Work shop on

Flow Cytometry and Cell Sorting"

(*Jointly organized by CCMB, IICT and Beckman Coulter)*

May 11-12, 2012

Protocol for

Sorting of SP cells from mice bone marrow.

Isolation of Bone Marrow Cells from Mice.

1) Euthanize the mice, 6-8 weeks of age by putting it into desiccator containing Diethyl Ether.
2) Ensure the death of the mice by cervical dislocation in the hood.
3) Wipe the animal with 70% alcohol and dissect it for both femurs and tibias. Wrap in Aluminum foil and keep in ice or 4 ^{0}C.
4) Make holes on both ends of the bones by using 24 gauge needle and flush the bone marrow with pre–warmed 1X HBSS buffer(see appendix). Remove the clumps by agitation or passing through 22 gauge needle.
5) If the suspension is highly red in colour, go for RBC lysis step.(see appendix)
6) Count the cells by using Hemocytometer.
7) Suspend the cells in pre–warmed 1 X HBSS at the concentration of 1 million cells/ml.

Staining of the bone marrow cells for SP

1) Add Hoechst-33342(see appendix) to a final concentration of 5µg/ml
2) Mix the cells well, and place in the 37 ^{0}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 ^{0}C. Tubes should be mixed several times during the incubation. DO NOT use a water bath which is constantly fluctuating in temperature due to heavy use

3). After 90 minutes, spin the cells down in the **COLD (4^0C)** and re-suspended in **COLD** HBSS+.

4) At this point samples may be run directly on the FACS. All further manipulations **MUST** be performed at **4^0C** to prohibit leakage of the Hoechst dye from the cells.

5) At the end of the staining, resuspend bone marrow cells in cold HBSS+ containing 2 µg/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.

Confirmation of SP cells

In order to confirm that you have identified the right cells, you can block the population with verapamil (see appendix). Verapamil is used at 50µM and make a 100x stock in 95% ethanol/ Milli Q), and is included during the entire Hoechst staining procedure.

Flow Cytometry Set Up

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 450/65 BP filter (Hoechst Blue) and a 670/30 BP Filter optical filter (Hoechst Red). A 510 DCLP is used to separate the emission wavelengths. Propidium iodide (PI) fluorescence is also measured through the 670/30 BP filter (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. Set the intra –laser delay on the Trigger Card by measuring the time delay using oscilloscope peak signals using the primary and secondary laser.

Appendix

1X HBSS preparation:

Add 10ml of 10X HBSS, 2 ml of FBS, 1 ml of 1M HEPES and 75 ml of milli Q water and adjust the P^H to 7.2-7.4 with HCl and finally make up the volume to 100ml.

RBC lysis:

- Add 2 ml of RBC lysis buffer (0.84% Ammonium Chloride in water) to the cell pellet.
- Leave for 2 minutes at room temperature.
- Dilute it with cold 1X HBSS to stop the reaction.
- Centrifuge the cells and collect the pellet.

Stock preparation

Hoechst 33342(bis benzimide)- Sigma Cat# 2261

5 mg/ml in Milli Q

Verapamil hydrochloride- Sigma Cat# V4629

2.45 mg/ml in Ethanol

10X HBSS (Hanks Balanced Salt Solution) composition:

0.4gms KCl

0.06gms K_2HPO_4

0.048gms $NaH2PO_4$

8gms NaCl

1gm Glucose

0.35gms Sodium bicarbonate.