

Chapter 3

Hematopoietic Stem Cell Characterization and Isolation

**Lara Rossi, Grant A. Challen, Olga Sirin, Karen Kuan-Yin Lin,
and Margaret A. Goodell**

Abstract

Hematopoietic stem cells (HSCs) are defined by the capabilities of multi-lineage differentiation and long-term self-renewal. Both these characteristics contribute to maintain the homeostasis of the system and allow the restoration of hematopoiesis after insults, such as infections or therapeutic ablation. Reconstitution after lethal irradiation strictly depends on a third, fundamental property of HSCs: the capability to migrate under the influence of specific chemokines. Directed by a chemotactic compass, after transplant HSCs find their way to the bone marrow, where they eventually home and engraft.

HSCs represent a rare population that primarily resides in the bone marrow with an estimated frequency of 0.01% of total nucleated cells. Separating HSCs from differentiated cells that reside in the bone marrow has been the focus of intense investigation for years. In this chapter, we will describe in detail the strategy routinely used by our laboratory to purify murine HSCs, by exploiting their antigenic phenotype (KSL), combined with the physiological capability to efficiently efflux the vital dye Hoechst 33342, generating the so-called Side Population, or SP.

Key words: Hematopoietic stem cells, Side population, Hoechst 33342, c-Kit⁺ Sca-1⁺ Lineage⁻ cells (KSL)

1. Introduction

HSCs represents by far the most extensively studied population of stem cells in the adult. In particular, the murine model represents an excellent investigation system, where putative HSCs can be tested for long-term reconstitution of the lympho-hematopoietic system in lethally irradiated recipients. As demonstrated by the first transplantation assays performed decades ago, the hematopoietic activity resides primarily in the bone marrow. However, the cellular composition of the bone marrow is extremely

heterogeneous and includes different populations of progenitors that can be hierarchically organized according to their self-renewing and differentiation potential. Long-term HSCs (LT-HSCs) represent the foundation pillars of hematopoiesis: their ability to self-renew indefinitely guarantees the homeostatic and continuous turn-over of blood cells that organisms require throughout life. LT-HSCs can also give rise to short-term HSCs (ST-HSCs), whose extensive proliferation and differentiation contributes to generate multipotent progenitors (MMPs) and all the downstream progenitors that will eventually produce terminally differentiated blood cells. Conversely to the subset of quiescent LT-HSCs, the highly proliferative ST-HSCs and MMPs, when transplanted, can only sustain hematopoiesis in the short-term and rapidly exhaust. Furthermore, deeper investigations have shown that the hematopoietic hierarchy might be more complicated than originally thought. Dykstra et al. (1) assessed single HSCs by serial transplantation and retrospectively classified them based on their pattern of peripheral blood reconstitution. Their analysis proved that even the LT-HSC compartment is a heterogeneous and multifaceted entity, comprising cells that are partly biased toward myeloid or lymphoid phenotypes. Identifying the rare cell population, on which the hematopoietic homeostasis is elegantly built, represents therefore one of the major challenges in the field (2–4). Nonetheless, despite the numerous efforts, a single specific marker, that can be employed alone to isolate HSCs, has yet to be discovered. Hence, investigators must turn to combinations of different markers or physiological properties. Benefiting from the advances in multicolor flow cytometry and monoclonal antibody development, several laboratories have proposed over the last two decades different isolation schemes that, however, lead to extremely similar HSC populations (5, 6).

Among the principal criteria utilized for HSC identification and isolation is the expression, or lack of expression, of specific cell surface markers. The isolation of one of the most thoroughly characterized populations of HSCs relies on the positive expression of the tyrosine kinase receptor c-Kit (CD117) and the membrane glycoprotein Sca-1 (7), concomitantly with the lack of markers of terminal differentiation (Ter119, Gr-1, Mac-1, B220, CD4, and CD8), collectively known as Lineage markers. The resulting c-Kit⁺ Sca-1⁺ Lineage⁻ population, commonly referred to as KSL cells, contains cells capable of hematopoietic reconstitution. However, different studies showed that the KSL fraction contains a variety of progenitors, including ST-HSCs. Thanks to the contribution of different groups, schemes to further enrich the KSL fraction in HSCs have been developed over time. These strategies are based on either the combination with other surface markers, such as Thy1.1 (KSL Thy1^{low} or KTSL), CD34 (KSL CD34^{neg/low}), and Flk2 (KSL CD34⁻ Flk2⁻) (8), or

on physiological properties, such as the capability to efflux Hoechst observed in SP cells (SP^{KSL} or SPKLS, pronounced *SParKLeS*) (4, 7, 9, 10).

More recently, alternative methods to identify HSCs have been described, that do not rely on the KSL scheme. These strategies include the use of markers such as Tie-2 (11), Endoglin (12), or endothelial protein C receptor (EPCR) (13). Morrison and colleagues recently described an alternative method based on markers from the signaling lymphocytic activation molecule (SLAM) family (CD150+ CD244- CD48-) (14). However, in order to obtain high purity, this strategy should be used in conjunction with other purification schemes.

In this chapter, we will focus on the purification of murine SP^{KLS} cells, based on the peculiar pattern that bone marrow cells acquire after Hoechst 33342 staining.

Hoechst 33342 fluorescent dye is a bisbenzimidazole derivative, capable of permeating through cell membranes and binding to nucleic acids. The emission of fluorescence is highly affected by DNA properties, such as chromatine rearrangements, DNA conformation, and nucleic acid composition. In particular, Hoechst dyes bind in a stoichiometric manner to AT-rich regions of the minor groove of double-stranded DNA (this property has been extensively used by genetists to develop the Q-bands staining for chromosomes).

Interestingly, when Hoechst dyes bind to DNA, their fluorescence undergoes a small spectral shift, that can be detected and used as a measurement of the amount of cellular DNA. This property has been exploited in flow cytometry to study ploidy and distribution in the different cell-cycle stages of a heterogeneous population, such as bone marrow samples. Traditionally, cell cycle studies have been performed by analyzing Hoechst emission at a short wavelength (450 nm), through a “blue” bandpass on a fluorescence-activated cell-sorter. However, Hoechst fluorescence can be detected with “red” (650 nm) bandpass optics as well. When Hoechst blue and red fluorescence signals are simultaneously collected and plotted against each other, a characteristic tail-shaped population, displaying low fluorescence, can be observed and distinguished from the main bulk that conversely emits high levels of fluorescence. This “tail” is the so-called Side Population, or SP, and comprises cells that display low Hoechst fluorescence. Conversely to the main bulk of bone marrow cells (whose Hoechst fluorescence is directly proportional to the DNA content), the atypical cytometric morphology of SP cells is a direct consequence of their capability to efflux with high efficiency the vital dye Hoechst 33342. However, what makes this peculiar bone marrow population so interesting for the stem cell field is the fact that SP cells are highly enriched in HSCs, capable of sustaining multilineage and long-term engraftment in the murine

model. Since the first description of SP cells in 1996 (10), follow-up studies also proved that the SP fraction encompasses entirely the hematopoietic activity that resides in the murine bone marrow, thus making Hoechst staining a unique experimental tool in stem cell biology (2, 4–6, 15).

The capability of SP cells to efflux vital dyes at a higher rate than other bone marrow cells is believed to reside in the activity of membrane pumps belonging to the superfamily of ATP-binding cassette (ABC) transporters. Members of this family are, for instance, multidrug resistance 1 (murine Mdr 1a/1b; human MDR1) and breast cancer resistance protein 1 (Bcrp1)/ABC, superfamily G, member2 (ABCG2). Interestingly, drugs such as verapamil block the activity of these transporters and concomitantly cause the SP profile to disappear.

Knock-out and retroviral-driven overexpression models helped shed some light onto the role ABC transporters play in HSC biology. MDR1 overexpression only slightly increases the SP fraction; on the other hand, Mdr 1a/1b^{-/-} bone marrow shows numbers of SP cells comparable to the *wild type*, thus indicating that this membrane transporter only plays a marginal role in the SP phenotype (16, 17). Conversely, the enforced expression of ABCG2 significantly expands SP cells, while loss of ABCG2 expression has been shown to drastically reduce the size of the SP fraction. Nonetheless, since HSC numbers and function in these mice are preserved, it is not yet clear whether the efflux plays a functional role in HSCs. Furthermore, ABCG2 knock-out mice still contain in their bone marrow a few residual SP cells, suggesting that multiple drug transporters are likely to be involved in the appearance of this phenotype (18–21).

However, if ABC membrane pumps are not crucial determinants of stem cell activity, why are they expressed at high levels in stem cells? This observation could be teleologically interpreted as a mechanism that biological systems adopt to protect from the environment crucial subsets of cells, like HSCs. Also, membrane pumps could play a role in extruding differentiation factors from HSCs, thus helping maintaining their stemness throughout the life of an organism.

2. Materials

2.1. Sample Preparation: Isolation of Murine Bone Marrow Cells

1. Murine bone marrow cells obtained from C57Bl/6 mice, 5–8 weeks old (see Note 1).
2. HBSS. Hanks Balanced Salt Solution, supplemented with 2% Fetal Bovine Serum and 10 mM HEPES buffer.

The solution so prepared will be hereafter referred to as HBSS+.

3. Needles (27 Gauge and 18 Gauge).
4. Cell strainer (70 μ m).
5. Red Blood Cells (RBC) lysis buffer. 0.17 M TrisCl, pH 7.6:0.16 M NH_4Cl = 1:9.

2.2. Staining of Murine Bone Marrow Cells with Hoechst 33342

1. DMEM. Dulbecco's Modified Eagle's Medium with High Glucose, supplemented with 2% Fetal Bovine Serum and 10 mM HEPES buffer. The solution so prepared will be referred to as DMEM+.
2. Hoechst 33342, bisBenzimide H33342 trihydrochloride (Sigma-Aldrich). To make concentrated stock solutions of Hoechst 33342, dissolve the powder in water (recommended concentration: 1 mg/mL, 200 \times solution) and filter-sterilize (see Note 2).
3. Verapamil (Sigma-Aldrich). Prepare a concentrated stock (100 \times) in 95% Ethanol and use at the final concentration of 50 μ M in the staining buffer (HBSS+ and Hoechst 33342) (see Note 3).
4. Circulating water bath at exactly 37°C (see Note 4).
5. Refrigerated centrifuge at 4°C (see Note 5).

2.3. Isolation of SP Sca-1⁺ c-Kit⁺ Lineage⁻ Cells

1. HBSS+ (as described in Subheading 2.1).
2. Anti-Sca-1 antibodies either biotinylated or FITC-conjugated (BD Pharmingen).
3. Anti-Biotin magnetic microbeads (Miltenyi Biotech).
4. AutoMACS separator (Miltenyi Biotech).
5. Anti-c-Kit antibody. We use a PE-conjugated antibody.
6. Anti-Lineage antibody cocktail. The cocktail comprises a mixture of the following PE-Cy5-conjugated antibodies (all from eBioscience): anti-B220, anti-CD4, anti-CD8, anti-Gr-1, anti-Mac-1, and anti-TER119.
7. Propidium Iodide (PI, Sigma-Aldrich). Prepare a stock solution at 10 mg/mL in water and store at -20°C. From this solution, prepare a working solution at 200 μ g/mL and keep it at 4°C, protected from light. The final concentration of PI in the sample should be 2 μ g/mL (100 \times dilution of the working solution).

2.4. Identification and Sorting of SP^{KL}S Cells

1. Flow cytometer equipped with a UV laser, such as a MoFlo sorter (Dako) or a FACSARIA (BD Biosciences) (see Note 6).

3. Methods

Because the method relies on detecting dye efflux from a cell, which is a dynamic biological process, a successful SP staining is highly dependent on cell and Hoechst concentration, as well as temperature and time of staining. Even small variations in any of these parameters can affect significantly the composition and purity of the SP. Here we illustrate the protocol as it was originally established for the staining of C57Bl/6 bone marrow and we recommend the protocol to be followed exactly as we describe before attempting the use in different species, tissues, or mouse strains.

3.1. Sample

Preparation: Isolation of Murine Bone Marrow Cells

1. Anesthetize the mouse and sacrifice it by cervical dislocation. Lay the mouse on its back and profusely spray with 70% Ethanol to sterilize.
2. Make a horizontal abdominal incision at the level of the knees and pull the skin until the legs are exposed completely.
3. Proceed to remove the tibias by cutting through the ankles and the knees. Clean the muscle off the tibias and place them in a Petri dish containing HBSS+ (5 mL) on ice.
4. Proceed now to remove the femurs, by cutting at the level of the hips. Carefully remove the muscle from the femurs and put them into the Petri dish with the tibias. Femurs are extremely rich in bone marrow, so we recommend to cut off the bone as close to the hip as possible.
5. Load a 10cc syringe with HBSS+ buffer and, holding a bone over a new Petri dish, insert the needle (27 Gauge) into one of the extremities and proceed to flush the bone marrow out of the bone. As the bone marrow is expelled, the bones will appear clearer. Repeat the same by inserting the needle into the second extremity of the same bone and flush thoroughly (see Note 7).
6. Using a syringe with an 18-Gauge needle, proceed to resuspend the bone marrow in the Petri dish. Repeat several times (four to five times), until the clusters of bone marrow will convert into a homogeneous single-cell suspension. Pay special attention to avoid the formation of air bubbles while resuspending cells, because of their detrimental effect on cell survival. Transfer the cell suspension into a 50 mL-conical tube and filter through a 70 μ m cell-strainer to remove from the sample cell clumps or bone fragments.
7. Carefully count the bone marrow cells, paying particular attention to exclude red blood cells (RBCs) (see Note 8). To do so, prepare a 1:20 dilution of an aliquot of bone marrow cell suspension (e.g., 5 μ L) in RBC-lysis buffer (95 μ L) for

counting. One C57Bl/6 mouse (5–8 weeks old) will averagely yield $5\text{--}7 \times 10^7$ nucleated cells. Note that, in order to proceed to the following staining, no Ficoll separation or lysis of red blood cells of the whole sample is necessary.

3.2. Staining of Murine Bone Marrow Cells with Hoechst 33342

1. Pre-warm the staining medium (DMEM+) in a circulating water bath at 37°C.
2. Spin down bone marrow cells and resuspend in pre-warmed DMEM+ at the concentration of 10^6 cells/mL (see Notes 8 and 9).
3. Add Hoechst 33342 to the cell suspension to a final concentration of 5 µg/mL (from the 200× working solution).
4. Incubate the sample for *exactly* 90 min at 37°C in a circulating water bath. During the incubation, periodically mix the tubes and always ensure that the tubes are fully immersed in the water.
5. Once the 90-min staining is completed, *always* keep your sample at 4°C and *always* use a refrigerated centrifuge to spin cells down, in order to prevent continuous Hoechst expulsion from the stained cells (see Note 10).
6. Spin down the Hoechst-stained cells in a refrigerated centrifuge and resuspend in iced HBSS+ buffer at the concentration of 10^8 cells/mL. Bone marrow cells are now stained with Hoechst and ready for the following staining procedures with monoclonal antibodies. Any further handling of the sample must be performed at 4°C or on ice (see Note 11).

3.3. Isolation of SP Sca-1⁺ c-Kit⁺ Lineage⁻ Cells

1. Sca-1 enrichment (see Note 12). Incubate cells on ice in the presence of anti-Sca-1 biotinylated antibody (0.5 µg/ 10^6 cells, 1:100 dilution) (see Note 13). After 10 min, wash out the unbound antibody by adding a tenfold volume of iced HBSS+. Spin cells down at 4°C and resuspend in HBSS+ buffer.
2. Label bone marrow cells with anti-biotin magnetic microbeads (1:5 dilution). Incubate for 15 min at 4°C.
3. Wash the sample with a tenfold volume of HBSS+ buffer and spin cells down at 4°C.
4. Resuspend at 2×10^8 cells/mL in HBSS+. The sample is now ready to be processed by AutoMACS (choose the program for stringent positive selections) (see Note 14).
5. Spin down at 4°C the Sca-1-enriched cells and resuspend in iced HBSS+ buffer.
6. Label the cells with anti-c-Kit antibody and with an anti-Lineage cocktail, comprising anti-B220, anti-CD4, anti-CD8, anti-Gr-1, anti-Mac-1, and anti-TER119 antibodies. Although the sample has been previously enriched for Sca-1⁺ cells,

we recommend staining the sample with an anti-Sca-1 antibody as well, as a control during the sorting. Incubate for 15 min on ice.

7. Wash the sample with a tenfold volume of HBSS+ buffer and spin cells down at 4°C. Resuspend HBSS+ buffer containing PI. The sample is now ready for sorting of SP c-Kit⁺ Lin⁻ Sca-1⁺ cells.

3.4. Identification and Sorting of SPKLS Cells

1. Excitation of Hoechst 33342. In order to view the SP, the flow cytometer must be equipped with a high power ultraviolet laser (35–100 mW), which is capable to excite both Hoechst 33342 and Propidium Iodide (PI) at 350 nm (see Note 15). A second laser is necessary to excite additional fluorochromes involved in the staining, such as a 488 nm laser for FITC and Phycoerthrin.
2. Detection of Hoechst 33342 emission. The emission of Hoechst 33342 is measured bimodally and commonly referred to as *Hoechst Blue* and *Hoechst Red*. Hoechst Blue is measured with a 450BP filter, whereas Hoechst Red is measured with a 675LP filter. In order to separate the different emission wavelength, a dichroic mirror is used (we use a 610 DMSP). PI emission is also measured with the 675LP filter, but its signal is significantly brighter than the one captured for Hoechst Red, so that PI-positive cells line up to the very far right side of the SP profile (Fig. 1).
3. FACS Analysis. The characteristic SP profile can be visualized by plotting Hoechst Blue emission (on the vertical axis) vs. Hoechst Red emission (on the horizontal axis). The detectors for both parameters must be set on linear mode. The voltage must be adjusted so that the PI-positive dead cells will appear at the far right vertical line. Also, if the voltage is set correctly, red blood cells should group together in the lower left corner. The majority of the bone marrow cells will be displayed in the central area or in the upper right quadrant of the plot. If the cytometer settings are arranged correctly, the SP profile should appear as displayed in Fig. 1.
4. Identification and gating of SP^{KLS} cells. Once the instrument set-up has been performed, follow the gating strategy described in Fig. 2. Briefly, start by drawing the first gate around the SP population. Proceed by checking the morphological phenotype of SP cells (FSC vs. SSC plot) and gate out all the events not compatible with stem cell morphology (low granularity and small/medium size). Finally, proceed to analyze the KSL phenotype: first, gate Lineage⁻ cells and then display these events as shown in the last panel of Fig. 2. The events that simultaneously fulfill the criteria of both c-Kit and Sca-1 positivity represent the desired SP^{KLS} population (see Note 16).

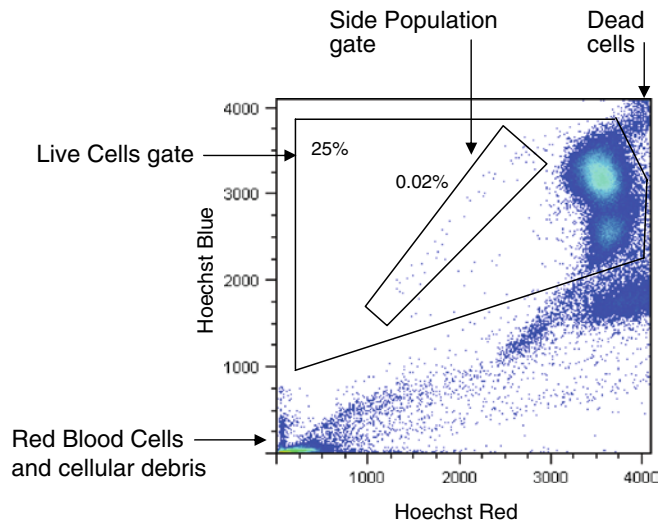


Fig. 1. Example of an SP population from an unenriched whole bone marrow sample. In order to visualize the characteristic SP pattern of bone marrow cells, the emission of Hoechst 33342 must be displayed bimodally as Hoechst Red vs. Hoechst Blue, both in a linear scale. The cells concentrated at the lower left corner represent red blood cells and cellular debris, while the rest of the sample is mainly grouped on the upper right side of the acquisition window. The SP gate is drawn around the tail that diagonally emerges from the main population and usually represents 0.02–0.05% of whole bone marrow cells.

4. Notes

1. This protocol was originally established and optimized for murine bone marrow cells, derived from normal C57Bl/6 mice. Because of the high sensitivity of Hoechst efflux to multiple parameters, we strongly recommend investigators, who are attempting this procedure for the first time, to follow the protocol *exactly* as we describe, until proficiency in SP staining and identification is achieved. In order to optimize the protocol for different species, we suggest to change one parameter at a time (for instance, duration of the staining or Hoechst concentration).
2. For long-term storage, prepare aliquots of the stock solution (e.g., 1 mL aliquots) and store them at -20°C , protected from light. Avoid, when possible, repeated thawing/freezing cycles. We strongly recommend using a new Hoechst aliquot for each experiment.
3. Verapamil is a drug that blocks the activity of the membrane transporters responsible for the efflux of Hoechst 33342. When Verapamil ($50\text{ }\mu\text{M}$) is included in the Hoechst staining solution and in the washing buffers, the SP fraction is no longer detectable and becomes part of the main population.

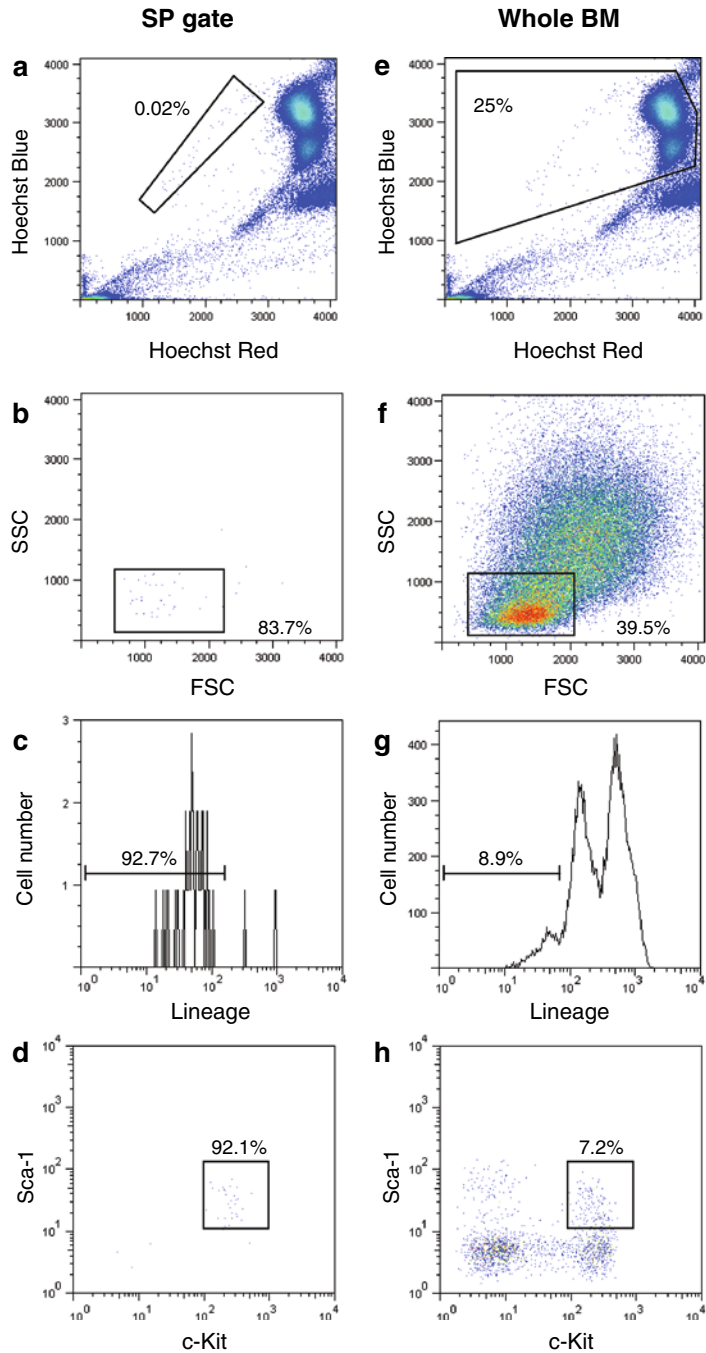


Fig. 2. Sorting strategy for SP^{KLS} (SP c-Kit $^{+}$ Lineage $^{-}$ Sca-1 $^{+}$) cells. **(a)** SP gate: the first step consists in displaying the Hoechst 33342 efflux pattern in linear mode (as Hoechst Red vs. Hoechst Blue) and gating the SP population. **(b)** Morphological characteristics: display the SP cells gated in the first panel as FSC (*forward scatter*) vs. SSC (*side scatter*) and draw a second gate as shown in figure. **(c)** Lineage staining (PE-Cy5): gate out cells that express markers of hematopoietic terminal differentiation and select Lineage-negative cells. **(d)** c-Kit vs. Sca-1: the last panel shows the expression of the stem cell markers c-Kit (PE) and Sca-1 (FITC) in SP/Lineage-negative cells. This is the sorting gate, comprising the SP^{KLS} population. **(e–h)** The panels on the right show, by comparison, how unenriched bone marrow cells (gated only on the live population from **(e)**) distribute on the same parameters.

We highly recommend the use of Verapamil-treated cells as negative control to help investigators identify the “true” SP population and draw the sorting gate. However, once the method has been routinely established, Verapamil treatment can be left out.

4. Hoechst staining is highly sensitive to temperature. Therefore, the water bath must be set at *precisely* 37°C. Avoid using water baths whose temperature fluctuates (we recommend using a circulating water bath) and avoid immersing ice-cold or frozen reagents into the water during the staining.
5. Use a refrigerated centrifuge for spinning cells down and *always* keep the sample at 4°C or on ice. In the case the stained cells are exposed to higher temperatures, they might expel Hoechst to the point they will become undistinguishable from the “true” SP cells. This will eventually affect the composition and decrease the purity of the SP.
6. Although it is possible to detect SP using a violet laser, in order to obtain optimal results, we recommend using a UV laser.
7. When isolating tibias and femurs, it is important to remove as much muscle as possible in order to prevent the bone marrow from sticking to it once it is flushed out of the bone.
8. Cell dilution, Hoechst concentration, and staining time are all critical factors in determining an optimal staining. In particular, dye concentration and number of nucleated cells should be carefully determined.
9. In order to prevent cells from sticking to the plastic, we recommend using polypropylene tubes while staining with Hoechst.
10. Because of the aforementioned sensitivity of the procedure to temperature, even when the staining process is over, the samples must be maintained at 4°C, in order to prohibit efflux of the dye from the cells. Therefore, whether you are going to directly sort SP cells or you are going to perform antibody staining, *always* keep your sample at 4°C.
11. If interested in SP isolation only, disregard the following KSL staining. Resuspend the sample in HBSS+ buffer and PI and proceed to sort. However, keep in mind that combination of SP staining with KSL markers significantly increases HSC purity, other than being an internal diagnostic parameter for optimal staining conditions. Likewise, if this protocol is used to isolate stem cells from other tissues, SP staining should be combined, whenever possible, with tissue-specific stem cell markers.

12. Enrichment of the bone marrow sample before sorting is not strictly necessary, but strongly recommended. Enrichment helps increase purity and yield after sorting and sensibly decreases sort time.
13. The antibody concentration of 0.5 $\mu\text{g}/10^6$ cells reflects the optimal staining conditions that have been identified in our laboratory and is consistently used for each antibody mentioned throughout this protocol. However, especially for samples different from murine bone marrow cells, we recommend to adjust the antibody titration ad hoc.
14. Alternatively, the Sca-1 enrichment can be performed manually using Miltenyi MS/LS columns for positive selection.
15. In the case that the sorting strategy relies also on conjugated antibodies (as in the case of SP^{KLS} purification), the flow cytometer must have the corresponding additional lasers (e.g., a 488 nm laser, if cells are stained with FITC and PE).
16. Despite the unique pattern of SP cells, uninitiated investigators usually are challenged by deciding where to draw the SP gate, especially when it comes to deciding how far toward the top of the tail it is possible to go, without including cells that are not “true” HSCs. In our laboratory, we tend to use a conservative gate, while attempting to maximize cell yield and minimize contamination from non-HSCs. An excellent internal quality control for drawing the SP gate in the correct position is provided by the KSL staining itself. Since SP cells are highly enriched in HSCs, the SP gate should not contain more than 25% Lineage⁺ cells. Also, approximately 85% of SP should be KSL. If these criteria are not matched, it generally means that a more restricted gate should be drawn. Another possible reason is that the protocol has been poorly performed and consequently a high percentage of non-SP cells are contaminating the SP gate.

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Stem Cell Migration

Methods and Protocols

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