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Development of the automated circulating tumor cell recovery system with microcavity array

Ryo Negishi [a], Masahito Hosokawa [a], Seita Nakamura [a], Hisashige Kanbara [b], Masafumi Kanetomo [c], Yoshihito Kikuhara [b], Tsuyoshi Tanaka [a], Tadashi Matsunaga [a], Tomoko Yoshino [a,*]

[a] Division of Biotechnology and Life science, Institute of Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan
[b] Hitachi Chemical Co., Ltd., 1-9-2, Marunouchi, Chiyoda, Tokyo 100-6606, Japan
[c] KOMEYA Professional engineer Japan office, 5-11-30, Kugayama, Suginami-ku, Tokyo 186-0081, Japan

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ABSTRACT

Circulating tumor cells (CTCs) are well recognized as useful biomarker for cancer diagnosis and potential target of drug discovery for metastatic cancer. Efficient and precise recovery of extremely low concentrations of CTCs from blood has been required to increase the detection sensitivity. Here, an automated system equipped with a microcavity array (MCA) was demonstrated for highly efficient and reproducible CTC recovery. The use of MCA allows selective recovery of cancer cells from whole blood on the basis of differences in size between tumor and blood cells. Intra- and inter-assays revealed that the automated system achieved high efficiency and reproducibility equal to the assay manually performed by well-trained operator. Under optimized assay workflow, the automated system allows efficient and precise cell recovery for non-small cell lung cancer cells spiked in whole blood. The automated CTC recovery system will contribute to high-throughput analysis in the further clinical studies on large cohort of cancer patients.

1. Introduction

Circulating tumor cells (CTCs) are rare cells found in peripheral blood of metastatic cancer patients. The number of CTCs has clinical parameter of cancer patient prognosis and therapeutic effects of anticancer drug (Budd et al., 2006; Cristofanilli et al., 2004; Nagrath et al., 2007). Thus, CTCs are well recognized as useful biomarker for cancer diagnosis and potential target of drug discovery for metastatic cancer. However, CTCs are extremely rare (1 in 5×10^9 blood cells), so the efficient and precise recovery of CTCs from blood has been required to increase the detection sensitivity (Ross et al., 1993).

The assay formats of CTCs recovery have been categorized into two types; immuno-based assay and cell size- and deformability-based assay. Immuno-based assay uses antibodies that recognize surface markers of CTCs or leukocytes for CTCs recovery. Epithelial cell-adhesion molecule (EpCAM) is the most commonly used CTC marker in this assay format. Anti-EpCAM antibody-coated magnetic particles are used in immunomagnetic separation-based systems such as CellSearch system (Velidex, Raritan, NJ) that has been approved by FDA for the diagnosis of breast, prostate and colon cancers (Allard et al., 2004; Xie et al., 2014; Xu et al., 2011). Many reports have been published on antibody-coated tools including microfluidic channel, needle, and magnetic nanoparticles with the aid of the antibodies, which recognize CTC specific markers (Durgadas et al., 2011; Kamande et al., 2013; Kirby et al., 2012; Nora Dickson et al., 2011; Park et al., 2014; Svensson et al., 2014; Wang et al., 2004). However, this assay format has a limitation to apply the basic protocol to various types of tumor cells because the expression level of surface makers, especially EpCAM (Punnoose et al., 2010), was varied with tumor types. Therefore, immuno-based assay could not achieve stable and reproducible CTCs recovery from all tumor types.

On the other hand, cell size- and/or deformability-based assays have been developed for CTCs recovery. In general, blood cells are smaller in size and shows higher deformability compared with epithelial tumor cells (Cook et al., 1998; Mohamed et al., 2004; Shelby et al., 2003). Isolation by size of epithelial tumor cells (ISET) system can be achieved by using filtration to separate individual tumor cells on a polycarbonate membrane, since tumor cells (>8 μm) are larger than leukocytes (Vona et al., 2000). Tumor cells are selectively recovered by the cell size and/or deformability,

* Corresponding author. Fax: +81 42 385 7713.
E-mail address: y-tomoko@cc.tuat.ac.jp (T. Yoshino).

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and subsequently detected by fluorescent microscopic analysis (Lin et al., 2010; Vona et al., 2000). The size- and/or deformability-based methods are generally inexpensive and simple method for CTCs recovery. Furthermore, antigen independent recovery was achieved by size-based assay formats. Some clinical studies indicated that ISET system has higher CTC detection sensitivity than immuno-based CellSearch system in some cancer types (Farace et al., 2011; Hofman et al., 2011; Hou et al., 2011).

Our group has demonstrated a novel miniaturized device integrated with microcavity array (MCA) for highly efficient recovery of cancer cells based on differences in cell size and deformability. Sizes of the circular microcavities were designed to capture tumor cells, while allowing blood cells to flow through during whole blood filtration. The detection efficiency of non-small cell lung cancer (NSCLC) cells in a 1 mL blood sample spiked with 10 – 100 cells was approximately 96% (Hosokawa et al., 2010). The structure of MCA was optimized as a rectangular shape for entrapment of small-sized tumor cells that found in small-cell lung cancer (SCLC) patients (Hosokawa et al., 2013b). Furthermore, recent clinical study showed that MCAs might be superior to the CellSearch System for detecting CTCs in patients with NSCLC and SCLC (Hosokawa et al., 2013a; Hosokawa et al., 2013b). However, trained operators had to carry out time-consuming and labor-intensive processes for blood separation and CTCs detection manually. Thus, the whole processes required for CTCs detection using the MCA should be automated to improve assay throughput, reliability and reproducibility of sample process for further clinical studies on large cohort of cancer patients at various institutions.

In this study, an automated CTC recovery system using MCA was developed toward high-throughput analysis with high assay accuracy and high efficiency for tumor cell recovery from whole blood. Especially, we focused on the evaluation of the performance of the automated system for efficient and reproducible cell recovery using lung cancer cells-spiked whole blood.

2. Material and methods

2.1. Cell culture and cell sample preparation

NCI-H69 cells were used as small cell lung cancer (SCLC) cell line, and NCI-H358, NCI-H1975, NCI-H1650 and HCC-827 cells were used as non-small cell lung cancer (NSCLC) cell line. Lung cancer cells were cultured in RPMI-1640 medium containing 2 mM L-glutamine (Sigma-Aldrich, Irvine, UK), 10% (v/v) fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) and 1% (v/v) penicillin/streptomycin (Invitrogen Corp.) for 3–4 days at 37 °C with 5% CO_2 supplementation. Immediately before each experiment, confluent cells are trypsinized and re-suspended in PBS (Gibco). To estimate the average size of cancer cells, size distribution analysis was carried out using CASY cell counter+Analyzer System Model TTC (Schärfe System GmbH, Reutlingen, Germany).

2.2. Fabrication of microcavity array (MCA) cartridge

MCA was made of nickel by electroforming in the same manner as previous reports (Hosokawa et al., 2010; Hosokawa et al., 2013b). Each rectangular microcavity (Fig. 1A; $18 \times 18\ mm^2$) was fabricated with a width of 8 μm and length of 100 μm, longitudinal pitch of 23 μm and transverse pitch of 115 μm. The MCA was integrated with poly(methyl methacrylate) (PMMA) substrates and silicon gasket (thickness of 0.1 mm) by ultrasonic welding. The upper substrate consists of a microchamber, sample inlet and reagents inlet (Fig. 1B). The outlet was fabricated in the lower substrate to produce a negative pressure, enabling blood filtration. The sample inlet was connected to a sample reservoir, while the reagents inlet and outlet were connected to the automated system.

2.3. Automated CTC recovery system

Fig. 1C shows the layout of the automated CTC recovery system. The automated CTC recovery system is equipped with peristaltic pump, stream selector and pinch valves to introduce blood sample and reagents into MCA cartridge. Stream selector is connected to 8 reagent bottles; no. 1–5: PBS containing 2 mM EDTA and 0.5% BSA (PBS with EDTA/BSA) for washing; no. 6: PBS with 4% paraformaldehyde (PFA) for fixation; no. 7: PBS with 0.2% Triton-X 100 for permeabilization; no. 8: staining solution for cell visualization. Sample reservoir is independent of the reagents flow line to avoid blood contamination. The automated process was controlled by manufactured software which could be used for various purposes by changing protocols and parameters. The automated CTC recovery system consists of 4 independent units to handle 4 samples simultaneously (Fig. 1D). Fig. 1E shows the platform for disposable unit. Sample reservoir can be shaken to mix sample solution.

2.4. Preparation of cell and blood samples

To evaluate the cell recovery rate and the assay precision by the automated system, fluorescent dye-stained cells were used. The lung cancer cells were labeled with 5 μM CellTracker Green CMFDA (Molecular Probes, Eugene, OR) for 30 min. The cells were then pelleted by centrifugation (400*g*, 3 min). After washing twice with PBS, the cells were re-suspended in PBS with EDTA/BSA.

To demonstrate the practical performance of the automated system, whole blood samples spiked with lung cancer cells were used for cell recovery. All human blood samples were collected from healthy donors at Tokyo University of Agriculture and Technology in accordance with Institutional Review Board procedure (approval number: 22-05). Samples were collected in a collection tube with EDTA to prevent coagulation and used within 24 h. Then, known number of lung cancer cells was spiked into 1 ml of whole blood.

2.5. Recovery and detection of cancer cells by the automated system

Table S1 shows scheme of cancer cell recovery and detection process. Blood samples or cell suspensions were introduced into the sample reservoir. Subsequently, negative pressure was applied by peristaltic pump to draw aqueous solutions. The sample solution (1 ml) was passed through the MCA at a flow rate of 200 μl/min to capture the cancer cells on MCA, and to remove blood cells from MCA (Step 1). To further eliminate the remaining blood cells on MCA, PBS with EDTA/BSA was introduced into MCA cartridge at a flow rate of 200 μl/min for 5 min (Step 2). Then, the cells were fixed with 4% PFA (Step 3 and 4). After washing (Step 5), permeabilization treatment was performed using 0.2% Triton-X 100 (Step 6 and 7). After washing (Step 8), cells were stained (Step 9 and 10) with 1 μg/ml Hoechst 33342 (Molecular Probes, Invitrogen Corp.), a cocktail of anti pan-cytokeratin antibodies (1 μg/ml Alexa Fluor 488-AE1/AE3 (eBioscience, San Diego, CA) and 1.7 μg/ml FITC-CK3-6H5 (Miltenyi Biotec, Auburn, CA)), and 0.2 μg/ml PE-labeled anti-CD45 antibody (BD Biosciences, San Diego, CA). After washing (Step 11), the cells were subjected to the following fluorescent microscopic analysis.

2.6. Fluorescent microscopic analysis

Fluorescent microscopic analysis was carried out using fluorescent microscope (BX53; Olympus Corporation, Tokyo, Japan) integrated with 10 × objective lens computer operated motorized stage, UNA, BNA, GW filter sets, a cooled digital camera (EXi Aqua;