A microfluidic platform for high-purity separating circulating tumor cells at the single-cell level

Kun Wang¹b, Lin Zhoua, Simin Zhao³, Zule Chengb, Shihui Qiu²b, Yunxing Lu¹b, Zhenhua Wu⁴, Abdel Hady A. Abdel Wahabd, Hongju Maoa,⁎ Jianlong Zhaoa,⁎

¹ State Key Laboratory of Transducer Technology, Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Sciences, Shanghai 200050, China
² University of Chinese Academy of Sciences, Beijing 100000, China
³ School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200050, China
⁴ Department of Cancer Biology, National Cancer Institute, Cairo University, Cairo 11796, Egypt

ARTICLE INFO

Keywords:
CTCs
Microvalves
High-purity isolation
On-chip analysis

ABSTRACT

Circulating tumor cells (CTCs) are rare cancer cells that are shed from the tumors into the peripheral blood and are instrumental in distant metastasis. Early detection of CTCs can therefore improve prognoses and help design patient-specific treatment regimen. However, the current CTC isolation techniques have poor efficacy and selectivity, owing to the rarity and heterogeneity of the CTCs. We designed a microchip for integrated single-cell isolation of CTCs – based on cell size and immuno-phenotype – and analysis. Each isolation unit consisted of a trap channel, a bypass channel, and a release channel. The larger cells were preferentially captured at the trap channels and flushed out selectively via release microvalves according to their immuno-phenotype. The average recovery rate and purity of lung cancer cells isolated from a spiked WBC population were respectively 92.5% and 94% using the microchip, which were significantly higher compared to that obtained using anti-CD45 magnetic beads. In addition, the isolated cancer cells were analyzed on chip for the surface markers of epithelial mesenchymal transition. Taken together, the integrated microchip is a promising tool for the isolation and analysis of CTCs in the clinical setting.

1. Introduction

Circulating tumor cells (CTCs) are rare tumor cells that are shed from the primary tumor into the bloodstream, and play a vital role in distant metastasis [1–3]. A number of studies in recent years have focused on the importance of CTCs in disease development, as well as their therapeutic and prognostic potential [4–6]. Since high CTC load is associated with significantly worse prognosis [2,7], and specific surface markers or mutations can help identify/enrich this rare population [6,8], the CTCs are a promising biomarker for cancer diagnosis and treatment. In addition, unlike traditional biopsies, enrichment of CTCs is non-invasive and allows dynamic monitoring of cancer progression and metastasis.

However, the clinical application of CTC-based diagnosis is limited at present due to the extreme rarity and heterogeneity of the CTCs [9]. The effective enrichment of CTCs from billions of red blood cells and millions of white blood cells (WBCs) is indispensable for subsequent analysis and applications, and several immunological and physical separation techniques have been developed to isolate CTCs. Physical methods, including size-based filtration [10–12], dielectrophoresis [13,14], density gradient centrifugation [15] and inertia-based isolation [16–18], have very low sensitivity due to the overlapping physical characteristics of CTCs and WBCs. Immuno-separation relies on CTC-specific surface proteins that can be targeted using antibodies conjugated to magnetic beads or immobilized on the surface of microstructures [19]. However, immuno-separation has also shown low efficacy due to the heterogeneity of the CTCs, especially in terms of the differential expression levels of the epithelial mesenchymal transition (EMT) markers. The established methods for CTCs enrichment are mainly based on bulk analysis, and therefore limited by WBCs contamination and low purity of CTCs, rendering them unsuitable for the downstream analysis.

Abbreviations: CTCs, circulating tumor cells; WBCs, white blood cells; EMT, epithelial to mesenchymal transition; EpCAM, epithelial cell adhesion molecule; PDMS, Polydimethylsiloxane; BSA, bovine serum albumin; PBS, phosphate buffer saline; 0.05% PBST, 0.05% Tween-20, PBS; MBs, magnetic beads

⁎ Correspondence to: Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Science, No. 865 Changning Rd., Shanghai 200050, China.

E-mail addresses: hjmao@mail.sim.ac.cn (H. Mao), jlzhao@mail.sim.ac.cn (J. Zhao).

https://doi.org/10.1016/j.talanta.2019.03.035

Received 18 December 2018; Received in revised form 3 March 2019; Accepted 6 March 2019
Available online 12 March 2019

0039-9140/ © 2019 Elsevier B.V. All rights reserved.
To address these challenges, several single-cell isolation technologies have been explored. For example, fluorescence activated cell sorters (FACS) and microneedle-based micromanipulation have been used to enrich CTCs [20–22], but showed poor yield due to the intrinsic cell loss associated with FACS and adherence to the operating implements respectively. FACS lacked the detection sensitivity to separate rare cells and was usually considered to be more suitable to isolate cells that occupied the majority of the mixture [23]. Recent advances in microfluidic chip-based technologies, such as droplet-in-oil-based isolation [24], pneumatic membrane valving [25], and hydrodynamic cell traps [26], have opened new possibilities for single CTC isolation. However, cell isolation and analysis are usually not possible on a single chip, resulting in complex operations and cells loss.

Here, we designed an integrated microfluidic chip for efficient single-cell isolation, in order to enrich CTCs at high purity from WBCs. In our study, CTCs were isolated at the single-cell level based on their large size and specific biomarker, leading to the higher isolation purity. Isolated CTCs analysis was integrated with the isolation part in the single microchip, simplifying experimental operations and avoiding cell loss. The microchip consisted of two microchannels with different flow resistances to preferentially trap larger cells with lower flow resistance, followed by further selection of the differentially labeled CTCs. The enriched target cells were then intercepted at the analysis module by micropillars. The flow microvalves helped coordinate multiple isolation steps on a single chip, which not only reduced cells loss but also simplified the process. We also tested this microchip to differentiate between two lung cancer cell lines H446 and H1975 on the basis of EpCAM (epithelial cell adhesion molecule) and vimentin surface expression, and cell typing was achieved on EMT condition. In conclusion, this microfluidic device achieved high-purity isolation of CTCs at low cost and with operational ease, and is therefore promising for future clinical applications.

2. Material and Methods

2.1. Reagents

Polydimethylsiloxane (PDMS) base and curing agent (Sylgard 184) were purchased from Dow Corning Co. (NY, USA). SU8 3050 photoresist was from Micro Chem Corp (USA). Trichloro-(1H,1H,2H,2H-perfluorooctyl) silane and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). H446 and H1975 tumor cell lines were purchased from Cell Bank (Shanghai Institutes for Biological Sciences, Shanghai, China). Healthy individuals samples were from Shanghai Ann Biomedical Technology Co. Ltd. (Shanghai, China). RPMI 1640 medium was purchased from GIBCO Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and anti-CD45 magnetic beads (MBs) were from ThermoFisher Scientific (Victoria, Australia). A nuclear dye, 4,6-diamidino-2-phenylindole (DAPI), was purchased from Beyotime Institute of Biotechnology (USA). Phosphate buffer saline (PBS, 10 mM, pH = 7.4), 4% paraformaldehyde, Triton X-100, and Tween-20 were provided by Sangon Biotech Co., Ltd. (Shanghai, China). Ficoll-Paque Plus was from GE Healthcare Bio-Sciences (Upppsala, Sweden). Anti-CD45 fluorescein isothiocyanate-conjugated antibody (anti-CD45-FITC) was from BD Bioscience (USA). Anti-pan cytokeratin PE-conjugated antibody (anti-CK-PE), anti-EpCAM-FITC, and anti-vimentin-FITC were purchased from Abcam (Hong Kong).

2.2. Microchip design

The microchip device for integrated single-cell isolation and analysis consisted of three layers – the glass slide, micro-valve layer and single-cell function layer – for CTC capture, release, retrieval and analysis (Fig. 1). The single-cell function layer consisted of three inlets, two outlets, a micropillar-based analysis region, and ten isolation units for cells capture and release. Each isolation unit contained a trap channel, a bypass channel and a release channel, and a filter was fitted in front of the first unit to prevent impurities from blocking the channels. Larger cells were captured at the trap channel due to their lower flow resistance compared to the bypass channel, and then discharged through the release microvalves. The inlets and outlets were switched on/off by controlling the four flow microvalves.

2.3. Microchip fabrication

All structures were manufactured by microfabrication technologies, as outlined in Fig. 2A. For the functional layer, the microstructures on
The flow microvalves were driven by syringes (Fig. S1A) which were modified by screws, and valves on/off were realized via rotating the screw. Four modified syringes were fixed on acrylic plate according to the positions of flow microvalves. The release microvalves were driven by air pressure and controlled by electromagnetic relays connected to a 24 V DC power supply through a printed circuit board (PCB) (Fig. S1B). There were ten button switches combined on the PCB for the separate control of release microvalves.

2.4. Control of microvalves

The H446 and H1975 tumor cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C with 5% CO2. The cells were harvested using trypsin and centrifuged at 1500 rpm for 10 min. After fixing with 4% paraformaldehyde, the cells were blocked with 1% BSA to prevent non-specific binding, and re-suspended in PBS. Tumor cells were permeabilized with 0.2% Triton X-100 for 10 min and stained with anti-CK-PE, and WBCs were stained with anti-CD45-FITC. All cells were counterstained with DAPI and then used for subsequent tests.

2.5. Cells culture and staining

The microchip was vacuumed for 10 min, and then filled with medium supplemented with 10% FBS at 37 °C with 5% CO2. The cells were harvested using trypsin and centrifuged at 1500 rpm for 10 min. After fixing with 4% paraformaldehyde, the cells were blocked with 1% BSA to prevent non-specific binding, and re-suspended in PBS. Tumor cells were permeabilized with 0.2% Triton X-100 for 10 min and stained with anti-CK-PE, and WBCs were stained with anti-CD45-FITC. All cells were counterstained with DAPI and then used for subsequent tests.

2.6. WBC extraction

Blood was collected from healthy individuals into EDTA-coated vacutainer tubes, and WBCs were isolated by density-gradient centrifugation in Ficoll-Paque Plus. Briefly, 2 ml whole blood was diluted 1:1 with PBS, and layered on 3 ml Ficoll-Paque Plus in a 15 ml centrifuge tube. The samples were centrifuged at 400 g for 30 min at room temperature, and the mononuclear WBC layer between the plasma and erythrocyte sediment was carefully aspirated. The cells were pelleted at 200 g for 10 min, fixed with 4% paraformaldehyde and re-suspended in PBS.

2.7. Optimization of injection pressure

The proposed microchip was driven by positive pressure pump (Prinzen Biomedical Inc., Shanghai, China). To determine the optimal positive pressure for sample injection, we analyzed the capture and release efficiencies for the H446 and H1975 cells using different pressure conditions (5, 10, 20, 40 and 60 mbar). Briefly, cancer cells suspension and 0.05% PBST (0.05% Tween-20, PBS) were injected into the chip via inlet 1 and 2 respectively through the positive pump. Ten cancer cells were allowed to flow into the chip per test (total five tests) by controlling the flow microvalves. Cells were captured at the functional unit. After the tenth cell flowing into the chip, closed inlet 1 and opened inlet 2, and the conditions of cells capture and release were recorded under an inverted fluorescence microscope (IX51, Olympus, Tokyo, Japan). Capture efficiency was defined as the ratio of the number of captured to that of loaded cells. Cells captured at the trapping location were released easily (Video S1). Occasionally however, the captured cells would squeeze into the middle of the trap channels and block the release channels (Fig. S2), which delayed their release in some cases. The release efficiency was defined as the ratio of the number of successfully released to that of captured cells.

Supplementary material related to this article can be found online at doi:10.1016/j.talanta.2019.03.035.
2.9. Isolation of spiked cancer cells via immuno-magnetic separation

CD45 is a pan-leukocyte marker and is routinely used for WBC exclusion in various processes, including CTC enrichment [27,28]. Therefore, we compared the efficacy of our integrated microchip with anti-CD45 MBs. Operations of anti-CD45 MBs separation were shown as follows. Firstly, 3 μL of anti-CD45 MBs blocked with 1% BSA was added into 30 μL of the spiked sample in a tube, and incubated for 30 min at room temperature with gentle tilting and rotation. WBCs were bound by anti-CD45 MBs. After incubation, 30 μL of PBS was added into the tube to limit trapping of unbound cells. The tube was placed in a magnet for 5 min, and the supernatant containing cancer cells were transferred to a new tube. Finally, repeated the magnet separation step and collected the supernatant. Under the inverted fluorescence microscope, the recovered cancer cells were enumerated.

2.10. Characterization of isolated cancer cells

After isolated cancer cells were collected at analysis module, to further explore potential application of our microchip, expression of EpCAM and vimentin on cells has been compared by fluorescent immunoassay. To ensure the consistency of the experiment, both kinds of antibodies were labeled with the same fluorescence (FITC), and acted on cells sequentially. All flow microvalves were initially closed and the anti-vimentin-FITC was pumped through inlet 3 (sealed prior by a dust film). The cells were incubated at room temperature for 20 min, and the chip was washed with 0.05% PBST. Considering the fluorescence decay of anti-vimentin-FITC with time, a brief (5 min) photobleaching was imposed on cells to remove the fluorescence (Fig. S4). Finally, the cells were incubated with anti-EPAM-FITC and washed as described. After each staining, the fluorescence images were taken with the exposure time of 250 ms, and the mean fluorescence optical densities of the target proteins were quantified by Gray Quantifier software.

3. Results and discussion

3.1. Mechanism of single-cell isolation chip

The Takeuchi microfluidic design [29] was adopted for trapping the cells, and the schematics of the isolation unit are shown in Fig. 3A. The bypass channels were designed with the width to allow passage of only a single row of cells, and with higher flow resistance compared to the empty trap channels. Since the CTCs are usually larger than WBCs [11,12], the trap channel would preferentially capture the CTCs and larger WBCs while filtering out the small cells. Once the trap channel is blocked by a cell, its flow resistance increases dramatically and leads the following cells to the bypass channel and next functional unit, thus ensuring single-cell trapping. We further improved the microfluidic design by adding release channels and microvalves, which enabled the trapped cells to be flushed out of the trap channel as needed, and allowed them to flow to the designated outlet by controlling the flow microvalves.

The geometric dimension of channels were determined based on the following flow resistance equation [30]:

\[ R_f = \frac{H \mu L}{2(W + H)(W \cdot H)} \]  

where \( R_f \) is the flow resistance of the channel, \( \mu \) is the fluid’s viscosity, and \( H, W \) and \( L \) are the height, width and length of the channel respectively.

The typical mean diameters of H1975 and H446 cell lines are 17 μm and 15 μm respectively. Based on previous studies, 8 μm was set as the minimum gap in the trap channel [31,32], and the trapping location was designed to accommodate only one cell with a width of 15 μm (Fig. 3B). The length of the trap channel (L_trap) was 50 μm for efficient release. In order to guarantee a single row of cells, the width and the height of bypass channels were 20 μm and 17 μm respectively. Since the cells could not block the trap channel completely, when the ratio of the flow resistance of trap channels to bypass channels \( R_{f,\text{trap}}/R_{f,\text{bypass}} \) was too small, multi-cell trapping was occurred [33]. Therefore, the \( R_{f,\text{trap}} \) was designed to be slightly lower than the \( R_{f,\text{bypass}} \) in our microchip, and according to the Eq. (1), the length of the bypass channel (L_bypass) was the only variable affecting \( R_{f,\text{bypass}} \). To simplify the calculation, the width of curve in trap channel was replaced by its minimum width, so that the calculated \( R_{f,\text{trap}} \) was over the actual value. Accordingly, the \( L_{\text{bypass}} \) was determined to be 650 μm \( (R_{f,\text{trap}}/R_{f,\text{bypass}} = 0.74/0.77) \).

3.2. Numerical simulation of the isolation unit

To better understand the flow characteristics around the trap channel and verify the validity of determined dimensions, a numerical simulation of the microchip was computed using the COMSOL Multiphysics software. A 3D laminar flow model based on the steady-state Navier-Stokes’ equation, and the particle tracing model for fluid flow were used for the simulation. Water, with density 1000 kg/m³ and dynamic viscosity 0.001 Pa.s, was selected as the fluid, and an incompressible flow model was applied. The boundary conditions of 300 μm/s and zero pressure were used at the inlet and outlet respectively, and no-slip conditions were applied to all walls. Particles introduced in the simulation were released one particle at a time with a coupling velocity from the inlet of the laminar flow model at the projected plane grid, and drag force based on Stokes’ equation was added in the particle tracing model. A convergence test based on the ratio of flow flux between bypass channels and trap channels was performed for various mesh sizes (Fig. S5), and physical-controlled type with normal elements size served as an optimal mesh for the geometric model.

The flow velocity profile and particles trajectory based on the above calculated dimensions are shown in Fig. 4A. The flow velocity profile was demonstrated at the center cross section of the chip, and the maximum flow velocity was achieved in the trap channel (Fig. 4A-1).
Small flow resistance meant a larger flow flux, which could be calculated by surface integration instrument. Consistent with the lower theoretical flow resistance, the trap channel had larger flow flux ($5.67 \times 10^{-8} \text{m}^3/\text{s}$) than that of the bypass channel ($2.82 \times 10^{-8} \text{m}^3/\text{s}$). While the trapping location was occupied (Fig. 4A-2), the maximum flow velocity was occurred in the bypass channel where the flow flux ($6.82 \times 10^{-8} \text{m}^3/\text{s}$) was larger than that of the trap channel ($1.97 \times 10^{-8} \text{m}^3/\text{s}$). The trajectory of the cell flow was computed in the particle tracing model, and preferential paths under vacant and occupied trapping location are shown in Fig. 4A-3 and A-4 respectively. Therefore, the determined dimensions of the isolation unit were feasible for single-cell trapping, and the capture and release of cells under the simulated conditions are shown in Fig. 4B and C. A lower flow velocity was used for better visualization of cell release.

3.3. Optimization of injection pressure

The effect of pressure on the capture and release efficiencies are shown in Fig. 5. For the H446 cells, both were highly stable at injection pressure lower than 10 mbar. However, when the pressure was increased to over 20 mbar, both capture and release efficiencies steadily declined with increasing pressure. The cells were subjected to the flow pressure of the fluid at all times, which also increased with the injection pressure till a certain threshold when the cells started to squeeze out of the trap channel, resulting in reduced capture efficiency. The larger H1975 cells on the other hand could not squeeze out of the trapping locations, resulting in higher capture and release efficiencies between 5 mbar and 60 mbar injection pressure. The injection pressure of 10 mbar resulted in capture and release efficiencies of over 97% for both cell lines, and was therefore used for subsequent experiments.

3.4. Evaluation of capture efficiency and purity in spiked samples

To determine the potential of our microchip in separating CTCs from numerous WBCs, we spiked WBC suspensions of varying cell densities (1000, 2000, 4000, 7000 and 10000 cells/µL) with 30 H446 cells/µL, in order to simulate the rare CTCs in the blood. The spiked samples were run under the optimized conditions described so far, and the cancer cells were captured accordingly. Occasionally, WBCs might be intercepted in the occupied trap channel, and collected with cancer cells. So purity was determined by the ratio of the number of cancer cells to that of total cells collected.

The capture efficiency and purity of the isolated cancer cells relative to contaminating WBCs are summarized in Fig. 6A. As shown in Video S1, the smaller WBCs passed through trap channels, while the larger WBCs clustered around the trapping locations, thus hampering cancer cell capture. Therefore, the capture efficiency declined from 99% to 92% with increasing WBC count. Furthermore, the trap channel could...
not be fully blocked by the cancer cells and some individual WBCs would be stuck inside especially at high WBC concentration. Consistent with this, the purity of the captured H446 cells decreased significantly from 92% to less than 80% as the WBC concentration increased from 2000 to 10,000 cell/μL. These “stuck” WBCs could be avoided by reducing the capability of trap location or increasing the flow resistance of trap channels. The bulk of the WBCs flowed through outlet 2, and captured cancer cells were collected at analysis module (Fig. 6B). Taken together, our modified chip successfully isolated rare cells from WBC-loaded samples with high capture efficiency and purity, indicating its potential in clinical CTCs separation.

3.5. Comparison of the capture efficiencies of the integrated microchip and immuno-magnetic separation

WBC suspensions (100,000 cells) were spiked with 5, 10, 20 and 30 H1975 or H446 cells, and the number and purity of the recovered cancer cells were evaluated following either microfluidic or immuno-magnetic separation. As shown in Fig. 7A, the average recovery rate for the microchip was > 92.5% for both cell lines, while that for MBs was only 65.2% for the H1975 cells. In addition, almost no H446 cells were recovered using the anti-CD45 MBs. The massive target cell loss in immuno-magnetic separation was mainly due to the non-specific adsorption between the MBs and cancer cells, and their negative migration due to bead motion. In addition, the purity of cells isolated by the microchip ranged from 83% to 94%, compared to the extremely low purity of < 1% in immuno-magnetic separation, a bulk isolation technique that cannot remove all extraneous cells, especially when the latter are present at high density. In conclusion, our microchip platform isolated rare cancer cells from WBC suspension with high purity compared to the standard immuno-magnetic separation using anti-CD45 MBs. However, the microchip took roughly 1–1.5 h to isolate 10 cancer cells from the spiked samples, compared to the 40–50 min operational time of MBs.

3.6. Characterization of the isolated cancer cells

Cancer cells undergo EMT to enhance their ability to invade the adjacent blood vessels, and result in distant metastasis. EpCAM and vimentin are the typical epithelial and mesenchymal biomarkers, and are respectively downregulated and upregulated during EMT. The expression levels of EpCAM and vimentin on the captured H446 and H1975 cells are shown in Fig. 8. While there were considerable variations across individual cells, the overall expression levels of vimentin were significantly higher compared to EpCAM in the H446 cells, and the opposite trend was observed for the H1975 cells. The EpCAM and vimentin levels were also measured by flow cytometry to verify the microchip results, and were found to be consistent. Therefore, the integrated microchip can not only isolate single rare cancer cells, but also type them on chip. This is highly promising for the study of heterogeneous cancer cell populations.

In summary, to better demonstrate the superiority of the microchip, injection pressures were optimized and capture and release efficiencies of over 97% were achieved for both cell lines at the injection pressure of 10 mbar. Under the optimal condition, H446 cells in spiked samples were isolated with high purity ranging from 92% to less than 80% as the WBC concentration increased from 2000 to 10,000 cell/μL. In comparison with immuno-magnetic separation, our microchip showed favorable performance, especially in isolation purity. Ultimately, EpCAM and vimentin on isolated cancer cells were successfully characterized by immunostaining in the microchip, and on-chip cells typing...
was achieved contributing to heterogeneity study of cancer cells.

4. Conclusion

An integrated microfluidic chip based on microvalves was fabricated for CTC isolation and analysis with high purity. Compared with other microfluidic systems for CTC isolation based on filter membrane, immunocapture and inertial separation, the proposed microchip achieved cells manipulation and analysis at the single-cell level, and introduced microvalves to enhance its operability. Integrated analysis module avoided potential cell loss and expanded functions of the microchip. High capture efficiency and release efficiency were achieved for H446 and H1975 cells under the optimal injection pressure. Compared to immuno-magnetic separation, the microchip achieved high recovery rate and purity of cancer cells from WBC suspension by minimizing cell loss. In addition, the H446 and H1975 cells were also successfully characterized on chip for EMT markers. In future work, in order to better apply our microchip in clinical CTC study, the size of channels in functional unit will be improved according to the size of clinical cells. Operations of flow microvalves will also be automated by electromagnetic relays and programmable logic devices for more convenient control. Our microchip is currently being further optimized for clinical application, and is a highly promising option for integrated CTC enrichment and analysis.

Acknowledgements

This study was supported by Grants from National Key Research and Development Program of China (No. 2018YFA0108202 and 2017YFA0205300), National Natural Science Foundation of China (Nos. 61571429, 61801464 and 61801465), the STS Project of the Chinese Academy of Sciences (KJF-STS-SCYD-120) and the Science and Technology Commission of Shanghai Municipality (16410711800, 14391901900 and 17511104402).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.03.035.

References


