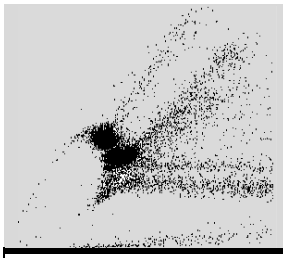

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Title	**Hoechst 33342 HSC Staining and Stem Cell Purification**	
Introduction	This protocol describes the use of the fluorescent DNA binding dye Hoechst 33342 to identify and purify murine hematopoietic stem cells, the so-called SP population in combination with the presence of HSC surface markers. The side population (SP) was first found in murine hematopoietic stem cells in bone marrow by their ability to pump out fluorescent DNA-binding dye Hoechst 33342 (1,2,3). Hoechst 33342 binds to the AT-rich region of the DNA and emits primarily in the blue range (around 450nm) but also has a weaker red emission (>675nm) component. When these two emission wavelengths are detected and plotted against each other, the "side population" can be easily resolved. In a dot plot of emission spectra they appear at the side of the rest of the cells and constitute a discrete population of cells with emission data that differs from that of the other cells (Figure 1). This side population is highly enriched for hematopoietic stem cells and comprises 0.02-0.15% of the whole mouse bone marrow cells depending on the age and mouse gender. The frequency of SP cells steadily increases with age.	
Materials	1. DMEM+ :Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Cat. No. 11965-092, Gibco Invitrogen) supplemented with 10 mM HEPES (Cat. No. 15630-080, Gibco Invitrogen) and 2% Fetal Bovine Serum (FBS). 2. HBSS+:Hank`s balanced salt solution (HBSS, Cat. No. 14170-112, Gibco Invitrogen) supplemented with 10 mM HEPES (Cat. No. 15630-080, Gibco Invitrogen) and 2% FBS 3. Hoechst 33342 : Hoechst 33342 powder (Cat. No. B2261, Sigma) is dissolved in distilled water and filter sterilized at 1 mg/ml concentration which makes 200X stock and frozen in small (~1 ml) aliquots at -20^{0}C. 4. Red blood cell Lysis buffer (D-5001, Gentra) 5. 18-G and 27-G needles 6. 70µm Cell strainers (Becton Dickinson "Cell Strainers") 7. 15 and 50 ml conical polypropylene centrifuge tubes, sterile (Falcon) 8. 250 ml polypropylene tubes (Cat. No 430776, Corning) 9. Circulating water bath at exactly 37°C. 10.Biotinylated Sca-1 antibody (Cat. No 553334, BD Pharmingen) 11.Anti-biotin magnetic microbeads from Miltenyi Biotech (Cat. No. 130-090-485). 12.Magnetic separation machine: autoMACS 13.PI: Propidium iodide (Cat. No. P-4170, Sigma) is dissolved at 200 µg/ml in	

	PBS as 100X stock and covered with aluminum foil and kept in 4^0 C fridge. Final concentration of PI in HBSS+ should be 2µg/ml. 14. Flow/sorting equipment with UV laser capable of excitation at 350 nm and detection with 450/20 and 675LP optical filters	
Protocol		*Notes*
1.	Prewarm DMEM+ (see below) while preparing the bone marrow.	*Ensure that a water bath is at precisely 37° C (check this with a thermometer!).*
2.	Using mice 8-10 weeks of age, prepare bone marrow from femurs and tibias and resuspend in ice-cold HBSS+	*Cut ends of the each bone and flush out bone marrow into a sterile tissue culture dish using a syringe (5-10 ml) with a 27G needle which is filled with ice-cold HBSS+. Flush from both ends to ensure all the marrow is removed. Bones should be very pale after flushing of the bone marrow. Change the needle to 18G and pass bone marrow-media mixture through 18G needle several times in order to make a single cell suspension.*
3.	Count the nucleated cells accurately	*Take out 5 µl of from bone marrow suspension and mix it with 95 µl of Red blood cell lysis buffer, vortex thoroughly and take 10 µl to count cells using a hemacytometer. Do not use RBC lysis for the whole bone marrow suspension. This procedure generally yields an average of 40 to 70 million nucleated cells per C57Bl/6 mouse (2*

		femur and 2tibias).
4.	Spin down the cells in a refrigerated centrifuge.	
5.	Resuspend cell pellet at 10^6 cells/ml in pre-warmed DMEM+.	*Polypropylene tubes must be used while staining with Hoechst to avoid retention of cells in tubes. For large volumes, staining in 250 ml polypropylene tubes is the most convenient method.*
6.	Add Hoechst to a final concentration of 5 µg/ml.	*Make a 200X (1mg/ml) stock, which is made by dissolving an entire bottle of Hoechst powder (Sigma) in water and stored in -20°C freezer.*
7.	Incubate cells in a circulating 37°C water bath for exactly 90 minutes.	
8.	Spin down the cells in a centrifuge at 2200 rpm for 5 minutes at 4°C and remove the supernatant. Resuspend cells at 10^8 cells/ml in ice-cold HBSS+.	

9.	For Sca-1 enrichment, add biotinylated Sca-1 antibody to the cell suspension at 1/100 dilution, and incubate on ice for 15 minutes.	*Hoechst stained cells can be enriched for progenitors by using biotinylated Sca-1 or c-Kit antibodies which will increase the yield and decrease the time required for sorting.*
10.	Wash out unbound antibodies with 10 fold volume of ice-cold HBSS+. Spin down the cells and remove the supernatant. Resuspend cells at 10^8 cells/ml in ice-cold HBSS+.	
11.	Label cells with magnetic beads. Incubate cells with 20% volume of microbeads and incubate at 4°C fridge for 15 minutes.	*Anti-biotin magnetic microbeads from Miltenyi Biotech can be used. It is recommended to incubate cells in the fridge instead of on ice, because of the low binding efficiency of microbeads on ice.Other manufacturers can also be used.*
12.	Wash the cells with a 10 fold volume of ice-cold HBSS+.Centrifuge the cells for 6 minutes at 2200 rpm, at 4°C and remove the supernatant.Resuspend cells at $2x10^8$ cells/ml in ice-cold HBSS+.	
13.	Load the cells onto autoMACS column. Take the positive fraction from the autoMACS column and wash with ice-cold HBSS+.	
14.	Centrifuge the cells for 6 minutes at 2200 rpm, at 4°C and remove the supernatant. Resuspend cells at $1x10^8$ cells/ml in ice-cold HBSS+.	

15.	Prepare lineage marker antibody cocktail. Mix monoclonal antibodies of anti-mouse c-Kit, anti-mouse CD150 , lineage markers of anti-mouse CD4, anti-mouse CD8a, anti-mouse B220, anti-mouse Gr-1, anti-mouse Mac-1, anti-mouse Ter-119 and anti-mouse CD150 at 1/100 dilution. Use streptavidin conjugated fluorescently labeled antibody.	
16.	Analysis of SP cells can be performed on any cytometry but a UV laser is critical for optimal resolution.	*Samples stained with Hoechst are placed on the cytometer and kept cold by a chilling apparatus if possible.*
17.	Hoechst fluorescence is displayed with Hoechst Blue (450BP filter) on the vertical axis vs. Hoechst Red on the horizontal axis, both in linear mode.	*Voltage adjustments are made so that red blood cells can be viewed in the lower left corner and dead cells which are stained with PI are seen against the far right vertical line. The majority of the cells can be viewed in the center, or in the upper right quarter.*
18.	A sample gate is drawn to exclude red blood cells and dead cells. 50,000-100,000 events should be collected within this sample gate for an un-enriched bone marrow sample.	*The SP prevalence is around 0.01%-0.05% of an un-enriched whole bone marrow in the mouse.*
19.	With a proper Hoechst staining, 60-80% of them are lineage negative and Sca-1 and c-Kit positive. These cells can be further separated for CD150 expression which marks for myeloid-biased HSC population.	*In young mice, 25%-40% of SPKSL cells express CD150 whereas in old mice this percentage is increased to 60%-85%. In order to confirm proper SP staining, verapamil can be included in a control sample.*

References.

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Fig. 1

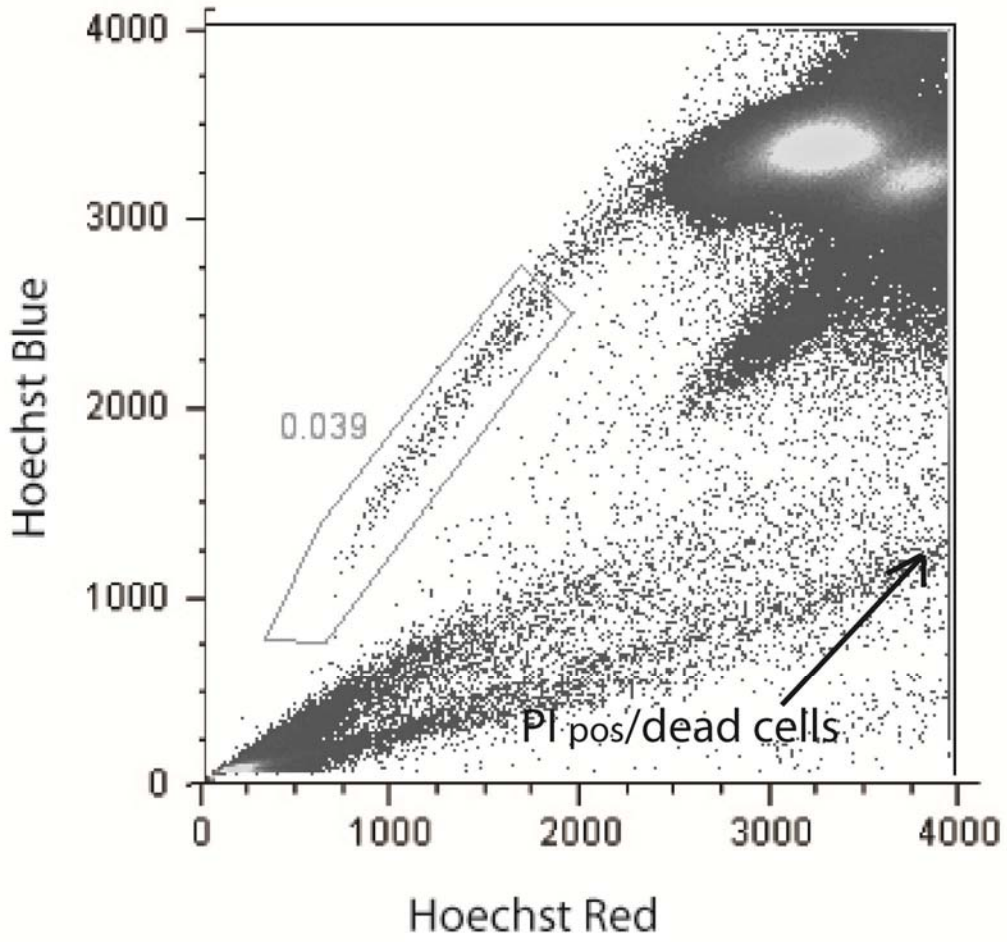

Fig. 1. SP profile of unenriched murine bone marrow sample. Flow cytometric profile of SP population is visualized after staining bone marrow cells with 5 μg/mL Hoechst 3342. Signals are displayed in a Hoechst Blue vs Hoechast Red dot plot. The PMT voltages are adjusted until the majority of cells are at the upper right corner whereas red blood cells and debris are at the lower left

corner. SP cells (~0.02-0.05% of whole bone marrow) are very distinct and small subset of cells at the left side of the plot. PI positive cells (dead cells) are much brighter in the Hoechst Red channel.