A HANDHELD DEVICE FOR RAPID VIABLE CIRCULATING TUMOR CELL ISOLATION USING MICROFABRICATED TAPERED-SLIT FILTERS

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ABSTRACT

We present a handheld device for the simple, stable, high-throughput, and viable circulating tumor cell (CTC) isolation with the whole blood processing capacity of 3.3ml/hr. The device uses the microfabricated tapered-slit filters having wider slit entrances and gradually narrower slit exits. Compared to the conventional straight-slit filters, the tapered-slit filters are capable to increase blood flowrate with the minimal cell stress. We verified the device's performance using the 4 different lung cancer cells at 5 different concentrations in the range of 5-100cells per 1ml of whole blood; thus achieving the average capture efficiency of 80.4% with the viability of 82.3%. Furthermore, the device captured 1 to 9 CTCs from the experiments using 5 different lung cancer patients' blood samples; thus, demonstrating the device's potential for clinical applications.

KEYWORDS

Circulating tumor cells, tapered-slit filters, membrane filters, viable cell isolation, high-throughput isolation

INTRODUCTION

CTCs are the rare cells, departing from the primary tumor site, circulating in blood stream, and causing metastasis by forming secondary tumors [1]. Due to their rarity and heterogeneity, 'viable' CTC isolation methods are demanded for CTC culture or characterization as well as for metastasis mechanism understanding [2]. The previous antibody-based CTC isolation methods [3, 4] have difficulty in maintaining the viability of CTC due to irreversible antibody bindings. The alternative size-based CTC isolation filters [5, 6] were proposed for viable CTC isolation. The conventional CTC filters of straight-slits, however, limit the throughput due to the cell stress concentration, resulting in the captured cell damages at high flow rate condition.

In order to solve this problem, the tapered-slit filters [7], having wider inlet entrance and gradually narrower slit exits, was recently proposed with the merit of minimizing stress concentration at the slit edge. This filter device showed the promising results in terms of high CTC capturing ability and viability, however, its three-layer design including two chamber layers and a filter layer-is not adequate for handheld applications due to large dead volume and inconvenient interconnection. In addition, their optimal flow-rate was low as 5ml/h because of low slit-density. Here, we present the handheld device having tapered-slit filters, inserted in a filter cassette and simply connected to commercial syringes to isolate the CTCs. The present device only uses 1 filter layer with enhanced throughput as much 2 times higher than previous device without significant reduction of capturing ability and viability. Using these merits, we tested the performance of our device varying 4 types of cancer cell lines and 5 different cell concentrations. These comprehensive studies showed that our device is ready to use for clinical applications.

DESIGN AND FABRICATION

A schematic diagram of the present handheld device is shown in Fig. 1. The present device is composed of three parts: two acryl filter cassettes and a 100μm-thick circular SU8 membrane filter. The circular membrane filter having the diameter of 20mm contains 625x625(=165,625) tapered-slits at the slit density of 52,747slits/cm^2. The tapered-slit designed with the wider slit inlet and the narrower outlet facilitates not only high-throughput viable isolation, but also selective CTC isolation from other blood cells having the same size but different deformability. The effects of the tapered-slit were showed theoretically and experimentally in our previous studies [7, 8]. The outlet width of the tapered-slits is 6 μm, which is identical to our previous work, however, slit density and overall shape are improved and changed for the handheld device applications.

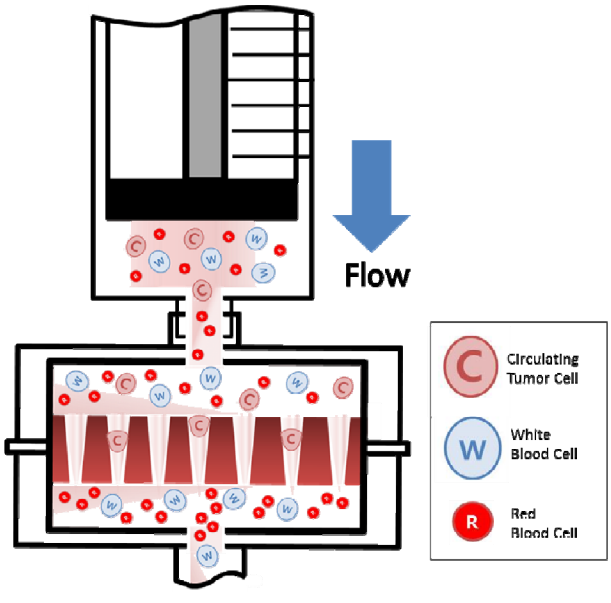

Figure 1: Schematics of the handheld device for rapid viable circulating tumor cell isolation using micro fabricated tapered-slit membrane filter.

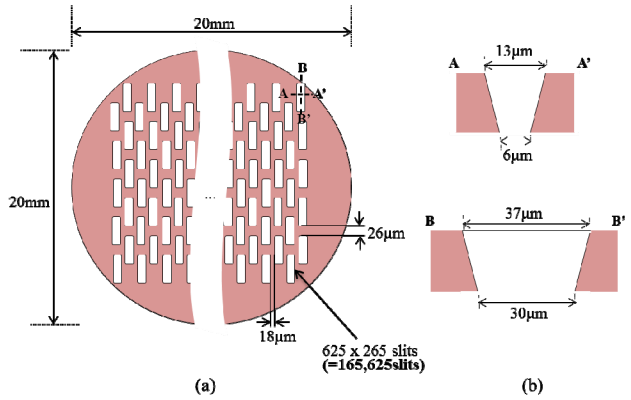

Figure 2: The tapered-slit filter: (a) top view; (b) cross sectional view across A-A' (upper) and B-B' (lower).

The tapered-slits are fabricated by the single-mask process of Fig.3a. The tapered slit angle of 2 degree is formed from the adjustment of the UV exposure dose on the spin-coated SU8 layer and the air gap between photomask and SU8 filter layer. Due to the effect of diffraction-reflection-refraction during photo lithography [9], tapered slits were uniformly formed on SU8 layers with high reproducibility (Fig. 3b). The fabricated tapered-slit filter is inserted between the filter cassettes with O-rings (Fig.4b) for bi-directional connections to commercial syringes (Fig.4a).

EXPERIMENTAL RESULTS

The performance of the present device has been verified by the experiments for the lung cancer cells spiked in normal blood and the lung cancer patients' blood samples at an identical sample flow rate of 10ml/h.

Experiment using Spiked Cancer Cells

We demonstrated the device performance using the 4 different lung cancer cells (H358G, A549G, H460G, and A549) at the higher concentration (50, 100 cells/ml) and the lower concentration (5, 10, 15 cells/ml).

For the higher cell concentration samples, we used the 3 different human lung cancer cells, H358G, A549G, and H460G, transfected with green fluorescent protein (GFP) in order to test the device performance independent to antibody staining of captured cells. We spiked the cancer cells in the 3ml of the samples diluted to 1:2 (Blood:PBS) at the cell concentration of 50, 100cells/1ml of whole blood. After then, we loaded the 3ml of the blood sample to the device at the flow rate of 10ml/h using syringe pump. We add an additional 1ml of the phosphate buffered saline (PBS) after finishing the sample flow for washing out remaining unwanted cells on filter. Then, we disassembled our device and observe the captured cell number using fluorescent microscope. In this process, our device captures 74.3%, 83.5%, and 82.7% of H358G, A549G, and H460G, respectively, at the cell concentration of 50(cells/ml). At the cell concentration of 100(cells/ml), 84.7% of H358G, 72.9% of A549G, and 84.4% of H460G were captured, respectively (Fig. 5a).

After measuring capture efficiency, we reassembled the device with the same tapered-slit filter used for CTC capture, then applied the reverse flow (flow rate: 50ml/h) to the device. We enumerated the remaining cells on the filter three times, then calculate the release rate of each cell lines at two cell concentrations. The present device can release the 64.7%, 73.3%, and 51.1% cells of H358G, A549G, and H460G, respectively at the cell concentration of 50(cells/ml). At the 100(cells/ml), 80.4% of H358G, 56.4% of A549G, and 56.7% of H460G were released from tapered-slit filters, respectively (Fig.5b).

In order to verify the viability of the cells during our process, we additionally tested the viability of captured cancer cells using live/dead kit (Calcein AM/Eth-D) at the identical conditions to those of capturing experiments. Immediately after cell capture process, we loaded the 200μm of live/dead staining solution and waited for thirty minutes to be stained. Then we disassembled the device, enumerate the live/dead cells on filter, and calculated the viability rate. The viability rate is defined by the ratio of the viable cancer cell number to the total cancer cell number captured by tapered-slit filter. The present device showed 80.9%, 83.8%, and 82.3% of viability for the A549G, H358G, and H460G, respectively. From these experiments, we verified that the present device captures the cancer cells with the average viability of 82.3±5.2%.

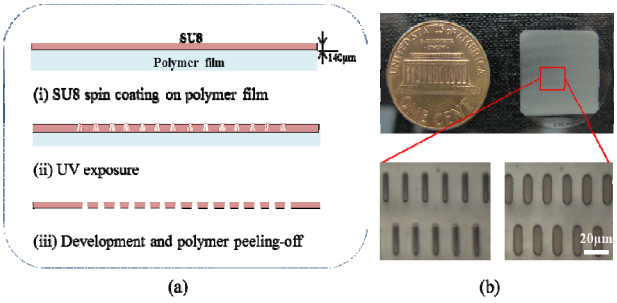

Figure 3: Tapered-slit filter fabrication: (a) fabrication procedure; (b) the enlarged views of front (left) and back (right) sides.

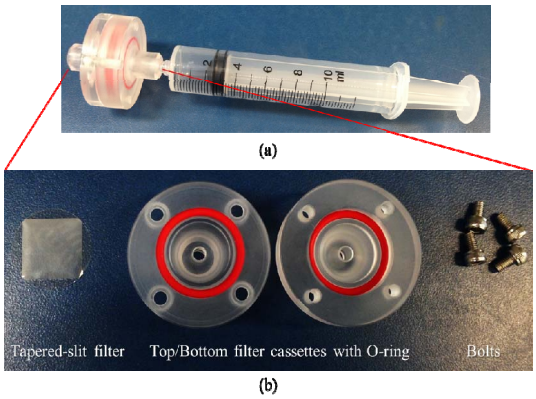

Figure 4: The handheld viable circulating tumor cell isolation device: (a) the device connected to a commercial syringe; (b) the device components before assembly.

For the lower cell concentration samples, we used a non-GFP human lung cancer cell line, A549, in order to verify the capturing ability of the present device using antibody-staining method at the more realistic conditions. In this experiments, we spiked the cancer cells in the 9ml of the blood samples diluted to 1:2 (Blood:PBS) at the cell concentration of 5, 10, and 15cells/1ml of whole blood, reflecting the CTC detection frequency from the previous studies [10,11]. We loaded the 9ml of samples to the device at the identical flow rate of 10ml/h, then washed out non-target cells using additional 1ml of PBS as like the experiments at the higher concentrations. After this procedure, we fixed the captured cells on filter using 500µl of 4% paraformaldehyde solution at the flow rate of 5ml/h, then washed out excess solution using 1ml of PBS.

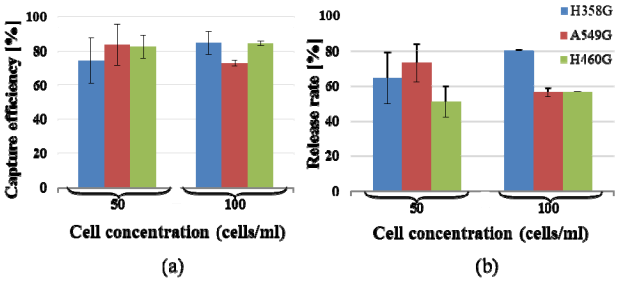

Figure 5: The experimental results for the higher cell concentration (50, 100 cells/ml) samples: (a) the capture efficiency and (b) the release rate for 3 different GFP lung cancer cells (H358G, A549G, H460G) depending on the concentration of the cells spiked in the 3ml of diluted blood (Blood : PBS=1:2).

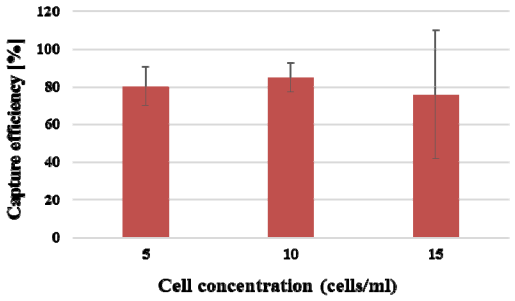

Figure 6: The capture efficiency for the lower cell concentration (5, 10, 15 cells/ml) of non-GFP lung cancer cell (A549) depending on the concentration of the cells, spiked in the 9ml of diluted blood.

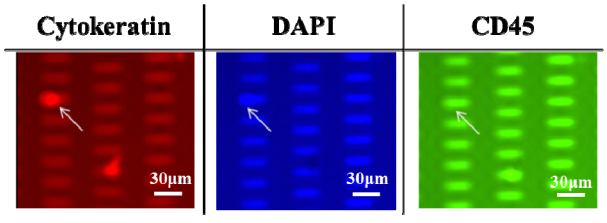

Figure 7: Fluorescent images of the circulating tumor cell, captured from the lung cancer patient blood (stage: CT1bN1M0) and identified by cytokeratin(+), DAPI(+), and CD45(-).

For the antibody staining-dye to be penetrated to cell membrane, we loaded 500µl of 0.5% Triton X-100 solutions. After that, we added 500µl of 1% bovine serum albumin (BSA) solution in order to eliminate the non-specific binding, generating false-positive results. After blocking, we stained the captured cells using the 600µl staining dye consisting of 10µl of PE-conjugated cytokeratin dye (Cat.# 347204, BD Bioscience), 10µl of FITC-conjugated CD45 dye (Cat.# 340664, BD Bioscience), and 5µl of DAPI staining dye (Cat.# D3571, Life technologies); each dye, cytokeratin, CD45, and DAPI stains epithelial cells (such as CTCs), white blood cells, and nucleus of eukaryotic cells, respectively. After staining the cells, we washed out excess dye using 1ml of PBS at the flow rate of 5ml/h, disassembled the devices, and then enumerated the CTCs using fluorescent microscope.

Our fluorescence microscope system (Eclipse Ti, Nikon) scanned and captured every points of our device three times with three different fluorescent filter, then captured images were carefully identified. From this experiments, the device showed the average capture efficiency of 80.3±16.9% (Fig.6) based on the cancer cell identification criteria of cytokeratin(+), DAPI(+), CD45(-) immunostaining.

Experiment using Cancer Patient Blood

The 5ml of 5 different blood samples were drawn from stage 3 lung cancer patients at the Samsung Medical Center (SMC) under an institutional review board (IRB) approved protocol. These blood samples were collected in vacutainer tubes containing anticoagulant, ethylenediaminetetraacetic acid (EDTA), transported, and then processed within 12 hours after sampling.

For the experiment using cancer patient bloods, we diluted the whole blood to 1:2 (Blood:PBS), loaded the sample at the flow rate of 10ml/h, and then stained the captured cells with the same conditions (dilution ratio, flow rate, and staining dye) as experiments using spiked non-GFP cancer cells.

Our fluorescence microscope system scanned all slits on filter, then scanned slide were carefully examined considering both staining criteria and morphological standard (such as size, nucleus-to-cytoplasm ratio, irregular shape) [12]. From this study, we confirmed 1 to 9 CTC from the 5 different lung cancer patients' samples (Fig.7). This result indicate that the present handheld device has the potentials for clinical applications.

CONCLUSION

We have designed, fabricated, and verified the performance of handheld device having microfabricated tapered-slit filter. From the experiments using 3 GFP cancer cells at higher cell concentration, we tested the essential performance of filter device in terms of capture efficiency, release rate, and viability. At the lower cell concentration of non-GFP cancer cells, we verified the CTC capturing ability considering positive immunostaining of cytokeratin and nucleus; thus achieving 80.4±12.3% of capture efficiency maintaining the 82.3±5.2% of viability. At the same device

and conditions, the present device captures average 4.2 CTCs per 1ml of 5 different lung cancer patients' samples. We envision that the present device can be used in various applications which would be helpful for CTC cultures and understanding metastasis mechanism from the captured viable CTCs.

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