

STAINING PROTOCOL FOR FACS

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1. Prepare the suspension single cells.
2. Filter the suspended cells through <70um cell strainer (Cat. 352350) and keep cells on ice.
3. Count the cell concentration.
4. Spin down the filtered cells at 1000~1500rpm for 5-10 min.
5. Resuspend the cells with ice-cold FACS staining buffer (HBSS+, ~2x10⁷ cells/mL if possible).
6. Aliquot 200ul suspended cells for staining. Prepare controls including unstained control, single color controls for FACS compensation.
7. Add 4ul antibody (for example, Pharmingen antibodies for FACS) to cells and slowly rock in 4°C cold room for 15 min.
8. Spin down the cells at 1000~1500rpm for 5-10 min and resuspend cell pellets with 200ul ice-cold HBSS+.
9. Spin down the cells again and resuspend with 200ul ice-cold HBSS+.
10. Add 10ul 7-AAD solution to 200ul cells
11. FACS analysis

Reagents

HBSS+: 1xHBSS, 2% Fetal calf serum, 10mM Hepes pH 7.2, 1% Pen/Strep

C. FACS analysis and sorting

This protocol was tested on MoFlo, BD FACSCalibur and Vantage sorter. An Innova 70 Argon laser was used to excite Rhodamine-123, PE and PI. An Innova Krypton-Argon mixed gas laser was used to excite APC. The protocol is yet to be adopted for a single-laser sorter.

<u>Marker-Dye</u>	<u>FACS channel</u>
Rhodamine-123	FL1
PE-Sca-1	FL2
PI (or PerCP CD45)	FL3
APC-Endoglin	FL4

- 1) Run the unstained cell sample. Adjust FSC (Forward Scatter) and SSC (side scatter) detector setting as well as FL1, FL2, FL3, and FL4 settings. Use FSC and SSC plot to gate out debris, aggregates and the majority of erythrocytes in the mouse bone marrow sample.
- 2) Compensate FL3 based on PE, Rhodamine-123, APC.
- 3) Run the PI stained cell sample, and set a gate to exclude dead cells.
- 4) While gating on the cells of interest, run all other single fluorophore stained samples. Set compensation based on single fluorophore stained samples.
- 5) Set a gate for Endoglin and Sca-1 double positive cells (Endo⁺ Sca-1⁺) using negative gate defined by single color controls. Typically, Endo⁺ Sca-1⁺ cells account for about 0.1% of the live nucleated bone marrow cells.
- 6) Set a Rhodamine-123 low gate. For this gate, we found it is more helpful to examine the Sca-1 PE/Rhodamine-123 FACS plot (Figure 5B) than to examine a Rhodamine-123 histogram plot. If proper compensation was done, the Sca-1⁺ Rh^{Med/Hi} (upright of Figure 5B) population should define an up-limit boundary for Rh^{Low} population. We found that this FACS profile is quite consistent between different experiments while the absolute fluorescent intensity of Rhodamine- 123 may vary.
- 7) Examine the Endo⁺ Sca-1⁺ Rh^{Low} population on either a Sca-1 PE/Rhodamine-123 FACS plot or a Rhodamine-123 histogram plot that are gated on live, nucleated, Endo⁺ Sca-1⁺ cell populations. The frequency of Rh^{Low} cells is typically 0.5 1% of the Endo⁺ Sca-1⁺ cell population.
- 8) Since only 0.1% of bone marrow cells are Endo⁺ Sca-1⁺ cells, the frequency of Endo⁺ Sca-1⁺ Rh^{Low} cells is less than 1 in 10⁵ of the total live nucleated

bone marrow cells. Under this condition, the majority of the Endo⁺ Sca-1⁺ Rh^{LOW} cells are Lin^{-/LOW} or CD34^{-/LOW} (Figure 5).

- 9) Endo⁺ Sca-1⁺ Rh^{LOW} cells can be resorted to further increase the purity.