Cell culture-based enrichment of mouse hematopoietic stem and progenitor cells

Kiyosumi Ochi
The University of Tokyo
Maiko Morita
The University of Tokyo
Adam C. Wilkinson
University of Oxford
Atsushi Iwama
The University of Tokyo
Satoshi Yamazaki (y-sato4@md.tsukuba.ac.jp)
The University of Tokyo; University of Tsukuba https://orcid.org/0000-0003-4249-3854

Method Article

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Abstract

Bone marrow (BM) chimeric mice are a valuable tool in the field of immunology and hematology, and genetic manipulation of donor cells is widely used to study gene function under physiological and pathological settings. Current BM chimera protocols generally require use of multicolor fluorescence-activated cell sorting (FACS) for donor hematopoietic stem and progenitor cell (HSPC) purification. Here, we describe a cell culture technique for the enrichment of functional HSPCs from mouse BM without the use of FACS purification. This technique is therefore expected to overcome current limitations in mouse BM chimera models.

Introduction

The generation and analysis of bone marrow (BM) chimeric mice is a common technique in the fields of immunology, hematology, and stem cell biology. Current applications include assaying the functional potential of hematopoietic stem and progenitor cells (HSPCs) and evaluating how genetic alterations influence hematopoietic and immune cell functions (1,2). Although a well-established technique, BM chimera assays currently have several limitations. These include the need to pre-condition recipient mice with irradiation or chemotherapy to achieve donor cell engraftment and the common use of multicolor fluorescence-activated cell sorting (FACS) to purify donor HSPCs, which requires expensive and complex equipment.

We recently developed a novel poly-vinyl alcohol (PVA) based cell culture media that could support the long-term expansion of functional hematopoietic stem cells (HSCs) (3,4). PVA is a chemically synthesized polymer that stabilized cytokines in vitro (5) and can replace the use of serum albumin supplements (e.g., fetal bovine serum or bovine serum albumin), which contains numerous biologically active contaminants that can induce loss of HSC function ex vivo (6). This HSC expansion method was initially validated using FACS purified HSCs (3). However, we have since discovered that this PVA-based media, containing only two cytokines (stem cell factor or SCF, and thrombopoietin or TPO), selectively supports the growth of HSPCs and can therefore be used to expand HSPCs from mouse bone marrow without the need for FACS purification (see Associated Publication). This new FACS-free protocol is described here. Potential applications of this protocol include ex vivo genetic manipulation of HSPCs and the generation of conditioning-free BM chimeric mice, which are described in our Associated Publication.

Reagents

Cell culture reagents and supplies:

1. 1X Ham's F-12 Nutrient Mix liquid media (Gibco 11765-054 or Wako 087-08335)
2. 1 M HEPES (Gibco 15630-080)

3. 100X Penicillin-Streptomycin-Glutamine (P/S/G) (Gibco 10378-016)

4. 100X Insulin-Transferrin-Selenium-ethanolamine (ITS-X) (Gibco 51500-056)

5. 100 mg/ml polyvinyl alcohol dissolved in sterile DI water* (Sigma P8136)

6. Alternative to reagents 1-5: HemEx-Type9A (NIPRO)

7. Recombinant animal-free murine thrombopoietin (TPO) stocked at 100 ug/ml in F-12 media (stored at -80°C) (Peprotech AF-315-14)

8. Recombinant animal-free murine stem cell factor (SCF) stocked at 10 ug/ml in F12 media (stored at -80°C) (Peprotech AF-250-03)

9. Sterile phosphate-buffered saline (PBS) (Gibco 10010-023)

10. Anti-mouse c-Kit MicroBeads (Miltenyi Biotech 130-091-224)

11. LS columns (Miltenyi Biotech 130-042-401)

12. MidiMACS separator magnet and MACS multistand (Miltenyi Biotech 130-042-302, 130-042-303)

13. 48-well CellBIND plates (Corning 3338)

14. Sterile 15 ml and 50 ml tubes

*Note: See other recent protocols for how to dissolve PVA in water (4).

Cell analysis reagents:

1. Trypan Blue solution (0.4%) (Gibco 15250-061)

2. PE anti-mouse CD150 antibody** (TC15-12F12.2, BioLegend)

3. APC anti-mouse c-Kit antibody** (2B8, eBioscience)

4. PE/Cy7 anti-mouse Sca-1 antibody** (D7, eBioscience)

5. APC/eFluor780 anti-mouse CD4 antibody** (RM4-5. eBioscience)

6. APC/eFluor780 anti-mouse CD8 antibody** (53-6.7, eBioscience)

7. APC/eFluor780 anti-mouse Mac-1 antibody** (M1/70, BioLegend)

8. APC/eFluor780 anti-mouse Gr-1 antibody** (RB6-8C5, eBioscience)
9. APC/eFluor780 anti-mouse B220 antibody** (RA3-6B2, eBioscience)
10. APC/eFluor780 anti-mouse Ter119 antibody** (TER-119, eBioscience)
11. APC/eFluor780 anti-mouse D127/IL-7Rα antibody** (A7R34, eBioscience)
12. Propidium iodide (PI) solution (Biolegend)
13. FACS tubes with cell strainer (Corning)
14. 1.5 ml and 15 ml tubes

**Note: Antibody concentration of should be individually titrated before use (antibody concentration will change between batches and vendors).

Mouse bone marrow:

1. Bone marrow from tibia, femur, and ilium bones should be isolated from freshly euthanized 8–12-week-old C57BL/6 mice (available from various vendors, e.g. The Jackson Laboratory).
2. Please see other published protocols for collection of mouse bone marrow (4, 7,8)
3. Please ensure all ethical approval is in place before initiating these studies.

Equipment

Equipment:

1. Laminar-flow sterile tissue culture hood
2. Sterile pipettes and filter tips
3. Sterile electric pipet-aid and sterile strip-pipettes
4. Calibrated laboratory scales
5. Laboratory autoclave
6. Laboratory water-bath set to 37°C

7. Tissue culture incubator, humified and set to 37°C, 5% CO_2, 20% O_2

8. Fluorescence-activated cell sorter (e.g., BD FACS AriaII, BD FACSVerse)

9. Bench-top centrifuge set to 4°C

Procedure

Preparing PVA-based HSC media:

1. Media should be prepared fresh for every use and media should be pre-warmed to 37°C before use.

2. Mix media reagents to make F12 media supplemented with 10mM HEPES, 1X P/S/G, 1X ITSX, 1 mg/ml PVA, 100 ng/ml TPO, and 10 ng/ml SCF. Prepare enough for 200 ul per well. Note that PVA solution is viscous and will need to be pipetted slowly. Mix media well by inversion before use.

3. Transfer 200 ul media into desired 96-well plate wells (or 1 ml media for 24-well plate wells). Fill remaining plate wells with PBS.

Isolating c-Kit+ cells from mouse bone marrow:

1. Count mouse bone marrow cells and spin down at 440 *g* for 5 minutes at 4°C.

2. Resuspend cells in 10^7 cells/ml in PBS and add anti-c-Kit Microbeads at a ratio of 20 ul per 10^7 cells.

3. Mix and incubate at 4°C for 15 minutes, then add 10 ml of PBS and spin down at 440 *g* for 5 minutes at 4°C.

4. Resuspend in 2 ml PBS and perform magnetic column enrichment using LS columns, according to the manufacturers' protocol.

5. Count the retrieved c-Kit-enriched cells by transferring 10 ul of the cells to a tube, mixing 1:1 with Trypan Blue and count using a hemocytometer.

6. Spin down cells at 440 *g* for 5 minutes at 4°C and resuspend in at 1 x 10^6 cells/ml. Seed 1 ml per 48-well plate well.

7. Fill any unused wells with PBS and incubate at 37°C with 5% CO_2 and 20% O_2.

Maintaining HSPC cultures:

1. Media changes must be performed every 2-3 days throughout the entire culture using pre-warmed and freshly-prepared media. Inspect the cell cultures before performing each media change using a light microscope.

2. Collect all the cell culture media in the well into a 15 ml tube using a pipette.

3. Spin down at 440 *g* for 5 minutes at RT. After removing supernatants, add new media and resuspend in at ~1 x 10^6 cells/ml. Seed 1 ml per well in 48-well plate.

4. After media changes, transfer back to the tissue culture incubator.

5. Once wells exhibit 80-90% confluency, cell cultures can be passaged at a ratio of 1:2-1:3.

6. Cells cultures can be analyzed at any time point as described below. Alternatively, cell cultures can be used in *in vivo* transplantation assays (see other published protocols on the HSC transplantation assay) (4,7,8).

Analyzing HSPC cultures:

1. To count the cell cultures, gently pipette the cultures to dissociate attached cells. Transfer 10 ul of the culture to a tube. Mix 1:1 with Trypan Blue and count using a hemocytometer. Alternatively, an automated cell counter can be used.

2. To analyze by flow cytometry, cells should be first stained with Sca1, cKit, and Lineage antibodies: Dissociate attached cells by gently pipetting the media over the plate bottom. Transfer cells to a tube and spin down at 1500 rpm (440 *g*) for 5 minutes.

3. Resuspend cells in PBS containing the antibodies against CD150, c-Kit, Sca-1, CD4, CD8, CD45R/B220, Ter119, Ly6G/Ly6C, CD127, and CD11b. Stain cells at 4°C for 30 minutes, wash with at least 10 volumes of PBS and spin down.

4. Resuspend cells in ~200 ul PBS containing 1X PI, transfer to a FACS tube, and store at 4°C until analysis.

5. Analyze on a FACS machine/flow cytometer using unstained and single stained samples as controls, and quantify the frequency of $CD150^+Kit^+Sca1^+Lineage^-$ live cell population.

Troubleshooting

Lack of cells after culture: Check that your starting population has high c-Kit$^+$ cell frequencies by antibody staining and flow cytometry. Make sure to gently perform media changes and check that you are not removing all cells during media changes.

Low KSL frequency after culture: Always prepare your media fresh. Confirm the activity of your cytokines (TPO and SCF). Check that your starting population has high c-Kit$^+$ cell frequencies by antibody staining and flow cytometry. Service your tissue culture incubator. Consider initiating the cultures at higher or lower densities.

Time Taken

Preparing PVA-based HSC media: 15 minutes

Isolating c-Kit$^+$ cells from mouse bone marrow: 1-2 hours

Maintaining HSPC cultures: 15-30 minutes every 2-3 days, depending on number of cells

Analyzing HSPC cultures: 1-4 hours, depending on the number of samples

Total ex vivo HSC culture: Usually 4 weeks, but depends on downstream application

Anticipated Results

Please see Associated Publication.

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