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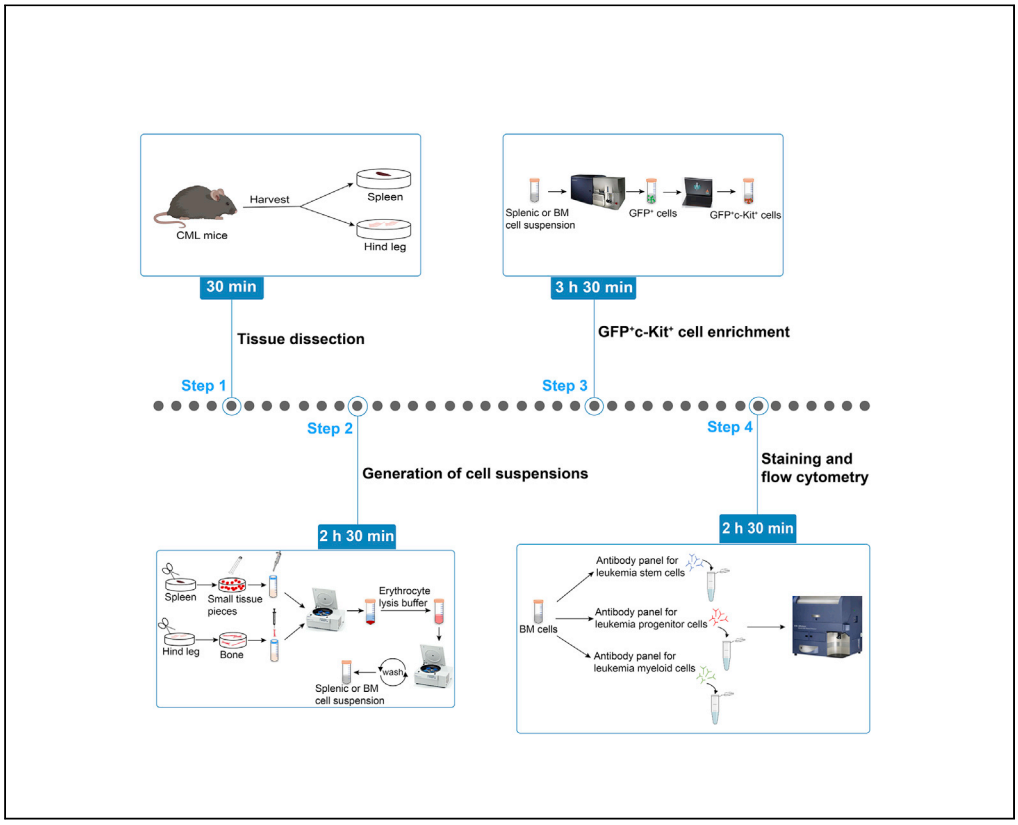
Protocol

Protocol for isolation and analysis of the leukemia stem cells in BCR-ABL-driven chronic myelogenous leukemia mice



Practical procedures for sorting and analysis of leukemia stem cells (LSCs) are to improve our understanding of chronic myelogenous leukemia (CML). Here, we present a detailed magnetic-bead-based sorting and flow-cytometry-based analysis protocol for LSCs in BCR-ABL-driven CML mice. We describe steps for sorting and functional analysis of BCR-ABL-expressing c-Kit$^+$ cells (GFP$^+$c-Kit$^+$) from CML mice as well as antibody staining and gating strategies for characterization of leukemia stem/progenitor cells and myeloid leukemia cells.

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

Chang Liu, Xiaoying Lin, Yanli Jin, Jingxuan Pan

yanlijin2014@jnu.edu.cn (Y.J.)
panjx2@mail.sysu.edu.cn (J.P.)

Highlights

FACS- and magnetic-bead-based protocol to isolate GFP$^+$c-Kit$^+$ cells from CML mice

Applicable to functional analysis of sorted GFP$^+$c-Kit$^+$ cells *in vitro*

Antibody staining of leukemia stem/progenitor cells prior to FACS running

Gating and quantitative analysis of leukemia stem/progenitor cells and myeloid cells

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Protocol for isolation and analysis of the leukemia stem cells in BCR-ABL-driven chronic myelogenous leukemia mice

Chang Liu,[1,3,4] Xiaoying Lin,[1,3,4] Yanli Jin,[1,*] and Jingxuan Pan[2,5,*]

[1]Jinan University Institute of Tumor Pharmacology, College of Pharmacy, Jinan University, Guangzhou 510632, China

[2]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China

[3]These authors contributed equally

[4]Technical contact: liuchang0206@126.com, 15217634117@163.com

[5]Lead contact

*Correspondence: yanlijin2014@jnu.edu.cn (Y.J.), panjx2@mail.sysu.edu.cn (J.P.)
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SUMMARY

Practical procedures for sorting and analysis of leukemia stem cells (LSCs) are to improve our understanding of chronic myelogenous leukemia (CML). Here, we present a detailed magnetic-bead-based sorting and flow-cytometry-based analysis protocol for LSCs in BCR-ABL-driven CML mice. We describe steps for sorting and functional analysis of BCR-ABL-expressing c-Kit$^+$ cells (GFP$^+$c-Kit$^+$) from CML mice as well as antibody staining and gating strategies for characterization of leukemia stem/progenitor cells and myeloid leukemia cells.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2022).[1]

BEFORE YOU BEGIN

Institutional permission

The C57BL/6 mice were kept in a specific pathogen-free environment at the Animal Experiment Center of Jinan University. All animal experiments described in this protocol were approved by the Institutional Animal Care and Use Committee of Jinan University and conducted in full accordance with the institutional guidelines.

Preparation of solutions

Timing: 1–2 h

All solutions and buffers used in this protocol can either be prepared in advance and stored as described, or be freshly prepared on the day of experiment. Please refer to materials and equipment for specific buffer recipes.

Preparation of scissors and tweezers

Timing: 2 h

Scissors and tweezers should be autoclaved sterilization in advance.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-mouse Lin antibody	BD Biosciences	Cat#558074;RRID: AB_1645213
PE anti-mouse c-Kit antibody	BD Biosciences	Cat#553355; RRID: AB_394806
APC-H7 anti-mouse c-Kit antibody	BD Biosciences	Cat#560185; RRID: AB_1645231
BV421 anti-mouse Sca-1 antibody	BioLegend	Cat#108127;RRID: AB_10898327
PE-CF594 anti-mouse Sca-1 antibody	BD Biosciences	Cat#562730; RRID: AB_2737751
PE-Cy5 anti-mouse CD135 antibody	eBioscience	Cat#15-1351-82; RRID: AB_494219
PE-Cy7 anti-mouse CD150 antibody	eBioscience	Cat#25-1502-82; RRID: AB_10805742
APC-Cy7 anti-mouse CD48 antibody	BD Biosciences	Cat#561242; RRID: AB_10644381
PE anti-mouse CD34 antibody	BD Biosciences	Cat#551387; RRID: AB_394176
PE-Cy7 anti-mouse CD16/32 antibody	eBioscience	Cat#25-0161-82; RRID: AB_469598
APC anti-mouse Gr-1 antibody	BD Biosciences	Cat#553129; RRID: AB_398532
Chemicals, peptides, and recombinant proteins		
NaCl	MilliporeSigma	Cat#S5886
KCl	MilliporeSigma	Cat#P5405
$Na_2HPO_4 \cdot 12H_2O$	MilliporeSigma	Cat#1065790500
KH_2PO_4	MilliporeSigma	Cat# P0662
NH_4Cl	MilliporeSigma	Cat#A9434
$KHCO_3$	MilliporeSigma	Cat#237205
EDTA	MilliporeSigma	Cat#E9884
BSA (bovine serum albumin)	MilliporeSigma	Cat#V900933
Penicillin streptomycin solution	Gibco	Cat#15140-122
5-Fluorouracil (5-FU)	MilliporeSigma	Cat#F6627
SCF	Pepro Tech	Cat#250-03
IL-3	Pepro Tech	Cat#213-13
IL-6	Pepro Tech	Cat#216-16
MethoCult™ GF M3434	STEMCELL Technologies	Cat# 03434
FBS (fetal bovine serum)	Transgen Biotec	Cat#FS301-02
Critical commercial assays		
CD117 Microbeads Kit	Miltenyi Biotec	Cat#130-091-224
Experimental models: Organisms/strains		
Mouse: C57BL/6 (donor), male, 6–8 weeks old. C57BL/6 (recipient), female, 6–8 weeks old.	Guangdong Medical Laboratory Animal Center	N/A
Other		
Falcon® 40 μm Cell Strainer	Corning	Cat#352340
6 cm cell culture dish	Corning	Cat#353002
50 mL tube	NEST	Cat#602052
15 mL tube	NEST	Cat#601052
1.5 mL Eppendorf tube	MilliporeSigma	Cat#T6649
1 mL syringe	N/A	N/A
MACS™ MuliStand	Miltenyi Biotec	Cat#130-042-303
MiniMACS Separator	Miltenyi Biotec	Cat#130-042-102
MS Columns	Miltenyi Biotec	Cat#130-042-201
Centrifuge 5427 R	Eppendorf	N/A
Centrifuge 5810 R	Eppendorf	N/A
BD LSRFortessa Flow Cytometer	BD Biosciences	N/A
BD Aria II Flow Cytometer	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Buffers

PBS		
Reagent	Final concentration	Amount
NaCl	136.7 mM	8 g
KCl	2.68 mM	0.2 g
$Na_2HPO_4 \cdot 12H_2O$	9.96 mM	3.57 g
KH_2PO_4	1.76 mM	0.24 g
ddH_2O	N/A	Adjust to 1 L
Total	**N/A**	**1 L**

Note: Dissolve NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4 in 900 mL double distilled H_2O. Adjust volume to 1L and autoclaved sterilization. Keep sterile and store the solution at 4°C for up to 6 months. PBS can also be commercially bought.

BM flush buffer		
Reagent	Final concentration	Amount
FBS	2% (v/v)	20 mL
PBS	N/A	Adjust to 1 L
Total	**N/A**	**1 L**

Note: Add FBS freshly before the experiment. Keep sterile and store the solution at 4°C for up to 1 month.

Erythrocyte lysis buffer		
Reagent	Final concentration	Amount
NH_4Cl	149.9 mM	8.02 g
$KHCO_3$	10.0 mM	1 g
EDTA	0.1 mM	0.0372 g
ddH_2O	N/A	Adjust to 1 L
Total	**N/A**	**1 L**

Note: Dissolve all reagents in 900 mL double distilled H_2O. Adjust volume to 1L and filter sterilize. Keep sterile and store the solution at 4°C for up to 6 months. Erythrocyte lysis buffer can also be commercially bought.

0.5% BSA buffer		
Reagent	Final concentration	Amount
BSA	0.5% (g/v)	5 g
EDTA	0.146 mM	293 µL (from 0.5 M solution)
PBS	N/A	Adjust to 1 L
Total	**N/A**	**1 L**

Note: Dissolve BSA in 900 mL PBS, add 293 µL 0.5 M EDTA. Adjust volume to 1L and filter sterilize. Keep sterile and store the solution at 4°C for up to 1 month.

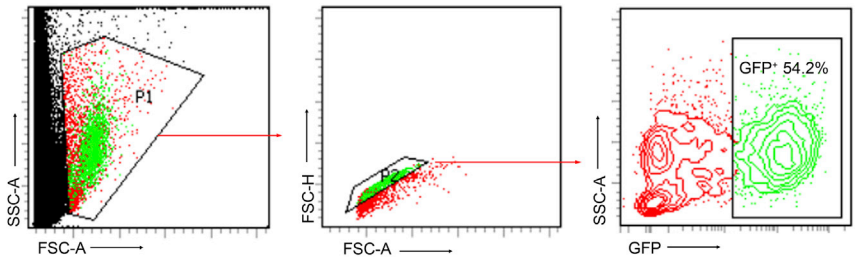


Figure 1. The percentage of GFP$^+$ cells in PB of a representative CML mouse at day 14 post transplantation

BD LSRFortessa flow cytometer

Samples were detected on a BD LSRFortessa flow cytometer (BD Biosciences). The panels used in this protocol were designed according to the configurations of the flow cytometer: 1 channel for the violet laser 405 nm (Brilliant Violet 421), 5 channels for the blue 488 nm laser (FITC, PE, PE-Cy7, PE-Cy5 and PE-CF594), 3 channels for the red 640 nm laser (APC, APC-H7 and APC-Cy7). Depending on the instrument configuration available to you, alternative antibodies and fluorochrome combinations may be required to probe your cells of interest.

*Similar reagents or equipment with similar characteristics from other companies can be used for this protocol.

STEP-BY-STEP METHOD DETAILS

Obtain the BCR-ABL-driven CML mice

Timing: ~3 weeks

1. Pretreat male donor C57BL/6 mice (6–8 weeks) with 5-fluorouracil (200 mg/kg) via tail vein injection for 5 days.
2. Collect BM cells and incubate them with cytokines including stem cell factor (SCF, 50 ng/mL), interleukin-3 (IL-3, 6 ng/mL), and IL-6 (6 ng/mL) for 24 h at 37°C in a humidified incubator with 5% CO_2.
3. Transduce BM cells twice with MSCV-BCR-ABL-IRES-GFP retrovirus (1,500 × *g*, 90 min, 32°C).
4. Transplant the infected BM cells into sublethally irradiated (550 cGy) female recipient C57BL/6 mice (6–8 weeks) via tail vein injection.
5. Obtain CML mice at day 14 post transplantation.

Note: For a detailed protocol of retrovirally BCR-ABL-driven CML mouse model, please refer to Gavrilescu and Van Etten[2]; Zhang and Li.[3]

Generating cell suspension

Timing: 3 h/mouse

In this part, we describe our approaches to harvest BM and splenic cells from CML mice.

Note: In order to keep cells sterile for further sorting, we usually work in a biosafety cabinet and use sterile reagents.

6. Sacrifice CML mice by cervical dislocation or other approved method.

Note: Typically, the percentage of leukemia cells (GFP$^+$) in peripheral blood (PB) from CML mice is more than 50% at day 14 post transplantation (Figure 1).

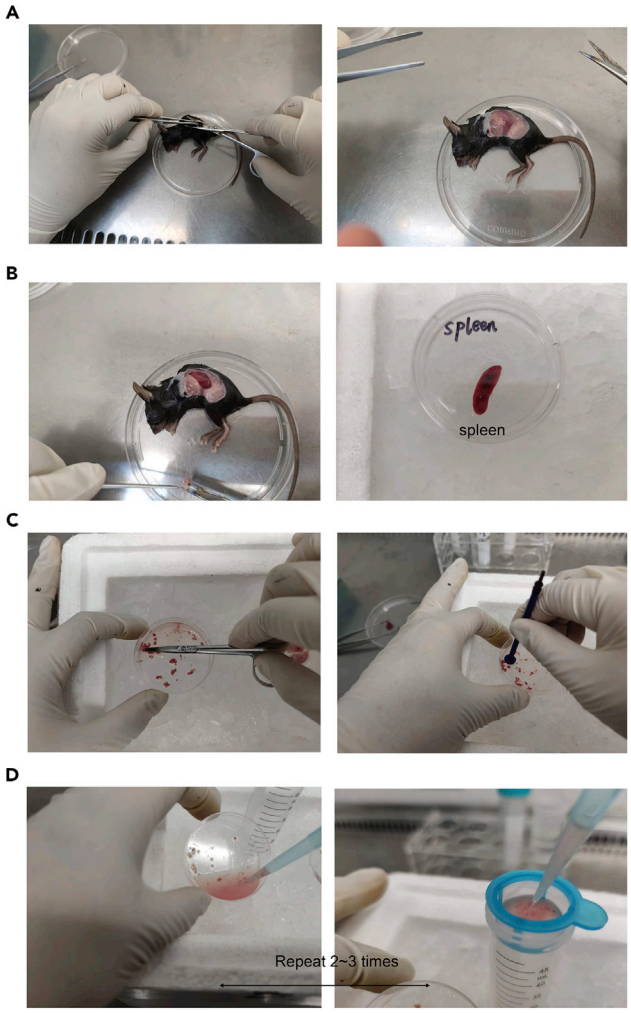


Figure 2. Harvest splenic cells from CML mice

(A) Expose the spleen.

(B) Isolate the spleen.

(C) Dissociate the spleen and grind it completely on ice.

(D) Collect splenic cell suspension through a 40 μm cell strainer on ice.

7. Harvest splenic cells.

Note: We recommend working on ice during the following steps.

a. Snip the skin over the midsection using scissors and tweezers (Figure 2A).
b. Isolate the spleen and place in a sterile 6 cm dish containing BM flush buffer (refer to materials and equipment) (Figure 2B).
c. Cut the spleen into small tissue pieces using scissors. Grind tissue pieces by the plunger of a 1 mL syringe in 6 cm dish (Figure 2C).
d. Add 2 mL of BM flush buffer into the dish, break down the tissue homogenates by pipetting several times with 1 mL pipette tip. Transfer the suspension into a sterile 50 mL tube through a 40 μm cell strainer (Figure 2D).

Note: Repeat 2–3 times for step 7d. We recommend working on ice during steps 7b–7d to maintain cell viability.

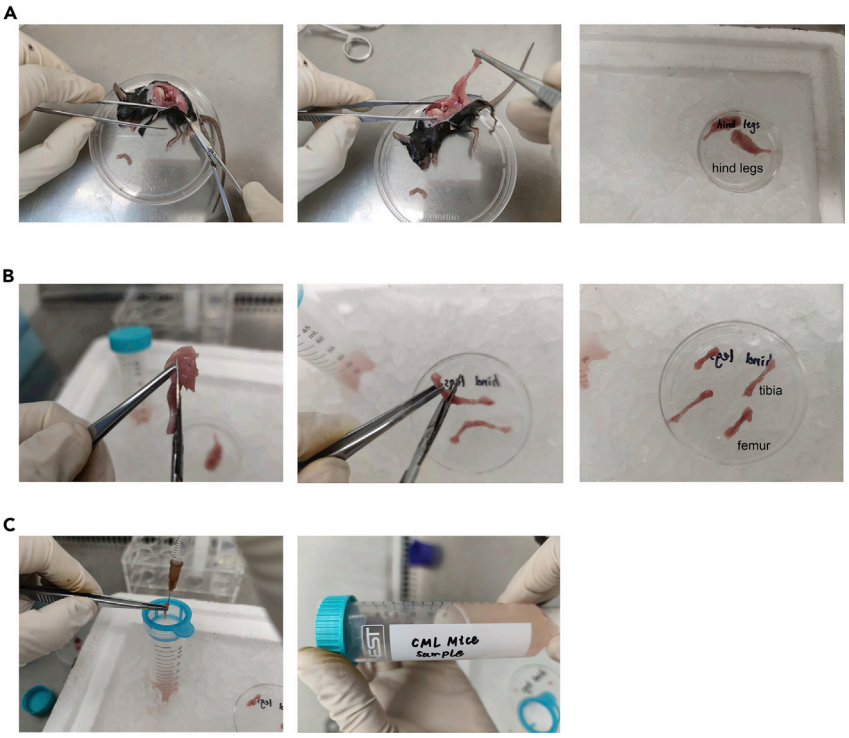


Figure 3. Harvest BM cells from CML mice

(A) Isolate hind legs.

(B) Dissect hind leg into femur and tibia on ice.

(C) Flush and collect BM cells through a 40 μm cell strainer on ice.

8. Harvest BM cells.

Note: We recommend working on ice during the following steps.

a. Use scissors to snip the skin over hind legs and pull the skin apart using tweezers.
   i. Hold one hind leg via tweezer and cut off the hip joint using scissors.
   ii. Transfer it into a sterile 6 cm dish containing 1 mL BM flush buffer.
   iii. Repeat the process for the other hind leg (Figure 3A).
b. Remove the adherent muscle from hind legs using scissors. Dissect hind legs into femurs and tibias (Figure 3B).
c. Hold each bone with tweezer.
   i. Insert the needle of a 1 mL syringe containing 1 mL of BM flush buffer into the marrow cavity.
   ii. Flush into the sterile 50 mL tube through a 40 μm cell strainer, repeat flush 6 times.
   iii. Repeat this procedure for all bones (Figure 3C).

Note: Perform step 8c on ice to maintain cell viability.

9. Remove erythrocytes from BM and splenic cell suspension, respectively.
   a. Centrifuge cells at 800 × *g* for 10 min.
   b. Discard supernatant, resuspend the cell pellet with 10 mL of erythrocyte lysis buffer (refer to materials and equipment), and incubate on ice for 5 min.
   c. Centrifuge cells at 800 × *g* for 10 min.

Note: The volume of erythrocyte lysis buffer is dependent on the number of erythrocytes. Increase the volume if necessary.

d. Discard supernatant, resuspend the cell pellet with 10 mL of PBS.
e. Centrifuge cells at 800 × *g* for 10 min.
f. Discard supernatant, resuspend the splenic cells with 5 mL of 0.5% BSA buffer and BM cells with 1 mL of 0.5% BSA buffer (refer to materials and equipment), respectively.

Enrichment of GFP$^+$c-Kit$^+$ cells

Timing: 3 h 30 min

In this part of the protocol, we describe how to isolate the GFP$^+$c-Kit$^+$ cells from the BM and splenic cell suspensions obtained in the previous steps. We firstly sorted the GFP$^+$ population from BM and splenic cells using flow cytometer. We then isolated c-Kit$^+$ population from the sorted GFP$^+$ cells using a CD117 Microbeads Kit (Miltenyi Biotec, Cat#130-091-224) according to manufacturer's instructions. (https://www.miltenyibiotec.com/HK-en/products/cd117-microbeads-mouse.html).

Note: Sterile conditions is necessary if GFP$^+$c-Kit$^+$ cells will be used for culture, colony-forming cell/replating (CFC/replating) assay, and BM transplantation into recipient mice.

10. Sort GFP$^+$ cells from BM and splenic cell suspension.
    a. Filter the cell suspension through a 40 μm cell strainer in order to remove clumps.
    b. Count the cells and prepare a suspension of 1 × 10^7 cells/mL of 0.5% BSA buffer in a falcon tube. Keep the samples on ice until cell sorting.
    c. Prepare a 15 mL tube containing 1 mL of 0.5% BSA buffer and 10 μL penicillin/streptomycin solution to collect the sorted cell populations. Keep all the tubes, solutions and sorted cells on ice during sorting process to maintain cell viability.
    d. Perform cell sorting on a BD FACS Aria II containing 407/488/633 lasers and use a 100 μm nozzle with a sorting speed of 6,000–8,000 events/second.
    e. Use the BM or splenic cells from normal mice for negative control to set appropriate photomultiplier tubes (PMT) voltages and gating strategy.
    f. According to the gating strategy (Figure 4), sort GFP$^+$ cells by flow cytometry into tubes and transfer onto ice.
    g. Analyze the percentage of GFP$^+$ cells in the sorted population by flow cytometry (Figure 4).

Note: It can be obtained approximately 1 × 10^7 of GFP$^+$ cells from one CML mouse. The users require previous experience with flow cytometry to design the panel.

11. Enrichment of c-Kit$^+$ population from the sorted GFP$^+$ cells.
    a. Centrifuge cell suspension at 800 × *g* for 10 min. Aspirate supernatant completely.
    b. Add 500 μL of 0.5% BSA buffer and count the cells.
    c. Place 10^7 cells in a 1.5 mL Eppendorf tube.
    d. Centrifuge cell suspension at 300 × *g* for 10 min. Aspirate supernatant completely.
    e. Resuspend cell pellet with 80 μL of 0.5% BSA buffer and add 20 μL of CD117 microbeads per 10^7 cells.
    f. Mix well and incubate on ice for 15 min.
    g. Wash the cells with 1 mL of 0.5% BSA buffer, then centrifuge at 300 × *g* for 10 min. Aspirate supernatant completely.
    h. Resuspend the cell pellet with 50 μL of 0.5% BSA buffer.
    i. Set up the MS column against the magnetic MACS™ Multistand and place a 15 mL tube under the MS column to collect c-Kit$^-$ cells.
    j. Add 500 μL of 0.5% BSA buffer to rinse the column.

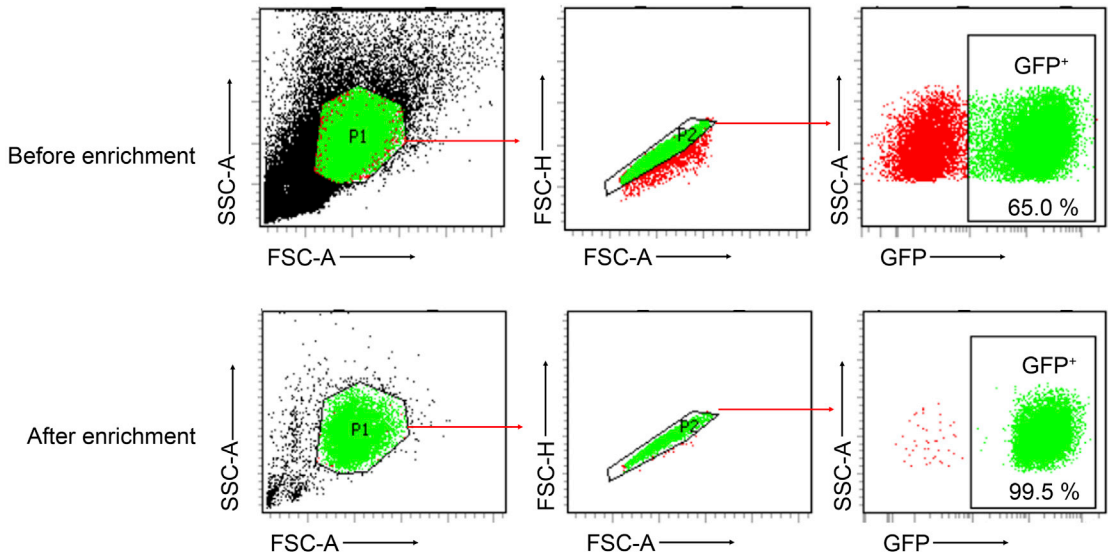


Figure 4. The purity of the sorted GFP$^+$ cells using flow cytometry is shown

Note: Always wait until the column is empty before adding cell suspension or buffer.

k. Add cell suspension into the column, collect the buffer containing unlabeled (GFP$^+$c-Kit$^-$) cells to a 15 mL tube. Wash column with 500 μL of 0.5% BSA buffer (Figure 5A).
l. Remove the column from the separator, add 500 μL of 0.5% BSA buffer to the column and immediately flush out the labeled c-Kit$^+$ cells to a new 15 mL tube (Figure 5B).

Note: This fraction contains GFP$^+$c-Kit$^+$ cells.

12. Determine the purity of sorted c-Kit$^+$ cells by flow cytometry.
    a. Count the cells, and use 10^6 cells/sample for antibody staining.
    b. Centrifuge the cells at 800 × *g* for 5 min.
    c. Resuspend the cell pellet in 100 μL of 0.5% BSA buffer and add 0.5 μL of isotype control (BioLegend, cat#565363) or c-Kit-PE antibody (eBioscience, cat#553355) at 4°C in dark for 30 min.
    d. Add 1 mL of 0.5% BSA buffer, then centrifuge the cells at 800 × *g* for 5 min.
    e. Resuspend the cell pellet in 400 μL of 0.5% BSA buffer.

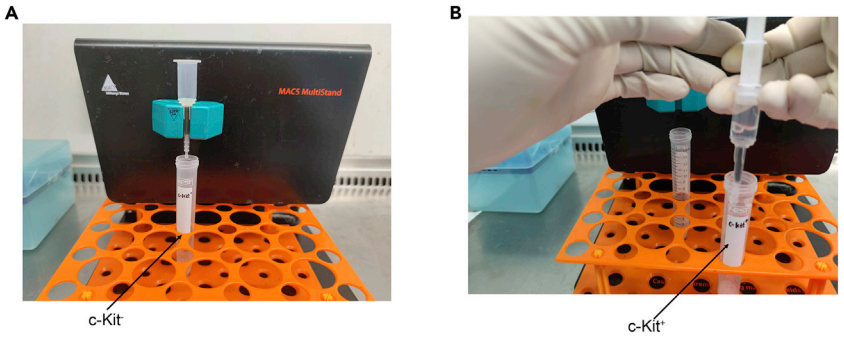


Figure 5. Enrichment of c-Kit$^+$ population using a CD117 Microbeads Kit
(A) Collect the unlabeled c-Kit$^-$ cells.
(B) Collect the c-Kit$^+$ cells.

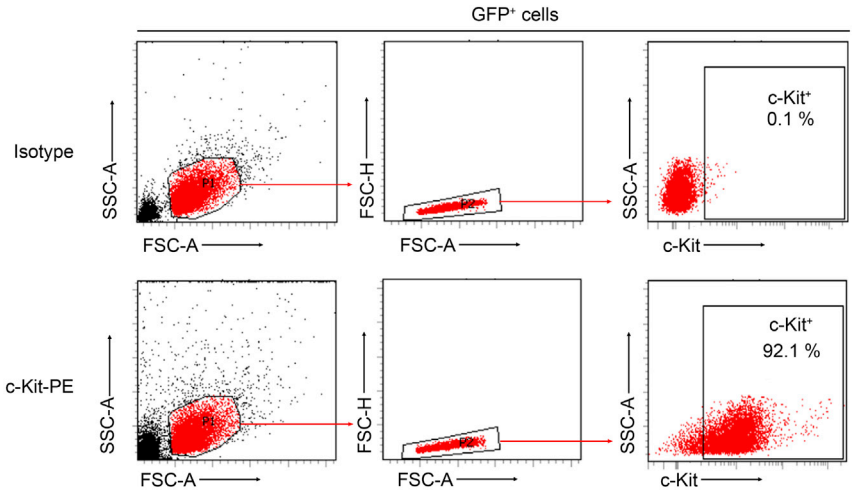


Figure 6. The purity of the sorted GFP$^+$c-Kit$^+$ cells is shown

f. Before acquiring samples, set appropriate PMT voltages and compensation by using isotype control.

g. Analyze the percentage of c-Kit$^+$ cells by flow cytometry (Figure 6).

13. Determine the function of sorted GFP$^+$c-Kit$^+$ cells by CFC/replating assay.

a. Count the sorted GFP$^+$c-Kit$^-$ and GFP$^+$c-Kit$^+$ cells.

b. Place 25,000 cells in a 1.5 mL Eppendorf tube.

c. Centrifuge the cells at 800 × *g* for 5 min.

d. For a quadruplicate assay, resuspend the cell pellet in 50 μL of PBS and add to 450 μL of MethoCult M3434.

e. Vortex the tube vigorously to mix the contents thoroughly.

f. Let the tube stand for at least 5 min to allow the bubbles to rise to the top.

g. Attach a sterile 16 gauge blunt-end needle to a sterile 1 mL syringe.

h. Draw up the MethoCult mixture containing cells into the syringe and dispense a volume of 100 μL into individual wells of a 96-well plate (5,000 cells/well).

i. Incubate at 37°C, in 5% CO_2 with ≥ 95% humidity.

j. After 7 days, count all the colonies in each well using microscope.

k. Using a 1 mL pipettor, add 200 μL of PBS to each well.

l. Pipette up and down gently to mix the methylcellulose-based medium with the cells.

m. Transfer the diluted methylcellulose and the cells into a 4 mL Eppendorf tube.

Note: Repeat 3 times for steps 13k–13m.

n. Add an additional 2 mL PBS to the tube to ensure that the methylcellulose is completely diluted.

o. Centrifuge the cells at 800 × *g* for 5 min.

p. Remove the supernatant. Resuspend the cell pellet with 1 mL of PBS.

q. Repeat steps 13a–13j (Figure 7).

Immunostaining of BM or splenic cells for flow cytometry analysis

Timing: 2 h 30 min

Note: In this procedure, we label BM or splenic cells with the relevant antibodies to analyze the leukemia stem/progenitor cells (Tables 1 and 2), and myeloid leukemia cells (Table 3) by flow cytometry. The volume of antibodies depends on the number of cells.

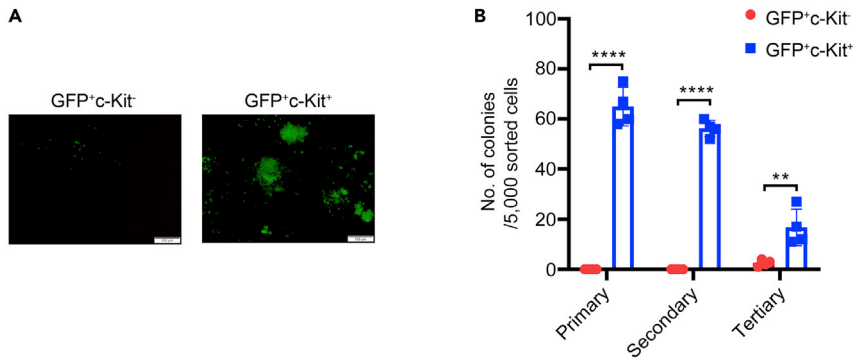


Figure 7. CFC/replating assay

$GFP^+c\text{-}Kit^+$ and $GFP^+c\text{-}Kit^-$ cells (5,000 cells/well) sorted from the primary CML mice were plated in methylcellulose medium (MethoCult™ M3434, STEMCELL Technologies) for 3 rounds of replating assay. Colonies were counted on day 7 as described previously.

(A) Representative photographs of colonies from each group. Scale bar: 100 μm.

(B) Quantitative analysis of the colonies is shown. Data are represented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$, Student's t test.

14. Centrifuge the BM or splenic cell suspension obtained in the previous steps at 800 × *g* for 10 min, aspirate the supernatant.
15. Resuspend the cell pellet in 100 μL of 0.5% BSA buffer.
16. Stain 10^6 cells/sample at 4°C in dark for 30 min using different anti-mouse antibodies combined with fluorophore listed in key resources table.

Note: It's important to titrate the antibody to determine the optimal concentration of the antibody before proceeding.

a. The antibody dilutions in this table are titrated for staining in a volume of 100 μL. The final concentration of the antibodies is listed in the table below.
b. Add 1 mL of 0.5% BSA buffer to the sample and centrifuge at 800 × *g* for 10 min, aspirate the supernatant.
c. Resuspend the cell pellet in 400 μL of 0.5% BSA buffer. The samples are now ready for analysis by flow cytometry.
d. Before acquiring samples, set appropriate PMT voltages and compensation by using unstained or single-stained controls (BM or splenic cells from CML mice) for every fluorochrome used in your panel. Adjust compensation using the same set of antibodies.
e. Define the gating strategy.
f. Collect data with BD LSRFortessa flow cytometry (BD Biosciences) (Figure 8).

EXPECTED OUTCOMES

We usually harvest ~4 × 10^7 total BM and splenic cells per CML mouse induced in our lab. Following this protocol, we obtain highly enriched $GFP^+c\text{-}Kit^+$ cells from CML mice. Purity of the sorted

Table 1. Antibody panel for flow cytometry analysis of leukemia stem cells

CD marker	Fluorophore	Final dilution	Volume of antibody (for 10^6 cells in 100 μL of BSA buffer)
Lineage	APC	1/200	0.5 μL
Sca-1	BV421	1/200	0.5 μL
c-Kit (CD117)	PE	1/200	0.5 μL
CD135	PE-Cy5	1/200	0.5 μL
CD150	PE-Cy7	1/200	0.5 μL
CD48	APC-Cy7	1/200	0.5 μL

Table 2. Antibody panel for flow cytometry analysis of leukemia progenitor cells

CD marker	Fluorophore	Final dilution	Volume of antibody (for 10^6 cells in 100 μL of BSA buffer)
Lineage	APC	1/200	0.5 μL
Sca-1	PE-CF594	1/200	0.5 μL
c-Kit (CD117)	APC-H7	1/200	0.5 μL
CD34	PE	1/200	0.5 μL
CD16/32	PE-Cy7	1/200	0.5 μL

$GFP^+c\text{-}Kit^+$ cells is ~92% (Figure 6). The total number of $GFP^+c\text{-}Kit^+$ cells sorted from one CML mouse is ~1.5 × 10^6 cells. The function of sorted $GFP^+c\text{-}Kit^+$ cells is determined by CFC/replating assay. The results showed that serially CFC/replating capacity was significantly higher in $GFP^+c\text{-}Kit^+$ cells than $GFP^+c\text{-}Kit^-$ cells (Figures 7A and 7B).

Additionally, we develop the gating strategies for analysis of populations of leukemia stem cells, including GFP^+LSK cells ($GFP^+Lin^-Sca\text{-}1^+c\text{-}Kit^+$), GFP^+ long-term HSCs (GFP^+LT-HSCs, GFP^+ LSK $Flt3^-CD150^+CD48^-$), GFP^+ short-term HSCs (GFP^+ST-HSCs, GFP^+LSK $Flt3^-CD150^-CD48^-$) (Figure 8A), leukemia progenitor cells, including GFP^+ granulocyte-macrophage progenitors (GFP^+GMP, $GFP^+Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma RII/III^{high}$) and GFP^+ common myeloid progenitors (GFP^+CMP, $GFP^+Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma RII/III^{low}$) cells (Figure 8B), and myeloid leukemia cells (GFP^+Gr1^+) (Figure 8C).

LIMITATIONS

This optimized protocol is applied to isolate $GFP^+c\text{-}Kit^+$ cells and analyze the populations of leukemia stem/progenitor cells and myeloid leukemia cells in BM and spleen of CML mice. Analysis of stem cells for other types of cancer will require different markers.

TROUBLESHOOTING

Problem 1

Mice show no symptoms of CML disease, such as low percentage of GFP^+ cells in PB and no splenomegaly at day 14 post transplantation (step 1).

Potential solution

This means that very few BCR-ABL-expressing cells were transplanted successfully. One possibility is that the retrovirus titer is too low to achieve a high transduction efficiency. A solution is to harvest fresh retrovirus present with strong GFP. Another possibility is that the transduced cells were failed to inject into recipient mice vail the tail vein.

Problem 2

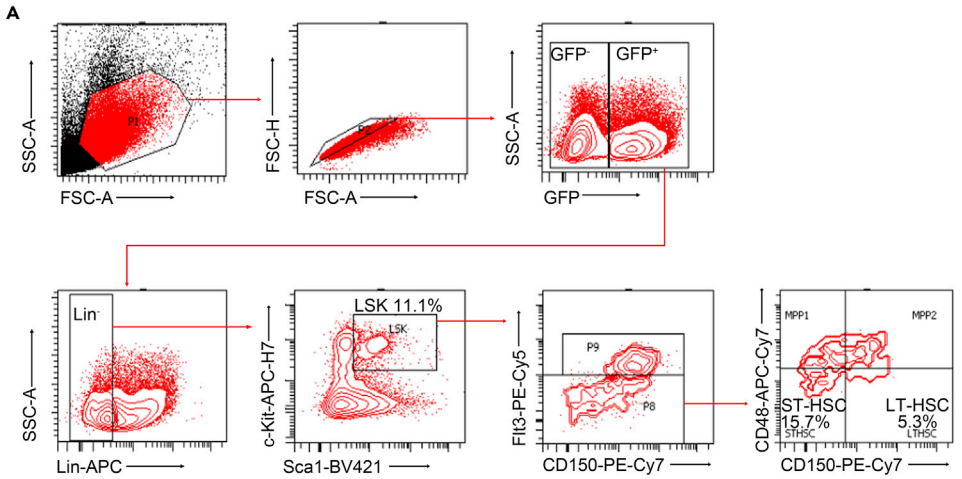
Insufficient quantity of GFP^+ cells for further sorting $c\text{-}Kit^+$ cells (step 10).

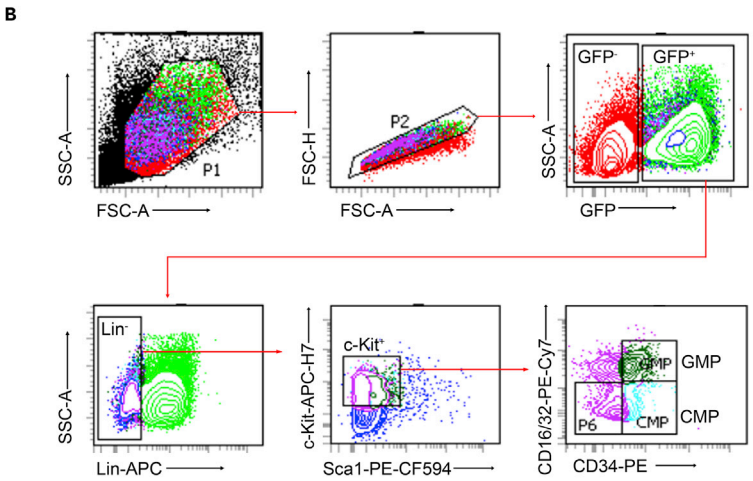
Potential solution

In this case, we strongly recommend mixing GFP^+ cells sorted from several CML mice for further sorting.

Table 3. Antibody panel for flow cytometry analysis of myeloid leukemia cells

CD marker	Fluorophore	Final dilution	Volume of antibody (for 10^6 cells in 100 μL of BSA buffer)
Gr-1	APC	1/200	0.5 μL


A
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-A
GFP
GFP-
GFP+
Lin-
SSC-A
Lin-APC
c-Kit-APC-H7
Sca1-BV421
LSK 11.1%
LSK
Flt3-PE-Cy5
CD150-PE-Cy7
P9
P8
CD48-APC-Cy7
CD150-PE-Cy7
MPP1
MPP2
ST-HSC
15.7%
LT-HSC
5.3%


B
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-A
GFP
GFP-
GFP+
Lin-
SSC-A
Lin-APC
c-Kit-APC-H7
Sca1-PE-CF594
c-Kit+
CD16/32-PE-Cy7
CD34-PE
GMP
CMP
P6

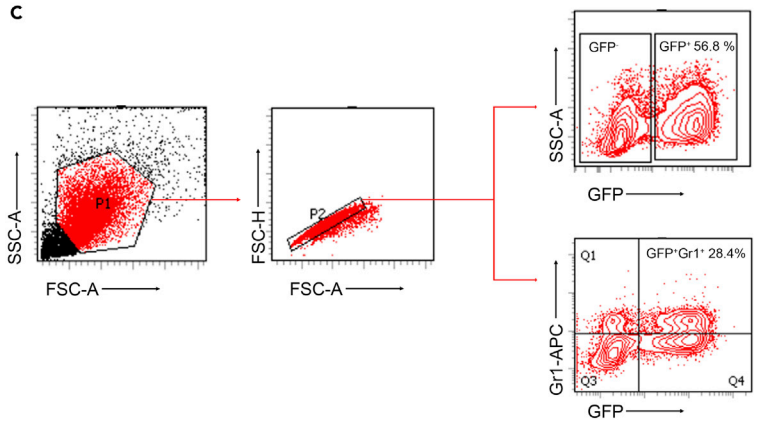

C
SSC-A
FSC-A
P1
FSC-H
FSC-A
P2
SSC-A
GFP
GFP-
GFP+ 56.8 %
Gr1-APC
GFP
Q1
GFP+Gr1+ 28.4%
Q3
Q4

Figure 8. Gating strategies for analysis of populations of leukemia stem/progenitor cells and myeloid leukemia cells in CML mice by flow cytometry

(A) Leukemia stem cells, including GFP$^+$LSK cells (GFP$^+$Lin$^-$Sca-1$^+$c-Kit$^+$), GFP$^+$LT-HSCs (GFP$^+$LSK Flt3$^-$CD150$^+$CD48$^-$), GFP$^+$ST-HSCs (GFP$^+$LSK Flt3$^-$CD150$^-$CD48$^-$).

(B) Leukemia progenitor cells, including GFP$^+$GMP (GFP$^+$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{high}) and GFP$^+$CMP (GFP$^+$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{low}) cells.

(C) Myeloid leukemia cells (GFP$^+$Gr1$^+$).

Problem 3

Poor cell viability of isolated cells.

Potential solution

The timing is critical to keep high cell viability. Time taken for start isolation and cell storage conditions, will affect cell viability. During generating cell suspension, ensure that the spleen, bone and cells are stored in FBS-containing buffer.

Problem 4

No positive staining (step 16).

Potential solution

Prior to the data acquisition, ensure proper flow cytometer settings. Using single staining to optimize the antibody dilution and the appropriate PMT voltage in the flow cytometer that all targeted populations are shown. Ensure that antibodies are correctly stored and not expired. Keep the samples on ice and in dark condition.

Problem 5

Incorrect flow cytometry profiles (step 16).

Potential solution

Excessive compensation can lead to unexpected flow cytometry profiles. A solution is to adjust compensation using compensation beads.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagent and resource sharing should be directed to and will be fulfilled by the lead contact, Jingxuan Pan (panjx2@mail.sysu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

C.L., X.Y.L., Y.L.J., and J.X.P. designed the research. C.L. and X.Y.L. performed the experiments and analyzed the data. C.L. and Y.L.J. wrote the manuscript. J.X.P. supervised the entire study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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