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Isolation of CD31$^+$ Bone Marrow Endothelial Cells (BMECs) from Mice

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[Abstract] In the bone marrow microenvironment, endothelial cells (ECs) play a pivotal role in regulating the production of both growth and inhibiting factors. They are held together by adherence molecules that interact with hematopoietic progenitor cells. The study of ECs in the hematopoietic stem cell niche is limited due to the lack of efficient protocols for isolation. In this protocol, we developed a two-step approach to extract bone marrow endothelial cells (BMECs) to unlock the challenges researchers face in understanding the function of the endothelial vascular niche in *in-vitro* studies.

Keywords: Bone marrow endothelial cells (BMECs), Endothelial cells (ECs), Extraction, Mouse bone marrow, Isolation, Endothelial-vascular niche

[Background] The bone marrow microenvironment (BMM) is the compilation of cells and vascular networks within the marrow that acts as a vital and dynamic support system to assist hematopoiesis. The cellular component of the bone marrow consists of hematopoietic and non-hematopoietic cells, such as endothelial cells (ECs), osteoblasts, osteoclasts, adipocytes, and fibroblasts (Mishra *et al.*, 2011; Birbrair and Frenette, 2016). The presence of ECs within the bone marrow environment regulates the trafficking and homing of the hematopoietic progenitor and stem cells. Alteration of the bone marrow endothelial cells (BMECs) due to malignant cells causes activation of endothelial-derived signaling pathways that favor the proliferation, differentiation, migration, and survival of neoplastic cells. It also stimulates angiogenesis, disrupting homeostasis within the BMM (Coşkun and Hirschi, 2015; Isern and Mendez-Ferrer, 2011; Passweg *et al.*, 2016). In this protocol, we describe the isolation of BMECs and characterize their morphology.

Materials and Reagents

1. 1.5 ml Eppendorf centrifuge tube (Eppendorf, catalog number: NA)
2. 0.5 ml Nest micro-centrifuge tube (Biosharp, Life Science, catalog number: 201214)
3. 18-gauge needle and syringe (Lujie®, 2015315175)
4. Sterile 15 ml and 50 ml conical tubes (Beijing Labgic Technology, catalog numbers: CT-002-15 and CT-002-50)

5. 100 mm cell culture dish (Thermo Fisher Scientific) and 12-48 well plates (Costar®, Corning, catalog number: 430161)
6. Micro-dissecting board and pins
7. Sterile gloves and gauze
8. 70 µm cell strainer (Biologix, catalog number: 15-1070)
9. Pipette calibrators P1000 (N253062), P200 (MZ26504), and P10 (N252362)
10. Magnetic separation column and MACS® Separator (MACS, Miltenyi Biotec)
11. Wild-type mice (C57BL/6)
    Male and female wild-type mice aged 8-12 weeks old were obtained from Jackson laboratory. Mice were housed under the regulation of Xuzhou Medical University, Jiangsu Province, China.
12. 70-80% ethanol
13. Milli-Q water (Quantum® Tix, catalog number: QTUMOTIX1)
14. Miltenyi Biotec CD 31 microbeads, MS column (miniMACS™ Separator, Miltenyi Biotec)
15. Fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific)
16. Bovine serum albumin (BSA) (Solarbio® Life Science)
17. Distilled water
18. Accutase solution (Absin, Biochemical Company/ Thermo Fisher Scientific)
19. Hydrochloride acid (HCl)
20. Potassium chloride (KCl)
21. Sodium chloride (NaCl)
22. Sodium phosphate Dibasic (Na_2HPO_4)
23. Potassium phosphate monobasic (KH_2PO_4)
24. 10,000 U/ml penicillin /streptomycin (Gibco, Thermo Fisher Scientific)
25. Rat-tail collagen type-I 3 mg/ml (Thermo Fisher Scientific, catalog number: A10483-01)
26. Human plasma fibronectin purified protein (EMD Millipore, catalog number: 3383980 or FC010)
27. PECAM-1 (CD31), rabbit-polyclonal (Affinity Bioscience, catalog number: AF6191)
28. VE-CADHERIN (CD144) rabbit-polyclonal (Affinity Bioscience, catalog number: AF6265)
29. Vascular endothelial growth factor receptor-2 (VEGFR2 (FIK-1) rabbit-polyclonal (Bioworld Technology, catalog number: Q1169)
30. Secondary antibodies: Goat Anti-rabbit (IgG) Alexa Fluor® 594 (green; Abcam, catalog number: Ab15008) and Alexa Fluor®488 (red; Abcam, catalog number: Ab15007)
31. Endothelial cells medium (EBM-2) (Lonza, Clonetics®)
32. 1× Dulbecco's phosphate buffer solution (DPBS) (see Recipes)
33. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) (see Recipes)
34. 0.5 M Disodium Ethylenediaminetetraacetic acid (EDTA)·$2H_2O$ (see Recipes)
35. 0.001 M Ethylenediaminetetraacetic acid (EDTA)/DBPS (see Recipes)
36. Sterile harvesting buffer solution (see Recipes)
37. Complete endothelial cell medium (see Recipes)
38. 4% Paraformaldehyde (PFA) solution (Sinopharm Chemical Reagent) (see Recipes)

39. 1% bovine serum albumin (BSA/DPBS) (see Recipes)
40. 0.5 M EDTA (see Recipes)
41. Dish pre-coating reagents (see Recipes)

Equipment

1. Surgical scissors/forceps (10-11 cm long stainless steel dissecting scissors and 10-11 cm long stainless steel dissecting straight forceps)
2. Cell culture incubator (CO_2 incubator) (Thermo Fisher Scientific, model: Heracell 150i, catalog number: 41629032)
3. Pipettes: P10, P200, and P1000 (Thermo Fisher Scientific, catalog numbers: 464000, 4640050, 4640060)
4. Cell counting chamber (hemocytometer)/automated cell counting machine (Mindray)
5. Refrigerator (2-8°C) (Haier, Bio-Medical)
6. -20°C freezer (Haier)
7. Laminar flow hook (Thermo Fisher Scientific, SN-194564, model -1381)
8. High-speed centrifuge (Beckman Coulter, model: Microfuge®20R, catalog number: MRZ14H028, and low-speed centrifuge AIRTECH-KDC-40 Anhui USTC Zonkia Scientific instrument co.Ltd, catalog number: 02241700049)
9. Nikon Eclipse microscope (Nikon, model: Eclipse Ti)
10. Sterilization machine (Hirayama, model: HICLAVE™ HVE-50, catalog number: 30613065826)
11. pH meter and automated weighing machine (Metter Toledo)
12. Magnetic stirrer (IKA® RCT Basic, 0317090005661)
13. Graduate cylinder

Software

1. Flow cytometry software
2. FlowJo software version 7.6.2

Procedure

Note: Sterilize all necessary equipment and reagents beforehand.

A. Preparation of adherent bone marrow cells

1. Euthanize mouse (aged 8-12 weeks, Figure 1-i) by cervical dislocation and soak the whole mouse in 70% ethanol for 2-5 min. Place the sterile microdissection board, forceps, and scissors in the laminar flow hook. Clip the mouse onto the dissecting board with the pins, which were disinfected with 70% ethanol. Peel the skin off the mouse from the top of the hind leg down to the foot. Separate the hind legs with scissors and gently cut off the foot delicately to maintain

the integrity of the bones. Place the hind legs in a 100 mm diameter dish containing 4-5 ml of the sterile room temperature DBPS for 2-3 min.

2. Detach the muscle from the bones with forceps and scissors, dissect the bones with sterile tooth forceps, and place them in a sterile 100 mm dish diameter containing 2-3 ml of the DPBS (Figure 1-ii). Clean the bones with separate sterile forceps in DPBS and transfer them into a sterile 100 mm diameter dish containing 3 ml of 0.002 M EDTA/DPBS/antibiotic solution. Cut off the distal end of the femora and tibias (Figure 1-iii).
3. Take the sterilized 0.5 ml nest microcentrifuge tube and push an 18-gauge needle down to the bottom of the tube to create a hole. Insert the cut edge of the femora and tibias downward, with a maximum of four bones per tube, and close the lid tightly (Figure 1-iv).
4. Place the closed 0.5 ml nest microcentrifuge tube into a 1.5 ml Eppendorf tube and seal with Parafilm disinfected with 70% ethanol. Centrifuge the 1.5 ml Eppendorf tube at 15,000 × *g* for 30 s at 37°C (Figure 1-v). Remove the Eppendorf tube and verify that the bones are white (Figure 1-vi); if this is not the case, repeat the centrifugation. Discard the 0.5 ml nest microcentrifuge tube, suspend the noticeable pellet at the bottom of the 1.5 ml Eppendorf tube gently with 100-200 µl of sterile room temperature DPBS/EDTA solution containing antibiotics, and transfer the cells into a new clean tube.
5. Gently pipet the suspended bone marrow cells with 3-4 ml of sterile room temperature DPBS/EDTA solution and filter the cells with a 70 µm cell strainer into a sterile 50 ml conical tube (Figure 1-vii). Transfer the purified bone marrow cells into a 10-15 ml clean tube (Figure 1-viii) and centrifuge at 300 × *g* for 5 min at room temperature (37°C) (Figure 1-ix). Discard the supernatant and resuspend the visible pellet with 2-3 ml of complete DMEM (see Recipes) (Figure 1-x). Count the total number of cells with an automated cell counter or hemocytometer.
6. Seed 50-100 million bone marrow cells into a sterile 100 mm diameter cell culture dish containing 10-12 ml of complete DMEM (see Recipes) and incubate in a 5% CO_2 humidified incubator for 8-12 h or overnight (Figure 1-xi).

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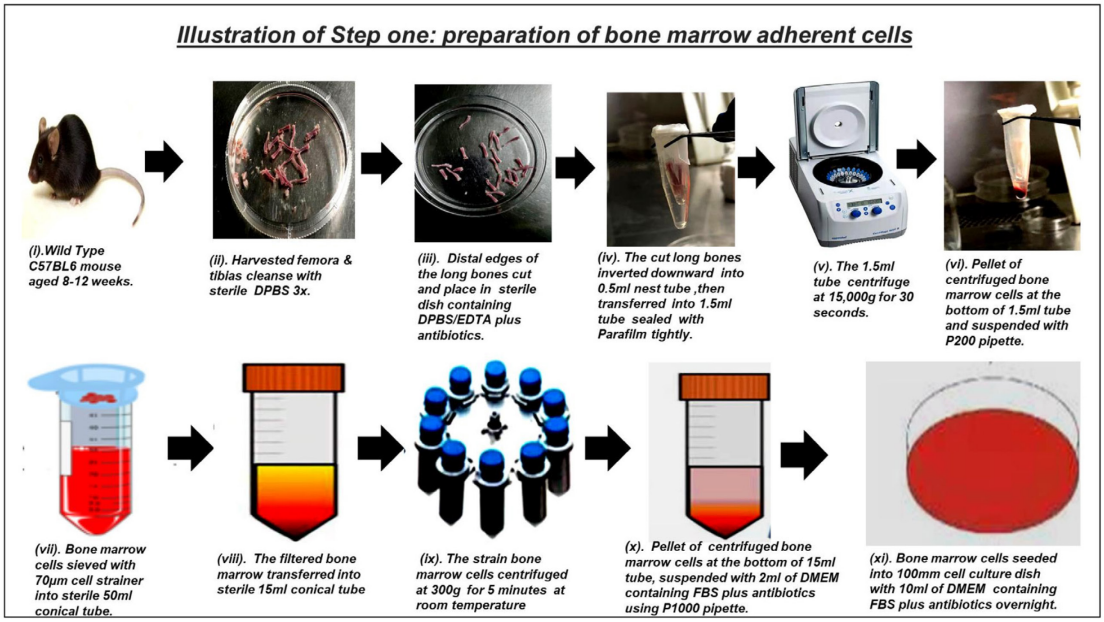

Figure 1. Illustration of Procedure A: preparation of bone marrow adherent cells.

B. Isolation of cultured adherent BMECs

1. Remove the 100 mm cell culture dish from the cell culture incubator after 8-12 h or overnight incubation. Patiently pipette the non-adherent cells from the cell culture dish with P1000 and discard them. Wash the cultured adherent bone marrow cells with 2-3 ml of sterile room temperature DPBS twice (Figure 2-i). Place 2-3 ml of accutase solution into a 100mm cell culture dish and incubate for 15 min at room temperature.
2. Observe the cultured adherent bone marrow cells under the light microscope. If the cells have detached from the dish, suspend them in 1-2 ml of the harvesting buffer solution. Transfer the cells into 10-15 ml tubes and add 2-3 ml of the harvesting buffer solution to resuspend the remaining cells from the cell culture dish (Figure 2-ii). Confirm that all cells have been detached by looking at the cell culture dish under the light microscope. Centrifuge the resuspended cells 300 × *g* for 10 min at room temperature (Figure 2-iii). Resuspended the pellet (Figure 2-iv) with 100-200 µl of harvesting buffer and determine the total number of cells by an automated cell counter or hemocytometer.
3. Aliquot approximately 10^7-10^9 cultured adherent marrow cells in 90 µl with 10 µl of CD31 microbeads into a 1.5 ml Eppendorf tube and mix gently with a P200 pipette. Then, incubate the mix at 4°C for 15 min in the dark (Figure 2-v).
4. After incubation, transfer the CD31 microbeads cells into 10-15 ml conical tubes and resuspend the cells with 10 ml of harvesting buffer solution (Figure 2-vi). Centrifuge at 300 × *g* for 10 min at room temperature to wash out the excess magnetic beads from the cells (Figure 2-vii). Resuspend the pellet with 500-1,000 µl of the harvesting buffer solution (Figure 2-viii).
5. Sanitize the magnetic (MACS™ Separator) and miniMACS™ Separator with 70% ethanol and place it in the laminar flow hook. Attach the MS column (miniMACS™ Separator) to the MACS™

Separator and rinse the column once with 500 µl of the harvesting buffer solution. Load the resuspended marrow cells into the column to allow single cells to pass through. Rinse the MS column three times with 500 µl of harvesting buffer solution, followed by a solitary wash with 100-200 µl of the complete endothelial cell medium (see Recipes) (Figure 2-ix).

Note: The flow-through of non-magnetic CD31 microbeads cells (CD31 negative cells) is comprised of other marrow adherent cells such as macrophages, mesenchymal cells, and endothelial progenitor cells, which can be stored for other experiments or discarded.

6. Push the magnetic beads cells into a new sterile tube with 1,000 µl of complete endothelial cell medium (see Recipes) (Figure 2-x). Determine the total number of BMECs by pipetting the cells into the well of the hemocytometer counting chamber or automated cell counter.

   Note: For the immediate experimental use of the isolated BMECs, we advise that the positive magnetic bead cells be resuspended with 1,000 µl of the harvesting buffer solution and repeat step 5. If the isolated cells are to remain in culture for several days after isolation, move to step 7. The minimal contaminated adherent cells with isolated BMECs will die off after 4-7 days in cell culture.

7. If the 12-48 well plates are pre-coated with rat-tail collagen type-I, rinse the plate with 0.5-1ml of sterile room temperature DPBS solution twice and once with 100-200 µl of complete endothelial cell medium (see Recipes). Seed approximately 2×10^5-3×10^5 cells per well (Figure 2-xi) and leave the cells undisturbed for 3-4 days to maintain their number. Then, change the medium without a wash and incubate the cells for an additional 7-10 days (Figure 2-xii). Alternatively, if the plates were pre-coated with human plasma fibronectin, there is no need to wash, and cells can be seeded directly into the well as described above.

   Notes:

   a. *The total number of BMECs per mouse is approximately 3×10^4-5×10^4 cells.*
   b. *The prepared complete endothelial cell medium (50 ml, see Recipes) should be pre-warmed at room temperature.*
   c. *The cells should be distributed at the side of the wells to have equivalent dispensation. Agitation or shaking of the plates must be avoided to prevent aggregation of the cells in some regions of the wells.*

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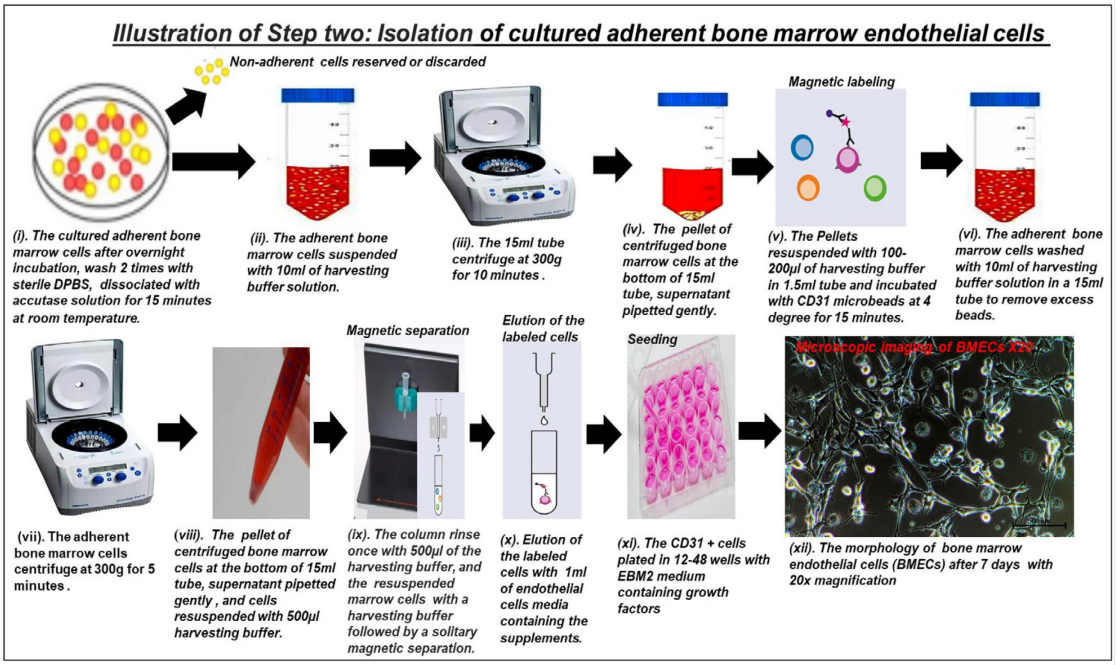

Figure 2. Illustration of Procedure B: isolation of cultured adherent BMECs.

C. Treatment of BMECs

1. Cells should be cultured in pre-coated 12-48 wells plate for seven days before being treated with pharmacological drugs or inhibitors. Before treatment of the cells, wash the cells with pre-warmed endothelial cells medium. Add 100-300 µl of the endothelial cell medium containing the inhibitor or drug and 100-300 µl of endothelial cell medium containing 2% FBS for the vehicle cells as described in our publication (Smith *et al.*, 2021). After the duration of treatment, replace the medium with complete endothelial cell medium (see Recipes). For external treatment such as radiation exposure, replace complete endothelial cell medium (see Recipes) with the pre-warmed medium at room temperature.

   Notes:

   a. *Aspiration and release treads of the media must be executed at the side of the wells to avoid aggregation of the cells, as shown in Figure 3.*
   b. *Perform a serial dilution of the drugs or inhibitors to ascertain the non-toxic concentration for the primary BMECs.*
   c. *The time scale for individual experiments needs to be established to attain optimal results.*
   d. *An experiment involving protein extraction from BMECs should be cultured in 12-48 wells plate without coating for excellent results. The coating of the wells with fibronectin/ rat tail collagen type I will affect the experimental outcome.*

Figure 3. Aspiration and release of the medium should be performed at the side of the wells.

Hold the plate at an approximate angle of 20-30 degrees and hold the pipette at 120 degrees to avoid detachment of the cells.

D. Fixation and imaging of BMECs (Figure 4)

1. Aspirate the supernatant from the adherent BMECs.
2. Drop 50-100 µl of 4% PFA solution (see Recipes) into the wells and incubate at room temperature for 20 min. Wash cells with DBPS and incubate with 50-100 µl of 90% methanol for 20-25 min. Fellow the link to see the entire procedure: https://rdcu.be/cni0O or https://doi.org/10.1186/s13287-021-02352-3.

   Note: Do not culture the primary BMECs in a glass slide. The cells do not adhere even to slides pre-coated with either fibronectin or rat tail collagen type I.

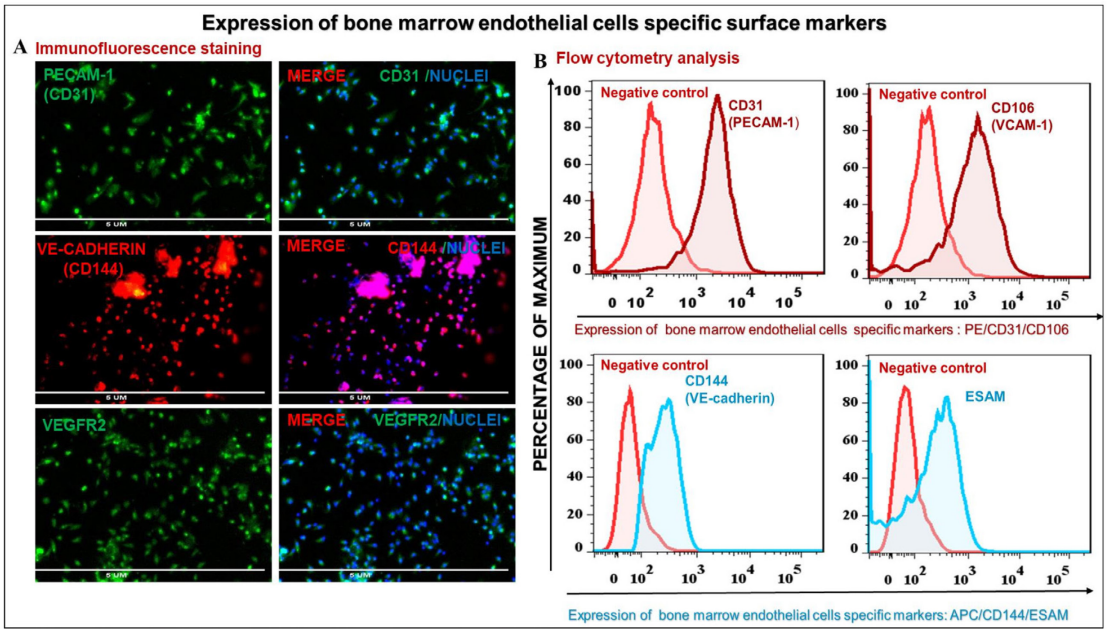

Figure 4. Characterization of BMECs by immunofluorescence staining and flow cytometry.

(A) Imaging of BMEC specific markers after seven days of cell culture. Primary antibodies: PECAM-1 (CD31) Rabbit-polyclonal, VE-CADHERIN (CD144) Rabbit-polyclonal, and vascular endothelial growth factor receptor-2 (VEGFR2/FlK-1) Rabbit-polyclonal. Secondary antibodies: Goat Anti-rabbit (IgG) Alexa Fluor® 594 (green, Ab15008) and Goat Anti-rabbit Alexa Fluor®488 (red, Ab15007). Dilution in 1% BSA/DPBS of 1:50 for primary antibodies and of 1:500 for secondary antibodies. Scale bars = 5 µm, 20× magnification. **(B)** Flow cytometry analysis of BMEC surface markers: CD31 (PE, anti-mouse, eBioscience™), CD106 (PE, Rat-anti-mouse, BD-Pharmingen™), CD144 (APC, anti-mouse, eBioscience™) and endothelial selective adhesion molecule (ESAM) (APC, anti-mouse, Biolegend®) antibodies. 5 × 10^5 cells were collected per tube, washed with DPBS, and centrifuged at 500 × *g* for 5 min at 37°C (this procedure was repeated twice). The cell was diluted with the antibodies or a blank control at 4°C for one hour. Data were analyzed by flow cytometry (BD LSRFortessa™) within 24 h. The lower frequency was used to exclude dead cells/debris by forward scatter (FSC) × side scatter (SSC), followed by graphical histogram presentation. Cytometry data were analyzed by FlowJo software version 7.6.2.

Data analysis

Each independent experiment should be executed with at least three experimental repeats and the data normalized with vehicle cells or untreated cells as described in our publication (Smith *et al.*, 2021). A bar graph and suitable statistical tests can be used to represent the data**.** *Follow the link to see statistical analysis: https://rdcu.be/cni0O or https://doi.org/10.1186/s13287-021-02352-3.*
Note: At least six (Smith et al., 2021) mice per group for each experimental setup are necessary to attain optimal results.

Recipes

1. 1× Dulbecco's phosphate buffer solution (DPBS) sodium (1 L)
   NaCl 8.0 g
   KCl 0.2 g
   KH_2PO_4 0.2 g
   Na_2HPO_4 1.15 g
   Dissolve in 1,000 ml of Milli-Q water
   Adjusted pH to 7.2-7.6 with HCl, sterilize, and store at 4°C
2. Complete DMEM (50 ml)
   Dulbecco's Modified Eagle Medium (DMEM)
   20% fetal bovine serum (FBS)

500 µl of 10,000 U/ml penicillin/streptomycin

Keep at 4°C

3. 0.5 M Ethylenediaminetetraacetic acid (EDTA) solution, pH 8.0 (500 ml)

   93.05 g of $Na_2EDTA{\cdot}2H_2O$

   400 ml of Milli-Q water

   Adjust pH with NaOH, sterilize and store at 4°C.

4. 0.001 M Ethylenediamine tetra-acetic acid (EDTA)/DBPS

   50 ml of DPBS

   100 µl of 0.5 M EDTA

   100 µl of 10,000 U/ml penicillin/streptomycin

   Store at 4°C

5. Sterile harvesting buffer solution

   0.002 M EDTA/DBPS

   2% FBS

   1% bovine serum albumin

   100 µl of 10,000 U/ml penicillin/streptomycin

   Store at 4°C for 4 weeks

6. Complete endothelial cell medium (50 ml)

   25 ml of Fetal bovine serum (FBS)

   0.2 ml of hydrocortisone

   0.5 ml of vascular endothelial growth factor (VEGF)

   Human fibroblast growth factor (hFGF)

   Ascorbic acid

   Human epidermal growth factor (hEGF)

   Heparin

   Gentamicin/Amphotericin

   Store at 4°C for 4 weeks only

7. 1% bovine serum albumin (BSA/DPBS)

   50 ml of sterile DPBS

   0.5 g of bovine serum albumin (BSA)

8. 4% Paraformaldehyde (PFA) solution

   Add 4 g of graded Paraformaldehyde to 50 ml of distilled water or Milli-Q-water.

   Add 1 ml of 1 M NaOH, stir gently with a magnetic stirrer at 60°C until the PFA has dissolved.

   Add 10 ml of 10× DPBS solution.

   Allow the mixture to cool at room temperature.

   Adjust the pH to 7.4 with 1 M HCl, then raise the volume to 100 ml.

   Filter solution through 0.45 µm membrane to get rid of any residual particles.

   Aliquot into appropriate volumes and store at 4°C for one month or at -20°C for several months.

9. 0.5 M EDTA

Add 93.05 g of $Na_2(EDTA)\cdot 2H_2O$ to 400 ml of distilled water and stir with a magnetic stirrer. Adjust pH to 8.0 with NaOH, raise the volume to 500 ml, and store the solution at 4°C.

10. Dish pre-coating reagents
    a. The Rat-tail collagen type-I 3 mg/ml plus 0.2 M sterile HCl (50-100 µg/ml).
    Preparation of 50 µg/ml of Rat-tail collagen type-I in 0.02 M HCl:
    Prepare 0.02 M HCl = 1 ml of 0.2 M HCl in 9 ml of distilled water
    Aliquots ~17 µl of 3 mg/ml Rat-tail collagen type-I add to 983 µl of 0.02 M HCl
    b. Alternatively, human fibronectin coating solution 1 mg/ml with 2-10 µg/cm^2.
    Preparation of 20 µg/ml of the human fibronectin coating solution:
    Aliquots 200 µl of human fibronectin solution and add to 3.8 ml of sterile room temperature DBPS.
    Note: Overnight pre-coating of the above coating reagents produced optimal results, and the table below shows the volume ranges of pre-coating solution recommended.

Plates/well	Recommended volume
48 well	250-300 µl
12 well	400-500 µl
24 well	0.5-1 ml
6 well	1-2 ml

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Competing interest

The authors declared no competing interest in these protocols.

Ethics

The National Institute of Health guide for the care and use of laboratory animals and use committee of Xuzhou Medical University (Xuzhou, China) and Jiangsu province, China, approved the studies with acceptance number: XZMC20130226.

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