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 μg/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

Other Species

The optimal Hoechst-staining protocols are similar for multiple species. We found 90 minutes to be optimal for mouse SP cells, whereas

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 50μM (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!