Multitarget magnetic activated cell sorter

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Magnetic selection allows high-throughput sorting of target cells based on surface markers, and it is extensively used in biotechnology for a wide range of applications from in vitro diagnostics to cell-based therapies. However, existing methods can only perform separation based on a single parameter (i.e., the presence or absence of magnetization), and therefore, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput remains a challenge. In this work, we present an alternative system, the multitarget magnetic activated cell sorter (MT-MACS), which makes use of microfluidics technology to achieve simultaneous spatially-addressable sorting of multiple target cell types in a continuous-flow manner. We used the MT-MACS device to purify 2 types of target cells, which had been labeled via target-specific affinity reagents with 2 different magnetic tags with distinct saturation magnetization and size. The device was engineered so that the combined effects of the hydrodynamic force produced from the laminar flow and the magnetophoretic force produced from patterned ferromagnetic structures within the microchannel result in the selective purification of the differentially labeled target cells into multiple independent outlets. We demonstrate here the capability to simultaneously sort multiple magnetic tags with >90% purity and >5,000-fold enrichment and multiple bacterial cell types with >90% purity and >500-fold enrichment at a throughput of 10¹⁰ cells per hour.

The capability to sort specific biological targets from complex mixtures with high purity, recovery, and throughput is critically important for many biomedical applications, ranging from in vitro diagnostics (1, 2) to cell-based therapies (3–5). In particular, FACS (6) has become an indispensable tool for the specific isolation of target cells from heterogeneous mixtures because of its ability to sort based on the simultaneous measurements of multiple optical parameters (7) (e.g., forward scatter, side scatter, and fluorescence). However, because of its serial nature of operation, where each cell must be quantitatively measured, it suffers from a fundamental limitation in throughput (8). In contrast, selection techniques such as magnetic activated cell sorting (MACS) (9–11) allow high-throughput separation of magnetically labeled target species. However, because the physics of separation is based on a single parameter (i.e., magnetization), this method is generally effective only for single-target selection.

A number of investigations have pursued multiparameter, multitarget magnetic separation methods to combine the advantages of screening and selection techniques. For example, Chalmers et al. (12) achieved separation of cells based on their degree of magnetic labeling by using conventional macroscale magnetic dipoles to generate high magnetic field gradients. This approach achieved a high level of purity but suffered from relatively low throughput. Several investigators have recently reported the feasibility of using microfluidics technology to accurately control the fluidic and magnetophoretic forces that ultimately govern separation performance (i.e., purity, recovery, throughput, and multiplexed operation). For example, Yellen et al. (13) used traveling-wave magnetophoresis to separate objects based on their magnetophoretic mobility; Liu et al. (14) demonstrated separation of magnetic particles by using a traveling magnetic field defined by staggered electromagnets; and Pamme et al. (15) used free-flow magnetophoresis to demonstrate controlled separation of mixtures of magnetic particles (16). Although these initial reports show promise, a quantitative demonstration of high-purity, multitarget magnetic sorting with high throughput has remained elusive.

In this work, we report the design, fabrication, and performance of the multitarget magnetic activated cell sorter (MT-MACS) (Fig. 1). This device incorporates microfabricated ferromagnetic strips (MFS) to generate large and reproducible magnetic field gradients within its microchannel and utilizes a multistream laminar flow architecture to accurately control the hydrodynamic forces to obtain continuous sorting of multiple target cells into independent spatially-addressable outlets with high purity and throughput.

Results

Device Fabrication and Architecture. The MT-MACS chips were fabricated by using a glass–polydimethylsiloxane (PDMS)-glass architecture [supporting information (SI) Fig. S1]. Excluding external magnets and fluidic connections, the assembled chip has width, length, and thickness of 15.7 × 64 × 1.5 mm, respectively. The microchannel has a height of 50 μm and width of 500 μm at the main flow path and contains 2 sets of MFS structures. Each set incorporates 20 200-nm-thick nickel patterns, which are 10 μm wide at a pitch of 20 μm. Each MFS region is connected to a corresponding outlet for the collection of deflected target cells; these channels are 50 μm tall and 300 μm wide. Before separation, each type of target cell is labeled with a corresponding superparamagnetic tag coupled to affinity reagents that recognize and bind to target-specific cell surface markers (Fig. 1A, step A). Each type of magnetic tag has a distinct magnetization (M) and radius (r). After labeling, the sample mixture and running buffer are volumetrically pumped into separate inlets of the device by 2 independently controlled syringe pumps. Inside the device, the 2 types of target cells become deflected at their corresponding MFS regions and elute through 2 independent outlets (Fig. 1A, step B). Fractions collected from each outlet are subsequently analyzed by flow cytometry to quantitatively measure purity and enrichment (Fig. 1A, step C).

The fluidic drag (Fd) on an object in the device can be approximated with the Stokes equation to be

$$F_d = 6\pi \eta a (v_f - v_p),$$

where $\eta$ is the fluid viscosity, a is a characteristic length of the object, $v_f$ is the velocity of the fluid, and $v_p$ is the velocity of the object. For spherical particles, a is equal to the radius of the particle. For nonspherical particles such as labeled cells, a may be determined empirically. Within the microchannel, the 2 MFS regions generate high magnetic field gradients (17), such

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... that superparamagnetically labeled cells traveling through these regions experience a magnetophoretic force $F_m$. This can be approximately calculated as

$$F_m = m \nabla B = \frac{4}{3} \pi Mr^3 \nabla B,$$

where $m$ is the magnetic moment of the tag, $r$ is the radius of the tag, and $B$ is the magnetic field. The 2 sets of MFS arrays are aligned at 2 different angles with respect to the flow direction (Fig. 1B). In this way, the net amplitude and direction of the force on the labeled cells are governed by the sum of $F_d$ and $F_m$, and selective deflection occurs when the magnitude of $F_m$ attracting the labeled cell toward the MFS exceeds the component of $F_d$ that pulls the object away from the MFS [i.e., $F_m > F_d \sin(\theta)$].

The simultaneous sorting of multiple target cells is achieved by exploiting the fact that the balance of the 2 forces has a nonlinear dependence on the radius: $F_d$ has a linear dependence on $r$ (Eq. 1), whereas $m$ (and hence $F_m$) has a cubic dependence (Eq. 2). Thus, at MFS 1 ($\theta_1 = 15^\circ$), cells labeled with tag 1, which is larger in magnetization and radius, are deflected because $F_{m1} > F_{d1} \sin(\theta_1)$ and glide along the strips to elute through outlet 1 (Fig. 1C Left). In contrast, cells labeled with tag 2, which is smaller in magnetization and radius, are not deflected here because $F_{m2} < F_{d2} \sin(\theta_1)$. Instead, they become deflected at MFS 2 ($\theta_2 = 5^\circ$), because $F_{m2} > F_{d2} \sin(\theta_2)$, and elute through outlet 2 (Fig. 1C Right). Unlabeled nontarget cells are not deflected by either MFS array and elute through the waste outlet. During this selective deflection process, a running buffer stream is introduced alongside the sample flow to act as a sheath, thus, helping to prevent nontargets from accidentally entering either of the target outlets.

**High-Precision Magnetophoresis.** The operation of MT-MACS depends on the precise and reproducible generation of magnetophoretic forces, which is achieved in 2 ways. First, a long-range magnetic field gradient is created with a custom magnetic fixture, consisting of a stack of neodymium–iron–boron (NeFeB) permanent magnets placed underneath the chip (Fig. 2A). This long-range gradient is designed to attract the labeled cells to the bottom plane of the microchannel in the MT-MACS device. Second, a short-range, high-precision, strong magnetic field gradient is created within the microchannel by the MFS arrays. This magnetic field gradient is automatically generated at the interface between the MFS and the buffer because nickel possesses significantly higher magnetic permeability compared with aqueous buffer ($\mu_r_{n i c k e l} = 200$, $\mu_r_{b u f f e r} \approx 1$), thereby creating a precise and reproducible $F_m$ (17).

The magnetic field gradients within the MT-MACS device were numerically calculated by using finite element analysis. The simultaneous sorting of multiple target cells is achieved by exploiting the fact that the balance of the 2 forces has a nonlinear dependence on the radius: $F_d$ has a linear dependence on $r$ (Eq. 1), whereas $m$ (and hence $F_m$) has a cubic dependence (Eq. 2). Thus, at MFS 1 ($\theta_1 = 15^\circ$), cells labeled with tag 1, which is larger in magnetization and radius, are deflected because $F_{m1} > F_{d1} \sin(\theta_1)$ and glide along the strips to elute through outlet 1 (Fig. 1C Left). In contrast, cells labeled with tag 2, which is smaller in magnetization and radius, are not deflected here because $F_{m2} < F_{d2} \sin(\theta_1)$. Instead, they become deflected at MFS 2 ($\theta_2 = 5^\circ$), because $F_{m2} > F_{d2} \sin(\theta_2)$, and elute through outlet 2 (Fig. 1C Right). Unlabeled nontarget cells are not deflected by either MFS array and elute through the waste outlet. During this selective deflection process, a running buffer stream is introduced alongside the sample flow to act as a sheath, thus, helping to prevent nontargets from accidentally entering either of the target outlets.

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in relative permittivity ($\varepsilon$) high ($\varepsilon > 200$) extends over the full length of the main fluidic channel. (Inset) See Fig. S1 for position of the external magnets. (B) An abrupt change in relative permittivity ($\varepsilon_r$) between the microfabricated nickel features ($\varepsilon_r = 200$) and the surrounding material ($\varepsilon_r = 1$) creates large short-range magnetic field gradients in the vicinity of the MFS. The magnitude of this gradient is high ($>10^4$ T/m) and it extends $-8 \mu m$ from the MFS. The large magnetophoretic forces for target deflection are generated by these short-range gradients. The height of the image corresponds to the height of the fluidic channel.

Magnetic Tag Separation Performance. We initially tested the MT-MACS device by simultaneously sorting two types of magnetic tags, tag 1 ($r = 2.25 \mu m, M = 14$ kA/m) and tag 2 ($r = 1.4 \mu m, M = 30$ kA/m), in the presence of an excess of polystyrene beads ($r = 2.5 \mu m$). The device and magnetic module were mounted under the objective of an epifluorescence microscope (DM4000B, Leica). Two dual-track programmable syringe pumps (PhD 2000, Harvard Apparatus) were used to deliver the sample mixture (flow rate $5$ mL/h) and buffer (flow rate $42$ mL/h) to the device. To facilitate analysis, tag 1 particles were fluorescently labeled with Alexa Fluor 488, which fluoresces at 519 nm, tag 2 particles were fluorescently labeled with R-phycocerythrin, which fluoresces at 578 nm, and nontarget particles were labeled with Firefly Green, which fluoresces at 508 nm. The ternary sample mixture (Fig. 3A), consisting of $0.020\%$ tag 1 (color = low green, low red), $0.006\%$ tag 2 (color = low green, high red), and $99.974\%$ nontarget particles (color = high green, high red), was sorted at an overall throughput of $10^9$ particles per hour. A total of $10^8$ particles were sorted, and the sorted fractions from each outlet were quantitatively analyzed via flow cytometry.

The results revealed that each of the tags was enriched several thousandfold at their respective outlets after a single round of purification. At outlet 1, the population of tag 1 was purified from $0.020\%$ to $95.876\%$ of the total population, an $15,000$-fold enrichment (Fig. 3B). The impurities in this fraction consisted of $3.125\%$ tag 2 and $1.150\%$ nontarget beads. Similarly, the population of tag 2 in outlet 2 was enriched $\sim 15,000$-fold to $99.997\%$. The two magnetic tags were efficiently separated by the device, and the waste elute contained only $0.002\%$ of tag 1 and $0.001\%$ of tag 2.

Multitarget Bacterial Cell Sorting Performance. We subsequently examined the separation performance of MT-MACS for sorting multiple bacterial strains by using 3 distinct subtypes of Escherichia coli MC1061 cells. The same 2 magnetic tags were used, with different surface functionalizations. Target 1 cells, which

![Image](image-url)
to facilitate visualization. Target 2 (GGQMG) on their surface (18), were labeled with tag 1 (MASTMTG-
0.83%) to 93.865% (Fig. 4C), corresponding to a 245-fold enrichment. The impurities in outlet 2 included some target 1 cells (6.123%) and nontarget cells (0.012%). The overwhelming majority of the cell population in the waste outlet were nontarget cells (99.621%), with a small amount of target 1 (0.102%) and target 2 cells (0.277%). Because we observed an insignificant number of magnetic tags in the waste outlet under similar experimental conditions in our previous experiment (Fig. 3D), we hypothesize that the target cells in the waste outlet are cells that were either unlabeled or became detached from their magnetic tags during the sorting process. It is also possible that multiple tags could label a single cell, however, such cases are expected to be rare and would not be detected with our experimental method.

Discussion
In this work, we describe MT-MACS, a system that takes advantage of a continuous-flow microfluidic architecture to simultaneously sort multiple targets labeled with magnetic tags of differing radius and saturation magnetization. In initial tests with a mixture of magnetic tags alone, we have shown ~5,000- to 15,000-fold enrichment for each type of tag in a single pass, at a throughput of 10^9 tags per hour through a single microchannel. Working with a mixture of tagged bacterial strains, we have demonstrated that even low concentrations of labeled target cells (< 0.4%) can be simultaneously enriched into highly purified fractions for each target cell type (>90%) at an equally high throughput.

MT-MACS separation relies on the capability of the device to precisely control and balance the fluidic and magnetophoretic forces, and the advantages conferred by microfluidics and microfabrication technology are critical in this regard. The microscale fluidic channel dimensions permit consistent, laminar flow of multiple streams at high velocities (v ≈ 0.5 m/s and Re ≈ 50), enabling precise control of hydrodynamic forces. The laminar flow at these low Reynolds numbers significantly improves the purity of the separation because it allows the use of a multistream fluidic architecture in which only actively deflected target cells are selectively transported across the sample/buffer stream boundary into the collection outlets.

To generate large magnetophoretic forces reproducibly within the microchannel, we developed a 2-step strategy that exploits both long-range and short-range magnetic field gradients in a complementary manner (21). In this scheme, the long-range magnetic field gradient from the external magnetic stack was used for 2 functions. First, it created a sufficiently large B field (~0.5 T) to magnetize the MFS patterns and superparamagnetic tags. Second, it imposed a significant downward field gradient (~200 T/m) throughout the cross-section of the microchannel to pull the magnetically labeled cells down to the bottom plane of the device where the MFS patterns are located. In that plane, the short-range high magnetic field gradients created by the MFS arrays generated tens of nanonewtons of force, effectively transporting the tagged cells across the interface of the sample and buffer streams.

This high degree of control over both the hydrodynamic and magnetophoretic forces also permits considerably higher throughput compared with reported work (13–15, 17, 22, 23); the large magnetic field gradients created locally within the microchannel allow the cells to be sorted at higher velocities. As an additional advantage, the high fluid velocities used in these experiments prevented cells and tags from sticking to the inner surfaces of the device. Although some cells were lost in the tubing and at the fluidic connections, the device itself was virtually lossless, and we observed negligible loss of tags and cells within the device because of adhesion (data not shown). We believe that the current device architecture could be modified to further increase the throughput considerably, for example, by
widening the microchannel and by operating multiple MT-MACS units in parallel to reach clinically relevant throughputs.

This approach can be scaled to sort $\geq 2$ targets simultaneously; the judicious selection of magnetic tags and MFS angles should enable the separation of 3 or more targets, further extending the versatility and potential applications of this technique. In its current implementation, the MT-MACS device is well suited for sorting targets that are smaller or comparable in size to the tags (e.g., molecules, viruses, and bacteria). However, because of the strong influence of the fluidic drag term, which is proportional to the size of the sorted object (Eq. 1), additional adaptations to this method will be required to enable multitag sorting of mammalian cells. We believe that modifications such as the use of magnetic tags with higher saturation magnetization, the labeling of each cell with many magnetic tags, and the modification of MFS regions to incorporate more defectors would be beneficial in this regard.

A small amount of nontarget particles was present in both target outlets, especially for the case where we sorted only the magnetic tags. It is possible that a hydrodynamic partitioning effect (24) may be the source of this contamination—particles that enter either of the target flow streams through nonmagnetic means will nonetheless be transported to the target outlets. In our design, we reduced this effect by ensuring that the sample flow stream does not enter either of the target outlets. However, it is possible that at high particle concentrations, some particles may have been sterically displaced into the target outlet flow streams and thus be sorted by hydrodynamic partitioning. This may also explain why we observe less contamination in the bacterial cell sorting case; because the E. coli cells are significantly smaller than magnetic tags, less displacement into the target flow streams is likely to occur. In addition to the entry of nontarget cells into the collection outlets, we note that the purity performance is also reduced by cross-over of targets between the two collection outlets (i.e., entry of target 1 into outlet 2, and target 2 into outlet 1). Additional gains in purity could be achieved by implementing multiple sorting stages within a single device, as we have demonstrated with dielectrophoretic sorting devices (25). However, we believe that an important source of this cross-contamination may not originate from the device but rather from heterogeneity in the magnetic properties of the tags (26, 27). Thus, the use of magnetic tags with less variability in saturation magnetization and size should further improve the separation performance. Furthermore, we note that the enrichment performances obtained with the tags (approximately a few percent for each target) are significantly higher than those achieved with nonmagnetic tags, labeled bacterial cells (approximately a few hundredfold). The difference is most likely because of the lower initial concentration of the tags alone (0.020% and 0.006%) compared with the concentration of the tag-target complexes (0.175% and 0.383%). Ultimately however, rare cell separation performance will be limited by the labeling efficiency of the target cells, and thus, the use of reagents with higher affinity and specificity is desirable.

We believe that our microfluidic separation technology could provide a useful means for sample preparation in advanced in vitro diagnostics. Although not demonstrated here, it is foreseeable to integrate the MT-MACS device with a wide range of chip-based genetic amplification (28) and multiplexed detection methods (29, 30). Such integration of sample preparation, amplification, and molecular detection processes into a single monolithic system may hold the key in bringing the promise of chip-based point-of-care diagnostics into reality.

Materials and Methods

Device Fabrication. Standard microfabrication techniques were used to fabricate the MT-MACS device. Briefly, the MFS patterns were defined on a glass wafer (Pyrex 7740 borosilicate, Corning) by using photolithography. Deposited by electron beam physical vapor deposition were 20 nm of Ti and 200 nm of Ni (Temescal), and standard lift-off technique created the MFS regions. A passivation layer of 100 nm of SiO2 was deposited by plasma-enhanced chemical vapor deposition (Plasma-Therm). Inlet and outlet fluidic connections were drilled by using a CNC mill (Flashcut CNC) with a diamond bit (Triple Ripple, Abrasive Technology). The microfluidic channels were created through soft lithography. The channel pattern was first defined on a silicon wafer with photolithography, and then the unpatterned sections of the wafer were etched 50 μm by using a standard Bosch Si etch process, creating a channel mold. PDMS was poured over the mold and cured at 65 °C for 3 h. The PDMS was removed from the mold and placed on a clean glass baking wafer and treated in a UV ozone oven for 10 min. The 2 halves of the device were then aligned and bonded together with a flip-chip bonder (M8-A, Research Devices). A brass eyeglet and microfluidic connector port (Labsmith) were used as the buffer and sample inlet ports, respectively, and Teflon tubing was used for the outlet fluidic ports. The external magnets consisted of 3 $1 \times 10 \times 10$ mm thick neodymium magnets (grade NA2, K&J Magnetics). All connections and magnets were bonded in place by using 5-min epoxy (Devcon).

Magnetic Tags, Cells, and Reagents. In both the magnetic tag separation and labeled cell separation cases, we used 4.5-μm-diameter, epoxy-coated M-450 magnetic beads (tag 1) and 2.8-μm-diameter, streptavidin-coated M-280 magnetic beads (tag 2) Invitrogen. The saturation magnetizations of the tags are 100 and 45 emu of magnetic moment per gram, respectively. The A/A1 beads were used as a nonmagnetic tag for cell separations and were fluorescently labeled for bead-only separations. A similar labeling protocol was used for both preparations. First, the beads were washed twice and resuspended in a solution of 0.1 M sodium phosphate, pH 7.8. Next, 1 μM BSA-conjugated Alexa Fluor 488 (Invitrogen) or 300 nM T7-tag monoclonal antibody (EMD Biosciences) was added for fluorescent or antibody labeling, respectively. After 15 min of incubation at room temperature, buffer was added to 0.1%, and the beads were incubated at room temperature for an additional 24 h. The beads were then washed 3 times in wash buffer (1× PBS, 0.1% BSA, pH 7.4). The tag 2 beads were used as is (i.e., streptavidin-coated) for cell sorting but were fluorescently labeled for bead-only separations. For the latter preparation, beads were first washed twice in binding and washing buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1.0 M NaCl). Next, 1.7 μM biotin-conjugated R-phycocerythrin (Invitrogen) was added, and the mixture was incubated at room temperature overnight. Before sorting, the device and tubing were thoroughly cleaned with running buffer, followed by ddH2O, and then allowed to dry. The remaining initial mixture and each of the outlet collections were subsequently analyzed by FACS (FACSAria, BD Biosciences).

Bacterial Cell-Sorting Protocol. Target cells were grown in LB medium with 34 μg/ml chloramphenicol (CM) for 4 h at 37 °C. The cells were then subcultured at a 1:50 dilution for 2 h at 37 °C. Protein expression was induced with the addition of 0.02% L-arabinose for 3 h. Tag 1 and 2 beads were washed three times with wash buffer. Next, a small number of target cells were added to a 10-fold excess of washed beads (target 1 cells to tag 1 beads, target 2 cells to tag 2 beads) and 1× PBS added such that the total volume of each mixture was $\approx 100 \mu l$. These mixtures were incubated overnight at 4 °C. The next morning, nontarget cells were grown in LB with CM for 6–7 h. Before sorting, the device was treated in a UV ozone cabinet for 10 min. The target cells were pelleted by centrifugation at 2,500 × g for 5 min and resuspended in 600 μl of 1× PBS. The nontarget cells were then combined with small volumes of each of the labeled target cell samples; these were...
mixed by pipette and then loaded into the sample inlet. We estimate the total cell concentration to be \( \sim 2 \times 10^8 \) cells per mL and the total tag concentration to be \( \sim 10^4 \) tags per mL for the mixture. Cold running buffer was loaded via the buffer inlet, and the sorted fractions were collected from each outlet in microcentrifuge tubes. After sorting, the device and tubing were cleaned as described in tag separation protocol. The collected cells were regrown to be highly purified human blood eosinophils. J Chromatogr B 843:105–110.


 20 nM streptavidin-conjugated phycoerythrin (Invitrogen) and incubated at 4 °C for 1 h. The cells were pelleted by centrifugation at 2,500 × g for 5 min, resuspended in 200 μL of PBS, and analyzed immediately with FACS.

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