

Side population Analysis - Dye Cycle Violet Stain

Development and Principles of the Side Population Assay:

In the late 1990's, Peggy Goodell and colleagues discovered the existence of a small homogenous populations of cells in murine, swine, rhesus and human bone marrow, with the capability to efflux the DNA-binding dye Hoechst 33342 in dual wavelength flow cytometric analysis experiments. Goodell termed these cells SP (side population) cells. These cells were CD34⁻ and lineage-marker negative but when cultured on bone marrow stroma, they formed colonies of cells that became CD34⁺ indicating that these cells are primitive hematopoietic progenitor cells. SP analysis is now a common technique in the identification and sorting of stem cells and early progenitors.

Hoechst 33342 side population analysis requires an ultraviolet laser for maximum excitation. Unfortunately, ultraviolet lasers are expensive. Violet diode lasers are less expensive. The cell-permeable DNA binding dye, Dye Cycle Violet (DCV) can be excited by a violet laser. SP cells will efflux DCV just as efficiently as they efflux Hoechst 33342.

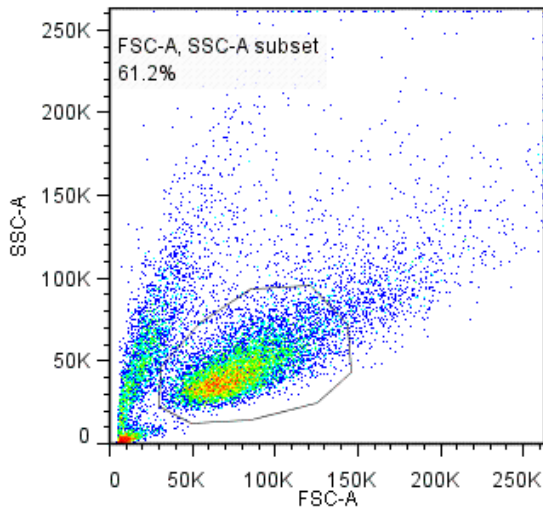
Hoechst and DCV SP efflux occurs because the ABCG2 transporter pumps out the dye in the stem cells. The compounds verapamil and fumetrimogin C have been demonstrated to block the ABCG2 transporter and make excellent controls for identifying SP cells by blocking the dye efflux.

Reagents and Protocol:

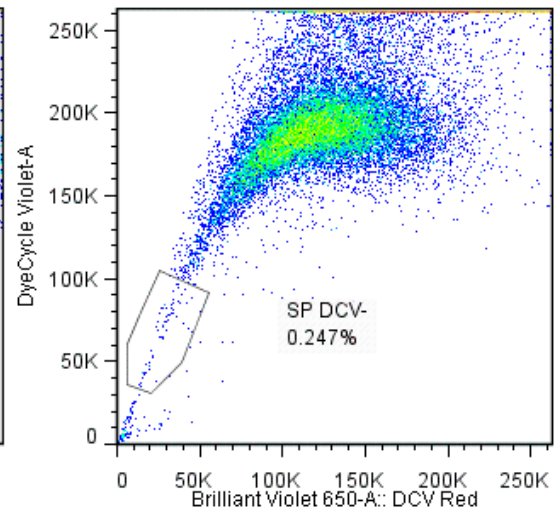
1. Cells are re-suspended at a density of 1×10^6 cells/ml in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 mM HEPES (pre-warmed to 37°C). Add verapamil 200 μ M, treat for 30 min.
2. Add Vybrant DyeCycle Violet Stain (DCV, Invitrogen cat # V35003) 2 μ l to each tube, stain for 30min. If cell viability is a concern, 7-AAD (1 μ g) can be added to gate out dead cells.
3. Wash cells twice with 1XPBS (RT). 1250 rpm, 5 min, RT.
4. Re-suspend at a density of 1×10^6 cells/ml in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 mM HEPES (pre-warmed to 37°C) for 1 h.
5. Centrifuge the cells and re-suspend with ice-cold HBSS/2%FBS/10mMHEPES buffer. Put on ice.
6. Analyze on the LSR II, using the 405 nm violet laser with the 450 nm \pm 50 nm DCV channel and the 660 \pm 40 nm BV 650 channel. Analyze on the Aria II, using the 405 nm violet laser with the 450 nm \pm 40 nm DCV channel and replace the second violet laser channel with a 635 nm longpass dichroic mirror and a 660 \pm 20 nm bandpass filter. All DCV channels should be run in the linear setting.

LSR II DCV SP Sample Data, MT2 Cells:

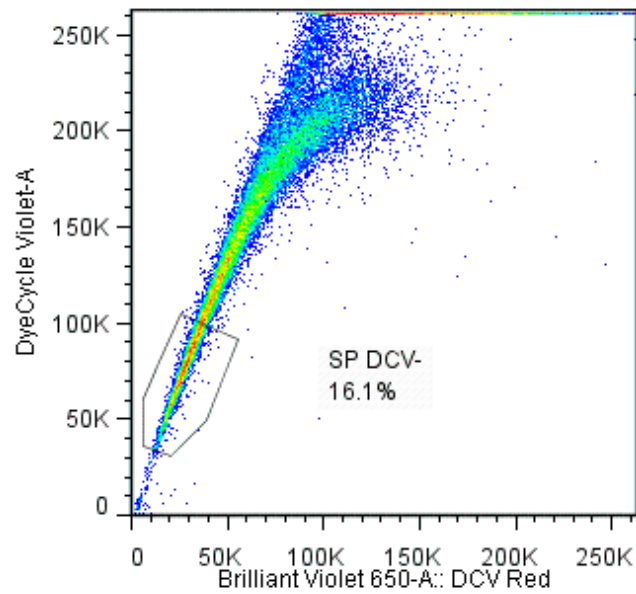
Gate on Live Cells:



Block SP efflux with Verapamil:

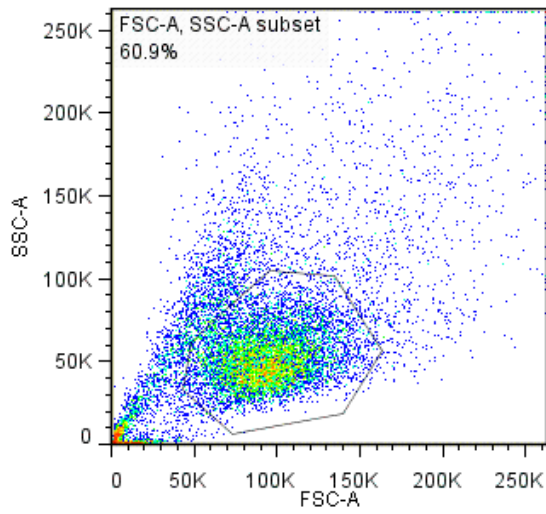


DCV only:

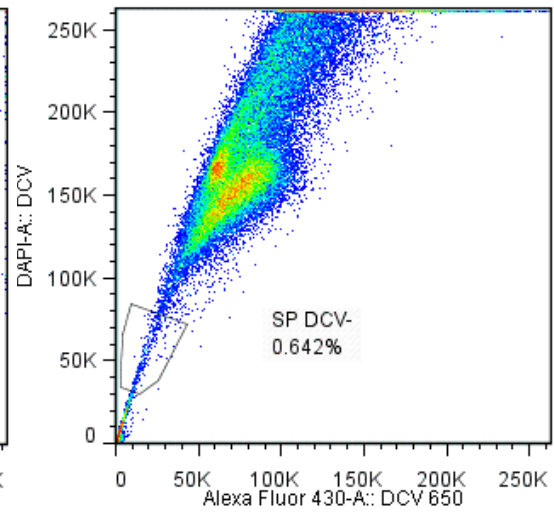


Aria II DCV SP Sample Data, ED Cells:

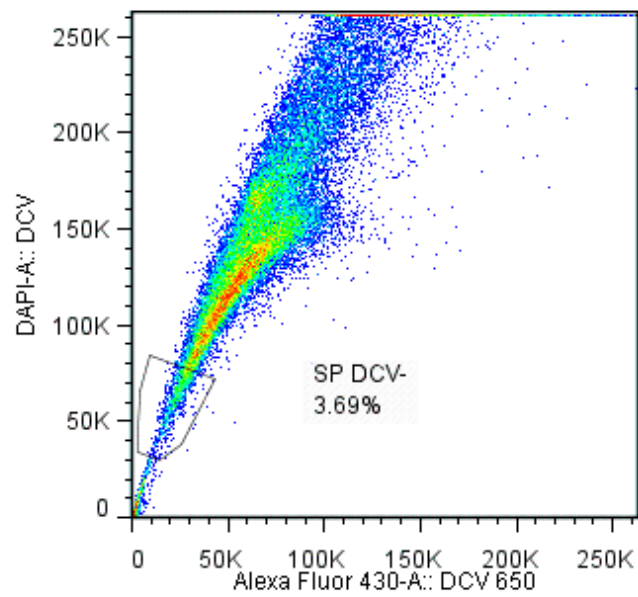
Gate on Live Cells:



Block SP efflux with Verapamil:



DCV only:



Notes on the technique:

The violet laser alignment is critical, the flow core will check the alignment with Spherotech Rainbow beads in the 450 nm \pm 50 nm channel on the LSR II and the 450 nm \pm 40 nm channel on the Aria II.

The LSR II has a 100 mW violet 405 nm laser; the Aria II has a 30 mW violet 405 nm laser. The extra laser power in the LSR II is quite helpful in discriminating the SP from DCV+ cells. If you need to sort, please do a preliminary experiment to make sure we can detect the SP on the Aria II.

You may want to probe your cell line for ABCG2 expression as high ABCG2 expression is necessary for SP analysis.

References:

1. Goodell, M. A. et al., Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species, *Nature Medicine*, 3, 12, pp 1337-1345; 1997.
2. Telford, W. G. et al. Side population analysis using a violet-excited cell-permeable DNA binding dye, *Stem Cells*, 25, pp 1029-1036; 2007.
3. Mathew, G. et al. ABCG2-mediated dye cycle violet efflux defined side population in benign and malignant prostate, *Cell Cycle*, 8, 7, pp 1053-1061; 2009.
4. Bleau, A. M. et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells, *Cell Stem Cell*, 4, pp 226-235; 2009.
5. Telford, W. G., Stem cell side population analysis and sorting using dye cycle violet, *Current Protocols in Cytometry*, 9, 30, pp 1-9; 2010.