

Mouse Bone Marrow Hoechst Staining for SP cells

1. Euthanize a C57/Bl6 8-12 week old mouse by CO₂ and/or cervical dislocation
2. Take both femurs and tibia and keep on ice in PBS+ (see below for buffer recipes) till ready to extract marrow.
3. Place all bones in a mortar with 5cc of PBS+ and crush with pestle to extract marrow (yields around 100-120 million marrow cells per mouse; can take pelvis, sternum or even humeri if more cells needed)
4. Filter through a 70 um filter into a 50cc conical tube on ice. Repeat step 3 until all red marrow pulp has been removed from the bones, then wash the filter with a few ml of PBS+
5. Pellet cells (e.g centrifuge at 1600 in sorvall RT-7 x 5 min, =530rcf), resuspend in HBSS+ and count non-RBC marrow cells (e.g on coulter counter with gates set to 6-10 microns or treat a small aliquot with RBC lysis buffer for 1 minute and count on hemocytometer.)
6. Dilute cells to 4.5 million/ml in HBSS+ and stain cells with 8.8 ug/ml of Hoechst 33342 dye (Molecular Probes) for 90 minutes in a 37C water bath.
7. After staining, Ficoll deplete RBCs from sample by: pelleting cells and resuspend in HBSS+ (5cc for every two mice harvested) and layer over 5cc of Ficoll-Paque Plus (Amersham) in a 15cc conical and spin for 20 minutes at 2000RPM (sorvall RT-7=830rcf). Take buffy coat and wash in PBS+.
8. If antibody surface staining is needed, count cells and stain with relevant antibodies of choice for 20-30 in on ice (e.g Sca1-PE from Pharmigen at 2mcl per million cells in 100mcl of PBS+). Wash. (NB: cells can not be fixed or permeabilized for antibody staining as Hoechst efflux relies on a live cells with intact cell membranes.)
9. Resuspend cells in PBS+ with 2ug/ml propidium iodide added (Molecular Probes) and filter through 40um filter into a FACS tube for cell sorting.

Comments:

This protocol is modified from the original by Goodell and Mulligan (MA Goodell. J. Exp Med 1996) as it Ficoll depletes RBCs from the sample and stains the cells with a higher Hoechst concentration and a higher cell density. As 80-90% of cells (mainly RBCs and granulocytes) are depleted from the sample the resulting frequency of SP cells on the FACS plot is 10 fold higher than with the original method. So one should expect an SP gate of 0.1% (for an 8 week old C57/BL6 mouse) to be equivalent to a 0.01% gate if the original Goodell method were used. In our hands, 200 marrow SP cells sorted from a 0.1% gate using this modified method, results in 70-80% long term blood chimerism when these cells are transplanted along with 200,000 unfractionated competitor marrow cells into a lethally irradiated C57/BL6 recipient.

Buffers used:

PBS+= PBS with 2% fetal bovine serum

HBSS+= HBSS with 2% fetal bovine serum, 10mM HEPES, and 1% pen/strep.