An integrated microfluidic device for rapid and high-sensitivity analysis of circulating tumor cells

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Recently there has been a more focus on the development of an efficient technique for detection of circulating tumor cells (CTCs), due to their significance in prognosis and therapy of metastatic cancer. However, it remains a challenge because of the low count of CTCs in the blood. Herein, a rapid and high-sensitivity approach for CTCs detection using an integrated microfluidic system, consisting of a deterministic lateral displacement (DLD) isolating structure, an automatic purifying device with CD45-labeled immunomagnetic beads and a capturing platform coated with rat-tail collagen was reported. We observed high capture rate of 90%, purity of about 50% and viability of more than 90% at the high throughput of 1 mL/min by capturing green fluorescent protein (GFP)-positive cells from blood. Further capturing of CTCs from metastatic cancers patients revealed a positive capture rate of 83.3%. Furthermore, our device was compared with CellSearch system via parallel analysis of 30 cancer patients, to find no significant difference between the capture efficiency of both methods. However, our device displayed advantage in terms of time, sample volume and cost for analysis. Thus, our integrated device with sterile environment and convenient use will be a promising platform for CTCs detection with potential clinical application.

Cancer has become a major public health problem, due to its association with most number of patient deaths worldwide1. Primarily, it involved tumor cell metastasis2,3, wherein tumor cells escape the primary lesions, and penetrate into lymphatic or blood vessel system, through which they migrate comfortably to distant places and form metastatic colonies4. Circulating tumor cells (CTCs) are defined as tumor cells typically present in the body circulating system. They have generally been considered as “liquid biopsies”, which can be used as a minimally invasive method for diagnosis and assessing cancer status, estimating prognosis, evaluating efficacy and instructing personal therapy5–7. However, there has been a tremendous challenge in detecting and capturing these CTCs due to their extreme rarity, with the presence of only 1 to 100 CTCs in 109 blood cells8.

Apparentely, in the past decade, numerous methods have been developed to isolate the CTCs population based on their properties, particularly their physical or biological properties that differentiate them from blood cells. These mainly include size filtration, density gradient and di-electrophoresis9–13, which are label-free, convenient and low cost, but have some limitations. For instance, in size filtration method, the clogging due to other blood cell types, makes CTCs capture difficult14. Recently the immune-based CTCs separation method involving the heterogeneous expression of surface markers, such as epithelial cell adhesion molecule (EpCAM) has been shown to be useful15. The CellSearch system is the only commercial detection system approved by the US Food and Drug Administration (FDA), which uses ferro-fluids conjugated with anti-EpCAM antibody, to magnetically enrich CTCs16, and has already been used in the clinic to monitor the response of patients with breast, prostate, and colorectal cancers to specific treatments. Nevertheless, a multi-institutional study involving 177 patients

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with measurable metastatic breast cancer, reported that CTCs were detected in only 61% of the patients by this system17. This has mainly been attributed to the heterogeneous expression of EpCAM18–24. Despite the presence of many potential approaches to isolate and purify CTCs, there several issues must be addressed to realize the full potential of CTCs as a diagnostic and research tool.

Thus, to overcome the issues, microfluidic technology, which literally represents lab on a chip, and has the advantages of high throughput, integration, adjustment and management, with low cost and small volume, may solve the problems. Until now, various CTC detection methods using microfluidic system have been analyzed, with different cell separation mechanisms, such as size or deformability based isolation, dielectrophoresis, affinity chromatography and magnetic forces25–28. These physical properties based separation systems offer the advantages of label-free sorting, high throughput, and low cost29–30. However, these methods often results in poor capture rate and poor purity by ignoring the interference of WBCs, and CTCs are also susceptible to damage from large mechanical stresses27. Moreover, the immune-based methods to detect CTCs also display few limitations. For instance, it costs too much time for CTCs to react with the antibodies coated on the chip due to the interference and hindrance by other blood components. Subsequently, these factors result in low capture efficiency. Thus, if blood samples could somehow be processed early, then affinity based capturing of CTCs can yield better results.

Hence, our group has developed an integrated microfluidic system that has the potential to overcome the obstacles of any individual method. In the past, we developed an integrated microfluidic system, that included a DLD and a fishbone structure31. However, to avoid clogging, blood had to be diluted before adding to the microchannels of the DLD structure. And some WBCs might get stuck in the corners of the fishbone structure. In this study, we have tried to improve the DLD structure, where blood samples needn’t to be diluted to have more convenient and rapid detection. In addition, we have also tried to develop an automatic magnetic purifying device for negative isolation, which decrease the false positive rate and enhance CTCs purity. In fact with our developed method, CTCs will be captured by the sequential elimination of blood cells in the novel method, where nearly all RBCs and platelets and more than 90% of the WBCs will be first depleted by DLD isolating chip. The remaining small percentage of WBCs will be purified and removed by the automatic immunomagnetic purifying device. Finally, CTCs will be captured on a platform coated with rat-tail collagen, where CTCs are distinguished based on the immune affinity31. The integrated system has demonstrated its usefulness not only in artificially spiked blood samples, but also in clinical samples obtained from various advance cancer patients. We observed a capture rate of around 90%, capture purity of about 50% at the high throughput of 1 mL/min, and the viability of CTCs detected through this method reached more than 90%. Furthermore capturing of CTCs using the novel device specifically from the peripheral blood of patients with various advanced metastatic cancers revealed a positive capture rate of 83.3%. In addition, the efficiency of the novel device was compared with CellSearch system, and we observed equal and excellent capture efficiency just as it. What’s more, the results of CTCs detection were in accordance with the clinical condition. Nevertheless, the novel device displayed the advantages in terms of less time, volume and cost required for the analysis. Finally, it is an integrated system which is automatic, sterile and has the convenience of manipulation for universal adaptation, based on the requirement.

Results

Design and functioning of the combined microfluidic chip. The newly developed DLD chip was shown in (Fig. 1b). We introduced one chamber with dimensions of 68 mm long, 3.5 mm wide and 50 μm high, two inlets, and two outlets, which was instead of the 8 parallel chambers with dimensions of 42 mm long, 3.5 mm wide and 30 μm high, only one inlet and outlet in the previous design. Moreover, the size of the microarray was also optimized. The new microarray consisted of microposts with 25 μm radius, 30 μm horizontal gaps and 20 μm vertical gaps, with a tilted angle of 3.2 degrees towards the fluid flow direction. The cell separation mechanism in DLD array was based on the interaction of cells with microposts under low Reynolds number conditions. As shown in (Fig. 1d,e), microposts can divide the flow of fluid into several narrow streams, and when cells pass through the gaps in microposts, smaller cells with radius less than the width of first stream L, are able to continue following the stream. On the contrary, large cells with radius larger than the width of first stream L, are forced to follow the second stream with a deterministic path. Based on this analysis, 2L can be regarded as the critical cells size. According to the theory proposed by Loutherback et al.32, the critical separation size of our DLD structure was 6.75 μm. Compared with the previous design, the optimization of the new structure led us to isolate blood samples without diluting with a high throughput of about 1 mL/min, and 3 mL blood would be detected within 3 min. After the first isolation, CTCs with the residual WBCs were collected and subjected to further purification.

Next, a negative isolation based automatic magnetic purifying device was developed instead of the fishbone chip labeled with anti-EpCAM antibody. The size of chip was automatic, sterile and has the convenience of manipulation for universal adaptation, based on the requirement. The chip chamber was fabricated with a 20 (Length) × 10 (Width) × 3 (Height) mm3 size, and a corresponding computer code was written to move the magnet within the borders of the chamber. The movement of the magnet was in a rectangular shape, as it moved from one length to another and one width to another, with a round trip.

In addition, the best parameters were also standardized, including the binding rate (the number of WBCs binding to CD45 dynabeads/all the WBCs) and the binding time. The human WBCs were isolated from the healthy volunteers by using RBC Lysis Buffer (1:10, eBioscience) and CD45 immunomagnetic dynabeads (Invitrogen, USA). As the recommended ratio and time were 5:1 (Dynabeads/WBCs) at 30 min, different ratios (5:1, 10:1, 15:1) and different time (10 min, 20 min, 30 min) points were tried. The binding time of 20 min displayed the binding rate of 40.09% (5:1), 82.95% (10:1) and 85.25% (15:1), (Fig. 2a). However at a ratio of 10:1, the binding rate was 36.9% (10 min), 82.95% (20 min) and 85.6% (30 min), (Fig. 2b) Finally, the 10:1 ratio and 20 min time were identified as best parameters, and under this condition, WBCs were binding to the dynabeads sufficiently (Fig. 2c). Further, to verify the advantage of the novel developed device, the similar experiments were
performed with the traditional method using DH-II Rotary mixing apparatus (Xinzhi, Ningbo, China) to remove WBCs, and observed that removal rate with the traditional method was about 10% lower than the automatic device.

**Analysis of the capture efficiency of lung cancer cell line by the novel device.** To mimic the capturing of actual CTCs from the blood samples, we generated a model where peripheral blood of healthy volunteer was spiked with H1299-GFP cell line at concentrations of 10¹ to 10⁵ cells/mL. The spiked blood samples were introduced smoothly into the integrated system, (Fig. 3a1–a3). The GFP expression in H1299 cells led us to easily track these cells from rest of the blood cells. The capture rate and capture purity were two important parameters tested to evaluate the system. We separately calculated the capture rate of DLD isolating chip, the automatic purifying device and the integrated system, respectively. The DLD isolating chip showed the H1299-GFP cells capture rate of 88.9%, 94.5%, 93.4%, 94.3% and 93.7% among the blood when spiked with a concentration of 10¹/mL to 10⁵/mL, respectively (Fig. 3b1). Similarly, the capture rate of automatic magnetic device was 87.5%, 97.0%, 95.5%, 96.7% and 95.5% respectively for different concentrations (Fig. 3b2). Moreover, the integrated system displayed a capture rate of 77.8%, 91.7%, 89.2%, 91.2% and 89.5%, respectively for similar concentrations of H1299-GFP cells (Fig. 3b3). Overall, the capture rate was around 90%. In terms of capture purity, we observed about 50% at the 10³/mL concentration, and it increased in parallel with increase in H1299-GFP cell concentration.

**Analysis of the viability of captured H1299-GFP cancer cells.** Next, the viability of the captured H1299-GFP cells were measured, using Hoechst/PI assay (Fig. 4a). The numbers of the viable H1299 cells in the blood samples were observed and counted under microscope, and later compared with the cells cultured in 24 well plates at room temperature. The results showed that captured H1299 cells had viability of 90.2% after 30 minutes culturing in a plate, but it decreased to 87.3% after 1 hr and 85.7% after 2 hrs of culturing at 37°C. This rate was similar to the cell viability of the control cells that did not pass through the integrated system. The viability of the control cells was 91.4% after 30 min, while it reduced to 88.5% after 1 hr and 86.5% after 2 hrs (Fig. 4b). Further culturing of H1299 cells captured via the integrated device at 37°C with 5% CO₂, led them to adhere to the surface of the plate and proliferate similar to the control cells, and thus suggesting that these cells were not
Figure 2. The binding rate of the purifying device under different ratios of dynabead/WBC and different binding time. (a) When the binding time was 20 minutes, at different ratios, the different binding rate was shown. (b) When the ratio was 10:1, at different time points, the different binding rate was shown. (c) The picture showed when CD45 dynabeads were mixed with WBCs for 20 minute at 10:1 ratio, magnification, X200. The smaller spheres were CD45 dynabeads with the diameter of 4.5 μm, while the bigger size structures were WBCs isolated from the peripheral blood of healthy donors.

Figure 3. Capture rate of H1299-GFP cells in the novel device (a1–a3) showed merged bright and fluorescent field images of cancer cells (H1299-GFP) and blood cells in the isolating system (a1) (magnification, X100), purifying system (a2) and capture system (a3) (magnification, X200). (b1–b3) showed capturing rate of different concentrations of H1299 cancer cells at DLD (b1); at CD45 immunomagnetic device (b2); and in the integrated device (b3). The original concentration of H1299 cancer cells in (a1–a3) was 10^5/mL.
damaged (Fig. 4c). The expression of green fluorescence further indicated that these originally spiked cells into the whole blood, can survive many generations.

**Detection of CTCs from patient blood samples.** Finally, we tested the developed integrated system for capturing CTCs from the advanced cancer patients' blood samples. The six cases of patients with advance cancers, including 4 cases with metastatic lung cancer, and 2 cases with metastatic liver cancer, along with 2 healthy volunteers as controls, were involved in the study. The 3 mL blood sample obtained from each subject for CTCs detection was run through the integrated device. The identity of the captured CTCs was confirmed by the positive expression of EpCAM or CK proteins as shown with a green fluorescent signal, and simultaneous negative expression of blood cell leukocyte marker, CD45, a red fluorescent signal (Fig. 5a). Among the 6 patients, we identified CTCs in 5 patients (83.3% positive detection rate), while the remaining patient showed no CTCs. The average numbers of CTC identified were between 3 and 6, with an average of 3.8/mL of peripheral blood. However, we did not observe any CTCs in both healthy controls (Fig. 5b).

**Comparison of the efficiency of the integrated device with CellSearch System.** To further testify the efficiency of the novel device, CTCs detection from 30 patients was compared in parallel with CellSearch
In the past decade, an increasing number of microfluidic devices have been developed to assist efficient CTC isolation and detection, with the positive rate of 70.0%, which corresponded to the clinical condition. CTCs were detected in patients at I or II stage. However, 16 in 23 patients at III or IV stage were positive in CTCs. Few studies have reported that the capture efficiency of CTCs is equally excellent as the CellSearch System. Moreover, from the results, it was found that few CTCs were captured in patients with I or II stage. However, 16 in 23 patients at III or IV stage were positive in CTCs detection, with the positive rate of 70.0%, which corresponded to the clinical condition.

However, the novel device still displayed the advantage in terms of requiring less volume of blood sample, less expense, and even less time for analysis as indicated in (Table 2).

### Discussion

CTCs acts as “seeds” of incurable metastasis and thus represent a promising and effective alternative to invasive tumor biopsies to detect, monitor and combat solid tumors in patients. However, the presence of very few numbers of CTCs in the blood makes their detection and capture hard and thus hinders the realization of them being an important indicator for diagnosis, prognosis and therapeutic targets of metastatic cancer.

In the past decade, an increasing number of microfluidic devices have been developed to assist efficient CTC isolation and detection, and are broadly based on physical and/or immune-based separation. Among physical properties, size-based isolation is the most widely used, which employs size-based membrane filters and size/deformation-based hydrodynamic methods. As alluded earlier, the disadvantages of these methods cannot be ignored. For instance, a study by Li et al., used surface-micromachined PDMS microfiltration membrane (PMM) to isolate WBCs from lysed blood. It resulted in the filtration throughput of 1 mL/h with the recovery rate of 27.4 ± 4.9%. Thus it consumed much time and resulted in low capture rate. Another study by Loutherback et al. developed a deterministic method to separate cancer cells from blood at 10 mL/min rate with the capture rate of 85%. This study, although had enough throughput, but the capture rate was not very efficient, and moreover resulted in capturing of similar-sized WBCs along with CTCs. Based on dielectrophoretic (DEP) force, Gupta et al. have designed a microfluidic CTC-isolation device that exploited the varying directionality of the DEP force and acted on cancer and blood cells in a suitable electric field with capture efficiency of around 70% and a throughput of 1 mL/h, but it also had some issues like low capture efficiency and throughput. However, on the other side, immune-based separation methods, for example, herringbone-chip instead of microarrays functionalized with antibodies by Li et al., increased the capture rate up to 90%. But, to achieve sufficient contact with the antibodies, the flow rate has to be as slow as 1 mL/h and blood has to be lysed. Thus it would lead to huge volumes and would require too much time.

In this study, we have developed an integrated microfluidic system, which overcame the defects of single platform, had high throughput of 1 mL/min, showed high capture efficiency and excellent viability of about 90%, a fine capture purity of about 50%, in comparison to most of the other developed methods. Also, this system integrates every single device together as sterile, automatic and more convenient, which not only can be translated to the clinical application, but can also help in further analysis of CTCs by single cell sequencing. In this integrated system, we improved the DLD design to be used rapidly and conveniently in high throughput conditions, without diluting the blood samples. Furthermore, compared to other negative isolation systems, our automatic immunomagnetic purifying device was able to efficiently remove WBCs and exclude their interference in CTCs purification. We showed that 20 min time period was sufficient for WBCs to bind with CD45 dynabeads. The reduced time showed decreased removal rate. When the incubation time was 10 min, the removal rate was further decreased by half. However, in most of the previous approaches, the mixing time was less than 10 min and this can explain the partial removal of WBCs. As we know, the presence of WBCs results in false positive, which subsequently led to misunderstanding of the patient condition, and indicate towards further analysis of
CTCs. The novel device has demonstrated the ability to capture pure CTCs. In addition, this device is automatic and small, and it not only substituted for the manual, tedious and heavy method, but also increased the removal rate by 10%.

Next, to assess the efficiency of our integrated device in clinic, we analyzed the peripheral blood of 6 patients with advanced metastatic cancers and 2 healthy volunteers. We reported that 5 out of 6 patients displayed an average of 3.8 CTCs/mL blood with a positive rate 83.3%, which was consistent with the clinical condition. Further analysis of the clinical history of the case, where we fail to capture CTCs, revealed that the patient suffered from liver cancer and had intrahepatic metastases, which could somehow restrict the availability of CTC to peripheral blood.

It is worth mentioning that we also tested the efficiency of the novel device against CellSearch System (Janssen Diagnostics, LLC.) for CTCs detection. The parallel analysis of blood samples from 30 cancer patients revealed that there was no significant difference in the capture efficiency by the two methods, with p value of >0.05.

Moreover, it was found that few CTC was detected in patients at I or II stage. However, 16 in 23 patients at III or IV stage were positive in CTCs detection, with the positive rate 70.0%, which corresponded to the clinical condition. And, the view that CTCs occurred in the peripheral blood of patients with metastatic cancer was confirmed.

Table 1. Comparison of the efficiency between CellSearch System and the novel device. Group A represents CellSearch system based CTCs analysis in 7.5 mL peripheral blood, while Group B represents for the same with the novel device in 3 mL. S denotes surgery, C denotes chemotherapy, E denotes Endocrinotherapy, M denotes Molecular targeting treatment, and R denotes radiotherapy.

| Patient | Gender | Age | Diagnosis | Stage | Treatment | Count(n) | A(7.5 mL) | B(3 mL) |
|---------|--------|-----|-----------|-------|-----------|---------|-----------|---------|
| 1       | Female | 57  | Breast Cancer | IIB   | S,E       | 0       | 2         |         |
| 2       | Female | 64  | Colon Cancer  | IIIIC  | S,C       | 0       | 16        |         |
| 3       | Male   | 62  | Thymoma      | IVB   | S,C       | 80      | 15        |         |
| 4       | Female | 55  | Breast Cancer | IIA   | S,C       | 0       | 0         |         |
| 5       | Male   | 82  | Lung Cancer   | IVA   | S,C,R     | 0       | 0         |         |
| 6       | Female | 45  | Breast Cancer | I     | S         | 0       | 0         |         |
| 7       | Female | 65  | Breast Cancer | IIA   | S,C       | 0       | 0         |         |
| 8       | Male   | 55  | Lung Cancer   | IIIA  | S,C       | 0       | 0         |         |
| 9       | Male   | 68  | Lung Cancer   | IIIB  | M         | 3       | 6         |         |
| 10      | Male   | 58  | Bladder Cancer | IIIA  | C         | 0       | 9         |         |
| 11      | Male   | 78  | Lung Cancer   | IVA   | S,M       | 0       | 0         |         |
| 12      | Female | 76  | Breast Cancer | IIB   | S,C       | 0       | 0         |         |
| 13      | Female | 46  | Breast Cancer | IIIB  | S,C       | 0       | 0         |         |
| 14      | Female | 60  | Lung Cancer   | IIIA  | S,C,M     | 0       | 0         |         |
| 15      | Female | 76  | Breast Cancer | IIB   | S,C,M     | 0       | 0         |         |
| 16      | Male   | 60  | Colon Cancer  | IIIIB  | S,C       | 30      | 15        |         |
| 17      | Female | 57  | Lung Cancer   | IVA   | C         | 2       | 9         |         |
| 18      | Male   | 44  | Lung Cancer   | IVA   | C,M       | 0       | 4         |         |
| 19      | Male   | 72  | Myeloma       | IVB   | C         | 0       | 0         |         |
| 20      | Female | 67  | Colon Cancer  | IVA   | C         | 3       | 2         |         |
| 21      | Male   | 72  | Lung Cancer   | IVB   | C         | 35      | 16        |         |
| 22      | Female | 63  | Lung Cancer   | IVB   | C         | 10      | 5         |         |
| 23      | Male   | 72  | Lung Cancer   | IVA   | C         | 0       | 3         |         |
| 24      | Male   | 69  | Lung Cancer   | IIIB  | C,R       | 3       | 4         |         |
| 25      | Male   | 64  | Colon Cancer  | IIIIA  | S,C       | 0       | 1         |         |
| 26      | Male   | 65  | Lung Cancer   | IIIA  | S,C       | 64      | 20        |         |
| 27      | Female | 70  | Breast Cancer | IIIA  | S,C       | 0       | 2         |         |
| 28      | Female | 68  | Lung Cancer   | IVA   | C         | 15      | 3         |         |
| 29      | Female | 57  | Lung Cancer   | IVA   | E         | 0       | 0         |         |
| 30      | Female | 72  | Myeloma       | IVB   | C         | 0       | 0         |         |

Table 2. Comparison of CellSearch System and Microfluidic System in CTCs detection. “A” stands for CellSearch System, “B” stands for the novel device.
Materials and Methods

Microfluidic chip fabrication. This entire device consisted of three parts. The DLD isolating structure and the automatic magnetic purifying device were fabricated on the microfluidic chip (Fig. 1a). The schematic structure of DLD chip and automatic purifying device were shown (Fig. 1b,c). The DLD chip had two inlets, two outlets with triangle pillar posts among them. The chip was fabricated using polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI, USA) which was molded on a master, prepared by spin-coating a 100-μm layer of SU8-2035 negative photo resist (Microchem Corp., Newton, MA, USA) onto a glass wafer and patterned by photolithography48. The Sylgard 184 PDMS base and curing agent were mixed thoroughly in a 10:1 mass ratio, degassed under vacuum, and poured onto the master. The polymer was oven-cured at 80 °C for 1 hr, and after cooling, the PDMS layer was gently peeled from the master and trimmed to specific size. Inlet and outlet holes were punched out of the PDMS to form reservoirs for the introduction of liquid. The PDMS was bonded irreversibly to a glass slide (1.2 mm thick) after 60 s oxygen plasma treatment. Next, the microfluidic device was sterilized under 110 kpa at 120 °C for 0.5 hr and UV sterilized for 30 min before use.

Additionally, similar to the structure developed by Grzybowski et al.49, an automatic purifying device consisting of a chip, an electronic control system, and a permanent magnet was developed. This chip was fabricated using Polymethylmethacrylate, and the microchannels were created using a Laser Engraving Machine (LS100CO2, Gravograph, Shanghai). The chip was fabricated with three layers, which were bonded to each other using a Thermocompressor (CARVER, USA) at 120 °C for approximately 10 minutes. The inlet was connected to the outlet of the DLD device. The outlet was used for adding CD45 dynabeads and for collecting CTCs. The reaction chamber was cuboid in shape. The diameter of the inlet and the outlet were both 1.5 mm, and movement of the magnet was regulated using an electrode controlled by a C Language-based computer program. The diameter of the magnet was 5 mm. The magnet attracted the dynabeads to the bottom of the chamber; thus, the CD45 dynabeads used to bind WBCs were removed with the magnet.

Rat-tail collagen coating. The standard CTCs purification protocol would leave few WBCs among them and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. To accomplish this, rat-tail collagen (0.012 mg/mL, Solarbio, China) was coated surface50. and thus they remain to be identified. 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Trypsin-ethylene diamine tetra acetic acid (EDTA) (Invitrogen, CA) at 37 °C for 2 min to suspended them and subsequently dilute them to the desired concentration.

**CTCs isolation and capture on cell line circulated in the healthy blood.** The blood samples from healthy individuals were provided by the Second Hospital of Dalian Medical University, PR China. The experimental protocol was approved by the Ethics Review Committee of the Second Hospital of Dalian Medical University, and this study was conducted in compliance with ethical and safe research practices involving human subjects or blood. Informed consent was obtained from all subjects. Clinical characteristics and information of the healthy volunteers were obtained from their medical records. All these blood specimens were collected in ethylene diamine tetra acetic acid (EDTA) containing tubes and were processed within 6 hours. In addition, these specimens were spiked with HI1299-GFP stable cells at concentrations from 10^3 to 10^5 cells/mL. Next, 5 samples were tested on the integrated system design, by pumping them onto the DLD chip with OB1 Pressure Controller (ELVEFLOW, France). This pressure controller had the advantage over the traditional injection pump, in terms of maintaining uniform force to move the fluid more stably, and also simultaneously providing different forces through different passageways.

First, under pressure exerted by a OB1 Pressure Controller (ELVEFLOW, France), the blood specimens were pumped into the Inlet A2, and PBS was injected into the Inlet A1 to balance the pressure. The section (waste) containing the RBCs and most WBCs was collected at Outlet B2, while CTCs and the residual WBCs passed through Outlet B1, which was subsequently connected to the inlet of the automatic purifying device by a flexible plastic tube (TYGON, USA). CD45 Dynabeads were then added into the purifying chip through the outlet. To improve the interaction between anti-CD-45 Dynabeads and WBCs, we developed the purifying device, in which Dynabeads were forced via magnet to move around the chamber to enhance their interaction with WBCs. Further, to ensure that captured WBCs and Dynabeads were removed, the magnet from the purifying device was allowed to rest at the bottom of the chamber, and the supernatant containing CTCs and uncaptured WBCs was sucked out. Finally, cells were captured on the rat-tail coated dish and counted under microscope. The capture rate and capture purity were calculated with the following equation: Capture rate = C1/N %; Capture purity = C1/C2%; where C1 is the number of cancer cells captured from the chip, and N is the number of all the cancer cells pumped at the starting point, C2 is the number of all the cells (cancer cells and white blood cells) captured from the device.

Next, the viability of the captured cells was measured by a standard Live/Dead fluorescent assay (Hoechst/PI staining), whose result was compared with the control cells that were never introduced into the microfluidic device. Both these cell types were cultured at 37 °C with 5% CO₂ and the medium was changed every 1–2 days, and the growth and proliferation were observed every day.

**CTCs isolation and capture from metastatic cancer patients.** Furthermore, the blood specimen (3 mL) of patients with advanced metastatic cancer, who were recruited at The Second Hospital Of Dalian Medical University, PR China, were collected for CTCs detection after their informed consent. The experimental protocol was approved by the Ethics Review Committee of the Second Hospital of Dalian Medical University, and this study was conducted in compliance with ethical and safe research practices involving human subjects or blood. Clinical characteristics and information of the patients were obtained from their medical records. These blood specimens were also collected into ethylene diamine tetracarboxylic acid (EDTA)-containing tubes and processed within 6 hours as outlined in the above paragraph. The clinical history of each patient was also considered, which contributed to the analysis of the results of CTCs detection by the novel device.

**Comparison of the novel device with CellSearch System for capture efficiency.** To further test the novel device, more blood samples from advanced metastatic cancer patients, were collected. Each patient donated 10.5 mL of blood for detection, wherein 7.5 mL was processed with Cell Search System, while 3 mL volume was analyzed in the novel device. Samples detected by CellSearch were processed within 96 hrs. Capture efficiency by the two methods were compared by the peripheral blood samples from 30 patients with advanced metastatic cancer. The condition of the each patient, including gender, age, diagnosis and therapy was also recorded, to correlate the results of CTCs detection by the two methods.

**Statistical analysis.** All statistical analyses were carried out using SPSS 13.0 statistical software package (Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Statistical significance of the differences between experimental groups was determined using Student's t-test. P < 0.05 was considered statistically significant.

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**Author Contributions**

J.J., H.Z., W.S. and J.T. performed the experiments. J.J. and Q.W. designed the study. Y.H., Y.S., R.W. and E.L. provided technical help. J.J., D.S., D.H., and X.D. performed statistical analysis. J.J., Q.W., Y.C. and L.Z. wrote the manuscript. L.Z., Y.C. and Q.W. provided scientific discussion. All authors contributed to the discussion and review of the manuscript.

**Additional Information**

**Competing financial interests:** The authors declare no competing financial interests.

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