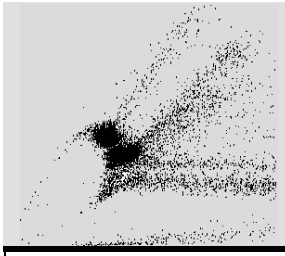

THE GOODELL LABORATORY

Author	Grant Challen	1/30/09
Title	**BrdU Labeling of Hematopoietic Stem Cells**	
Introduction	This protocol describes the use of BrdU incorporation to determine the rate of turnover of hematopoietic stem cells (HSCs). BrdU is incorporated into the DNA of replicating cells so the percentage of BrdU+ cells over a time period can be used to determine the relative rate of turnover. For this assay, mouse HSCs are labelled with BrdU *in vivo*, the HSCs are purified (along with a carrier cell population to minimize cells loss when dealing with limiting numbers of HSCs), and then fixed and reanalyzed for BrdU incorporation.	
Materials	1. BrdU (Sigma; B5002-500MG) 2. PBS 3. HANKS+ buffer = Hanks Balanced Salt Solution (Gibco #14170) + 2% FBS + 10 mM HEPES (Gibco #15630) 4. DMEM+ buffer for Hoechst staining = DMEM (Gibco #11965) + 2% FBS + 10 mM HEPES (Gibco #15630) 5. Hoechst 33342 (Sigma; B2261-500MG) 6. Sca-1 or c-Kit microbeads (Miltenyi Biotec) 7. Fluroescent-conjugated antibodies for Sca-1, c-Kit, and Lineage markers 8. Propidium iodide (PI) stock = 200 μl / mL. Dissolve 10 mg PI (Sigma; P1470-25mg) in 50 mL dI H_2O. 9. PI solution = 1:100 dilution of PI stock in HANKS+ buffer. 10. BD Biosciences BrdU-FITC analysis kit (cat. # 559619).	
Protocol		*Notes*
1.	Make up BrdU solution for injection. Need 3.33mg BrdU dissolved in 500μL PBS per mouse (e.g. for 5 mice dissolve 0.0167g BrdU in 2500μL PBS, inject 500μL). This is for an average 8-week old C57Bl/6 mouse. This amount works well for most applications but for large variations in weight the amount of BrdU injected must be adjusted accordingly.	*BrdU takes up to a few hours to dissolve at 37°C depending on amount.*
2.	Prepare BrdU for supplementation in drinking water (if necessary). Make up solution of 0.8mg/mL BrdU in water (e.g. 0.240g in 300mL).	

3.	Inject mice intraperitoneally with 500 μL of BrdU / PBS solution and substitute BrdU-drinking water.	*Supplementation of drinking water not necessary for timepoints less than 12 hours.*
4.	Sac mice and prepare bone marrow at desired timepoints.	*Typical timepoints are 12 hours, 3 days and 6 days.*
5.	Prepare spleenocytes from unlabelled mouse for carrier cells.	*Prepared by manual dissociation with scissors and filtration.*
6.	Prepare bone marrow for HSC isolation using the Hoechst staining protocol. Perform positive-selection magnetic enrichment if desired using Sca-1+ or c-Kit+ microbeads and AUTOMACS magnetic separation. Following enrichment, stain the cells with the following antibodies - Sca-1 / c-Kit-PE, Sca-1 / c-Kit-APC, Lineage-PeCy5. Resuspend cells in PI for FACS sorting.	*For Sca-1 enrichment, use c-Kit-PE and Sca-1-APC; for c-Kit enrichment use Sca-1-PE and c-Kit-APC. Cannot use any FITC antibodies, this channel is required for the BrdU reanalysis*
7.	Stain spleenocytes with B220-PeCy5 antibody, wash and resuspend in PI solution for FACS sorting.	
8.	Pre-sort 250,000-500,000 B220-PeCy5+ spleenocyte carrier cells into required number of FACS collection tubes.	*Number of carrier cells is not critical. Take the brightest PeCy5+ cells.*
9.	Sort HSCs into FACS tubes containing carrier cells as SP+Sca-1+c-Kit+Lineage-	*Be careful with the cutoffs for PeCy5 so as not to confuse HSCs with carrier cells in the reanalysis.*
10.	Following sorting, spin down cells and prepare for BrdU incorporation analysis with the BrdU-FITC flow kit (BD Biosciences).	*Probably will not be able to see a cell pellet, be careful pouring off supernatant.*
11.	Perform BrdU staining as per kit instructions.	*After fixation, cells can be kept overnight at 4^oC in the dark if desired (usually convenient due to time required for bone marrow prep and sort).*
12.	Resuspend cells in 500 μL staining buffer for reflow analysis.	
13.	In flow cytometric reanalysis, HSCs can be identified as PE+ (Sca-1 or c-Kit+) PeCy5-negative cells and can be discriminated from B220-PeCy5+ carrier cells.	

14.	Determine rate of turnover of HSCs by determining percentage of BrdU-FITC+ HSCs.	*Approximate normal HSC rate of turnover 4-10% per day*

Figure: Analysis of HSCs turnover by BrdU labeling using flow cytometry. (A) HSCs from mice injected with BrdU are purified from Sca-1-enriched bone marrow (increasing the proportion of SP cells 10-fold) and then fixed and permeabilized overnight. (B) Reanalysis of the sorted cells following intracellular staining for BrdU. A PE versus PeCy5 dot-plot allows for discrimination between sorted HSCs and carrier B cells (the majority of carrier cells are lined up against the x-axis). The HSCs can then be gated to either a stem cells marker versus BrdU dot-plot or to a histogram to determine BrdU incorporation.

References.

1. Hock et al., Nature, 2004. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells.
2. Feng et al., Cell Stem Cell, 2008. The p47 GTPase Lrg-47 (Irgm1) links host defense and hematopoietic stem cell proliferation.