Identification and Functionality of Adult Mouse Hematopoietic Stem Cell Side Populations after Enrichment on the BD FACSAria II Flow Cytometer Equipped with a 375-nm Near UV Laser

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Application Note

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Abstract

Cells of the hematopoietic system develop in the bone marrow from a population of self-renewing progenitor cells known as hematopoietic stem cells (HSCs). These cells make up only 0.01–0.10% of bone marrow, and a recent focus of stem cell biology has been isolating, characterizing, and using these HSCs to replenish normal bone marrow function.

Goodell and coworkers have described a fluorescence-activated cell sorting (FACS) technique to identify the most primitive HSCs present in human, murine, and primate bone marrow based on their specific ability to efflux the supravital dye Hoechst 33342.[1] This population of HSCs, called "side population (SP) cells", is able to replenish normal bone marrow function in vivo. These cells have traditionally been identified using a flow cytometer fitted with a 350-nm UV laser. Recently, however, Telford and Frolova determined that SP cells can also be identified by exciting the Hoechst dye with a 375-nm near UV laser.[2]

Osawa et al, developed an alternative method to identify and purify the HSCs based on cell surface marker expression.[3,4] This combination of markers is composed of CD34$^{-/low}$ c-kit^{+} Sca-1^{+} Lin^{-}, and the cells are referred to as CD34KSL. Using these markers, it is possible to achieve a high degree of HSC enrichment of an extremely small and homogeneous population of HSCs with a long-term capability for self renewal (LT-HSCs).

Here we present a protocol developed to isolate and purify LT-HSCs on the basis of Hoechst dye efflux in combination with KSL surface staining. We used the BD FACSAria™ II flow cytometer equipped with a 375-nm near UV laser. Purified cells were then assayed for the presence of colony-forming cells (CFCs) using a CFC assay that demonstrated the presence of one or more types of hematopoietic lineage cells within the colonies generated.[5]

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Introduction

The ability to characterize and isolate stem cell populations and monitor the dynamic changes that occur in both intracellular and surface proteins during differentiation is essential to understanding underlying developmental pathways. A main focus in stem cell biology has been isolating, characterizing, and using hematopoietic stem cells to replenish normal bone marrow function. Cells of the hematopoietic system develop in the bone marrow from a population of self-renewing progenitor cells known as hematopoietic stem cells (HSCs).

Flow cytometry and cell sorting are valuable research tools for using cellular markers to isolate and characterize HSCs. HSCs represent approximately 1 in 10^3–10^4 bone marrow cells in the adult mouse. Goodell and coworkers have described a method using dual wavelength flow cytometry, which defines a small (0.01–0.10%) population of the most primitive HSCs that selectively efflux the supravital dye Hoechst 33342 (bis-benzimide) in murine, primate, and human bone marrow, as well as in a variety of other tissues.[1] The cells of the population of HSCs identified by this method are called side population (SP) cells. Since the enriched SP cells can be used to replenish normal bone marrow function, it is important that SP cells are functional after sorting. This can be assessed using a colony-forming cell (CFC) assay to investigate the growth of lineage-restricted colonies.

Traditionally, these cells have been enumerated by exciting Hoechst-labeled SP cells with the 350-nm line of a UV laser and simultaneously measuring blue and far red light emission. Experiments performed in 2003 by Telford and Frolova[2] at the National Institutes of Health (NIH) determined that SP cells could also be analyzed by exciting the Hoechst (HO) dye with a 375-nm near UV laser.

Recently, another method that uses a combination of cell surface markers has also proven useful for HSC purification. This combination of markers is composed of $CD34^{-/low}$ $c\text{-}kit^{+}$ $Sca\text{-}1^{+}$ Lin^{-}, and is referred to as CD34KSL. In mice, the majority of hematopoietic activity takes place within this rare bone marrow population. It has been reported that using this phenotype yields one of the highest degrees of HSC enrichment of an extremely small and homogeneous population with a long-term capability for self renewal (LT-HSCs).[3,4] In this proof of principle study, we present a protocol developed to isolate and purify prospective LT-HSCs on the basis of Hoechst dye efflux in combination with KSL surface staining. We use the 375-nm near UV laser option on a BD FACSAria II flow cytometer.

In adult mouse bone marrow, a small number of HSCs produce heterogeneous populations of actively-dividing hematopoietic progenitors that proliferate and differentiate into mature blood cells. When cultured in a semi-solid matrix, individual progenitors called colony-forming cells (CFCs) proliferate to form discrete cell clusters or colonies. To assess the functionality of enriched cells, a CFC assay was performed. Here we show that one or more types of hematopoietic lineage cells within the colonies generated from enriched cells were identified using flow cytometric methods.

Objective

The objective of this application note is to demonstrate that the BD FACSAria II cell sorter, equipped with a 375-nm near UV laser, can be used to identify and enrich functional mouse HSC side populations from mouse bone marrow by identifying HSCs through their ability to efflux Hoechst dye in combination with cell surface marker expression.

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Methods

Reagents and materials

Product Number	Product Description	Vendor
555815	BD Via-Probe™ solution	BD Biosciences
352070	50-mL centrifuge tubes	BD Biosciences
309585	3-mL syringe	BD Medical
309602	1-mL syringe	BD Medical
305143	23G 3/4 needle	BD Medical
553129	Ly-6G and Ly-6C-APC	BD Biosciences
553142	Purified Rat anti-mouse CD16/CD32 (Mouse BD Fc Block™)	BD Biosciences
553355	CD117 (c-kit) PE (Clone 2B8)	BD Biosciences
553733	CD34 (gp105-120) FITC (Clone RAM34)	BD Biosciences
554657	Stain Buffer (BSA)	BD Biosciences
557397	CD11b (Integrin α_M chain) PE	BD Biosciences
558074	Lineage Cocktail APC with Isotype control (CD3e clone 145-2C11, CD11b clone M1/70, CD45R/B220 clone RA3/6B2, Ly-6G and Ly-6C clone RB6-8C5, TER-119 clone TER-119)	BD Biosciences
558162	Ly-6A (Sca-1) PE-Cy™7 (Clone D7)	BD Biosciences
H1399	Hoechst 33342, trihydrochloride, trihydrate (Molecular Probes)	Invitrogen
07700	Iscove's MDM with 2% FBS	StemCell Technologies
03534	MethoCult® GF M3534	StemCell Technologies
10-013-CV	Dulbecco's Modified Eagles Medium (DMEM) (Mediatech)	VWR
20-031-CV	Dulbecco's Phospate-Buffered Saline (DPBS), (10X) (Mediatech)	VWR
25900-CI	Trypan Blue, 0.4% Solution (Mediatech)	VWR
RLBSA-30	BSA, Fraction V, 30% solution (Rockland Immunochemical)	VWR
BDH1164-4LP	Ethanol, denatured solution (BDH)	VWR
SH30237.01	HEPES Free Acid, 1M solution (HyQ)	VWR
16777-230	Fetal Clone I (FBS) (Thermo-Fisher Scientific)	VWR
25384-326	VWR petri dish, sterile	VWR

Instruments

The stained cells were sorted and analyzed on a BD FACSAria II cell sorter equipped with a 488-nm laser, a 633-nm laser, and the 375-nm near UV laser option. The 375-nm near UV laser option includes a 670 LP filter to detect Hoechst red and a 450/20 BP filter to detect Hoechst blue, with a 610 DM LP filter between the two detectors. The cytometer was also equipped with the Temperature Control Option. Analysis and sorting were performed using BD FACSDiva™ software.

Mice

C57BL/6 mice 8 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, Maine), and bone marrow cells were obtained from both femurs and tibias.

Methods

Isolation of bone marrow cells
Mouse bone marrow cells were isolated as outlined in the MethoCult Technical Manual.[5] Cells were resuspended at 1 x 10^6 cells/mL in DMEM + 10% FBS.

Cell staining

Bone marrow cells were stained with Hoechst dye by incubation in 5 µg/mL of HO 33342 for 90 minutes at 37°C, as described by Goodell et al.[1] Cells were washed twice (335*g*, 10 min, 4°C) in cold DPBS + 2% FBS + 10 mM of HEPES. Surface Mouse Fc receptors were blocked by incubating the cell pellet with 5 µL of purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) for 5 minutes on ice. The HO-stained bone marrow cells were then co-stained with the recommended amounts of surface markers CD34, c-kit, Sca-1, and Lineage Cocktail (CD34KSL) for 30 minutes. Cells were washed (335*g*, 10 min, 4°C), resuspended in cold HBSS + 2% BSA, and stored on ice until use.

Enrichment of HSCs using FACS

Labeled cells were loaded onto the BD FACSAria II cell sorter and analyzed as shown in Figure 1, panel A. This analysis was performed to confirm the Hoechst labeling of the bone marrow cells and that HSCs could efflux the supravital dye HO 33342. The gating strategy used to enrich SP/KSL cells in the mouse bone marrow is outlined in Figure 1. Cells were discriminated using scatter and viability (panels B and C) and doublet discrimination (panels D and E). The cells were then gated for Lin⁻(panel F) and Sca-1⁺ c-Kit⁺ expression (panel G). The final sorting gate (SP) was set on the HO plot (panel H).

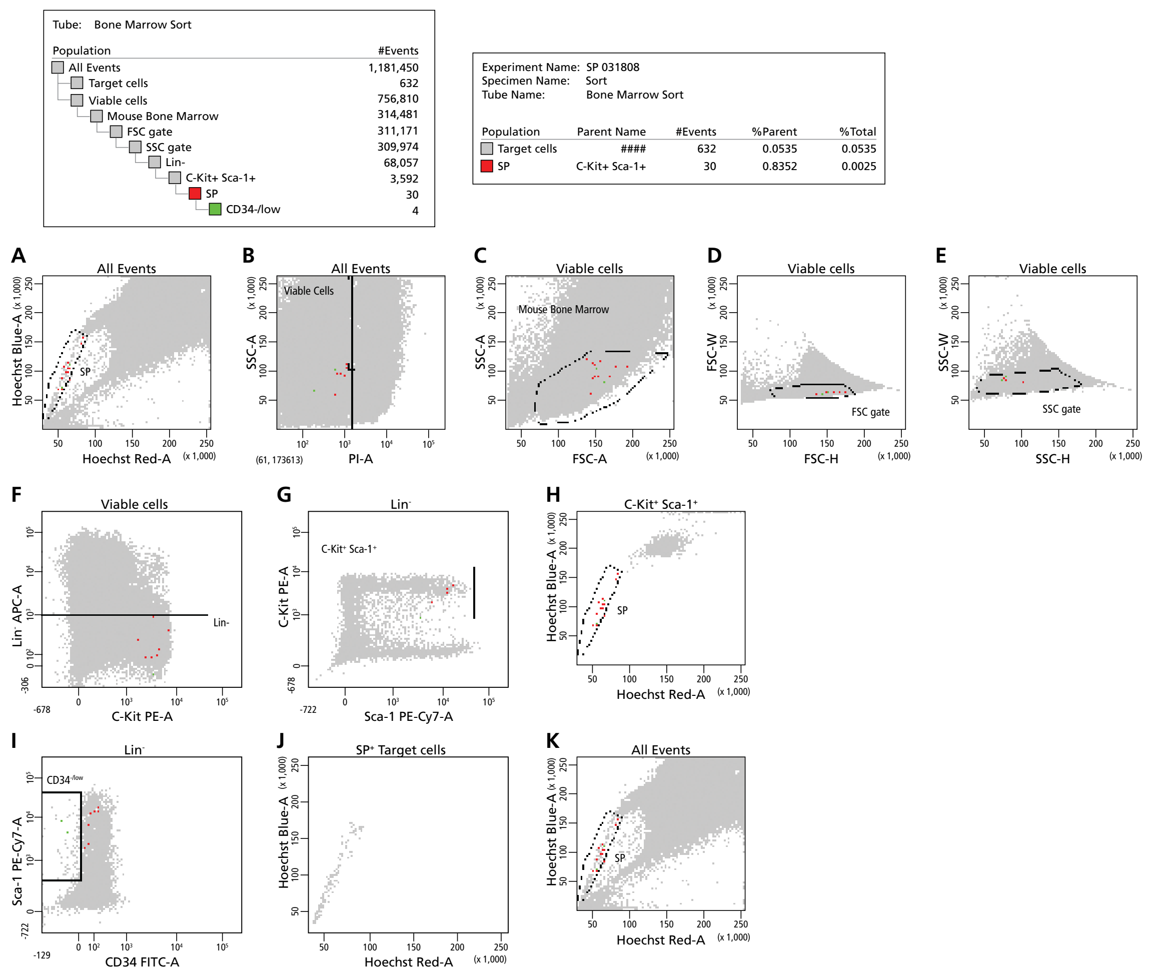

Figure 1: Analysis of mouse bone marrow (panel A) gating strategy used to enrich SP/KSL cells (panels B through H) and analysis of the enriched cell population (panels I through K).

The cytometer was set up to sort using a 70-µm nozzle at 35 psi with a frequency of 60 kHz. To maintain high purity, the SP cells were sorted using a maximum purity sort mode, with a one-drop envelope. To keep both the cell sample and the sort collection tubes chilled, the sample temperature and temperature control option were maintained at 4°C.

Analysis of sorted cells

Flow cytometric analysis of the enriched cells was used to check the CD34 status and Hoechst dye efflux ability as shown in Figure 1, panels I through K.

Colony-forming assays (CFC assays)

To assess the functionality of the enriched SP/KSL cell population, mouse colony-forming cell assays were performed using the Mouse Colony-Forming Cell Assay MethoCult® GF M3534 (StemCell Technologies).[5] This product is an assay for mouse clonogenic hematopoietic progenitor cells in samples of mouse bone marrow and other hematopoietic tissues. Three different cultures were set up to determine the presence of granulocyte (CFU-G), macrophage (CFU-M), and granulocyte and macrophage (CFU-GM) progenitors by the addition of appropriate growth factors or cytokines as outlined in the Technical Manual.[5]

Enriched SP/KSL cells were cultured for 12 to 14 days according to the manufacturer's protocol.[5] After the 12 to 14-day culture period, individual cultures (CFU-G, CFU-M, and CFU-GM) were assessed visually and photographs taken. Cells were then harvested from the culture. Culture dishes were placed in the refrigerator for two hours to liquefy the methylcellulose. One mL of Iscove's MDM + 2% FBS was added to each dish, and the suspension was slowly pipetted up and down to mix the methylcellulose and medium. The contents of each dish culture condition (CFU-G, CFU-M, and CFU-GM) were pooled in a 50-mL centrifuge tube, and the cells were washed in Iscove's MDM + 2% FBS (335*g*, 10 min, 4°C).

Analysis of colonies

The pooled cells were counted using the Trypan blue exclusion method, resuspended in stain buffer (FBS) at a concentration of 1×10^6 cells/mL, and then stained for the presence of stem cell–derived granulocyte progenitors using anti-mouse Ly-6G and Ly-6C APC, and granulocyte and macrophage progenitors using anti-mouse CD11b PE. Flow cytometric acquisition and analysis were performed using a BD FACS Canto II™ system and BD FACSDiva software v6.

Results

Enrichment of HSCs using FACS

As shown in Figure 1, panel A, the side population target cells with the ability to efflux the supravital dye Hoechst 33342 composed 0.00535% of the nucleated cells found in the original bone marrow population.

After enrichment, when the Lin^- cells (panel F), $Sca\text{-}1^+$ $c\text{-}kit^+$ cells (panel G), and Hoechst dye efflux active (panel H) enriched SP cells (SP/KSL) were analyzed on the BD FACSAria II instrument (using the same settings as those used in the cell sorting), the SP cells overlapped the original target cell population (panels J and K). More than 97% expressed the SP/KSL phenotype and composed 0.0025% of the nucleated cells found in the original bone marrow population (panel K). A small number of these SP/KSL cells were also $CD34^{-/low}$ (panel I).

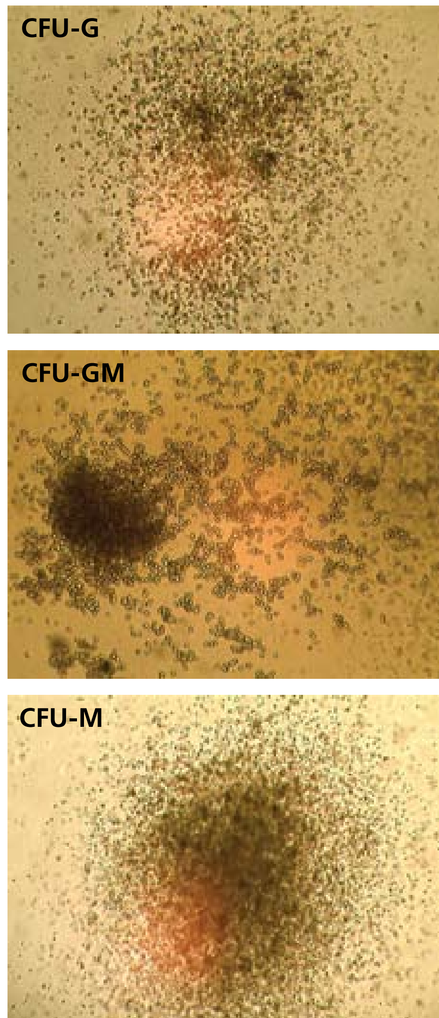

Figure 2: Photographs of mouse hematopoietic colonies.

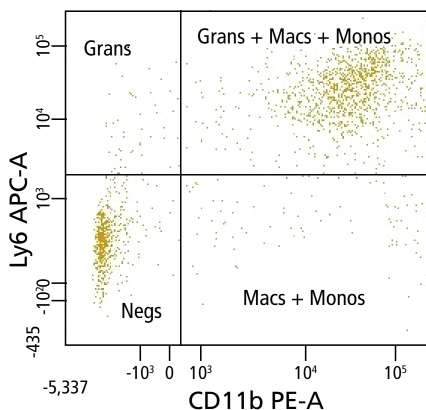

Figure 3: Phenotypic analysis of cultured SP/KSL cells.

Colony forming ability of the isolated SP/KSL cells

As shown in Figure 2, the sorted SP/KSL cells formed cell colonies when cultured in MethoCult GF M3534 medium supplemented with growth factors and/or cytokines to support the growth of granulocyte, macrophage, or granulocyte and macrophage progenitors. These colonies were visually similar to the reference photographs provided by StemCell Technologies in the Mouse Colony-Forming Assay Technical Manual.[5]

Phenotypic analysis of cultured SP/KSL cells

As shown in Figure 3, phenotypic analysis of the progenitor cells isolated from the different cultures confirmed that the cultures were composed of the expected granulocyte and macrophage lineages: 65.7% of the cells stained positive for Ly-6G/Ly6C and CD11b, indicating that the majority of the progenitor cells within the colonies were either granulocytes, macrophages, or a mixture.

Discussion

Previous studies have shown that CD34 KSL cells are responsible for most of the hematopoietic activity within mouse bone marrow and may mark the LT-HSCs.[3-5] In adult mouse bone marrow, these LT-HSCs produce heterogeneous populations of actively-dividing hematopoietic progenitors that proliferate and differentiate into mature blood cells. When cultured in a semi-solid matrix, individual progenitors called CFCs proliferate to form discrete cell clusters or colonies.

In this application note, we demonstrated that we have developed a protocol for the isolation of HSCs using a combination of cell surface markers that are unique to HSCs, in combination with Hoechst dye efflux and use of the 375-nm near UV laser option on the BD FACSAria II cell sorter. The data demonstrates that the SP cells are phenotypically c-kit^{+}Sca-1^{+}Lin^{-} (KSL), showing that this strategy can be used for the isolation and enrichment of a rare and homogeneous population of potential LT-HSCs that have the ability to differentiate into hematopoietic cell lineages (CFU-G, CFU-GM, and CFU-M).

We had anticipated that the fresh SP/KSL cells would have been CD34$^{-/low}$, and we did see a very small number of SP/KSL cells expressing this phenotype. However, the majority of the SP/KSL cells were found to be CD34^{+}. The expression of CD34 depends on the developmental stage of the mouse, and CD34 is known to be expressed on HSCs of mice younger than 8 weeks of age. It appears that the mice used in this study might have been slightly younger than 8 weeks of age, thus explaining the CD34^{+} expression observed.[6]

The relationship between SP cells and HSCs isolated on the basis of cell-surface markers has not been thoroughly described.[7] Further isolation studies with the inclusion of the Single Lymphocyte Activation Molecule (SLAM) family of surface markers (CD150, CD244, and CD48) should be performed to determine if the addition of these markers can not only increase HSC purity, but lead to the establishment of a more definitive protocol for the isolation and study of the most primitive HSCs contained within murine bone marrow.[8]

***Note:** CD34 expression of LT-HSCs in mouse bone marrow*

Caution should be observed when using CD34 for the identification and isolation of LT-HSCs, since the level of CD34 expression depends on the developmental stage of the mouse. CD34 is expressed on HSCs of mice younger than 8 weeks of age. This expression changes to CD34$^{-/low}$ at 8 weeks of age and becomes CD34^{-} in mice older than 8 weeks. See Purton and Scadden for more guidance in this area.[6]

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Conclusions

The data presented in this application note demonstrates that:

- A BD FACSAria II cell sorter, equipped with a 375-nm near UV laser, can be used to identify and enrich functional mouse hematopoietic stem cell side populations from mouse bone marrow that have been stained with HO 33342.
- When the HO 33342 staining is combined with the cell surface markers c-kit, Sca-1, and Lin, a pure and homogenous population of hematopoietic stem cells can be obtained (SP/KSL).
- The enriched SP/KSL population contains granulocyte and macrophage progenitors as demonstrated using a mouse colony-forming assay followed by phenotypic analysis of the cells generated.
- Further isolation studies with the inclusion of other cell surface markers (SLAM family) could be performed to determine if the addition of these markers can not only further increase HSC purity but lead to a more definitive protocol for the isolation of the most primitive HSCs contained within the bone marrow.

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