Label-free isolation of circulating tumor cells (CTCs) from breast cancer patients using parallel multi-orifice flow fractionation (p-MOFF)

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ABSTRACT

Circulating tumor cells (CTCs), which are detached from primary cancer and circulate in peripheral blood, are known to cause the metastatic cancer. Therefore isolation of CTCs can serve as a powerful tool for cancer prognosis, diagnosis of minimal residual disease, assessment of tumor sensitivity to anticancer drugs, and personalization of anticancer therapy. In this paper, we introduce the parallel multi-orifice flow fractionation (p-MOFF) device which is connected by four single MOFF channel for the isolation of CTCs from whole blood of the metastatic breast cancer patients. For the experimentation of feasibility, we separated over 90% of human breast cancer cell line (MCF-7, MDA_MB_231 cells), and eliminate 90.80 % of WBCs from individual experiment at 600 μL/min inlet flow rate and 240 μL/min outlet flow rate. Based on this result, we have attempted to isolate CTCs from whole blood under the same conditions. Then we identify CTCs using immune staining method. Because our devices do not require any labeling processes (e.g. EpCAM antibody), heterogeneous CTCs can be isolated regardless of EpCAM expression.

KEYWORDS

Circulating tumor cell, Metastasis, Flow fractionation, and Breast cancer

INTRODUCTION

Circulating tumor cells (CTCs) are shed by both primary and metastatic cancers and circulate in peripheral blood. They are known to mediate the hematogenous spread of cancer to distant sites. Consequently, pathological and molecular analysis of these cells can be exploited to find the characteristics of cancer. However it is difficult to isolate CTCs from whole blood because these cells are extremely rare with counts of 1 to 10 CTCs per ml in the whole blood of metastatic cancer patients. [1]

Most approaches of CTC isolation are grounded on epithelial cell adhesion molecule (EpCAM) antibodies based detection. Because CTCs have heterogenetic features, the EpCAM expression on cell surface is downregulated in some tumor types. [2] Detection techniques that use the anti-EpCAM affinity molecule may lose considerable amounts of EpCAM negative expressed CTCs. This can be a very crucial limitation in CTC analysis as the cancer cells are very heterogeneous.

In a previous study, we introduced a novel hydrodynamic method for size-based particle separation, multi-orifice flow fractionation (MOFF), in which the microparticle is moved laterally due to inertial force generated in a series of contraction/expansion micro structure. [3, 4] Different sizes of particle can be separated depending on the range of the channel Reynolds number (Re_c). This method is expedient for CTC isolation because of the fast operational flow rate (100~300μl per min) and non-labeling separation.

In this study, we designed the parallel MOFF device (p-MOFF), which is connected by four single MOFF channels, to handle the large volume of blood sample (>7.5ml) within half an hour and isolate CTCs from whole blood of the metastatic breast cancer patient.

PRINCIPLE

The parallel multi-orifice flow fractionation (p-MOFF) consists of an inlet, a filter, four parallel multi-orifice segment and outlets. The multi-orifice segment is an alternating series of contraction and expansion channels, through which particles are separated by the momentum-change-induced inertial force. Within each of MOFF channels, relatively smaller WBCs than CTCs split into two positions, and CTCs focused at the center of the channel. Consequently, at the end of the channels, the WBCs go to the outlets for waste, and the CTCs were collected in the outlet for CTC (Fig. 1). In previous research, we studied the separable flow rate zones of differently sized particles according to varying dimensions of the orifice channel [3]. When the channel Reynolds number reached 70, MCF-7 cells are successfully separated from a spiked blood cell sample. Based on this study, we chose a channel dimension of 70 channel Reynolds number. The width and length of the contraction channels are 60μm and 150μm, respectively, while those of the expansion channels are 300μm in both of them. The designed depth of the micro-channel is 60μm.

EXPERIMENTAL

Breast cancer cell lines (MCF-7, MDA_MB_231 cells) were incubated under ordinary cell culture conditions. Cells were trypsinized, washed with PBS and resuspended in PBS containing 3% BSA. WBCs were processed using density gradient separation. Blood of metastatic breast cancer patient under a IRB-approved protocol were used for CTC analysis. Human blood sample (7.5 ml) was collected into vacutainer tubes containing the EDTA and was processed within 6 hours. For the p-MOFF separation using lysed blood, RBC lysis buffer (Qiagen, Chatsworth, CA) was added to whole blood in 1:10 v/v ratio and incubated for 10 minutes at room temperature. Following

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centrifugation at 600G for 10 minutes, the supernatant was removed and the pellet re-suspended in 10 ml PBS containing 3 % BSA.

We used two syringe pump (KDS200, KD Scientific, Massachusetts) to generate a continuous and stable micro flow. The 10 mL syringes were connected to inlet for the prepared sample injection and outlet for withdrawal. The flow rate of inlet was 600 μL/min, and the flow rate of outlet was 240 μL/min. The flow rate of inlet was derermined by the channel Reynolds number as 70 at MOFF's contraction channel and the flow rate of outlet was adjusted as 40 % of total inflow.

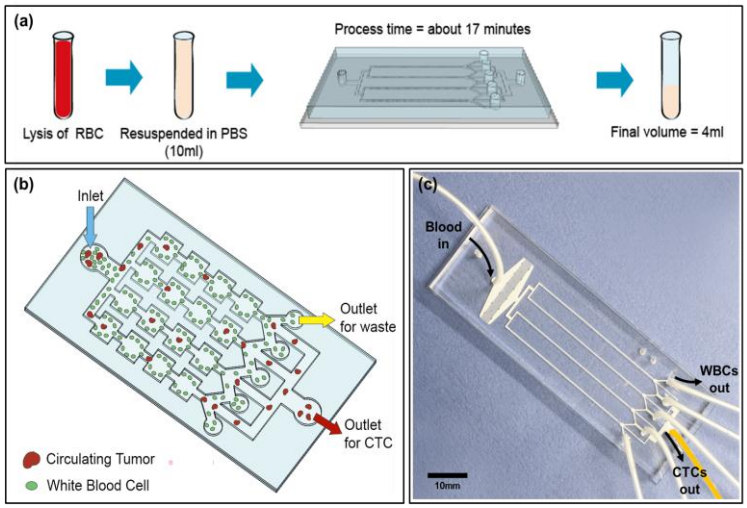
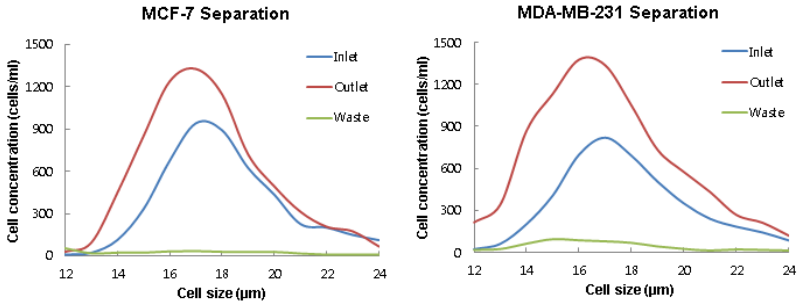


Figure 1 (a) Schematic drawing of the overall process for the isolation of CTCs from cancer patients. (b) A cartoon presentation of separation principle. (c) Photographic image of the fabricated p-MOFF device.

RESULTS AND DISCUSSION

We evaluated the separation performance of p-MOFF device with breast cancer cell line and WBC by counting the number of cells in each outlet. In the case of cancer cells larger than WBC, the streamlines were finely focused in the middle of the channel. We separated 93.75% of MCF-7 cells and 91.60 % of MDA_MB_231 cells and eliminate 90.8 % of WBCs from individual experiment (Fig. 2). These values verify that the newly proposed p-MOFF is well-designed for parallel channel network.



Sample name		WBC (Blood cell)	MCF-7 EpCAM(+)	MDA_MB_231 EpCAM(-)
recovery	Outlet for CTC	9.2%	93.75%	91.60%
	Outlet for waste	90.8%	6.25%	8.4%

Figure 2. Separation yields and size distributions of isolated cancer cell lines.

Based on these results, we have attempted to isolate CTCs from whole blood (7.5ml) of the breast cancer patients under the same conditions (Fig. 3). After processing of blood from metastatic breast cancer patients on the p-MOFF device, cells were fixed on a slide glass and stained with DAPI for DNA content, PE conjugated pan-Cytokeratin for CTC, and APC conjugated CD45 for WBC (Fig. 2). Following imaging the slide glass, captured images were examined carefully with predefined criteria. Cell size and shape were also considered in cell characterization. We found the CTCs which are positive for DAPI, negative for CD45 and positive for Cytokeratin. When the blood samples of 24 breast cancer patients were subjected to our device, 1 to 21 CTCs were identified in 19 patients (Table 1).

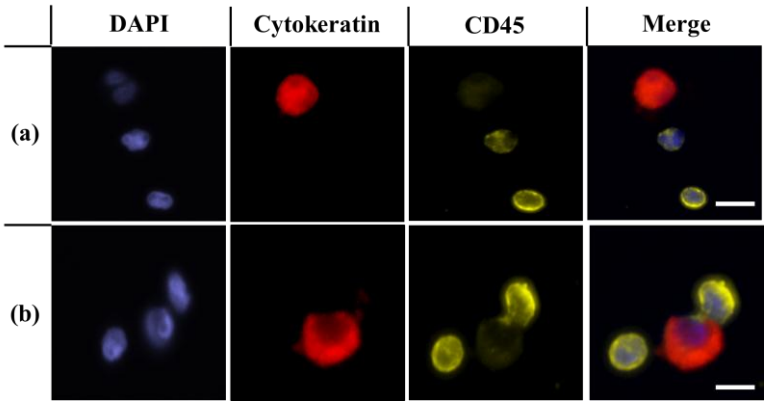


Figure 3. Fluorescent images of cells isolated using the p-MOFF device from (a) a metastatic breast cancer patient and (b) an adjuvant breast cancer patient.

Metastatic patients	J1	P1	K1	P2	P3	K2	M1	C1	K3	K4	P4	O1
CTC No.	0	2	3	4-15	6-16	0	4-5	3	6-15	1-4	5-7	5-12
Adjuvant patients	**k1**	**o1**	**b1**	**y1**	**p1**	**l1**	**b2**	**y2**	**j1**	**h1**	**k2**	**j2**
CTC No.	2	4-7	0	1-4	0	2-6	1	2-15	2-7	0	1	2-21

Table 1 The number of CTCs in the blood of breast cancer patient counted using immunofluorescence staining after the p-MOFF device process.

CONCLUSION

The major advantages of the p-MOFF device include hydrophoresis and non-labeling. The number of intact and heterogeneous CTCs continuously collected by the device is expected to give researchers many opportunities to investigate the molecular nature of CTCs.

Using breast cancer cell line, we separated 93.75% of MCF-7 cells and 91.60 % of MDA_MB_231 cells, and eliminate 90.80 % of WBCs from individual experiment at 600 μL/min inlet flow rate and 240 μL/min outlet flow rate. Based on this result, we isolated the CTCs from whole blood of the metastatic breast cancer patients. When the blood samples of 24 breast cancer patients were subjected to our device, 1 to 21 CTCs were identified in 19 patients. We believe the p-MOFF provides evidence for the potential as a powerful tool for cancer diagnosis and prognosis.

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