

Hoechst 33342 HSC Staining and Stem Cell Purification Protocol

(see Goodell, M., *et al.* (1996) J Exp Med 183, 1797-806)

The Hoechst purification was established for murine hematopoietic stem cells (HSC) on normal C57Bl/6 bone marrow (NBM). We suggest that initial experiments be performed using this marrow exactly as we describe in order to establish the procedure in your laboratory and to definitively identify the side population (SP) on the flow cytometer.

Hoechst Staining of C57Bl/6 bone marrow

Note that the ability to discriminate Hoechst SP cells is based on the *differential efflux* of Hoechst 33342 by a multi-drug-like transporter. This is an *active biological process*. Therefore, optimal resolution of the profile is obtained with great attention to the staining conditions. The Hoechst concentration, staining time, and staining temperature are all **CRITICAL**. Likewise, when the staining process is over, the cells should be maintained at 4°C in order to prohibit further dye efflux. If you adhere rigorously to the protocol below, you should easily find SP cells.

- 1) Ensure that a water bath is at precisely 37°C (check this with a thermometer!). Pre-warm DMEM+ (see below) while preparing the bone marrow.
- 2) Using mice 5-8 weeks of age, prepare bone marrow from femurs and tibias and resuspend in HBSS+ (see below).
- 3) Count the nucleated cells accurately. We find an average of 5×10^7 nucleated cells per C57Bl/6 mouse. This number varies from strain to strain.
- 4) Spin bone marrow down. Resuspend at **10^6 cells per ml** in pre-warmed DMEM+. Mix well.
- 5) Add Hoechst to a final concentration of $5 \mu\text{g}/\text{ml}$ (a 200x dilution of the stock).
- 6) Mix the cells well, and place in the 37°C water bath for 90 minutes EXACTLY. Make sure the staining tubes are well submerged in the bath water to ensure that the temperature of the cells is maintained at 37°C. Tubes should be mixed several times during the incubation.

We find staining large amounts of bone marrow most convenient in Corning 250 ml polypropylene centrifuge tubes. Because of the sensitivity of the staining to temperature, DO NOT use a water bath which is constantly fluctuating in temperature due to heavy use. Water baths next to your tissue culture hoods are

constantly dropping temperature as your friends put 500 ml bottles of ice cold medium in, or worse, 500 ml of frozen serum.

- 7) After 90 minutes, spin the cells down in the **COLD** and re-suspended in **COLD HBSS+**.
- 8) At this point samples may be run directly on the FACS or further stained with antibodies*. All further manipulations **MUST** be performed at **4°C** to prohibit leakage of the Hoechst dye from the cells. Magnetic enrichments may also be employed at this stage if the entire procedure is carried out at 4°C. Alternatively, perform magnetic enrichments prior to Hoechst staining.
- 9) At the end of the staining, resuspend bone marrow cells in cold HBSS+ containing 2 $\mu\text{g}/\text{ml}$ **propidium iodide** (PI) for dead cell discrimination. This is not required to see the SP cells, but will help. Hoechst is somewhat toxic to the bone marrow, and the PI will allow you exclude the dead cells from the profile (see Figure 1).

*Antibody Staining of Hoechst-stained cells

In order to confirm to your satisfaction that you have the correct population, you may want to co-stain the Hoechst-stained bone marrow with antibodies. We find the mouse SP population to be very homogeneous with respect to cell surface markers. We recommend staining with two antibodies, one which positively stains most SP cells (Sca-1 or c-kit) and one which does not stain SP cells but stains a large fraction of the bone marrow (for example Gr-1, or B220). These antibodies are available from Pharmingen. We suggest Sca-1-FITC and Gr-1-PE. Figure 2 shows typical staining of whole marrow and SP cells with these markers.

Hoechst 33342

We obtain from Sigma (called Bis-Benzimide) as a powder and resuspend at 1 mg/ml in water, filter sterilize, and freeze in small aliquots. Since Hoechst is not costly, there is no reason to reuse old or frozen dye.

HBSS+

Hanks Balanced Salt Solution (from Gibco) with 2% Fetal Calf Serum and 10 mM HEPES buffer (Gibco).

DMEM+

DMEM (Gibco) with 2% Fetal Calf Serum and 10mM HEPES buffer (Gibco).

Propidium Iodide

We obtain from Sigma. Our frozen stock is at 10 mg/ml in water. Our working stock (covered with aluminum foil and kept in the fridge) is at 200 micrograms/ml in PBS. Final concentration of PI in your sample should be 2 micrograms/ml.

Other Species

The optimal Hoechst-staining protocols are similar for multiple species. We found 90 minutes to be optimal for mouse SP cells, whereas *120 minutes is optimal for human*, rhesus, and swine cells. Follow the protocol exactly as described above, but stain the bone marrow for 120 minutes.

Storage of Cells

If bone marrow preparation and sorting cannot be performed the same day, we recommend that the BM be kept in the refrigerator overnight **prior** to Hoechst staining. In our experience, cell viability is best when unficolled bone marrow is kept at 4°C. [Note that mouse marrow does not need to be ficolled] In the morning, ficolled or unficolled bone marrow may be warmed to 37°C, resuspended at 10⁶ cells/ml, and stained with Hoechst as described above. We do not recommend plating the bone marrow on tissue culture plastic and leaving in the incubator overnight.

Flow Cytometry

Set Up

We have now used this set-up on multiple cytometers from both BD (Facstar-plus and Vantage) and Cytomation (MoFlow). You need an ultraviolet laser to excite the Hoechst dye and propidium iodide. A second laser can be used to excite additional fluorochromes (eg. FITC and phycoerythrin with a 488 laser).

The Hoechst dye is excited with the UV laser at 350 nm and its fluorescence is measured with a 450/20 BP filter (Hoechst Blue) and a 675 EFLP optical filter (Hoechst Red) (Omega Optical, Brattleboro VT). A 610 DMSP is used to separate the emission wavelengths. Propidium iodide (PI) fluorescence is also measured through the 675 EFLP (having been excited at 350 nm). Note that PI is much **BRIGHTER** than the Hoechst red signal. Hoechst blue is the standard analysis wavelength for Hoechst 33342 DNA content analysis. We have tried other filter sets/combinations. While others work sufficiently, we have found these to give the best results.

Running

Hoechst-stained cells are placed on the flow cytometer and preferably kept cold by the use of a chilling apparatus. It is not necessary to establish live gates on forward vs. side scatter parameters. First, the Hoechst **BLUE** vs. **RED** profile is displayed, with BLUE (450 BP filter) on the vertical axis and RED (675 LP) on the horizontal axis. With the detectors in **LINEAR** mode, the voltages are adjusted so that the red blood cells are seen in the lower left corner and the dead cells line up on a vertical line to the far right (very bright for the PI wavelength, thus dead cells: SEE FIGURE 1). The bulk of the rest of the cells can be centered. It should be possible to identify a major G0-G1 population with S-G2M cells going off to the upper right corner.

Once you can see a profile similar to that shown in Figure 1, draw a live gate to exclude the red and dead cells. Then, collect a large file within this window. In order to identify the SP region definitively, **50,000-100,000 events MUST be collected within this live gate**. The SP region should appear as shown in the figure. The prevalence is LOW: it is around 0.05% of whole bone marrow in the mouse. In human samples, the prevalence is lower (0.03% of ficolled marrow).

Confirmation

In order to confirm that you have identified the right cells, you can 1) block the population with verapamil, or 2) co-stain with antibodies. Verapamil is used at 50 μ M (buy it from Sigma, and make a 100x stock in 95% ethanol), and is included during the entire Hoechst staining procedure. To confirm the mouse SP population, good antibodies to co-stain with are Sca-1 and Gr-1 or another lineage antigen. Figure 2 shows whole C57Bl/6 bone marrow and SP cells stained with Gr-1 and Sca-1.

For human SP cells, you can also block with verapamil. For antibody staining, you can use CD34 and some other marker. The most consistent feature of human SP cells is their *lack* of CD34 expression. They express low but variable level of CD38.

Other tips for optimal resolution of the multiple Hoechst populations

Since analysis of the Hoechst dye is performed in linear mode, we have found that **good C.V.s are critical**. We perform alignments in linear mode with particles which have a very tight distribution (e.g. DNA Check beads from Coulter). Furthermore, we have used the UV laser in the "first" position for optimal C.V.s (this has the added benefit of allowing thresholding on DNA (Hoechst blue) and thus red blood cells are irrelevant). However, this is not necessary.

In keeping with having good C.V.s, the sample differential pressure must be as low as possible. Preferably, the maximum sample differential pressure is calibrated with your alignment particle. In other words if your C.V. for your alignment particle is 3% with a low differential pressure then determine the maximum differential pressure that will still give you good % C.V. s and do not ever exceed that pressure.

Finally, a relatively high power on the UV laser gives the best CVs. We find 50-100 mW to give the best Hoechst signal. Less power will suffice, but the populations may not be as clearly resolved.

Other comments about Hoechst Fluorescence

Many people have asked us why we even see Hoechst fluorescence in the far red (>675 nm). This is indeed surprising. None of the great flow cytometry textbooks document Hoechst fluorescence out this far. We do NOT think that this represents a separate emission peak for Hoechst fluorescence, but rather the fact that Hoechst stains cells VERY brightly, and we still manage to detect significant signal this far because the overall quantity of signal is so great. However, although you can easily detect a signal,

it is not very bright in the red wavelengths, relative to the blue. The voltage on our red PMT is usually cranked fairly high.

Note that the red signal is NOT propidium iodide. PI positive cells are even brighter than these Hoechst-red cells and can be seen lining up at the far right of the profile (see Figure 1). Of course, if you also have a 488 laser running, you will also see these PI positive cells in the PE and PI channels until you gate them out on the basis of the Hoechst profile.

Given that we DO see red Hoechst fluorescence, what is going on? And why are so many populations resolved? Hoechst is doing several things at once:

- 1) It IS a DNA binding dye, and can be used for DNA cell cycle analysis. Some of the cells that reach into the upper right of your plot are in S-G2M. And if you had a homogeneous population of cells, you should get a simple cell cycle profile if you look at Hoechst fluorescence at only one wavelength (usually the blue).
- 2) Hoechst is pumped out by hematopoietic stem cells. That is why we see the LOW Hoechst fluorescence in the SP population
- 3) Hoechst also has some property that we don't understand, that we think of as a chromatin effect. If you do a literature search, you can find out more about how Hoechst binds AT base pairs and think about how the binding and emission spectra might be affected by chromatin conformation

The best paper we have found that explores this dual wavelength phenomenon in any detail is:

Watson, J. V.; Nakeff, A.; Chambers, S. H.; Smith, P. J. (1985) Flow cytometric fluorescence emission spectrum analysis of Hoechst-33342-stained DNA in chicken thymocytes. *Cytometry* 6 310-5.

GOOD LUCK!

MOUSE BONE MARROW

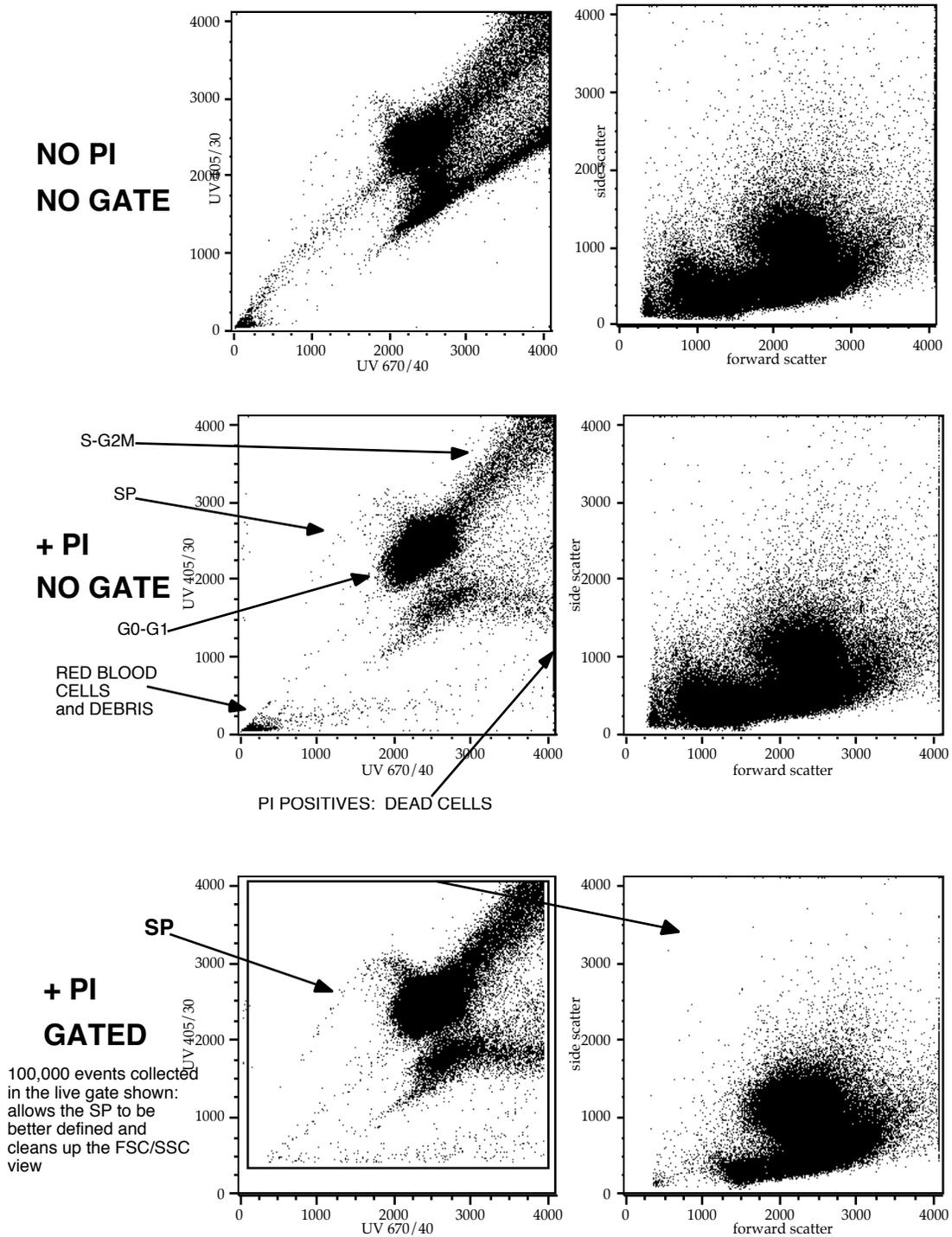
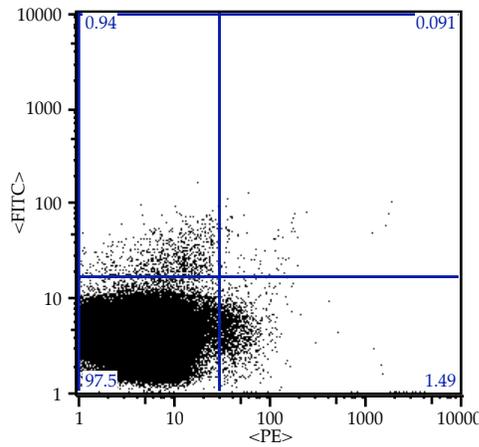


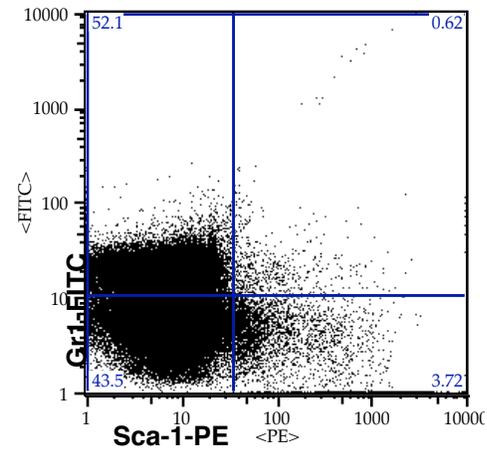
Figure 1

Mouse Bone Marrow (C57BI/6)

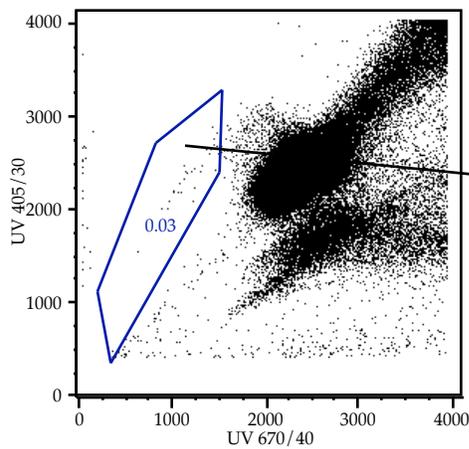
Whole BM NO STAIN (Gated from Fig 1)



Whole BM: Sca-PE+ Gr1-FITC (Gated from Fig 1))



Whole BM



SP Cells

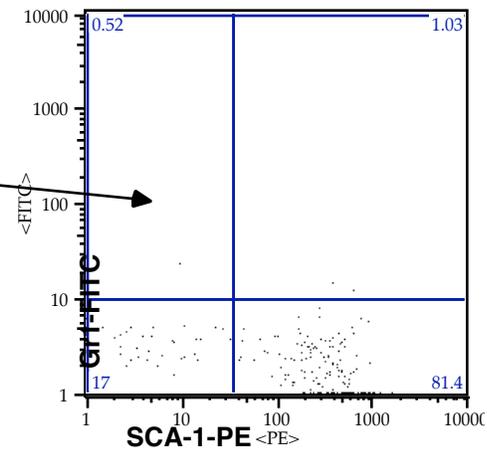


Figure 2