The Fleischman Lab

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Title: Hematopoietic Stem Cell Differentiation Assay

Introduction

While myeloid cells are relatively easy to obtain *in vitro*, lymphoid cell development from hematopoietic stem or progenitor cells requires culturing the cells in the presence of bone marrow stroma. This assay utilizes the mouse stromal cell line OP9 to support the differentiation of stem or progenitor cells to both lymphoid and myeloid lineages. This assay is particularly useful to investigate the effects of certain mutations on the differentiation of HSCs. The culture conditions defined in this protocol may be altered to address other questions as well. As lymphoid development can be difficult, it is important to optimize the FBS and cytokines.

Materials

1. OP-9 cells
2. RPMI
3. αMEM20 (MEM-α without nucleosides + 20% FBS + penicillin/streptomycin/L-glutamine)
4. 0.05% trypsin-EDTA
5. I10 (IMDM + 10% FBS [we use Sigma lot# 164382 for lymphoid colony development] + penicillin/streptomycin/L-glutamine)
6. 2x cytokine mixture in I10 (the cytokines used will depend on your experiment)
7. Mouse bone marrow, stained for LKS or progenitor cells
8. 60 mm cell culture dishes
9. 15 cm cell culture dishes
10. 50 ml conical tubes
11. 96-well cell culture plates
12. microFACS tubes
13. Serological pipettes and pipet-aid
14. Micropipettes, sterile pipette tips, multichannel pipette, sterile reagent reservoir
15. Cell sorter
16. Flow cytometer
17. 37°C water bath
18. Swinging bucket centrifuge
19. 37°C 5% CO_2 incubator with ≥95% humidity

It is crucial to always maintain good aseptic technique.
Work in the hood, wear a lab coat and sterilize often!

Protocol	A. Culture and plate OP9 cells	*Notes*
1.	Warm up 30 ml of RPMI in a 50 ml conical tube. Remove cells from freezer and thaw. Pour cells into pre-warmed RPMI and wash cryovial with RPMI to remove remaining cells. Centrifuge for 10 minutes at 1200 rpm (400g).	*Thaw cells by swirling in a 37°C water bath until just thawed enough to pour the frozen pellet into the RPMI. Dry tube and sterilize with 70% EtOH.*
2.	Discard supernatant and resuspend in 3 ml αMEM20. Transfer to a 60 mm dish and place in the incubator. Monitor cells daily to prevent the cells from becoming overcrowded.	*It is very important to prevent OP9 cells from overcrowding! Confluent OP9 cultures transform from stroma cells to fat cells, evidenced by large vacuoles in the cytoplasm. Discard cells and take fresh aliquot if cells become overcrowded.*

<table>
<tr><td>3.</td><td>Once the 60 mm dish is ~80% confluent, trypsinize and expand into a 15 cm dish:
1. Aspirate media.
2. Add 3 ml RPMI and wash by gently tilting dish back and forth to cover the entire cell surface with media.
3. Aspirate media.
4. Add 0.5 ml trypsin-EDTA and gently tilt dish back and forth to cover the entire cell surface with trypsin.
5. Place dish in the incubator until all of the cells have lifted, about 5 minutes.
6. Add 2 ml αMEM20 and pipette up and down vigorously to obtain a single-cell suspension.</td><td>Trypsin is inactivated by FBS. Do not add trypsin without first washing with serum-free media.

Check cells on the microscope frequently to ensure that cells have lifted.

Trypsin can be toxic to cells. Do not incubate for more than 10 minutes. If cells still do not lift, remove trypsin, wash, and add fresh trypsin.</td></tr>
<tr><td>4.</td><td>Transfer cells to a 15 cm dish and add 15 ml of αMEM20. Return to incubator. Monitor cells daily to prevent the cells from becoming overcrowded.</td><td></td></tr>
<tr><td>5.</td><td>Once the 15 cm dish is ~80% confluent, trypsinize as above and transfer cells to a 50 ml conical tube.</td><td>Scale the volumes of media and trypsin up accordingly.</td></tr>
<tr><td>6.</td><td>Centrifuge cells for 10 minutes at 1200 rpm (400g). Discard supernatant and resuspend in 30 ml I10.</td><td></td></tr>
<tr><td>7.</td><td>Pour cells into a sterile reagent reservoir. Use a multichannel pipette to plate 70 µl of cells into each well of a 96-well plate. Place in the incubator.</td><td>Each 15 cm dish should fill 4x 96-well plates.</td></tr>
<tr><td></td><td>B. Add mouse stem or progenitor cells</td><td></td></tr>
<tr><td>1.</td><td>One day after plating OP9 cells, harvest bone marrow and stain for the population(s) of interest (e.g. LKS, CMP, GMP, MEP, etc.).</td><td>See our Isolation of Bone Marrow from the Major Leg Bones and Cell Surface Staining protocols.</td></tr>
<tr><td>2.</td><td>Sort cells directly into the 96-well plate containing the OP9 cells. The number of cells per well will depend on your experimental conditions and the type of cell.</td><td>For LKS, sorting 1 cell per well will yield about 20-40% positive wells.</td></tr>
<tr><td>3.</td><td>Prepare the 2x cytokine mixture. Below are some common formulations:
<table>
<tr><th>Cytokine</th><th>Myeloid</th><th>Lymphoid</th><th>Myeloid + Lymphoid</th></tr>
<tr><td>SCF</td><td>50 ng/ml</td><td>50 ng/ml</td><td>50 ng/ml</td></tr>
<tr><td>Flt3 ligand</td><td>10 ng/ml</td><td>10 ng/ml</td><td>10 ng/ml</td></tr>
<tr><td>IL-7</td><td>--</td><td>20 ng/ml</td><td>20 ng/ml</td></tr>
<tr><td>IL-3</td><td>5 ng/ml</td><td>--</td><td>5 ng/ml</td></tr>
<tr><td>β-ME</td><td>--</td><td>100 µM</td><td>100 µM</td></tr>
</table></td><td>The cytokine formulation and serum used must be optimized for different conditions.

For lymphoid colony development, it is important to use Sigma brand FBS, lot# 164382. If lymphocytes are not desired, other sera may be used.</td></tr>
<tr><td>4.</td><td>Pour the 2x cytokine mixture into a sterile reagent reservoir. Use a multichannel pipette to add 70 µl to each well.</td><td></td></tr>
<tr><td>5.</td><td>Return the plates to the incubator. This is day 0. Monitor cell growth by examining at each well under the microscope daily. Add more I10 media as needed.</td><td></td></tr>
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	C. Analyze colonies	
1.	Monitor cell growth daily. By day 4-5, colonies can be observed with the microscope. Positive wells are best identified on days 5-6.	
2.	On day 10-11, harvest cells from positive wells into microFACS tubes.	
3.	Stain cells with myeloid and lymphoid markers (e.g. Gr-1, NK1.1, CD11b, B220) and analyze by flow cytometry.	*See our Cell Surface Staining protocol.*
4.	Analyze the percent of positive wells in each experimental condition. Of the positive wells, determine the frequency with which each lineage is represented.	