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Flow Cytometry-Based Analysis of the Mouse Bone Marrow Stromal and Perivascular Compartment

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Abstract

Bone marrow stromal cells (BMSCs) account for an extremely small percentage of total bone marrow cells; therefore, it is technically challenging to harvest a good quantity of BMSCs with good viability using fluorescence-activated cell sorting (FACS). Here, we describe the methods to effectively isolate BMSCs for flow cytometry analyses and subsequent FACS. Use of transgenic reporter lines facilitates FACS-based isolation of BMSCs, aiding to uncover fundamental characteristics of these diverse cell populations.

Keywords

Bone marrow stromal cells (BMSCs); Skeletal stem cells (SSCs); Mesenchymal stem cells (MSCs); Fluorescence-activated cell sorting (FACS); In vivo lineage-tracing experiments; Single cell RNA-seq; C-X-C motif chemokine ligand 12 (CXCL12); Transgenic reporter mouse lines

1 Introduction

Bone marrow contains a diverse array of cells, including hematopoietic cells, vascular endothelial cells, and skeletal (mesenchymal) cells [1, 2]. Among them, skeletal cells account for an extremely small percentage of total bone marrow cells [3, 4]. Skeletal cells encompass bone-making osteoblasts, bone marrow stromal cells (BMSCs) and other skeletal progenitor and precursor cells. BMSCs are mainly located in contact with blood vessels [5], a small subset of which assumingly function as skeletal stem cells [6, 7]. A large majority of BMSCs express two important genes, C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor 1, SDF1) [8–10] and leptin receptor (LepR), a receptor for a fat-specific hormone leptin [11]. BMSCs can be marked by their reporter strains, including *Cxcl12-GFP* [12], *Cxcl12-DsRed* [13], *Cxcl12-creER*; *R26^{tdTomato}* [10], and *Lepr-cre*; *R26^{tdTomato}* [11] mice; as a result, these cells are also termed as CXCL12⁺ or CXCL12⁺LepR⁺ cells [14]. BMSCs can be isolated from these transgenic fluorescent reporter strains by flow cytometry and fluorescence-activated cell sorting (FACS) in a highly reproducible manner, without involving technique-sensitive steps of antibody staining. With this approach, BMSCs can be clearly identified by strong signals emanating from products of fluorescent reporter genes, the existence of which can be further confirmed by subsequent transcriptomic analyses. Moreover, if a cell type-specific tamoxifen-inducible *creER* line is

utilized, for example in *Cxcl12-creER; R26^{tdTomato}* mice, cell fates of a specific subset of BMSCs can be interrogated through in vivo lineage-tracing experiments.

Here as an example, we describe the methods to isolate BMSCs effectively from the femurs of *Cxcl12^{GFP/+}; Cxcl12-creER; R26^{tdTomato}* mice, which were treated with tamoxifen at postnatal day (P) 21 and dissected at P28 (Fig. 1). We further describe the methods to harvest BMSCs using FACS and subsequently apply these cells to a droplet-based single-cell RNA-sequencing analysis. We also describe the method to culture lineage-marked BMSCs ex vivo [10, 15] to circumvent the common problem associated with FACS-isolated BMSCs that do not survive well in cultured conditions [16].

2 Materials

2.1 Cell Preparation

1. Transgenic reporter mice (here, *Cxcl12^{GFP/+}; Cxcl12-creER; R26^{tdTomato}* mice, as an example).
2. Tamoxifen.
3. Sunflower seed oil.
4. Absolute ethanol.
5. Isoflurane.
6. Ice.
7. Scissors (sharp and dull).
8. Forceps (1 × 2 teeth).
9. Razor (single edge blade).
10. Paper wipe.
11. 6-well plate
12. LiberaseTM TM (collagenase, Sigma/Roche).
13. Molecular biology-grade water.
14. Pronase (neutral protease, Sigma/Roche).
15. Ca²⁺, Mg²⁺-free Hank's balanced salt solution (HBSS).
16. HBSS medium: HBSS supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) solution and 2% fetal bovine serum.
17. Mortar and pestle.
18. Luer-lock syringe 1 ml.
19. 18G Needle.
20. 70 µm cell strainer.
21. 50 ml conical tube.

22. DPBS–1% BSA: Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 1% bovine serum albumin (BSA).
23. DMEM medium: Low-glucose DMEM with GlutaMAX™ supplement (DMEM).
supplemented with 10% mesenchymal stem cell–qualified FBS and 1% penicillin–streptomycin.
24. Flow cytometry staining buffer (eBioscience).
25. Allophycocyanin (APC)-conjugated CD45 antibody (30F-11).
26. 5 ml polystyrene round-bottom tube with cell-strainer cap.
27. Shaking incubator.
28. Centrifuge.

2.2 Cell Sorting and Single-Cell RNA-Seq Analysis

1. BD FACS Aria III (Ex.407/488/561/640 nm).
2. RNase zap.
3. Dulbecco’s phosphate-buffered saline (DPBS).
4. Bovine serum albumin (BSA).
5. Protein LoBind 1.5 ml tube.
6. Trypan blue solution.
7. Centrifuge.
8. Automated Cell Counter.
9. Chromium Single Cell 3’ v3 microfluidics chip (10× Genomics Inc).
10. Sequencer (for example NovaSeq 6000 from Illumina).
11. R (ver 3.5) and Rstudio (ver 1.1).
12. Seurat R package [17].

2.3 Colony-Forming Assay, Subsequent Subcloning and In Vitro Trilineage Differentiation

1. Counting chambers (Neubauer-improved).
2. 6-well plate.
3. DMEM medium: DMEM supplemented with 10% mesenchymal stem cell-qualified FBS and 1% penicillin–streptomycin (mix #3, #4, and #5).
4. 70% ethanol.
5. 50% ethanol.
6. Methylene blue.

7. Cloning cylinders.
8. Dow high vacuum grease.
9. 0.5% trypsin–EDTA.
10. Non-tissue culture-coated V-bottom 96-well plate.
11. StemPro™ Chondrogenesis differentiation kit (Gibco).
12. Alcian Blue Staining Solution.
13. 48-well plate.
14. α MEM medium: α MEM with GlutaMAX™ supplemented with 10% FBS and 1% penicillin–streptomycin.
15. Osteogenic medium: α MEM medium with 1 μ M dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/ml ascorbic acid.
16. 4% paraformaldehyde solution.
17. Alizarin Red S.
18. IBMX (3-isobutyl-1-methylxanthine).
19. Adipogenic medium: α MEM medium with 1 μ M dexamethasone, 0.5 mM and 1 μ g/ml insulin.
20. LipidTOX Green (Invitrogen).
21. Oil Red O.
22. Vortex.
23. Centrifuge.
24. Automated inverted fluorescence microscope with a structured illumination system (for example the Zeiss Axio Observer Z1 with ApoTome.2 system).
25. Zeiss Zen 2 software (blue edition).

3 Methods

3.1 Cell Preparation

1. Label CXCL12⁺ BMSCs with green and red fluorescent proteins, by administering tamoxifen to *Cxcl12*^{GFP/+}; *Cxcl12-creER*; *R26R*^{tdTomato} mice: typically, a single dose of 1 mg tamoxifen is injected intraperitoneally (100 μ l of 10 mg/ml tamoxifen-oil mix) for mice at 3–6 weeks of age (see Note 1).

¹To make 10 mg/ml tamoxifen solution, dissolve 100 mg tamoxifen in 2.5 ml absolute ethanol in a 50 ml conical tube. Vortex persistently until crystals become completely dissolved. Add 10 ml sunflower seed oil and vortex persistently again. Open the cap and incubate at 65 °C overnight in a chemical fume hood until ethanol completely evaporates. Close the cap and keep the tamoxifen-oil mix at room temperature until use. A dose of 25–175 mg/kg body weight is typically approved by an IACUC protocol. Average body weights of male C57BL/6J are 10.6 $\pm$ 1.9 g at 3-week, 16.5 $\pm$ 2.6 g at 4-week, 21.9 $\pm$ 1.8 g at 6-week and 25.0 $\pm$ 1.4 g at 8-week, according to The Jackson Laboratory. Tamoxifen should be administered to mice under a certified biosafety cabinet, and the mice should be housed in a special containment space.

2. Sacrifice the mice after a short-chase (typically 2–7 days after tamoxifen injection) using carbon dioxide overdose or isoflurane overdose in a drop jar followed by induction of bilateral pneumothorax.
3. Make an incision in the skin of the leg by scissors and peel it off. Dissect the femur by excising the ligament at the femur head and at the knee joint using sharp scissors. Remove soft tissues carefully from dissected femurs using scissors and paper wipes.
4. Remove distal epiphyseal growth plates using dull scissors and cutting off proximal ends (*see* Note 2).
5. Open up the marrow cavity and expose bone marrow. To achieve this, cut the cortex of the femurs longitudinally, roughly by a razor for 1–2 times, into several pieces. Do not cut the femur into small pieces.
6. Transfer the roughly cut bone pieces into a 6-well plate containing 2 ml HBSS.
7. Digest the bone pieces with enzymes, by adding 2 Wunsch units of Liberase™ TM with or without 1 mg of Pronase to 2 ml HBSS in the 6-well plate (*see* Notes 3 and 4).
8. Incubate the solution at 37 °C, 300 rpm for 60 min on a shaking incubator (*see* Note 5).
9. Aspirate and mix the supernatant containing dissociated cells using an 18-gauge needle with a 1 ml Luer-Lok syringe, and filter it through a 70 µm cell strainer into a 50 ml tube on ice.
10. Add 2 ml fresh HBSS to the 6-well plate containing digested bone pieces. Aspirate and mix the supernatant rigorously using the needle with the syringe used above, and filter it through the 70 µm cell strainer into the 50 ml tube on ice.
11. Transfer the digested bone pieces to a mortar containing 2 ml fresh HBSS medium, then mechanically triturate the bone pieces using the 18-gauge needle with the 1 ml Luer-Lok syringe used above, as well as using a pestle, to release any remaining cells on bone pieces. Filter dissociated cells through the 70 µm cell strainer into the 50 ml tube on ice to prepare single cell suspension. Repeat this step for five times and collect the supernatant into the same tube.
12. Centrifuge, $280 \times g$, at 4 °C for 10 min, then discard supernatant by decanting (*see* Note 6).

².Epiphyses of femurs can be easily popped out using dull scissors.

³.Reconstitute and aliquot Liberase solution according to the manufacturer's protocol. Add 10 ml molecular biology-grade water to the amber bottle of Liberase™ TM (50 mg), and swirl the bottle well. Keep it on ice for 30 min, while inverting the bottle for every 5 min (do not use vortex). Aliquot 500 µl enzyme solution in a 1.5 ml tube, and keep it at –30 °C until use.

⁴.Adding Pronase in the digestion cocktail increases the yield of BMSCs, especially those located in the central marrow space, without compromising RNA integrity number. However, Pronase digestion degrades protease-sensitive antigens such as CD31 (encoded by *Pecam1*).

⁵.Length of digestion needs to be optimized for the age of mice. A 60 min digestion is sufficient for mice at 3–8 weeks of age.

⁶.For standard flow cytometry analysis, cells should be resuspended in a 500 µl flow cytometry staining buffer containing appropriately diluted antibodies (such as 1:500 CD45-APC), then incubate the cells at 4 °C for 30 min. Add 2 ml flow cytometry

13. Resuspend the pellet in 2 ml ice-cold DPBS–1% BSA (for single-cell RNA-seq) or 10 ml DMEM medium (for cell culture, skip to Subheading 3.3 below).
14. Filter cell suspension into a 5 ml polystyrene round-bottom tube with a cell-strainer cap, then keep it on ice until loading onto a cell sorter.

3.2 Cell Sorting and Single-Cell RNA-Seq Analysis of FACS-Isolated Cells

1. Set up a four-laser BD FACS Aria III (Ex.407/488/561/640 nm) high-speed cell sorter with a 100 μ m nozzle (*see* Note 7).
2. Rinse the nozzle with RNase zap then backflush.
3. Run no-fluorescence control, and GFP⁺ or tdTomato⁺ singlecolor controls to set up the gate and the compensation to sort the desired target cells. Single-color samples are separately collected from control mice carrying only single colors (*see* Note 8) (Fig. 2).
4. Load the sample onto a cell sorter and collect the target cells (sorting speed should be less than 10,000 events/s).
5. Sort GFP⁺ cells and/or tdTomato⁺ cells directly into 600 μ l ice-cold DPBS–1% BSA in a Protein LoBind 1.5 ml tube (*see* Note 9).
6. Pellet cells by centrifugation (300 $\times$ *g*, at 4 °C for 10 min) and resuspend the pellet in 10–30 μ l DPBS–1% BSA to achieve a concentration of 1000 cells/ μ l (*see* Note 10).
7. Mix 5 μ l sample (or cell count control) and 5 μ l trypan blue solution for cell counting.
8. Count total and live cell numbers by automated Cell Counter.
9. Load cells onto the Chromium Single Cell 3' v3 microfluidics according to the manufacturer's protocol.
10. Sequence cDNA libraries by Illumina NovaSeq 6000.
11. Preprocess the sequencing data using the 10 $\times$ Genomics software Cell Ranger.

staining buffer to wash. Centrifuge, 280 $\times$ *g*, at 4 °C for 10 min, then discard supernatant by decanting. Resuspend the cells in flow cytometry staining buffer (500–1000 μ l, then run on flow cytometer.

⁷RNA quality of sorted cells depends on the size of the nozzle. Wider nozzles, such as 100 μ m or 130 μ m nozzles, give much better results than narrower nozzles, such as 70 or 85 μ m nozzles. The sheath pressure is 20 psi using a 100 μ m nozzle, which is close to the atmospheric pressure (15 psi). A 130 μ m nozzle (10 psi) is ideal to prevent damage to the cells; however, the sorting speed becomes substantially slow. It may be difficult to harvest a sufficient number of cells within a limited amount of sorting time. A 70 μ m nozzle (70 psi) or an 85 μ m nozzle (45 psi) may inflict damage to the cells and compromise the RNA quality.

⁸Exclude FSC^{high}/SSC^{high} events as they contain duplets or multiplets of cells. We often experience that Cxcl12-GFP⁺ BMSCs are physically coupled with hematopoietic cells in a way indistinguishable from purely single cells.

⁹Sort more than 50,000 cells ideally. If less than 50,000 cells are harvested, a separate control tube should be set up solely for the purpose of cell counting, to estimate the cell number in an experimental tube. For the cell counting control tube, sort at least 10,000 GFP-low or tdTomato-low cells and dilute it at the same ratio as the experimental tube.

¹⁰Generally, 30% of the cells counted on FACS are lost due to centrifugation, between the sorting step and the actual cell counting step. As the ideal cell concentration for a 10 $\times$ microfluidics device is 1000 cells/ μ l, sorted cells should be diluted to approximately 1400 cells/ μ l.

12. Generate and use a custom genome fasta and index file by including the sequences of *eGFP* and *tdTomato-WPRE* to the mouse genome (mm10) for alignment purposes.
13. Perform further downstream analyses using the Seurat R package.
14. Filter out cells with less than 1000 genes per cell and with more than 15% mitochondrial read content.
15. Perform downstream analyses, including normalization, identification of highly variable genes across the single cells, scaling based on number of UMI, dimensionality reduction (PCA, CCA, and UMAP), unsupervised clustering, and the discovery of differentially expressed cell-type specific markers (Fig. 3).
16. Perform differential gene expression analyses to identify cell-type specific genes.

3.3 Colony-Forming Assay, Subcloning, and In Vitro Trilineage Differentiation

1. Vortex DMEM-suspended cells.
2. Count cells manually using a hemocytometer.
3. Plate cells into a 6-well plate at a density of 10^5 cells/cm² with DMEM medium for 10–14 days (*see* Note 11).
4. Locate tdTomato⁺ colonies in a 6-well plate: capture tdTomato epifluorescence from live cultured cells by tile-scanning the entire 6-well plates with an inverted fluorescence microscope equipped with a fully automated stage (Fig. 4). For subcloning, skip to **step 7**.
5. Aspirate the supernatant, then fix cells by adding 70% ethanol, for 5 min.
6. Discard the ethanol, then add 2% methylene blue–50% ethanol (methylene blue staining) (Fig. 4).
7. To subclone individual tdTomato⁺ colonies, isolate each tdTomato⁺ colony using cloning cylinders (*see* Note 12).
8. Add 50 μ l of 0.25% trypsin-EDTA into the cylinder, then incubate it at 37 °C for 5 min.
9. Add 50 μ l of a basal medium, and transfer the cell suspension to a new 6-well plate.
10. Culture a single cell-derived clone of tdTomato⁺ cells in a 6-well plate with a basal medium described above at 37 °C with 5% CO₂ with exchanges into fresh media every 3–4 days.

¹¹Cell cultures are maintained at 37 °C in a 5% CO₂ incubator.

¹²Print tile-scanned tdTomato⁺ colony images as the actual size of a 6-well plate. For this purpose, make 1.42 $\times$ 1.42-in. circles in 10 $\times$ 7.5-in. PowerPoint slide, and print it to a letter size paper. Then insert the printed images under the 6-well plate. After aspirating the medium, attach a cloning cylinder using grease to the position of the plate corresponding to the position of tdTomato⁺ colonies.

11. Passage each clone when the culture reaches subconfluency to confluency, transferring approximately 1/10 of an old 6-well plate to a new 6-well plate with a fresh basal medium.
12. Continue to passage clones to analyze self-renewability, or use passage 4–7 clones for trilineage differentiation assays (*see steps 13, 19, or 22*).
13. To induce chondrocyte differentiation, transfer cells (approximately 1/6 of a confluent 6-well plate) into a non-tissue culture-coated V-bottom 96-well plate.
14. Centrifuge at $150 \times g$ for 5 min at room temperature to pellet the cells, then aspirate the supernatant carefully.
15. Add StemPro Chondrogenesis medium, and centrifuge the plate at $150 \times g$ for 5 min at room temperature to pellet the cells.
16. Culture the pellet in the differentiation medium with exchanges into fresh media every 3–4 days for up to 3 weeks, each time with centrifugation at $150 \times g$ for 5 min at room temperature to repellet the cells.
17. Fix cell pellets with 70% ethanol for 5 min.
18. Stain for Alcian-Blue Staining Solution for 30 min.
19. To induce osteoblast differentiation, plate cells (approximately 1/6 of a confluent 6-well plate) on a 48-well plate and cultured with α MEM medium until confluency.
20. Change to the osteogenic medium with exchanges into fresh media every 3–4 days for up to 3 weeks.
21. Fix cells with 4% paraformaldehyde for 5 min and stain for 2% Alizarin Red S for 30 min.
22. To induce adipocyte differentiation, plate cells (approximately 1/6 of a confluent 6-well plate) on a 48-well plate and cultured with α MEM medium until confluency.
23. Change to the adipogenic medium with exchanges into fresh media every 3–4 days for up to 2 weeks.
24. Stain cells with LipidTOX Green (1:200 in basal medium) for 3 h at 37 °C, or fix cells with 4% paraformaldehyde for 5 min and stain for Oil Red O for 30 min.

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Fig. 1. Histological images of *Cxcl12*^{GFP/+}; *Cxcl12-creER*; *R26R*^{tdTomato} femurs. *Cxcl12*-GFP⁺ and/or *Cxcl12-creER*;tdTomato⁺ cells, which are located in contact with Endomucin (Emcn)⁺ blood vessels

Fig. 2.

Gating strategy for cell sorting of Cxcl12-GFP⁺Cxcl12-creER⁺ cells isolated from *Cxcl12*^{GFP/+}; *Cxcl12-creER*; *R26R*^{tdTomato} femurs. (Figures modified and adapted with permission from [10])

Fig. 3.

Single cell RNA-seq analysis of Cxcl12-GFP⁺ (including Cxcl12-creER;tdTomato⁺) BMSCs harvested from *Cxcl12*^{GFP/+}; *Cxcl12-creER*; *R26R*^{tdTomato} femur bone marrow at P28 (tamoxifen injected at P21). Left panel: UMAP-based visualization of major classes of FACS-sorted cells, center panel: feature plots of *tdTomato* expression, right center panels: violin plots, *tdTomato* expression in each cluster. (Figures modified and adapted with permission from [10])

Fig. 4.
tdTomato⁺ cells and total CFU-Fs