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Hematopoietic Stem Cell Cultures and Assays

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Summary

The adult hematopoietic system is repopulated in its entirety from a rare cell type known as hematopoietic stem cells (HSCs) that reside in the marrow space throughout the skeletal system. Here we describe the isolation and identification of HSCs both phenotypically and functionally.

Keywords

Hematopoietic Stem Cell; HSC; HSPC; Flow Cytometric Analysis; FACS; CFC; LTC-IC

1. Introduction

A single HSC is capable of repopulating the entire hematopoietic system [1]. This requires both unlimited self-renewal as well as the ability to differentiate into every type of hematopoietic cell. In mammals hematopoiesis occurs, and HSCs reside, in the marrow cavity of the skeletal system. In humans, direct evaluation of HSC function is very limited. As a surrogate, in vitro assays have been developed to functionally evaluate immature hematopoietic cells [2].

Multiple methods of in vitro analysis have been developed for the identification and quantification of immature hematopoietic cells. The fastest method, flow cytometric analysis, is the only method that can prospectively identify and isolate HSCs, however it is also the one that provides no functional data. It has however been strongly correlated with repopulating ability and is therefore widely accepted in the field [3, 4]. Colony Forming Cell assays (CFCs) are in vitro functional assays that represent the second most rapid method of identifying hematopoietic progenitors. These provide some limited functional analysis, as the ability to form a multi-lineage colony requires both the ability to differentiate as well as some limited self-renewal. Much more time consuming assays, cobblestone area-forming cells (CAFC) and long-term culture initiating cells (LTC-IC) are used to represent the most primitive hematopoietic cell population that can be functionally assayed in vitro [5]. Both of these assays require more extensive self-renewal capacity than the CFC assay. To date however the ability to serially repopulate myeloablated recipients is the only true way to determine both unlimited self-renewal, and multi-potentiality.

2. MATERIALS

2.1 Immunophenotypic Identification by Flow Cytometric Analysis

1. Antibodies: CD3e, B220, CD11b, GR1, TER119, Flt3, CD48, c-Kit, Sca-1, CD150. (See Note 1)
2. FACS staining buffer: 1X Phosphate Buffered Saline (PBS) and 2% Fetal Bovine Serum (FBS)
3. Red blood cell (RBC) lysis buffer: 156 mM NH_4Cl , 127 μM EDTA, and 12 mM NaHCO_3
4. Vital dye to distinguish live cells from dead/dying cells E.g. 4',6-diamidino-2-phenylindole (DAPI).
5. Flow cytometer

2.2 Colony Forming Cell Assays

1. Biosafety cabinet
2. 5% CO_2 incubator set to 37°C with humidity 95%
3. 35mm petri dishes that have not been coated for tissue culture
4. 100mm petri dishes
5. Sterile syringes
6. Sterile blunt end 16 gauge needle
7. Semi-solid methylcellulose media: Multiple formulations are commercially available. (See Table 1)
8. Complete Iscove's Minimum Essential Medium (IMEM). IMEM should be supplemented with 2% (by volume) FBS.
9. Red blood cell (RBC) lysis buffer: 156 mM NH_4Cl , 127 μM EDTA, and 12 mM NaHCO_3
10. Recombinant CSF-1, SCF, IL1, and IL3.

2.3 Co-culture Assays

1. Biosafety cabinet
2. 5% CO_2 incubator set to 37°C with humidity 95%
3. 5% CO_2 incubator set to 33°C with humidity 95%
4. 96 well flat bottomed tissue culture plates
5. M2-10B4 cells or primary bone marrow stromal cells

¹Proper panel design is critical to the success of flow cytometric analysis. There are several software programs available to aid in panel design, one such program is Fluorish (Fluorish, LLC, Ashland, OR).

6. 35mm petri dishes that have not been coated for tissue culture
7. H5100 cell culture medium (Stem Cell Technologies)
8. 10^{-3} M Hydrocortisone sodium hemisuccinate in A-MEM (prepared weekly)
9. H4435 methylcellulose containing medium (Stem Cell Technologies)
10. 0.25% Trypsin
11. X-ray or gamma-radiation source
12. All materials listed for CFC assays
13. Light microscope capable of phase contrast imaging
14. L-Calce™ software (Stem Cell Technologies cat#28600)

2.4 Competitive Repopulation

1. FACS staining buffer: 1X PBS and 2% FBS
2. 0.5 mL insulin syringes with 29G beveled needles
3. X-ray or gamma-radiation source
4. Recipient mice: For mouse bone marrow cells the most commonly used strain is C57bl/6 mice. These mice are available with 2 different alleles of CD45 that are distinguishable by flow cytometric analysis. CD45.2: WT C57bl/6 mice and CD45.1 (Jackson Labs, Bar Harbor, ME: Strain # 002014)
5. Antibodies for analysis of engraftment can be obtained from multiple commercial sources including: eBioscience, and BD Biosciences.

3. METHODS

3.1 Immunophenotypic Identification by Flow Cytometric Analysis

Putative HSCs can be identified by the expression (or lack thereof) of specific cell surface antigens. This allows for rapid quantification of populations of cells that are otherwise costly and time-consuming to assay. Moreover, most assays quantify HSCs post facto, therefore flow cytometry is the only method allowing for prospective identification of HSCs and is therefore necessary for any effort attempting to isolate HSCs. The caveat to flow cytometric analysis (FACS) is that it is not a functional assay. The cell surface antigen phenotypes reported have been well documented to correlate to a population of cells that is capable of repopulating the hematopoietic system of a myeloablated recipient [1, 3, 4, 6–9].

1. An appropriate source of cells must be obtained. Depending on the details of each particular experiment the source may vary. Typical sources are cells of the marrow, spleen or peripheral blood.
2. RBCs should be removed. One recommended method is by the use of RBC lysis buffer. Cells should be suspended in RBC lysis buffer at a concentration of approximately 1×10^7 cells per mL. They should be incubated at room temperature

for 5 minutes and then promptly washed with an equal volume of FACS staining buffer.

3. Following the removal of RBCs up to 1×10^7 mononuclear cells should be resuspended in 100 μ L FACS staining buffer.
4. An appropriate amount of each antibody should be added to the cell suspensions. Typical amounts are 0.02–0.2 μ g per test, though it is recommended to titrate each antibody to determine an optimum amount. (See Note 2)
5. Putative long-term repopulating HSCs are identified by the following surface antigens in mice. They are negative for markers such as: CD3e, B220, CD11b, GR1, TER119, Flt3, CD48. They are positive for markers such as: c-Kit, Sca-1, CD150. (See Note 1)
6. Putative short-term repopulating HSCs are identified by the following surface antigens in mice. They are negative for markers such as: CD3e, B220, CD11b, GR1, TER119, CD48, CD150. They are positive for markers such as: c-Kit, Sca-1.
7. Putative multi-potent progenitors (MPPs) are identified by the following surface antigens in mice. They are negative for markers such as: CD3e, B220, CD11b, GR1, TER119,. They are positive for markers such as: c-Kit, Sca-1, Flt3.
8. Putative HSCs in humans are enriched by selecting for the population that is CD34 positive and CD38 negative. (See Note 1)

3.2 Colony Forming Cell Assays

Hematopoietic progenitor cell frequencies can be determined by performing colony forming cell assays[10]. Using these assays progenitors such as colony forming unit erythrocytic (CFU-E), blast forming unit erythrocytic (BFU-E), colony forming unit granulocytic and monocytic (CFU-GM), colony forming unit granulocytic, erythrocytic, monocytic and megakaryocytic (CFU-GEMM), and highly proliferative progenitors (HPP) can be quantified.

1. An appropriate source of cells must be obtained. Depending on the details of each particular experiment the source may vary. Typical sources are cells of the marrow, spleen or peripheral blood.
2. Red blood cells should be removed. One recommended method is by the use of RBC lysis buffer. Cells should be suspended in RBC lysis buffer at a concentration of approximately 1×10^7 cells per mL. They should be incubated at room temperature for 5 minutes and then promptly washed with an equal volume of complete IMEM.
3. Cells should be resuspended in complete IMEM at 10x the final desired concentration. If performing mouse HPP cultures media should be supplemented

²All conjugated antibodies are prone to contamination, and degradation following exposure to room temperature and/or light (particularly tandem dyes such as PE-Cy7). Therefore it is good practice to use all conjugated antibodies on ice, in a biosafety cabinet with the lights off.

with 250ng/mL of recombinant CSF-1 and 50ng/mL of recombinant SCF, IL-1 and IL-3.

4. For each individual sample a 15mL conical tube should be filled with 3mLs of methylcellulose containing media if cultures are to be performed in duplicate. Add 4mLs if they are to be performed in triplicate. (See Notes 3 and ⁴)
5. Add the stock solution of cells to the methylcellulose containing media and vortex thoroughly. Add 300μLs if cultures are to be performed in duplicate. Add 400μLs if cultures are to be performed in triplicate.
6. Allow tubes to rest for 5 minutes at room temperature.
7. Using a blunt end 16 gauge needle attached to a syringe dispense 1.1mL of cell containing methylcellulose media into each 35mm dish.
8. Evenly distribute the methylcellulose containing media across the bottom of the dish.
9. Place 2 35mm sample containing dishes along with 1 35mm dish containing 3mLs of sterile ultrapure water into 1 100mm dish and place in the 37° incubator.
10. After the indicated amount of time for the culture type analyze the cultures by counting the number of colonies present per dish. (See Table 1)

3.3 Co-culture assays

HSCs require a specific microenvironment or niche to properly regulate their function. Therefore, since transplantation of HSCs in humans for research purposes is unethical, in order to evaluate numbers of hematopoietic progenitors that are less mature than those found in CFC assays, a co-culture assay is required. The two co-cultures most commonly used measure the frequency of cell types that act as surrogates for HSCs; these are cobblestone area forming cells (CAFC), and long-term culture initiating cells (LTC-IC) [11–13]. Both use a stromal cell feeder layer to support immature hematopoietic cells however the readout is different. These cultures are somewhat controversial as they do not measure the true functional potential of HSCs to repopulate a marrow, and variations in procedures and stromal cell layers can result in different outcomes from different laboratory environments. The feeder layer used can be either primary bone marrow stromal cells, or a cell line. One well established cell line used for these cultures is M2-10B4 cells.

1. Prepare an appropriate stromal layer. If you are using M2-10B4 cells: Inoculate flat-bottomed 96-well tissue culture plates with M2-10B4 cells and maintain at 37° C. When cells have reached confluency irradiate the plates with a 20GY dose of either X-ray or gamma radiation. This serves the purpose of preventing over-proliferation of the feeder layer, while still allowing support for hematopoietic progenitors.

³To limit the number of freeze thaw cycles methylcellulose containing media should be aliquoted into 3 or 4 mL volumes depending on whether you are performing your assay in duplicate or triplicate respectively.

⁴Methylcellulose containing media should be thawed at room temperature the day of use, or at 4° C overnight.

2. Suspend test cell population in H5100 supplemented 1:100 with 10^{-3} Hydrocortisone sodium hemisuccinate. For bone marrow cells an initial concentration of 8×10^5 mononuclear cells per mL should be used. If performing a limiting dilution analysis it is recommended that 4–8 different concentrations be used with 12 wells/concentration.
3. 100 μ L of cell suspension should be added to each well.
4. Cultures should be maintained at 33° C for 5 weeks with a half media change performed each week in which the non-adherent cells removed with the media are discarded. Aseptic technique must be strictly followed for media changes. (See Note 5)
5. Following 5 weeks in culture CAFCs can be counted. Cobblestone areas are identified by phase contrast microscopy. Cobblestone areas appear as dark centered circles as they are located below the stromal layer.[11]
6. In order to measure LTC-ICs the cultured cells must be removed from the 96 well plates. Remove the non-adherent cells from the plates with the cells from each well being placed into separate tubes. Trypsin should be used to release the adherent cells from the wells. Following trypsinization adherent cells should be added to the same tubes as the non-adherent cells. CFCs should be set up using the cell population of each individual well of the 96 well plates to seed a 35mm dish. The CFC protocol should be followed for the setup of these cultures with the use of H4435 methylcellulose containing media. After 18–20 days in culture, dishes should be scored as positive if they contain at least 1 BFU-E, CFU-GM, or CFU-GEMM. If no colonies are present the dish should be scored as negative. (See Notes 3 and 4)
7. If a limiting dilution was performed the number of CAFCs or LTC-ICs can be calculated based on the number of positive wells per dilution and using L-CALC™ software.

3.4 Competitive Repopulation

The best method by which to measure functional HSCs is by their ability to serially transplant myeloablated recipient mice[14–16]. In mice, congenic strains result in little to no tissue rejection. To transplant human HSCs however, immune compromised strains of mice must be used to prevent tissue rejection. These experiments are timely and costly, leading to the use of the previously described methods as screening tools and competitive repopulation as functional validation.

1. One day before transplantation recipient mice should be irradiated with a dose of 5GY from either an X-ray or gamma-radiation source. The recipient mice should be CD45.2 if donor cells are CD45.1 or the recipient mice should be CD45.1 if donor cells are CD45.2. (See Note 6)

⁵Bacterial or fungal contamination can become an issue in LTC-IC assays as the cultures are long and the media used contains no antibiotic or antifungal treatment. Therefore the strictest aseptic technique must be maintained throughout the culture.

2. An appropriate source of donor cells must be obtained. In mice, bone marrow cells are used almost exclusively.
3. An appropriate source of competitor marrow cells must be obtained. Competitor cells should be CD45.2 if donor cells are CD45.1 or the competitor cells should be CD45.1 if donor cells are CD45.2.
4. Donor cells and competitor cells should be resuspended in FACS staining buffer at an appropriate ratio. A donor:competitor ratio of 1:1 or 1:2 is commonly used. Donor and competitor cell mixtures should be resuspended at an appropriate cell concentration so that the total injection volume does not exceed 200 μ L. The minimum number of competitor cells transplanted to ensure survival of recipient mice is 1×10^5 . Example: If donor cells are CD45.2 and a ratio of 1:2 donor:competitor is desired then 5×10^4 CD45.2 donor cells and 1×10^5 CD45.1 competitor cells will be combined and resuspended in 100–200 μ L of FACS staining buffer.
5. Recipient mice should be irradiated a second time with a dose of 5GY 24 hours following the first dose of radiation. The total dose of radiation should be 10GY to achieve a lethal dose. (See Note 6)
6. No more than 2 hours following the second dose of radiation the donor and competitor cell mixture should be intravenously injected. This can be achieved by injecting directly into the lateral tail veins, or by injecting into the retro-orbital sinus. If injecting by tail vein warming recipient mice with a heat lamp and using an appropriate restraining cone or tube is highly recommended. If injecting by retro-orbital sinus an anesthetic or sedative must be used to avoid permanent damage to the eye. (See Note 7)
7. Following transplantation the level of engraftment can be determined by flow cytometric analysis of the peripheral blood using the appropriate CD45.1 and CD45.2 antibodies. Analysis should be performed for short-term engraftment, 4–12 weeks, and long term engraftment, greater than 16 weeks. Multi-lineage engraftment of donor cells should also be assessed using the following antibodies: CD3e for T-Cell lineage, B220 for B-Cell lineage, and CD11b for Myeloid lineage. (See Note 1)
8. To truly measure long-term engraftment secondary transplantations should be performed. Marrow from primary recipients should be obtained and injected into secondary recipients that have been lethally irradiated as previously described. Secondary recipients should be of the same strain as primary recipients. Marrow cells obtained should not be transplanted with competitor cells. At least 1×10^5 whole marrow cells from the original donors should be transplanted. Successful

⁶When irradiating mice it is recommended to immobilize them in some way to prevent variable doses of radiation that can lead to variable engraftment rates.

⁷Both tail vein injections and injections into the retro-orbital sinus require considerable skill. It is highly recommended that they be practiced prior to the use of any experimental animals.

multi-lineage engraftment of a secondary recipient is the gold standard for determination of a functional long term repopulating HSC.

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Figure. Gating strategy to identify phenotypic LSK cells, MPPs, ST-HSCs, and LT-HSCs by flow cytometric analysis. Analysis was performed using FlowJo Ver. 8.8.7 (Treestar).

Table 1

Cell # per dish, media type, and culture time for various CFC cultures started with total mono-nuclear bone marrow cells from either mice or humans. Media is commercially available from Stem Cell Technologies, Vancouver, BC. Media listed as M is for mouse cultures, media listed as H is for human cultures.

Culture Type	Cell #	Media	Culture Time (days)
CFU-E	2×10^4	M3434/H4434	12
BFU-E	2×10^4	M3434/H4434	7–10
CFU-GM	2×10^4	M3434/H4434	12
CFU-GEMM	2×10^4	M3434/H4434	12
CFU Pre-B	5×10^4	M3630	7
HPP	4×10^5	M3231/H4535	21