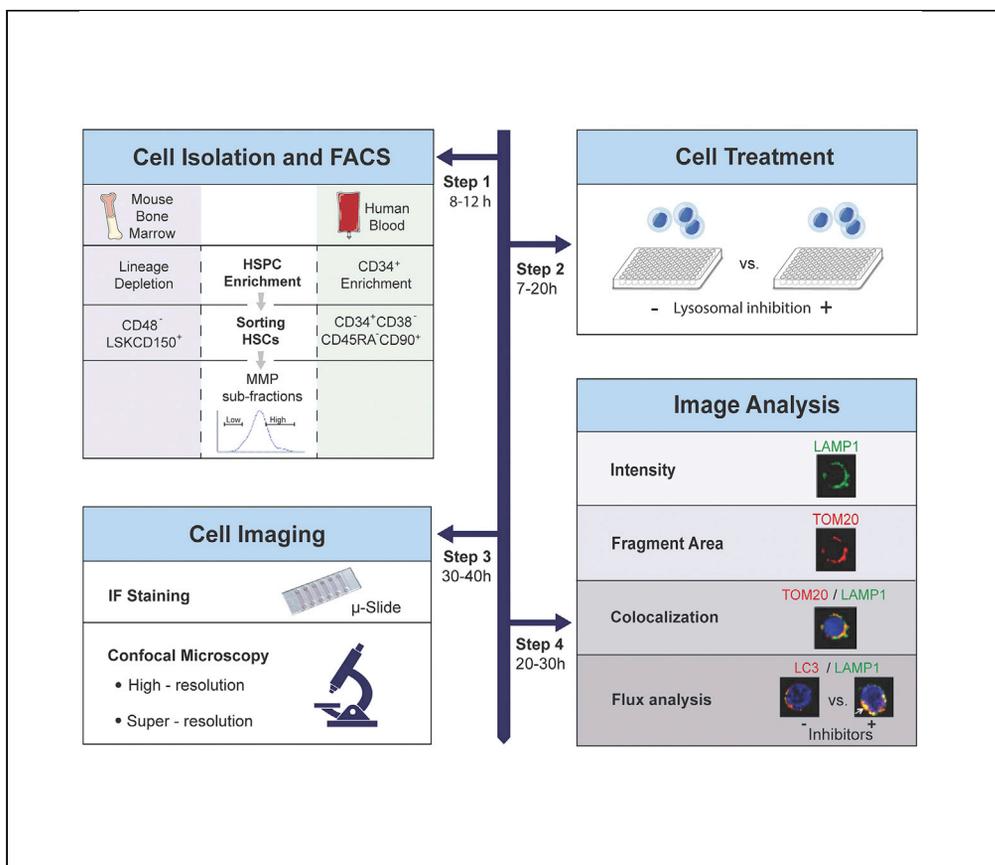


## Protocol

# Protocol to identify and analyze mouse and human quiescent hematopoietic stem cells using flow cytometry combined with confocal imaging



Jiajing Qiu, Vijay Menon, Nikolaos Tzavaras, Raymond Liang, Saghi Ghaffari  
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### Highlights

Using mitochondrial membrane potential to isolate HSC subsets with distinct potency

Immunofluorescence staining of rare HSC subpopulations using ibid  $\mu$ -slides

Imaging HSC organelles using confocal/super-resolution microscopy

Analyzing lysosomal content and flux activity in HSCs based on confocal images

Mitochondrial membrane potential (MMP) segregates functionally distinct subsets within highly purified hematopoietic stem cells (HSCs). Here, we detail a protocol for FACS isolation of MMP sub-fractions of phenotypically defined mouse and human HSCs. These steps are followed by high-/super-resolution immunofluorescence microscopy of HSCs' lysosomes. While the protocol describes the isolation of quiescent HSCs, which are the most potent subsets, it could also be applied to other HSC subsets. This protocol overcomes some experimental challenges associated with low HSC numbers.

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

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## Protocol

## Protocol to identify and analyze mouse and human quiescent hematopoietic stem cells using flow cytometry combined with confocal imaging

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## SUMMARY

Mitochondrial membrane potential (MMP) segregates functionally distinct subsets within highly purified hematopoietic stem cells (HSCs). Here, we detail a protocol for FACS isolation of MMP sub-fractions of phenotypically defined mouse and human HSCs. These steps are followed by high-/super-resolution immunofluorescence microscopy of HSCs' lysosomes. While the protocol describes the isolation of quiescent HSCs, which are the most potent subsets, it could also be applied to other HSC subsets. This protocol overcomes some experimental challenges associated with low HSC numbers.

For complete details on the use and execution of this protocol, please refer to Liang et al. (2020) and Qiu et al. (2021).

## BEFORE YOU BEGIN

## Introduction

The most potent, quiescent hematopoietic stem cells (HSCs) are actively maintained at low metabolic state through complex but inter-related sets of regulatory processes (Shyh-Chang and Ng, 2017; Chakrabarty and Chandel, 2021; Chandel et al., 2016; Bigarella et al., 2014). The underlying mechanism that controls the switch between quiescence and proliferation and how it influences stem cell potential is central to adult stem cell research.

Mitochondrial membrane potential (MMP) is the electric (or charge) gradient across the mitochondrial inner membrane. It is generated through mitochondrial electron transport chain (ETC) activity during oxidative phosphorylation (OXPHOS) coupled with adenosine-5'-triphosphate (ATP) production. Tetramethylrhodamine ethyl ester (TMRE), a rhodamine 123 derivative, is a positively charged, membrane permeable fluorescent probe. TMRE is driven into the mitochondrial matrix proportional to the level of MMP. Greater amount of dye accumulates in more polarized mitochondria (higher level of MMP) (Kim et al., 1998; Perry et al., 2011; Qiu and Ghaffari, 2022). MMP is a key indicator



of mitochondrial activity (Schieke et al., 2008; Brand and Nicholls, 2011). The level of MMP has been shown to be reversely correlated with HSC functional capacity (Rimmele et al., 2015; Sukumar et al., 2016; Vannini et al., 2016; Anso et al., 2017). The MMP heterogeneity segregates functionally distinct mouse and human HSC subsets within highly purified phenotypically-defined primitive HSCs (Sukumar et al., 2016; Vannini et al., 2016; Liang et al., 2020; Qiu et al., 2021; Hinge et al., 2020; Mansell et al., 2021; Loeffler et al., 2022).

As the digestion centers of the cell, lysosomes mediate autophagy to maintain stem cell potential. In the process of autophagy and the clearance of mitochondria by means of mitochondrial specific autophagy (known as mitophagy) lysosomes were thought to constitute the final destination of the cargo to be degraded, passively mediating the autophagy processes, including in HSCs (Ito et al., 2016; Ho et al., 2017; Loeffler et al., 2019, 2022). Recent evidence suggest that lysosomes actively and dynamically influence the output of the degradation process and autophagy (Fujimaki et al., 2019; Lawrence and Zoncu, 2019) and have distinct properties in quiescent relative to dividing cells (Garcia-Prat et al., 2021; Liang et al., 2020). Lysosomes were found to be critical in reversible switching of HSC quiescence versus activation (Liang et al., 2020; Garcia-Prat et al., 2021; Ghaffari, 2021; Qiu and Ghaffari, 2022). The maintenance of cellular quiescence in HSCs was shown to be dependent specifically on slow and greatly regulated lysosomal degradation ability (Liang et al., 2020; Ghaffari, 2021; Qiu and Ghaffari, 2022).

The protocol below describes a method that fractionates phenotypically defined highly potent (mouse and human) HSCs based on MMP levels. It provides tools to study specific properties in HSC subpopulations. Particularly, using immunofluorescence staining and confocal microscopy, lysosomal properties in HSC subpopulations that differ in their mitochondrial activities (MMP levels) are studied. This approach overcomes challenges associated with biochemical methods and enables the examination of organelles in rare HSC subpopulations.

### Institutional permissions

All experiments were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine and conform to the relevant regulatory standards.

Human peripheral blood samples of normal donors were obtained from New York blood center. Human subject experiments were approved by the Committee for Human Investigations at the Icahn School of Medicine at Mount Sinai. Researchers who would be applying this protocol for their own work would need to acquire permission from their respective institutions.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Streptavidin APC-Cy7	BD Biosciences	Cat#554063
APC-Cy7 Hamster Anti-Mouse CD48; Clone HM48-1	BD Biosciences	Cat#561242
Pacific Blue Anti-mouse Ly-6A/E (Sca-1); Clone D7	BioLegend	Cat#108120
APC Rat Anti-Mouse CD117; Clone 2B8	BD Biosciences	Cat#553356
PE/Cyanine7 Anti-mouse CD150 (SLAM); Clone TC15-12F12.2	BioLegend	Cat#115914
APC Mouse Anti-Human CD34; Clone 581	BD Biosciences	Cat#555824
PerCP-Cy5.5 Mouse Anti-Human CD38; Clone HIT2	BD Biosciences	Cat#561106
FITC Mouse Anti-Human CD90; Clone 5E10	BD Biosciences	Cat#555595
PE/Cyanine7 Anti-human CD45RA; Clone H100	BioLegend	Cat#304125
Anti-LAMP-1 Mouse mAb; Clone H4A3	Santa Cruz Biotechnology	Cat#sc-20011

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<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-TOM20 Rabbit mAb	ABclonal	Cat#A19403
Anti-LC3B Rabbit mAb	Cell Signaling Technology	Cat#3868
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	Cat# A-11012
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11001
<b>Chemicals, peptides, and recombinant proteins</b>		
PBS, pH 7.2, Ca <sup>2+</sup> /Mg <sup>2+</sup> free; Gibco	Thermo Fisher Scientific	Cat#20012-043
IMDM (Glutamine (+) HEPES (+))	Thermo Fisher Scientific	Cat#12440-053
Fetal bovine serum, qualified, heat inactivated, USDA-approved regions	Thermo Fisher Scientific	Cat#10438-026
Gibco Penicillin-Streptomycin (Pen Strep) solution, 100x	Thermo Fisher Scientific	Cat#15140-122
EDTA (0.5 M), pH 8.0	Thermo Fisher Scientific	Cat#AM9260G
Triton X-100 Wetting Agent, 100 mL	PerkinElmer	Cat# N9300260
BD Pharm Lyse lysing solution	BD Biosciences	Cat#555899
Tween 20	VWR	Cat#BDH4210
Paraformaldehyde, 16% w/v aq. soln., methanol free	Thermo Scientific	Cat#43368.9M
Bovine serum albumin (BSA)	Fisher Scientific	Cat#BP1600-1
Ficoll Paque Plus	MilliporeSigma	Cat#GE17-1440-03
Tetramethylrhodamine ethyl ester (TMRE)	MilliporeSigma	Cat#87917
DAPI	MilliporeSigma	Cat#D9542
Mounting Medium With DAPI - Aqueous, FluoroShield	Abcam	Cat#ab104139
UltraComp eBeads Plus Compensation Beads	Thermo Fisher Scientific	Cat#01-3333-42
StemSpan SFEM	STEMCELL Technologies	Cat#09650
Leupeptin	MilliporeSigma	Cat#L2884
Concanamycin A (ConA)	Santa Cruz Biotechnology	Cat#sc-202111
Chloroquine diphosphate salt	MilliporeSigma	Cat#C6628
Dimethyl sulfoxide (DMSO)	MilliporeSigma	Cat#D8418
Fibronectin	Corning	Cat#354008
50 mM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Thermo Fisher Scientific	Cat#M34152
Oligomycin A	MilliporeSigma	Cat#75351
Recombinant Mouse SCF Protein	R&D Systems	Cat#455-MC
Recombinant Human Thrombopoietin Protein	R&D Systems	Cat#288-TP
<b>Critical commercial assays</b>		
MACS CD34 microbead kit (human)	Miltenyi Biotec	Cat#130-046
EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit: Immunomagnetic negative selection kit	STEMCELL Technologies	Cat#19856
<b>Software and algorithms</b>		
ImageJ (2.1.0/1.53c)		<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
JACoP v2.0 plugin		<a href="https://imagej.nih.gov/ij/plugins/track/jacop2.html">https://imagej.nih.gov/ij/plugins/track/jacop2.html</a>
ZEN 2 Black software (2.3 SP1 version 14.0.21.201)	ZEISS	

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<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ZEN 2.6 (Blue edition)		<a href="https://www.zeiss.com/microscopy/en/products/software/zeiss-zen-lite.html">https://www.zeiss.com/microscopy/en/products/software/zeiss-zen-lite.html</a> - downloads
FCS Express		<a href="https://denovosoftware.com/">https://denovosoftware.com/</a>
GraphPad Prism		<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
Falcon 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap, 25/Pack, 500/Case	Corning	Cat#352235
LS columns, 25 columns	Miltenyi Biotec	Cat#130-042-401
MidiMACS Separator	Miltenyi Biotec	Cat#130-042-302
Falcon 40 µm Cell Strainer, Blue, Sterile, Individually Packaged, 50/Case	Corning	Cat#352340
BD Insulin Syringe with Detachable Needle	Fisher Scientific	Cat#BD 329652
BD PrecisionGlide Single-use Needles: Regular Bevel - Regular Wall	Fisher Scientific	Cat#BD 305120
Falcon 70 µm Cell Strainer, White, Sterile, Individually Packaged, 50/Case	Corning	Cat#352350
EasySep Magnet	STEMCELL Technologies	Cat#18000
Milllex-HV Syringe Filter Unit, 0.45 µm	MilliporeSigma	Cat#SLHV013SL
Falcon 96-Well, Non-Treated, U-Shaped-Bottom Microplate	Fisher Scientific	Cat#351177
µ-Slide VI - Flat	ibidi	Cat#80626
Dako Coverglass 24 × 50 mm	Agilent Technologies	Cat#CS70430-2
Transparent Nail Polish	Various (Can be purchased from any pharmacies)	N/A

## MATERIALS AND EQUIPMENT

Thermo Shandon Cytospin 3 centrifuge with Cytoclips (Used in this protocol). Alternatively, Thermo Scientific Cytospin 4 Centrifuge can also be used (Available at Marshall Scientific, Product Code: TH-CYTO4).

<b>Antibody dilution</b>	
Antibodies	Dilution
Anti-LAMP-1 Mouse mAb	1:150
Anti-TOM20 Rabbit mAb	1:150
Anti-LC3B Rabbit mAb	1:150
Goat anti-Rabbit IgG (H+L), Alexa Fluor 594	1:1,000
Goat anti-Mouse IgG (H+L), Alexa Fluor 488	1:1,000

<b>2%FBS/PBS buffer</b>		
Reagent	Final concentration	Amount
FBS	2%	20 mL
0.5 M EDTA	2 mM	4 mL
(Gibco Pen-Strep Solution 100×)	1 × (100 U/mL penicillin, 100 µg/mL streptomycin)	10 mL
PBS Ca <sup>2+</sup> /Mg <sup>2+</sup> free	1 ×	
<b>Total</b>		<b>1 L</b>

Storage: Prepare in sterilized condition. Store at 4°C < 1 month. Use within one week after adding Pen-Strep.

### MACS buffer

Reagent	Final concentration	Amount
BSA	0.5%	2.5 g
0.5 M EDTA	2 mM	2 mL
(Gibco Pen-Strep Solution 100×)	1× (100 U/mL penicillin, 100 µg/mL streptomycin)	5 mL
PBS Ca <sup>2+</sup> /Mg <sup>2+</sup> free	1×	
<b>Total</b>		<b>500 mL</b>

Storage: Filter to sterilize. Store at 4°C, < 6 month. Use within one week after adding Pen-Strep.

### Freezing buffer

Reagent	Final concentration	Amount
FBS	50%	1 mL
DMSO	10%	200 µL
PBS	1×	800 µL
<b>Total</b>		<b>2 mL</b>

Storage: Freshly prepared in sterilized condition.

### 10%FBS/IMDM medium

Reagent	Final concentration	Amount
IMDM Medium		17.8 mL
FBS	10%	2 mL
Gibco Pen-Strep Solution 100×	1× (100 U/mL penicillin, 100 µg/mL streptomycin)	200 µL
<b>Total</b>		<b>20 mL</b>

Storage: Prepare in sterilized condition. Store at 4°C < 1 month. Use within one week after adding Pen-Strep.

### TMRE master stock

Reagent	Final concentration	Amount
TMRE	2 mM	10 mg
DMSO		10 mL
<b>Total</b>		<b>10 mL</b>

Storage: -20°C.

### TMRE working stock

Reagent	Final concentration	Amount
TMRE master stock (2 mM)	100 µM	500 µL
PBS	1×	9.5 mL
<b>Total</b>		<b>10 mL</b>

Storage: -20°C.

### TMRE staining medium

Reagent	Final concentration	Amount
100 µM TMRE stock	100 nM	2 µL
10%FBS/IMDM Medium	1×	2 mL
<b>Total</b>		<b>2 mL</b>

Storage: Freshly prepared.

#### Oligomycin A stock

Reagent	Final concentration	Amount
Oligomycin A	5 mg/mL	5 mg
DMSO		1 mL
<b>Total</b>		<b>1 mL</b>

Storage:  $-20^{\circ}\text{C}$ .

#### Oligomycin A working stock

Reagent	Final concentration	Amount
5 mg/mL Oligomycin A	200 $\mu\text{g/mL}$	1 $\mu\text{L}$
DMSO		24 $\mu\text{L}$
<b>Total</b>		<b>25 <math>\mu\text{L}</math></b>

Storage: Freshly prepared.

#### DAPI stock

Reagent	Final concentration	Amount
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	1 mg/mL	10 mg
DMSO		10 mL
<b>Total</b>		<b>10 mL</b>

Storage:  $-20^{\circ}\text{C}$ .

#### 100 $\times$ DAPI staining solution

Reagent	Final concentration	Amount
1 mg/mL DAPI stock	100 $\mu\text{g/mL}$	1 $\mu\text{L}$
PBS	1 $\times$	9 $\mu\text{L}$
<b>Total</b>		<b>10 <math>\mu\text{L}</math></b>

Storage: Freshly prepared.

#### StemSpan working medium

Reagent	Final concentration	Amount
StemSpan	1 $\times$	9.9 mL
Gibco Pen-Strep Solution 100 $\times$	1 $\times$ (100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin)	100 $\mu\text{L}$
<b>Total</b>		<b>10 mL</b>

Storage: Freshly prepared.

#### SCF stock

Reagent	Final concentration	Amount
SCF	50 $\mu\text{g/mL}$	50 $\mu\text{g}$
0.1% BSA in PBS (sterilized)	1 $\times$	1 mL
<b>Total</b>		<b>1 mL</b>

Storage: Store at  $-20^{\circ}\text{C}$ , aliquoted.

#### TPO stock

Reagent	Final concentration	Amount
TPO	20 $\mu\text{g/mL}$	50 $\mu\text{g}$
0.1% BSA in 4 mM HCl (sterilized)	1 $\times$	2.5 mL
<b>Total</b>		<b>2.5 mL</b>

Storage: Store at  $-20^{\circ}\text{C}$ , aliquoted.

### Cytokine mix 100×

Reagent	Final concentration	Amount
50 µg/mL SCF stock	10 µg/mL	2 µL
20 µg/mL TPO stock	2 µg/mL	1 µL
StemSpan Working Medium	1×	7 µL
<b>Total</b>		<b>10 µL</b>

Storage: Freshly prepared.

### Leupeptin stock (health hazard, wear glove)

Reagent	Final concentration	Amount
Leupeptin	10 mM	1 mg
DMSO		210 µL
<b>Total</b>		

Storage: Store at –20°C.

### Concanamycin A master stock (acute toxicity, wear glove)

Reagent	Final concentration	Amount
Concanamycin A	100 µM	50 µg
DMSO		577 µL
<b>Total</b>		<b>577 µL</b>

Storage: Store at –20°C.

### Concanamycin A working concentrate

Reagent	Final concentration	Amount
Concanamycin A Master Stock (100 µM)	4 µM	1 µL
StemSpan medium	1×	24 µL
<b>Total</b>		<b>25 µL</b>

Storage: Freshly prepared.

### Chloroquine stock (irritate, wear glove)

Reagent	Final concentration	Amount
Chloroquine	10 mM	51.6 mg
H <sub>2</sub> O		10 mL
<b>Total</b>		<b>10 mL</b>

Storage: Store at –20°C.

### Chloroquine concentrated working solution

Reagent	Final concentration	Amount
Chloroquine Stock (10 mM)	4 mM	4 µL
StemSpan Medium	1×	6 µL
<b>Total</b>		<b>10 µL</b>

Storage: Freshly prepared.

### Fibronectin stock

Reagent	Final concentration	Amount
Fibronectin	1 mg/mL	1 mg
H <sub>2</sub> O		1 mL
<b>Total</b>		<b>1 mL</b>

**Note:** Reconstitute lyophilized powder in ultra-pure water for 30 min at 37°C.

Avoid swirling or agitating. A small amount of undissolved product might remain.

Store small aliquots at –20°C. Avoid multiple freeze-thaw cycles.

#### Fibronectin coating solution

Reagent	Final concentration	Amount
Fibronectin Stock (1 mg/mL)	75 µg/mL	30 µL
PBS Ca <sup>2+</sup> /Mg <sup>2+</sup> free	1×	370 µL
<b>Total</b>		<b>400 µL</b>

Storage: Freshly prepared.

#### Paraformaldehyde solution (flammable, health hazard, wear glove, handle in a fume hood)

Reagent	Final concentration	Amount
16% Paraformaldehyde	4%	250 µL
PBS	1×	750 µL
<b>Total</b>		<b>1 mL</b>

Storage: Freshly prepared.

#### Permeabilization solution (corrosive, wear PPE)

Reagent	Final concentration	Amount
10% Triton X-100	0.25%	1.25 mL
PBS	1×	48.75 mL
<b>Total</b>		<b>50 mL</b>

Storage: Store at room temperature.

#### Washing buffer (Tween 20/PBS; PBST)

Reagent	Final concentration	Amount
1% Tween 20	0.01%	500 µL
PBS	1×	49.5 mL
<b>Total</b>		<b>50 mL</b>

Storage: Store at room temperature and use within 1 week. Prepare new one if buffer becomes cloudy.

#### 30% BSA solution

Reagent	Final concentration	Amount
BSA	30%	3 g
PBS	1×	10 mL
<b>Total</b>		<b>10 mL</b>

Storage: Store at –20°C.

#### Blocking buffer

Reagent	Final concentration	Amount
BSA (30% BSA solution)	3%	100 µL
Tween 20/PBS	1×	900 µL
<b>Total</b>		<b>1 mL</b>

Storage: Prepare freshly. Store remaining at 4°C and use within 1 or 2 days.

Antibody diluent		
Reagent	Final concentration	Amount
BSA (30% BSA solution)	1%	100 $\mu$ L
Tween 20/PBS	1 x	2.9 mL
<b>Total</b>		<b>3 mL</b>

Storage: Prepare freshly. Store remaining at 4°C and use within 1 or 2 days.

### STEP-BY-STEP METHOD DETAILS

#### Step one: Cell isolation and fluorescence-activated cell sorting (FACS)

##### Part I. From mouse bone marrow (BM)

⌚ Timing: 7–9 h (for 10–12 mice)

In this step, BM cells are extracted from mouse hind leg bones. Red blood cell free and lineage deleted live cells are then stained with HSC specific fluorescently-labeled antibodies, and labeled with MMP-specific probe TMRE. Distinct MMP fractions of CD48<sup>+</sup> LSK CD150<sup>+</sup> murine HSCs (CD150<sup>+</sup> HSCs) are sorted using flow cytometry sorter.

#### 1. Harvest BM.

10–12 adult (8–10 weeks old) wildtype male/female mice (C57BL/6J) are required for this step.

- Sacrifice, sanitize, and dissect the mice following standard operating procedure for harvesting murine tissue biopsies.
- Remove the entire tibias and femurs from both hind legs. Slice off from the joint with intact metaphysis. Try not to break the bones.

**Note:** Higher number of HSCs are located in the trabecular region of epiphysis and metaphysis (Morrison and Scadden, 2014). To ensure both high yield and nonbiased results, the entire bone should be preserved as much as possible.

- Trim off the large muscles attached to the bones.
- Clean off any remaining muscles attached to the bones by either scraping with a scalpel or rubbing off with Kimwipes. Discard the knee cap.
- Rinse the bones to remove any attached hair using 2%FBS/PBS. Keep the bones in the buffer on ice.
- Move to a biosafety cabinet to maintain sterile conditions.
- Harvest BM using flushing approach (or alternative crushing approach below).
  - Cut off only a thin piece of epiphysis at the end of the bone.
  - Hold the bone shaft with a forceps and insert a 23-gauge needle attached to a syringe into the end of the bone.
  - Completely flush BM into a 15 mL conical tube using no less than 3 mL (per mouse) 2%FBS/PBS. Flush multiple times until bone shafts turn white.

**Note:** Using a finer needle to flush the BM or trying to disperse it by passing through a syringe could introduce extra shear force that is harmful to cells.

- Gently pipet flushed BM up and down using a 1 mL micropipette to disperse BM aggregates.
  - Wash the cells with an additional 3 mL of 2% FBS/PBS by spinning down at 300 g for 5 min at 4°C.
- h. Crushing approach (alternative to flushing approach above):
- Briefly crush the bones, up to 5 legs a group, using a mortar and a pestle in a small volume of 2% FBS/PBS just enough to cover the bones.

**Note:** Do not grind bones into fine pieces. Just briefly fragment bones to release BM.

- ii. Add ~10 mL 2% FBS/PBS to the crushed bones. Swirl around to gently release BM from bone fragments. Pipet up and down to disperse BM aggregates using a 10 mL pipette.
- iii. Pass the cell suspension through a 70  $\mu$ m cell strainer (white) placed in a 50 mL conical tube to remove bone debris.

**Note:** 70  $\mu$ m strainer is used for its bigger pore size that avoid being clogged by debris.

- iv. Add another 10 mL 2% FBS/PBS to the mortar. Collect BM cells as above. Repeat until the suspension becomes clear and colorless.
- v. Centrifuge at 300 g for 5 min at 4°C.

**Note:** Using flushing approach, a piece of epiphysis has to be cut off to flush the BM. And it is hard to thoroughly flush the trabecular area. For this reason, crushing may be recommended over flushing for harvesting BM from the entire trabeculae.

2. Isolate Lineage negative cells from BM.
  - a. Resuspend BM cells in 2 mL of 1 $\times$  BD Pharm Lyse buffer to lyse RBCs.
  - b. Incubate for 3 min in a 37°C water bath.

**Alternatives:** Cells can be incubated for 15 min on ice. Both approaches work well.

- c. Centrifuge at 300 g for 5 min. Wash the pellet once with at least 10 mL 2% FBS/PBS.
- d. Resuspend RBC lysed BMs (designated as total BM in the rest of the text) in 2 mL of 2% FBS/PBS.
- e. Filter this suspension through a 40  $\mu$ m cell strainer (blue cap) to generate single cell suspension into a 5 mL FACS tube.
- f. Take 5  $\mu$ L and make a 200-fold dilution. Count the total number of cells.

**Note:** Expect to obtain 3–5  $\times 10^8$  RBC<sup>-</sup> BM pooled from 10–12 mice (2–5  $\times 10^7$  per mouse).

- g. Adjust the cell concentration to 1  $\times 10^8$  cells/mL in a maximum volume of 2 mL (total cells less than 2  $\times 10^8$ ). Use multiple tubes if the volume exceeds 2 mL.
- h. Save an aliquot of 1.5  $\times 10^6$  cells for FACS controls.
- i. To isolate lineage negative cells (Lin<sup>-</sup>) enriched BM (designated as Lin<sup>-</sup> BM), perform lineage depletion using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit following manufacturer's instruction ([EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit](#)).

**Note:** The Isolation Cocktail in the kit is a biotin conjugated, anti-lineage antibody mixture (Lin cocktail). Biotin labeled cells are bound by streptavidin coated magnetic beads (RapidSphere) and negatively selected using a magnet.

- j. Count the number of isolated cells. About 1–3  $\times 10^6$  Lin<sup>-</sup> BM cells per mouse (both hind legs) are expected, 5%–10% of total BM.

**Note:** The purpose of lineage depletion is to enrich for stem and progenitor cells to increase sorting efficiency. After depletion, about 60%–80% cells are expected to be lineage negative. The remaining un-depleted lineage<sup>+</sup> cells will be stained and gated out during FACS sorting procedures as follows.

**Table 1. Sample preparation and staining panel—Mouse HSC**

	Lin <sup>-</sup> BM	Total BM				UltraComp beads			
	Full stain sample	Lin/CD48 positive control	Single stain control (probes)		Single stain control (antibodies)				
Cell Number	1 mL $1 \times 10^7$	100 $\mu$ L $1 \times 10^6$	$> 10^4$						
Antibody/Probe	Full Stain	APCCy7	TMRE	Unstained	DAPI	CD48-APCCy7	Sca-PB	c-Kit-APC	CD150-PECy7
Lin-biotin	+								
SV-APCCy7	2 $\mu$ L	0.5 $\mu$ L							
CD48-APCCy7	10 $\mu$ L	1 $\mu$ L					1 $\mu$ L		
Sca-PB	10 $\mu$ L						1 $\mu$ L		
c-Kit-APC	10 $\mu$ L							1 $\mu$ L	
CD150-PECy7	10 $\mu$ L								1 $\mu$ L
TMRE	+	+							
DAPI	+	+	+						

3. Label mouse HSCs using fluorochrome conjugated antibodies and mitochondrial membrane potential specific probe, TMRE.

a. Prepare sample/controls:

Full Stain Sample (Lin<sup>-</sup> BM):

- i. Resuspend Lin<sup>-</sup> BM cells at a concentration of  $1 \times 10^7$  cells/mL in 2% FBS/PBS for immunostaining.

Lin/CD48-APCCy7 positive staining control (total BM):

- ii. Resuspend  $\sim 1 \times 10^6$  total BM (from the saved aliquot in 2.h.) in 100  $\mu$ L of 2% FBS/PBS for immunostaining.

Single staining controls (probes) for compensation (total BM):

- iii. Prepare 3 FACS tubes labeled as TMRE, DAPI, and unstained.
- iv. Transfer  $\sim 10^4$  total BM (from the saved aliquot in 2.h.) to each of the three tubes.
- v. Add 200  $\mu$ L of 2% FBS/PBS to the two tubes labeled as unstained and DAPI.

Single staining controls (antibodies) for compensation (beads):

- vi. Prepare 4 tubes of UltraComp Plus compensation beads, labeled as: CD48-APCCy7, Sca-1-PB (Pacific Blue), c-Kit-APC, CD150-PECy7.
- vii. Thoroughly vortex UltraComp Plus compensation beads. Add one droplet to each tube.
- viii. Add 1  $\mu$ L corresponding antibody as labeled on the tube. No incubation or wash is needed.
- ix. Protect the tubes from light. The beads are ready for flow cytometry analysis.

b. Immunofluorescent staining (Table 1):

**Note:** Based on previously conducted titrations for mouse HSCs, use 1  $\mu$ L antibody per  $10^6$  cells per 100  $\mu$ L in this protocol.

**△ CRITICAL:** Make sure that cells are suspended at  $\sim 1 \times 10^7$  cells/mL and sufficient antibodies are added. Because the majority of BM are lineage cells and CD48<sup>+</sup> cells, these cells may not be thoroughly gated out if staining is insufficient. See troubleshooting [problem 4: Insufficient Lin/CD48 staining](#).

Full Stain Sample (Lin<sup>-</sup> BM):

- i. For every  $1 \times 10^7$ /mL Lin<sup>-</sup> BM cells, add 2  $\mu$ L Streptavidin-APCCy7 (SA-APCCy7) and 10  $\mu$ L each of the following antibodies: anti CD48-APCCy7, Sca-1-PB, c-Kit-APC, CD150-PECy7.
- ii. Mix well and incubate for 20 min on ice. Protect from light.
- iii. Wash cells with 2% FBS/PBS by centrifuging at 300 g for 5 min at 4°C.
- iv. Pour off the supernatant quickly by tilting once.

- v. While keeping the tube inverted, aspirate off the last hanging droplet to remove all the supernatant.
- vi. Proceed to TMRE staining.

**Note:** The remaining un-depleted, biotin labeled lineage cells will be stained by SA-APCCy7. In this protocol, both Lin<sup>+</sup> cells and CD48<sup>+</sup> cells are stained with APCCy7 and gated out altogether during FACS. The benefit of this approach is that it spares one fluorescent color. An additional surface marker of interest can be added if needed. This also simplifies the flow cytometry algorithm and increases sorting efficiency. However, this approach relies on sufficient Lin and CD48 staining and a good Lin, CD48 double negative FACS gating.

Lin/CD48-APCCy7 positive staining control (total BM):

- vii. Add 1  $\mu$ L Anti CD48-APCCy7 antibody and 0.5  $\mu$ L SA-APCCy7 to 100  $\mu$ L of  $10^6$  total BM cells.
- viii. Mix well and incubate for 20 min on ice. Protect from light.
- ix. Wash the cells with 2% FBS/PBS by centrifuging at 300 g for 5 min at 4°C.
- x. Pour off the supernatant quickly by tilting once. Do not remove the last droplet.

**Note:** This will leave  $\sim$ 200  $\mu$ L of buffer in the tube, which should be enough to resuspend the cells.

- xi. Place cells on ice and protect from light.

c. TMRE staining:

- i. Prepare 2 mL of 100 nM TMRE staining medium in 10% FBS/IMDM pre-warmed at 37°C.
- ii. Resuspend full stain sample cell pellet and cells from the tube marked as TMRE, each in  $\sim$ 500  $\mu$ L pre-warmed TMRE staining medium.
- iii. Incubate for 15 min at 37°C in humidified atmosphere with 5% CO<sub>2</sub>.
- iv. Wash with 2% FBS/PBS by centrifuging at 300 g for 5 min at 4°C.
- v. Pour off the supernatant without aspirating the last hanging droplet, leaving  $\sim$  200  $\mu$ L in the tube.

d. DAPI staining:

Full stain sample (Lin<sup>-</sup> BM):

- i. Add 2% FBS/PBS to the above  $\sim$  200  $\mu$ L cell suspension to reach  $1 \times 10^7$ /mL.
- ii. Add 1  $\mu$ L of 1 mg/mL DAPI to 1 mL of full stain sample.
- iii. Filter through a Falcon cell strainer cap into a 5 mL FACS tube.

DAPI single staining control:

- iv. Add 2  $\mu$ L of 100 $\times$  DAPI staining solution into  $\sim$  200  $\mu$ L cells in the tube.

Lin/CD48-APCCy7 positive staining control (total BM):

- v. Add 2  $\mu$ L of 100 $\times$  DAPI staining solution into  $\sim$  200  $\mu$ L cells in each tube.

e. All samples and controls are now ready for flow cytometry analysis. Keep cells on ice and protect from light.

4. Sort distinct MMP fractions of CD48<sup>-</sup>LSK CD150<sup>+</sup> HSCs (CD150<sup>+</sup> HSCs).

Sort cells using a 5 laser BD FACSAria sorter with a 100  $\mu$ m nozzle and 4-way purity sorting mode. Set the temperature of the sample injection chamber to 4°C.

- a. Make plots over FSC/SSC, FSC-A/FSC-H, SSC-A/SSC-H, DAPI, APCCy7, APC/PB, PECy7.

**Note:** FSC, SSC are plotted on linear scale, fluorescence parameters are plotted on biexponential scale.

b. Draw tentative gates subsequently on each of the plots in the following order:

Debris<sup>-</sup> gate (FSC-A/SSC-A) -> doublet<sup>-</sup> gate (FSC -A/-H) -> doublet<sup>-</sup> gate (SSC -A/-H) -> DAPI<sup>-</sup> gate -> APCCy7<sup>-</sup> gate -> APC<sup>+</sup>PB<sup>+</sup> gate -> PECy7<sup>+</sup> gate.

c. Set voltage for each parameter.

- i. Load the full stain sample (Lin<sup>-</sup> BM).
  - ii. Acquire just enough events to reveal populations in all gates.
  - iii. Adjust the voltages to appropriate ranges for each parameter.
  - iv. Remove the tube once the voltage has been set and place it on ice.
- d. Set compensation.
- i. Acquire the following single staining controls for compensation.  
Single staining controls with probes:  
Unstained.  
DAPI.  
TMRE.  
Single staining controls with antibodies:  
CD48-APCCy7.  
Sca-PB.  
cKit-APC.  
CD150-PECy7.
  - ii. Calculate spill over matrix and apply compensation to all.
- e. Set the gates for CD150<sup>+</sup> HSCs (CD48<sup>-</sup> LSK CD150<sup>+</sup>):
- Determine Lin<sup>-</sup>CD48<sup>-</sup> gate (Gate 1):
- i. Acquire Lin/CD48 positive staining control (total BM).
  - ii. On a plot of APCCy7, the majority of total BM should be APCCy7<sup>+</sup> (Lin<sup>+</sup> and /or CD48<sup>+</sup>).
  - iii. Set Lin<sup>-</sup>CD48<sup>-</sup> gate according to the clear-cut bottom edge of the APCCy7 positive population, where no more than 1% should fall into the negative gate (Figure 1Ai).
  - iv. Acquire minimal sufficient number (100,000–200,000) of fully stained Lin<sup>-</sup> BM sample.
  - v. Between 10%–15% cells are expected to fall into Lin<sup>-</sup>CD48<sup>-</sup> gate (Figures 1Aii and 1Bv).

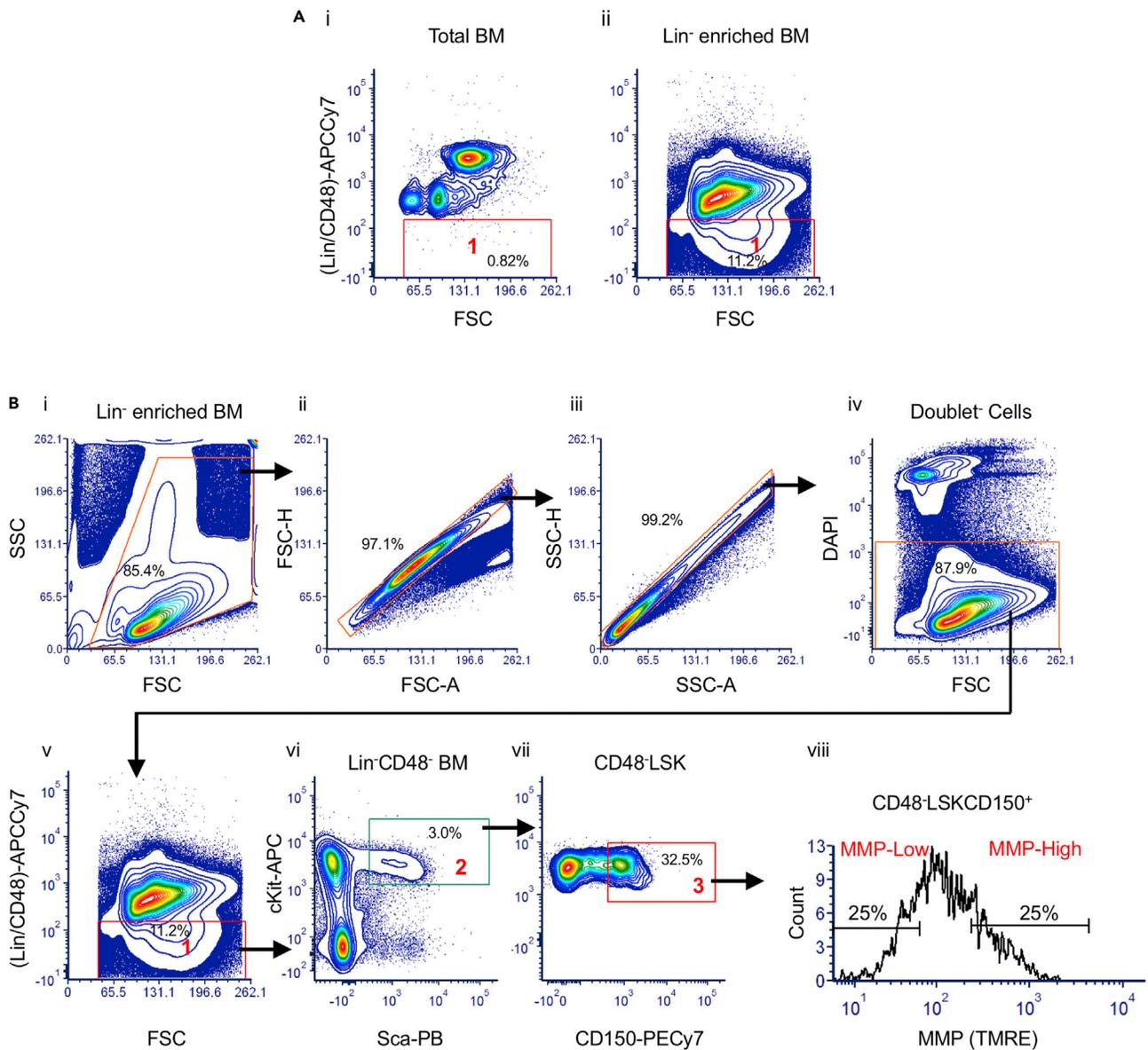
**Note:** The majority of BM are lineage cells or CD48<sup>+</sup> cells. If insufficiently stained, these cells will be gated as Lin<sup>-</sup>CD48<sup>-</sup>, indicated by unusually high percentage in this gate. Follow Troubleshooting Problem 4: Insufficient Lin/CD48 staining for this.

- Determine Sca-1<sup>+</sup>c-Kit<sup>+</sup> gate (Gate 2):
- vi. On a plot of APC against PB, four distinguished populations are revealed under Lin<sup>-</sup>CD48<sup>-</sup> gate (Gate 1) (Figure 1Bvi).
  - vii. Set Sca-1<sup>+</sup>c-Kit<sup>+</sup> gate to include clearly separated, the most positive population. Normally 1%–4% of Lin<sup>-</sup>CD48<sup>-</sup> cells are expected in this gate (Figure 1Bvi).

**Note:** If the remaining lineage<sup>+</sup> cells and/or CD48<sup>+</sup> cells are not sufficiently stained and gated out, Sca-1/c-Kit profile will become mostly double negative. In that case, the percentage of Sca-1<sup>+</sup>/c-Kit<sup>+</sup> will be unusually low (below 1%) and might be contaminated with CD48<sup>+</sup> cells. Follow Troubleshooting Problem 4: Insufficient Lin/CD48 staining for this.

- Determine CD150<sup>+</sup> gate (Gate 3):
- viii. On a plot of PECy7, set CD150<sup>+</sup> gate under Sca-1<sup>+</sup>c-Kit<sup>+</sup> gate (Gate 2) to include clearly separated, the most positive population. Normally 20%–30% of CD48<sup>-</sup> LSK cells are expected to be CD150<sup>+</sup> (Figure 1Bvii).
- f. CD150<sup>+</sup> HSCs (CD48<sup>-</sup> LSK CD150<sup>+</sup>) can now be determined as following:  
Gate 1 (Lin<sup>-</sup>CD48<sup>-</sup>) -> Gate 2 (Sca-1<sup>+</sup>c-Kit<sup>+</sup>) -> Gate 3 (CD150<sup>+</sup>) (Figures 1Bv–1Bvii).
- g. Plot TMRE histogram from 100 CD150<sup>+</sup> HSCs. Gate top and bottom most 25% as MMP-High and MMP-Low (Figure 1Bviii).

**△ CRITICAL:** TMRE histogram keeps fluctuating until sufficient number of parental cells (100 cells recommended; minimal 50) are acquired. Wait until a stable and fully distributed TMRE profile is shown before determining MMP gates.



**Figure 1. FACS gating strategy to sort MMP-Low or -High CD48-LSK CD150<sup>+</sup> mouse HSCs**

(A) Determine Lin<sup>-</sup>CD48<sup>-</sup> gate (Gate 1). Flow cytometry APCCy7 contour plots of total BM (Lin/CD48)-APCCy7 positive staining control (i), and Lin<sup>-</sup> enriched BM full stain sample (ii).

(B) Flow cytometry gating scheme towards CD48<sup>-</sup>LSKCD150<sup>+</sup> from Lin<sup>-</sup> enriched mouse BM. i, ii, iv, gating debris<sup>-</sup> (i), doublets<sup>-</sup> (ii, iii), DAPI<sup>-</sup> (iv) single viable cells. v, vi, vii, sequential gating of Lin<sup>-</sup>CD48<sup>-</sup> BM (Gate 1; v); Sca1<sup>+</sup>c-Kit<sup>+</sup> (Gate 2;vi), CD150<sup>+</sup> (Gate 3; vii). viii, TMRE Flow histogram of CD48<sup>-</sup>LSKCD150<sup>+</sup> HSCs. Set bottom 25% MMP-Low and top 25% MMP-High gates.

h. Sort MMP-Low and -High HSCs into 1.5 mL microcentrifuge tubes containing 200  $\mu$ L cold StemSpan.

$\Delta$  **CRITICAL:** Even though the sample chamber of FACS sorter is set at 4°C, cells are not cooled as efficiently as on ice (depending on the instrument). As temperature increases, TMRE profile might slowly shifts towards lower signal intensity, likely as a result of HSC dye efflux activity. Try not to leave samples in the sample chamber longer than needed. If the volume of a sample is large, divide it into multiple tubes and keep the rest on ice.

△ **CRITICAL:** MMP-Low and -High gates might need to be re-gated according to the MMP profile of each new tube loaded, if multiple tubes are used for samples with large volume. Monitor the shift of TMRE profile during the entire sorting. Finely re-adjust MMP-Low/-High gates whenever MMP-Low gate comprises more than 30% and MMP-High gate less than 20% of the entire profile.

**Note:** The absolute level of MMP or the relative difference of MMP between cell types is not the focus in the studies presented in this protocol. MMP-Low and -High here are defined as the lowest and the highest 25% MMP, respectively, of a given population. Depending on the goal of the study, MMP gating strategy may vary (e.g., comparing between normal and malignant HSCs, or between young and old HSCs which may differ greatly in MMP levels) and the results should be interpreted accordingly (Liang et al., CSC, 2020; Mansell et al., CSC, 2021; Qiu and Ghaffari, Exp Hematol, 2022).

**Note:** Real-time MMP gate adjustment demands a reliable TMRE profile based on sufficient cell numbers. During sorting, the number of parental cells (CD150<sup>+</sup> HSCs) displayed for TMRE profile is limited by the maximal total events allowed for each screen refresh. Since only less than 0.1% Lin<sup>-</sup> BM are CD150<sup>+</sup> HSCs, at least 50,000 Lin<sup>-</sup> BM cells need to be displayed with each screen refresh to give a TMRE profile of > 50 CD150<sup>+</sup> HSCs. Set Events to Display to maximum (Displaying > 50 CD150<sup>+</sup> HSCs is possible using BD FACSDiVa software). This also emphasizes the necessity of progenitor cell enrichment using lineage depletion kit prior to sorting. See troubleshooting [problem 5: Unstable TMRE histogram of CD150<sup>+</sup> HSCs](#).

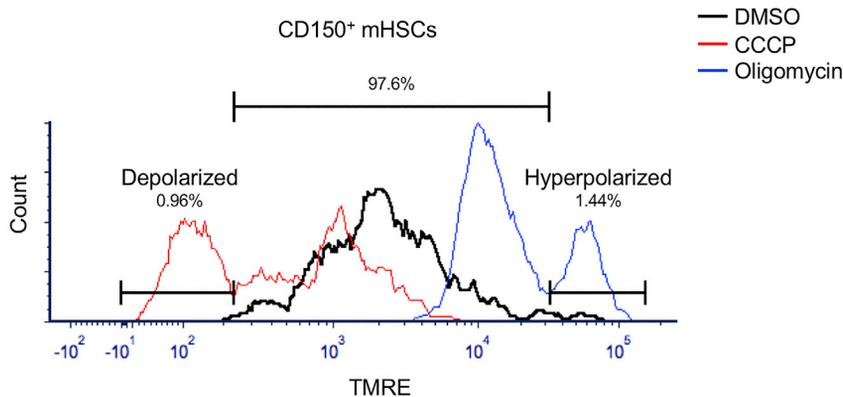
- i. After sorting, 4,000–5,000 MMP-Low or -High CD150<sup>+</sup> HSCs are expected from 10–12 mice.

**Optional:** The following step is only required for initial setup of TMRE analysis for a new cell type or staining strategy:

5. Validate TMRE staining using CCCP and oligomycin.

**Note:** The fluorescence intensity increases proportionally with the concentration of TMRE before reaching a self-quenching dose. The optimal TMRE concentration for non-quenching FACS analysis is typically between 20 and 200 nM, 15–30 min at 37°C. During the initial setup of the experiment, the specificity and responsiveness of TMRE staining to changes in MMP should be validated using inhibitor controls such as CCCP (carbonyl cyanide 3-chlorophenylhydrazone) and oligomycin for each cell type of interest and/or staining approach (dose, incubation time).

- a. Take a proportion of pooled BM equivalent to the amount of one bone for this analysis.
- b. Perform RBC lysis, lineage depletion and immunofluorescent staining of Full Stain Sample and controls as described in 2.a. – 3.b.
- c. Resuspend washed Full Stain Lin<sup>-</sup> BM in 1.5 mL pre-warmed 10% FBS/IMDM. Divide the cell suspension into 3 FACS tubes, 500 µL each. Mark the tubes as NT (non-treated), CCCP, or Oligomycin.
- d. In 500 µL cell suspension marked as CCCP, add 5 µL of 10 mM CCCP stock to 100 µM final.
- e. In 500 µL cell suspension marked as NT, or oligomycin, add 5 µL DMSO each.
- f. Incubate for 10 min at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.
- g. During this time, prepare 20 µL of 10 µM TMRE (1:10 diluted from 100 µM working stock) in 10% FBS/IMDM.
- h. At the end of incubation, without washing, add 5 µL of 10 µM TMRE into each of 500 µL cell suspension (NT, CCCP, oligomycin) to a final concentration of 100 nM. Return cells to the incubator, and incubate for another 15 min.



**Figure 2. Validation of the specificity and responsiveness of TMRE staining to changes in MMP level using CCCP and oligomycin**

Flow cytometry histogram of TMRE for CD48<sup>+</sup> LSKCD150<sup>+</sup> HSCs treated with CCCP (100  $\mu$ M, red), or oligomycin (2  $\mu$ g/mL, blue), or untreated (DMSO, 10 min, black). Depolarized and hyperpolarized TMRE gates were determined according to the peak separation on TMRE profile of CCCP and oligomycin treated samples respectively. Only 0.96% or 1.44% untreated HSCs are depolarized or hyperpolarized. TMRE intensity of 97.6% of cells are within physiological range.

- i. During this time, prepare 25  $\mu$ L of 200  $\mu$ g/mL Oligomycin working stock (1:25 diluted from 5 mg/mL stock) in 10% FBS/IMDM.
- j. 5 min before the end of incubation, add 5  $\mu$ L of 200  $\mu$ g/mL Oligomycin into 500  $\mu$ L cell suspension labeled as oligomycin to a final concentration of 2  $\mu$ g/mL. Return cell to the incubator and incubate for the rest of 5 min.

**Note:** The response to oligomycin is transient. Do not leave cells with oligomycin for longer than 15 min.

- k. At the end of incubation, immediately fill the tubes with ice cold 2% FBS/PBS and centrifuge at 300 g for 5 min at 4°C.
- l. Pour off the supernatant without aspirating the last hanging droplet, leaving  $\sim$ 200  $\mu$ L in the tube.
- m. Add 2  $\mu$ L of 100 $\times$  DAPI staining solution into  $\sim$ 200  $\mu$ L cells in each tube. Samples are ready for flow cytometry analysis. Keep the cells on ice and protect from light.
- n. Plot TMRE histogram for CD150<sup>+</sup> HSCs gated as previously described. Overlay TMRE profiles of NT, CCCP and Oligomycin treated samples.

**Note:** The physiological range of TMRE fluorescence intensity should fall between the depolarized (CCCP treated) and hyperpolarized (oligomycin treated) level. CCCP increases proton permeability by decoupling the electron transfer chain from ATP synthase, which depolarizes the mitochondrial membrane (loss of proton gradient) and results in reduced TMRE accumulation. Oligomycin blocks proton re-entry into mitochondria through inhibiting mitochondrial ATP synthase, leading to hyperpolarization of mitochondrial membrane as exhibited by increased TMRE accumulation. The indication of physiological MMP in CD150<sup>+</sup> HSCs using TMRE staining approach adapted in this protocol was validated in [Figure 2](#). Close to 98% of TMRE fluorescence intensity of non-treated sample is within the depolarized (CCCP treated) and hyperpolarized (Oligomycin treated) level.

## Part II. From human peripheral blood (PB)

© Timing: 10–12 h total

⌚ Timing: 3–4 h (for 5–6 pouches of blood) for step 6

⌚ Timing: ~2 h for step 7

⌚ Timing: 4–6 h for step 8

In this step, mononuclear cells (MNCs) are isolated from human peripheral blood. CD34<sup>+</sup> MNCs are enriched through immunomagnetic column separation. Live cells are then stained with HSC specific immunofluorescent antibodies, and labeled with mitochondrial membrane potential – MMP – specific probe TMRE. Distinct MMP fractions of CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup> Human HSCs (CD90<sup>+</sup> HSCs) are sorted using flow cytometric sorter.

6. Isolate human mononuclear cells (MNCs) from peripheral blood (PB).

- a. Fresh, non-mobilized whole PB from normal donors are obtained from NY blood center (available as ~ 50 mL/pouch/donor for research purpose).

**Note:** Similar procedure applies to fresh cord blood (CB) and mobilized PB. Details for processing leukocyte reduction filter or frozen-thawed blood products are not included here.

**Note:** Blood samples are treated as potentially infectious and are collected and processed by following standard for proper handling of blood borne biohazard.

- b. Process blood samples the same day upon receipt.

⚠ **CRITICAL:** If same-day process is not possible, keep the blood in a cool environment and process as soon as possible. Hemolysis, aggregation, and low yield are expected if blood is not fresh. Follow Troubleshooting [Problem 1: Hemolysis](#).

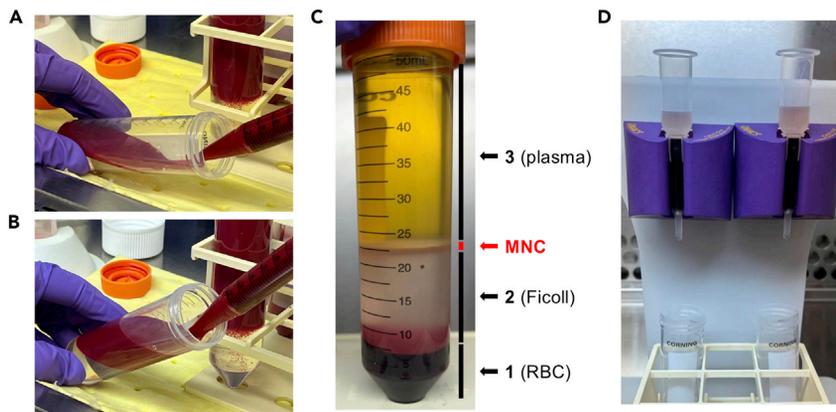
- c. Spray blood pouches with 70% ethanol and wipe it down.
- d. In a biological safety cabinet, disinfect the tubing on the pouches using alcohol prep pads.
- e. Cut open the tubing using a clean, disinfected razor blade and release blood into 50 mL conical tubes or other sterile containers.

**Note:** To minimize blood spill, do not cut off the entire tip of the tubing directly. Make a small cut first, just enough to let in some air. Once the entering air pushes the blood back into the pouch, it is safe to cut off the entire tip without spilling.

- f. Dilute the whole blood at least 1:1 with 1 × PBS.

⚠ **CRITICAL:** Sufficient dilution of RBCs is critical for a good Ficoll separation. If poor separation occurred due to high RBC concentration, follow Troubleshooting [Problem 2: Poor Ficoll Separation](#).

- g. Add 15 mL Ficoll-Paque PLUS in each 50 mL conical tube.
- h. Layer ~30 mL of diluted blood on the Ficoll-Paque PLUS ([Figures 3A and 3B](#)).
  - i. Tilt the tube as low as possible toward the bench top.
  - ii. Gently release the diluted blood using a 25 mL pipette.
  - iii. Adjust the dispensing mode on the pipetman to slow.
  - iv. Allow the blood to flow slowly instead of gushing along the tube until it spreads over the Ficoll surface.
- i. Set the centrifuge brake to “off”, or the deceleration to the minimum level (varies depending on the centrifuge model).
- j. Centrifuge the blood loaded Ficoll at 800 g for 30 min at 18°C–20°C.



**Figure 3. Isolating MNCs from human PB and enrichment of CD34<sup>+</sup> PBMCs**

(A and B) Layering blood on Ficoll.

(C) Layers after Ficoll separation.

(D) Passing CD34-MicroBead labeled PBMCs through LS columns on magnetic MidiMACS separator.

△ **CRITICAL:** 18°C–20°C is the optimal temperature for Ficoll separation. The efficiency of Ficoll separation is reduced at lower temperature (4°C), whereas RBCs tend to aggregate at a higher temperature (37°C). If poor separation occurred due to high RBC concentration, follow Troubleshooting [Problem 2: Poor Ficoll Separation](#).

- k. After centrifugation, the blood-Ficoll cocktail is separated into three layers plus a middle ring ([Figure 3C](#)).

Bottom layer:

Red blood cell pellet.

(~7.5 mL).

Middle layer:

Colorless Ficoll containing granulocytes.

(~15 mL).

Top layer:

Yellow and clear plasma containing platelet.

(~25–30 mL).

MNCs:

A white, thin ring between Ficoll and plasma layers.

(At ~20–25 mL mark in a 50 mL conical tube).

**Note:** With good handling, the MNC ring generated by Ficoll separation from fresh blood is expected to be white and homogeneous. The top plasma layer should appear lightly yellow and clear. Red plasma and dark red clumps in the MNC ring indicate hemolysis. If this happens, follow Troubleshooting [Problem 1: Hemolysis](#).

- l. Gently vacuum aspirate off most of the upper plasma layer without disturbing MNC layer, leaving ~5 mL above.

**Note:** Small amount of platelet contamination from residual plasma is allowed, for HSCs are to be FACS sorted in the following steps.

- m. Insert a 10 mL pipette through the remaining plasma layer and collect the entire MNC layer, pooling them into 3–4 new 50 mL tubes.

**Note:** Small amount of Ficoll carryover is allowed in the type of study focused in this protocol.

**Note:** From this step forward, keep cells on ice, set centrifuge to 4°C and work with pre-cooled buffers.

- n. Pellet MNCs.
  - i. Set the centrifuge brake back to “on”, or the deceleration to the maximum level.
  - ii. Centrifuge pooled MNC suspensions at 300 g for 10 min at 4°C.
  - iii. Remove most supernatant without disturbing the pellets, leaving ~ 5 mL.

**Note:** Do not try to aspirate all supernatant completely, for MNC pellets tend not to be very compact at this step.

- iv. Resuspend the pellets with the remaining supernatant.
- v. Pool all MNC suspensions into one tube.
- vi. Thoroughly rinse off any remaining cells in all tubes with small amount of 2% FBS/PBS and combine the rinse-off with pooled MNC suspension.
- vii. Centrifuge again at 300 g for 10 min at 4°C.

**Note:** MNC pellet from Ficoll separation of fresh blood is expected to be white or slightly pink (small number of residual RBCs are expected). Pellet containing too much broken RBC membrane as a result of hemolysis tends to be slimy. If this happens, follow Troubleshooting [Problem 1: Hemolysis](#).

- o. Fill up the tube to 50 mL with buffer and mix well. Take 5 µL, dilute 1:20, and count the number of cells.

**Note:** If MNC suspension appears slushy and full of fine debris, follow Troubleshooting [Problem 1: Hemolysis](#).

- p.  $\sim 1 \times 10^8$  MNCs are expected from every 50 mL of fresh whole blood.

**Note:** If excessive hemolysis happened, cells will be trapped within dead cell aggregates and easily get lost during washing step. This will result in lower yield MNCs than expected.

- q. Cryopreserve aliquots of  $5 \times 10^6$  or  $1 \times 10^7$  MNCs in freezing buffer at  $-80^\circ\text{C}$  (short term) or liquid nitrogen (long term) to use as controls during FACS sorting setup.

▮▮▮ **Pause point:** Alternatively, cryopreserve the rest of MNCs. Isolate CD34<sup>+</sup> cells in another day.

**Note:** Recommended: Isolate CD34<sup>+</sup> cells from freshly harvested PB MNCs. CD34<sup>+</sup> yield from frozen-thawed MNCs is expected to be lower.

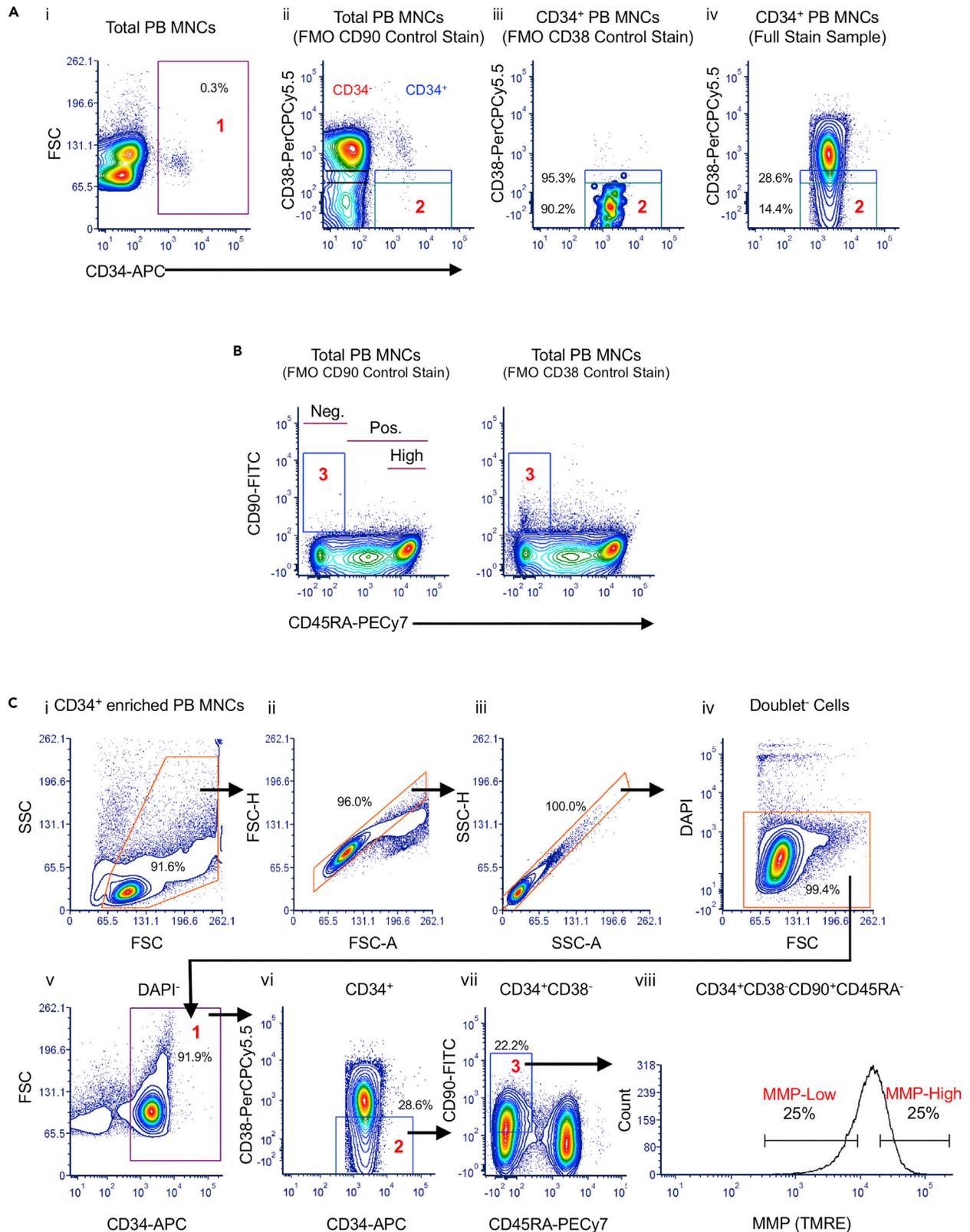
## 7. Isolate CD34<sup>+</sup> cells from PB MNCs.

### CD34 Labeling:

- a. Centrifuge MNCs at 300 g for 10 min at 4°C.
- b. Remove most of the supernatant, leaving a thin layer unstirred.

**Note:** Do not try to aspirate the entire supernatant, for the pellet may not be very compact.

- c. Add 1 mL MACS buffer to resuspend the pellet. Adjust the final volume to approximately 1.4 mL per  $1 \times 10^9$  cells with additional MACS buffer if needed.
- d. Add 300 µL FcR blocking reagent and subsequently 300 µL microbeads from CD34 MicroBead kit in every 1.4 mL cell suspension, making the total volume ~2 mL, at  $5 \times 10^8$  cells/mL.



**Figure 4. FACS gating strategy to sort MMP-Low or -High CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>CD90<sup>+</sup> human HSCs**

(A) Determine CD38<sup>-</sup> gate (Gate 2). i. Flow cytometry contour plot of APC of total PB MNCs. ii-iv, Flow cytometry contour plots of PerCPy5.5 vs. APC. ii, Plot of total PB MNCs (using FMO-CD90 control stain) to determine CD38<sup>-</sup> gate within CD34<sup>+</sup> cells (Gate 2) based on positive or negative population of CD38 within CD34<sup>+</sup> cells (black lines). Stringent gate (green), based on negative CD38 population (lower black line); Less stringent gate (blue), based on positive CD38 population (higher black line). iii. Plots of CD34<sup>+</sup> PB MNCs (using FMO-CD38 control stain) to validate CD38<sup>-</sup> gating. Above 90% (green gate) or 95% (blue gate) CD34<sup>+</sup> cells are included in Gate 2. iv, Plot of CD34<sup>+</sup> PB MNC Full Stain. (B) Determine CD90<sup>+</sup>CD45RA<sup>-</sup> gate (Gate 3). Flow cytometry contour plots of FITC vs. PECy7. Left, CD90 negative control: using FMO-CD90 control stain of total PB MNCs; Right, CD90 positive control: using FMO-CD38 control stain of PB MNCs. (C) Flow cytometry gating scheme towards CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>CD90<sup>+</sup> from CD34<sup>+</sup> enriched PB MNCs. i, ii, iv, gating debris<sup>-</sup> (i), doublets<sup>-</sup> (ii, iii), DAPI<sup>-</sup> (iv) single viable cells. v, vi, vii, sequential gating of CD34<sup>+</sup> (Gate 1, v), CD38<sup>-</sup> (Gate 2, vi), CD45RA<sup>+</sup>CD90<sup>+</sup> (Gate 3, vii). viii, TMRE flow histogram of CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>CD90<sup>+</sup> HSCs. Set bottom 25% MMP-Low and top 25% MMP-High gates.

**Note:** According to manufacturer's instruction, 100  $\mu$ L CD34 microbeads is required for every  $10^8$  MNCs. However, it is unfeasible and not necessary to purify rare HSCs from large amount of whole blood using this amount of microbeads. Based on our bench experience, 300  $\mu$ L CD34 beads per  $1 \times 10^9$  MNCs is sufficient to yield  $\sim 1 \times 10^6$  CD34<sup>+</sup> cells as expected (0.1%–0.3% CD34 in PB MNCs, [Figure 4Ai](#)).

- e. Mix well and incubate on ice for 30 min. Swirl the mixture once in the middle.
- f. Wash cells in 50 mL MACS buffer by centrifuging at 300 g for 5 min at 4°C.

Magnetic Column Separation:

- g. Attach magnetic MidiMACS separators to any stable iron stand/board that can hold the separators straight.

**Alternatives:** Purchase a MACS multistand from Miltenyi Biotec.

- h. Insert an LS column into the separator. Place a collection tube underneath the column ([Figure 3D](#)).
- i. To rinse LS columns, apply 2 mL MACS buffer to the column reservoir and let the buffer run through.
- j. Resuspend the cell pellet in  $\sim 5$  mL MACS buffer to a concentration around  $1 \times 10^8$  cells/500  $\mu$ L.
- k. Filter the cells through a Falcon cell strainer to remove any clumps.
- l. Pass the cells through the column.

**△ CRITICAL:** To ensure smooth magnetic column separation, it is critical to filter the cells prior to load them on column. Clumps larger than 30  $\mu$ m will block LS column. Keep cell density below  $1 \times 10^8$  cells/500  $\mu$ L. The maximum capacity of each LS column is  $2 \times 10^9$ . For separating more than  $2 \times 10^9$  cells, use multiple columns. If slow flow or no flow through column is observed, follow [Troubleshooting Problem 3: Column blockage](#).

**Note:** CD34<sup>+</sup> cells bound to the magnetic beads will be trapped within the column. Unlabeled cells will pass through the column and flow into the collecting tube.

**△ CRITICAL:** Make sure cells are maintained cool. Add cell suspension to the column in small batches at a time and work quickly. Keep the remaining cells on ice.

- m. Once all the cells are processed, wash the column twice with 5 mL MACS buffer.

**Note:** Wait for the column to empty completely each time before adding the buffer. According to the manufacturer's instruction, the column needs to be washed 3 times but this is not necessary for this protocol since it is followed by FACS sorting. A second round of column separation can further improve purity. However, it is not necessary in this protocol since cells will be FACS-sorted. Furthermore, additional rounds of column purification reduce overall yield. At this stage, yield is more important than purity.

- n. Remove the column from the separator and place it over a 15 mL conical tube.
- o. Add 5 mL MACS buffer into the column. Push the plunger into the column and elute the magnetically labeled cells.
- p. Count the number of eluted CD34<sup>+</sup> MNCs without dilution.

**Note:** Alternatively, if available, one may consider to use AutoMACS to perform automatic column separation. It is especially useful when multiple clinical samples are being processed individually and simultaneously. The process of using AutoMACS is not included in this protocol.

- q. Centrifuge the cells at 300 g for 10 min to pellet the cells.
- r. Resuspend the cells in freezing buffer and cryopreserve aliquots at  $1 \times 10^6$  or  $5 \times 10^5$  cells/vial in liquid nitrogen (> 1 month) or at  $-80^\circ\text{C}$  (< 1 month).
- s.  $1\text{--}3 \times 10^5$  CD34<sup>+</sup> MNCs are expected per 50 mL pouch of fresh whole blood.

**Note:** CD34<sup>+</sup> enriched cells are later stained and analyzed by FACS. Above 90% of the final elutes are expected to be CD34<sup>+</sup> (Figure 4Cv).

**△ CRITICAL:** For following FACS analysis, one must use an anti CD34 antibody recognizing an epitope other than the one recognized by the antibody included in the CD34 MicroBead kit.

- 8. Label human HSCs using fluorochrome conjugated antibodies and mitochondrial membrane potential specific probe, TMRE.
  - a. Thaw one vial of  $0.5 \times 10^6$  or  $1 \times 10^6$  CD34<sup>+</sup> MNCs and one vial of  $1 \times 10^6$  MNCs.
  - b. Gently transfer cells to 20 mL 2% PBS/FBS buffer in a biosafety cabinet.
  - c. Centrifuge at 300 g for 5 min.
  - d. Carefully aspirate off most of the supernatant without stirring up cell pellet, leaving about 1 mL at the bottom.

**△ CRITICAL:** Pellet from less than a million cells is not visible in a 15 mL tube. To avoid losing cells during washing steps, do not completely aspirate supernatant. Keep the tip of the aspirating pipette just above the surface of supernatant.

- e. Resuspend the cells and filter through a 40  $\mu\text{m}$  cell strainer cap into 5 mL FACS tubes.
- f. Centrifuge at 300 g for 10 min at  $4^\circ\text{C}$ .
- g. Prepare sample/controls:
  - Full stain sample (CD34<sup>+</sup> MNCs):
    - i. Aspirate off most supernatant of CD34<sup>+</sup> MNCs, leaving  $\sim 200 \mu\text{L}$ .
  - FMO (Fluorescence Minus One) staining controls (MNCs):
    - ii. Aspirate off most supernatant of MNCs, leaving  $\sim 500 \mu\text{L}$ , and resuspend the pellets. Transfer  $200 \mu\text{L}$  MNCs to each of the following FACS tubes:
      - FMO (CD38-PerCPCy5.5).
      - FMO (CD90-FITC).
  - Single staining controls (probes) for compensation (MNCs):
    - iii. Prepare 3 FACS tubes labeled as TMRE, DAPI, or unstained.
    - iv. Transfer  $30 \mu\text{L}$  of the remaining MNCs to each of the labeled tubes.
  - Single staining controls (antibodies) for compensation (beads):
    - v. Prepare four tubes of UltraComp PLUS beads, labeled as CD34-APC, CD38-PerCPCy5.5, CD90-FITC, and CD45RA-PECy7.
    - vi. Thoroughly vortex UltraComp PLUS compensation beads. Add one droplet of these beads to each tube.
    - vii. Add  $1 \mu\text{L}$  each antibody to the respective labeled tube.

**Table 2. Sample preparation and staining panel—Human HSC**

	CD34 <sup>+</sup> MNCs	MNCs					UltraComp beads			
	Full Stain Sample	FMO Gating Reference	Single stain control (probes)				Single stain control (antibodies)			
Cell number	200 $\mu$ L $1 \times 10^6$	0.4 $\times 10^6$	5 $\times 10^4$							
Antibody/Probe	Full stain	CD38-PerCPCy5.5	CD90-FITC	TMRE	DAPI	Unstain	CD34-APC	CD38-PerCPCy5.5	CD90-FITC	CD45RA-PECy7
CD34-APC	5 $\mu$ L	5 $\mu$ L	5 $\mu$ L				1 $\mu$ L			
CD38-PerCPCy5.5	5 $\mu$ L	–	5 $\mu$ L					1 $\mu$ L		
CD90-FITC	10 $\mu$ L	10 $\mu$ L	–						1 $\mu$ L	
CD45RA-PECy7	5 $\mu$ L	5 $\mu$ L	5 $\mu$ L							1 $\mu$ L
TMRE	+	+	+	+						
DAPI	+	+	+		+					

- viii. No incubation or wash is needed. Keep all the tubes on ice and protect from light. The beads are ready for subsequent flow cytometry analysis.
- h. Immunofluorescent staining (Table 2):
  - i. Add the following antibodies to  $0.5\text{--}2 \times 10^6$  cells/200  $\mu$ L.

**Note:** The amount of BD antibodies used for human HSC staining is 5  $\mu$ L–20  $\mu$ L per  $10^6$  cells per 100–200  $\mu$ L according to manufacturer’s instruction. The following antibody concentrations are determined based on titrations conducted previously in the lab.

Full stain sample (CD34<sup>+</sup> MNCs):

- 5  $\mu$ L anti-CD34-APC.
- 5  $\mu$ L anti-CD38-PerCPCy5.5.
- 5  $\mu$ L anti-CD45RA-PECy7.
- 10  $\mu$ L anti-CD90-FITC.

FMO (CD38-PerCPCy5.5) staining control:

- 5  $\mu$ L anti-CD34-APC.
- 5  $\mu$ L anti-CD45RA-PECy7.
- 10  $\mu$ L anti-CD90-FITC.

FMO (CD90-FITC) staining control:

- 5  $\mu$ L anti-CD34-APC.
- 5  $\mu$ L anti-CD38-PerCPCy5.5.
- 5  $\mu$ L anti-CD45RA-PECy7.

- ii. Mix well and incubate on ice for 20 min, protected from light.
- iii. Fill FACS tubes with 2% FBS/PBS and centrifuge at 300 g for 5 min at 4°C.
- iv. Pour off the supernatant quickly in one smooth motion. While the tubes are in the inverted position, aspirate off the last hanging droplet.
- i. TMRE staining.
  - i. Prepare 2 mL of 100 nM TMRE staining medium in 10% FBS/IMDM (pre-warmed at 37°C).
  - ii. Resuspend full stain sample, both of FMO staining controls, and TMRE-single staining control, each in  $\sim$ 500  $\mu$ L pre-warmed TMRE staining medium.
  - iii. Incubate at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 15 min.
  - iv. Fill the FACS tubes with 2% FBS/PBS and centrifuge at 300 g for 5 min at 4°C.
  - v. Aspirate off most of the supernatant, leaving  $\sim$ 200  $\mu$ L for FMO gating controls or TMRE staining control, and  $\sim$ 500  $\mu$ L for CD34<sup>+</sup> cells.
- j. DAPI staining.
  - i. Resuspend the remaining two single staining controls (DAPI, unstained) in 200  $\mu$ L 2% FBS/PBS.
  - ii. Prepare 20  $\mu$ L of 100 $\times$  DAPI staining solution.
  - iii. Add 2  $\mu$ L of 100  $\times$  DAPI staining solution in each of the two 200  $\mu$ L FMO controls and DAPI-single staining control.

- iv. Add 5  $\mu\text{L}$  of 100 $\times$  DAPI to 500  $\mu\text{L}$  of CD34<sup>+</sup> cells.
  - k. All samples and controls are now ready for flow cytometry analysis. Keep the cells on ice and protect from the light.
9. Sort distinct MMP fractions of CD90<sup>+</sup> HSCs (CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup> HSCs).  
Sort cells using a 5 laser BD FACSAria sorter with a 100  $\mu\text{m}$  nozzle and 4-way purity sorting mode. Set the temperature of the sample injection chamber to 4°C.
- a. Make plots over FSC/SSC, FSC-A/FSC-H, SSC-A/SSC-H, DAPI, APC, PerCPCy5.5, FITC/PECy7.

**Note:** FSC, SSC are plotted on linear scale, fluorescence parameters are plotted on biexponential scale.

- b. Draw tentative gates subsequently on each of the plots in the following order: Debris<sup>-</sup> gate (FSC-A/SSC-A) -> doublet<sup>-</sup> gate (FSC -A/-H) -> doublet<sup>-</sup> gate (SSC -A/-H) -> DAPI<sup>-</sup> gate -> APC<sup>+</sup> gate -> PerCPCy5.5<sup>-</sup> gate -> FITC<sup>+</sup>PECy7<sup>-</sup> gate.
- c. Set voltage for each of the parameters.
  - i. Load the full stain sample (CD34<sup>+</sup> MNCs).
  - ii. Acquire just enough events to reveal population in all gates.
  - iii. Adjust the voltages to appropriate ranges for each parameter.
  - iv. Remove the tube once the voltages are set and place it on ice.
- d. Set compensation.
  - i. Acquire the following single staining controls. Calculate spill overs and apply compensation matrix to all:
 

Single staining controls with probes:

    - Unstained.
    - DAPI.
    - TMRE.

Single staining controls with antibodies:

    - CD34-APC.
    - CD38-PerCPCy5.5.
    - CD90-FITC.
    - CD45RA-PECy7.
  - ii. Calculate spill over matrix and apply compensation to all.
- e. Set the gates for CD90<sup>+</sup> HSCs (CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup> Human HSCs).
  - i. Acquire FMO (CD38-PerCPCy5.5) for CD38<sup>-</sup> gating reference (Figure 4Aii).
  - ii. Acquire FMO (CD90-FITC) for CD90<sup>+</sup> gating reference (Figure 4B, left).

**Note:** Since only 0.3%–0.5% of total PB MNCs are CD34<sup>+</sup> cells (Figure 4Ai), acquire at least 20,000 cells of FMO (CD38-PerCPCy5.5) staining control to display a reliable profile of at least 50 CD34<sup>+</sup> cells.

Determine CD38<sup>-</sup> gate (Gate 2):

**Note:** Since the expression of CD38 within CD34<sup>+</sup> population is continuous, the differential expression of CD38 within CD34<sup>+</sup> population can be used as a reference to determine CD38<sup>-</sup> gate.

- iii. On a plot of PerCPCy5.5 against APC, adjust CD38<sup>-</sup> gate according to the clear separation between positive and negative within CD34<sup>+</sup> population of PB MNCs (Using FMO (CD90) staining control) (Figure 4Aii).
- iv. To verify, this CD38<sup>-</sup> gate should comprise 90%–95% CD34<sup>+</sup> cells of PB MNCs (Using FMO (CD38) staining control) (Figure 4Aiii).

**Note:** If quantity is considered a priority over purity, CD38<sup>-</sup> gate can be set at the bottom edge of positive CD38 population, as in [Figure 4Aii](#), blue gate, resulting in ~30% CD38<sup>-</sup> cells among total CD34<sup>+</sup> cells ([Figure 4Aiv](#), blue gate). If purity is considered over quantity, a more stringent CD38<sup>-</sup> gating can be applied by gating at the top edge of negative CD38 population ([Figure 4Aii](#), green gate), resulting in ~14% CD38<sup>-</sup> cells ([Figure 4Aiv](#), green gate).

Determine CD90<sup>+</sup>CD45RA<sup>-</sup> gate (Gate 3):

- v. On a plot of FITC against PEcy7, CD45RA labeling reveals three well separated populations in total PB MNCs: negative, positive, high.
- vi. Determine the threshold between negative and positive CD90 based on the negative and positive CD90 controls, correspondingly FMO (CD90) and FMO (CD38) staining controls of total PB MNCs ([Figure 4B](#), left and right panel).
- vii. Acquire minimal sufficient number (3,000–5,000 cells) of fully stained CD34<sup>+</sup> PB MNCs.
- viii. CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>CD45RA<sup>-</sup> HSCs (CD90<sup>+</sup> HSCs) can now be determined as following:
  - ix. Gate 1 (CD34<sup>+</sup>) -> Gate 2 (CD38<sup>-</sup>) -> Gate 3 (CD90<sup>+</sup>CD45RA<sup>-</sup>) ([Figures 4Cv–4Cvii](#)).
- f. Plot TMRE histogram from at least 100 CD90<sup>+</sup> HSCs. Gate top and bottom 25% as MMP-Low and MMP-High ([Figure 4Cviii](#)).

**Note:** TMRE histogram keeps fluctuating until sufficient number of parental cells (100 cells are recommended) are acquired. Wait until a stable and fully distributed TMRE profile is shown before determining MMP gates.

- g. Sort MMP -Low and -High HSCs into 1.5 mL microcentrifuge tubes containing 200  $\mu$ L cold StemSpan.
- h. After sorting, 1,000–2,000 MMP-Low or -High CD90<sup>+</sup> HSCs (per 5 pouches of 50 mL fresh PB) are expected ([Qiu et al., 2021](#)).

**Note:** Even though the sample chamber of FACS sorter is set at 4°C, cells are not cooled as efficiently as on ice. As temperature increases, TMRE profile slowly shifts toward lower signal intensity, likely as a result of HSC dye efflux activity. Try not to leave samples in the sample chamber longer than needed. If the volume of a sample is large, divide it into multiple tubes and keep the rest on ice. Follow Troubleshooting [Problem 6](#): TMRE intensity dropping for this.

**Note:** MMP-low and -high gates need to be re-gated each time the next tube is loaded according to the new MMP profile. Monitor the shift of TMRE profile during sorting. Finely re-adjust MMP-Low/-High gates whenever MMP-low gate comprises more than 30% and MMP-high gate less than 20% of the entire profile.

**Note:** Relative to Lin<sup>-</sup> BM, human CD34<sup>+</sup> MNCs are more enriched in primitive HSCs. Displaying limit is not a problem. However, due to limited availability of blood samples, regularly only  $0.5\text{--}1 \times 10^6$  CD34<sup>+</sup> cells are sorted for the type of experiments in this protocol. Low acquirement rate as a result of low cell concentration gives poor real-time TMRE profiling. Concentrate samples to under 500  $\mu$ L to raise acquirement rate close to or above 100 events/second. Raising flow rate at a cost of reduced signal resolution is not recommended, since none of CD38 and CD90 profile gives well separated populations in CD34<sup>+</sup> cells.

## Step two: Cell treatment

© Timing: 7–20 h

FACS-sorted murine HSC fractions are treated with one of the lysosomal modulators, Concanamycin A (ConA), Leupeptin (Leu), Chloroquine (CQ), or incubated with DMSO as a control. After 5 or 18 h culture, lysosomes are examined using confocal microscopy in Step Three.

Concanamycin A is a specific inhibitor of vacuolar H<sup>+</sup>-adenosine triphosphatase (v-ATPase) essential for lysosomal acidification that is required for lysosomal digestion and release of digested molecules. Protease inhibitor Leupeptin reduces lysosomal degradation capacity. Chloroquine impairs autophagosome-lysosome fusion (Mizushima et al., 2010; Kania et al., 2017; Mauthe et al., 2018). Transfer 4,000–5,000 FACS-sorted cells into a 1.5 mL microcentrifuge tube.

10. Prepare cell cultures for lysosomal inhibitor treatment.

- a. Spin down the cells at 500 g for 5 min at 4°C.

**Note:** To avoid losing cells, use higher speed at 500 g instead of 300 g to spin down cells for better pelleting.

- b. Carefully aspirate off most of the supernatant without disturbing the cells at the bottom, leaving about 100  $\mu$ L medium.
- c. Resuspend the pellet with StemSpan/Pen Strep to 1.5 mL. Spin down at 500 g for 5 min at 4°C.
- d. Carefully aspirate off most of the supernatant, leaving about 100  $\mu$ L medium at the bottom.
- e. Add StemSpan/Pen Strep to a total volume slightly above 800  $\mu$ L. Gently pipet up and down to resuspend the cells.
- f. Transfer 800  $\mu$ L cell suspension to a new tube.
- g. Add 8  $\mu$ L 100  $\times$  cytokine mix to a final concentration of SCF 100 ng/mL, TPO 20 ng/mL. Mix well gently.
- h. Transfer 200  $\mu$ L cells from above to each of the 4 wells of a U-bottom 96 well plate. Mark the wells as DMSO, Leu (Leupeptin), ConA (Concanamycin), CQ (chloroquine).
- i. In each of the 4 wells, add 2  $\mu$ L one of the following:
  - DMSO (to 1% final concentration).
  - 10 mM Leupeptin stock (to 100  $\mu$ M final concentration).
  - 4  $\mu$ M ConA working concentrate (to 40 nM final concentration).
  - 4 mM Chloroquine concentrated working solution (to 40  $\mu$ M final concentration).

11. Incubate the cells at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 4 h or 18 h according to the assay.

12. Harvest cells at the end of the incubation.

- a. Transfer all cells into a 1.5 mL microcentrifuge tube. Rinse the plate twice with 200  $\mu$ L cold StemSpan/Pen Strep (without cytokine) and collect all remaining cells.
- b. Wash cells with cold StemSpan/Pen Strep. Centrifuge at 500 g for 5 min at 4°C.
- c. Carefully aspirate most of the supernatant without disturbing the cells at the bottom, leaving about 200  $\mu$ L medium.

### Step three: Cell imaging

⌚ Timing: 30–40 h

In this step, HSCs treated with lysosomal modulators (in Step Two) are seeded onto channel  $\mu$ -slides, stained with immunofluorescent antibodies and imaged using confocal microscopy in high-resolution or super-resolution mode.

Turnover efficiency of autophagic vacuoles (mitochondrial specific) is assessed by quantifying mitochondria (marked by TOM20, translocase of the outer membrane 20) and the flux, lysosomal accumulation of autophagosomes (marked by LC3, Microtubule-associated protein 1A/1B-light



**Figure 5. Adding solution/buffer/cell suspension into  $\mu$ -slide channels**

chain 3) in response to lysosomal modulators. Association of TOM20 or LC3 with lysosome is indicated by their colocalization with lysosome marker, lysosome-associated membrane protein 1 (LAMP1).

### Immunofluorescence staining

⌚ Timing: 8–10 h

Channel  $\mu$ -slide technology from ibidi is utilized for carrying out immunofluorescence on a few number of HSCs (Figure 5). These slides used for immunofluorescence assays (VI-Flat) contain 6 parallel channels allowing small number of cells of multiple samples to be processed in minimal volume at the same time.

Key approaches for manipulating  $\mu$ -slides:

**Replacing solutions:** To replace previous solutions in the wells with fresh solutions (wash buffer, stains, etc.), pipette at least 50  $\mu$ L of the required solution into each channel in a slanting position and simultaneously dab off the overflow from the other side with a kimwipe. This replaces the solutions already present in the channels. Repeat the process to fill in the channels with 50  $\mu$ L of the fresh solution.

**Rinsing/Washing:** To rinse or wash the channels, pipette 200  $\mu$ L of the required wash buffer, 3–5 times the volume in each channel in a slanting position and simultaneously dab off the overflow.

All incubation steps are conducted in sealed containers lined with damp paper towels to retain moisture. Rack slides on sticks. Alternatively, purchase a standard slide humidity incubation box. Both ends of the channel should always be filled with liquid to prevent drying out and air bubble formation within the channels.

13. Prepare ibidi  $\mu$ -slides.

- a. Coat ibidi  $\mu$ -slides by pipetting 50  $\mu$ L fibronectin coating solution (75  $\mu$ g/mL in  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  free PBS, pH 7–9) in each channel of the slide in a slanting position.

**Note:** The concentration of coating solutions are calculated according to the required amount of protein per area (1.5  $\mu\text{g}/\text{cm}^2$  fibronectin recommended by ibidi manufacturer) and the maximum volume per channel (30  $\mu\text{L}$  for  $\mu$ -slide VI flat). For coating a different type of ibidi slide, re-calculate the concentration required according to the surface area and total volume specific to that slide.

**Note:** Fibronectin coating solution should be prepared in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free PBS, pH 7–9. Insoluble metal hydroxide may form at pH > 8.0.

- b. Incubate the slides at room temperature for 1 h.
- c. Wash twice by pipetting 200  $\mu\text{L}$  PBS into one end of the channel in a slanting position and simultaneously dabbing off the overflow as described in the beginning.

**△ CRITICAL:** It is important to thoroughly rinse off unbound fibronectin. Any remaining unbound protein could prohibit cells binding to the surface.

- d. Coated slides can be stored at 4°C in a sealed humidity container. Use them as soon as possible.

14. Fix cells on the slides.

- a. Seeding cells.
  - i. For cells cultured in the presence of inhibitors, transfer the entire 200  $\mu\text{L}$  of washed cells into a 1.5 mL or 500  $\mu\text{L}$  microcentrifuge tube.
  - ii. For freshly sorted cells, transfer 500–1,000 freshly sorted/treated cells into a 1.5 mL or 500  $\mu\text{L}$  microcentrifuge tube.
  - iii. Spin down the cells at 500 g for 5 min at 4°C.
  - iv. Carefully aspirate most of the supernatant without disturbing the pellet, leaving only a small volume (30–50  $\mu\text{L}$ ) at the bottom. Mix to resuspend.

**△ CRITICAL:** To avoid losing cells, use higher speed at 500 g instead of 300 g to spin down cells for better pelleting.

**△ CRITICAL:** To avoid losing cells when aspirating the supernatant, keep the tip slightly above the surface of supernatant when the volume becomes low. Each channel of the  $\mu$ -slide holds only 30  $\mu\text{L}$ . Cells resuspended in a volume larger than that will not be all seeded in the slide channel.

- v. Pipette 30  $\mu\text{L}$  of cell suspension into the channel of the slide.
- b. Cytospin.
  - i. Carefully insert the slide into a Cytospin clip without filter paper, facing up. Gently fasten the clip.
  - ii. Place the set in the spinner recess, balanced, tilted forward and facing down.
  - iii. Spin at 200 rpm for 2 min at room temperature. Use medium acceleration.
  - iv. Carefully remove the slides from the binder without stirring up the cells.
- c. Incubate the slides for 30 min at 37°C with 5%  $\text{CO}_2$  in a sealed humidity container.
- d. Fixing cells.
  - i. During the incubation time, prepare fresh 4% PFA from commercially available 16% PFA stock solution.
  - ii. At end of the incubation, rinse off the medium with 200  $\mu\text{L}$  PBS as described in the beginning.
  - iii. In a chemical fume hood, pipette at least 50  $\mu\text{L}$  of freshly prepared 4% PFA in a slanting position to replace PBS in the channel of the slide.
  - iv. Briefly dab off the overflow onto a tissue. Refill the channel with 4% PFA.
  - v. Incubate for 15 min at  $\sim 23^\circ\text{C}$ .

- vi. Wash three times for 5 min each with 200  $\mu$ L PBS.
- e. Check under the microscope if enough cells (> 40 per channel) are present on the slide.

**Note:** Follow Troubleshooting [Problem 7](#). Few cells to reduce cell lost.

- f. Slides can be stored for a short duration at 4°C in a sealed humidity container.
- 15. Perform immunofluorescence staining of the cells.
  - a. Pipette 50  $\mu$ L permeabilization buffer (0.25% Triton-X 100) into the channel and replace PBS as described in the beginning.
  - b. Fill the channel with another 50  $\mu$ L permeabilization buffer.
  - c. Incubate the slide in a sealed humidity container for 15 min at  $\sim$  23°C.
  - d. Wash three times for 5 min each with 200  $\mu$ L PBS.
  - e. Pipette 50  $\mu$ L blocking buffer (3% BSA/PBST) into the channel and replace PBS as described previously.
  - f. Fill the channel with another 50  $\mu$ L blocking buffer.
  - g. Incubate the slide in a sealed humidity container for 1 h at  $\sim$  23°C (or 12–18 h at 4°C).
  - h. Wash three times for 5 min each with 200  $\mu$ L PBST.
  - i. During incubation or washing, dilute the following primary antibody cocktails in 1% BSA/PBST:
    - Cocktail #1:
      - rabbit anti-TOM20 (1:150 for murine BM samples).
      - mouse anti-LAMP1 (1:150 for murine BM samples).
    - Cocktail #2:
      - mouse anti-LAMP1 (1:150 for murine BM samples).
      - or, mouse anti-LC3 (1:150 for murine BM samples).
  - j. Pipette 50  $\mu$ L of the desired primary antibody cocktail into the channel and replace PBST as described previously.
  - k. Fill the channel with another 50  $\mu$ L antibody cocktail.
  - l. Incubate the slide in a sealed humidity container for 12–18 h at 4°C.
  - m. Wash three times for 5 min each with 200  $\mu$ L PBST.
  - n. During incubation or washing, dilute the following secondary antibody cocktail in 1% BSA/PBST:
    - Goat anti-rabbit IgG: Alexa Fluor 594 (1:1,000).
    - Goat anti-mouse IgG: Alexa Fluor 488 (1:1,000).
  - o. Pipette 50  $\mu$ L of the secondary antibody cocktail into the channel and replace PBST as described previously.
  - p. Fill the channel with another 50  $\mu$ L secondary antibody cocktail.
  - q. Incubate the slide in a sealed humidity container for 1 h at  $\sim$  23°C in the dark.
  - r. Wash three times for 5 min each with 200  $\mu$ L PBST in the dark.

**Note:** If there are any bubbles trapped within the channel, flush it out with extra PBST. Avoid blowing bubbles into the channel during the final pipetting.

- s. Replace PBST with sufficient mounting medium with DAPI as described previously. Aspirate off any bubbles at the opening of the channel. Fill the channel and its openings with mounting medium, avoiding any bubble.
- t. Place a piece of a coverslip at one edge of the slide, and push toward the other end without drawing in bubbles. Briefly dab off extra liquid.
- u. Seal the coverslip with a transparent nail polisher. Allow to dry at  $\sim$  23°C. Keep slides at 4°C. Take images as soon as possible.

**Note:** Bubble may form during sealing or slides are stored for a long period. Follow Troubleshooting [Problem 8](#). Bubbles.

## Confocal laser scanning microscopy imaging

© Timing: 20–30 h

Immunofluorescence confocal microscopy images in this protocol are acquired using the ZEISS ZEN 2 Black software (2.3 SP1 version 14.0.21.201) on a Zeiss LSM880 (Carl Zeiss Microscopy GmbH, Jena, Germany) inverted confocal microscope equipped with a 32-channel Airyscan GaAsP-PMT detector. Imaging is performed using a 100×/1.46 alpha-Plan-Apochromat lens (Carl Zeiss Microscopy GmbH, Jena, Germany). Images are exported and processed using ZEN Blue software.

The following procedure uses microscopy settings that are used for acquiring the image shown in [Figure 6](#).

16. Mount the slide on the microscope.
  - a. Open Zen (black edition) software on the computer and start the system start.
  - b. Apply immersion oil to both the 40× and 100× oil immersion objectives.
 

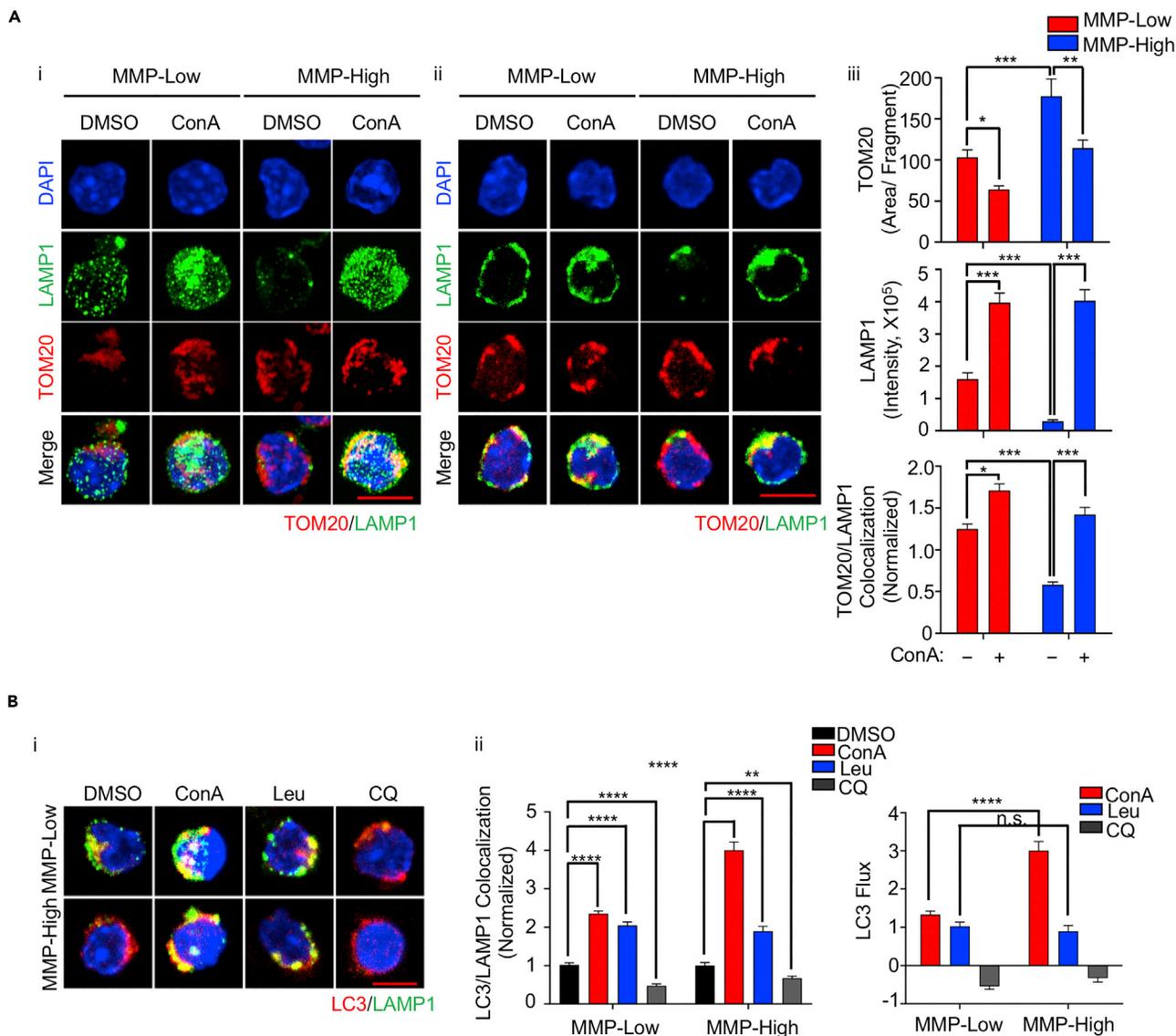
△ **CRITICAL:** Use only manufacturer recommended oil for slides (e.g., Zeiss Immersol 518F for ibidi  $\mu$ -slides), otherwise the plastic bottom of the ibidi slides may be damaged.
  - c. Insert the channel  $\mu$ -slide on the microscope stage facing up.
 

△ **CRITICAL:** While regular microscopy slides should be positioned upside down on an inverted microscope, ibidi  $\mu$ -slides should face up. The bottom of an ibidi  $\mu$ -slide where cells attach, is a No. 1.5 (180  $\mu$ m) polymer coverslip equivalent to No 1.5 glass coverslips. They should face toward the objective.
17. Locate the cell.
  - a. In Zen workspace on the computer, click “Locate” tab in the left tool area. Turn on the DAPI illumination and detection light path.
  - b. Locate the cells by using the 20× objective.
  - c. Switch to the 40× oil immersion objective (N.A. 1.4). Center one of the cells in the field and then switch to the 100× oil immersion objective (N.A. 1.46). Focus using the fine adjustment knob.
18. Setup experiment.
  - a. In Zen workspace, click “Acquisition” tab. Then click on “Smart Setup”.
  - b. Click “+” in the dye drop down menu and add fluorescent labels: DAPI, A488, A594 (PE Texas Red) in here.
  - c. Click on “Best Signal” imaging option and press the “Apply” button.
 

**Note:** The “Best Signal” imaging option (Frame Sequential acquisition) assigns each channel to a separate track and scanned sequentially. It takes longer time.

**Alternatives:** Place DAPI under the same track of any of the other two channels to increase imaging speed.
19. Open “Laser” window from the “Setup Manager” tool group. Turn on the excitation lasers: Diode laser 405 nm (DAPI), HeNe laser 594 nm. Argon laser 488 nm will turn on automatically.
20. Open “Acquisition Mode” window from “Acquisition Parameter” tool group. Set the following parameters:

Scan Mode: Frame.



Frame Size: 1024 × 1024.

Scan Speed: 8.

Scan step: 1.

Averaging Number: 2.

Averaging Mode: Frame.

Averaging Mode: Mean or Medium.

Bit Depth: 12.

Direction: Unidirectional.

Scan Area: Zoom 4.

**Note:** Larger frame size and slower scanning speed increase resolution, but takes longer time and increases photobleaching. Averaging helps increase signal-to noise ratio and smoothen the image. Averaging line by line smoothen the image better than frame by frame but increases photobleaching. A higher scan speed with averaging gives the best signal-to noise ratio. Bit depth can be chosen from 8, 12, or 16 bit, corresponding to 256, 4,096, or 85,536 gray levels. 12 bit above is used in the work presented in this protocol. However, ImageJ software can only handle 8, 16, 32 bit. Images taken at 12 bit will be converted to 8 bit by ImageJ during processing. Zooming is adjusted so that an individual cell fits most of the space of one frame. The default scan direction is unidirectional. Bi-directional scanning increases speed but requires precise calibration of the scanner.

21. Set gain/laser power value for each channel.
  - a. Channel selection:
    - i. Open “Channels” window.
    - ii. In “Tracks” list, select the checkbox of the channel to be set and deselect the rest of the options.
    - iii. Begin with the channel of the longest wavelength (A594 here).
    - iv. Proceed settings with one channel at a time.
  - b. Click on “Live”. Start live preview scanning of the cell in focus.
  - c. In the lasers field under the selected track, move the slider to adjust laser power, start with 2%.
  - d. In the channel field, move the slider of “Gain” (Master) to adjust detector sensitivity (between 600–800).
  - e. Monitor the pixel intensity of the image in the center screen area.
    - i. In the “Dimensions” tab in view options area at the bottom, click on “Range Indicator”.
    - ii. A gray scale image appears in the “Image Area”.
    - iii. Red pixels indicate saturated intensity.
    - iv. In the “Display” tab in view options area, monitor the histogram of pixel intensity.
  - f. Adjust the values of Gain and Laser Power based on following principles:
    - i. There is no red pixel in the range indicating image.
    - ii. The signal of pixels ranges to 2/3 of the scale in the histogram.
    - iii. If the image is still very dim at a gain above 800, slightly increase the laser power.
    - iv. If the image is already saturated at a gain below 600, decrease laser power.
- Note:** For far reds, it is not unlikely to set laser powers to 20% or 30% if signals are very dim, as the detectors have very low efficiency at those wavelengths.
- g. Click “Stop” when satisfied. Record the gain / laser power values.
- h. Test a few cells across experiment groups. Set the optimal gain / laser power according to the specimen with the highest signal of a particular channel.
  - i. Switch to the next channel and repeat the above steps.
22. Adjust pinhole size.
  - a. Select the channel of the longest wavelength.
  - b. In “Pinhole” field, click on “1AU” (one Airy Unit).
  - c. A value is automatically calculated and shown in the gray box. Record this value.
  - d. Input the recorded pinhole value in the gray box of all the remaining channels.

△ **CRITICAL:** It is especially important for colocalization analysis to keep the same pinhole size through all channels.

23. Once all the parameters are set, click “Snap” to acquire single plane 2D images.
24. Adjust image display settings on site with ZEN (black edition) or off site with ZEN (blue edition) software.
  - a. In the bottom view options area, activate “Single Channel” checkbox on “Dimensions” tab.
  - b. Click on the icon of the channel to be adjusted.
  - c. A single channel image shows in the middle view area.
  - d. On the “Display” tab, click the icon of the channel to be adjusted.
  - e. A histogram of intensity versus pixel appears.
  - f. Move the Black point and White point control (lowest 0, highest 4,096 for 12-bit images) beneath the histogram to adjust the contrast and brightness.
  - g. Test multiple images to optimize the settings.
  - h. Once the optimal black/white levels are determined, click the setting icon above the histogram. Then click “Copy” in the pull-down menu.

**Note:** If the icon is not visible, select the checkbox of “show all” on the top of the tab.

- i. For all images acquired, click “Paste” from the setting menu to apply the same display settings to all images.

**Alternatives:** Manually input the value of black and white threshold in the box underneath the histogram of each image.

**Note:** The display setting will be transferred to the images acquired if “Apply Display Settings and Channel Color” is chosen during image exportation (step 14). But this will not change the original raw data.

- j. Save the images to .czi files as raw data.
25. Export images to .tif files.
  - a. Open Zen (Blue edition) software.
  - b. In “Processing” tab in the left tool area of the workspace, press “Batch” button.
  - c. In the “Batch Method” pull down menu, choose “Image Export”.
  - d. Proceed to the “Batch Processing” area in the middle of the workspace.
  - e. Click “+ Add” and upload the .czi files to be exported.
  - f. Click and highlight any one of the files.
  - g. Go back to the left tool area, open “Parameters” window and set the following parameters:
    - i. File Type: Tagged Image.
    - ii. File: (.tiff).
    - iii. Convert to 8-bit: Deselect.
    - iv. Compression: None.
    - v. Resize: 100%.
    - vi. Original Data: Deselect except required.
    - vii. Apply Display Settings and Channel Color: Select.
    - viii. Choose Merged Channels Image and/or Individual Channel Image.
    - ix. Use Full Set of Dimensions: Select.
  - h. Go back to “Batch Processing” area. Click “Copy Parameters”. Select all image files and click “Paste Parameters”. Click “Run Selected”.
  - i. Raw images are now exported as .tiff files.

### Microscopy imaging in super-resolution (SR) mode

26. In “Acquisition” tab, assign DAPI (Channel 1), A488 (Channel 2), A594 (Channel 3) to separate tracks and turn on excitation lasers as in Confocal Laser Scanning Microscopy Imaging steps 7 and 8 (Channel 1: Diode 405, Channel 2: Argon 488, Channel 3: HeNe 594).
27. Open “Imaging Setup” window and set the beam path:
  - a. Click on “Ch.A” checkbox in the beam path and activate the Airyscan detector.
  - b. Choose dual band path filters:  
BP 420–480 + BP 495–620 (Channel 1, 2, 3).
  - c. Replace mirror with a plate.
  - d. Choose Beam Splitters/dichroics:  
Main Visible Beam Splitter – MBS 488 (Channel 2)/594 (Channel 3).  
Main UV Beam Splitter – MBS 405 (Channel 1).
  - e. Duplicate and create a total of three tracks, one for each channel.

**Note:** The only difference between the tracks should be the active laser line. This setup can be used in line- or frame-scan mode.

28. Open “Channels” window, set the following parameters for each channel:

Airyscan Mode:

SR (Super Resolution).

Pinhole:

3.5AU (minimum 1.8AU).

Detection gain and laser power:

Refers to [confocal laser scanning microscopy imaging](#), step 10.

**Note:** Pinhole diameter value is automatically calculated depending on the mode of Airyscan, lens, and zoom values (128  $\mu\text{m}$  for the images in [Figure 6Ai](#)).

**Note:** Set excitation parameters that give the best signal vs. noise ration while utilizing 50% of the full dynamic signal range and avoid saturation. Keep detector gain below 850V to ensure linearity of the response (750V–850V is ideal for Airyscan). Laser power for Airyscan rarely exceed 1%.

29. Open “Acquisition Mode” window from “Acquisition Parameters” tool group and set the following parameters:

Objective:

Alpha Plan Aplanachromat 100 $\times$ /1.46 N.A.

Scan Mode:

Frame.

Frame Size:

Click on “Optimal” (Auto-calculated lateral pixel scaling: 0.034  $\times$  0.034  $\mu\text{m}$ /pixel as for the image in [Figure 6Ai](#)).

**Note:** The “Optimal” button automatically sets the proper number of pixels to achieve optimal acquisition for the given objective and zoom settings based on Nyquist criteria. Make sure SR mode is activated in the Channel window prior to frame size adjustment. SR mode requires larger pixel number (smaller pixel size) to achieve high resolution.

Scan Speed:

Click on “Max” (Auto-calculated pixel dwell time: 2.12  $\mu\text{sec}$ /pixel as for the image in [Figure 6Ai](#)).

**Note:** The “Max” button sets the maximum speed for the given zoom.

Zoom:

Minimum 4.5×–6× (as for the image in [Figure 6Ai](#)) depending on sample size (at least 1.8×).

Averaging:

1.

Bit Depth:

16.

30. Acquire z-stack series of images.
  - a. Click on “z-stack” checkbox in the multidimensional input field underneath the action buttons.
  - b. This activates “z-stack” tool bars in “multidimensional acquisition” tool group.
  - c. Open “z-stack” window from “Multidimensional Acquisition” tool group.
  - d. Set up z-stack range using First/Last mode.
    - i. Press the “First/Last mode” button.
    - ii. Click “Continuous” to keep scanning the specimen.
    - iii. Turn focus control knob towards yourself (lens moving upward) until reaching the first plane in focus, top of the specimen.
    - iv. Click “Set First”.
    - v. Then turning focus control in the opposite direction (lens moving downward) until reaching the last plane in focus, bottom of the specimen.
    - vi. Click “Set Last”.
    - vii. Once set, click “Stop”.

**Alternatives:** One can set the bottom of specimen as “first” and top of the specimen as “last”.

**Note:** The blue frame in the z-stack Navigation field indicates the current focus position. The microscopy always moves up during acquisition regardless which plane is assigned as “first” or “last”.

- e. With SR mode activated, press “Optimal” button to determine z-Step interval.

**Note:** z-step size is set as half the axial resolution to ensure Nyquist sampling for high resolution in SR mode. The auto-calculated interval for the images in [Figure 6Ai](#) is 0.145 μm/step.

- f. Press “Start Experiment” button in multidimensional input field, and acquire 3D image.
31. Process z-stack images.
    - a. In “Processing” tab, choose “Airyscan Processing” in the pull down “Method” list.
    - b. In “Method Parameters” window, press “Select” button. The highlighted image series in the Central View area is then loaded in “Input of Preview” window.
    - c. Click on “Auto” checkbox in “Processing Strength” area.

**Note:** A Wiener Filter value is set at 5–7 by default.

- d. Select “3D”. Press “Run Batch” button and select the files.
- e. Click “Apply” button at the top of “Processing” tab.
- f. All images in the z-stack series are processed.

### Step four: Image analysis

⌚ Timing: 20–30 h

For the images presented in this protocol, mitochondria is marked by TOM20 (Alexa Fluor 594, red channel). Lysosome is marked by LAMP1 (Alexa Fluor 594, green channel). Autophagosome is marked by LC3 (Alexa Fluor 594, red channel). Nucleus is stained by DAPI (blue channel). All image analysis are based on 2D images.

Confocal microscopy images were acquired, processed and exported using ZEISS' ZEN 2 Black software (2.3 SP1 version 14.0.21.201) and ZEN 2.6 (Blue edition) software. Image analysis is performed using Image J 2.1.0/1.53c software (Schindelin et al., 2012) downloaded at <https://imagej.nih.gov/ij/download.html>.

32. Measure fluorescence intensity of labeled lysosomes.

- a. In ImageJ, open .tif file of a merged channel image.
- b. Use a selection tool to select the cell (e.g., 160 × 160 pixel square).
- c. Go to Edit > Copy to System to save the selection.
- d. Go to Analyze > Set Scale. Click "remove scale" to revert to pixel measurement.
- e. Go to Analyze > Set Measurements. Select Integrated Density (IntDen).
- f. Go to Image > Color > Split Channels.

**Note:** The color image will be split into three gray scale images of each channel (red, green, blue). After split, the images are converted to 8 bit.

- g. Activate the gray image of green channel (LAMP1-Alexa Fluor 488; lysosome). Close red (LC3-Alexa Fluor 594) and blue (DAPI) channel images.
- h. Go to Analyze > Measure. In the popup window, the result box shows the values of IntDen, and RawIntDen.

**Note:** RawIntDen: The sum of gray values (intensity) of all the pixels in selection. IntDen: RawIntDen converted from pixel measurement to calibrated units e.g., cm, inch. When scale is removed, the gray value under IntDen and RawIntDen are the same.

- i. Open the next image file.
- j. Go to Edit > Selection > Restore Selection. The same selection frame as the previous image appears.
- k. Move the frame to select the cell.
- l. Repeat steps f-k and analyze 30–50 cell images per experiment group.
- m. Record all values under RawIntDen as the fluorescence intensity of LAMP1 (Figure 6Aiii, middle).

33. Measure average fragment area of mitochondria.

- a. In ImageJ, open .tif file of a merged channel image.
- b. Use a selection tool to select the cell (e.g., 160 × 160 pixel square).
- c. Go to Edit > Copy to System to save the selection.
- d. Go to Analyze > Set Scale, click "remove scale" to revert to pixel measurement.
- e. Go to Analyze > Set Measurements. Select Area.
- f. Go to Image > Color > Split Channel.
- g. Activate the gray image of red channel (TOM20- Alexa Fluor 594; mitochondria). Close green and blue channel images.
- h. Go to Image > Adjust > Threshold. A "Threshold" window pops out.
- i. From the dropdown menu, select "Default" as the black and white threshold method.

**Alternatives:** Choose from threshold methods other than "Default".

- j. Click Auto button to set threshold automatically using the selected method.

**Alternatives:** To manually set threshold by sliding bars: Adjust the top and bottom slide bars for black and white threshold.

**Alternatives:** To manually set threshold by inputting numbers: Click Set button and type in numbers in the pop-out “Set Threshold Levels” window.

- k. Once the threshold is set, click Apply button. The threshold will be signed to a scale of 0–255 (for 8 bit images). Close the window.
- l. Go to Process > binary > Convert to Mask.
- m. Go to Analyze > Analyze Particles.
- n. Set parameters in the pop out window:
  - Unit size:
    - n to infinity (In this protocol, n is set to 8).
  - Circularity:
    - 0–1.
  - Show:
    - Choose “outlines” from the drop down menu.
  - Click check:
    - Summarize.

**Note:** Different values of unit size should be tested to determine the best n by examining the resulted outline image. N set too small will include noise particles. N set too large will result in lower resolution and lost smaller fragments.

- o. Click OK button. A “Summary” window pops out, showing the number of fragments, total area of all fragments, and the average area of fragments.
- p. Record the average fragment area (Figure 6Aiii, top).
- q. Open the next image.
- r. Go to Edit > Selection > Restore Selection. The same selection frame as the previous image appears.
- s. Move the frame to select the cell.
- t. Repeat steps f-s and analyze 30–50 cell images per experiment group.

#### 34. Analyze colocalization of mitochondria with lysosomes.

Colocalization of mitochondria or autophagosome with lysosome is determined by the fraction of TOM20 or LC3 (both Alexa Fluor 594, red channel) accumulated in LAMP1 (Alexa Fluor 488, green channel) positive areas among total cellular TOM20 or LC3.

Colocalization is measured by Mander’s overlap coefficient value M1 or M2, ranging between 0 and 1 (Bolte and Cordelieres, 2006). 0 indicates no colocalization and 1 indicates 100% colocalization between the two color channels. M is defined as the ratio of total signal intensity in one channel from pixels that also show signals above the threshold of another color channel.

- a. Download JACoP v2.0 plugin (NIH ImageJ) for Mander’s overlap coefficient from <https://imagej.nih.gov/ij/plugins/track/jacop2.html>.
- b. Open ImageJ, File > Show Folder > Plugins. Plugin folder window opens. Copy-paste downloaded JACoP (.jar) in Plugin folder.
- c. In ImageJ, open merged channel images.
- d. Use a selection tool to select the cell (e.g., 160 × 160 pixel square).
- e. Go to Edit > Copy to System to save the selection.
- f. Go to Analyze > Set Scale, click “remove scale” to revert to pixel measurement.
- g. Go to Image > Color > Split Channels.
- h. Color the gray images according to their channel color.
  - i. Go to Image > Type, choose RGB color.
  - ii. Image > Color > Make composite.
  - iii. Image > Color > Channel tools > Color.

- iv. Choose the corresponding color of the channel.
- i. Open both green and red individual color images.
- j. Go to Plugins > JACoP.
- k. Choose an image file from drop-down list (red or green channel) for Image A or B (Figures 6Aii and 6Bi).
  - Image A: red channel; TOM20.
  - Image B: green channel; LAMP1.
- l. Under Analysis to perform, click check M1 & M2 coefficients (Manders' Coefficients).
- m. Click Threshold tab at the bottom. Threshold will be automatically set for each channel.
- n. Click Analyze button. A "Log" window will pop out showing Manders' Coefficients M1 and M2 either using threshold values or without (original):
  - M1: fraction of image A overlapping with image B.  
(fraction of TOM20 or LC3 overlapping with LAMP1 among total TOM20 or LC3).
  - M2: image B overlapping with image A.  
(fraction of LAMP1 overlapping with TOM20 or LC3 among total LAMP1).
- o. Open the next image.
- p. Go to Edit > Selection > Restore Selection. The same selection frame as the previous image appears.
- q. Move the frame to select the cell.
- r. Repeat steps g–n and analyze 30–50 cell images per condition per experiment.
- s. Record all M1 values (using threshold). The values indicate the ratio of TOM20 or LC3 that colocalizes with LAMP1 versus total TOM20 or LC3.
- t. Colocalization is presented as M1 value of each cell normalized to the average of all cells across experiment groups (Figures 6Aiii, bottom and 6Bii, left).

35. Analyze LC3 flux based on confocal images.

LC3 flux is evaluated as the increased autophagosome (LC3) that colocalizes with lysosome (LAMP1) in inhibitor-treated (ConA, Leu) relative to non-treated (DMSO) cells (Mizushima et al., 2010; Kania et al., 2017). Since it is challenging to measure LC3 flux of rare HSCs using traditional methods that demand a large number of cells, we calculate LC3 flux based on confocal images in this protocol.

- a. Open a merged channel image.
- b. Use a selection tool to select the cell (e.g., 160 × 160 pixel square).
- c. Go to Edit > Copy to System to save the selection.
- d. Go to Analyze > Set Scale. Click "remove scale" to revert to pixel measurement.
- e. Go to Analyze > Set Measurements. Select Integrated Density.
- f. Go to Image > Color > Split Channels.
- g. Activate the gray image of green channel (LAMP1- Alexa Fluor 488). Keep the red channel image (LC3-Alexa Fluor 594) open. Close the blue channel image.
- h. Go to Image > Adjust > Threshold. Select Default threshold. Click Auto to set threshold.
- i. DO NOT click Apply. Close Threshold window.
- j. Go to Edit > Selection > Create Selection. Pixels within the threshold will be turned into the selection, shown as yellow.
- k. Go to Analyze > Tools > ROI Manager. Press Add. The selection is added to ROI Manager.
- l. Select the ROI and rename it as LAMP1.
- m. Activate the gray image of red channel (LC3- Alexa Fluor 594).
- n. Repeat step h-k.
- o. Select the newly added ROI and rename it as LC3.
- p. Select both ROIs of LAMP1 and LC3. Click More. Choose "And" for overlapping between LAMP1 and LC3. Click Add.
- q. A new ROI will appear. Rename it as "coloc" (colocalization).
- r. Select ROI "coloc" in ROI Manager. Click activate the red channel image (LC3). Click Measure in ROI Manager.

**Note:** Make sure that before clicking Measure, the correct channel to be measured (red channel LC3) is active.

- s. A Results window pops out. Record IntDen value. This is the fluorescence intensity of LC3 colocalized with LAMP1 (FI-coloc).
- t. Open the next image.
- u. Go to Edit > Selection > Restore Selection. The same selection frame as the previous image appears.
- v. Move the frame to select the cell.
- w. Repeat steps f-v and analyze 30–50 cell images per condition per experiment.
- x. Calculate the average FI-coloc of un-treated (DMSO) cells in each of MMP-Low or -High HSC group:
  - FI-coloc Ave DMSO.
- y. Normalize FI-coloc of each cell to the average FI-coloc of un-treated cells within the corresponding MMP-Low or -High group (Figure 6Bii, left).

$$\text{FI-coloc Norm} = \text{FI-coloc} / \text{FI-coloc Ave DMSO}$$

- z. Determine LC3 flux value by subtracting the average of normalized FI-colo value of un-treated cells from that of each treated (Leu/ConA) cells (Figure 6Bii, right).

$$\text{LC3 Flux} = \text{FI-coloc Norm}_\text{(Leu/ConA)} - \text{FI-coloc Ave\_Norm}_\text{(DMSO)}$$

$$= \text{FI-coloc Norm}_\text{(Leu/ConA)} - 1$$

## EXPECTED OUTCOMES

Figure 6A shows the immunofluorescent confocal images of lysosomes (LAMP1), mitochondria (TOM20) and the colocalization between the two in murine CD150<sup>+</sup> HSCs with or without ConA treatment (Liang et al., 2020) (i, Representative super-resolution images; ii, Representative single plane high resolution images; iii, The quantification of the average fragment area of TOM20 labeled mitochondria, LAMP1 fluorescence intensity and colocalization of TOM20 with LAMP1 based on single plane images).

Lysosomes are abundant in MMP-Low HSCs, as indicated by LAMP1 fluorescence intensity, but barely detected in MMP-High HSCs (Figure 6Aiii, middle). The average size of mitochondrial fragments is smaller in MMP-Low relative to MMP-High HSCs, indicating greater fragmentation (Figure 6Aiii, top). ConA treatment led to increased amount of lysosomes (LAMP1) and mitochondrial fragmentation in both MMP -Low and -High HSCs (Figure 6Aiii, middle and top).

The colocalization of TOM20 with LAMP1 is increased after ConA treatment in both MMP-Low and -High HSCs (Figure 6Aiii, bottom).

An autolysosome is formed from an autophagosome (marked by LC3) fusing with a lysosome, indicated by the colocalization of LC3 with LAMP1 (Liang et al., 2020). Figure 6Bi shows LC3-LAMP1 colocalization in murine CD150<sup>+</sup> HSCs treated with or without lysosomal modulator (ConA, Leupeptin, or Chloroquine). The treatment with ConA or Leupeptin, which each independently impairs lysosomal degradation capacity, resulted in significantly increased colocalization of LC3 with LAMP1 in both MMP-Low and -High HSCs. In contrast, Chloroquine, which alters autophagosome-lysosome fusion, blocked LC3-LAMP1 colocalization (Figure 6Bii, left). LC3 flux is significantly higher in MMP-High HSCs as compared to MMP-Low in response to ConA treatment (Figure 6Bii, right).

All images were captured at the Microscopy and Advanced Bioimaging CoRE in Icahn School of Medicine at Mount Sinai.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data quantification is presented as mean  $\pm$  SEM. Statistical significance is determined by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . At least 30–50 cells per condition per experiment are imaged and analyzed for quantification.

## LIMITATIONS

This protocol is limited to studies focusing on subsets of HSCs only. Due to HSCs' dye/drug efflux phenomenon, the staining bias of TMRE should be taken into account when comparing HSCs with other blood compartments, and between HSC samples with significantly different dye efflux properties [e.g., HSCs of different age (Kim et al., 1998; Qiu and Ghaffari, 2022)]. Verapamil, an inhibitor of MDR (Multidrug resistance) efflux pumps, can be included using a different TMRE staining strategy to eliminate such a bias (Morganti et al., 2019; de Almeida et al., 2017). Accordingly, results may be interpreted differently. In addition, potential effects of verapamil on autophagy flux (Mizushima et al., 2010; Kania et al., 2017) in HSCs should be tested for studies that also focus on lysosomes.

If samples are not maintained at low temperature, TMRE profiles might shift toward lower intensity during prolonged sorting. In that case, adjusting gates based on TMRE profile shift will be necessary throughout cell sorting. Manually adjusting the gates may be challenging in real-time if the cell number is limited and the percentage of cells within the final gate is low, resulting in possibly wider range of gated MMP fractions than what was originally set. This may not be a major concern in experiments in which the gating of the highest vs. lowest TMRE is relative rather than absolute. One could include verapamil (50  $\mu\text{g}/\text{mL}$ ) in post-staining cell suspension throughout washing and sorting to block HSC dye efflux activity, if cells cannot be maintained at low 4°C–10°C temperature.

Single plane confocal images do not provide a global view of cellular details in HSCs given their spherical morphology. In addition, variability can be introduced depending on the choice of focal plane, the distribution of organelles of interest and the positioning of each individual cell on the slide. To avoid inconsistency a large number of cells need to be imaged to determine subtle differences or to preclude bias. Although Z-stacks of multi-plane images provide a global view, acquiring large amount of 3D images for quantification purposes is time consuming and costly.

Another limitation comes from the paucity of purified HSCs and the availability of sample resources (especially human samples). The use of ibidi  $\mu$ -slides greatly increases the efficiency of locating small number of cells under microscopy. However, it remains challenging to concentrate small number of FACS-sorted cells to 30  $\mu\text{L}$  per channel with minimal loss.

## TROUBLESHOOTING

### Problem 1

Hemolysis (Related to step 6. Isolate PB MNCs).

Description:

MNC layer appears dark red with clumps and top plasma layer is red (step 6. k.).

MNC pellets are dark red and slimy (step 6. n.).

MNC suspension appears slushy and full of fine debris, which is difficult to be removed by centrifugation or filtering through cell strainer (step 6. o.).

Lower number of MNCs than expected (step 6. p.).

Cause:

Hemolysis of old or mechanically damaged RBCs.

MNCs are trapped in the aggregates of dead RBCs. Excessive broken RBC membranes make MNC pellet slimy. Cells are easy to be lost during the washing steps.

(Extensive hemolysis is expected if leukocyte reduction filters instead of whole blood are processed).

### Potential solution

To prevent:

Process blood the same day upon receipt. If same day process is not possible, keep blood at 4°C–10°C. Process as soon as possible the next day.

Solution:

Allow cell suspension to sediment for 1–2 min on the bench.

Collect the lighter upper suspension by decanting into new tubes.

Add 50 mL 2% FBS buffer to the remaining sediment.

Repeat above steps until upper suspension is mostly clear.

Discard the sediments.

Combine all collected upper suspension and centrifuge.

Debris in MNC suspension will be mostly removed.

### Problem 2

Poor Ficoll Separation (Related to step 6. Isolate PB MNCs).

Description:

Red but clean MNC layer without clumps.

Cause 1:

Overloading of blood, exceeding the maximal capacity of Ficoll separation (step 6. f.).

Cause 2:

The centrifuge temperature is set too low (step 6. j.).

### Potential solution

To prevent:

Dilute blood further before layering on Ficoll, increase centrifugation time, increase temperature to 18°C–20°C.

Solution:

Collect the entire MNC layer, combine and run a second round Ficoll separation.

Alternatively, lyse the residual RBCs in MNC pellet with RBC lysis buffer for 3 min in 37°C water bath. Wash with 50 mL 2% FBS buffer.

### Problem 3

Column blockage (Related to step 7. Isolate CD34<sup>+</sup> cells from PB MNCs).

Description:

Column blocked, slow flow, or no flow through (step 7. I.).

Cause 1:

Overloading of highly concentrated cells, exceeding the maximum column capacity ( $2 \times 10^9$  cells).

Cause 2:

Column blocked by clumps of dead RBC debris produced from hemolysis.

### Potential solution

To prevent:

Dilute cells sufficiently. Filter the cells before loading.

Solution:

Transfer the remaining cell suspension from column reservoir to a new tube.

Add MACS buffer to the column. Flush all the cells inside the column into the tube.

Remove the debris in MNCs as much as possible by sediment-decanting approach described in [Problem 1](#).

Before re-load onto new columns, dilute the cell suspension extensively until not slurry.

Keep stirring the cell suspension in column reservoir and gently pipet up and down without blowing bubbles.

(This will be time consuming and could result in low yield and low purity).

### Problem 4

Insufficient Lin/CD48 staining (Related to step 4. Sort MMP fractions of CD150<sup>+</sup> HSCs).

Description:

The percentage of Lin<sup>-</sup>CD48<sup>-</sup> cells in Lin<sup>-</sup> enriched BM abnormally high. (step 4. e.).

The percentage of Sca-1<sup>+</sup>/c-Kit<sup>+</sup> will be unusually low (below 1%) (step 4. e.).

Cause:

Poor lineage depletion and insufficient Lin cocktail staining or CD48 staining. The population is likely contaminated with Lin<sup>+</sup> or CD48<sup>+</sup> improperly stained.

### Potential solution

To prevent:

Make sure to add 50  $\mu$ L Lin cocktail for every 1 mL of  $1 \times 10^8$  RBC<sup>-</sup> total BM during Lin depletion.

Count cell numbers after Lin depletion.

Add 10  $\mu$ L anti-CD48 antibody per 1 mL of  $1 \times 10^7$  Lin<sup>-</sup> BM.

Solution:

Spin down the cells, count, and resuspend at  $1 \times 10^8$ /mL.

Add 50  $\mu$ L additional lineage cocktail per mL cells. Incubate for 15 min on ice.

Wash cells without going through the rest steps of lineage depletion.

Re-stain cells with proper amount of antibodies and SA-APCCy7.

### Problem 5

Unstable TMRE histogram (Related to step 4. Sort MMP fractions of CD150<sup>+</sup> HSCs).

Description:

TMRE histogram of CD150<sup>+</sup> HSCs keep fluctuating (step 4. g.).

Cause:

Fewer than 50 parental events (CD150<sup>+</sup> HSCs) are available to be displayed to give a reliable TMRE profile each screen refresh.

### Potential solution

To prevent:

Enrich HSCs by improving lineage depletion efficiency.

Run an enrichment sort for CD48<sup>-</sup>LSK using enrichment sort mode prior to sorting for MMP fractions of CD150<sup>+</sup> HSCs using purity sort mode.

### Problem 6

TMRE intensity dropping (Related to step 4. Sort MMP fractions of CD150<sup>+</sup> HSCs, and step 9. Sort MMP fractions of CD90<sup>+</sup> HSCs).

Description:

TMRE profile shifts in large scale towards lower signal intensity during sorting (step 4. h. step 9. f.).

Cause:

The sample chamber of FACS sorter does not cool efficiently. As temperature rises, TMRE might be pumped out as a result of HSC's dye efflux activity.

**Potential solution**

To prevent:

Keep samples ice-cooled during the entire sorting.

Set sorter sample chamber at 4°C. The chamber needs at least 30 min to cool down.

Avoid sorting for a long period of time which might warm up the samples.

Divide sample of big volume into multiple tubes. Keep the ones that are not sorted on ice.

If sorter sample chamber is not cool enough, prepare a bag of ice slur and rap around the FACS tube.

Consider adding verapamil to a final concentration of 50 µg/mL just before running FACS to inhibit ABC transporter mediated dye efflux of HSCs.

**Problem 7**

Few cells on slide (Related to step 15. [Immunofluorescence staining](#)).

Description:

Very few cells are found on the slide.

Cause 1:

Available number of cells to be seeded are low (< 1,000). Slide channel only fills up to 50 µL. After spinning down, reducing volume below 50 µL without losing cells is challenging.

Cause 2:

Insufficient coating of slides. Cells are not attached to the ibidi µ-slide well and could be easily flushed off during washing steps.

**Potential solution**

To prevent:

After spinning down cells, aspirate supernatant gently at a low vacuum strength.

Avoid touching the surface of supernatant.

Alternatively, remove the supernatant using a pipet.

Increase centrifugation time and speed. Avoid stirring up cell pellets when removing tubes from the centrifuge.

Increase the number of cells to begin with, which may require processing more blood or sacrificing more mice.

### Problem 8

Bubbles in channel (Related to step 15. [Immunofluorescence staining](#)).

Description:

Bubbles might form within the sealed  $\mu$ -slide channel after a few days (steps 15. t. r.).

Cause:

Channels are not completely sealed.

### Potential solution

To prevent:

Avoid using the first and the last channel since the coverslip may not completely cover both ends.

Add plenty of mounting medium.

Aspirate off any bubbles that appear.

Mount the coverslip in a gliding motion, pushing through from one end to the other end of the slide.

Dap off extra medium before sealing with nail polisher. Apply nail polisher a second round if necessary.

Finish imaging the slides as soon as possible. If slides are not completely sealed, bubbles may form over time when medium start to dry.

Solution:

If bubble forms after sealing:

Cut open the nail polish seal.

Remove the cover slip. Add more loading medium to the channels.

Aspirate off bubbles and re-seal the slide with a new cover slip.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Saghi Ghaffari ([Saghi.Ghaffari@mssm.edu](mailto:Saghi.Ghaffari@mssm.edu)).

### Materials availability

All reagents associated with this protocol are available from the [lead contact](#) upon request.

### Data and code availability

This study did not generate any unique datasets or code.

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### AUTHOR CONTRIBUTIONS

J.Q. and S.G. conceived the manuscript and J.Q. wrote the manuscript. V.M. made figures. N.T. contributed to the writing of the imaging section of the manuscript. R.L. provided information, suggestions, and comments. V.M. and J.Q. packaged the manuscript. J.Q., V.M., and S.G. reviewed and edited the manuscript.

### DECLARATION OF INTERESTS

Some elements of the original work published in Liang et al., *Cell Stem Cell*, 2020, have been filed in a patent application to the United States Patent and Trademark Office by S.G. and R.L.

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