Rare cell isolation and profiling on a hybrid magnetic/size-sorting chip

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We present a hybrid magnetic/size-sorting (HMSS) chip for isolation and molecular analyses of circulating tumor cells (CTCs). The chip employs both negative and positive cell selection in order to provide high throughput, unbiased CTC enrichment. Specifically, the system utilizes a self-assembled magnet to generate high magnetic forces and a weir-style structure for cell sorting. The resulting device thus can perform multiple functions, including magnetic depletion, size-selective cell capture, and on-chip molecular staining. With such capacities, the HMSS device allowed one-step CTC isolation and single cell detection from whole blood, tested with spiked cancer cells. The system further facilitated the study of individual CTCs for heterogeneity in molecular marker expression. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4821923>]

Circulating tumor cells (CTCs) have emerged as an important biomarker in clinical practice as well as in fundamental research.^{1,2} CTCs, shed from primary tumors, have been shown to be an early harbinger of tumor expansion and metastasis³ and have been used to predict disease progression, response to treatment, relapse, and overall survival.^{4–6} Recent work has shown that CTCs display distinct proteomic and genetic profiles; for example, CTCs in pancreatic cancer, have increased RNA expression of Wnt, implicating this pathway in metastasis.⁷ Proteomic characterization of proliferative markers such as Ki-67, and hormonal markers such as androgen receptor in prostate cancer, also have been shown to be predictive of treatment outcome.^{8,9}

Despite such clinical potential of CTCs, their routine detection and characterization still remains a significant technical challenge.¹⁰ The task requires screening of a large number of cells (e.g., $> 10^7$ cells in 10 ml blood) and enrichment of heterogeneous targets against a complex biological background. Two main methods of CTC isolation are typically used: positive and negative selection. In positive selection, CTCs are directly isolated from blood *via* size-based filtration^{11–20} or antibody-based capture.^{1,8,21} Negative depletion reduces abundant blood cells, often by immunomagnetic separation, for downstream CTC enrichment.²² Both approaches have been used for high throughput CTC isolation from whole blood (SI Table 1).²³ Each method, however, has its own inherent limitations. Positive enrichment could be biased by its selection criteria (e.g., cell size and cell surface markers). Negative selection, albeit unbiased, often requires complex sample processing (e.g., multiple washing steps for CTC isolation) that could result in cell loss.

We hypothesized that both positive and negative selection could be combined in a single platform to enable (1) highly efficient and unbiased CTC purification, and (2) *in-situ* molecular analyses of collected cells. As a proof-of-concept, we herein describe a hybrid magnetic/size-sorting (HMSS) system that integrates magnetic and size-based isolation into a compact

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microfluidic chip. The HMSS first uses a magnetic filter to deplete leukocytes through immuno-magnetic capture. Samples then pass through a size-sorter region that traps individual cells at predefined locations. Since abundant leukocytes are removed by the magnetic filter, the size-sorter could have a low size cut-off ($\sim 5 \mu\text{m}$), which allows for the unbiased capture of even small cancer cells. Furthermore, molecular probes can be introduced to perform on-chip, multiplexed analyses at single-cell resolution. We evaluated the utility of the developed system by capturing and profiling tumor cells in whole blood. The HMSS offers the advantages of both negative and positive selection and thereby differs from the recently reported iChip system²⁴ which can operate only in either a negative or a positive selection mode.

EXPERIMENTS

Hybrid magnetic and size-sorting chip

The structure of the HMSS chip (Fig. 1) is based on two distinct miniaturized components that have been developed in our laboratory: a self-assembled magnet filter²⁵ and a cell-size sorter.²⁶ The magnet filter consists of a layer of magnetic grains ($125 \mu\text{m}$, NdFeB), which spontaneously self-assemble into an array of anti-aligned magnetic dipoles (Fig. 1(b)).²⁵ Such configuration creates a magnetic field pattern that is confined tightly above the surface of the array. The field strength (B) falls off exponentially with distance from the magnet surface, producing steep field gradients (∇B). A large trapping force ($\sim B \cdot \nabla B$) can thus be imparted on magnetically labeled cells near the magnet surface.²⁵ In tandem with the magnetic filter, a size-based cell sorter is implemented (Fig. 1(c)).²⁶ The structure has a weir-style physical barrier with an underpass-gap that allows flow-through of small cells (e.g., red blood cells; RBCs) and effluent fluids. Bigger cells (e.g., CTCs), however, are trapped at the capture sites, which enables *in-situ* analyses of single cells (Fig. 1(d)).^{26,27}

Compared to our previous magnetic filter and size-based sorter, the HMSS chip incorporates new design features that significantly improve the throughput of the device. First, 64 parallel chaotic-mixing channels (length, 12.5 mm) are implemented in the magnetic separation

FIG. 1. Design of a hybrid magnetic / size-sorting chip. (a) A herringbone pattern was integrated on main parallel channels to force cells near top surface of channels onto the bottom surface. The design parameters for the herringbone mixer were: $W_C = 380 \mu\text{m}$; $H_C = 50 \mu\text{m}$; $L_{H1} = 250 \mu\text{m}$; $L_{H2} = 130 \mu\text{m}$; $W_{H1} = 150 \mu\text{m}$; $W_{H2} = 150 \mu\text{m}$; $H_H = 50 \mu\text{m}$. (b) Magnetic grains were self-assembled on the bottom surface of the channels to generate a large magnetic field and field gradients. (c) Schematics of HMSS sorter. Two separate PDMS blocks were permanently bonded. The top block included parallel channels with herringbone structures, size-based capture sites, embedded glass sled, and connecting ports. The self-assembled magnetic layer was embedded in the bottom block. (d) Schematics of size-based capture structure. Cells (red) larger than a underpass gap could be captured while smaller cells (green) would pass through the gap. The capture sites were repeated with the center-to-center distance of $40 \mu\text{m}$, and 900 sites were incorporated. Gap height, $5 \mu\text{m}$; passage width (w), $6 \mu\text{m}$; diameter of a capture site, $15 \mu\text{m}$. (e) A micrograph of a HMSS-sorter ($35 \text{ mm} \times 36 \text{ mm}$).

region (Fig. 1(a)). Unlike a simple laminar flow, the chaotic advection brings cells more frequently in contact with the channel bottom, enhancing the capture efficiency by the magnetic filter.^{28,29} The channel height, therefore, is no longer limited by the reach of the magnetic field into the flow channel, and tall channels could be implemented to increase the throughput while reducing fluidic resistance. Second, the structural strength of the size-sorter is reinforced by embedding a thin glass slip in the channel top (Fig. 1(c)). By providing rigid support to the physical barrier, this design helps the size-sorter maintain a consistent gap height during high flow-rate operation, and thus minimizes potential escape of CTCs.

Figure 1(e) shows a prototype HMSS device. To fabricate the magnetic layer, we first suspended NdFeB particles (MQFP-B-20076, Magnequench) in uncured PDMS (polydimethylsiloxane, Dow Corning), and magnetized them using a high-field magnet (~ 1.5 T). The mixture was then slowly cured (~ 3 h) to allow NdFeB grains to self-assemble. The fluidic mold had three layers that defined the underpass-gap ($5\text{ }\mu\text{m}$ in height), fluidic channels, and herringbone mixers (see supplementary methods and Fig. S1 for design details).²³ Cell capturing structure was implemented in the size sorter region (Fig. 1(d)). The capture sites were designed to trap even small cancer cells ($>5\text{ }\mu\text{m}$ in diameter). Prior to PDMS curing, a thin glass slab ($150\text{ }\mu\text{m}$ thick) was placed above the cell capture unit. The HMSS was then assembled by permanently bonding the magnet layer with the fluidic structure. The channel inside of the fabricated devices was treated with F127 Pluronic copolymer (Sigma Aldrich) to minimize non-specific cell adhesion.³⁰ Liquid injection (e.g., cell suspension, antibody-mixture) was driven by an external peristaltic pump.

System operation

The sequence of HMSS operation is demonstrated in Fig. 2. Samples were prepared by spiking ~ 5000 cancer cells (HT29, colorectal adenocarcinoma) into RBC-lysed whole blood samples (1 ml). Prior to mixing, cancer cells and human leukocytes were stained in red (Cell Tracker Red CMPTX, Life Technologies) and blue (Hoescht 33258, Invitrogen), respectively. Samples were then incubated with CD45-specific magnetic particles (EasySep[®] CD45 Depletion Cocktail) to label leukocytes (see Methods: Demonstration of system operation for details). In the initial cell mixture (Fig. 2(a)), cancer cells (red) were difficult to detect due to the presence of abundant leukocytes (blue). Following negative depletion of leukocytes with the magnetic filter, cancer cells were efficiently enriched (Fig. 2(b)). The size-sorter then collected cancer cells along the capture sites, which allowed for easy identification and counting of individual cells (Fig. 2(c)). Note that the entire assay was performed without off-chip washing steps; after incubating samples with leukocyte-specific magnetic particles, the mixture was flown through the HMSS chip to complete the assay. Such on-chip processing minimized potential cell-loss and simplified the system operation.

METHODS

Samples containing blood cells and cancer cells were prepared in two different methods (summarized in the workflows shown in Fig. S2)²³ according to the purpose of experiments. For initial device characterization (enrichment, recovery rate, and titration experiments), we used RBC-lysed human blood and pre-stained leukocytes and cancer cells (Fig. S2a).²³ For all other experiments, we used anti-coagulated human whole blood without a pre-staining process (Fig. S2b).²³

Preparation of RBC-lysed blood for device characterization

Human whole blood were lysed and fixed with pre-warmed $1\times$ Lyse/Fix (BD Biosciences) for 10 min, shaking at 37°C . Samples were then washed twice with PBS + 2% BSA. Leukocytes were labeled blue by adding Hoechst 33258 (Invitrogen) (1:2000 dilution).

Preparation of cancer cells for device characterization

Cancer cells (HT29) were pre-fixed in the same manner as described above. The fixed cells were then labeled with Cell Tracker Red CMPTX (Life Technologies) at $25\text{ }\mu\text{M}$.

FIG. 2. Operation of the HMSS chip. Following the magnetic labeling of leukocytes, samples were introduced into the HMSS chip for one-step CTC isolation. (a) Before the magnetic depletion, the initial sample was highly heterogeneous, and cancer cells (arrows) were difficult to find. (b) After the depletion of leukocytes by the magnetic filter, cancer cells were significantly enriched. (c) The size-sorter (gap height, 5 μm) trapped cells at predefined capture sites for easy cell counting. Captured cells could be further stained on-chip. Cancer cells and leukocytes were pre-stained in red and blue, respectively, for visualization.

Magnetic labeling of leukocytes

Leukocytes were labeled using EasySepTM Human CD45 Depletion Kit (Stemcell Technology). First, EasySep[®] CD45 Depletion Cocktail at 100 $\mu\text{l}/\text{ml}$ cells (e.g., for 1 ml of cell suspension, add 100 μl of cocktail) was added in prepared whole blood. The mixture was incubated at room temperature for 15 min. EasySep[®] magnetic nanoparticles were then added and mixed at 220 $\mu\text{l}/\text{ml}$ cells for 10 min.

Demonstration of system operation

Pre-stained red-fluorescent HT29 cells (~ 5000) were spiked into RBC-lysed blood (1 ml), and leukocytes were magnetically labeled. Without washing, the mixture (1 ml) was introduced to the HMSS device at a flow rate of 3 ml/h. The operation was monitored with an inverted fluorescent microscope (Nikon Eclipse Ti).

Enrichment testing

Samples were prepared by adding $\sim 10^5$ cancer cells per 1 ml RBC-lysed blood which contained $\sim 2 \times 10^7$ leukocytes. We flowed a minimum of 250 μl of the prepared sample to the magnetic filtering device to estimate the enrichment ratio. The numbers of cancer cells ($N_{C,i}$) and leukocytes ($N_{L,i}$) were measured using a flow cytometer (LSRII, BD Biosciences), before ($i = 1$) and after ($i = 2$) the magnetic depletion. The enrichment ratio was then calculated from $(N_{C,2}/N_{L,2}) \cdot (N_{C,1}/N_{L,1})^{-1}$.²⁵ All experiments were performed in triplicate under the same conditions.

Titration experiments

Confluent cancer cells in culture were suspended ($>10^7$ cells/ml), and the cell concentration was determined through standard hemacytometer cell counting. We then serially diluted ($10\times$) the stock solution. Final samples were then prepared by separately adding a precise volume of dilution series into 10 ml of RBC-lysed blood. The final concentrations of cancer cells were 10, 50, 100, and 250 cells/ml. Note that this approach minimized the logistic errors coming from pipetting. For each experiment, we used 1 ml of prepared samples. The cancer cells captured by the HMSS were counted via microscopy.

On-chip staining of EpCAM and vimentin

Three cell lines (SW872, liposarcoma; SKOV3, ovarian carcinoma; MCF-7, breast adenocarcinoma) were chosen for their varying expression levels of EpCAM and vimentin. Cells in culture ($\sim 70\%$ confluence) were detached and subsequently fixed as described above. Whole blood was obtained from healthy volunteers and placed in tubes containing ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lakes, NJ). Approximately, 3500 cancer cells were added to 3.5 ml of whole blood. After magnetic labeling of leukocytes (see above), samples were flown through the HMSS device at a flow rate of 3 ml/h. Captured cancer cells were then stained on-chip by injecting a cocktail of molecular probes (0.1 ml/h, 6 h). The following probes were mixed: Hoescht 33258 (Invitrogen), a vimentin (D21H3)-Alexa Fluor[®] 555 antibody (Cell Signaling) and an EpCAM (VU1D9)-Alexa Fluor[®] 488 antibody (Cell Signaling). These probes were suspended in $1\times$ Perm/Wash buffer (BD Biosciences) supplemented with 2% BSA (PW+). The final concentration of each antibody was $2\text{ }\mu\text{g/ml}$. After staining, cells were washed on-chip by flowing PW+ buffer (0.1 ml/h, 30 min) and imaged under an inverted microscope (Nikon Eclipse Ti).

On-chip staining of Ki-67

Cancer cells (A431, epidermoid carcinoma) were cultured to 70% confluence, detached and fixed, following the same protocol described above. Cells were then pre-stained with 1:2000 Hoescht 33258 (Invitrogen). Approximately, 1000 cancer cells were mixed into whole blood (3.5 ml), and the prepared samples were processed by the HMSS. We first treated the captured cancer cells with PW+ buffer (0.5 ml/h, 30–40 min) to permeabilize their membrane. Next, Ki-67 antibody (BD Biosciences) in PW+ buffer ($1\text{ }\mu\text{g/ml}$) was introduced (0.1 ml/h, 8 h). Fluorescein isothiocyanate (FITC)-labeled secondary antibody ($4\text{ }\mu\text{g/ml}$) against Mouse IgG1 (BD Biosciences) was then injected (0.1 ml/h, 1 h), followed by a final wash with PW+ (1 h).

RESULTS AND DISCUSSION

Device characterization

The performance of the HMSS chip was evaluated by determining two parameters, enrichment ratio and recovery rate. The enrichment ratio was defined by measuring how many times the population of non-magnetic cells (e.g., cancer cells) is enhanced relative to that of magnetically-labeled host cells (e.g., leukocytes). The recovery rate, which measures how many target cells are inadvertently trapped by the system, was obtained from the relative changes of target cell population before and after HMSS operation.

We first assessed the effect of chaotic mixing on target cell enrichment. We performed 3-dimensional fluidic simulations (COMSOL Multiphysics ver. 4.3a) to map out the trajectory of magnetic objects in the magnetic filter region. Actual geometry of devices was used. The fluidic field $\mathbf{U}$ in the microfluidic channel was first obtained by solving the Navier-Stokes equation for low Reynolds number:

$$\rho_f \mathbf{U} \cdot \nabla \mathbf{U} = -\nabla p + \mu \nabla^2 \mathbf{U}, \quad (1)$$

where ρ_f and μ are fluidic density and viscosity, respectively, and p is the pressure. The magnetophoretic force $F_{MP,z}$ on a particle by the self-assembled magnet was calculated based on the following equation:²⁵

$$F_{MP,z} = m_p \nabla B = -\frac{2\pi m_p B_0}{a} e^{-\frac{2\pi z}{a}} a_z, \quad (2)$$

where m_p is the particle magnetic moment, a is the mean diameter of magnetic grains in the self-assembled magnet, and B_0 is the field strength on the magnet surface. The boundary conditions assumed that the magnetic field B goes to zero at very large distances $z \gg a$ from the self-assembled magnet. The following values, which were previously characterized for the self-assembled magnets were used: $a = 125 \mu\text{m}$, $B_0 = 0.5 \text{ T}$.²⁵ We then solved the velocity field $\mathbf{V}$ of a magnetic object from the coupled equation

$$\rho_p \frac{d\mathbf{V}}{dt} + 6\pi\mu r_p (\mathbf{U} - \mathbf{V}) + \mathbf{F}_{MP} = 0, \quad (3)$$

where ρ_p and r_p are the density and the diameter of a magnetic object. We used the following parameters for simulation:²⁵ $m_p = 1 \times 10^{-12} \text{ emu}$ (assuming ~ 5000 per cell³¹); $\rho_p = 1000 \text{ kg/m}^3$; $r_p = 5 \mu\text{m}$; flow rate, 10 ml/h .

Computational simulation revealed that magnetic objects in the mixing channel can be more effectively captured by the magnetic filter than those in the laminar flow device (Fig. 3(a)). This trend was more pronounced at higher flow rate. In the laminar flow system, high speed objects could easily escape the magnetic filter region before significantly pulled down towards the channel bottom. Inside the herringbone channel, however, particles were

FIG. 3. Characterization of the HMSS chip. (a) Computational simulation of magnetic capture in the laminar (top) and the chaotic (bottom) fluidic systems. Each line represents a trajectory of a magnetic object under magnetic and hydrodynamic forces. Magnetic objects inside the chaotic mixer more frequently contact the magnet surface ($z = 0$), leading to higher capture yield. (b) Comparison of enrichment ratios with and without chaotic mixing. The enhancement due to the chaotic mixing is > 30 -fold. (c) Enrichment ratios at varying flow rates. Consistent cell-enrichment ($> 10^3$ enrichment ratio) could be achieved at various flow rates. (d) Titration experiments. Known amounts of cancer cells were spiked in blood and were captured by the HMSS. The measured counts were in good agreement with the expected numbers. Data are displayed as mean $\pm$ SD from triplicate (b, c) and quintuplicate (d) measurements.

forced to make frequent contact with the channel bottom (Fig. S3).²³ Consequently, the chaotic mixer system could maintain a consistent capturing efficiency even at higher flow rate.

To test the computational prediction, we fabricated two types of magnetic filter chips that were with and without herringbone structure. Samples were prepared by adding $\sim 10^3$ cancer cells (HT29) in RBC-lysed blood (1 ml) and subsequently labeling leukocytes with magnetic particles. Samples (1 mL) were then introduced to the devices at the flow rate of 3 ml/h. The number of leukocytes before and after magnetic depletion was measured by flow cytometry. As expected, the herringbone pattern-assisted magnetic filter achieved significantly higher (> 30 -fold) enrichment ratio (~ 1800) than the simple-laminar flow device (enrichment ratio: ~ 50 ; Fig. 3(b)). The capture efficiency was also stable over wide range of flow rates. For instance, even though the flow rate of whole blood samples was varied from 1 to 12 ml/h, the overall enrichment ratio remained nearly constant with values $> 10^3$ (Fig. 3(c)). This can be attributed to the deterministic nature of the fluidic flow in the herringbone mixer.³² Namely, the flow pattern inside the mixer is determined by the herringbone geometry, not by the flow speed. The chance of contact between magnetic objects and the magnetic surface is thus maintained regardless of the flow rate, leading to consistent capture rate.

We next characterized the recovery performance of HMSS chip. Samples containing varying concentrations of cancer cells (HT29; 10, 50, 100 and 250 cells/ml) were prepared in 10 ml of RBC-lyses blood (see Methods: Titration experiments for details). Leukocytes were then magnetically labeled, and samples (1 ml) were introduced to the HMSS chip at a flow rate of 5 ml/h. Cancer cells trapped at pre-defined capture sites were enumerated. The measured cell counts showed excellent agreement with expected cell counts (Fig. 3(d)); the slope between the measured and the expected cell counts was statistically equal to 1 ($P > 0.59$, two-tailed t-test) after linear regression. The average recovery rate was 87% for 50 cells and increased to 96% for 250 cells.

Molecular profiling on individual cancer cells

The HMSS chip can be an effective platform not only for CTC isolation and counting but also for its characterization. By flowing a mixture of molecular probes, captured cells can be molecularly screened at a single cell resolution. As an representative example, we profiled the expression of EpCAM (epithelial cell adhesion molecule) and vimentin in tumor cells. These markers were chosen for their clinical implications: EpCAM is a widely used epithelial marker for CTC isolation; vimentin is a mesenchymal marker, whose level is up-regulated during the epithelial-to-mesenchymal transition of CTCs.^{21,33} Samples were prepared by spiking different types of cancer cells (SW872, liposarcoma; SKOV3, ovarian serous adenocarcinoma; MCF-7, breast adenocarcinoma) into whole human blood (see Methods: On-chip staining and Fig. S2b for details²³). Following cell enrichment and trapping, a cocktail of fluorescent probes was introduced to stain captured cells for nucleus, EpCAM and vimentin. The assay revealed distinct marker profiles among different cell lines (Fig. 4(a)). For the epithelial cancer cells (SKOV3, MCF-7), EpCAM expression was strongly positive; vimentin was positive in SKOV3 but was negative in luminal-like MCF-7, which is consistent with previous reports.^{34,35} The mesenchymal cancer cells (SW872), on the other hand, were stained positive for vimentin and negative for EpCAM.

The HMSS also facilitated molecular analyses at a single cell level. For instance, Fig. 4(b) shows the expression profiling of Ki-67, a cell-proliferation marker, in A431 cells (epidermoid carcinoma) spiked into whole human blood. With the cancer cells stably trapped in the HMSS, we could resolve nuclear localization, and heterogeneity amongst cells. Some cells showed strong Ki-67 staining, suggesting their high proliferative potential. Proliferative markers such as Ki-67 have been shown to correlate to disease progression and survival; dual staining of CTCs with PSA and Ki-67 in prostate cancer patients showed a broad range of proliferative index in CTCs, even under castrate conditions.⁸ Note that other diagnostic markers could be readily incorporated into the HMSS platform, including DNA damage markers (e.g., histone γ H2AX) and signaling proteins (e.g., phosphorylated EGFR).^{36,37} Such multiplexed assays on CTC

FIG. 4. On-chip analyses on captured cancer cells. (a) Molecular analyses on three different types of cells (SW872, SKOV3, and MCF-7) showed differential staining of EpCAM and vimentin. The staining was performed on-chip after capturing cancer cells in whole blood. DAPI, 4',6-diamidino-2-phenylindole. (b) With cancer cells stably trapped, the HMSS facilitated the identification and higher resolution imaging of individual cells. Ki-67 staining of A431 cells revealed subcellular nuclear localization and heterogeneity amongst cells.

molecular status would be of significant clinical importance in monitoring and rationalizing patient treatment.

CONCLUSION

In summary, the HMSS is a promising tool for CTC detection and analysis. The integration of two distinct cell separation technologies, namely a self-assembled magnetic filter and a size-based sorter, allows for the efficient isolation of CTCs in an unbiased manner. Subsequent on-chip profiling enables cellular studies at single cell resolution. Furthermore, by optimizing the fluidic design, we have significantly improved the throughput and the cell-capture accuracy. With such advanced features, the HMSS chip could play an important role in translational cancer research. We plan on using the HMSS system with clinical samples to look at a variety of markers that are relevant to pathway inhibition in molecularly targeted treatments. Ultimately, we envision that the technological advancements in HMSS device would aid in understanding the underlying biology of CTCs as well as in establishing their clinical utility.

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