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Notes & Tips

Enrichment of cancer cells from whole blood using a microfabricated porous filter

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ABSTRACT

Enrichment of circulating tumor cells (CTCs) from whole blood is very challenging due to its rarity. We have developed a new CTC enrichment method using a microfabricated filter. The filter was designed to fractionate tumor cells by cell size and optimized to have high porosity and proper pore distribution. When cancer cells were spiked in whole blood, the average recovery rate was 82.0 to 86.7% and the limit of detection by filtration process was approximately 2 cancer cells in a testing volume of blood. The results indicate that the microfabricated filter-based enrichment would be useful to retrieve and analyze CTCs in practice.

Circulating tumor cells (CTCs)[2] is a prospective prognostic marker for progression-free and overall survival in several types of cancer [1,2] and a potential source of the metastatic tumor cells. Isolation and characterization of CTCs are regarded as a promising tool for cancer prognosis, monitoring after drug treatment as well as early detection of cancer [3]. However, isolating CTCs in the peripheral blood of cancer patients is still technically challenging due to their rarity (~1 in 10^9 blood cells) and heterogeneity. Various methods have been developed, mostly positive selection based on cell surface markers or nonspecific negative selection by physical properties such as density and size [4]. However, to achieve high levels of sensitivity, purity, recovery rate, and other factors such as reproducibility and cost effectiveness [5], still further improvement is needed.

This study describes a CTC enrichment method using a newly designed microfabricated porous filter. The principle of isolating CTCs by filtration is based on the fact that the sizes of most CTCs are larger than those of most blood cells. Most cancer cells measure more than 15 μm in size (data not shown), whereas most peripheral blood leukocytes (with some exceptions) measure from 8 to 11 μm. Currently, most filtration methods use 6.5- to 8-μm pore membrane filters [6–8] because leukocytes are deformable and can maneuver to pass through the pore size smaller than their resting sizes. To reduce the possible loss of any cancer cells of smaller size, we decided to take the lower limit of the pore size (6.5 μm). The gap distance between pores was another important design factor because it sets the porosity of filter, which in turn affects the purity of desired target cancer cells. We took the two factors into consideration to determine the upper limit of gap distance: the size of leukocytes and the distribution of streamlines. Using a commercial CFD (computational fluid dynamics) analysis program, ANSYS–CFX, we estimated how the gap distance influences the streamline distributions near filter pores. We assumed that the flow is laminar and that the density and viscosity of the cell suspension are 1.0 g/cm^3 and 0.1 poise, respectively. Then we imposed symmetry boundary conditions on the model, as shown in Fig. 1A. From this simulation, streamline distributions and dead zones were predicted, as in Fig. 1B. Periodic appearance of dead zones between the pores was also observed when flow velocity was near zero. To measure the size of a dead zone in accordance with the change in the gap distance, we defined the size of the dead zone as the diameter of a sphere that could fit in the dead zone (Fig. 1B). We found that the size of the dead zone (D) was in proportion to the ratio of gap distance (δ) to pore size (L), as in Fig. 1C. This implies that leukocytes fallen in the dead zone can be pulled toward and passed through the pores by the influence of fluid flow, which can give further improvement in CTC enrichment. This positive flow effect seems to increase when the size of the dead zone is less than half of that of the pore size. Therefore, we determined the gap distance to be equal to the pore size as the upper limit.

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[2] *Abbreviations used:* CTC, circulating tumor cell; GFP, green fluorescence protein; PBMC, peripheral blood mononuclear cell; IF, immunofluorescence; DAPI, 4′6-diamidino-2-phenylindole-2HCl.

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Initially, we designed a membrane filter of square pore array (pore size of 6.5 × 6.5 μm and gap size of 6 μm) based on the simulation results (Fig. 1D). Because the parylene-C coating process would increase the gap distance and decrease the pore size of the filter, and the coating thickness is 1 μm in general, we finally adjusted the size of the square pores to 8.5 × 8.5 μm for the nickel electroformed membrane filter. For the remaining processes of microfabrication, we followed the standard protocol.

We measured a recovery rate by loading onto the filter the known number of green fluorescence protein (GFP)-labeled cancer cells spiked in peripheral blood mononuclear cells (PBMCs), filtering, recovering the cells on the filter, and then counting the number of cells recovered. Three cancer cells of the GFP-labeled H358 cell line were spiked in 1 ml of normal whole blood (see Supplementary Table 1 in supplementary material), and then erythrocytes were removed by Ficoll–Hypaque density gradient centrifugation. The resulting PBMCs containing cancer cells were passed through the filter, all the retaining cells were recovered, and GFP-labeled cancer cells were counted under a fluorescence microscope (Nikon Eclipse Ti-S, Nikon, Tokyo, Japan). To increase the reliability of the experiments, six independent spiking tests were performed. Our filtration device showed the average recovery rate to be 72% even at a load of 3 cancer cells in total (Supplementary Table 1). This can be interpreted as indicating that for any successful recovery of cancer cells with our device, the cancer cell load should be approximately 2 or higher.

To check the average recovery rate over the range of the cell load, various numbers of GFP-labeled H358 cells were spiked in 1 ml of whole blood and processed as before (Fig. 2A). We found that the average recovery rate was 82.0 to 86.7% and that a higher number of cancer cells spiked in the blood produced lower coefficients of variation (CVs). This confirmed that our filtration device provides sensitive and reproducible recovery at least within the range of 1 to 100 cancer cell load.

In reality, CTCs are unlabeled and should be detected by immunofluorescence (IF) staining after collecting by an additional step of cytospin. For this reason, we compared the recovery rate at two different steps (i.e., up to filtration and up to cytospin) using two different cell loads: 30 and 100 GFP-labeled H358 cells spiked in 3 ml of whole blood (Fig. 2B). The recovery rates were more than 85% at up to the filtration step, which again confirmed the observation in Fig. 2A. But the recovery rates after the cytospin step were 50.7 and 61.5%, respectively. Because the higher load of cancer cells produced a higher overall efficiency, we presumed that an inevitable loss of 20 to 30% cancer cells was experienced in the step of cytospin.

To check the purity of cancer cells as an indication of degree of enrichment, leukocytes that survived this overall process were counted after cytospin at two different cell loads: 30 and 100 GFP-labeled cells spiked in whole blood (Fig. 2C). Because the numbers of recovered GFP-labeled H358 cells were 18 and 60, whereas those of leukocytes were approximately 400 and 500, the purity was found to be 4.5 ± 0.07% and 11.8 ± 1.04%, which could be made available for some molecular analysis.

Next, we moved to the unlabeled cancer cells to check the overall efficiency of the entire enrichment and characterization process, including IF. This would give us the practical recovery rate of cancer cells in the patient's blood using our filtration device. We spiked 10, 30, and 100 PC-9 cells in 3 ml of whole blood and performed our enrichment process (Fig. 2D and E). The resulting enriched PC-9 cells were recovered and processed as in the protocol for the IF staining with antibodies against CD45, cytokeratins, and then counterstained with DAPI (4′6-diamidino-2-phenylindole-2HCl). When the PC-9 cells (which were supposed to be $DAPI^+$, $CD45^-$, and $cytokeratins^+$) were counted, the average recovery rates at three different loads of cells were 40.6, 45.1, and 64.2%, respectively, which evidently showed the comparable recovery

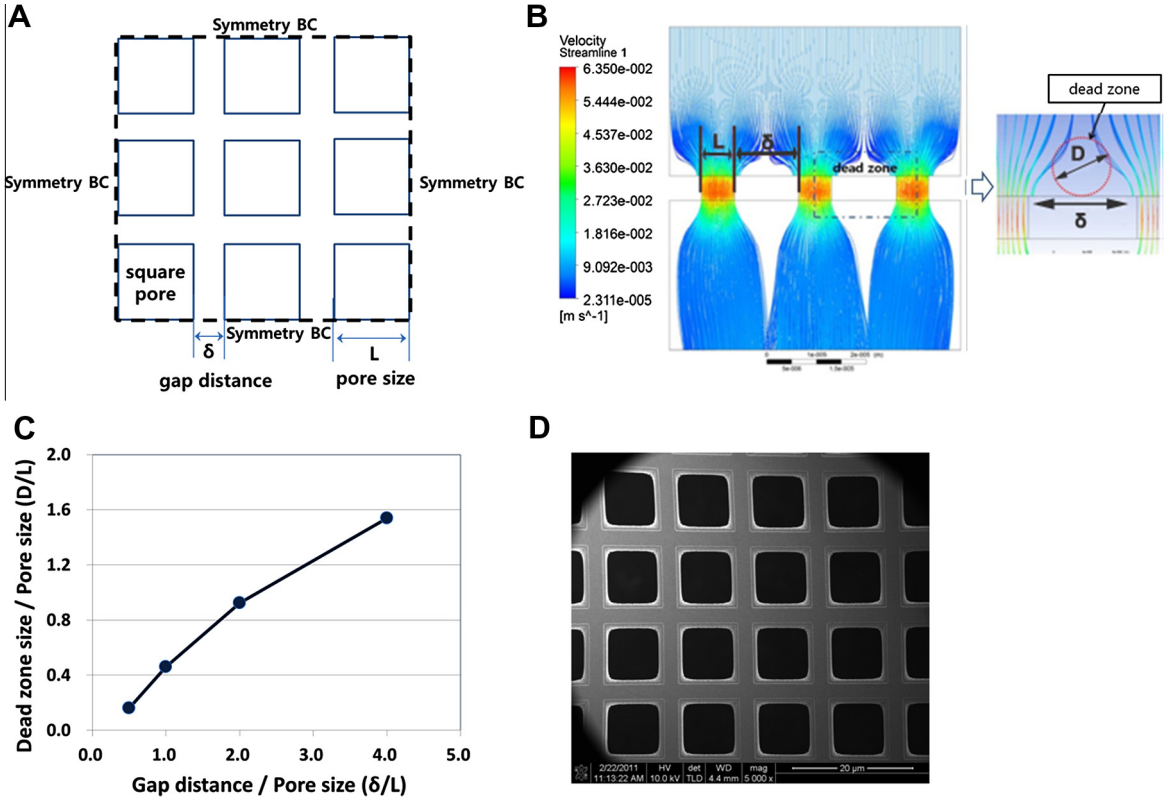

Fig.1. Optimal design of pore size and gap distance by reducing the dead zone. (A) Concept diagram of filter pore array (top view) and boundary condition (BC) for simulation. (B) Predicted streamline distribution and dead zones (L = 6.5 μm, δ/L = 2.0). (C) Predicted size of dead zone according to the gap distance (L = 6.5 μm). (D) Electroformed porous filter after the coating process considered.

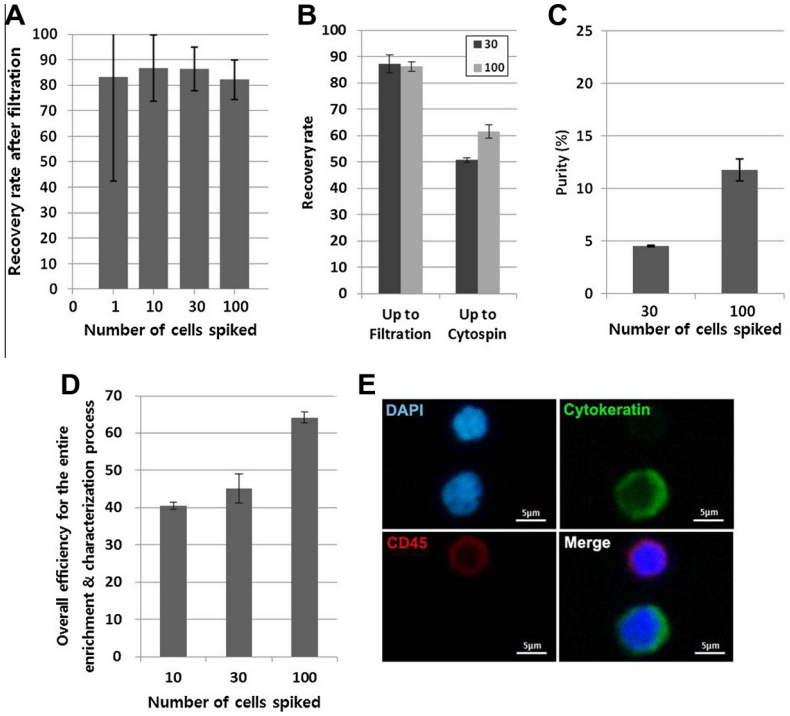

Fig.2. Recovery rates for the filtration process and overall efficiency of the entire enrichment and characterization process. (A) Recovery rates after filtration over the range of cancer cell load. Error bars indicate means ± standard deviations of twice with triplicate determinations. (B) Recovery rates at two different points in the process: up to filtration and up to cytospin. (C) Purity after cytospin at two different cancer cell loads: 30 and 100 GFP-labeled H358 cells spiked. (D) Overall efficiency of the entire enrichment and characterization process at three different cell loads: 10, 30, and 100 PC-9 cancer cells spiked in whole blood. (E) Immunofluorescent images of recovered PC-9 cancer cells. PC-9 cancer cells ($DAPI^+$, $CD45^-$, and $cytokeratins^+$) and leukocytes ($DAPI^+$, $CD45^+$, and $cytokeratins^-$). Error bars indicate means ± standard deviations of duplicate (B, C) or triplicate (D) determinations. All scale bars represent 5 μm.

rate at 30- and 100-cell loads regardless of GFP labeling. As a result, the overall efficiency of the entire enrichment and characterization process was equivalent to 48 to 76% of the recovery rate for the filtration and recovery step, increasing in proportion to the size of the cell load. To eliminate any possible involvement of the inherent property of lung cancer cell lines for this level of recovery rate, we tested the human breast cancer cell line, MDA–MB468, and the human colon cancer cell line, SW480, for the comparable overall efficiency of the entire enrichment (see Supplementary Fig. 1 in supplementary material). When 100 MDA–MB468 or SW480 cells were spiked in 3 ml of whole blood, the average recovery rates were approximately 50% in both cancer cell lines. These results can be interpreted as indicating that the high recovery rate with our filtration device is entirely due to the size of cancer cells. To further prove that the cell size is the primary determinant factor in this device, we used as a negative control the erythrocytes that are small enough to pass through the filter. Here, 20 μl of normal whole blood containing approximately 5×10^6 erythrocytes/μl was spiked in PBMCs and passed through our filtration device (see Supplementary Fig. 2 in supplementary material). We found no remaining erythrocytes after filtration. Based on our result that the average recovery rate of 40% is maintained at 1 cell load using our filtration device, the minimum required blood volume for detection of any cancer cells would be 3 ml provided that 1 ml of the patient's blood contains as little as 1 cancer cell.

In conclusion, we have developed a method for efficient CTC enrichment using a microfabricated filter that has high porosity and the optimized pore size and gaps distance for efficient negative selection of cancer cells in whole blood. High efficiency of cancer cell recovery was repeatedly confirmed through several independent tests with a range of cancer cells spiked in whole blood. This suggests that our microfabricated porous filter would be useful to enrich CTCs from a minimum load of 3 cells in a testing volume of patients' blood for subsequent characterization and molecular analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.05.016.

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