purity between an antibody-aptamer ensemble (Ab + Ap) and antibody (Ab) or aptamer (Ap) alone. The error bars indicate one standard deviation of three repeat experiments.

CTC Enumeration for Cancer Treatment Monitoring

We have employed microfluidic devices for isolating and enumerating CTCs from peripheral blood samples of pancreatic cancer patients. The FDA-approved CTC definition was used, i.e., the channel surfaces were immobilized with antibodies against EpCAM. The isolated cells were then verified as CTCs by a composite identification process using a nuclear DNA stain (DAPI) to exclude red blood cells, anti-cytokeratin (CK) to confirm epithelial origin, and anti-CD45 to exclude white blood cells, as shown in Fig. 6a.

To demonstrate the functions of our microfluidic devices, we have processed de-identified blood samples from 37 patients with pancreatic cancer undergoing chemotherapy. Blood samples were collected at the first day of each treatment cycle (14 days per cycle) by oncologists or nurses, followed by an appropriate de-identification process before being sent to researchers. Fig. 6b show the number of CTCs detected at different treatment cycles of patient #3, whose CTC number decreased with continuation of the anti-cancer treatment over 1 year. Note that the CTC number in some patients did not change much, indicating that these patients do not respond to the particular chemotherapy. We found that the change (or non-change) in the CTC number is in the agreement with the change (or non-change) in the tumor size measured by X-ray computed tomography (CT) scans. The inset of Fig. 4b show that tumor size decreased as treatment progressed for patient #3. Since CTC quantification using our devices is noninvasive, and it causes significantly less harms to patients (thus can be used much more frequently) than standard clinical radiographic measurements, our platform could be a powerful tool for monitoring early response or failure to cancer treatment and potentially early cancer diagnosis and relapse prediction.

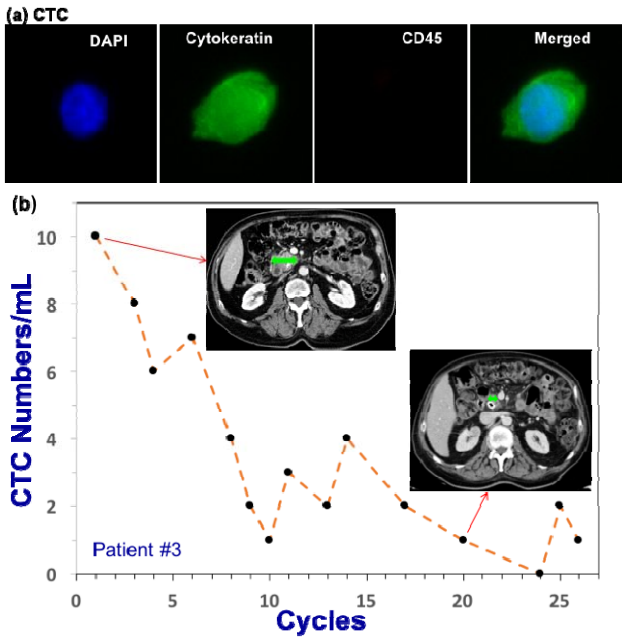

Figure 6: (a) Images of a CTC captured on channel surfaces after staining with DAPI and immunocytochemistry using anti-cytokeratin and anti-CD45, as well as a merged image of three pictures. (b) The number of CTCs per mL of blood from a pancreatic cancer patient at different treatment cycles for patient #3. Each treatment cycle is 2-week. The insets are two CT scan images at cycle 1 and 20, with green arrows indicating regression of tumor.

CONCLUSIONS

In summary, we demonstrated two microfluidic devices for isolating CTCs in blood samples of pancreatic cancer patients. An optimized ensemble of aptamers and antibodies was found to have strong multivalent binding with tumor cells, leading to higher capture efficiency and cell purity than aptamers or antibodies alone when they were used for the isolation of cancer cells in the microfluidic devices. Our devices were also used to enumerate CTCs in clinic samples, and we were able to correlate the CTC number with the anti-cancer treatment responses (indicated by CT scans) in a clinical trial.

ACKNOWLEDGEMENTS

This work was supported by National Cancer Institute of USA (K25CA149080), University of Florida (UF), and UF Health Cancer Center. The authors also acknowledge Drs. Weihong Tan, Chen Liu, and Weian Sheng for their contribution related to this work.

REFERENCES

[1] S. L. Stott, R. J. Lee, S. Nagrath, M. Yu, D. T. Miyamoto, L. Ulkus, E. J. Inserra, M. Ulman, S. Springer, Z. Nakamura, A. L. Moore, D. I. Tsukrov, M. E. Kempner, D. M. Dahl, C. L. Wu, A. J. Iafrate, M. R. Smith, R. G. Tompkins, L. V. Sequist, M. Toner, D. A. Haber, S. Maheswaran, "Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer", *Sci. Transl. Med.* 2010, 2, 25ra23.

[2] Z. H. Fan, *Circulating Tumor Cells: Isolation and Analysis*. J. Wiley: New York, 2016.

[3] W. Sheng, O. O. Ogunwobi, T. Chen, J. Zhang, T. J. George, C. Liu, Z. H. Fan, "Capture, release and culture of circulating tumor cells from pancreatic cancer patients using an enhanced mixing chip", *Lab Chip*, 2014, 14, 89-98.

[4] S. Riethdorf, H. Fritsche, V. Muller, T. Rau, C. Schindlbeck, B. Rack, W. Janni, C. Coith, K. Beck, F. Janicke, S. Jackson, T. Gornet, M. Cristofanilli, K. Pantel, "Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system", *Clin. Cancer Res.* 2007, 13, 920-928.

[5] L. R. Huang, E. C. Cox, R. H. Austin, J. C. Sturm, "Continuous particle separation through deterministic lateral displacement", *Science,* 2004, 304. 987-90.

[6] Stroock, A. D.; Dertinger, S. K. W.; Ajdari, A.; Mezic, I.; Stone, H. A.; Whitesides, G. M, "Chaotic mixer for microchannels", *Science,* 2002, 295, 647-651.

CONTACT

*Z. H. Fan, tel: +1-352-846-3021; hfan@ufl.edu