Flow Cytometry of the Side Population (SP)

This protocol describes the application of Hoechst 33342 (bisbenzimide, H 33342; Ho342) staining to identify and purify side-population (SP) stem cells from various human and murine tissue sources (Goodell et al., 1996, 1997; Storms et al., 2000; Jackson et al., 2001). This is a functional assay that uses living cells, allowing investigators to study an active biological process based on the differential efflux of Ho342 by the ABCG2 multidrug transporter (Zhou et al., 2001). UV laser power and alignment, filter settings, sample collection, cell viability, Ho342 concentration, and the staining procedure as a whole are crucial for the optimal resolution of the side population.

Ho342 is a specific stain for AT-rich regions of double-stranded DNA. The absorption maximum is 346 nm and the emission maximum 460 nm. When using Ho342, which has two emission wavelengths, one blue and one red, a small fraction of these Ho342-stained cells form a dim tail extending from the G_0/G_1 cells, if SP cells exist in the sample.

Keep in mind that not all tissues examined may have apparent SP cells. In order to rule out artifacts, the author strongly recommends that the protocols in this unit be followed exactly as described, and that novice experimenters start with murine bone marrow.

The Basic Protocol presents flow cytometric procedures (gating and sample acquisition) for flow cytometric identification of SP cells. Support Protocol 1 describes preparation of samples (hematopoietic cells, tissue/tumor samples, cultured cells) for the flow cytometric analysis. Support Protocol 2 describes Hoechst 33342 labeling of such cells in preparation for flow cytometry, and Support Protocol 3 describes an optional procedure for simultaneously staining the cells with antibodies. Finally, Support Protocol 4 provides information on laser alignment and quality control for the flow cytometric analysis.

GATING STRATEGY AND SAMPLE ACQUISITION FOR FLOW CYTOMETRIC IDENTIFICATION OF SIDE-POPULATION STEM CELLS

BASIC PROTOCOL

The original protocol developed by Margaret Goodell's laboratory (see *UNIT 9.18*) measures the PI emission through the same filter used for the Ho342 red. However, based on the emission properties of this dye, and for a more precise discrimination of dead cells, an alternative procedure was chosen in the author's laboratory. PI emission is measured on a logarithmic scale using a different PMT (BP 630/30) and 488-nm excitation, instead of using the same PMT for both PI and Ho342 red emission, resulting in better resolution of the SP. In the author's opinion, a feasible live gate based on use of these conditions should better resolve the SP, even if apoptotic/prenecrotic cells are present in the sample.

Materials

Stained cells (see Support Protocols 2 and 3)
Flow cytometer equipped with UV laser operating at ~350 nm and standard 488-nm laser or laser diode
Filter combination consisting of 405/30 band-pass (blue), 670/40 band-pass (red), and 440 dichroic long-pass filters *or* 450/65 band-pass (blue), 670/40 band-pass (red) and 510 dichroic long-pass filters

NOTE: Keep the sample at 4°C and protected from light during analysis.

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1. First create a dot plot displaying forward scatter (FS) on a linear scale and PI on a logarithmic scale.

   A region representing PI-negative cells is used to exclude all dead (PI-positive) cells. Pre-necrotic cells should also be excluded within the live-gate region.

   Support Protocol 4 provides information on laser alignment and quality control for the flow cytometric analysis.

2. Create a second dot plot region representing side scatter (SS) and Ho342-blue cells (both on a linear scale) to include all live DNA-positive cells, excluding erythrocytes and debris.

3. Create a third dot plot displaying SS and Ho342-red cells (linear scale) to set the emission for determining red DNA labeling in live cells.

4. Also create the following two-parameter plots:

   FS versus SS
   Ho342-red versus Ho342-blue.

5. Run sample while viewing the FS versus PI plot. Adjust voltages of both parameters until all the cells are captured on the dot plot.

6. Adjust SS while viewing the FS versus SS plot.

   Special care should be taken to include low-SS and low-FS events, which can be enriched in small SP cells.

7. Adjust voltages of Ho342-red and Ho342-blue (both on a linear scale) until cells representing the G_0/G_1 population appear visible in the center of the Ho342-red versus Ho342-blue dot plot.

   If they exist in the sample, SP cells appear as a dim tail extending on the left side of G_0/G_1 cells. A minimum of 100,000 live-gated events should be acquired. Use the maximum allowed pressure differential giving the best resolution (the lowest G_0/G_1 coefficient of variation).

SUPPORT PROTOCOL 1

SAMPLE PREPARATION FOR FLOW CYTOMETRY OF THE SIDE POPULATION

This protocol describes preparation of nucleated cells for flow cytometric analysis of the side population.

Materials

Hematopoietic sample, tissue/tumor sample, or adherent cultured cells to be stained
Ammonium chloride lysing solution (see recipe)
BME-HEPES: Basal Medium Eagle (e.g., Invitrogen) supplemented with 10 mM HEPES
Enzyme medium (see recipe)
DMEM+ (see recipe)
DMEM+ (see recipe) supplemented with 500 Kunitz units/ml DNase (prepare fresh daily; store at 4°C)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Refrigerated centrifuge
Curved-edge scalpels
75-ml trypsinization flasks (e.g., Fisher)
50-μm nylon mesh
Additional reagents and equipment for preparation of white blood cell samples for flow cytometry (*UNIT 5.1*), counting cells (*APPENDIX 3A*), and trypsinization of cultured adherent cells (*APPENDIX 3B*)

For hematopoietic samples

The original Hoechst staining protocol describes the use of this dye to identify and purify murine hematopoietic stem cells. Pre-enrichment techniques, such as those aimed to concentrate mononuclear cells (i.e., density gradients) as well as lineage positive–depletion protocols, are not recommended for small-volume samples (e.g., pediatric samples and bone marrow aspirates). Keep in mind that SP cells are very rare cells and it is conceivable that cell loss through sample manipulation may alter the final results. Classic density gradients (density of 1.077 g/ml), which can be useful for mononuclear cell enrichment, can be suboptimal for the accurate identification and isolation of the SP compartment. If needed, and for especially small sample volumes, red cell lysis is recommended instead of gradient-density enrichment protocols.

1a. Process hematopoietic sample immediately or within 2 hr after stem cell harvest.

Cord-blood specimens usually are maintained at 4°C at cord-blood banks, being collected and processed within the first 24 hr.

Murine bone-marrow (BM) samples (e.g., C57BL6/J mouse) can be obtained from 8- to 12-week-old mice by flushing their femora using a 21-G needle and a 5-ml syringe. BM from two femurs of a mouse yields an average of 3×10^7 nucleated cells (UNIT 9.18).

If sample processing and sorting cannot not be performed on the same day, the hematopoietic samples can be depleted of erythrocytes by lysis as described in step 2a, stained, and kept at 4°C overnight prior to cell sorting.

Human bone-marrow samples should be anticoagulated 1:3 with sterile room-temperature PBS containing 9 mM EDTA. Cord blood is usually anticoagulated 1:3 with PBS containing 2 mM EDTA.

2a. Prepare a suspension of white blood cells by lysis of erythrocytes as described in UNIT 5.1, Basic Protocol 1, with the following variations:

i. Use the ammonium chloride lysis solution described in Reagents and Solutions of this unit.

This solution contains only 1 mM EDTA, in contrast to the 10 mM EDTA in the corresponding recipe in UNIT 5.1.

ii. Centrifuge cells 5 min at $300 \times g$, room temperature.

The lysis procedure is preferred when it is desirable to avoid gradient density enrichment, especially in the case of low-volume hematopoietic samples, such as bone-marrow aspirates. The procedure is also useful for removing erythrocytes from blood-contaminated samples, such as those coming from highly vascularized tissues or tumors.

3a. Alternatively, use a Ficoll gradient with a density of 1.077 g/ml as described in UNIT 5.1 to prepare a mononuclear cell suspension.

Gradients can be adjusted using different densities for a further enrichment in SP cells (1.069 g/ml, 1.077 g/ml, 1.084 g/ml).

For solid tissues and tumors

Surgically excised solid tissues or tumors ~1 to 3 cm in diameter can be used. All surgical procedures should be carried out under sterile conditions.

1b. Transfer excised tissues to 100-mm petri dishes containing BME-HEPES.

2b. Cut tissues into pieces ~2 to 3 mm across the greatest dimension using two curved-edge scalpels in a cross-cutting motion.

3b. Rinse fragments thoroughly in BME-HEPES.

4b. Place the fragments into 75-ml trypsinizing flasks. Add 20 to 40 ml enzyme medium at 37°C.

5b. Stir tissues 20 to 30 min, 37°C, on a magnetic stirrer at a speed that causes tumbling of the tissue but does not produce foaming.

6b. After incubation, decant free cells into a 50-ml conical polypropylene centrifuge tube through several layers of sterile 50-μm nylon mesh that has been wetted with DMEM+.

7b. Centrifuge the cells 10 min at 225 × *g*, 4°C. Remove supernatant by aspiration. Resuspend cells in 5 to 10 ml of DMEM+ containing 500 Kunitz units/ml DNase and incubate 10 min at 37°C with shaking.

The final incubation with DNase is included to avoid clumps that may clog the flow cytometer nozzle.

This disaggregation process may be repeated for two to five incubations, depending on the tissue. Other soft or delicate tissues (i.e., brain, liver, fetal) may require only mechanic dissociation using a sterile tissue sieve partially submerged in DMEM+. For sterilization of the tissue sieve, 70% ethanol can be used; after sterilization, rinse the sieve by immersion in sterile DMEM+.

For cells attached to tissue culture plates

1c. Collect cells from flasks or petri dishes by trypsinization. As soon as cells are detached, rinse with medium containing serum for trypsin inactivation.

Special cell types may need a scraper to be detached.

2c. Pool the detached cells with cells floating in the medium (mostly detached mitotic and dead cells). Centrifuge 10 min at 225 × *g*, 21°C, and remove supernatant.

3c. Resuspend cells in ice-cold PBS at 10^6 cells/ml.

SUPPORT PROTOCOL 2

HOECHST 33342 LABELING OF CELLS FOR FLOW CYTOMETRY OF THE SIDE POPULATION

This protocol describes the application of Ho342 staining to identify and purify side-population stem cells from different human and murine tissue sources. Optimal staining time for murine samples is 90 min, whereas 120 min is optimal for human cells.

All human samples should be stained using the same incubation times (2 hr). Specimens obtained from other species may require different incubation times, which should be determined in pilot experiments. Murine samples should be stained for 90 min. After red-cell lysis, cells are resuspended at a density of 1-2 × 10^6 cells/ml in DMEM supplemented with 2% heat-inactivated fetal bovine serum (FBS) and 10 mM HEPES, prewarmed to 37°C. Hoechst 33342 (Ho342) is added at a concentration of 5 or 10 μg/ml, depending on the density of cells per ml. Cells are incubated 2 hr (human samples) or 90 min (murine samples) in a water bath at 37°C with periodic agitation. Cells are then centrifuged 6 min at 483 × *g*, 4°C, and resuspended at a concentration of 1-2 × 10^7 cells/ml in cold HBSS containing 2% FBS and 10 mM HEPES. Samples should be kept at 4°C until analysis. Propidium iodide (PI) is added at a concentration of 5 μg/ml to exclude dead cells. In order to remove cellular aggregates, cells should be filtered through a 50-μm nylon mesh prior to analysis.

Materials

DMEM+ (see recipe)
Sample of nucleated cells, ~1–2 ×10^6 cells/ml (Support Protocol 1)
1× ammonium chloride lysing solution (optional; see recipe for 10×)
Türk solution (see recipe; optional)

1 mg/ml stock solution of Hoechst 33342 (Ho342; Sigma) in H_2O (filter sterilize and store in 1-ml single-use aliquots at –20°C)

5 mM stock solution of verapamil (Sigma) in H_2O (filter sterilize and store up to 1 month at 4°C) *or* 5 mM stock solution of fumitremorgin C (FTC; available through NIH drug screen, *http://dtp.nci.nih.gov/branches/dscb/repo_request.html#spec-request*) in DMSO (filter sterilize and store up to 1 month at 4°C)

HBSS+ (see recipe)

1 mg/ml stock solution of propidium iodide (Sigma) in H_2O (store up to 1 month at 4°C)

Light-protected circulating water bath kept exactly at 37°C

Refrigerated centrifuge

50-ml conical polypropylene centrifuge tubes

50-μm nylon mesh

Additional reagents and equipment for simultaneous staining with antibodies (optional; Support Protocol 3)

NOTE: All incubations should be carried out with periodic mixing. Incubations must be done in the dark, but the incubation tubes should not be covered with aluminum foil, which can inhibit heat transfer to the cells.

Prepare cells for staining

1. Set a circulating water bath in a light-protected area exactly at 37°C. Prewarm DMEM+ to 37°C.

   In order to avoid temperature fluctuations, a dedicated water bath and mercury thermometer should be used. It is also possible to use a static water bath. In any case, consistency of temperature is necessary.

2. If RBC contamination is noted in the cell sample, lyse red cells by adding 10 ml of 1× ammonium chloride lysing solution per ml of sample and agitating ~3 min at room temperature. Centrifuge cells 5 min at 483 × *g*, room temperature, and remove the supernatant.

3. Count nucleated cells accurately (*APPENDIX 3A*). Resuspend pellet at 1×10^6 cells/ml in prewarmed DMEM+.

   IMPORTANT NOTE: *Always count cells accurately using a Neubauer chamber (hemacytometer) prior to Hoechst 33342 staining. If red cell lysis has not been performed during sample preparation, lysing erythrocytes with Türk solution (see Reagents and Solutions) will aid in counting of nucleated cells. To perform this lysis, first filter the Türk solution through a 0.22-μm filter. Prepare a 1:10 dilution of the sample by adding 100 μl of the cell suspension to 900 μl of the filtered Türk solution and incubate 5 min at room temperature. Load hemacytometer with 20 μl of the diluted cells and cover with an appropriate coverslip, then count nucleated cells under low-power objective (4× to 10×) using a bright-field microscope (also see APPENDIX 3A).*

Stain cells

4. Add Ho342 stock solution to the cell suspension for a final concentration of 5 μg/ml. Transfer the cell/dye suspension to 50-ml conical polypropylene centrifuge tubes.

   For larger volumes, use 250-ml tubes.

   The Ho342 concentration of 5 μg/ml is optimal for resolution of the side population of most human and mouse cells at a density of 10^6 cells/ml. This concentration has been empirically determined; other species may require testing of different Ho342 concentrations. Using euploid cells, optimal CVs should not exceed 6% to 7%, and CVs >15% should be considered inadequate. The degree of acceptable side shift should be confirmed by using specific ABCG2 reversal agents, e.g., verapamil or fumitremorgin C (see Commentary).

5. Prepare a parallel sample aliquot in the presence of a specific inhibitor, e.g., verapamil at 50 μM or FTC at 1 to 10 μM, to confirm ABCG2 inhibition.
6. Gently mix the tubes and place them in the 37°C water bath prepared in step 1. Incubate 2 hr, keeping the tubes well submerged to ensure that the temperature is maintained at 37°C, and gently inverting several times during the course of the incubation to ensure homogeneous cell staining.

   When the staining process is over, the cells should be maintained at 4°C in order to inhibit further dye efflux.
7. Centrifuge 6 min at 483 × *g*, 4°C, and remove the supernatant. Resuspend cells at 20×10^6 cells/ml in ice-cold HBSS+.
8. Add PI stock solution to the cell suspension for a final concentration of 5 μg/ml. Incubate 5 min at 4°C.
9. *Optional:* Perform simultaneous staining with antibodies as described in Support Protocol 3.

   All further manipulations must be performed at 4°C.
10. Filter the sample through a double 50-μm nylon mesh.

    At this point samples may be run directly on the flow cytometer.

SUPPORT PROTOCOL 3

SIMULTANEOUS STAINING WITH ANTIBODIES

For immunofluorescence assays, directly conjugated antibodies are preferred. However, indirect immunofluorescence techniques can also be applied for polychromatic flow cytometry. Staining with directly conjugated antibodies allows a feasible multicolor analysis, even using high-power laser settings. If low antigen expression is expected, use monoclonal antibodies conjugated to bright fluorochromes (e.g., phycoerythrin-conjugated anti-CD34 antibody). Conversely, and for the highest expected antigen expression, use monoclonal antibodies conjugated to less bright fluorochromes (e.g., fluorescein-conjugated CD45 antibody). If needed for high-power lasers, a set of neutral-density filters can be used to avoid saturation of the photomultiplier tubes. Conversely, in order to detect low-fluorescence signals, neutral-density filters can be removed if they are being used.

***Additional Materials** (also see Support Protocol 2)*

Conjugated antibodies (BD Biosciences, Beckman Coulter, Invitrogen, Miltenyi Biotec), e.g.:
FITC-CD45
APC-CD38
PE-CD34
PE-CD90
PE-CD117
PE-CD133
HBSS+ (see recipe) containing 5 μg/ml propidium iodide (PI; Sigma)

1. Incubate samples with the appropriate concentration of antibody (usually 10 μl of the undiluted antibody per 10^6 cells in 50 μl). Incubate 30 min at 4°C in the dark.
2. Following incubation with antibodies, add 450 μl cold HBSS+ containing 5 μg/ml PI directly to the samples without washing and incubate 5 min at 4°C in the dark.

   Propidium iodide should be maintained in the medium used to dilute the cells even if the cells have been previously stained with PI. Dilution of samples without PI will decrease the number of prestained dead cells, which may significantly alter the SP results.

LASER ALIGNMENT AND QUALITY CONTROL

For the SP application, the laser beam paths should be exhaustively checked. UV alignment should be adjusted to remove, if present, any clipping (or distortion), and beam-shaping optics also should be cleaned.

Materials

Fluorescent beads (also see *UNIT 1.3*):
For UV laser alignment: 4.5-μm diameter, emission maximum, ~405 nm (Fluoresbrite BB Carboxylate microspheres; Polysciences)
For 488-nm laser alignment: Flow-Check fluorospheres (Beckman Coulter)
Flow cytometer with 405/30 band-pass filter and 440 dichroic long-pass (DLP) filter for analyzing beads

1. Run beads and check the pulse shape on the UV parameters, especially for blue emission.
2. Improve the UV parameter pulse shape until the pulse width is uniform.

   The overall effect should be an improvement in blue and red sensitivity and lowers CVs.
3. Again run beads on the system and acquire data against time to monitor the stability of the UV path over time.
4. Check the stability from a "cold start-up" of the system and also following restart after a 1-hr shutdown.
5. Try to find the minimum time of laser warm-up for beam stability.

   Over this warm-up period (typically at least 30 min) the beam quality improves, giving more homogeneous bead populations and increased sensitivity.
6. After the warm-up period, check for any laser drift in the system over time.

   Drift should not exceed 10 channels (on a 1024 linear scale) after warm-up.
7. If possible and for laser alignment, try to set the power low to seek for the best CV.

   Too low power can result in poor CVs (too few photons for accurate reproducibility in counting); some lasers may also result in lower CVs when the power is increased beyond what is optimal.
8. Maximize the UV fluorescence and scatter signals using beads.

   Use small beads (i.e., 4.5 μm diameter fluorescent particles) with an emission maximum of ~405 nm and analyze through a band pass filter of 405/30 and a 440 DLP filter.
9. Adjust the detector in linear mode. Adjust the flow rate to less than 300 events per sec.

   Acceptable CVs with operating conditions of high pressure (60 psi; 70-μm nozzle tip; 0.1 psi pressure differential) are ≤3.5%. However, each laboratory must establish its own expected ranges based on its instrument, and results may vary slightly owing to instrument differences such as the narrowness of the band-pass filters, laser power, laser emission wavelength, nozzle type, pressure differential, light collection system (cuvette versus jet-in-air), and beam-shaping optics.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ammonium chloride lysing solution, 10×

1.5 M NH_4Cl
100 mM $NaHCO_3$
1 mM disodium EDTA
Distilled H_2O to 900 ml
Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
Distilled water to 1 liter

Stock solution should be diluted 1:10 in distilled water immediately before use to make the 1× working lysing solution. Working lysing solution should be sterilized by filtration through a 0.22-μm filter and used at room temperature.

Other lysing solutions are also commercially available. However, keep in mind that some lysing solutions may contain fixative agents (e.g., paraformaldehyde) and should not be used for the SP assay.

DMEM+

Dulbecco's modified Eagle medium (Invitrogen) containing:
2% (v/v) fetal bovine serum (FBS; *APPENDIX 3B*)
10 mM HEPES (Sigma)
Store up to 4 weeks at 4°C

Enzyme medium

Basal Medium Eagle (BME) containing:
10 mM HEPES
200 U/ml collagenase type I
500 Kunitz units/ml DNase

HBSS+

Hanks' balanced salt solution (Invitrogen) containing:
2% (v/v) fetal bovine serum (FBS; *APPENDIX 3B*)
10 mM HEPES (Sigma)
Store up to 4 weeks at 4°C

Türk solution

0.01% (v/v) Giemsa stain
3% (v/v) acetic acid
Prepare 100 ml; store up 1 month at room temperature

COMMENTARY

Background Information

The blue-fluorescent Hoechst dyes are viable, cell-permeant nucleic acid stains that have multiple applications. They can interact with DNA, and the binding properties of Hoechst dyes have been classically used as a tool to study and visualize DNA in living cells. Other viability measurements allow investigators to analyze the emission spectral shifts of the Hoechst dyes, as well as the multidrug-resistant phenotype in cancer cells. Reduced accumulation of several fluorescent dyes has also been observed in stem cells, and increased dye efflux is associated with long-term repopulating ability. The highest levels of multidrug-transporter expression are found in $CD34^+$ pluripotent cells (Chaudhary and Roninson, 1991) but also in $CD34^{neg}$ cells (Goodell et al., 1997).

ABCG2 (also known as BCRP) is a half-transporter that belongs to the ATP binding cassette transporter superfamily and is

involved in drug resistance (Litman et al., 2000). ABCG2 expression has also been reported in some types of stem cells, and it is a key determinant of the side-population (SP) phenotype, which includes cells with the characteristics of primitive stem cells. SP cells have been found in virtually all adult tissues examined so far (Jackson et al., 2001; Uchida et al., 2001; Lechner et al 2002; Murayama et al., 2002; Montanaro et al., 2003) and are present in very low number, usually representing <0.1% of the total cell content. Hematopoietic SP are primarily CD34 negative, lack markers of mature hematopoietic cell types (lineage^{neg}), and have been shown to reconstitute the bone-marrow function of lethally irradiated recipients in multiple species (Goodell et al., 1997).

ABCG2 is responsible for the formation of the Hoechst 33342 (Ho342) flow cytometric fluorescent profile (Zhou et al., 2001). This profile is blocked in the presence of multidrug reversal agents because Ho342 efflux is reduced in the presence of fumitremorgin C (FTC), verapamil (VPL), and other drugs. The ability to block the Hoechst efflux also allows determination of the DNA content as well as the phenotype ($CD34^-$ and lineage-marker negative) of the SP cells (Goodell et al., 1996).

The SP displays a unique low Hoechst fluorescence emission property (Fig 9.23.1). An elegant explanation of the complex Ho342 emission pattern was published by the group of Dr. van den Engh (Petersen et al., 2004). Typical Ho342 concentrations for SP isolation are on the order of 8 μmol (5 μg/ml = 8.12 μmol). Using murine thymocytes and Ho342, they analyzed blue fluorescence intensity versus red fluorescence intensity at dye concentrations ranging from 0.1 to 30 μmol. How the chromatic shift occurs is explained by changes in the cellular concentration of Ho342. As Ho342 moves across the cellular membrane because of the ABCG2 activity, the blue/red fluorescence ratio follows a curve as shown in typical Ho342-red versus Ho342-blue dot plots.

Critical Parameters and Troubleshooting

Although the method works as originally described, the author of this unit believes that the experiments using Ho342 may be difficult for many investigators—first, because the ability to discriminate SP cells is based on the differential retention of Ho342 during a functional assay; second, because of the difficulties in setting the right experimental and acquisition conditions; and third, because analysis of the acquired data requires extensive expertise in flow cytometry to accurately detect the SP events (Sales-Pardo et al., 2006). However, it should not be difficult to find the SP if the whole procedure is followed exactly as described.

The author would like to emphasize that the problem with Ho342 is the red emission. Although the blue Ho342 emission can be easily detected, resolution of the red emission is not as good. Laser alignment is critical to optimize the resolution in red, and should contribute to decreasing the coefficient of variation (CV) for the red emission. Better resolution is found when the coefficients of variation, both in blue and in red, are the lowest and nearly identical. A significantly greater coefficient of variation for the red emission gives elliptic clusters for the G_0/G_1 population and poor SP resolution. However, some other factors may contribute to elliptic clusters, even when the protocol has been exhaustively reproduced. The author has observed that specific Ho342 preparations may be suboptimal for the SP staining. As a result, both blue and red linear CVs are higher and of poor quality for the far-red emission. In this case, one should neither increase the PMT voltages nor switch the scale from linear to logarithmic. Adjust the laser again and consider checking different Ho342 sources. Discard Ho342 preparations that give a weak emission signal (Fig. 9.23.2). In addition, for new batch preparation, storing the dye in its original powder form is recommended. On the other hand, sensitivity of the PMT used for the red emission measurement may also have an effect on the resolution in red. Hence, the more sensitive PMT will provide the lowest coefficients of variation for the Ho342-red. A good recommendation is to use the most sensitive PMT for the red emission. Hamamatsu #15 photomultiplier tubes have a spectral range of 185 to 900 nm, and provide extremely high sensitivity, ultrafast response, and improved far-red sensitivity.

Band-pass filters may also to contribute to enhancing the resolution of the side population (Fig. 9.23.3). In the author's laboratory, different combination of filters have been used, and it has been found that detection of Hoechst blue and red signals, respectively, through a 405/30- and a 670/40-nm bandpass filter gives the best resolution. A 440-nm long-pass dichroic mirror is also used to split the emission wavelengths. This filter setup is able to fit the chromatic shift of Ho342 more

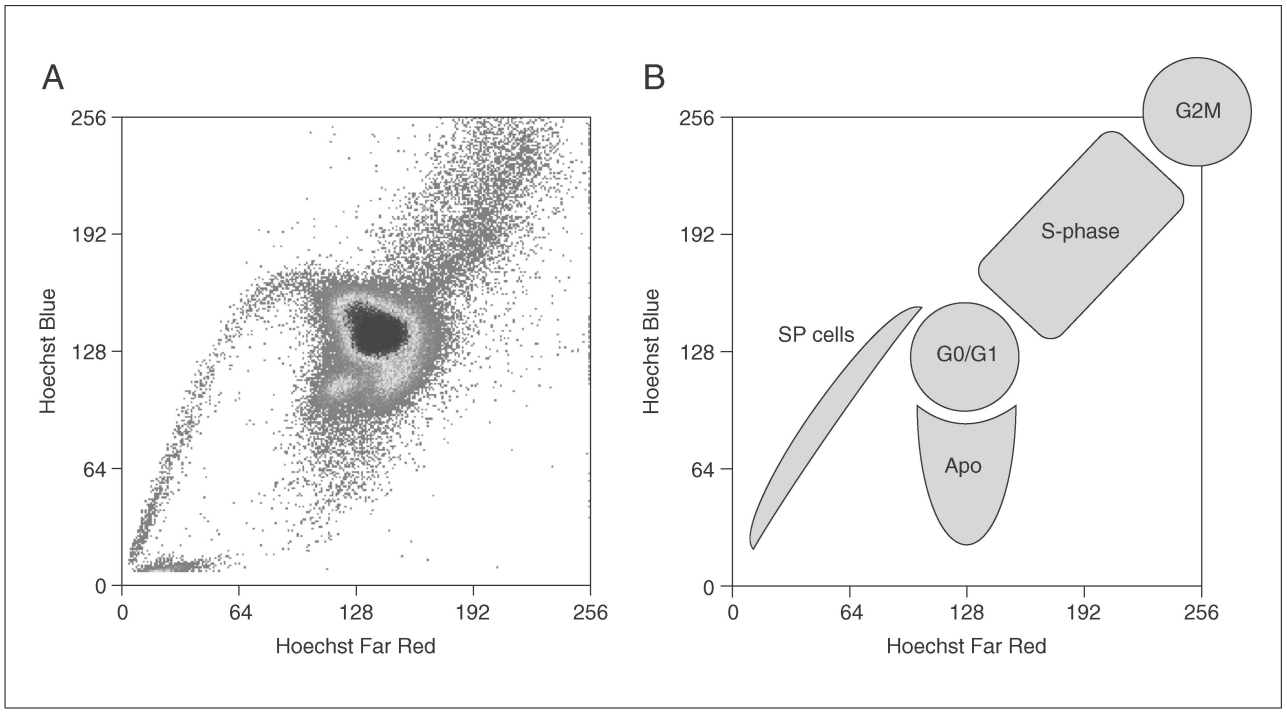

Figure 9.23.1 Hoechst 33342 SP analysis of unpurified human bone marrow on a MoFlo cell sorter (Dako) equipped with an Enterprise II water-cooled ion laser emitting at 351 nm (30 mW of laser power). (**A**) Representative dot plot for Hoechst Blue versus Hoechst Red simultaneous emission from a human bone-marrow sample containing SP cells. (**B**) Schematic dot plot showing distribution of events.

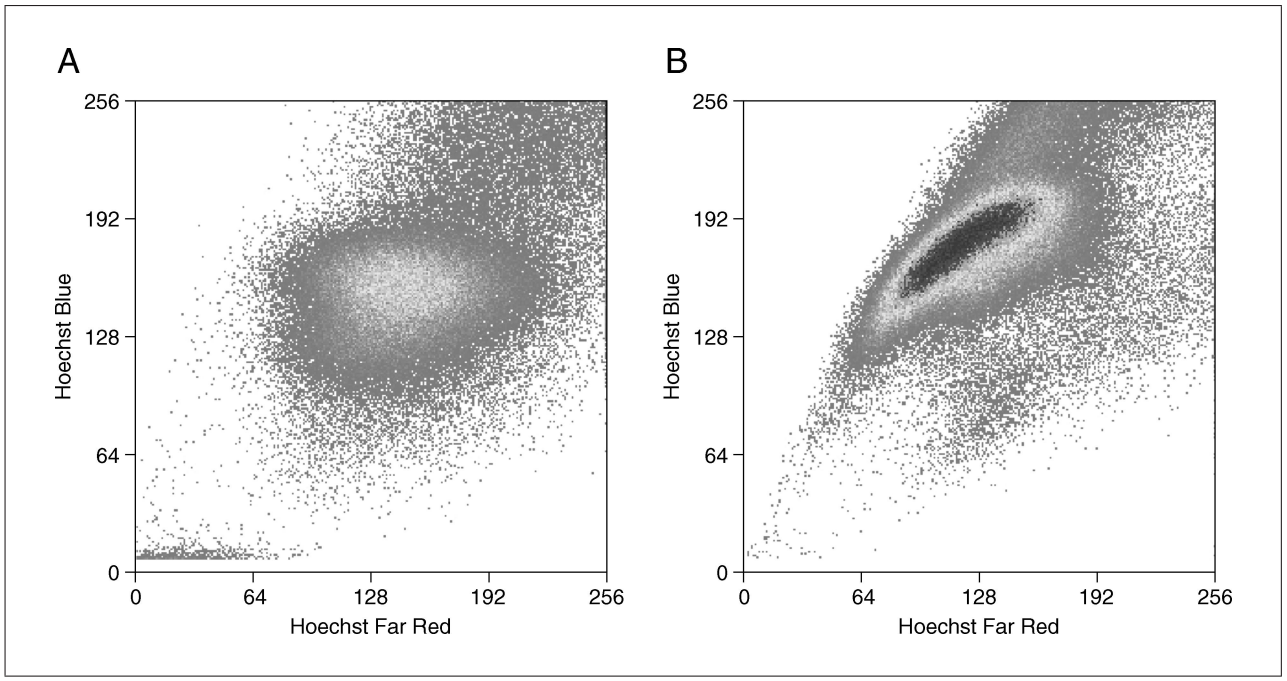

Figure 9.23.2 Hoechst 33342 SP analysis of unpurified human bone marrow on a MoFlo cell sorter (Dako) equipped with an Enterprise II water-cooled ion laser emitting at 351 nm. Since the red signal is lower in intensity by default (because of the intrinsic physical properties of this dye), resolution in the red emission is not as good as in the blue, and relatively higher laser power is needed. (**A**) Effect of UV laser misalignment on resolution of the SP. (**B**) Analysis of a bone-marrow sample stained with a suboptimal source (different batch).

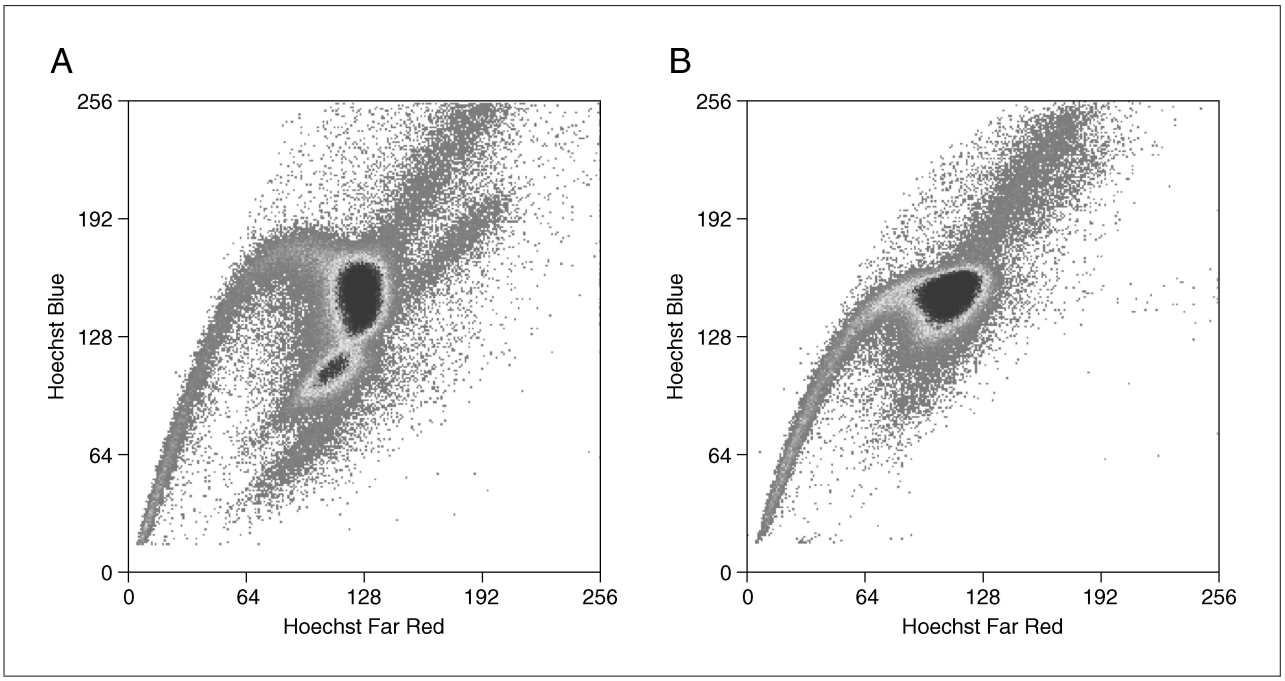

Figure 9.23.3 Hoechst 33342 SP analysis of unpurified human bone marrow. The analysis was carried out using (**A**) a filter setup consisting of BP 405/30 (blue), BP 670/40 (red), and 440 DLP filters, and (**B**) an alternative combination consisting of BP 450/65 (blue), BP 670/40 (red), and 510 DLP filters.

accurately than other filter combinations. Depending on the instrument used, blue fluorescence can be collected with a 450/20 BP filter and red fluorescence with a 675 edge filter long-pass (EFLP). A 610 dichroic mirror short-pass (DMSP) can be used to separate the emission wavelengths. Other combination consists of BP 450/65 (blue), BP 670/40 (red), and 510 DLP filters.

Note that the original protocol developed by Margaret Goodell's laboratory (see *UNIT 9.18*) measures the PI emission through the same filter for the Ho342-red. However, based on the emission properties of this dye, and for a more precise discrimination of dead cells, in the author's laboratory an alternative analysis was chosen. PI emission is measured on a logarithmic scale using a different PMT (BP 630/30) and 488-nm excitation, instead of using the same PMT for both PI and Ho342-red emission. In the author's opinion, a feasible live gate using this condition should better resolve the SP, even if apoptotic/prenecrotic cells are present in the sample.

Sample manipulation heavily affects the SP resolution. Efforts should be addressed towards minimizing the sample processing time. Excess time spent on isolation and preparation of cells can result in suboptimal SP analysis. For this reason, samples should be processed immediately. The SP protocol should be started as soon as possible after obtaining the sample, or performed in less than 2 hr. In addition, if sample processing and sorting cannot not be performed on the same day, samples can be stained and kept at 4°C overnight prior to cell sorting. While processing, avoid keeping cells in ice, because temperatures below 4°C may result in dramatically reduced cell viability. It is the author's opinion that a drop in total cell viability below 90% should be considered suboptimal for the SP analyses. Keep in mind that manipulation can cause cellular depletion and ABCG2 malfunction and may result in incorrect measurements, especially when target cells are very rare. Since Ho342 uptake, retention, and efflux depend on cell membrane integrity, this integrity must be assessed in Ho342 staining experiments by parameters other than light scatter (life gate). Simultaneous staining with PI, an impermeant dye that does not stain living cells, is used to overcome artifactual retention phenomena.

Note that Hoechst 33342 may exert cytotoxic effects by inducing DNA single-strand breaks (Chen et al., 1993). This cytotoxic effect is increased following exposure to UV lasers. Therefore, caution is needed when cells are sorted for further functional experiments. Although SP cells will retain a low concentration of the dye, genotoxic damage to low-tip cells (i.e., the SP cells with the highest ability to efflux Ho342) cannot be ruled out. In

order to minimize exposure to light, keep the cell sample covered during the whole staining procedure, even when cells are being run on the flow cytometer. Use the lowest UV laser power that gives good resolution for the SP to aid in minimizing cell damage. In addition, the distribution of SP cells within the tail may vary substantially among normal individuals, even from a common tissue origin (e.g., bone marrow). There is no doubt that different factors may alter the SP distribution of cells from the tip to the G_0/G_1 cluster. It is generally accepted that Ho342-low cells are the more primitive and have a higher clonogenic potential than the rest of the SP. However, those cells presenting the low Ho342 concentration would be more viable and functional than the rest. The author of this unit suggests that this would constitute an alternative explanation on why SP-low cells display a higher repopulation capacity than the SP-high subset.

In many situations, specimens assayed for the SP have a low cell number. This is not unusual for tissue biopsies and pediatric samples. The author's laboratory has adapted the methodology for investigating SP cells to low-volume samples. Such samples are usually subjected to extensive manipulation that includes disaggregation of tissues, centrifugation, washing steps, and in some cases, enrichment of the target cells by density gradients. In order to minimize cell loss, erythrocytes can simply be lysed and the remaining white blood cells prepared for Ho342 staining. Note that some fixative-containing red-cell lysing solutions, e.g., those with paraformaldehyde, are not useful for functional studies. Erythrocyte lysis using ammonium chloride does not hamper the resolution of the dim tail of the side population and it can be applied for the simultaneous staining with antibodies.

Cells can be prepared for immunofluorescence by using directly conjugated antibodies. Although indirect immunofluorescence techniques can be also applied for polychromatic flow cytometry, the author does not recommend the use of secondary antibodies. If possible, try to use a monoclonal antibody conjugated to a bright fluorochrome (i.e., phycoerythrin) for the lowest expected antigen expression (i.e., CD34, to define negativity). Conversely, use a monoclonal antibody conjugated to a less bright fluorochrome (i.e., FITC) for the highest expected antigen expression (i.e., CD45, for leukocytes). Thus, staining with directly conjugated antibodies allows a feasible multicolor analysis, even using high-power laser settings. In the author's laboratory, up to seven fluorescences have been measured simultaneously from individual SP events in a single sample tube using surface expression markers. Optimal multicolor combinations for human bone marrow SP analysis are as follows: Ho-Blue, Ho-Red, PI, FITC-CD45, APC-CD38, PerCP-CD34 (fixed panel); PE only–conjugated antibodies were replaced by the different markers: CD90, CD117, and CD133. The SP fraction gives CD45 positive/dim and CD38 negative/dim. SP cells are also negative for the CD34 marker, and for CD90, CD117, and CD133, which is in agreement with previous studies.

SP analysis represents a continuously growing field, especially in terms of understanding stem-cell biology, as well as for the study of cancer stem cells. In fact, chemotherapy contributes to increasing the heterogeneity of cancer cells instead of eliminating the malignant subset overexpressing active ATP-binding cassette transporters, such as ABCG2. In the author's opinion, ABCG2-based functional data may lend support to the fact that a hierarchy of stem cells originating in the side population can be identified in both cancer (Patrawala et al., 2005) and leukemias (Wulf et al., 2001) and probably in other distant tissues, as a potential source for long-distance metastasis. Several studies have shown that ABCG2 is expressed and functionally active in small subpopulations in acute myeloid leukemia (Abbott et al., 2002; Benderra et al., 2005; Raaijmakers et al., 2005). Thus, leukemic stem cells originating in this compartment may represent an important source of cells that present a challenge to achieving long-term remission. Although a precise relation between stem-cell and SP-cell fractions is still lacking, the analysis of SP cells is a potential new approach to demonstrate the complexity of the human stem-cell compartment.

In normal human bone marrow, the SP is composed of very rare cells, which account for ~0.03% of all nucleated cells. However, in bone-marrow preparations from leukemia patients, SP cells can be found in large numbers (up to 15% of viable nucleated cells). In addition, multiple G_0/G_1 peaks can be observed. Although the ability to block the Ho342 efflux also allows the determination of the DNA content, the author of this unit recommends analyzing the DNA content by PI staining. DNA index and cell cycle analysis are then used to rule out the presence of artifacts and to confirm the existence of one or more subpopulations

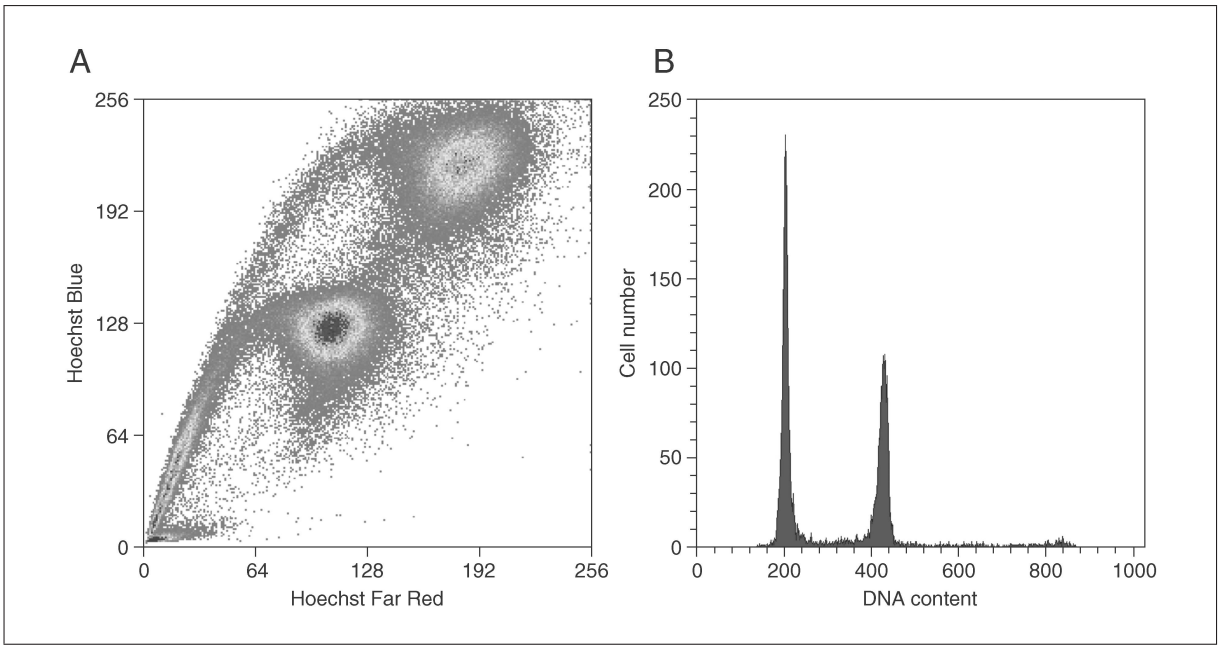

Figure 9.23.4 Side population (SP) pattern of Hoechst 33342 efflux in the bone marrow of a patient with acute myeloid leukemia (AML) at diagnosis. Using Hoechst 33342 dye and dual-wavelength flow cytometry analysis, an SP profile is found to be present in AML samples. The SP contains increasing levels of ABCG2-mediated dye efflux, from high to low fluorescence. (**A**) A representative flow cytometry dot plot of diploid and near-tetraploid AML (M2) cells also stained with Hoechst 33342 to identify SP+ cells (diploid SP+ cells: 13.31%; near-tetraploid SP+ cells: 1.89%). (**B**) DNA content through high-resolution cell cycle analysis, corresponding to the patient diagnosed with AML (M2), showing a secondary near-tetraploid subpopulation (DNA index = 2.1) with individual $S+G_2M$ phase, as calculated from the linear relationship between DNA content and propidium iodide fluorescence.

with a different DNA index (Fig. 9.23.4). Note that DNA/chromatin conformation may also affect Ho342 retention, especially when testing heterogeneous cell specimens and during apoptotic events, because of chromatin condensation. However, these multiple peaks cannot be considered as different cell subpopulations with different DNA indices. In order to rule out artifacts and to confirm the existence of multiple peaks, always consider an alternative DNA content analysis (e.g., Vindelov assay).

In addition, when looking at cancer or leukemic samples, the expression of other multidrug-resistance transporters involved in Ho342 efflux cannot be ruled out. It has been observed in the author's laboratory that cells carrying mutations in the *ABCB1* gene appear to be SP cells. However, this efflux is not observed in those cells with expression of wild-type *ABCB1*, such as human CD34-positive cells (J. Petriz, unpub. observ.).

Although the identification of the SP is based on Ho342 staining, alternative assays to examine the SP in cancer samples are needed, e.g., costaining with a monoclonal antibody against the external domain of ABCG2.

Anticipated Results

The prevalence of SP cells is very low: 0.05% of normal whole bone marrow in the mouse and 0.9% in normal human samples. Keep in mind that this is a functional assay and that sample manipulation heavily affects the SP resolution. However, it should not be difficult to find the SP if the whole procedure is followed exactly as described.

Time Considerations

Setting up the staining environment and preparation of the buffers requires ~45 min. Setting up both the flow cytometer and the cell sorter requires between 30 and 60 min. The time requirement for sample preparation depends on the specimen used. Note that solid tissues need to be disaggregated, a procedure usually taking up to 60 min. Once the cells are obtained from the desired tissue for the SP analysis in sufficient number, the whole staining procedure usually takes up to 3 hr. Incubation with antibodies will require additional time, up to 90 min depending on the immunostaining technique used.

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