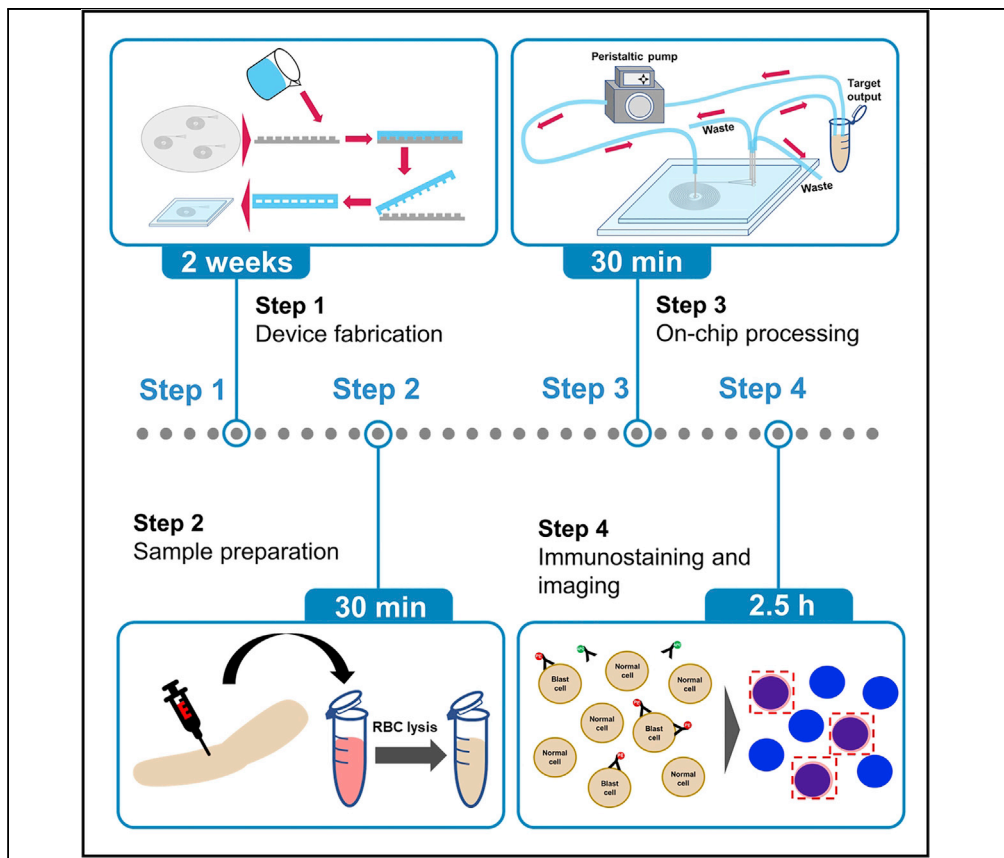


Protocol

Label-free enrichment of human blast cells from whole blood for leukemia monitoring



Liquid biopsy is an alternative to invasive bone marrow biopsy for leukemia detection and management. However, no robust technology is available for enriching leukemic blast cells from the blood. Here, we present a simple and effective protocol for vigorous enrichment of blast cells from whole blood using a one-step microfluidic blast cell biochip (BCB) that exploits distinct cell mechanical properties between diseased and healthy leukocytes. The BCB system achieves higher sensitivity than flow cytometry in detecting blasts.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A label-free biochip to enrich leukemic blasts from liquid biopsy (blood) samples

Detailed approaches for fabricating the microfluidic blast cell biochip (BCB)

Detailed steps for preparing blood samples to isolate leukemic blasts from blood

Operational procedure for biochip enrichment of leukemic blasts from whole blood

Fu et al., STAR Protocols 3, 101584
September 16, 2022 © 2022
The Author(s).
<https://doi.org/10.1016/j.xpro.2022.101584>



Protocol

Label-free enrichment of human blast cells from whole blood for leukemia monitoring

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<https://doi.org/10.1016/j.xpro.2022.101584>

SUMMARY

Liquid biopsy is an alternative to invasive bone marrow biopsy for leukemia detection and management. However, no robust technology is available for enriching leukemic blast cells from the blood. Here, we present a simple and effective protocol for vigorous enrichment of blast cells from whole blood using a one-step microfluidic blast cell biochip (BCB) that exploits distinct cell mechanical properties between diseased and healthy leukocytes. The BCB system achieves higher sensitivity than flow cytometry in detecting blasts.

For complete details on the use and execution of this protocol, please refer to Khoo et al. (2019).

BEFORE YOU BEGIN

Protocol overview

Inertial focusing-based microfluidic chips effectively enrich particles of distinct physical attributes from samples in a label-free manner and are therefore promising tools in biomedical applications (Chen et al., 2022; Khoo et al., 2019; Liao et al., 2021). This protocol demonstrates a unique procedure for enriching and detecting liquid biopsy blasts using a continuous flow BCB system. The fabrication of the BCB system is dependent on polydimethylsiloxane (PDMS) as the core material and pretreatment with surfactants such as Bovine serum albumin (BSA) or Pluronic acid, followed by extraction. The system can be multiplexed to facilitate the concentration of large samples such as blood and urine or integrate a manual feedback loop to continuously remove non-targeted particles while concentrating and purifying rare target cells. Rare blast cells recovered from BCB can then be identified using immunofluorescence staining and photography for CD34⁺CD38⁻ identification, a procedure that would detect low blast cell counts and aid in detecting minimal residual disease (10⁻⁶). This protocol also describes robust procedures that can be used to thaw, culture, and passage various cell types that mimic leukemia subtypes and to obtain nucleated cells from the blood.

Preparation one: Buffer preparation

⌚ Timing: 35 min

1. Prepare 25% BSA-PBS solution.



- a. Diluting BSA (Thermo Fisher Scientific, Cat#B14) with Phosphate buffered saline (PBS) (Thermo Fisher Scientific, Cat#70011-044) in biological safety cabinet (BSC) (NuAire, NU-543-400) 1:3 by volume.
- b. Store unused BSA-PBS solution at 2°C–8°C.
2. Prepare 0.1% Pluronic acid.
 - a. Mix 10% Pluronic acid stock solution (Thermo Fisher Scientific, Cat#24040-032) with PBS in BSC 1:99 by volume.
 - b. Before processing the cell samples, inject 0.1% Pluronic acid into the microfluidic chip and incubate for 30 min.
 - c. Wash the spiral microfluidic chip with PBS to remove Pluronic acid before use.
 - d. Store unused Pluronic acid stock solution at 2°C–8°C.

Preparation two: Preparations for optimization runs

⌚ Timing: 2 days

Human lymphoblastic leukemia cell line MOLT-4 (ATCC, CRL-1582), promyelocytic HL-60 (ATCC, CCL-240), and chronic myeloid leukemia cells KU812E (ATCC, CRL-2099) can be used for cell spiking experiments. The cell lines can be maintained in complete Roswell Park Memorial Institute (RPMI) containing basic RPMI 1640 medium (Thermo Fisher Scientific, Cat#11875-085) medium, 1% penicillin-streptomycin (Thermo Fisher Scientific, Cat#15140122), and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Cat#10270106) in suspension culture.

3. Thaw cryopreserved cells.
 - a. Pre-warm the complete medium in a 37°C water bath (Enyi, Cat#HH-1S).
 - b. Add 4 mL of prewarmed complete medium to a 15 mL tube (Jet Biofil, Cat#CFT011150).
 - c. Quickly place the cryovial containing the cell suspension into a 37°C water bath and swirl the cryovial constantly to thaw the ice.
 - d. When the frozen cell suspension is thawed, quickly transfer the thawed suspension from the cryovial into a 15 mL tube containing 4 mL pre-warmed medium. Gently mix the suspension.
 - e. Centrifuge (Dynamica, Cat#Velocity 14R) the suspension at 260 × g for 3 min and aspirate the supernatant through a single channel pipette (Eppendorf, Cat#3120000909) to remove the cryopreservation medium containing dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Cat#472301).
 - f. Resuspend the cell pellet with 5 mL of complete RPMI medium, then transfer the 5 mL cell suspension in complete RPMI into an empty T25 culture flask (SPL Life Science, Cat#70025).
 - g. Incubate cells at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Cat#381).
4. Culturing the thawed cells.
 - a. Replace the culture medium 16–24 h after thawing. After that, change the culture medium every 2–3 days.

⚠ **CRITICAL:** Pre-warm the fresh medium to 37°C before replacing the culture medium.

- b. When changing the medium, transfer the cell suspension into a 15 mL centrifuge tube and centrifuge at 260 × g for 3 min (if there are too many cells attached to the wall of the culture flask, add 1–2 mL of PBS to wash the attached cells and combine them into the cell suspension).
- c. After centrifugation, remove the supernatant, resuspend the cells in 10–15 mL of fresh complete RPMI medium, and distribute the cell suspension into 2 or 3 new T25 culture flasks.
- d. Make up the cell suspension to 5 mL in each T25 flask.

⚠ **CRITICAL:** Perform the process inside the BSC, and sterilize all items with 75% ethanol before placing them into the BSC.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CD34-PE (1:250)	Miltenyi Biotec	Cat#130-098-140
anti-CD38-APC (1:250)	Miltenyi Biotec	Cat#130-099-217
Biological samples		
Peripheral blood	ATCC	Cat#IWB1K2E10ML
Chemicals, peptides, and recombinant proteins		
Bovine serum albumin	Thermo Fisher Scientific	Cat#B14
Pluronic F-68 Non-ionic Surfactant (100×)	Thermo Fisher Scientific	Cat#24040-032
Roswell Park Memorial Institute (RPMI) medium 1640	Thermo Fisher Scientific	Cat#11875-085
Penicillin-streptomycin	Thermo Fisher Scientific	Cat#15140122
Fetal bovine serum	Thermo Fisher Scientific	Cat#10270106
Red blood cell lysis buffer	Thermo Fisher Scientific	Cat#00-4333-57
Dimethyl sulfoxide	Sigma-Aldrich	Cat#472301
Phosphate buffered saline	Thermo Fisher Scientific	Cat#70011-044
Negative photoresist	MicroChemicals	Cat#SU-8-2100
SU-8 photoresist developer	MicroChemicals	Cat#Y020100
Isopropyl alcohol (100%)	VWR Chemicals	Cat#470301-480
SYLGARDTM 184 Silicone Elastomer Kit (PDMS)	Dow	Cat#01673921
Hoechst	Thermo Fisher Scientific	Cat#62249
Experimental models: Cell lines		
MOLT-4 cell line	ATCC	Cat#CRL-1582
Software and algorithms		
ImageJ	ImageJ	https://imagej.nih.gov/ij/download.html
AutoCAD	Autodesk	https://www.autodesk.com/products
Other		
Spinner	SETCAS Electronics	Cat# KW-4A
Level hotplate	Shenzhen Xinhaomai Electronic Technology	Cat#X200
Mask aligner	The Institute of Optics and Electronics, Chinese Academy of Sciences	Cat#URE2000/35
Oven	JEIO TECH	Cat#AAH14012K
Plasma cleaner	Harrick Plasma	Cat#PDC-002
CO ₂ Incubator	Thermo Fisher Scientific	Cat#381
Water bath	Enyi	Cat#HH-1S
Silicon wafer (4 inch)	Shenzhen Weina Electronic Technology	Cat#WNRL-GPSIO136
Biological safety cabinet (class II)	NuAire	Cat#NU-543-400
Fluorescent microscope	Nikon	Cat#ECLIPSE-Ci
Microscopic camera	Nikon	Cat#DS-Ri2
Syringe pump	New Era Pump Systems	Cat#NE-4000
Centrifuge	Dynamica	Cat#Velocity 14R
Rotor	Dynamica	Cat#TA14-4-50T Max Speed 14,000 rpm 012-066
Pipette	Eppendorf	Cat#3120000909
Vacuum pump	Rocker	Cat#167300-22
Vacuum Desiccator	SP Bel-Art	Cat#F42020-0000
Biopsy puncher	Miltex	Cat#15110-15
Silicon hose (ID: 0.51 mm, OD: 1.52 mm)	Tygon Microbore	Cat#EW-06419-01
T25 culture flask	SPL Life Science	Cat#70025
15 mL centrifugation tube	Jet BifFil	Cat#CFT011150
1.5 mL centrifugation tube	Jet Biofil	Cat#CFT001015
Clamp	Deli	Cat#8562

MATERIALS AND EQUIPMENT

Complete RPMI medium		
Reagent	Final concentration	Amount
RPMI 1640 Medium	89%	445 mL
FBS	10%	50 mL
Penicillin-streptomycin	1%	5 mL
Total	N/A	500 mL

△ **CRITICAL:** Three items should be used within 12 months of manufacture. RPMI 1640 medium should be stored in the dark at 2°C–8°C. FBS should be stored at a temperature below or equal to –10°C. Penicillin-streptomycin should be stored at –5°C~–20°C. All of these items need to be warmed to around 25°C before mixing. Complete RPMI medium needs to be stored at 2°C–8°C.

STEP-BY-STEP METHOD DETAILS

Device fabrication

⌚ **Timing:** 2 weeks

This part describes the protocol for fabricating the blast cell biochip. The major steps include fabricating each element and assembling them to form a working device.

1. Draw the pattern of the device with the AutoCAD software, using the following steps in detail:
 - a. Draw a helix under the 2D-drawing mode of AutoCAD using the “Helix” tool.

Note: The parameters of the helix should be tuned according to the information below: base radius = 3200 μm, top radius = 12200 μm, turns = 10, twist = clockwise (CW), height = 0.

- b. After creating the helix, draw a line perpendicular to the helix terminal in the width of 600 μm.
 - c. Switch to the 3D-drawing mode, using the “pull” method to transfer the perpendicular line at the helix terminal to a rectangle.
 - d. Use the “Sweep” command to create a spiral pipe with the 600-μm-wide rectangle and the 2D helix.
 - e. Use the “flatten” command to flatten the pattern into a 2D channel [Figure 1A](#).
 - f. Supplement inlet and outlets following the instructions shown in [Figure 1B](#).
2. Submit the design as an AutoCAD file of the final pattern ([Figure 2A](#)) to a local vendor for mask printing.

Note: Typically, local microelectronic equipment providers would likely provide mask printing and silicon mold fabrication services. Examples are Shenzhen Weina Electronic Technology (China), SIMTech Microfluidics Foundry (Singapore), and MicruX (Spain). It usually takes two weeks to receive the printed mask (the detailed timing between different vendors in the same region usually is similar).

3. Conduct photolithography to fabricate the master mold.
 - a. Dispense 4–6 mL of SU-8 2100 negative photoresist (MicroChemicals, Cat#SU-8-2100) onto a cleaned and dehydrated 4-inch silicon wafer (Shenzhen Weina Electronic Technology, Cat#WNRL-GPSIO136).
 - b. Spin-coat using a spinner (SETCAS Electronics, Cat# KW-4A).

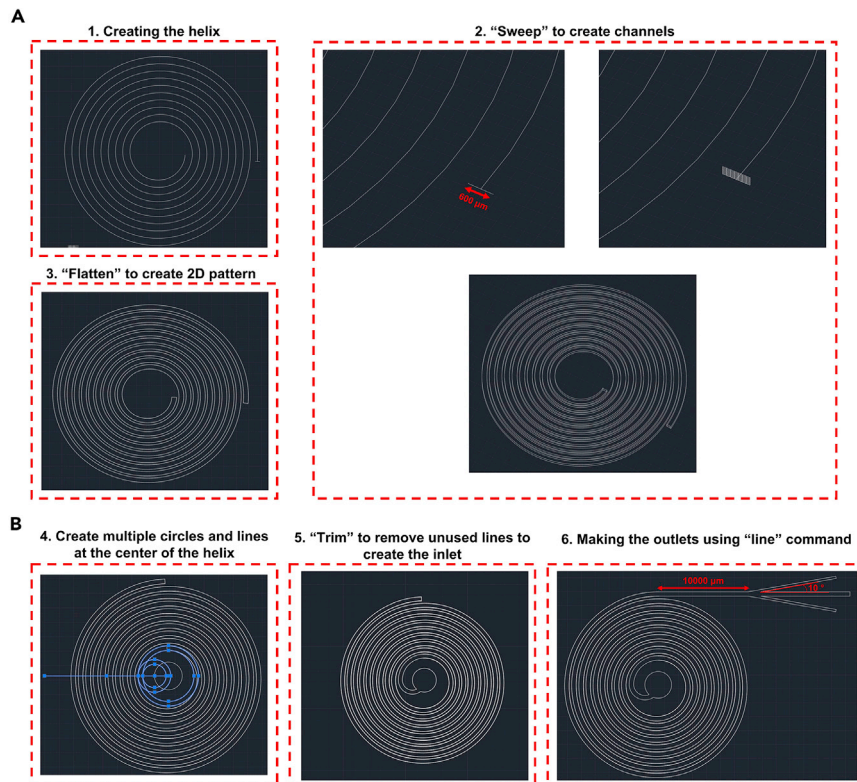


Figure 1. Pattern drawing in AutoCAD

(A) Creating the spiral channel using “helix” and “sweep” commands.

(B) Creating inlet and outlets using “circles”, “lines”, and “trim” commands. The details of the channel diameters are shown in the figure.

Note: Conduct the spinning by ramping to the rotational speed of 500 revolutions per minute (rpm) for 5–10 s with an acceleration of 100 rpm/s, and then ramping to 1750 rpm at 300 rpm/s acceleration for 30 s.

- c. Conduct soft baking for the coated wafer by placing it on a level hotplate (Shenzhen Xinhao-mai Electronic Technology, Cat#X200) and bake at 65°C for 7 min, followed by 95°C for 45 min.

△ **CRITICAL:** Do not use an oven for baking, as it would lead to incomplete drying.

- d. Turn on the mask aligner (The Institute of Optics and Electronics, Chinese Academy of Sciences, Cat#URE2000/35).
- e. Place the baked wafer onto the substrate holder of the mask aligner, and install the feature-printed film mask produced in step 2 onto the mask aligner.
- f. Expose the wafer to the UV light for 9–10 s.
- g. Conduct post-exposure baking on a level hotplate at 65°C for 5 min, followed by 95°C for 15 min.
- h. Fill a glass dish (150 mm in diameter) with around 100 mL photoresist developer (MicroChemicals, Cat#Y020100).
- i. Immerse the exposed wafer into the photoresist developer for 17 min to develop patterns.

Note: Gently shake the glass dish during pattern development to wash away the un-cross-linked photoresist.

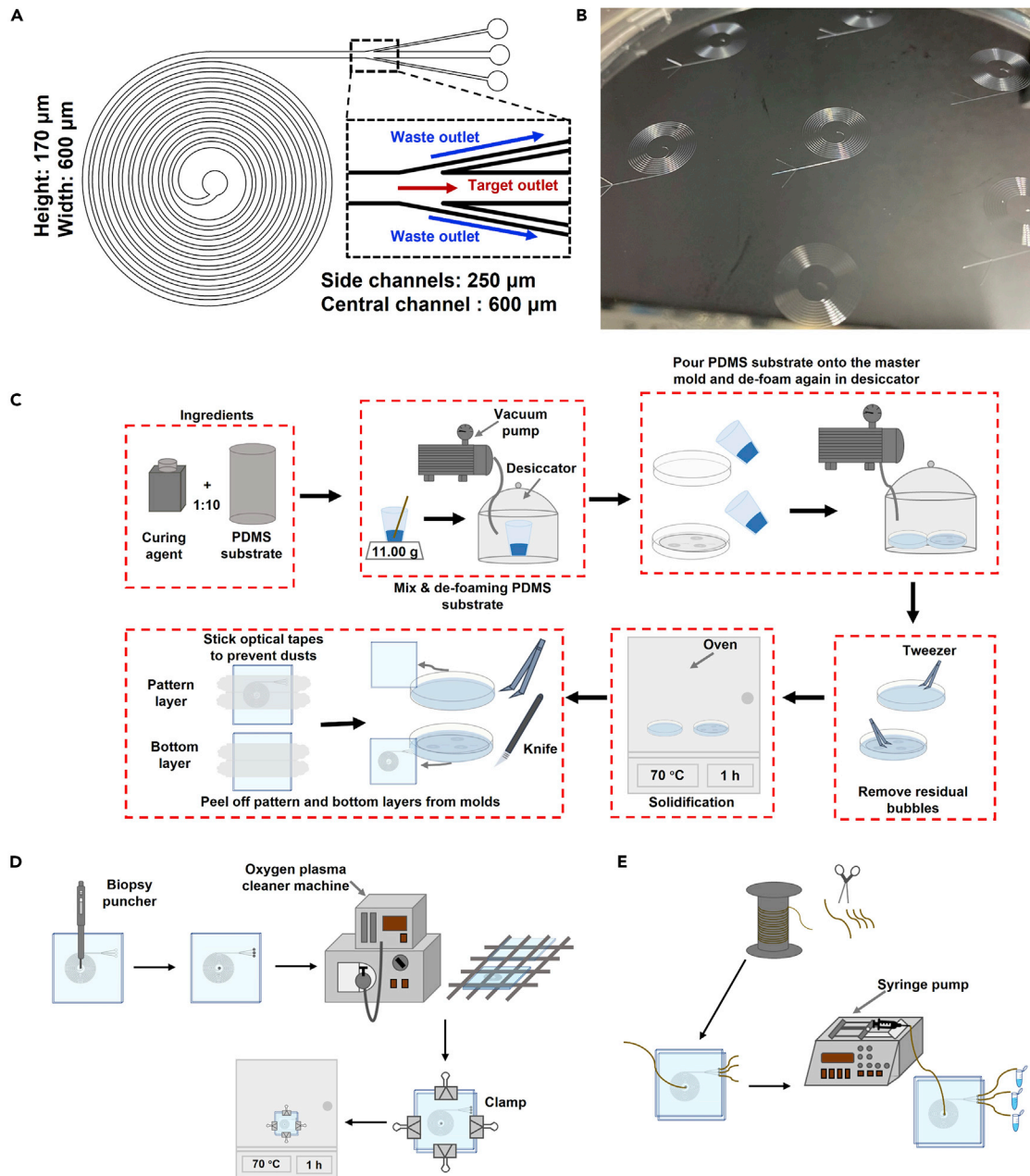


Figure 2. Device fabrication

- (A) Pattern drawing of the chip.
 (B) Master mold on the silicon wafer.
 (C) Preparing PDMS and making pattern layers and bottom layers.
 (D) Bonding a pattern layer and a bottom layer through oxygen plasma.
 (E) Assembling experimental setup by connecting hoses to the microfluidic inlets and outlets and priming the chip with a syringe pump.

- j. Take two glass dishes (150 mm in diameter), fill one dish with a fresh solution of photoresist developer, and the other with isopropyl alcohol (VWR Chemicals, Cat#470301-480).
- k. Immerse the developed wafer into the fresh photoresist developer to wash for approximately 10 s.
- l. Take the wafer and immerse it into isopropyl alcohol for another 10 s.
- m. Bake the wafer on a level hotplate at 95°C for 30–40 min.

Note: Step 3m (final baking) aims at drying the isopropyl alcohol and strengthening the crosslinked photoresist. The final product would be a silicon mold with patterns of the micro-channels (Figure 2B).

Pause point: Fabricated master mold can be stored at room temperature (24°C–26°C) until use.

4. Before soft lithography, prepare the PDMS (Dow, Cat#01673921) mixture.
 - a. Place a disposable cup on balance and zero the balance.
 - b. Weigh and mix 10 g of the PDMS substrate and 1 g of curing agent (ratio of 10: 1).

CRITICAL: Mix the PDMS substrate and curing agent thoroughly by stirring the mixture (with any tools that wouldn't pollute the mixture, including a clean plastic pipette or a disposable spoon).

- c. Place the mixed PDMS substrate and curing agent into a desiccator.
- d. Link the desiccator to a vacuum pump (Cat#167300-22) through a hose.
- e. Turn on the vacuum pump to draw out the gas inside the desiccator for approximately 30 min to remove bubbles inside the PDMS substrate.

Note: Working pressure of vacuum pump in step 4e is around 100 kPa.

Pause point: At this step, the prepared PDMS can be stored in the refrigerator at –20°C for a maximum of 2 weeks.

5. Fabricate the pattern layer of the spiral microfluidics (Figure 2C).
 - a. Pour the prepared PDMS directly on the silicon master mold and distribute it evenly.
 - b. Place the silicon mold with PDMS into a desiccator, and degas with the same method described in step 4.
 - c. After complete defoaming, check the PDMS substrate carefully.

Note: If residual bubbles are in the PDMS substrate, the user can gently use a tweezer to remove residual air bubbles on the PDMS surface.

- d. Incubate the device in a 70°C oven (JEIO TECH, Cat#AAH14012K) to solidify for an hour.
- e. Cut the four sides of the solidified pattern layer with a knife and separate it from the mold with tweezers to obtain a complete spiral microfluidic pattern layer.

CRITICAL: The unused pattern layers should be covered with tape to avoid the adhesion of oil residue, fingerprints, soil, dust particles, or other micro fragments.

6. Fabricate the bottom layer of the spiral microfluidics (Figure 2C).
 - a. Pour the prepared PDMS on a 120 mm plate and distribute the PDMS evenly.
 - b. Use a desiccator and a vacuum pump to remove bubbles in the PDMS for 10–20 min (using the same method described in step 4).
 - c. After desiccation, use tweezers to remove the residual air bubbles on the PDMS surface.
 - d. Incubate the bottom layer in a 70°C oven for an hour to solidify the PDMS.
 - e. Use a knife to cut out an area equivalent to the size of the pattern layer.

Note: If there are oil residue, fingerprints, soil, dust particles, or other micro fragments (such as pollen or spores) on the pattern and bottom layers, the user can use an optical tape to remove them by sticking the tape to the layers repeatedly and gently.

7. Punching inlet and outlets on the pattern layer (Figure 2D).
 - a. Hold the plastic handle of a biopsy puncher (Miltex, Cat#15110-15) and vertically align the steel tip to an inlet or outlet.
 - b. Increase the force to vertically insert the tip of the biopsy puncher into the PDMS layer until it penetrates the whole layer.
 - c. Press the plunger to expel the PDMS specimen and gently pull out the tip from the PDMS layer, remaining a 1.5 mm crack-free hole on the pattern layer.
 - d. Repeat steps a to c for all the one inlet and three outlets.
8. Assembling pattern layer and bottom layer (Figure 2D).
 - a. Turn on the power of the oxygen plasma cleaner machine (Harrick Plasma, Cat#PDC-002).
 - b. Select HI mode at a pressure of 700–800 mTorr, and perform plasma treatment for 5 min.
 - c. After oxygen plasma treatment, align the pattern and bottom layers and press firmly.
 - d. At the same time as step c, use clamps (Deli, Cat#8562) to clamp the periphery of the finished product to enhance adhesion.
 - e. After finishing the operations above, quickly place the product into a 70°C oven to enhance the bonding and incubate for an hour to bond the device firmly.
9. Verify the newly fabricated devices (Figure 2E).
 - a. Insert silicon hoses (outer diameter = 1.5 mm; inner diameter = 0.5 mm) (Tygon Microbore, Cat#EW-06419-01) into the inlet and three outlets.
 - b. Connect the hose at the inlet to a 10 mL syringe.
 - c. Use a syringe pump (New Era Pump Systems, Cat#NE-4000) to inject water into the spiral microfluidic device at a 1.5 mL/min flow rate.
 - d. Verify that there is no blockage or leakage in the device.

⚠ **CRITICAL:** During the device checking, attention should be paid to the liquid distribution of the three outlets. The output volume ratio of waste outlet 1: target outlet: waste outlet 2 should be similar to the ratio of their channel widths (5:12:5).

⏸ **Pause point:** Fabricated devices can be stored at room temperature (24°C–26°C) until use.

Preparation of blood samples

⌚ **Timing:** 30 min

Red blood cells in the sample need to be lysed and eliminated prior to treatment with BCB to isolate blast cells from clinical blood samples.

10. Pre-warm the blood (ATCC, Cat#IWB1K2E10ML) sample to restore room temperature (24°C–26°C).
11. Mix 1 mL blood sample with 9 mL red blood cell lysis buffer (RBC lysis buffer) (Thermo Fisher Scientific, Cat#00–4333–57) inside a 15 mL falcon tube.
12. Shake the tube for 3 min until the contents are dark red to ensure complete RBC lysis.
13. Centrifuge at 1000 × *g* for 5 min (25°C) to deposit white blood cells (WBCs) on the bottom of the tube.
14. Aspirate the supernatant by a single channel pipette and add 1 mL PBS to mix quickly by pipetting for the wash.
15. Transfer the sample to a 1.5 mL tube (Jet Biofil, Cat#CFT001015) and centrifuge (260 × *g*, 3 min).
16. Remove the supernatant with a single channel pipette.
17. Add 1 mL fresh PBS into the tube to resuspend the pellet by a pipette (Figure 3A).

⚠ **CRITICAL:** The pellets from completely lysed blood samples should be white. If the pellet is still red, the sample should be lysed again. Large counts of RBCs can generate clots that

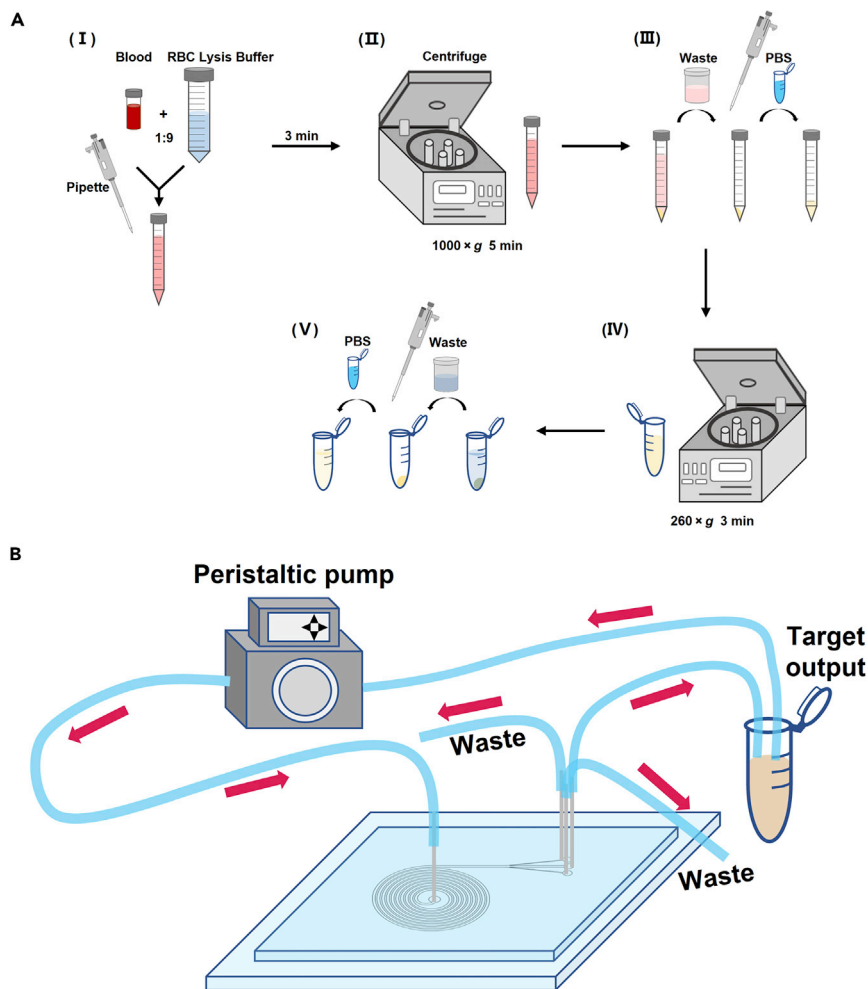


Figure 3. Blood collection and processing

(A) Schematic workflow of blood collection and lysis.

(B) General setup of the biochip for processing lysed blood samples in a feedback-loop mode.

would lead to biofouling within the device and generate background noise during immunostaining.

▣ **Pause point:** WBCs can be stored in liquid nitrogen for a maximum of 6 months in complete RPMI medium containing 10% DMSO.

Sample processing

⌚ **Timing:** 20 min for a single run, 30 min for a feedback-loop run

This part of the protocol describes the detailed operations of processing blood samples with the BCB device. Before processing blood samples, the device needs to be pre-treated with BSA to reduce the adhesion of cells to the channel walls and flushed to ensure that no blockage is present in the channels. During the on-chip processing, the flow rate needs to be kept stable to ensure the effective separation of target cells.

18. Before processing, add BSA-PBS solution to the sample to achieve a final BSA concentration of 2.5%.

Note: Alternative to BSA-PBS in step 18, the user can also dissolve the sample with 0.1% Pluronic acid.

19. Fill a 10 mL syringe with PBS, and connect the syringe to the inlet of the spiral biochip.
20. Install the syringe onto a syringe pump and prime the chip by flushing with PBS under a 1.5 mL/min flow rate for 60 s ([troubleshooting 1](#)).

△ CRITICAL: During priming of the device, observe the channels under a microscope (4× magnification) to ensure no leaks in the device and contaminants are not blocking the channels.

21. Single-run blast cell isolation:
 - a. Estimate the cell concentration of the lysed blood sample with a hemocytometer.
 - b. Centrifuge the lysed blood sample at $260 \times g$ for 5 min to remove the supernatant.
 - c. Resuspend the cells to a final concentration of $\leq 100,000$ cells per mL in PBS buffer with 0.1% Pluronic acid.
 - d. Load the sample into a 10 mL syringe, connect it to the primed chip, and connect the three outlets to three 5 mL centrifuge tubes.
 - e. After assembling the setup, prime the sample into the biochip under a 1.5 mL/min flow rate and collect the sample from the target outlet. ([troubleshooting 2](#)).
22. For isolation and concentration of blast cells through feedback-loop mode:
 - a. After finishing the single isolation run in the last step, resuspend the collected sample from the target (central) outlet in 10 mL PBS buffer with 0.1% Pluronic acid or 2.5% BSA-PBS.
 - b. The resuspended sample is processed on the biochip at a 1.5 mL/min flow rate.
 - c. Feed the sample from the target outlet back to the biochip and repeat steps a to c until the remaining sample volume from the target outlet is approximately 0.5 mL ([Figure 3B](#)). ([troubleshooting 3](#)).

Note: The feedback-loop mode can increase the purity and concentration of blast cells in the final enriched sample. This repeated feedback-loop process can be achieved by manually re-directing samples from targeted outlets with a syringe pump or directly linking the target outlet to the inlet with a peristaltic pump.

23. Flush the biochip with PBS immediately after the end of processing. The device can be reused if the channels remain clean without biofouling.

Immunostaining

⌚ Timing: 1.5 h

After processing the blood sample with the BCB device, the operator needs to check and identify the target leukemic blast cells from the output using immunostaining and imaging. CD34 and CD38 were used as biomarkers for discriminating blast cells and other leukocytes.

24. Centrifuge the sample obtained from the target outlet ($260 \times g$, 5 min).
25. Reduce the suspension volume to 100 μ L by removing the excessive supernatant with a pipette.
26. Add Hoechst (0.1 μ L) (Thermo Fisher Scientific, Cat#62249), anti-rabbit CD34-PE (0.4 μ L) (Miltenyi Biotec, Cat#130-098-140), and anti-mouse CD38-APC (0.4 μ L) (Miltenyi Biotec, Cat#130-099-217) to the 100 μ L suspension.
27. Resuspend the solution thoroughly by pipetting.
28. Incubate the mixed solution in the dark at 37°C for an hour.

Note: If the cell concentration is too high, more PBS buffer can be added, with the amounts of Hoechst and antibody added revised according to their respective diluting ratio (1:1000 (v/v) for Hoechst and 1:250 (v/v) for anti-CD34 and anti-CD38).

29. Centrifuge ($260 \times g$, 5 min) removes the unbound antibodies.
30. Add 1 mL PBS to resuspend the pellet. Centrifuge ($260 \times g$, 5 min) and remove the supernatant with a pipette.
31. Resuspend the cells with 50 μ L PBS and transfer the suspension into a 96-well plate by a pipette.

△ CRITICAL: Before imaging, keep the 96-well plate in the dark for about 10 min to allow cells to settle to the bottom of the well.

Imaging

⌚ Timing: 1 h

32. Immobilize the 96-well plate mentioned in step 31 on a fluorescent microscope (Nikon, Cat#ECLIPSE-Ci) holder, and adjust the focus under brightfield with a 10 \times objective lens.
33. Turn on the laser using the operating software.
34. Adjust gain and exposure time, and keep the setting consistent for the imaging process (Figure 4A).

Note: Emission filters DAPI, AF594, and AF633 are used for imaging Hoechst, CD-34-PE, and CD38-APC.

35. Taking images using the three filters mentioned in step 34 for each field of view. Gently move the field of view to scan all sample wells thoroughly. (troubleshooting 4) (troubleshooting 5) (troubleshooting 6).
36. To better visualize the fluorescent images, merge figures taken by different fluorescent channels at the same position were merged by ImageJ, following the procedures shown in Figure 4B.

Image analysis

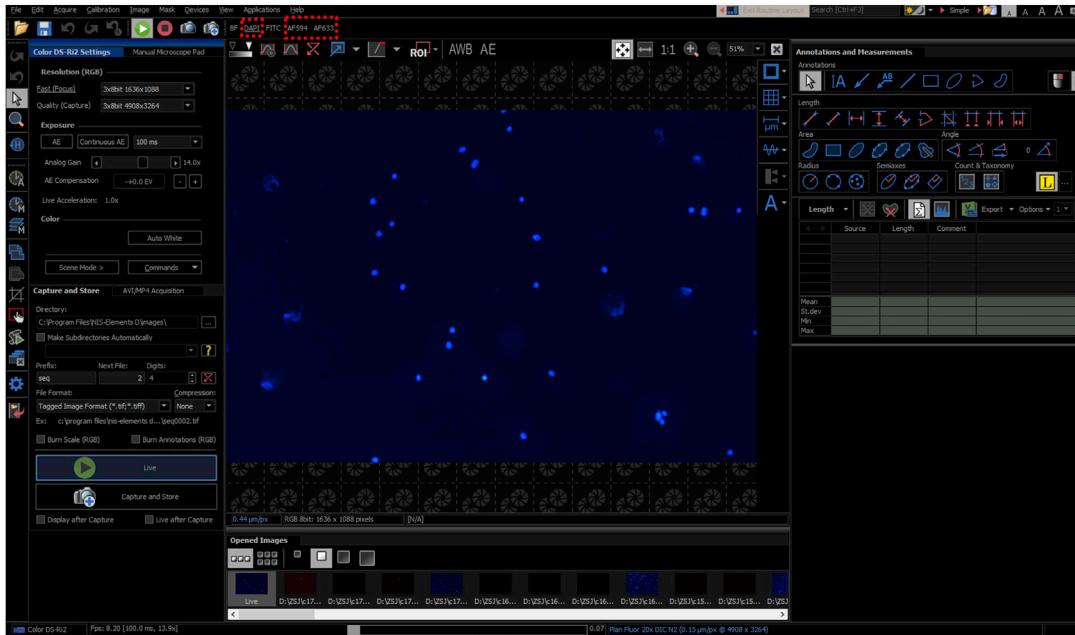
⌚ Timing: 1 h

This part describes the protocol for processing the fluorescent images in ImageJ to obtain counts of the target cells (CD34⁺/CD38⁺/Hoechst) from microfluidic outlets.

37. Open the file containing the fluorescent image of Hoechst staining using ImageJ.
38. Run the following macro in the ImageJ.

```
run('8-bit');
run('Auto Local Threshold...', 'method=Bernsen radius=15 parameter_1=0 parameter_2=0 white');
setOption('BlackBackground', false);
run('Make Binary');
run('Fill Holes');
run('Watershed');
run('Analyze Particles...', 'size=20-250 circularity=0.3-1.00 display clear summarize add');
```

A



B

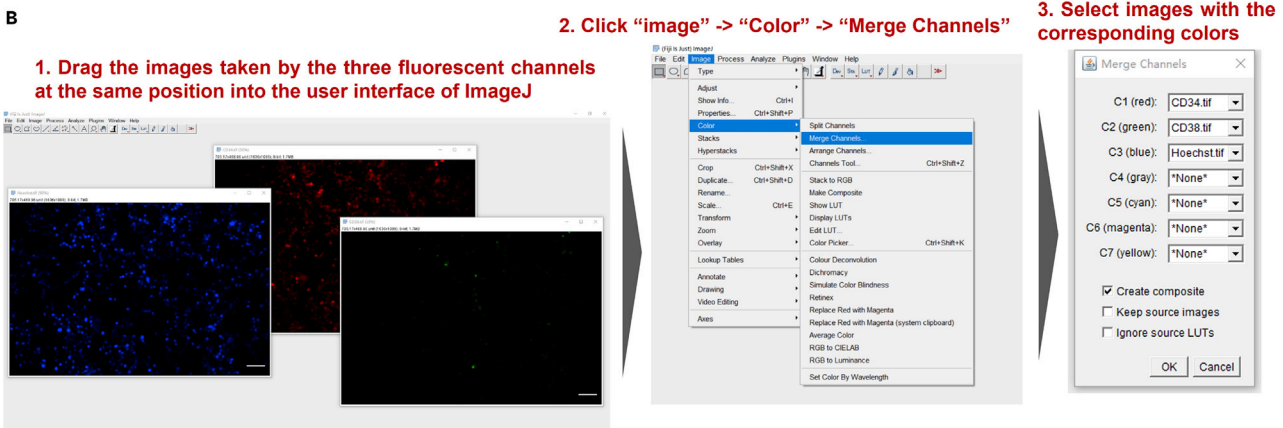


Figure 4. Imaging and merging of fluorescent images

(A) The interface of imaging in the software for operating fluorescent microscope.
(B) Merging images from multiple fluorescent channels.

Note: The macro helps identify the Hoechst-stained cells and extract the regions with positive signals, regions-of-interest (ROI). Automatically, the macro would record and display the ROIs in the “ROI Manager” of ImageJ (Figure 5A).

39. Keep the “ROI Manager” open. Using ImageJ, open the file containing the fluorescent image of CD34 corresponding to Hoechst staining of the same field of view, as described in step 37.
40. Select “Measure” in the “ROI Manager” to obtain the raw signal intensity of CD34.
41. Use the rectangular selection tool of ImageJ to measure the background intensity at positions without cells (step 4a in Figure 5B) and calculate the mean area of the background intensity.
42. Copy the background intensity and raw CD34 intensity into an Excel table.
43. Repeat steps 39–42 on the CD38 fluorescent image of the same cells (Figure 5B).

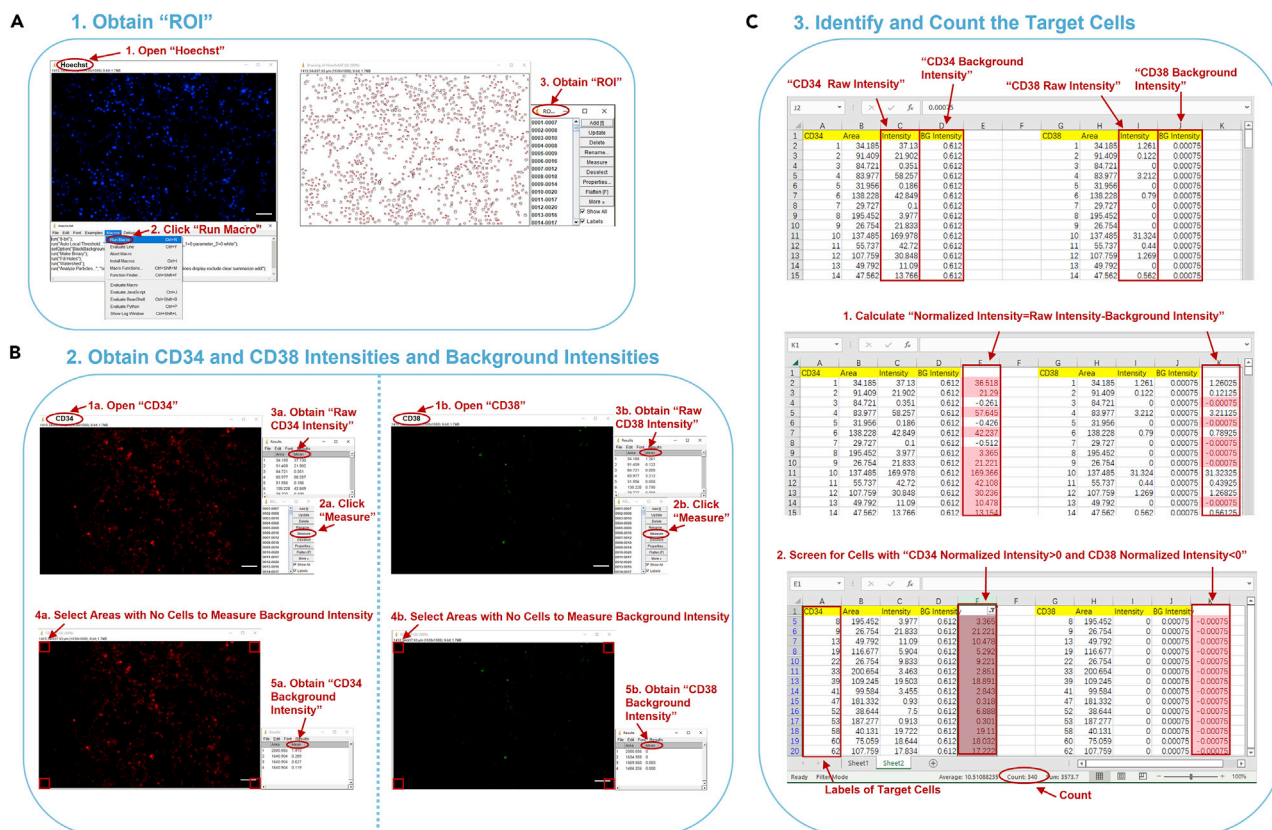


Figure 5. Image analysis with ImageJ

(A) Obtaining region-of-interest (ROI) using macro based on a fluorescent image of Hoechst.
 (B) Using the obtained ROIs to quantify signal intensities of CD34 and CD38 and their corresponding background signal intensity.
 (C) Calculate the normalized intensity of each ROI and obtain counts of target cells (CD34⁺/CD38⁻/Hoechst cells).

44. In the Excel table containing the raw intensity and background intensity of CD34 and CD38, align the data based on ROI identities, and calculate the normalized intensity of each ROI according to the formulation shown in Figure 5C.
45. Identify ROIs with CD34 Normalized Intensity > 0 and CD38 Normalized Intensity < 0 as target cells (Figure 5C).
46. Obtain counts of the target cells in each field of view.
47. Repeat steps 37–46 for all the images taken from the same outlet. Obtain the total counts of target cells in an outlet by summing the counts of its corresponding images.

EXPECTED OUTCOMES

Microfluidic chips based on inertial focusing can efficiently sort particles (Chen et al., 2022). Similar applications capitalizing on the intrinsic mechanical and morphological properties of microparticles, including stiffness, size, and shape, have been reported for the label-free enrichment of heterogeneous cell suspensions in liquid biopsies (Khoo et al., 2019; Liao et al., 2021).

This protocol is a guideline for detecting and enriching leukemic blasts from liquid blood biopsies using a designated microfluidic device with curvilinear channels (BCB platform). By processing liquid biopsy samples at a flow rate of 1.5 mL/min using the BCB, targeted blasts are expected to be enriched at the central outlet due to their higher stiffness than healthy leukocytes (Khoo et al., 2019; Zheng et al., 2015). Immunostaining combined with ImageJ image analysis can identify enriched blasts and quantify the effect of enrichment. CD34 is a biomarker associated with leukocyte

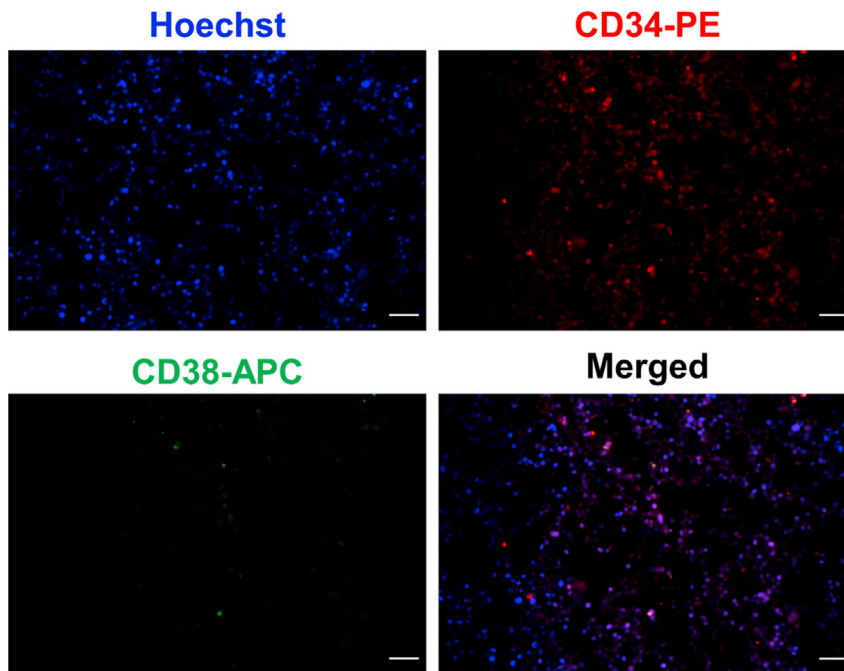


Figure 6. Immunostaining of enriched blast cells

Enriched cells were stained with anti-CD34-PE and anti-CD38-APC antibodies to identify $CD34^+CD38^-$ blast cell subpopulations. Hoechst dye was used to mark the position of cells. The scale bar is 50 μm .

stemness. It has been used to label blast cells in leukemia patients (Satterthwaite et al., 1992). Ideally, $CD34^+/CD38^-/Hoechst$ cells could only be found in the output of the central outlet by immunostaining. Hoechst staining is used to identify all cells through binding to DNA in the nucleus, and Hoechst-stained cells would therefore be distributed relatively equally among the outlets (Latt et al., 1975).

To demonstrate the expected results of characterizing blast cell enrichment from liquid biopsies, we simulated liquid biopsy samples by spiking MOLT-4 cells into healthy whole blood and carried out the enrichment process with BCB. Enriched cells in the target outlet were stained with Hoechst, CD38, and CD34 antibodies to distinguish leukemic blasts from healthy leukocytes. We identified cells expressing target surface antigens by combining the signals from the fluorescence channels corresponding to the three stains. $CD34^+/CD38^-/Hoechst$ cells were identified as leukemic blasts (Figure 6).

In performance evaluation, the enrichment efficiency was assessed as the recovery rate calculated as the ratio of the target cells obtained from the target outlet to the total number of target cells in all outlets. Ideally, $CD34^+/CD38^-/Hoechst$ blasts should only appear at the central outlet. Cell recoveries could be higher than 90% for samples with high blast ratios (>5%) and remained around 65% in samples with < 5% blasts.

LIMITATIONS

While the microfluidic device could focus target cells at the central outlet, non-target cells (primarily healthy white blood cells) were evenly distributed. Non-target cells within the central outlet can affect the purity of the enriched blast cells. These problems can be resolved by repeatedly adding a feedback loop to process the output from the target outlet. Recirculation of the feedback loop can significantly increase the concentration and purity of the target cells, at the expense of cell recovery, due to the inevitable loss of cells in each run. Users should consider all of these factors when planning the analysis setup.

TROUBLESHOOTING

Problem 1

Microchannels blocked by PDMS fragments or air bubbles (step 20):

PDMS fragments can be generated during punching inlets and outlets, while air bubbles can be introduced from buffer or sample. If these blockages are present in the microchannel, they would cause a change in the fluidic features or even block the flow, causing failures in target cell enriching.

Potential solution

The operator can examine the chip under a microscope (4 × magnification) to identify and monitor the site of obstruction. Flushing the channel at high flow rates may flush out the blockage. The operator should replace the chip if the blockage cannot be removed.

Problem 2

The target cell is not enriched in the central outlet (steps 19 and 20):

In some cases, the target cells are not enriched in the target outlet as expected. If no apparent blockages are identified, this is very likely due to dimensional problems of the device. For example, the channel height might not be the same as designed, or the bottom layer is not flat. These problems cause changes in inertial forces and thereby affect the sorting of cells.

Potential solution

The operator can carefully repeat the soft lithography and plasma bonding and test the new device. If the problem persists, the operator must fabricate a new master mold.

Problem 3

Enrichment at the target outlet is unstable (steps 21 and 22):

There might be bubbles in the channel, or the flow rate is not adjusted to a suitable value.

Potential solution

Examine the entire device under a microscope and remove air bubbles if they are found. Ensure that the flow rate is properly adjusted to 1.3–1.5 mL/min.

Problem 4

False-positive during immunostaining (step 35):

If an excess amount of antibody is added in step 26, non-specific binding may occur, causing false-positive results.

Potential solution

Decrease antibody concentration. Lowering the antibody concentration can avoid non-specific binding. Antibody titration can be performed on new antibody vials. Titration can be based on concentrations from 1:50 to 1:400 (antibody: cell suspension, v/v) to choose the optimal concentration.

Problem 5

High background signal during imaging of immunostaining (step 35):

If the concentration of the antibodies is too high in step 26 or washing is insufficient in steps 29 and 30, there would be a high fluorescent signal in the background.

Potential solution

Reduce the concentration of antibodies, and increase the wash buffer volume and washing time to wash away the excessive probes. Doing antibody titration may help to identify an optimal concentration.

Problem 6

No signals detected after immunostaining (step 35):

If the concentration of antibodies is too low in step 26, the signal could be very weak, especially when the abundance of the target cell is low.

Potential solution

Use a higher concentration of antibodies for staining (for example, 1:100). Doing antibody titration may help to identify an optimal concentration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Bee Luan Khoo (blkhoo@cityu.edu.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The ImageJ macro for image analysis in this study is available in the in-line text box at step 38.

This study did not generate datasets.

ACKNOWLEDGMENTS

This study was supported by the City University of Hong Kong, which is funded by the Research Grants Council (RGC). This work was also supported by the City University of Hong Kong (9610430, 7005208, 7005464, 7020002, 9667220); Hong Kong Center for Cerebro-Cardiovascular Health Engineering (COCHE); Research Grants Council of the Hong Kong Special Administrative Region (9048206); Pneumoconiosis Compensation Fund Board (9211276); and the Hetao Shenzhen-Hong Kong Science and Technology Innovation Cooperation Zone Shenzhen Park Project (HZQB-KCZYZ-2021017).

AUTHOR CONTRIBUTIONS

Y.F., S.Z., and B.L.K. conceived and designed experiments. Y.F. and S.Z. performed experiments. Y.F., S.Z., and B.L.K. wrote the manuscript. All authors have read the manuscript.

DECLARATION OF INTERESTS

One or more authors have a patent related to this work.

REFERENCES

- Chen, C.K., Zhang, J., Bhingarde, A., Matotek, T., Barrett, J., Hardesty, B.D., Banaszak Holl, M.M., and Khoo, B.L. (2022). A portable purification system for the rapid removal of microplastics from environmental samples. *Chem. Eng. J.* 428, 132614.
- Khoo, B.L., Shang, M., Ng, C.H., Lim, C.T., Chng, W.J., and Han, J. (2019). Liquid biopsy for minimal residual disease detection in leukemia using a portable blast cell biochip. *NPJ Precis. Oncol.* 3, 30.
- Latt, S.A., Stetten, G., Juergens, L.A., Willard, H.F., and Scher, C.D. (1975). Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. *J. Histochem. Cytochem.* 23, 493–505.
- Liao, J., Ren, J., Wei, H., Lam, R.H.W., Chua, S.L., and Khoo, B.L. (2021). Label-free biosensor of phagocytosis for diagnosing bacterial infections. *Biosens. Bioelectron.* 191, 113412.
- Satterthwaite, A.B., Burn, T.C., Le Beau, M.M., and Tenen, D.G. (1992). Structure of the gene encoding CD34, a human hematopoietic stem cell antigen. *Genomics* 12, 788–794.
- Zheng, Y., Wen, J., Nguyen, J., Cachia, M.A., Wang, C., and Sun, Y. (2015). Decreased deformability of lymphocytes in chronic lymphocytic leukemia. *Sci. Rep.* 5, 7613.