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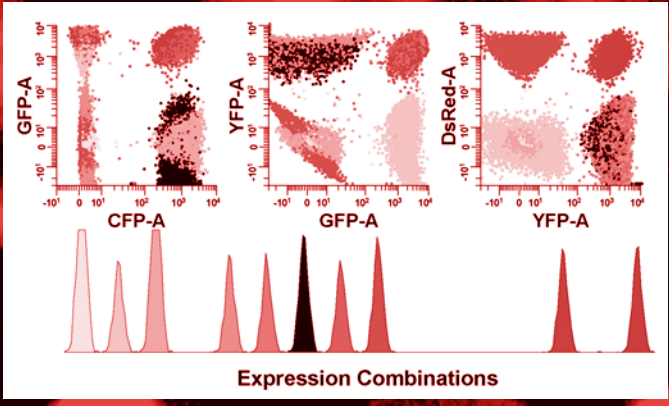

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Hematopoietic Stem Cell Characterization by Hoechst 33342 and Rhodamine 123 Staining

Ivan Bertoncello and Brenda Williams

Summary

A dual-dye efflux strategy utilizing the supravital dyes Hoechst 33342 (Ho) and rhodamine 123 (Rh123) is described and illustrated for the detection and analysis of hematopoietic stem cells in murine bone marrow. Mononuclear cells from bone marrow cell suspensions were incubated in a cocktail of Rh123 plus Ho, and both dyes were effluxed by two 15-min incubations in dye-free buffer prior to sorting. Compared to our original prototype method in which Rh123, but not Ho, was effluxed, this dual-dye efflux protocol more rapidly and efficiently resolves the most primitive Ho^{dull}/Rh^{dull} hematopoietic stem cells. Moreover, under conditions of optimal dual-dye uptake and efflux, Ho^{dull}/Rh^{dull} cells map to the subfraction of side population (SP) cells with the highest efflux of Ho, which were previously demonstrated to possess the highest hematopoietic stem cell activity.

Key Words

Dual-dye efflux, Ho^{dull}/Rh^{dull} cells, Hoechst 33342, primitive hematopoietic stem cells, rhodamine 123.

1. Introduction

The supravital dyes rhodamine 123 (Rh123) and Hoechst 33342 (Ho) have proven to be remarkably powerful probes for the characterization, resolution, isolation, and purification of primitive hematopoietic stem cells (PHSCs). When used alone ***(1–10)*** or together ***(11–17)***, and in combination with antibodies specifying stem cell associated cell surface antigens ***(18–22)***, lectins ***(23,24)***, or other supravital dyes ***(25,26)***, Rh123 and Ho precisely dissect and order the murine PHSC compartment, discriminating between closely related stem cell cohorts differing in long-term transplantation potential ***(11,13)***, cell cycle kinetic status and turnover rate ***(13)***, rate of entry into cell cycle ***(17)***, cytokine

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receptor repertoire and gene expression patterns, cytokine preferences and responsiveness ***(16*** and ***17)***, and in vitro clonogenic capacity ***(11–13,17)***.

The fidelity of Rh123 and Ho as stem cell probes resides in their individual and combined ability to hierarchically order the hematopoietic stem cells on the basis of their probability of cycling by probing individual traits that define the quiescent state ***(27–29)***, and by exploiting the overlapping activity of transmembrane efflux pumps belonging to the ABC transporter superfamily ***(30)***, including MDR-1 ***(31,32)***, MRP1 ***(33)***, and Bcrp1/ABCG2 ***(33–35)***, for which they are preferential substrates.

1.1. Rh123 and Ho As Stem Cell Probes

Rh123 was first characterized as a mitochondrial membrane specific dye that bound to mitochondria in proportion to their negative membrane potential ***(36,37)***. Rh123 is also a preferential substrate for the P-glycoprotein membrane efflux pump, encoded by the murine *mdr1a* and *mdr1b* homologs of human MDR1, which are highly expressed by PHSCs ***(31,32)***. Rh123 fluorescence intensity is an index of mitochondrial mass, number and activation state ***(38)***, and P-glycoprotein efflux pump activity. The Rh^{dull} phenotype distinguishes primitive, metabolically inactive, relatively quiescent PHSCs with long-term transplant potential from developmentally more mature transplantable cells capable only of short-term regeneration of the hematopoietic system of myeloablated mice ***(1,6,18,21,22)***.

Ho is a supravital DNA stain that stoichiometrically binds to the AT-rich regions of the minor groove of DNA ***(39–41)***. Ho fluorescence intensity is an index of DNA content, chromatin structure and conformation, and discriminates between cells in different phases of cell cycle ***(41)***. Second, although Ho is also a substrate for P-glycoprotein and MRP1 membrane pumps, it is predominantly effluxed by a recently described novel member of the ABC transporter superfamily, bcrp1/ABCG2, that is also highly expressed in PHSCs ***(33–35)***. Thus, the Ho^{dull} phenotype also characterizes relatively quiescent PHSCs with long-term transplant potential. PHSCs and primitive hematopoietic progenitor cells are preferentially enriched in the sub-G_1 region of the Ho fluorescence histogram ***(5)***, and this supravital dye has also been utilized for stem cell enrichment by many investigators over the last 20 yr ***(4,7,8)***. In recent years, the potency and utility of Ho as a stem cell probe has been greatly enhanced by exploitation of long recognized characteristics of Ho binding to DNA.

On binding to DNA, the Ho fluorescence emission undergoes a violet-to-red spectral shift ***(42–44)*** contingent on Ho affinity and binding energy, and on the ratio of Ho binding sites to intracellular dye concentration. In addition, Ho dye uptake can be resolved into a concentration-dependent–time-independent component that reaches a plateau minutes after incubation with Ho, and a

time-dependent component that reaches a plateau approx 90 min after incubation. Optimization of Ho dye incubation time to account for these differing binding dynamics, and display of Ho fluorescence as a bivariate Ho-blue vs Ho-red dot-plot to visualize the violet-to-red spectral shift in Ho fluorescence emission has led to further resolution of the sub-G_1 binding fraction to reveal a side population (SP cells) highly enriched in stem cell activity ***(9,10)*** (*see also* Chapter 9 by Eaker et al., *this volume*).

1.2. The Relationship of Ho^{dull}/Rh^{dull} Cells and Ho-SP Cells

On the other hand, the dual-dye cell separative strategy combining Rh123 and Ho dye uptake and efflux exploits the complementary properties of the two dyes and the overlapping roles of different ABC transporter superfamily members ***(30,33)***. The bivariate dot-plot display of Rh123 vs Ho fluorescence reveals a Ho^{dull}/Rh^{dull} cluster (**Figs. 1A** and **2D**) that contains PHSCs able to clonally regenerate the hematopoietic system of lethally irradiated mice ***(11)***, and contribute to steady-state blood cell production long-term when transplanted in nonablated recipients ***(14)***. The dual-dye approach to stem cell sorting has also proven a powerful tool for kinetic analysis of PHSCs, and the analysis of gene expression patterns and cytokine receptor repertoire within the stem cell continuum in steady-state hematopoiesis *in situ* ***(13,16,17)***.

In essence, we believe that Rh123 effectively subsets and orders Hoechst-SP cells to define highly synchronous stem cell targets that are superior candidates for the definition of early events in the process of hematopoietic stem cell activation, proliferation, and commitment. However, the precise relationship between Ho^{dull}/Rh^{dull} and SP cells is uncertain as optimal detection of each target cell population utilizes incubation conditions that exploit different characteristics of Rh123 and Ho binding and efflux. For example, incubation conditions for identification of Ho^{dull}/Rh^{dull} cells do not fully exploit the time-dependent Ho efflux component required for optimal revelation of the SP region (**Fig. 1A**,**B**). And conversely, the extended efflux time required for optimal resolution of SP cells leads to some loss of definition of the Ho^{dull}/Rh^{dull} cell cluster (**Fig. 1D**,**E**). However, it is pertinent to note that in both cases Ho^{dull}/Rh^{dull} cells have an SP phenotype (**Fig. 1C**,**F**), and that murine Ho^{dull}/Rh^{dull} cells are predominantly CD34^{-} *(unpublished observation)*.

The studies cited thus far describe the use of Rh123 and Ho to separate murine PHSC. Rh123 and Ho have also been used alone, and together to isolate and characterize primitive human hematopoietic stem and progenitor cells with similar outcomes ***(20,45,46)***. However, the fluorescence profile of primitive human Ho^{dull}/Rh^{dull} hematopoietic cells are not as clearly resolved and visualized in bivariate fluorescent dot-plots and histograms as the comparable profiles of murine hematopoietic cells. Whether this is related to the differential activity of

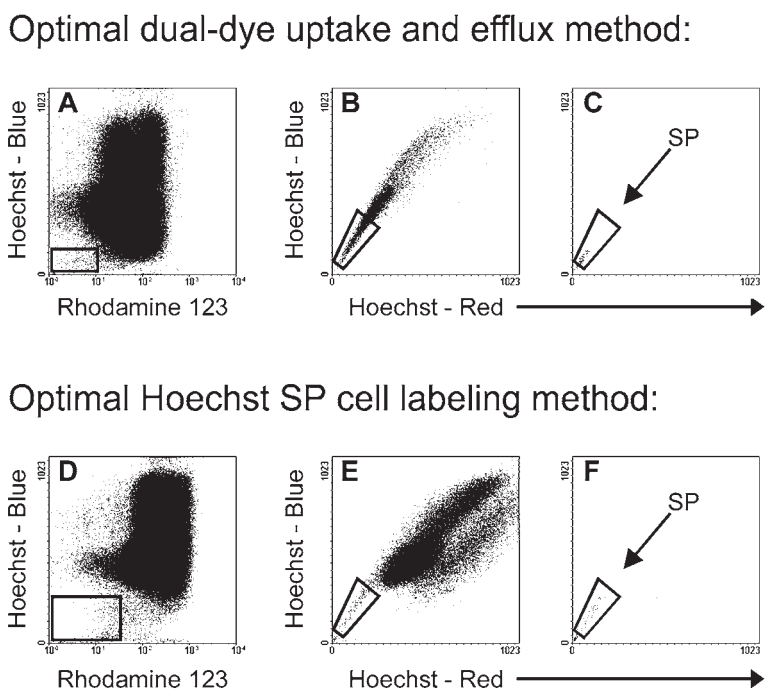

Fig. 1. The relationship between the Ho^{dull}/Rh^{dull} phenotype and the Hoechst SP phenotype in Lin^- murine bone marrow cell suspensions. Cells were labeled with a cocktail of both fluorochromes using incubation conditions optimized for **(A–C)** the detection of Ho^{dull}/Rh^{dull} PHSC (30 min dye uptake at 37°C followed by two 15-min incubation in dye-free medium) or **(D–F)** the resolution of SP cells (90-min dye uptake at 37°C followed by washing in ice-cold buffer). In each instance, gated Ho^{dull}/Rh^{dull} cells (**A**,**D**) are identified as a subset of Hoechst SP cells (**C**,**F**) defined with reference to the Hoechst-blue vs Hoechst-red bivariate dot-plot of each Lin^- bone marrow cell suspension (**B**,**E**).

human and murine membrane efflux pumps or the less efficient preenrichment of human hematopoietic stem cell targets is unclear.

1.3. Caveats and Limitations in the Application of Dye-Based Cell Sorting

Despite the robustness of dye-based approaches to stem cell sorting, investigators using these methods must be aware of their limitations, and be vigilant in controlling the critical variables affecting the reproducibility of the technique.

Rh123 and Ho are "functional probes" that exploit the kinetic and metabolic inertia of PHSCs. They are most effective when applied in the steady state,

and less so following perturbation ***(47)*** when cell activation, the recruitment of stem cells into the cycle, and the modulation of membrane pump activity distort the relationship between cells of differing developmental potential and maturational age. Mouse strain differences have not been noted. However, it has been reported that the Rh^{dull} phenotype is predominantly attributed to inactive mitochondria in young mice, and to enhanced P-glycoprotein activity in old mice ***(21,22)***. Significantly, PHSCs continue to be resolved in the Rh^{dull} cell fraction at all ages.

The significant variables that determine the binding efficiency of RH123 and Ho and the activity of membrane efflux pumps are: time of incubation and efflux, incubation temperature, dye concentration, density of cell suspensions, and the colligative properties of buffers ***(48)*** used for cell manipulation. All have the potential to influence the resolution and homogeneity of sorted stem cell targets. In this context it is interesting to note that the dual-dye staining protocols originally developed or adopted by ourselves ***(13)*** and others ***(11,14,15,25,46)*** have used different dye-loading and efflux incubation conditions that have exploited membrane pump activity to different degrees. These methods have either allowed for efflux of Rh123 but not Ho ***(13,25)***, or have followed a protocol ***(11,14,15,46)*** in which cells incubated with Rh123 and Ho were washed in ice-cold dye-free buffer without allowing for efflux of either dye.

The protocol we describe in this chapter is a recent modification ***(17)***. Target cells are incubated in a cocktail of Rh123 plus Ho, and both dyes are effluxed by two 15-min incubations in dye-free buffer prior to sorting. This method appears to resolve and discriminate Ho^{dull}/Rh^{dull} cells more rapidly and efficiently than our prototype method ***(13)*** in which Rh123, but not Ho, was effluxed.

2. Materials

1. Hoechst 33342 powder (Molecular Probes, Inc., Eugene, OR): Make up a 20 m*M* stock solution in sterile distilled water and store frozen at –20°C in 50-µL aliquots. Prepare a 1 m*M* working solution on the day of use by diluting 50 µL of stock solution in 950 µL of phosphate-buffered saline (PBS) (*see* **Note 1**). The working solution is protected from light and should be used within a few hours of preparation.
2. Rh123 powder (Molecular Probes, Inc.): Make up a 10 mg/mL stock solution in methanol and store frozen at –20°C in 100-µL aliquots. Make up an intermediate 1 mg/mL dilution by diluting 100 µL of the stock solution in 900 µL of PBS; store frozen in 10-µL aliquots. Prepare a working solution of 10 µg/mL Rh123 on the day of use by diluting 10 µL of the intermediate stock solution in 990 µL of PBS. The working solution is protected from light and should be used within a few hours of preparation.
3. 7-Aminoactinomycin D (7-AAD) powder (Sigma-Aldrich Pty. Ltd., Sydney, Australia): Make up a 1 mg/mL solution in ethanol, followed by a 1 : 10 dilution

in PBS to give a 100 µg/mL stock solution that is stored frozen at –20°C in 1-mL aliquots. This stock solution is added to cells suspended in PBS–2% heat-inactived serum (HiSe) at a 1:50 dilution to give a final working concentration of 2 µg/mL 7-AAD for the flow cytometric detection of nonviable cells.

4. Nycoprep™ 1.077A (265 mosM) (Axis-Shield PoC AS, Oslo, Norway) (*see* **Note 2**).
5. PBS (*see* **Note 3**): Prepare 1 L of PBS with 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of K_2HPO_4, and 0.2 g of glucose (*see* **Note 4**). Dissolve NaCl in approx 800 mL of distilled H_2O. Add the remaining salts and glucose sequentially, ensuring that each has fully dissolved before adding the next ingredient. Adjust the buffer to pH 7.4 using 1 *N* HCl or 1 *N* NaOH. Make up the volume to 1 L, check the osmolarity by osmometer and adjust to 310 mosM by addition of the required volume of distilled H_2O. Sterile filter the buffer through a 0.2-µm filter and store refrigerated.
6. Heat-inactivated fetal or newborn bovine serum (HiSe): Heat-inactivate by incubation at 56°C for a minimum of 1 h, cool, and filter through coarse filter paper and filters of progressively smaller pore size. Sterile filter through a 0.2-µm filter, aliquot, and store frozen for use as required. HiSe is added to buffers used for bone marrow cell harvesting and cell manipulation (*see* **Note 5**).
7. Collagenase/dispase: Make up 3 mg/mL collagenase type 1 (Worthington Biochemical Corp., Lakeside, NJ) and 3 mg/mL of dispase II (neutral protease) from *Bacillus polymyxa*, grade II (Roche Applied Science, Indianapolis, IN) in PBS, separately. Pool the two enzyme solutions 1:1 (v/v) and store frozen for use as required.
8. Antibody reagents: The panel of hematopoietic lineage antibody reagents and isotype control antibodies used for lineage negative selection of PHSCs are listed in **Table 1**. Dilute these antibodies in PBS–2% HiSe containing 0.1% sodium azide (w/v), and pretiter to establish optimal working concentrations for flow cytometric analysis and immunomagnetic selection. Typically we have found that the optimal dilutions for these commercially sourced antibodies range between 1:80 and 1:640 (v/v).
9. Ceramic mortar (approx 10 cm diameter) and pestle.
10. Sterile 40-µm nylon cell strainers (Falcon brand, BD Biosciences Discovery Labware, Bedford, MA).
11. Reagents for immunomagnetic preenrichment using a magnetic activated cell sorter (MACS) (*see* **Note 6**):
    a. Streptavidin microbeads (*see* **Note 7**).
    b. Columns and MACS devices (Miltenyi Biotec Australia Pty. Ltd., North Ryde, NSW) suitable for this application are listed in **Table 2**.
    c. Labeling buffer (PBS–2 m*M* EDTA, pH 7.2) and separation buffer (PBS–2 m*M* EDTA supplemented with 0.5% bovine serum albumin [BSA], pH 7.2) (*see* **Note 8**).
    d. Streptavidin–phycoerythrin (Strep–PE) conjugated rat antimouse antibody.
12. Fluorescence-activated cell sorter (FACS): A dual-laser instrument fitted with appropriate detectors and filter sets (*see* **Table 3**), and equipped with a 200-mW

Table 1
Description and Source of Antibody Reagents for Negative Immunomagnetic Selection of Murine Hematopoietic Stem and Progenitor Cells

Biotinylated antibodies[a]	Bone marrow specificity and alternate nomenclature	Clone	Supplier cat. no.	Isotype	Supplier[b] cat. no.
CD3e	T lymphocytes and NK-T cells	145-2C11	Pharmingen 553059	Hamster IgG1,κ	Pharmingen 553970
CD4	L_3T_4: T helper cells	GK1.5	Pharmingen 553728	Rat IgG2b,κ	Pharmingen 553987
CD5	Mature T lymphocytes and a subset of B lymphocytes	53-7.3	Pharmingen 553018	Rat IgG2a,κ	Pharmingen 553928
CD8a	Ly2: T-suppressor cells	53-6.7	Pharmingen 553028	Rat IgG2a,κ	Pharmingen 553928
B220	CD45R: Pro-B cells through to mature and activated B-cells, but not plasma cells	RA3.6B2	Pharmingen 553085	Rat IgG2a,κ	Pharmingen 553928
Gr-1	Ly6G: Neutrophils, and transiently by bone marrow cells of the monocyte lineage	RB6-8C5	Pharmingen 553124	Rat IgG2b,κ	Pharmingen 553987
Mac-1 (CD11b)	CD11b: Macrophages, granulocytes, dendritic cells, and NK cells	M1/70	Pharmingen 557395	Rat IgG2b,κ	Pharmingen 553987
Ter-119	Ly76: Erythroblasts through to mature erythrocytes, but not BFU-e and CFU-e	Ter-119	Pharmingen 553672	Rat IgG2b,κ	Pharmingen 553987

[a]All antibodies are rat antimouse, with the exception of anti-CD3 which is Armenian hamster antimouse.
[b]The supplier is BD Biosciences Pharmingen (San Diego, CA).

laser (such as an argon laser) capable of exciting at 488 nm, and an ultraviolet (UV) laser running at 50 mW power.
13. FACS collection tubes: Sterile 1.5-mL Eppendorf tubes or sterile 12 × 75 mm polypropylene tissue culture tubes.

3. Methods

3.1. Bone Marrow Cell Harvesting

1. Excise femurs, tibiae, and iliac crests. Scrape with a sterile surgical blade to remove adherent muscle and tissues. Place in cold PBS–2% HiSe.
2. Crush the bones using a sterile mortar and pestle, and filter the cell suspension through a 40-μm nylon cell strainer to remove bone debris (*see* **Note 9**).
3. Resuspend the bone fragments in a sterile 50-mL centrifuge tube in equal volumes of 3 mg/mL collagenase and 3 mg/mL dispase (*see* **Note 10**); incubate at 37°C for at least 5 min in an orbital shaker. Top up the tube with 25 mL of

Table 2
Maximum Capacity and Optimal Configuration of MACS Separation Columns Used for Negative Immunomagnetic Selection of Murine PHSCs

Column type	Magnetic separation device	Optimal flow resistor	Maximum column capacity (magnetically labeled cells)	Maximum column capacity (total cells)
LD	MidiMACS VarioMACS or SuperMACS with column adaptors		10^8	5×10^8
CS	VarioMACS SuperMACS	Three-way stopcock and 22G flow resistor	2×10^8	10^9
D	SuperMACS	Three-way stopcock and 21G flow resistor	10^9	5×10^9

PBS–2% HiSe, shake vigorously, and carefully decant the supernatant cells through a 40-μm nylon cell strainer. Resuspend the bone fragments in 25 mL of PBS–2% HiSe a second time and decant supernatant cells as before.

4. Centrifuge the cell suspension at 400*g* for 5 min at 4°C. Resuspend the cell pellet in PBS–2% HiSe. Pool all bone marrow cells, centrifuge, and wash twice in excess PBS–2% HiSe prior to dilution to an approximate cell density of 10^7 cells/mL.

3.2. Preparation of Low-Density Cells

1. Carefully layer 20-mL aliquots of bone marrow cell suspension over 10 mL of Nycoprep 1.077A in 50-mL centrifuge tubes (*see* **Note 11**). Centrifuge in a refrigerated centrifuge (600*g* at 4°C for 20 min) with the brake off.
2. Collect low density mononuclear cells at the Nycoprep interface in excess PBS–2% HiSe, wash, centrifuge twice, and resuspend in PBS–2% HiSe. Determine the cell count.

3.3. Negative Immunomagnetic Selection

1. Pellet low-density bone marrow cells and resuspend at 10^8 cells/mL in an optimally pretitered cocktail of biotinylated antibodies directed against CD3, CD4, CD5, CD8, B220, Gr1, Mac-1, and TER119 antigens (*see* **Table 1**). Incubate for 30 min on ice.

Table 3
Instrument Settings and Optical Filter Selection for the Isolation of Ho^{dull}/Rh^{dull} PHSC

Fluorochrome/ conjugate	PMT parameter	Excitation wavelength	Emission wavelength	Optical filter set characteristics
		(nm)	(nm)	(nm)
Rhodamine 123	FL1	488	530	530/30
PE-conjugated antibodies	FL2	488	575	575/26
7-AAD	FL3	488	650	675/20
Hoechst 33342	FL4	350	450	424/44

2. At the same time, set aside two small aliquots of low-density bone marrow cells to be used as reference samples for setting a Lin$^-$ sort gate (*see* **Subheading 3.6., step 3**). Incubate one aliquot with Strep–PE alone, the other aliquot with Strep–PE and a cocktail of relevant isotype control antibodies made up at the same protein concentration as the lineage antibodies.
3. Wash labeled cells in excess MACS labeling buffer and centrifuge (400*g* at 4°C for 5 min) to remove residual unbound antibody. Resuspend in MACS labeling buffer.
4. Perform a cell count. Set aside a small aliquot of labeled cells (~2.5 × 10^5) for incubation with Strep–PE as a pre-MACS control sample to be used for: (a) flow cytometric evaluation of the efficacy of negative immunomagnetic selection, (b) adjustment of fluorescence compensation settings, and (c) setting a sort gate excluding contaminating lineage positive cells in the Ho^{dull}/Rh^{dull} cell separation strategy.
5. Centrifuge the remainder of the cell suspension a second time (400*g* at 4°C for 5 min) and decant the supernatant, leaving a dry cell pellet.
6. Resuspend the antibody labeled cell pellet in 90 µL of labeling buffer plus 10 µL of streptavidin microbeads per 10^7 cells according to the manufacturer's instructions (*see* **Note 12**). Mix well and incubate on ice for 15 min with periodic agitation.
7. Following microbead incubation, wash labeled cells in 1–2 mL of MACS separation buffer per 10^7 cells (400*g* at 4°C for 5 min). Resuspend the cell pellet in MACS separation buffer at a cell density of 10^8 cells/500 µL.
8. During the antibody and microbead labeling steps described in the preceding, select an appropriate MACS column (*see* **Table 2**), prepare, assemble, and place in the magnetic separator following the instructions provided in the manufacturer's data sheets (*see* **Note 13**).
9. In brief, apply the cell suspension to the washed column and allow the cells to penetrate the matrix by turning the stopcock to run position. Collect the nonmagnetic, hematopoietic lineage negative cells in the column effluent by washing the column with three to five column volumes of MACS separation buffer (*see* **Note 14**).

3.4. Rh123 and Ho Staining

1. Prepare working solutions of 10 μg/mL of Rh123 and 1 m*M* Ho from frozen stock solutions immediately prior to use.
2. Following determination of lineage-negative cell yield, centrifuge the cell suspension (400*g* at 4°C for 5 min) and resuspend the cell pellet at a density of 1×10^6 cells/mL in PBS–5% HiSe in preparation for staining with Rh123 and Ho.
3. Add a 10-μL volume of each fluorochrome per milliliter of low-density, Lin^-, cell suspension to give a final concentration of 0.1 μg of Rh123 and 10 μ*M* Hoechst. Gently agitate the cell suspension and incubate in a 37°C water bath for 30 min in the dark (*see* **Note 15**).
4. Follow Rh123 and Ho dye staining by two incubations at 37°C in dye-free buffer to exploit membrane efflux pump activity in the resolution of Ho^{dull}/Rh^{dull} cells.
   a. Pellet the Rh123/Ho-stained cells by centrifugation at room temperature (400*g* for 5 min), resuspend at 1×10^6 cells/mL in prewarmed dye-free PBS–5% HiSe, and return to the 37°C water for 15 min in the dark.
   b. Pellet the cells again by centrifugation and repeat the dye-efflux step a second time.
5. Following the second dye-efflux step, set aside an aliquot of Rh123/Ho-stained cells (2.5×10^5) on ice as a flow cytometry control.
6. Pellet the remainder of the cell suspension, resuspend in Strep–PE at 10^8 cells/mL, and incubate on ice for 30 min in the dark to detect residual contaminating hematopoietic lineage positive cells.
7. Add excess PBS–0.25% HiSe. Pellet the cell suspension by centrifugation (400*g* at 4°C for 5 min) and resuspend in PBS–0.25% HiSe at $5–10 \times 10^6$ cells/mL. Add 7-AAD stock solution (1:50 v/v) to give a final 7-AAD concentration of 2 μg/mL. Store the labeled cells on ice in preparation for flow cytometric analysis and sorting.

3.5. FACS Instrument Settings

Aliquots of the following cell samples are required for selection of instrument settings and fluorescence compensation.

1. Unfractionated bone marrow cells stained with 7-AAD for determining cell viability settings and for setting a lymphoblastoid light scatter gate.
2. Low-density bone marrow cells labeled with biotinylated isotype control antibody cocktail and Strep–PE to determine background fluorescence and quantify nonspecific labeling.
3. Pre-MACS control cells labeled with biotinylated antibody cocktail followed by Strep–PE to be used in conjunction with low-density cells labeled with Strep–PE alone to set the Lin^- cell gate.
4. Rh/Ho-stained and effluxed nonmagnetic Lin^- cells to determine photomultiplier tube (PMT) settings for optimal detection and resolution of Ho^{dull}/Rh^{dull} cells.

Table 4
Flow Cytometric Gating Strategy for Flow Cytometric Analysis and Sorting of Murine Ho^{dull}/Rh^{dull} PHSC

Region	Display	PMT parameter and scaling
R1	7-AAD dye uptake	FL3 histogram (logarithmic)
R2	Cell size and structure	FSC vs SSC (linear vs linear)
R3	Lineage antibody expression	FL2 histogram (logarithmic)
R4	Rh123 vs. Hoechst dye uptake	FL1 vs FL4 (logarithmic vs linear)

Sequential gating strategy	Gate	Target cell description
R1	G1	Viable cells
R1 and R2	G2	Viable lymphoblastoid-like cells
R1 and R2 and R3	G3	Viable, Lin$^-$, lymphoblastoid-like cells
R1 and R2 and R3 and R4	G4	Viable, Lin$^-$, Ho^{dull}/Rh^{dull} lymphoblastoid-like cells

5. Adjust fluorescence compensation settings using the above control cell samples to account for the overlapping spectral emissions of Rh123, PE, and 7-AAD excited by the argon laser.
   a. Rh123 (FL1) vs PE (FL2): Rh/Ho-stained Lin$^-$ cells.
   b. PE (FL2) vs Rh123 (FL1): Biotinylated antibody/Strep–PE labeled pre-MACS control cells.
   c. PE (FL2) vs 7-AAD (FL3): Biotinylated antibody/Strep–PE labeled pre-MACS control cells.
   d. 7-AAD (FL3) vs PE (FL2): Unfractionated, 7-AAD stained bone marrow cells.
6. Ho fluorescence is excited by the second (UV) laser and does not require compensation.

3.6. Sort Gating Strategy for Isolation of Ho^{dull}/Rh^{dull} Cells

The sequential gating strategy for the isolation of Ho^{dull}/Rh^{dull} PHSCs is described in **Table 4** and illustrated in **Fig. 2**.

1. Set an appropriate gate on the 7-AAD histogram profile to exclude nonviable (7-AAD positive) cells.
2. Select a forward scatter (FSC) vs side scatter (SSC) window defining viable lymphoblastoid cells, and excluding debris and cell doublets.

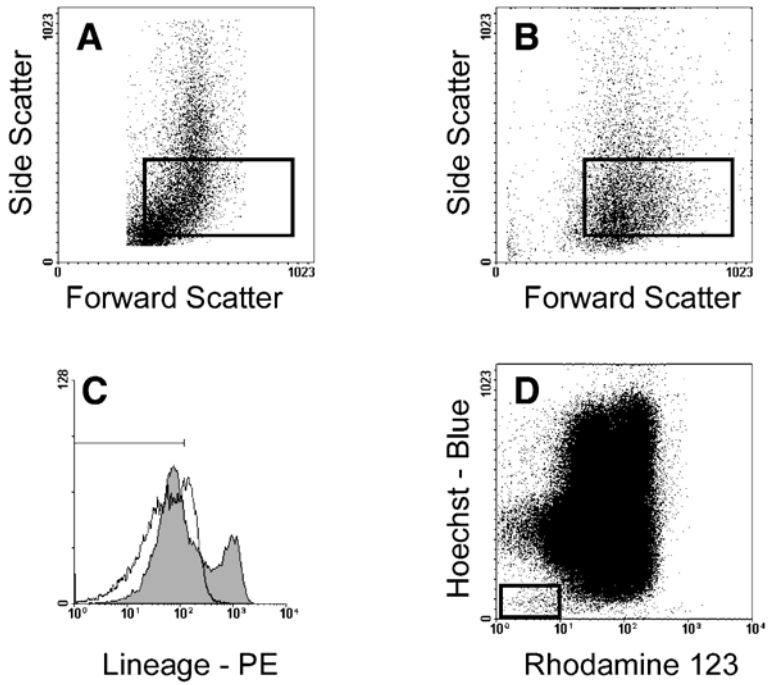

Fig. 2. The sequential gating strategy for the isolation of Lin^- Ho^{dull}/Rh^{dull} PHSC. Unseparated murine bone marrow **(A)** is used to define a FSC vs SSC lymphoblastoid gate that contains the majority of hematopoietic stem and progenitor cells ***(1)***. This gate is then imposed on the FSC vs SSC bivariate plot of viable low density Lin^- cells prepared by negative immunomagnetic selection using the MACS system **(B)**. Residual Lin^+ cells within the light scatter gate are excluded by setting a histogram region **(C)** with reference to the fluorescence profiles of an antibody labeled pre-MACS control sample *(shaded histogram)* and low-density cells labeled with a cocktail of relevant biotinylated isotype control antibodies and streptavidin–PE *(solid line)*. A sort window defining the Ho^{dull}/Rh^{dull} phenotype **(D)** is then set with reference to the bivariate Rh123 vs Ho fluorescence dot-plot of cells within these conditional gates.

3. Within this light scatter window, exclude contaminating Lin^+ cells in the Lin^- cell fraction (prepared by negative immunomagnetic selection) by setting a histogram gate with reference to the fluorescence histogram profile of low-density cells labeled with a cocktail of relevant biotinylated isotype control antibodies and Strep–PE.
4. Set a sort window defining the Ho^{dull}/Rh^{dull} phenotype within these conditional gates with reference to the bivariate Rh123 vs Ho fluorescence dot-plot in which Ho fluorescence is displayed on a linear scale, and Rh123 fluorescence is displayed on a logarithmic scale (*see* **Note 16**).

3.7. Sorting Ho^{dull}/Rh^{dull} PHSC

1. Selection of sort mode is instrument dependent. For the FACStar^{Plus} cell sorter (BD Biosciences, San Jose, CA), we sort cells in a two-droplet packet in "Normal R" mode. For the FACS Vantage SE (BD Biosciences), sort with a purity mask.
2. The Ho^{dull}/Rh^{dull} cell cluster typically comprises no more than 0.5% of listed events in the conditionally gated Rh123 vs Ho bivariate dot-plot. Consequently, it is recommended that the sort sample be run at low to medium differential pressures/flow rates to improve the definition and resolution of this subpopulation. For cell suspensions of 5×10^6 cells/mL we generally sort at rates of 2500–5000 events per second even when using a high-speed cell sorter.
3. The choice of collection vessel for sorted cells is determined by the expected cell recovery. Typically cells are sorted into sterile 1.5-mL Eppendorf tubes or 12×75 mm sterile polypropylene tissue culture tubes. The collection tubes are coated with a small volume of sterile HiSe or with PBS–5% HiSe into which the cells are collected.
4. Low-density Lin$^-$ Ho^{dull}/Rh^{dull} cells comprise approx 0.007% of cells in starting unfractionated murine bone marrow cell suspensions. Theoretical cell yields at each step of this cell separative protocol are given in the flowchart in **Fig. 3**. In practice, the actual number of sorted Ho^{dull}/Rh^{dull} cells recovered is generally lower. The yield is dependent on nonspecific cell losses during cell manipulation (which are proportionately more significant when processing small bone marrow harvests), day-to-day variation in cell sorting efficiency, and the precise setting of the Rh123 vs Ho sort window (*see* **Note 16**).

4. Notes

1. Hoechst 33342 can also be purchased from Molecular Probes Inc., as a 10 mg/mL solution dissolved in water. Some investigators have noted that Hoechst 33342 can precipitate in PBS at concentrations >30 μ*M* ***(26)***. We have occasionally noticed this when using serum-supplemented PBS. Precipitation is not instantaneous and can be avoided by making serial dilutions quickly. If precipitation is observed, the working solution should be discarded.
2. Murine mononuclear cells, like those of most mammalian species, are denser than human mononuclear cells. While commercial density gradient media for isolation of human mononuclear cells are generally formulated at osmolarities isotonic with human plasma, Nycoprep 1.077A is specifically formulated at a lower osmolarity (265 mos*m*) for optimal isolation of nonhuman mammalian mononuclear cells.
3. We routinely use PBS for all cell manipulations prior to cell culture. It is possible to use bicarbonate buffered balanced salt solutions, or more complex media, but these should be buffered using *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (HEPES). It is preferable to avoid use of buffers containing phenol red, as this dye can be excited at 488 nm and can cause cells to autofluoresce. However, these fluorescent emissions can be compensated, and we have used these buffers in the past without adversely affecting the detection of Ho^{dull}/Rh^{dull} target cells.

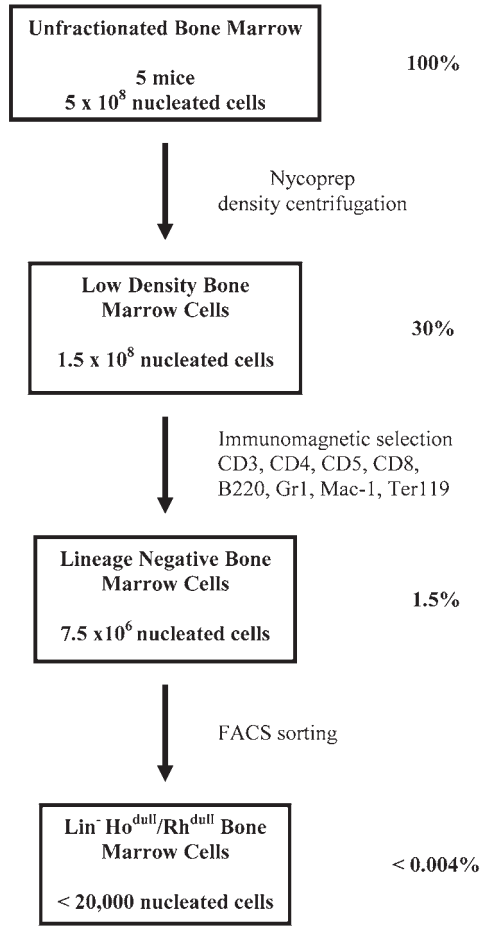

Fig. 3. Flowchart showing typical yields of nucleated cells at each step of the multiparameter cell separative strategy devised for the isolation of Ho^{dull}/Rh^{dull} PHSC from normal murine bone marrow.

4. The weights of chemical reagents used for making up PBS are for the anhydrous form of buffer ingredients. If hydrated forms of these reagents are used the weights need to be adjusted accordingly.
5. Heat inactivation of serum for this application is adopted as a precautionary measure. Although is not strictly necessary when using highly purified antibody reagents, we do so because we have occasionally experienced significant nonspecific cell losses when using buffers supplemented with non-heat-inactivated serum especially when incubating bone marrow cells with unpurified or partially purified

antibody supernatants. Alternately, 0.5% BSA (fraction V, tissue culture grade) can be substituted for serum as a buffer constituent.

6. We use MACS systems as the immunomagnetic cell separation platform in our laboratory. It is equally valid to use Dynabeads for this purpose. Alternatively, cells can be labeled with a cocktail of PE-conjugated primary antibodies. In this case, hematopoietic lineage antigen positive cells can be excluded flow cytometrically in the sort gating strategy. We have often used this approach for flow cytometric analysis of Ho^{dull}/Rh^{dull} bone marrow cells. However, because lineage positive cells constitute up to 95% of the low density cell fraction this alternative significantly extends the amount of cell sorter time required to isolate Ho^{dull}/Rh^{dull} PHSC.
7. We have chosen to use biotinylated antibody reagents and streptavidin beads for immunomagnetic selection based on the versatility of the primary antibody reagents in other applications in our laboratory. It is equally valid to use purified primary rat antimouse antibodies and microbead-conjugated antirat IgGs. The choice of fluorochrome conjugates will also ultimately be guided by laser configuration and fluorescence emission detection capabilities of the cell sorter.
8. It is imperative that biotin-free buffer is used for labeling with biotinylated antibodies to prevent competition of free biotin with biotinylated antibody in the binding of the streptavidin microbeads. This buffer can be supplemented with certified biotin-free BSA, but not serum or BSA of unknown status. BSA-supplemented buffer is recommended for the immunomagnetic separation steps following antibody and immunomagnetic bead conjugation.
9. The collection of bone marrow cells by crushing bones using a mortar and pestle rather than solely by flushing the bone shafts from both ends with cold PBS–2% HiSe using a 1-mL syringe fitted with a 23-gage needle significantly reduces the number of donor mice. Lambert et al. ***(49)*** have shown that approx 5×10^8 cells can be recovered from the entire skeleton of single adult mouse using this method. We typically harvest approx 10^8 cells from the pooled femurs, tibiae, and iliac crest of a single adult mouse.
10. PHSC are intimately associated with endosteal marrow ***(50)***. Digestion of crushed bone fragments using collagenase/dispase also maximizes stem cell yield. Periodic agitation of the tube will aid dispersion of the cells if a 37°C water bath is used in place of a shaker.
11. We routinely use 50-mL tubes for density gradient centrifugation. If preferred, gradients can be prepared in 15-mL centrifuge tubes by overlaying 6 mL of cell suspension on a 5-mL Nycoprep cushion. On average, a single 50-mL gradient tube is required for the bone marrow harvested from two mice.
12. Although it is advisable to follow the manufacturer's advice, a lower bead/cell ratio can be used; but the likelihood of contamination of the nonmagnetic fraction with cells expressing low levels of hematopoietic lineage antigens is increased.
13. Typically, a CS column (capacity 2×10^8 magnetically labeled cells) is required for the bone marrow harvested from five or six mice. The manufacturer's data sheets specify a number of flow resistors that are suitable for use with various

MACS columns. We have found that optimal resolution of Lin^- cells is achieved using a 22G flow resistor with a CS column and a 21G flow resistor if using a D column.

14. There are a number of critical variables that must be controlled to ensure an optimal enrichment of lineage-negative cells using the MACS system. The separator must be chilled, and buffers and cell suspensions kept cold. Degassing of buffers used to prime and wash MACS columns is essential to prevent the formation of air bubbles in the column or flow resistor with consequent impairment of flow rate. Likewise, it is essential that the column not be allowed to run dry during the separation procedure.
15. Temperature is an important variable that must be precisely controlled for optimal performance of efflux pumps. It is essential that buffers and media used in dye loading and efflux steps be prewarmed; and that tubes be immersed in a 37°C water bath to at least the level of the meniscus of the cell suspension during this procedure.
16. In contrast to the gating strategy used by others to isolate Ho^{dull}/Rh^{dull} cells ***(11)*** in which this fraction is delimited by the lowest 15 percentiles of Rh123 fluorescence within the lowest 3 percentiles of Ho fluorescence, we have defined Ho^{dull}/Rh^{dull} cells with reference to the bivariate Rh123 vs Ho fluorescence dot-plot ***(13)***. In the first strategy placement of the Rh123 sort gate is contingent on the distribution of Ho fluorescent cells. Although our approach by-and-large defines a similar population (0–14th percentile of Rh123 fluorescence within the 0–5th percentile of Ho fluorescence), we believe that definition Ho^{dull}/Rh^{dull} cells with reference to the bivariate plot provides a more accurate and reproducible definition of the Ho^{dull}/Rh^{dull} phenotype. However, in those instances in which the Ho^{dull}/Rh^{dull} cell cluster is difficult to visualize in the bivariate dot-plot, we defer to the former method.

References

1. Bertoncello, I., Hodgson, G. S., and Bradley, T. R. (1985) Multiparameter analysis of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of Rhodamine 123 fluorescence. *Exp. Hematol.* **13,** 999–1006.
2. Bertoncello, I., Hodgson, G. S., and Bradley, T. R. (1988) Multiparameter analysis of transplantable hemopoietic stem cells. II. Stem cells of long-term bone marrow reconstituted recipients. *Exp. Hematol.* **16,** 245–249.
3. Bertoncello, I., Bradley, T. R., Hodgson, G. S., and Dunlop, J. M. (1991) The resolution, enrichment, and organisation of normal bone marrow high proliferative potential colony-forming cell subsets on the basis of rhodamine 123 fluorescence. *Exp. Hematol.* **19,** 174–178.
4. Visser, J. W., Bol, S. J., and van den Engh, G. (1981) Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting. *Exp. Hematol.* **9,** 644–655.
5. Baines, P. and Visser, J. W. (1983) Analysis and separation of murine bone marrow stem cells by H33342 fluorescence-activated cell sorting. *Exp. Hematol.* **11,** 701–708.

6. Ploemacher, R. E. and Brons, N. H. (1988) Cells with marrow and spleen repopulating ability and forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. *J. Cell. Physiol.* **136,** 531–536.
7. Pallavicini, M. G., Summers, L. J., Dean, P. N., and Gray, J. W. (1985) Enrichment of murine hemopoietic clonogenic cells by multivariate analyses and sorting. *Exp. Hematol.* **13,** 1173–1181.
8. Neben, S., Redfearn, W. J., Parra, M., Brecher, G., and Pallavicini, M. (1991) Short- and long-term repopulation of lethally irradiated mice by bone marrow stem cells enriched on the basis of light scatter and Hoechst 33342 fluorescence. *Exp. Hematol.* **19,** 958–967.
9. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183,** 1797–1806.
10. Goodell, M. A., Rosenzweig, M., Kim, H., et al. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3,** 1337–1345.
11. Wolf, N. S., Kone, A., Priestley, G. V., and Bartelmez, S. H. (1993) In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. *Exp. Hematol.* **21,** 614–622.
12. Sitnicka, E., Ruscetti, F. W., Priestley, G. V., Wolf, N. S., and Bartelmez, S. H. (1996) Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. *Blood* **88,** 82–88.
13. Bradford, G. B., Williams, B., Rossi, R., and Bertoncello, I. (1997) Quiescence, cycling and turnover in the primitive hematopoietic stem cell compartment. *Exp. Hematol.* **25,** 445–453.
14. Nilsson. S., Dooner, M., Tiarks, C., Heinz-Ulrich, W., and Quesenberry, P. J. (1997) Potential and distribution of transplanted hematopoietic stem cells in a non-ablated mouse model. *Blood* **89,** 4013–4020.
15. Reddy, G. P., Tiarks, C. Y., Pang, L., Wuu, J., Hsieh, C. C., and Quesenberry, P. J. (1997) Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells. *Blood* **90,** 2293–2299.
16. Micallef, S, Ramsay, R., Williams, B., Rossi, R., Mucenski, M., and Bertoncello, I. (2000) The role of c-myb and BCL-2 in hematopoietic stem cell regulation. *Exp. Hematol.* 28(Suppl. 1), **68.**
17. Reddy, G. P. V., McAuliffe, C. I., Pang, L., Quesenberry, P. J., and Bertoncello, I. (2002) Cytokine receptor repertoire and cytokine responsiveness of Ho^{dull}/Rh^{dull} stem cells with differing potentials for G_1/S phase progression. *Exp. Hematol.* **30,** 792–800.
18. Spangrude, G. J. and Johnson, G. R. (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **87,** 7433–7437.
19. Li, C. L. and Johnson, G. R. (1995) Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. *Blood* **85,** 1472–1479.

20. Uchida, N., Combs, J., Chen, S., Zanjani, E., Hoffman, R., and Tsukamoto, A. (1996) Primitive human hematopoietic cells displaying differential efflux of the rhodamine 123 dye have distinct biological activities. *Blood* **88,** 1297–1305.
21. Kim, M., Cooper, D. D., Hayes, S. F., and Spangrude, G. J. (1998) Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood* **91,** 4106–4117.
22. Kim, M., Moon, H. B., and Spangrude, G. J. (2003) Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann. NY Acad. Sci.* **996,** 195–208.
23. Zijlmans, J. M., Visser, J. W., Kleiverda, K., Kluin, P. M., Willemze, R., and Fibbe, W. E. (1995) Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. *Proc. Natl. Acad. Sci. USA* **92,** 8901–8905.
24. Visser, J. W. and de Vries, P. (1988) Isolation of spleen-colony forming cells (CFU-s) using wheat germ agglutinin and rhodamine 123 labeling. *Blood Cells* **14,** 369–384.
25. Huttmann, A., Liu, S. L., Boyd, A. W., and Li, C. L. (2001) Functional heterogeneity within rhodamine123(lo) Hoechst33342(lo/sp) primitive hemopoietic stem cells revealed by pyronin Y. *Exp. Hematol.* **29,** 1109–1116.
26. Srour, E. F. and Jordan, C. T. (2002) Isolation and characterization of primitive hematopoietic cells based on their position in the cell cycle, in *Hematopoietic Stem Cell Protocols* (Klug, C. A. and Jordan, C. T., eds.), Humana Press, Totowa, NJ, pp. 93–111.
27. Hellman, S., Botnick, L. E., Hannon, E. C., and Vigneulle, R. M. (1978) Proliferative capacity of murine hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **75,** 490–494.
28. Lajtha, L. G. (1979) Stem cell concepts. *Differentiation* **14,** 23–34.
29. Hodgson, G. S., Bradley, T. R., and Radley, J. M. (1982) The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. *Exp. Hematol.* **10,** 26–35.
30. Bunting, K. D. (2002) ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* **20,** 11–20.
31. Schinkel, A. H., Smit, J. J., van Tellingen, O., et al. (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell* **77,** 491–502.
32. Schinkel, A. H., Mayer, U., Wagenaar, E., et al. (1997) Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc. Natl. Acad. Sci. USA* **94,** 4028–4033.
33. Scharenberg, C. W., Harkey, M. A., and Torok-Storb, B. (2002) The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* **99,** 507–512.
34. Zhou, S., Schuetz, J. D., Bunting, K. D., et al. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat. Med.* **7,** 1028–1034.

35. Zhou, S., Morris, J. J., Barnes, Y., Lan, L., Schuetz, J. D., and Sorrentino, B. P. (2002) Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc. Natl. Acad. Sci. USA* **99,** 12,339–12,344.
36. Johnson, L. V., Walsh, M. L., and Chen, L. B. (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA* **77,** 990–994.
37. Johnson, L. V., Walsh, M. L., Bockus, B. J., and Chen, L. B. (1981) Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J. Cell Biol.* **88,** 526–535.
38. Darzynkiewicz, Z., Staiano-Coico, L., and Melamed, M. R. (1981) Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc. Natl. Acad. Sci. USA* **78,** 2383–2387.
39. Latt, S. A. and Stetten, G. (1976) Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J. Histochem. Cytochem.* **24,** 24–33.
40. Lalande, M. E. and Miller, R. G. (1979) Fluorescence flow analysis of lymphocyte activation using Hoechst 33342 dye. *J. Histochem. Cytochem.* **27,** 394–397.
41. Arndt-Jovin, D. J. and Jovin, T. M. (1977) Analysis and sorting of living cells according to deoxyribonucleic acid content. *J. Histochem. Cytochem.* **25,** 585–589.
42. Watson, J. V., Nakeff, A., Chambers, S. H., and Smith, P. J. (1985) Flow cytometric fluorescence emission spectrum analysis of Hoechst-33342-stained DNA in chicken thymocytes. *Cytometry* **6,** 310–315.
43. Smith, P. J., Nakeff, A., and Watson, J. V. (1985) Flow-cytometric detection of changes in the fluorescence emission spectrum of a vital DNA-specific dye in human tumour cells. *Exp. Cell Res.* **159,** 37–46.
44. Smith, P. J., Morgan, S. A., and Watson, J. V. (1991) Detection of multidrug resistance and quantification of responses of human tumour cells to cytotoxic agents using flow cytometric spectral shift analysis of Hoechst 33342-DNA fluorescence. *Cancer Chemother. Pharmacol.* **27,** 445–450.
45. Ratajczak, M. Z., Pletcher, C. H., Marlicz, W., et al. (1998) CD34+, kit+, rhodamine123(low) phenotype identifies a marrow cell population highly enriched for human hematopoietic stem cells. *Leukemia* **12,** 942–950.
46. Leemhuis, T., Yoder, M. C., Grigsby, S., Aguero, B., Eder, P., and Srour, E. F. (1996) Isolation of primitive human bone marrow hematopoietic progenitor cells using Hoechst 33342 and Rhodamine 123. *Exp. Hematol.* **24,** 1215–1224.
47. Szilvassy, S. J. and Cory, S. (1993) Phenotypic and functional characterization of competitive long-term repopulating hematopoietic stem cells enriched from 5-fluorouracil-treated murine marrow. *Blood* **81,** 2310–2320.
48. Borth, N., Kral, G., and Katinger, H. (1993) Rhodamine 123 fluorescence of immortal hybridoma cell lines as a function of glucose concentration. *Cytometry* **14,** 70–73.

49. Lambert, J. F., Carlson, J. E., Colvin, G. A., and Quesenberry, P. J. (2000) Evaluation of mouse whole body bone marrow cellularity and distribution of hematopoietic progenitors. *Exp. Hematol.* **28,** 1493.
50. Nilsson, S. K., Johnston, H. M., and Coverdale, J. A. (2001) Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* **97,** 2293–2299.