Electronic Supplementary Material (ESI) for Lab on a Chip.

Exponential magnetophoretic gradient for the direct isolation of basophils from whole blood in a microfluidic system

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Supplementary Information

Materials and Methods

Blood sample preparation

Blood was collected by intravenous blood draw into heparinized tubes from donors under Institutional Review Boards IRB# 52850. For experiments to characterize purity and recovery, blood samples were kept on ice for at most 3 hours prior to injection into the microfluidic device. To assess basophil activation status, we processed blood samples (kept on ice) within 24 hours of collection.

For experiments characterizing the fully integrated i-BID, we mixed 100 µL of unwashed heparinized whole blood with 1-2 µL of 500 mM EDTA (final EDTA concentration ~5 mM) prior to injection into the i-BID. An EDTA concentration of 3-5 mM in blood was suggested by the manufacturer of the isolation reagents, STEMCELL. EDTA chelates calcium to mitigate coagulation and the formation of clots in the DLD channels.[1] Heparin inhibits free thrombin to reduce platelet aggregation and coagulation.

For a subset of experiments characterizing the operating conditions of the standalone MSD, we enriched WBCs from 500-600 µL of blood at a time in DLD channels. Because this subset of experiments was not aimed at rapid, fully-integrated basophil isolation with the i-BID, we washed the blood samples to dilute the clotting factors (e.g., fibrinogen and thrombin) and mitigate clogging in the DLD channels. We had observed that injecting whole blood directly into the DLD channels led to considerable build-up of cells at the DLD entrance. The wash was performed by diluting blood ~3000x in 5 mM EDTA in PBS (Ca^-/Mg^-), centrifuging the suspension (500 G, 5 min), and aspirating the supernatant for a final volume and hematocrit approximately equivalent to that of the starting whole blood sample.

For all experiments, we added stains to the blood samples to identify basophils in flow cytometry to characterize their purity and recovery after the microfluidic operation. Per 100 µL of blood, we added 3 µL of anti-CD123-APC (clone 7G3, BD Bioscience), 4 µL of anti-CD45-PE (clone HI30, BioLegend), and 4 µL of anti-CD193-PerCP/Cy5.5 (clone 5E8, BioLegend), and incubated the sample for ~18 minutes on ice. Following the stain incubation and prior to injection into the microchannels, we added 22 µL of DLD running buffer (2% FBS, 1 mM EDTA in PBS) for each 100 µL of blood to obtain a final sample volume of 133 µL (blood concentration of ~75% v/v). This step was performed to reduce the chance of clogging.

Design and fabrication of the deterministic lateral displacement (DLD) device and the mixer

Both the DLD and the mixer were fabricated in poly(dimethylsiloxane) (PDMS) using soft lithography. The master molds were fabricated in SU8-2050 using transparency masks (CAD/Art Services Inc.) and a Quintel Q-4000 mask aligner. The DLD/mixer microfluidic chip comprised two layers of PDMS. The bottom layer contained the DLD channels, and the top layer contained the mixer channel. The two-layer design was necessary for parallelizing the DLD channels as described below.

For the DLD, we adapted the design from Feng *et al.*[2] To improve the throughput and to reduce the rate of clogging at the entrance of the DLD arrays, we used four parallel DLD channels. Each DLD channel had a height of 50 µm, a pillar diameter of 22 µm, a gap of 13 µm, a row shift fraction of 1/30, and an approximate critical diameter of ~3.6 µm (see Fig. 1B).[2–4] We coated the DLD channels with Pluronic F68 (3% w/v, Alfa Aesar J66087 Poloxamer 188) to reduce the incidence of cell adhesion and the likelihood of clogging.[5] Pluronic F68 was left in the channels for at least one hour, after which it was thoroughly flushed with the DLD running buffer (2% FBS, 1 mM EDTA in PBS) prior to use. Basophil recovery from the DLD was >99%

for all blood injection flow rates tested (2-6 mL/hr) when injecting blood that was washed with 5 mM EDTA in PBS (Ca^-/Mg^-) (Fig. S1).

After the DLD, MNP and NSAb were introduced and mixed with the WBCs from the DLD using a mixer consisting of a Herringbone-grooved serpentine channel.[6] The MNP and NSAb were obtained from the EasySep™ Human Basophil Isolation Kit supplied by STEMCELL (catalog #17969). The NSAb mixture negatively selected for basophils with a mixture of antibodies targeting non-basophil surface antigens (e.g., CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56, glycophorin A).[7] The mixer channels were 200 μm wide and 70 μm tall with groove heights of 30 μm. In our experiments, the flow running through the device was between 3-5 mL/hr, corresponding to a Reynolds number between ~6 to 10, well within the mixer design's operating conditions.[6] MNP and NSAb reagents were injected at a rate necessary to maintain a target MNP/NSAb:WBC ratio of 50 μL:1 mL. Their injection rate was thus a function of the expected flow rate of the enriched WBC suspension exiting the DLD channels. For example, to achieve this target ratio for a WBC suspension exiting the DLD stage at 3 mL/hr, we injected MNP/NSAb at 0.15 mL/hr. After use, we flushed the channels with 10% bleach and deionized water. If all debris was removed, the channels were re-coated with F68 and reused.

Fabrication of the magnetic separation device (MSD)

The Halbach array consisted of five N52 bar magnets (1.5" by 0.75" by 0.75", Super Magnet Man, catalog #Rect1526). We used a stereolithography 3D printer (Form2, Formlabs) to print the MSD scaffold in Grey Pro resin with a layer resolution of 50 μm. The scaffold consisted of a slot to secure the Halbach array, passage tunnels to position a fluidic tubing (medical grade polyethylene tubing, 762 μm inner diameter, Scientific Commodities, catalog #BB31695) and steel wire MFCs (low carbon 1008 steel, 0.029" dia., McMaster, catalog #8870K15), and openings along the passages to facilitate the removal of uncured resin (Fig. S4). We chose a tubing with an inner diameter of 762 μm because its cross-sectional area and the corresponding internal surface area were sufficiently large to retain captured non-basophils and unbound MNP without obstructing the flow of basophils, while it was sufficiently small so that the magnetic flux from the MFCs could reach the center axis of the tubing.

We chose to have passes 1 and 4 traverse over magnet 3 (Fig. 1C) because the far-reaching magnetic field over magnet 3 could accommodate low magnetic forces in pass 1 and high magnetic forces in pass 4. Despite the adjacency of passes 1 and 4, no interference occurred in the magnetic field due to the proximity of the MFCs in passes 1 and 4. To prevent tubing intersection over magnet 3, it followed that passes 2 and 3 must span magnets 1 and 5, respectively.

Flow control of the integrated basophil isolation device (i-BID)

In all experiments, we loaded the blood cells and the basophil isolation reagents in medical grade polyethylene tubing, which were connected to syringes filled with DLD running buffer. To prevent the cell suspensions and reagents from back flowing into the syringes, we used air plugs (5-10 mm long) to separate them from the DLD running buffer which filled the rest of the tubing and syringes. While the air plugs could introduce a source of compressibility into the i-BID, their effects were not observed during steady-state operation.

All isolation experiments were performed at room temperature. Blood flow was actuated with a 1 mL syringe (HSW Norm-ject) and MNP/NSAb flows were each actuated with 250 μL glass syringes (SGE Analytical Science) to prevent syringe deformation under pressure. DLD

buffer injected into the DLD/mixer and isolated basophils withdrawn from the outlet of the MSD were actuated with 10 mL syringes (BD Biosciences).

We wrote a custom control program in Python to operate the syringe pumps (Chemyx Inc.). The control code facilitated loading the blood sample, ramping up the flow rate to a constant steady state, and stepping through a series of user-defined operation modes (i.e., injecting blood, flushing the DLD channels, and passing the cells through the MSD). A slight latency in writing and running commands to the pumps across USB serial ports was expected and could lead the contamination of WBC product by RBCs. To avoid this lag, we implemented threading in Python to instantiate multiple processes in parallel and update all pumps simultaneously.

Prior to injecting blood, we initiated the injection of MNP/NSAb and the withdrawal from the DLD/mixer outlet while gradually stepping up the DLD buffer flow until steady state was reached. We fixed the DLD buffer flow rate (Q_{buffer}) at 5x the flow rate at which the blood sample was injected (Q_{blood}) into the DLD. The flow rates were chosen to keep the average velocities the same in all channels feeding into and out of the DLD arrays. We fixed the flow rate of the mixed WBC-MNP/NSAb suspension (Q_{out}) by withdrawing from the outlet of the MSD at a rate equal to the sum of the injection rates of MNP, NSAb, and the WBC outlet flow rate (see Fig. 1A). The RBC waste outlet tubing was submerged in the fluid of the waste collection container to avoid pressure fluctuations due to dripping and maintain a constant pressure at the waste outlet. After the entire volume of blood was injected into the DLD/mixer channels and right before the air plug entered the channel, we advanced the Python control program to the next step to halt blood injection, flushed the DLD channels with DLD running buffer, and ramped down the injection of MNP/NSAb as the WBC-MNP/NSAb suspension continued through MSD.

Characterization of the performance of the standalone MSD

In a subset of experiments to evaluate the effectiveness of the MSD in isolating basophils as a function of flow rate, we enriched WBCs using the same DLD channels that comprised the i-BID. Aliquots of the DLD product (100 µL, with ~2-3×10^5 WBCs) were mixed with 5 µL of NSAb and 5 µL of MNP in a 5 mL round-bottom tube with a gentle shake. After 5 min of incubation at room temperature, we injected the mixture into the standalone MSD with a syringe pump in withdrawal mode from 3-18 mL/hr (n = 5 runs for all flowrates except for 12 and 15 mL/hr where n = 6 runs).

For a subset of experiments, we tested the operation of the standalone MSD by injecting the enriched WBCs manually from a syringe. Briefly, we asked 6 participants to withdraw the WBC-MNP/NSAb suspension from the outlet of the MSD by hand using a 1 mL syringe. Participants were instructed to steadily withdraw the sample through the MSD in 2-3 minutes resulting in approximate flow rates of 6-8 mL/hr

Comparison with commercial basophil isolation kit

We compared the performance of the MSD and manual MSD to that of the STEMCELL EasySep™ immunomagnetic column-free magnet (catalog #18000). We followed the same process detailed above for enriching WBCs from blood and adding MNP/NSAb to 100 µL of WBC suspension. After 5 min of incubation with MNP/NSAb, we added 3 mL of DLD running buffer and placed the tube in the EasySep™ magnet. We allowed 5 minutes for the removal of magnetically tagged cells following the EasySep™ protocol. After that, with the tube remaining in the EasySep™ magnet, the suspension containing purified basophils was poured into a new 5 mL tube. The cell suspension was then centrifuged at 500 G for 5 min to remove the excess buffer and prepare the cells for flow cytometry.

For direct comparison with a gold-standard commercial isolation kit that involved no microfluidic steps, we selected the STEMCELL Direct Basophil Isolation Kit™ (catalog #19667) that can isolate basophils directly from whole blood. Our aim was to assess if this kit was capable of isolating basophils from a small volume of blood (<1 mL), which is desirable for diagnostic applications. A starting blood volume of 300 µL was the smallest volume that we could process without significant challenges in pipetting and other bulk fluid handling steps. The isolation protocol began with adding EDTA to whole blood in a 5 mL round-bottom tube to reach a final EDTA concentration of 3 mM. Next, 15 µL of the direct kit's NSAb and MNP were added (50 µL per mL of blood) and incubated at room temperature for 5 minutes. The blood was then diluted with 3.7 mL of 1 mM EDTA in PBS and placed in the EasySep™ magnet for 5 min. With the tube remaining in the magnet, the suspension was poured into a new 5 mL tube and another 7.5 µL of MNP was added (25 µL per mL of starting volume). Following another 5 min incubation, the tube was placed in the EasySep™ magnet for another 5 min. The process of pouring into a new tube, adding MNP, and letting the tube sit in the magnet was repeated once more (3 total incubation in the magnet), and the suspension of isolated basophils was collected. We centrifuged the suspension at 500 Gs for 5 min to concentrate the cells in a 200-300 µL pellet for staining and flow cytometry.

Characterization of the purity and recovery of basophils

We quantified the purity and recovery of basophils from the product of our device using flow cytometry (BD FACScan, Cytek Biosciences). Purity was quantified by performing flow cytometry on the MSD product to determine the percentage of events that were $SSC^{low}/CD45^{+}/CD123^{+}/CCR3^{+}$ (Fig. S5) Gating was done in FlowJo v10.8. We followed the convention established by the STEMCELL basophil isolation kit protocol in which purity is determined as the percent of basophils within cells expressing the leukocyte common antigen (CD45).[8]

For experiments characterizing the performance of the standalone or manual MSD and the usage of the EasySep™ magnet, recovery was quantified by dividing the count of basophils in the MSD product by the count of basophils in the control volume of enriched WBC suspension. Enriched WBC suspensions obtained by DLD were split into 5-6 aliquots each with a volume of 100 µL. One of these aliquots was set aside as the control to determine the expected number of basophils in 100 µL of the WBC suspension. In our recovery quantification, we assumed a homogenous distribution of basophils in WBC suspensions.

For experiments characterizing the performance of the fully integrated i-BID, recovery was approximated by dividing the count of basophils produced by the i-BID by the count of basophils in a control sample of whole blood with a volume equivalent to that injected into the i-BID. This control was stained for CD123, CCR3, and CD45, and WBCs were enriched by lysing RBCs. In our recovery quantification, we assumed a homogenous distribution of basophils in whole blood.

In all experiments, we also collected the RBC waste from the DLD channel, lysed the RBCs, and injected any remaining WBCs through the flow cytometer to verify that <1% of the total expected basophil count was lost to the DLD channel waste (Fig. S1).

To quantify the recovery of basophils using the EasySep™ Direct Human Basophil Isolation Kit™ by STEMCELL (catalog #19667), we used a 100 µL control volume of whole blood to count the expected number of basophils and multiplied the basophil count by 3 to compare to the direct kit's recovered count from 300 µL of blood. For the direct isolation kit, we started with 300 µL of whole blood based on our experience with getting better recovery starting with 300 µL compared to 100 µL.

Characterization of basophil activation status

We performed experiments to examine whether the isolated basophils exhibited any unintended activation in our on-chip isolation process, and whether they could still undergo activation in response to a stimulus. For these experiments, the surface marker stains were not added until after isolated basophils were recovered and subjected to stimulus. Basophils, when recovered, were suspended in DLD running buffer which contained EDTA. Because EDTA chelates calcium ions and basophil activation is a calcium and magnesium dependent process (29), we resuspended the basophils in RPMI with 1 μM of $CaCl_{2(aq)}$ and $MgCl_{2(aq)}$ prior to subjecting them to any stimulus. Following this step, ~100 μL of basophil suspensions were mixed with 100 μL of RPMI or anti-IgE (2 μg/mL) dissolved in 100 μL of RPMI. After incubation for 30 minutes at 37˚C in 5% CO_2, the activation was halted by adding 1 mL of cold 2.5 mM EDTA in PBS (Ca^-/Mg^-) to each sample. The samples were centrifuged at 500 G for 5 min at 4 ˚C. The supernatant was aspirated, and the pellet was resuspended by vortex mixing. We then added 2 μL per stain per sample for identifying basophils (i.e., anti-CD123-APC and anti-HLA-DR-PE/Cy7 (clone L243, BD Biosciences) and for identifying activated basophils (i.e., anti-CD63-FITC (clone H5C6, BD Biosciences) and anti-CD203c-PE (clone NP4D6, BD Biosciences)). The cells were incubated with the stains for 20 min on ice followed by a wash with 3 mL of a stain buffer (2mM EDTA and 0.5% BSA in PBS). Cells were centrifuged at 500 G for 5 min at 4 ˚C. The supernatant was aspirated, and the pellet was resuspended in ~200 μL of stain buffer for flow cytometry.

We compared the activation of basophils isolated with the i-BID with that in the whole blood control samples. The control samples were subjected to the same conditions as the samples which passed through the i-BID, i.e., EDTA was added to the blood for a 5 mM final concentration, the control samples were left at room temperature for the duration of the i-BID run, and the sample was resuspended in RPMI with 1 μM of $CaCl_{2(aq)}$ and $MgCl_{2(aq)}$ prior to activation. For measuring the activation status of basophils from the whole blood control sample, we used the same procedure for measuring activation in purified basophil and included an RBC lysis step after the stain. We lysed RBCs by adding 4 mL of 1X RBC lysis buffer (Biolegend, catalog #420301) to the resuspended pellet. We incubated the lysis buffer with the cells for 15-20 minutes in the dark at room temperature, and centrifuged the lysed suspension at 800 G for 10 min at 4 ˚C. The supernatant was aspirated, and the cells were resuspended in 3 mL of stain buffer before another centrifugation at 500 G for 5 min at 4 ˚C to collect WBCs for flow cytometry.

Statistical analysis

To determine the significance of improvements in purity and recovery between the syringe pump-driven MSD, the manual MSD, and the EasySep™ magnet, and between the i-BID and the Direct Basophil Isolation Kit™, we used unpaired two-tailed t-tests assuming unequal variances. The same type of t-test was applied to show insignificance in CD203c ΔMFI between i-BID and the whole blood control. We used a two-tailed two-sample F-test to determine if there was any significance in the variances found for purity and recovery using each mode of isolation. In our results, we reported the F-value with the degrees of freedom indicated in the subscript. For t-test and F-test comparisons, we chose $\alpha = 0.05$ as the minimum type-1 error rate. Tukey's box plots were used to report purity and recovery across MSD flowrates (Fig. 4 B and C) and isolation modes (Fig. 4 E and F). The centerlines show the median, the box bounds represent the interquartile ranges, and the whiskers extend out to the maximum and minimum data points, excluding outliers. Plots were produced with Python and statistical analysis was performed in Excel.

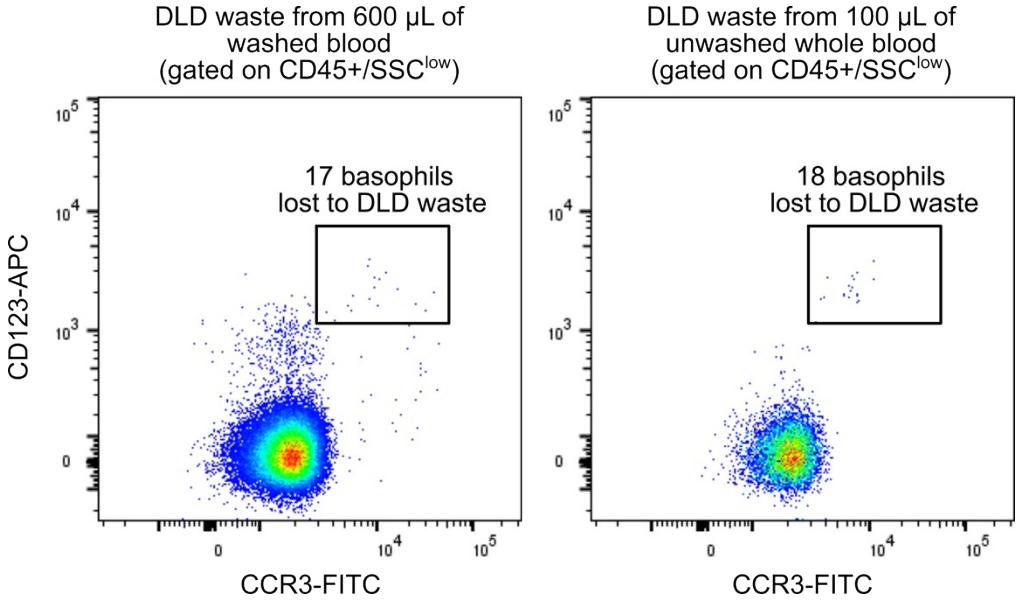


Fig. S1.
Representative data showing minimal basophils lost to the DLD channels' waste. *Left*: The performance of the deterministic lateral displacement (DLD) channels using blood that was pre-washed in 5 mM EDTA in PBS to dilute clotting factors. This method of blood preparation, used for characterizing standalone magnetic separation device (MSD) runs, allowed the DLD channels to process blood volumes as high as 600 µL with no clogging in the channel and minimal loss of basophils. *Right*: Unwashed whole blood was more easily affected by clogging at the entrance of the DLD array, but over the course of 100 µL of blood volume, minimal basophil losses were observed. In both cases, <1% of total expected basophils were lost to the DLD channels.

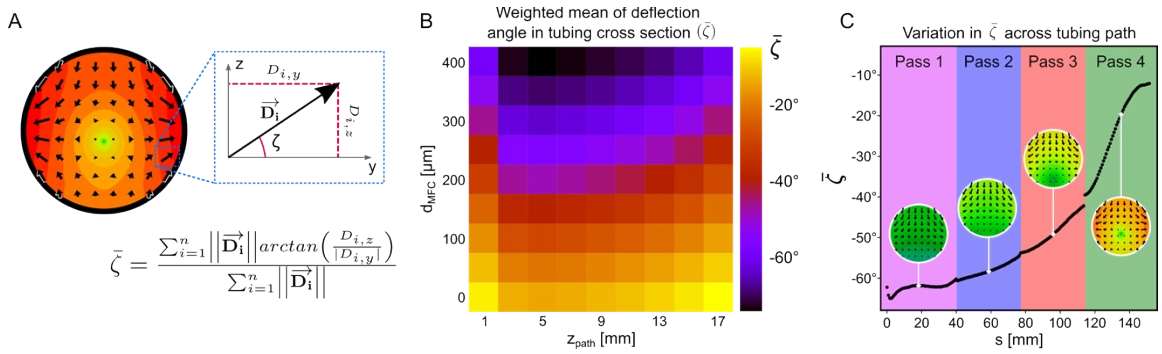


Fig. S2.
Variation in the mean deflection angle across the parameter space and the tubing path. (*A*) All deflection angles of *n* vectors in the tubing cross-section domain are averaged and weighed by their deflection magnitude to give a weighted mean angle, $\bar{\zeta}$. Angles greater than -45˚ (closer to 0˚) indicate a horizontally dominated deflection, i.e., along the global z-axis, that is influenced by the magnetic flux concentrators (MFCs) more than by the Halbach array. (*B*) Heatmap illustrating the range of horizontally deflected (yellow) to vertically deflected (black) configurations as a function of z_{path} and d_{MFC}. (*C*) $\bar{\zeta}$ approximated along the tubing path. We used the known target maximum magnetic force field value and d_{MFC} at position *s* to look up z_{path}, and we used z_{path} and d_{MFC} to look up the corresponding $\bar{\zeta}$ from a smooth cubic interpolation of the heatmap in B. The inflection points are attributed to changes in domain discretization when the values of z_{path} and d_{MFC} are varied and the model is re-meshed. The discontinuities between passes are attributed to edge effects at the ends of the magnets where the tubing turns to run across the Halbach array again.

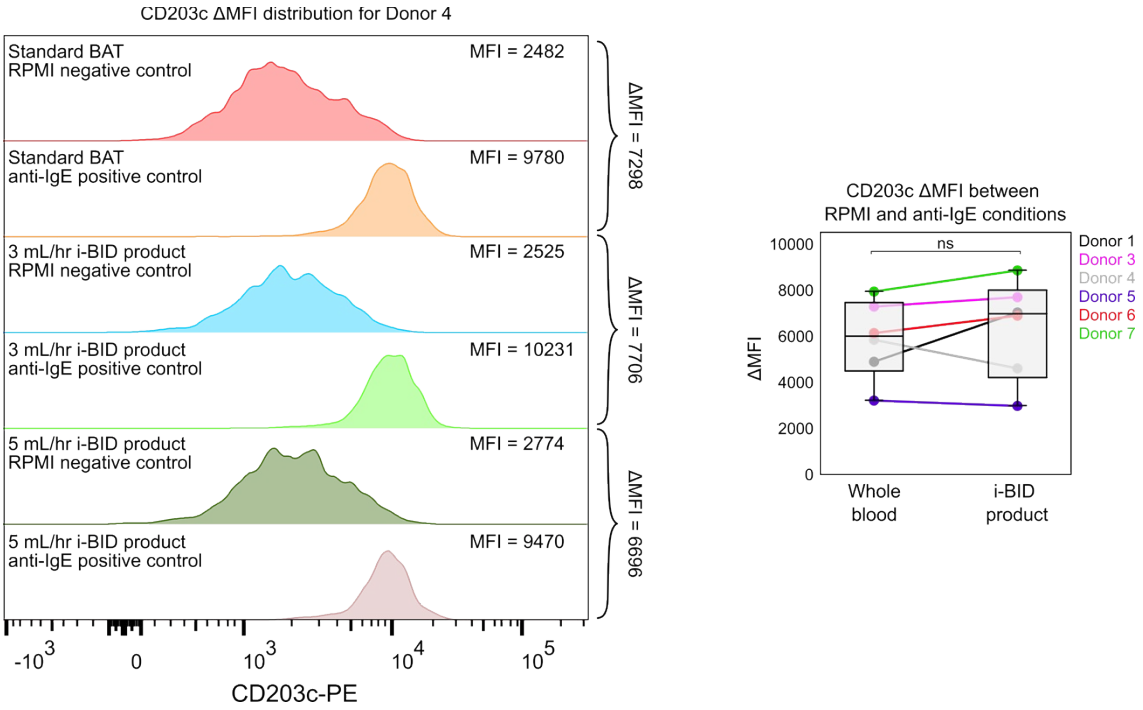


Fig. S3.
CD203c expression on allergic donor's basophils was unaffected by the i-BID. CD203c is a commonly used basophil activation marker in addition to CD63. There was an insignificant difference (P = 0.69) in CD203c delta mean fluorescence intensity ($\Delta MFI = MFI_{anti\text{-}IgE} - MFI_{RPMI}$) between i-BID basophils and whole blood control which was processed with a standard basophil activation protocol (see Methods).

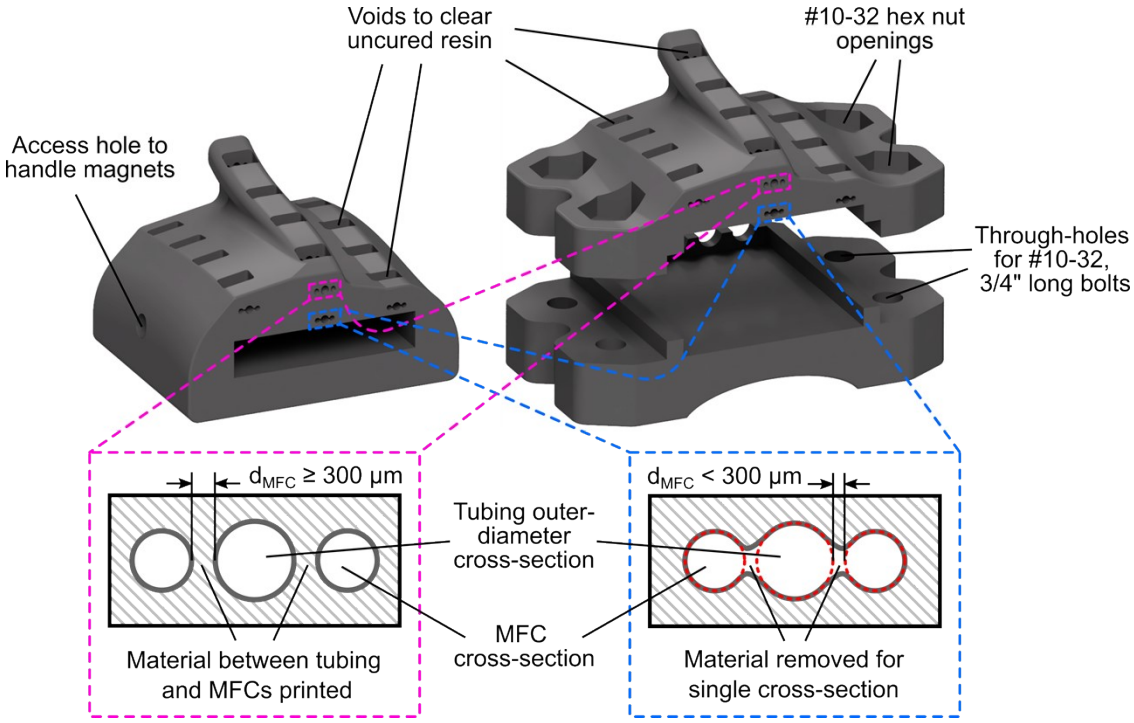


Fig. S4.
Computer-aided design (CAD) models of two versions of the magnetic separation device (MSD). We used Formlab's Grey Pro™ resin for its rigidity. The 3D-printed scaffold surrounded the magnet housing with ample material so that the magnets would not deform the MSD when repelling each other in a Halbach configuration. Both CAD models were used in this work interchangeably exhibiting no difference in performance. They both produced identical magnetic force field profiles with the same tubing path and relative positions between the MFCs and the magnets. The tolerances on the single-piece version (left) allowed for a snug fit around the magnets. The two-piece version (right) ensured the Halbach array could be securely sandwiched using aluminum bolts and nuts, and it facilitated loading and unloading the magnets. For $d_{MFC} < 300$ µm, to circumvent the minimum feature resolution limit of the Form2 printer that we used, we joined the holes to thread the tubing and MFC. The CAD design is available at: https://grabcad.com/library/magnetic-separation-device-1

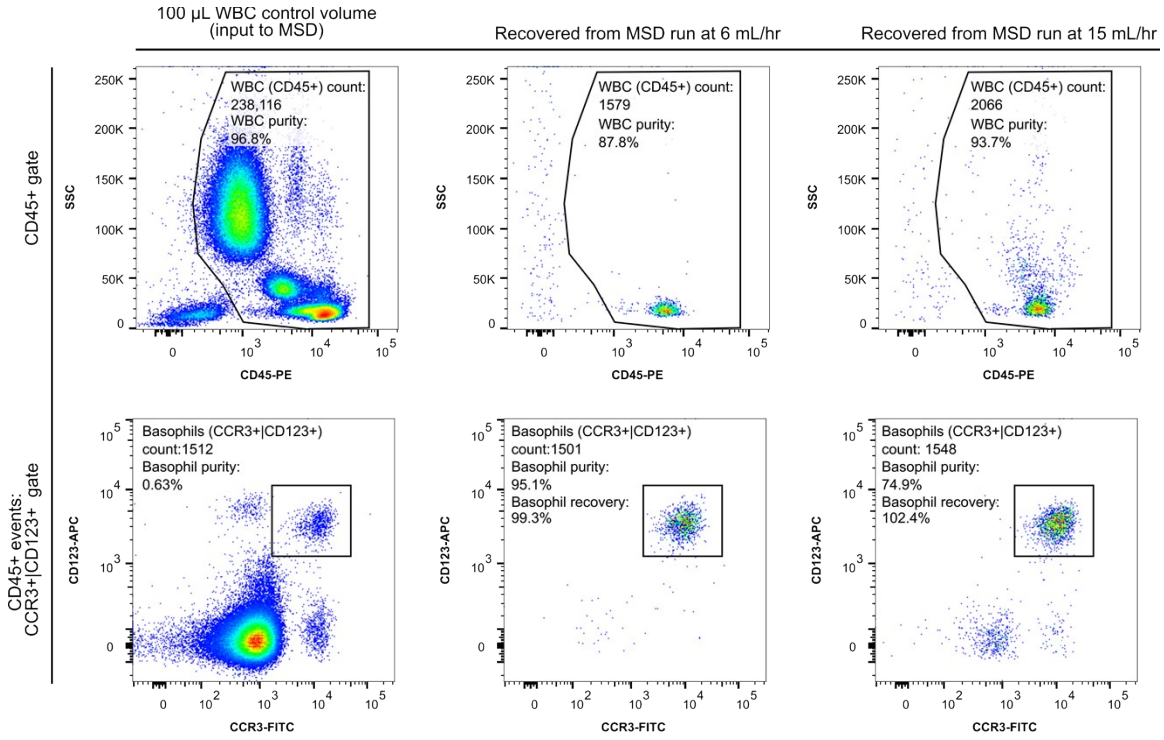


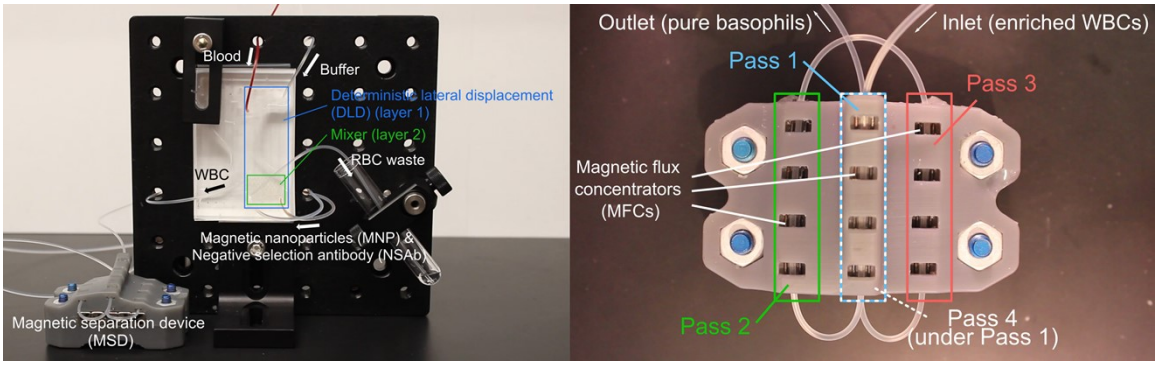
Fig. S5.
The gating process used for evaluating purity and recovery. Enriched WBCs from deterministic lateral displacement (DLD) channels were split into 100 µL volumes for testing different flow conditions in the magnetic separation device (MSD). One volume acted as the control to set the CD45 gate and determine the expected number of basophils in the MSD product. Following the convention established by STEMCELL for assessing the purity, basophil purity was determined considering events that were CD45+ only. Events outside this gate in the MSD samples were due to bubbles or debris entering the flow cytometer.

Method	Target Cell	Starting Volume [mL]	Time to isolate [min]	Purity	Recovery	Ref.
DLD and positive or negative immunomagnetic selection	CTC	6-12	45-90	7.8%	97.0%	[9]
Spiral inertial sorter (diluted blood) and negative immunomagnetic selection	CTC	--	--	4.4-51.5%	93.8%	[10]
Spiral inertial sorter and DLD	CTC	--	--	91.3%	17.7%	[11]
Immunoaffinity leukocytes capture and filtration of CTCs	CTC	10	~38	90.0%	90.0%	[12]
STEMCELL direct immunomagnetic negative selection isolation kit	Basophils	2-6	45	97.3%	--	[13]
		0.3	45	84.6%	52.4%	This work
DLD and negative immunomagnetic selection	Basophils	0.1	8-10	93.9%	95.6%	This work

Table S1.
Comparison between i-BID, a commercial basophil isolation kit (STEMCELL), and state-of-the-art microfluidic isolation platforms. The table only included approaches that isolate a target cell directly from whole blood, like the i-BID, and not from lysed or pre-purified blood. It should be noted that microfluidic cell isolation studies tend to focus on CTCs as the target cell. "--" indicates values not reported.

Parameters	
Tubing outer diameter	1.22 mm
Tubing inner diameter	762 μm
Magnets' remanent flux density norm	1.44 T
Magnetic flux concentrator (MFC) diameter	711.2 μm
Magnets' square cross section side length	6.35 mm
Out of plane thickness	38.1 mm
Materials (all built into COMSOL)	
Low Carbon Steel 1006	MFCs
N52 (Sintered NdFeB)	Magnets
Air	All non-magnetic domains (i.e., tubing wall, inner tubing area).
Physics	
Magnetic Fields, No Currents to solve magnetic field	Remnant flux density magnetization model used under Magnetic Flux Conservation domain condition.
Coefficient Form PDE to solve magnetic field gradient	Absorption coefficient matrix was cast to an identity matrix. Source Term vector components were defined by the magnetic flux density components. Other coefficient terms were set to zero.
Mesh	
Discretization	Quadratic Lagrange shape functions
Mesh resolution	To capture the highly nonlinear magnetic field we partitioned the region near the tubing and MFC for a refined mesh with element sizes from ~2-50 μm.

Table S2.
Details of the COMSOL simulation. Details of the physical parameters, materials, physics, and mesh are listed above. These values were used for parametric sweeps in 2D to define the parametric space $max\|(\vec{B}\cdot\nabla)\vec{B}\|$ and to solve for the 3D magnetic field.



Movie S1.
Demonstration of the i-BID operation with annotations. 100 µL of whole blood was injected at 5 mL/hr at the deterministic lateral displacement (DLD) channel inlet along with a running buffer and the NSAb and magnetic nanoparticles (MNP) isolation reagents. At 5 mL/hr, with a 3-minute incubation period for NSAb/MNP binding that occurred in a tubing between the poly(dimethylsiloxane) (PDMS) chip and the magnetic separation device (MSD) (not filmed), the isolation took ~8 minutes in total. The air plug that marked the end of the NSAb/MNP-WBC mixture can be seen entering the MSD. The magnetic force in the MSD was strong enough to pull all magnetic material through the air-liquid interface at this air plug, evident by the clear fluid that exits the MSD outlet, compared with the brown-tinted inlet mixture.

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